Specificity of the hyaluronate lyase of group-B streptococcus toward unsulphated regions of chondroitin sulphate

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The purification and properties of a hyaluronate lyase secreted by *Streptococcus agalactiae*, which is believed to facilitate the invasion of host tissues by the organism, have been described previously [Pritchard, Lin, Willingham and Baker (1994) Arch. Biochem. Biophys. **315**, 431–436]. The specificity of the limited cleavage of chondroitin sulphate by the enzyme is the subject of this report. To simplify the task, a chondroitin sulphate from the Swarm rat chondrosarcoma, which contains only 4-sulphated and unsulphated disaccharide repeats, was used in this study. Tetrasaccharides from an ovine testicular hyaluronidase digest of the chondroitin sulphate were isolated, identified and tested as substrates of the streptococcal hyaluronate lyase. Only tetrasaccharides with an unsulphated disaccharide at the reducing end were cleaved (by elimination at the *N*-acetylgalactosaminidic bond). Thus chondroitin sulphate chains are cleaved by the action of this lyase at every unsulphated disaccharide repeat, but release of unsaturated unsulphated disaccharides only occurs from sites where two or more sequential unsulphated disaccharide repeats are present. Analysis of the chondrosarcoma chondroitin sulphate showed that of approximately five unsulphated disaccharide repeats per chain, two are clustered. The ability of group-B streptococcal hyaluronate lyase to cleave chondroitin sulphate may allow the organisms to invade tissues more efficiently. The demonstrated specific and highly limited cleavage of chondroitin sulphate by this bacterial lyase promises to be a useful tool in the determination of chondroitin sulphate structure and variability.

INTRODUCTION

By 1951, two isomers of chondroitin sulphate (CS), now termed chondroitins 4- and 6-sulphate, had been identified [1]. Analyses of the two isomers of CS, from cartilage and other sources, gave near-equimolar values for their major constituents: galactosamine, glucuronic acid and inorganic sulphate. Therefore it was considered that these glycosaminoglycans have a regular consistent disaccharide repeat sequence. CS proteoglycans (PGs) are known to bind considerable amounts of water and serve to protect and cushion surrounding structures. PGs also limit the diffusion of other macromolecules [2]. Such general functions may well be performed by a polymer of regular structure.

Many recent studies of CS PGs emphasized the variability of glycosaminoglycan structure and related this variability to much more specific functions. Twenty-three different disaccharide repeat structures, including the unsulphated disaccharide, have been found in chondroitinase digests of CS/dermatan sulphates from various sources [3]. Most occur at only low levels, but still may affect the binding of cations and other macromolecules by the PG. For example, without some degree of oversulphation (i.e. more than one sulphate/disaccharide), there is no demonstrable binding of low density lipoproteins by CS [4]. The one or two chains attached to thrombomodulin are heavily oversulphated [5] and are involved in the thrombin/antithrombin-binding properties of this endothelial-cell-surface PG. There is also some evidence that the oversulphated CS PG at the cell surface of macrophages modulates synthesis and secretion of apoE-containing lipoproteins [6].

There is a need for methods for mapping the variability and distribution of CS disaccharide repeat structures, in order better to understand and explain CS function. There is little chance that satisfactory chemical methods will be developed as there is such great potential for variability in carbohydrate structures (cf. nucleic acids and proteins). Enzymic procedures are more promising. The different specificities of chondroitinases ABC and AC allow the separate estimation of glucuronate- and iduronate-containing disaccharides from chondroitin/dermatan sulphates [7]. Chondroitinase ACII acts as an exoglycosidase [8], so it could possibly be of help in determining the distribution of types of disaccharide repeat from the non-reducing to the reducing end of a CS chain. Using specific chondroitinases, it has been shown that 4- and 6-sulphated *N*-acetylgalactosamine moieties in CSs from a number of sources are not randomly arranged, and the arrangement varies according to source [9].

In our previous study [10], we showed that a hyaluronate lyase (GBS hyase) secreted by group-B streptococci (*Streptococcus agalactiae*, strain 3502) can cleave CS chains in a limited fashion. The average size of CS released by digestion of bovine cartilage aggrecan with GBS hyase was a little smaller than that of intact CS chains released by alkaline elimination, as determined by gel chromatography on Sepharose CL-6B. Thus most cleavages would appear to occur close to the linkage region of each glycosaminoglycan chain. Furthermore we showed that the only disaccharide released during the digestion of CS from rat chondrosarcoma aggrecan is 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi0S), despite the low percentage of 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (Di0S) disaccharides (12%) in the CS from this source. This result indicated that GBS hyase cleaves CS at some or all Di0S sites, i.e. it exercises considerable specificity in cleaving CS. The present work was undertaken to

Abbreviations used: ΔDi0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; Di0S, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose; ΔDi4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; CS, chondroitin sulphate; GBS hyase, hyaluronate lyase from group-B streptococcus; PG, proteoglycan; $\overline{DP}$, degree of polymerization.

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define further the extent and specificity of the cleavage, and to begin to determine the distribution of unsulphated residues in CS chains.

EXPERIMENTAL

Materials

Ovine testicular hyaluronidase (220 units/mg) was from ICN. Papain (2 × crystallized; 16–40 units/mg), protease-type XXI (Pronase, 15–25 units/mg), chondroitinase ABC, chondroitinase ACII and chondro-4-sulphatase were purchased from Sigma. Sephadex G-25 (superfine) and CM-Sepharose CL-6B were obtained from Pharmacia. AG 50 (X8) was from Bio-Rad. Ultrafree-MC filter units (5000 Da cut-off) were from Millipore, and a Partisil-SAX column (250 mm × 1.6 mm; 10 μm) was purchased from Alltech. CS oligosaccharides, which were prepared by testicular hyaluronidase digestion of CS and fractionated according to size by gel chromatography on Sephadex G-50, were a gift from Dr Jim Christner, Environmental Test Systems Inc., Elkart, IN, U.S.A.

The capillary electrophoresis system employed (model 270A-HT) was from Applied Biosystems.

Purification of GBS hyase

The construction of plasmid pLIN101 in which the complete GBS hyase gene (*hylB*) was cloned in the phagemid vector pBK-CMV was described previously [11]. The gene was expressed in *Escherichia coli* strain INVαF′ (Invitrogen Corp., San Diego, CA, U.S.A.). Recombinant GBS hyase was purified from an overnight culture (4 litres) of the organism grown at 37 °C in Terrific Broth (Life Technologies, Grand Island, NY, U.S.A.) containing 50 μg of kanamycin/ml. Bacterial cells were harvested by centrifugation (1000 ***g***), washed three times with 50 mM Hepes/10 mM EDTA, pH 8.0, and then resuspended in the same buffer (200 ml). The suspension was passed through a continuous-flow cell breaker operated at 103.5 MPa. The cell lysate was centrifuged at 10000 ***g*** for 60 min and the supernatant loaded on to a column (26 cm × 2.5 cm) of CM-Sepharose CL-6B. The column was washed with 50 mM Hepes/10 mM EDTA, pH 8.0 (200 ml) and then the enzyme was eluted in a linear gradient (0–0.3 M NaCl) in the same buffer. Fractions containing the peak of enzyme activity were pooled and concentrated using a Centricon 30 ultrafiltration device (Amicon, Beverly, MA, U.S.A.).

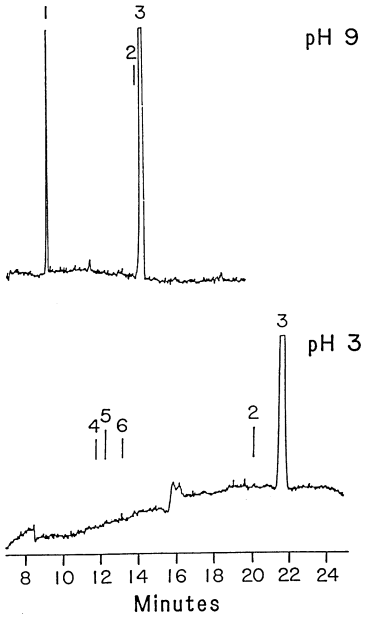

Figure 1 Disaccharide repeat composition of chondrosarcoma CS

Chondrosarcoma CS (10 μg) was digested with chondroitinase ABC and the resulting unsaturated disaccharides were analysed by capillary electrophoresis as described under 'Determination of the disaccharide composition of CS and CS oligosaccharides'. Separations of unsulphated and monosulphated disaccharides were at normal polarity and pH 9.0, and separations of mono- and di-sulphated disaccharides were at pH 3.0 using reverse polarity. Migration times of standard disaccharides are identified by numbers 1, ΔDi0S; 2, ΔDi6S; 3, ΔDi4S; 4, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; 5, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; 6, 2-acetamido-2-deoxy-3-*O*-β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulpho-D-galactose.

Isolation and composition of CS from Swarm rat chondrosarcoma

PGs were extracted from the Swarm rat chondrosarcoma using 4 M guanidinium chloride, and the PG-subunit fraction (A1D1) was isolated by density-gradient centrifugation under dissociative conditions as described previously [12]. CS chains were isolated after papain and Pronase digestion of A1D1 [13]. The disaccharide repeats of chondrosarcoma CS are only of Di4S and Di0S (Figure 1). There are no repeats of Di6S or oversulphated disaccharides.

Testicular hyaluronidase digestion of A1D1 and fraction of CS oligosaccharides by chromatography on Sephadex G-25

A1D1 (250 mg) was dissolved in 0.5 M NaCl/0.1 M sodium acetate, pH 5.0 (5.0 ml) ovine testicular hyaluronidase (10 mg) was added and the mixture was incubated at 37 °C for 45 h. The hyaluronidase digest of A1D1, supplemented with sucrose (250 mg), was applied to a column (2.5 cm × 200 cm) of Sephadex G-25 which had been pre-equilibrated and was subsequently developed with 0.5 M NaCl in aq. 10% methanol. The flow rate was maintained at 20 ml/h and 20 min fractions were collected. Elution of oligosaccharides was monitored at 206 nm.

CS disaccharides

Pool 5 (Figure 2) was desalted as described below, taken to dryness and redissolved in HPLC-grade water at 1 μmol of disaccharide/ml (Di4S). An aliquot of Di4S was digested in 0.05 M Tris/acetate, pH 7.5 (10 μl) with chondro-4-sulphatase (2 m-units) at 37 °C for 1 h to yield Di0S.

Fractionation of tetrasaccharides from CS

The tetrasaccharide pool (pool 4), recovered from the fractionation on Sephadex G-25 of the testicular hyaluronidase digest of A1D1 (Figure 2), was fractionated further by anion-exchange HPLC on a Partisil-SAX column (250 mm × 1.6 mm) in three stepwise gradients of 0–60 mM, 60–100 mM and 100–200 mM KH_2PO_4 in aq. 10% methanol. Each successive gradient was designed to elute and resolve unsulphated, monosulphated and disulphated tetrasaccharides respectively. The flow rate was 0.4 ml/min and saccharide peaks were detected at 200 nm. This system is a variant of that previously employed for the fractionation of CS oligosaccharides [14]. Fractions were pooled (A, B, C and D) as shown in Figure 3.

Desalting

CS or oligosaccharide samples (1 ml) to be desalted were applied to a column (2.0 cm × 20 cm) of Sephadex G-10 developed in aq.

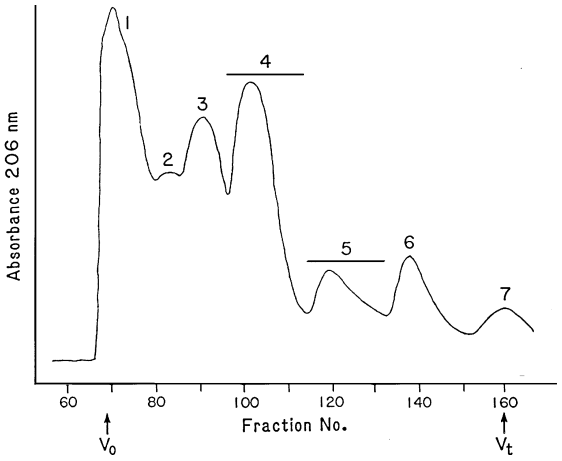

Figure 2 Fractionation of hyaluronidase-digested A1D1 by gel chromatography on Sephadex G-25

Conditions of the separation are given in the Experimental section. The horizontal bars above peaks 4 and 5 indicate fractions pooled to give the tetra- and di-saccharide pools respectively. Peak 6 is of sucrose.

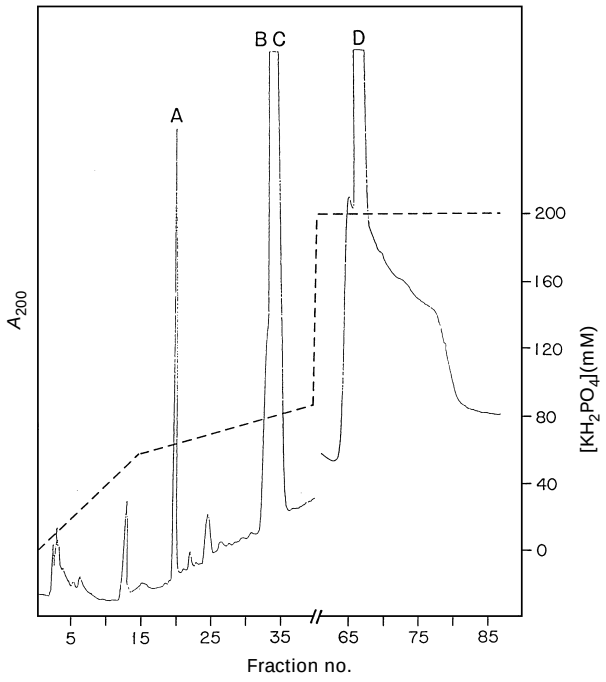

Figure 3 Fractionation of chondrosarcoma CS tetrasaccharides by anion-exchange HPLC

The tetrasaccharide pool (see Figure 2) was fractionated on a Partisil-SAX column using a gradient elution system as described in the Experimental section. Tetrasaccharides, resolved into peaks A, B (a shoulder preceding peak C), C and D, were separately pooled. ——, A_{200}; – – – –, $[KH_2PO_4]$.

20% methanol at a flow rate of 0.5 ml/min and monitored at 200 nm. Oligosaccharides of degree of polymerization ($\overline{DP}$) > 4 were eluted at V_0 and dried using a Savant SpeedVac.

Determination of the disaccharide composition of CS and CS oligosaccharides

Desalted CS samples (containing approx. 0.5 nmol of uronic acid) were taken to dryness, dissolved in 10 μl of buffer (0.1 M Tris/HCl, 0.03 M sodium acetate, 0.01 M EDTA, pH 7.4) containing chondroitinase AC (2.5 m-units) and incubated at 37 °C for 2 h. Desalted oligosaccharides (0.5 nmol of uronic acid) were digested with chondroitinase ABC (1 m-unit) in 0.1 M Tris/HCl/0.03 M sodium acetate, pH 8.0 (10 μl) at 37 °C for 1 h. Each digest was diluted with HPLC-grade water (40 μl) and centrifugally filtered through an Ultrafree-MC filter unit. Filtrates were analysed for disaccharides using capillary electrophoresis as described [15]. Samples were loaded hydrostatically (2 s) at 500 mm Hg and separated at pH 9.0, except where stated otherwise. Detection of peaks was at 200 nm (saturated and unsaturated disaccharides) and at 232 nm (unsaturated disaccharides).

Digestion with GBS hyase

Each tetrasaccharide (0.2–0.5 nmol) was incubated in 0.05 M ammonium acetate/0.01 M $CaCl_2$, pH 6.5 (hyase buffer; 10 μl) containing GBS hyase (9 units) at 37 °C for 1 h. Then the hyase digests were treated similarly to the chondroitinase digests before analysis by capillary electrophoresis.

RESULTS

Strategy

The strategy for determining the specificity of CS cleavage by GBS hyase is illustrated in Figure 4. A CS PG of relatively simple composition, from the Swarm rat chondrosarcoma A1D1, was digested with testicular hyaluronidase, and the tetrasaccharide fraction separated from the oligosaccharide products. Four different tetrasaccharides may be generated from this CS and they can be resolved by anion-exchange HPLC. The susceptibility of each of these tetrasaccharides to digestion by GBS hyase was tested. Thus the specificity of GBS hyase for cleavage of the glycosidic bonds present in chondrosarcoma CS could be defined.

Identification of tetrasaccharides

The identities of tetrasaccharides in pools A, B, C and D (Figure 3) were determined by digesting aliquots of each pool with chondroitinase ABC and analysing for the product disaccharides using capillary electrophoresis. Cleavage of a CS tetrasaccharide by this lyase yields a saturated disaccharide from the non-reducing end, and an unsaturated disaccharide from the reducing end. The four possible disaccharide products are Di0S, ΔDi0S, Di4S and ΔDi4S. They could be separated and distinguished as shown in Figure 5. Both saturated disaccharides, Di0S and Di4S, had shorter migration times than their unsaturated counterparts, ΔDi0S and ΔDi4S respectively. Also useful for distinguishing between saturated and unsaturated disaccharides was dual monitoring at 200 nm and 232 nm. The unsaturated disaccharides were detected at both wavelengths, whereas saturated disaccharides were detected at 200 nm only (Figure 5).

Aliquots of tetrasaccharide pools A, B, C and D were analysed by capillary electrophoresis and each gave single peaks at 10.0 min, 12.8 min, 12.7 min and 16.6 min respectively (results not shown). A, B, C and D were each completely digested by chondroitinase ABC and analysed by capillary electrophoresis. Only two peaks, identified as Di0S and ΔDi0S, were present in the digest of A, so it is evident that the tetrasaccharide of pool A is Di0S–Di0S. The disaccharide products from C were Di4S and ΔDi0S, and from D the only disaccharides produced were Di4S and ΔDi4S. Therefore the tetrasaccharides of C and D are Di4S–Di0S and Di4S–Di4S respectively. The chondroitinase

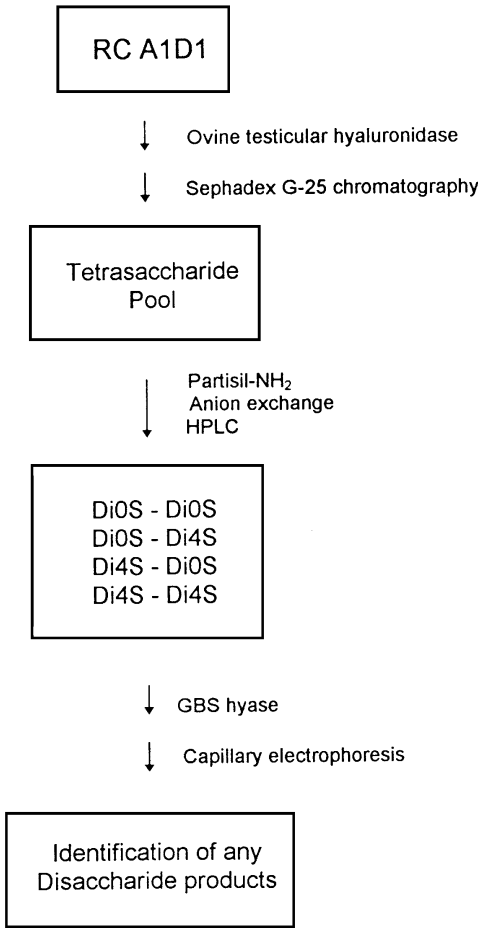

Figure 4 Isolation of tetrasaccharides from rat chondrosarcoma (RC) A1D1 and their selective cleavage by GBS hyase

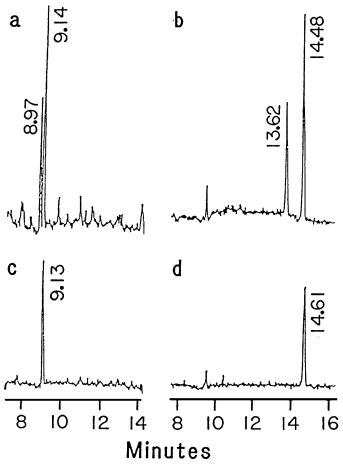

Figure 5 Separation of standard disaccharides Di0S, ΔDi0S, Di4S and ΔDi4S by capillary electrophoresis

Di0S (see the Experimental section) and ΔDi0S (**a** and **c**); Di4S (also see the Experimental section) and ΔDi4S (**b** and **d**), each at 1–5 μg/ml, were separated by capillary electrophoresis. Disaccharide peaks were detected at 200 nm (**a** and **b**) and 232 nm (**c** and **d**). (**a**) The migration time of Di0S (8.97 min) is less than that of ΔDi0S (9.14 min). The identities of these two peaks are confirmed by the absence of the former peak, but the presence of the latter peak, at 232 nm (**c**). (**b**) The migration time of Di4S (13.62 min) is less than that of ΔDi4S (14.48 min). The identities of these two peaks are also confirmed by the absence of the former peak, but the presence of the latter peak, at 232 nm (**d**).

ABC digest of pool B contained Di0S and ΔDi4S, which are derived from the tetrasaccharide Di0S–Di4S. Also present in this digest are the disaccharides from Di4S–Di0S (i.e. pool B contains some pool C material. B is a shoulder on the peak of C, see Figure 3).

Susceptibility of CS tetrasaccharides to digestion by GBS hyase

Samples of each of the isolated tetrasaccharides A, B, C and D were digested with GBS hyase, and the digests were analysed using capillary electrophoresis. By this means, both loss of tetrasaccharide substrate and appearance of disaccharide products could be monitored (Figure 6). Identifications of peaks seen in Figure 6 were based on migration times and absorbance at 200 nm and 232 nm. Results from the GBS hyase digestion of tetrasaccharides A, C and D were clearcut. Under the conditions employed, digestions of A (Di0S–Di0S) and C (Di4S–Di0S) went to completion (Figures 6e and 6i and 6g and 6k respectively). D (Di4S–Di4S) remained intact (Figures 6h and 6l). As reported above, B contains Di0S–Di4S and Di4S–Di0S. Digestion of Di0S–Di4S would be accompanied by the liberation of Di0S and ΔDi4S, but incubation of B with GBS hyase gave neither of these disaccharides. Only Di4S and ΔDi0S were seen (identified by asterisks, Figures 6f and 6j), which must be derived from Di4S–Di0S (as from C). Therefore Di0S–Di4S is not cleaved by the GBS hyase. When digestions of B and D were repeated using a 10-fold higher concentration of enzyme, there was still no liberation of disaccharides from Di0S–Di4S or Di4S–Di4S, respectively (results not shown).

Unsulphated disaccharide repeats in chondrosarcoma CS: their number and distribution

Based on the findings reported above, the glycosidic bonds of chondrosarcoma CS that are susceptible to cleavage by GBS hyase are illustrated below. (The distribution of the disaccharide repeats, Di4S and Di0S, along a chain of chondrosarcoma CS is also shown below.)

$$(Di4S)_n\text{–}Di0S\text{–}(Di4S)_p\text{–}Di0S\text{–}(Di4S)_q\text{–}Di0S\text{–}Di0S\text{–}Di0S\text{–}U\text{–}G\text{–}G\text{–}X\text{–}S$$
$$\uparrow \qquad\qquad \uparrow \qquad\qquad \uparrow \quad \uparrow \quad \uparrow \quad \uparrow$$

Where ↑ marks a bond cleaved by GBS hyase. n, p and q are the numbers of consecutive Di4S repeats occurring in different regions of the chain. U, G, X and S are the linkage region residues of glucuronic acid, galactose, xylose and serine respectively.

Then, from what we know of the specificity of the GBS hyase towards CS, it can be deduced that: (1) ΔDi0S will be released only from regions of two or more consecutive Di0S repeats. The ΔDi0S released by GBS hyase may be determined and equals the Di0Ss which occur in clusters. (A cluster is defined as two or more identical disaccharide repeats which occur consecutively.) (2) Oligosaccharides released, except that from the non-reducing end of the chain, will fit the formula $\Delta Di0S\text{-}(Di4S)_{p\text{ or }q}$ (i.e. they will have a non-reducing terminal ΔDi0S).

It is evident from this model that Di0S units are released as ΔDi0S by GBS hyase from a CS chain only if they occur in clusters of two or more. The repeat disaccharide of chondrosarcoma CS that is adjacent to the linkage region is undersulphated [16] and is not eliminated from the linkage region by chondroitinase ABC digestion [17]. Whether it can be eliminated by GBS hyase digestion, and possibly be part of a Di0S cluster,

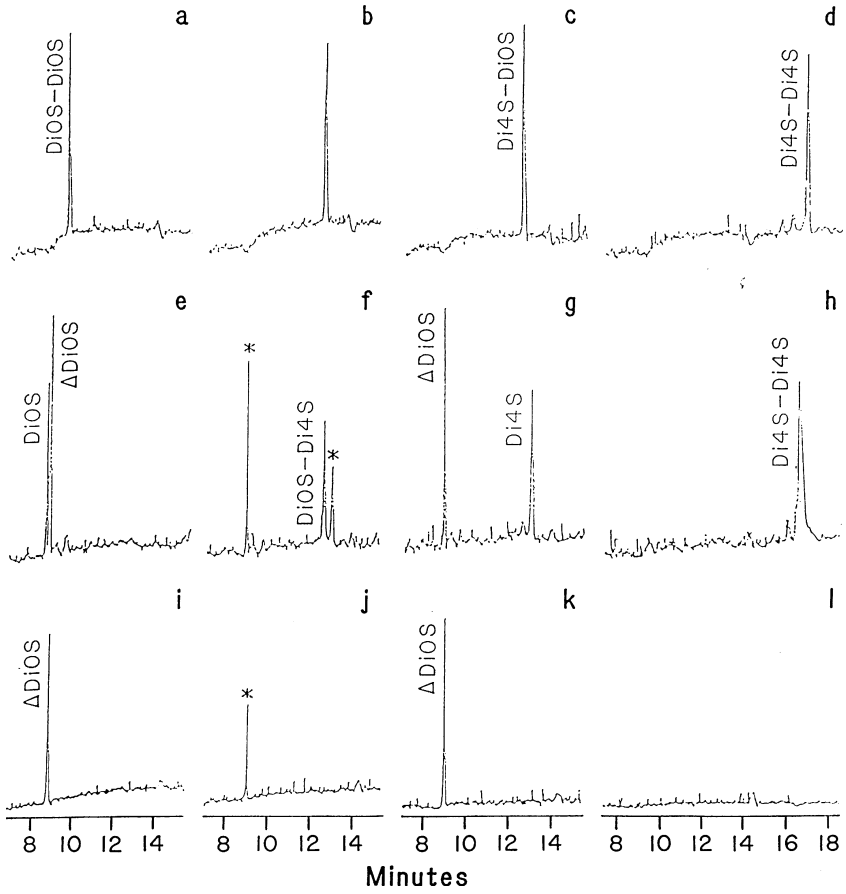

Figure 6 Analysis by capillary electrophoresis of GBS hyase digests of CS tetrasaccharides

Each CS tetrasaccharide preparation (A, B, C and D. See Figure 3) was digested by GBS hyase and analysed by capillary electrophoresis (see the Experimental section). The separations of A, B, C and D, undigested by GBS hyase and detected at 200 nm, are shown in (**a**), (**b**), (**c**) and (**d**) respectively. Separations of the GBS hyase digests of A, B, C and D, detected at 200 nm, are given in (**e**), (**f**), (**g**) and (**h**) respectively. The same separations, detected at 232 nm, are shown in (**i**), (**j**), (**k**) and (**l**) respectively. * denotes a peak derived from C in B. The tetrasaccharides Di0S–Di4S and Di4S–Di0S in B are unresolved (**b**).

is not clear. To determine this, chondrosarcoma A1D1 was fully digested with chondroitinase ABC and dialysed until no further release of disaccharides was detected. Then aliquots of this chondrosarcoma A1D1 core were incubated with GBS hyase, chondroitinase ACII or chondroitinase ABC (Figure 7). No further elimination of disaccharide was caused by incubation with chondroitinase ABC (Figure 7a). As a result of GBS hyase digestion (Figure 7b), further elimination of disaccharide was seen, which was identical in quantity and composition to the product from chondroitinase ACII digestion (Figure 7c). This result suggests that GBS hyase, in common with chondroitinase ACII, can remove the last disaccharide from the linkage region of chondrosarcoma CS.

Digestion of chondrosarcoma CS with GBS hyase would be expected to convert all Di0S units into non-reducing terminal ΔDi0S units or to release them as ΔDi0S. It was possible to test this prediction. A GBS hyase digest of chondrosarcoma CS was subjected to oxymercuration [18], a procedure that cleaves the glycosidic linkages of unsaturated uronic acid moieties. The oxymercurated preparation was digested using chondroitinase ACII and then analysed for disaccharides by capillary electrophoresis (Figure 8). This digest contained no ΔDi0S, which is in agreement with the proposed model of chondrosarcoma CS cleavage by GBS hyase.

We determined the proportion of Di0S units in a chain of chondrosarcoma CS that are clustered (Table 1). First, the total Di0S and Di4S content of the chondrosarcoma CS (1.85 μmol/mg of CS) was determined after digestion with chondroitinase ACII. From the total Di0S content (239 mmol/mg of CS) and the average number of disaccharide repeats per chondrosarcoma CS chain (approx. 40, see [19]), it was calculated that there are on average five Di0S residues/CS chain. However, only 97 nmol of Di0S/mg of CS is released by GBS hyase digestion (Table 1), which represents two Di0S residues per chain. Therefore approximately two of five Di0S units per chain of chondrosarcoma CS are clustered.

The Di0S units may be distributed within the chains of chondrosarcoma CS in a specific or a random manner. To help distinguish between these two possibilities, the oligosaccharides in a GBS hyase digest of chondrosarcoma CS were fractionated by PAGE (Figure 9). Release of a full range of oligosaccharides ($\overline{DP}$ 2–80) would indicate that Di0S units are distributed randomly, whereas a specific arrangement of Di0S units within the chain would give only a few released oligosaccharides. In that region of the profile where oligosaccharides are well resolved ($\overline{DP} < 60$), a full range of oligosaccharides is seen (Figure 9). Therefore a random distribution of unsulphated disaccharides within CS chains is suggested by this result.

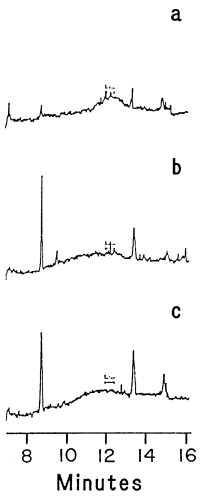

Figure 7 Cleavage of the last disaccharide repeat from the linkage region of chondrosarcoma CS by GBS hyase

A1D1 (1 mg) was digested with chondroitinase ABC (250 m-units) at pH 8.0 (200 μl) at 37 °C for 2 h. The digest was dialysed exhaustively against water and 0.2 M acetic acid, taken to dryness and redissolved in water (10 μl). Aliquots (2 μl) were incubated with chondroitinase ABC (**a**), GBS hyase (**b**) or chondroitinase ACII (**c**), and prepared for analysis by capillary electrophoresis as described in the Experimental section. Detection was at 232 nm. The major peak at 8.8 min and the lesser peak at 13.5 min, seen in (**b**) and (**c**) are of ΔDi0S and ΔDi4S respectively.

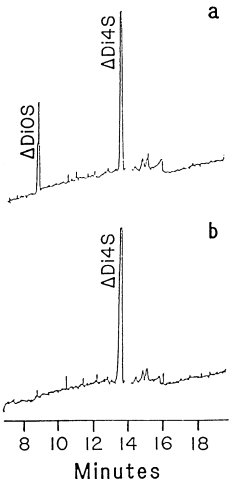

Figure 8 Loss of all Di0Ss of GBS hyase-digested chondrosarcoma CS after oxymercuration

(**a**) chondrosarcoma CS (0.5 mg) was digested with GBS hyase (9 k-units) in hyase buffer (100 μl) at 30 °C for 17 h. The digest was incubated with 20 mM mercuric acetate at pH 5, treated with AG 50 ($\times$8) (H^+) resin to remove Hg^{2+} as described [18], lyophilized and redissolved in water (1.0 ml). An aliquot (4 μl) was digested with chondroitinase (1 m-unit) and prepared for capillary electrophoresis as described in the Experimental section. (**b**) No treatment with mercuric acetate [otherwise, prepared as for (**a**)]. Detection was at 232 nm.

DISCUSSION

Group-B streptococci are the most common cause of potentially fatal infections of newborn babies in the United States. Most strains of the organism produce a hyaluronate lyase capable of degrading hyaluronan and CS in the extracellular matrix of tissues [10], an activity that appears likely to contribute to the invasive properties of the bacteria.

Table 1 'Clustered' unsulphated disaccharides (Di0Ss) in chondrosarcoma CS

Conditions for the digestion of chondrosarcoma CS with chondroitinase ACII and with GBS hyase were determined to be optimal for the release of ΔDi0S and ΔDi4S. The number of residues/mol of CS was calculated based on 40 disaccharide repeats/chondrosarcoma CS chain.

Enzyme treatment of CS	Content (nmol/mg of CS)		Content (residues/mol of CS)	
	ΔDi0S	ΔDi4S	ΔDi0S	ΔDi4S
Chondroitinase ACII	239	1609	5	35
GBS hyase	97	0	2	0

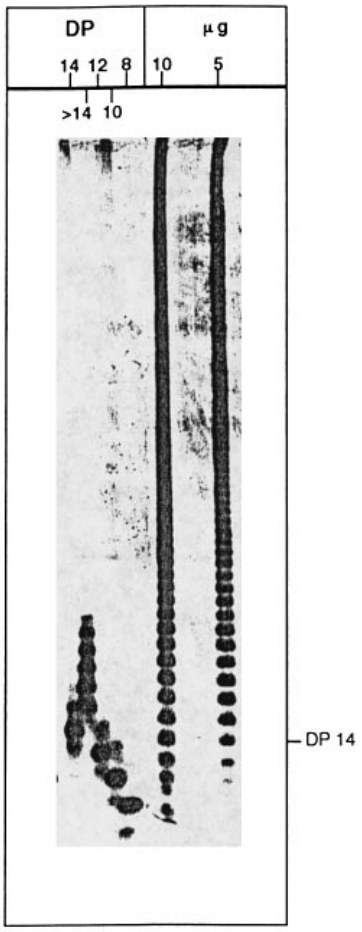

Figure 9 Oligosaccharides in a GBS hyase digest of A1D1: analysis by PAGE

A1D1 (250 μg) was digested with GBS hyase (9.6 k-units) in hyase buffer (50 μl) at 30 °C for 17 h. Aliquots of 1 and 2 μl (5 and 10 μg of CS respectively) were fractionated by PAGE (two wells on the right), and oligosaccharides located by silver staining as described [10]. CS oligosaccharides of known degree of polymerization (DP) (8–14 and $>$ 14) were included as markers (five wells on the left).

A low level of 6-sulphated disaccharide in rat chondrosarcoma CS (one Di6S per 14 chains or approximately one Di6S per 560 disaccharide repeats) has been reported recently [20]. In the present study, chondroitinase AC digests of chondrosarcoma CS separated by capillary electrophoresis at pH 9 or 3 show no peak

for ΔDi6S (Figure 1). From the data it is evident that if any 6-sulphated disaccharide units are present in this chondrosarcoma CS, they represent less than two Di6S units per 1000 disaccharide repeats. If there is a real but small difference in the composition of these two chondrosarcoma CS preparations, it may be a reflection of differences in the chondrosarcoma as maintained by the two laboratories. As the chondrosarcoma CS isolated in this laboratory contains only unsulphated and 4-sulphated disaccharide repeats, it is particularly suitable for testing the substrate specificity of GBS hyase.

The simplest GBS hyase substrates that can be derived from CS are tetrasaccharides. They were isolated from the mixture of oligosaccharides produced by testicular hyaluronidase digestion of CS. It has been reported [21] that the hydrolase activity of testicular hyaluronidase is favoured over its transglycosidase activity by incubation conditions of low pH (pH 5) and high salt (0.5 M NaCl). Similar conditions were employed in the present study to minimize the generation of large CS oligosaccharides. Unsulphated, monosulphated and disulphated tetrasaccharides from chondrosarcoma CS could be readily resolved preparatively by anion-exchange HPLC, but the two isomeric CS tetrasaccharides, Di0S–Di4S and Di4S–Di0S, were not completely separated by these means or analytically by capillary electrophoresis at pH 9.

When a tetrasaccharide is cleaved hydrolytically to yield disaccharides, it is not evident which disaccharide is derived from the reducing end of the tetrasaccharide, unless there is prior specific chemical tagging of that reducing group. Cleavage of a CS tetrasaccharide by GBS hyase, however, gives two disaccharide products which are readily distinguishable: the disaccharide derived from the reducing end of the tetrasaccharide is unsaturated, whereas that from the non-reducing end remains saturated. By exploiting this difference, we have determined that the GBS hyase cleaves Di4S–Di0S, as incubation of the enzyme with this CS tetrasaccharide yields Di4S and ΔDi0S. On incubation of a mixture of Di0S–Di4S and Di4S–Di0S with GBS hyase, no Di0S or ΔDi4S was produced, so it is evident that the former tetrasaccharide is not cleaved. Evidence presented indicates that the GBS hyase, in contrast with chondroitinase ABC, removes the last repeat disaccharide from attachment to the linkage tetrasaccharide. These results may be summarized:

Di0S ↓ GlcA-GalNAc
Di4S ↓ GlcA-GalNAc
ΔDi0S ↓ GlcA-Gal-Gal-Xyl-Ser-(peptide)
ΔDi4S ↓ GlcA-Gal-Gal-Xyl-Ser-(peptide)
(↓ denotes the bond cleaved by GBS hyase)

It appears that the enzyme requires that the disaccharide sequence to the right of the bond cleaved is unsulphated. Replacement of GalNac by Gal in this disaccharide sequence still permits cleavage of the same bond by GBS hyase.

GBS hyase digestion of CS from the rat chondrosarcoma yielded oligosaccharides of a wide range of sizes. This result can be interpreted to indicate that Di0S units are distributed throughout chondrosarcoma CS in a random manner. However, it must be remembered that many chondrosarcoma CS chains are linked, each at different sites, to a common protein core. CS from a given site may exhibit a more regular arrangement of Di0S units. Similar analysis of a PG that has only one CS chain per molecule (e.g. type-IX collagen or decorin) should indicate whether a regular arrangement of Di0S units along a CS chain does indeed occur.

CSs vary greatly in chain length and degree of sulphation, according to the identity of the PG to which they are attached, tissue, stage of development, species and disease or health. Different structures equip CSs to perform quite different specific functions. To aid in the task of interpreting functions of CS in terms of structure, techniques for sequencing CS chains are needed. Chondroitinases have been employed to examine the distribution of 4- and 6-sulphated disaccharide repeats in CS, and it was clear that their arrangement is non-random [9]. In contrast with the activity of chondroitinase ABC, that of chondroitinase ACII was diminished when an oversulphated CS, from squid skin, was employed as substrate [22]. It may prove possible to utilize this difference for mapping oversulphated disaccharide repeats in a CS chain. The limited specific cleavage of CS by GBS hyase, as detailed in the present work, may be exploited to examine the distribution patterns of unsulphated disaccharide repeats in CS. The digestion of chondroitin 6-sulphate by GBS hyase caused slow release of ΔDi6S (J. R. Baker and H. Yu, unpublished work). We are at present determining the enzyme's specificity for chondroitin 6-sulphate in more detail, with a view to its possible use in the analysis of 6-sulphated domains of CS.

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REFERENCES

1 Meyer, K. and Rapport, M. M. (1951) Science **113**, 596–599
2 Comper, W. D. and Laurent, T. C. (1978) Physiol. Rev. **58**, 255–315
3 Karamanos, N. K., Syrokou, A., Vanky, P., Nurminen, M. and Hjerpe, A. (1994) Anal. Biochem. **221**, 189–199
4 Sambandam, T., Baker, J. R., Christner, J. E. and Ekborg, S. L. (1991) Arteriosclerosis **11**, 561–568
5 Bourin, M. C., Lundgren-Akerlund, E. and Lindahl, U. (1990) J. Biol. Chem. **265**, 15424–15431
6 Lucas, M. and Mazzone, T. (1996) J. Biol. Chem. **271**, 13545–13460
7 Saito, H., Yamagata, T. and Suzuki, S. (1968) J. Biol. Chem. **243**, 1536–1542.
8 Jandik, K. A., Gu, K. and Linhardt, R. J. (1994) Glycobiology **4**, 289–296
9 Cheng, F., Yoshida, K., Heinegard, D. and Fransson, L. A. (1992) Glycobiology **2**, 553–561
10 Pritchard, D. G., Lin, B., Willingham, T. R. and Baker, J. R. (1994) Arch. Biochem. Biophys. **315**, 431–437
11 Lin, B., Hollingshead, S. K., Coligan, J. E., Egan, M. L., Baker, J. R. and Pritchard, D. G. (1994) J. Biol. Chem. **269**, 30113–30116
12 Caterson, B. and Baker, J. R. (1979) J. Biol. Chem. **254**, 2394–2399
13 Roden, L., Baker, J. R., Cifonelli, J. A. and Mathews, M. B. (1972) Methods Enzymol. **28**, 73–140
14 Delaney, S. R., Conrad, E. H. and Glaser, J. H. (1980) Anal. Biochem. **108**, 25–34
15 Carney, S. L. and Osborne, D. J. (1991) Anal. Biochem. **195**, 132–140
16 Baker, J. R., Christner, J. E. and Ekborg, S. L. (1991) Biochem. J. **273**, 237–239
17 Hascall, V. C., Riolo, R. L., Hayward, Jr., J. and Reynolds, C. C. (1972) J. Biol. Chem. **247**, 4521–4528
18 Chai, W. G., Kogelberg, H. and Lawson, A. M. (1996) Anal. Biochem. **237**, 88–102
19 Oegema, T. R., Hascall, V. C. and Dziewiatkowski, D. D. (1975) J. Biol. Chem. **250**, 6151–6159
20 Midura, R. J., Calabro, A., Yanagishita, M. and Hascall, V. C. (1995) J. Biol. Chem. **270**, 8009–8015
21 Takagaki, K., Nakamura, T., Izumi, J., Saitoh, H. and Endo, M. (1994) Biochemistry **33**, 6503–6507
22 Tsegenidis, T. (1992) Comp. Biochem. Physiol. [B] **103**, 275–279

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