

Action pattern and substrate specificity of the hyaluronan lyase from group B streptococci

John R. BAKER¹ and David G. PRITCHARD

Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

The hyaluronan lyase of group B streptococci rapidly cleaves hyaluronan by an elimination mechanism to yield the unsaturated disaccharide 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-glucose. Additionally, it has been shown that the enzyme has limited specificity for a chondroitin sulphate and cleaves the chain at unsulphated sites [Baker, Yu, Morrison, Averett and Pritchard (1997) *Biochem. J.* 327, 65–71]. In the present extension of that study it was found that 6-sulphated regions of chondroitin sulphate are also susceptible to cleavage by this hyaluronan lyase. Of the four 6- and/or 4-sulphated tetrasaccharides which can be isolated from testicular hyaluronidase digests of chondroitin sulphate, only those two tetrasaccharides with a 6-sulphated disaccharide at the reducing end

were cleaved. From this and other data, a model is proposed for the cleavage specificity of hyaluronan lyase on a chondroitin sulphate. Evidence is presented in support of an action pattern for hyaluronan lyase which involves an initial random endolytic cleavage followed by rapid exolytic and processive release of unsaturated disaccharide. Since the only oligosaccharides which tend to accumulate in near-complete digests of hyaluronan are unsaturated, it is argued that the processive cleavage occurs from the non-reducing to the reducing end of a hyaluronan chain. This detailed knowledge of substrate specificity contributes to our understanding of the enzyme's role in Group B streptococcal pathogenesis. In addition, the hyaluronan lyase may find application in sequence studies of chondroitin sulphates.

INTRODUCTION

Group B streptococci (GBS), which are frequently present in the microflora of the human vagina, are a common cause of fatal infections of the newborn [1]. The large majority of vaginal GBS isolates secrete a hyaluronan lyase (hyase) [2]. It has been suggested that the GBS hyase facilitates bacterial invasion by degrading extracellular hyaluronan (HA) and may promote persistent colonization of the vagina by GBS (D. Pritchard, B. Lin, X. Li, J. Ware and J. Philips, unpublished work).

Studies of this enzyme during the past 4 years have included a partial definition of its substrate specificity. The activity of GBS hyase is not restricted to the cleavage of HA. It was shown that a chondroitin 4-sulphate, which contains some unsulphated disaccharide repeats, is cleaved to a limited extent [4]. Cleavage occurred between Di0S and Di0S, and between Di4S and Di0S repeats, but Di0S-Di4S and Di4S-Di4S could not be cleaved by this enzyme (for brevity the saccharides Di0S etc. are defined only in the abbreviations footnote).

When acting on HA, GBS hyase appears to make an initial random cleavage and then to rapidly and processively degrade the chains to yield unsaturated disaccharide [5]. This is the first reported example of a glycosaminoglycan lyase acting processively. Enzymes that degrade biopolymers may act in one of two ways, or, like GBS hyase, employ both pathways. A

'distributive' enzyme will bind to a biopolymer and, after a single cleavage reaction, release the two biopolymer chains. (The term 'random endolytic cleavage' also describes this kind of activity). A 'processive' enzyme acts differently [6]. Although the initial cleavage by an enzyme that acts processively may not be at one end of the chain (i.e. it may not be exolytic), subsequent cleavages while the enzyme is bound to the polymer chain are exolytic. After binding to the biopolymer, it catalyses a series of identical cleavage reactions and simultaneously releases a series of small identical products into the medium. Among the known lyases which cleave glycosaminoglycan chains, there are examples of both endolytic and exolytic modes of action [7,8].

In the present study, evidence of the direction of processive cleavage (i.e. non-reducing end to reducing end or vice versa) by GBS hyase is presented. Also, the specificity of GBS hyase towards segments of 6-sulphated chondroitin is investigated and, as a result, a more complete description of chondroitin sulphate (CS) degradation by this enzyme can be presented.

EXPERIMENTAL

Materials

Ovine testicular hyase was obtained from ICN. Hyaluronan (from human umbilical cord), chondroitinase ACII, and chondro-4- and -6-sulphatases were purchased from Sigma.

Abbreviations used: Δ DiHA, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-glucose; DiHA, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-glucose; Δ Di0S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose; Di0S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose; Δ Di4S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose; Di4S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose; Δ Di6S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulpho-D-galactose; Di6S, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulpho-D-galactose; CE, capillary electrophoresis; CS, chondroitin sulphate; ESI-MS, electrospray-ionization MS; GBS hyase, hyaluronan lyase from group B streptococci; HA, hyaluronan; Δ HA₄ and Δ HA₆, unsaturated HA oligosaccharides of degree of polymerization (DP) 4 and 6 respectively; HA₄-HA₁₂, HA oligosaccharides of DP 4–12.

¹ To whom correspondence should be sent, at the following address: 786 BDB (Box 9), University of Alabama at Birmingham, 1530, 3rd Avenue South, Birmingham, AL 35294-0012, U.S.A. (e-mail: jrbaker@bmg.bhs.uab.edu).

Table 1 Relative rates of release of DiHA and Δ DiHA during digestion of HA₁₂ by GBS hyase

The percentage digestion of HA₁₂ by GBS hyase was determined spectroscopically at 232 nm. The methods of analyses of HA₁₂ digests for DiHA and Δ DiHA by CE and ESI-MS are outlined in the Experimental section.

Digestion of HA ₁₂ (%)	Analysis by ...	DiHA/ Δ DiHA molar ratio	
		CE	ESI-MS
10		0.96	0.73
20		0.67	0.54
30		0.62	0.49
40		0.46	—
50		0.45	0.39
100		0.20	0.17

Sephadex G-25 (superfine grade) was obtained from Pharmacia. Standard unsaturated chondro- and hyalurono-disaccharides were from Seikagaku Kogyo, Tokyo, Japan. HA oligosaccharides, HA₄–HA₁₂, prepared by digestion of HA using testicular hyase and fractionated according to size by gel chromatography on Sephadex G-50, were a gift from Dr. Jim Christner, Environmental Test Systems Inc., Elkhart, IN, U.S.A.

CS

The disaccharide compositions of four different commercially available CSs were determined. CS A (Calbiochem), with the composition 6.3% Di0S, 35.4% Di6S and 58.3% Di4S, was selected as a preparative source of CS tetrasaccharides, as its Di6S/Di4S ratio was closest to 1.0. A CS (5.3% Di0S/13.7% Di6S/81.0% Di4S) isolated from bovine nasal cartilage following exhaustive proteolytic digestion [9] and a CS (3.6% Di0S/86.6% Di6S/9.7% Di4S) similarly isolated from gill cartilage of the basking shark were employed for digestion by the GBS hyase.

GBS hyase

The preparation and purification of the recombinant GBS hyase have been described previously [4]. The working solution of pure enzyme (12 mg/ml in 50 mM Hepes/10 mM EDTA, pH 8.0) was stored at 4 °C.

Digestion of CS using testicular hyase and recovery of di- and tetrasaccharides

CS (253 mg) was dissolved in 0.15 M NaCl/0.05 M sodium phosphate, pH 6.0 (10 ml), ovine testicular hyase (25 mg) was added and the mixture incubated at 37 °C for 27 h. A further 10 mg of enzyme was added at 22 h. At the end of the incubation, the mixture was deproteinized by addition of trichloroacetic acid at 0 °C to a final concentration of 5%. Protein was removed by centrifugation and the supernatant brought to pH 6.2 by the gradual addition, with vigorous stirring, of 10 M NaOH. The neutralized supernatant (approx. 12 ml) was applied to a column (192 cm × 2.5 cm) of Sephadex G-25, developed in 0.5 M NaCl/aq. 10% (v/v) methanol at a flow rate of 20 ml/h. The column effluent was monitored at 206 nm and fractions (30 min) were collected. Six peaks were detected in advance of the large salt peak (*V*_r). Peaks 5 and 6 were identified, using electrospray-ionization (ESI)-MS analysis, as tetra- and disaccharides from CS respectively. Fractions from both peaks were pooled separately and applied to a column (2.0 cm × 20 cm) of Sephadex G-

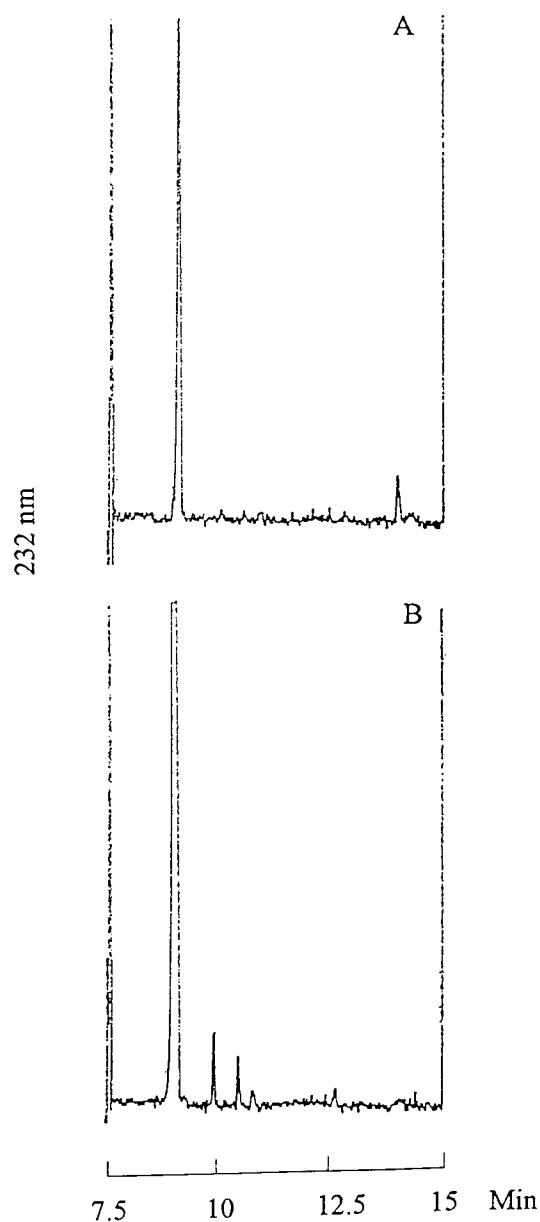


Figure 1 CE analyses of hyaluronan after partial enzymic digestion

HA was digested with (A) GBS hyase and (B) chondroitinase ACII to 10% of completion. The major peak at 9.08 min, present in (A) and (B), is of Δ DiHA. The peak at 10.24 min, seen in (B) only, is of Δ HA₄.

10 developed in aq. 20% (v/v) methanol at a flow rate of 0.5 ml/min and monitored at 200 nm. Both tetra- and disaccharide pools, separated from salt, were concentrated using a Savant SpeedVac to 1–10 mg of saccharide/ml. Yields were 43 mg of tetrasaccharide and 2.4 mg of disaccharide.

Determination of disaccharides released by enzymic digestion of HA and CS

CS or CS oligosaccharide samples (< 0.25 μ g) were dried, dissolved in 0.1 M Tris/HCl, 0.03 M sodium acetate and 0.01 M EDTA, pH 7.4 (10 μ l) containing chondroitinase ACII (2.5 m-units) and incubated at 37 °C for 1 h. To determine susceptibility of CS and CS oligosaccharides to digestion by GBS

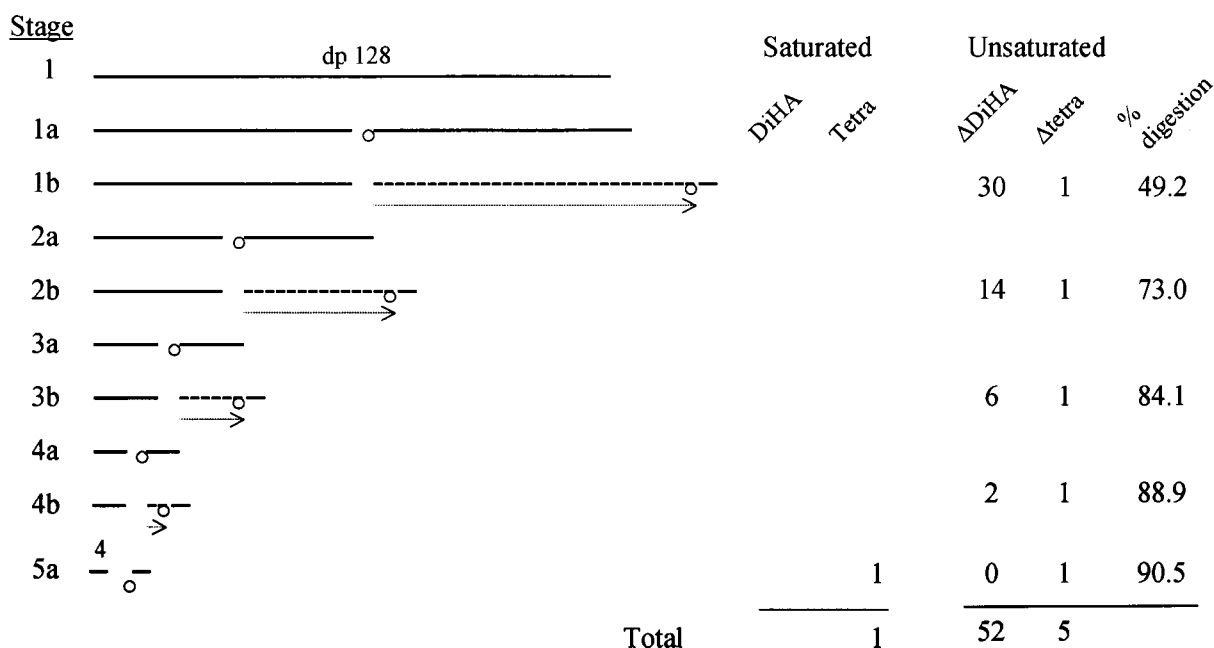


Figure 2 Proposed action pattern of GBS hyase

For convenience, the cleavage of an HA oligosaccharide of DP 128 is illustrated, and endolytic cleavages are shown as splitting a chain into equal parts, although it is understood that they occur randomly. In this model, it is assumed that processive cleavage by GBS hyase is from the non-reducing to the reducing end of an HA oligosaccharide. According to this model, an enzyme molecule binds to a random position along the chain and catalyses a single endolytic cleavage (stage 1a). The enzyme remains bound to the new non-reducing end at this cleavage site and, during a rapid series of processive exolytic cleavages, remains substrate-bound (stage 1b). During this process, Δ DiHA alone is released. When the substrate is decreased to the size of a tetra- or hexasaccharide, the enzyme disengages from the substrate. [It is suggested that the enzyme releases substrate at this stage, as only tetra- and hexa-saccharide are seen to accumulate in a partial digest (Figure 1); for simplicity, only release of tetrasaccharide is illustrated.] As illustrated, 30 molecules of Δ DiHA, each represented by a single dash in a broken line, and a single unsaturated tetrasaccharide, represented by a double-length dash, are released at stage 1b. This pattern of action repeats through stages 2–4; at each stage there is another random cleavage of the remaining HA chain and rapid processive cleavage from the new non-reducing end. At stage 5, following a random cleavage, the enzyme disengages from the substrate, as the latter is too short to bind the enzyme or to permit any further processive cleavage. The tetra- and hexa-saccharides from HA appear in partial digests because they are not cleaved processively and therefore are cleaved more slowly, but they are finally cleaved. Thus, in a digest of HA by GBS hyase which has been allowed to proceed to completion, the only product which is observed is Δ DiHA. If, as depicted in this Figure, processive cleavage proceeds from a new non-reducing end towards its reducing end, small unsaturated oligosaccharides will tend to accumulate. [In this simplified example, five unsaturated tetrasaccharides per one saturated tetrasaccharide; from a much longer HA, the relative accumulation of the unsaturated small oligosaccharide(s) will be more marked.] If processive cleavage occurred in the reverse direction, it would be small saturated oligosaccharide(s) that accumulate in partial digests. Beginning with a longer HA chain than depicted here, many more stages or random cleavages, some occurring simultaneously, would be required, but the essential features of this model would apply.

hyase, samples ($< 0.25 \mu\text{g}$) were incubated in 0.05 M ammonium acetate, 0.01 M calcium chloride, pH 6.5 ($10 \mu\text{l}$) containing GBS hyase (9 units) at 37°C for 1 h. Both chondroitinase and hyase digests were subsequently diluted with HPLC-grade water ($40 \mu\text{l}$) and centrifugally filtered through Ultrafree-MC filter units (Millipore). Filtrates were analysed for disaccharides using capillary electrophoresis (CE) at pH 9.8 as described in [4,10]. Samples were loaded hydrostatically (2 s) at 500 mmHg (1 mmHg \equiv 133.3 Pa) and separated at pH 9.8. Detection of peaks was at 200 nm (saturated and unsaturated disaccharides) and at 232 nm (unsaturated disaccharides).

The same CE separation system gave a near-baseline separation of DiHA (8.67 min) from Δ DiHA (8.78 min) in GBS hyase digests of HA oligosaccharides.

MS

Samples were analysed using the ESI mode on a PE Sciex API III triple quadrupole mass spectrometer. Aqueous samples were injected into a $25 \mu\text{l}/\text{min}$ flow of aq. 1:1 (v/v) acetonitrile containing 0.1% formic acid. A Harvard Apparatus model 22 syringe pump was used to deliver the flow to the electrospray interface.

Desulphation of CS disaccharides

The saturated disaccharides Di4S and Di6S at 13.48 min were not separated by CE at pH 9.8, although they were well resolved from their unsaturated counterparts, Δ Di6S and Δ Di4S, at 14.00 min and 14.23 min respectively. GBS hyase digests of CS oligosaccharides, which contained Di4S and/or Di6S, were dried using the Savant SpeedVac and redissolved in 0.1 M Tris/HCl, 0.03 M sodium acetate and 0.01 M EDTA, pH 7.4 ($10 \mu\text{l}$), and incubated with chondro 4- or 6-sulphatase (0.2 m-units) at 37°C for 1 h. The digests were processed and separated by CE as described above. Under the conditions employed, the 4-sulphatase showed absolute specificity for digestion of Di4S (and Δ Di4S) and the 6-sulphatase was specific for the desulphation of Di6S (and Δ Di6S). From both Di4S and Di6S, the product of digestion, as identified by CE, was Di0S.

RESULTS AND DISCUSSION

GBS hyase can act on HA in a random endolytic manner and this can be demonstrated directly by an experiment employing HA₁₂. All disaccharide repeats, with the exception of that from the non-

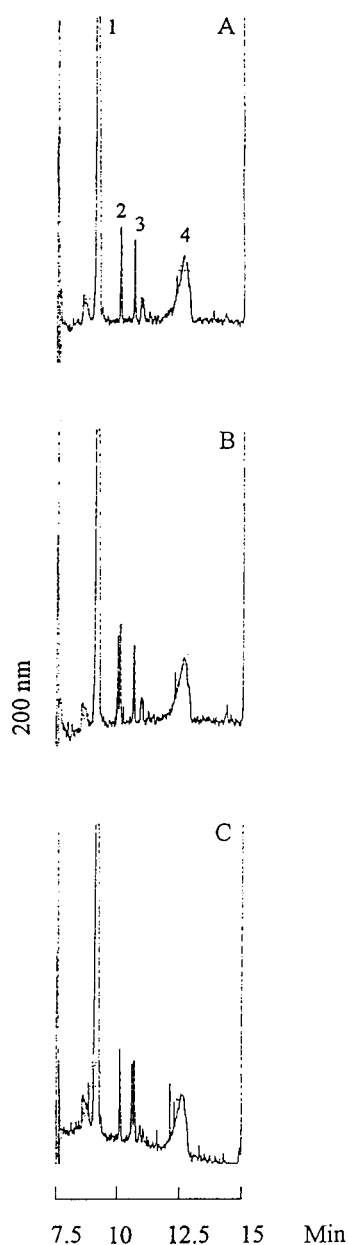


Figure 3 Analyses by CE of hyaluronan partially digested by GBS hyase

(A) A GBS hyase digest of HA, taken to 82.5% of completion, was analysed by CE at 200 nm. The off-scale peak at 9 min, and labelled 1, is of Δ DiHA. The peaks labelled 2 and 3 have mobilities similar to those of HA_4 and HA_6 respectively. The broad peak labelled 4 is of partially digested HA. (B) As (A), plus authentic HA_4 . The added HA_4 has a slightly shorter retention time than 2. (C) As (A), plus authentic HA_6 . The added HA_6 has a slightly shorter retention time than 3. At 232 nm, peaks 1, 2 and 3 were detected. Peak 4, and authentic HA_4 and HA_6 , added to (B) and (C), were not seen at this wavelength (not shown). Therefore the partial GBS hyase digest of HA, as observed in (A), does not contain HA_4 and HA_6 , but does contain their unsaturated counterparts, ΔHA_4 and ΔHA_6 .

reducing end, are liberated as Δ DiHA during the digestion of HA_{12} by GBS hyase. The saturated disaccharide DiHA is a marker of the non-reducing end. The rate of release of the disaccharide DiHA from the non-reducing end relative to the rate of release of other disaccharide repeats (Δ DiHA) provides information about the enzyme's action pattern. Thus, if the action pattern is exclusively random endolytic, HA_{12} with five

cleavage sites will give, at < 20% digestion, a DiHA/ Δ DiHA ratio of 1.0. This ratio would decline to 0.2 at complete digestion. By contrast, exclusively exolytic and processive action would give a constant ratio of 0.2 during digestion (assuming that the cleavage of an HA chain is extremely rapid compared with the time required for enzyme molecules to bind to another chain). The release of DiHA and Δ DiHA at different times were determined by CE analysis of digests. Analyses for species of 378 m/z (Δ DiHA) and 396 m/z (DiHA) were performed on the same digests by ESI-MS. The determined ratios of DiHA/ Δ DiHA from both analyses (Table 1) are compatible with a random endolytic mode of enzyme action.

Although, as demonstrated by the previous experiment, cleavage by GBS hyase of HA_{12} is primarily random endolytic, cleavage of a long HA chain appears to occur predominantly by other means. That the principal mode of action of GBS hyase on HA, following initial random endolytic cleavage, is exolytic is strikingly demonstrated by CE analysis of a partial digest, i.e. the only peak seen is of Δ DiHA (Figure 1A). Also shown (Figure 1B) is a partial digest of HA by chondroitinase ACII, which has been reported to act in an exolytic manner [7]. Again, the predominant product is Δ DiHA.

It is not known whether the exolytic and processive cleavage of HA by GBS hyase occurs from the non-reducing to the reducing end or vice versa. Unfortunately, to distinguish between these two possibilities by monitoring the rates of DiHA and Δ DiHA release is not experimentally feasible, as the ratio of DiHA to Δ DiHA from HA is too low (< 0.0004 in a complete digest). Instead, we have used CE analysis to determine whether small saturated or unsaturated oligosaccharides tend to accumulate during the digestion of HA by GBS hyase. As proposed in Figure 2, accumulation of small unsaturated oligosaccharides from HA is indicative of a non-reducing-to-reducing-end action pattern. In a preliminary experiment it was found that maximal accumulation of intermediate small oligosaccharides (2.0–2.5% relative to Δ DiHA) occurred at 60–90% of complete digestion (results not shown). CE analysis of an 82% digest (Figure 3) revealed low levels of two small HA oligosaccharides (labelled 2 and 3 in Figure 3A) in addition to the predominant Δ DiHA product (labelled 1). Both 2 and 3 could be detected at 232 nm, as well as at 200 nm, and their mobilities are similar to, but not identical with, those of HA_4 and HA_6 respectively (Figures 3B and 3C). It is evident that 2 and 3 are ΔHA_4 and ΔHA_6 respectively. The accumulation of these two small unsaturated, but no small saturated, HA oligosaccharides during digestion of HA by GBS hyase strongly suggests that the action of the enzyme is processive from the non-reducing to the reducing end of the chain.

Preliminary data, reported previously [4], suggests that 6-sulphated regions of a CS can be slowly cleaved by GBS hyase with the release of Δ Di6S. In order to define this activity properly, we determined whether the tetrasaccharides Di6S-Di4S, Di4S-Di6S, Di6S-Di6S and Di4S-Di4S are substrates for GBS hyase. Accordingly, a hybrid chondroitin 4-/6-sulphate was digested using testicular hyase, the digest fractionated by gel chromatography on a column of Sephadex G-25 and a peak containing the tetrasaccharides was identified and recovered (pool 5), as described in the Experimental section. Analysis of pool 5 by CE (Figure 4A) revealed a peak of rapid mobility, peak 1, followed by a cluster of three peaks, numbered 2, 3 and 5. A small shoulder on peak 3 was indicative of an additional component, numbered 4. This cluster of peaks was within the range of mobilities expected for disulphated tetrasaccharides from CS. Therefore the presence of all four possible disulphated tetrasaccharides listed above could be predicted. Pool 5 was

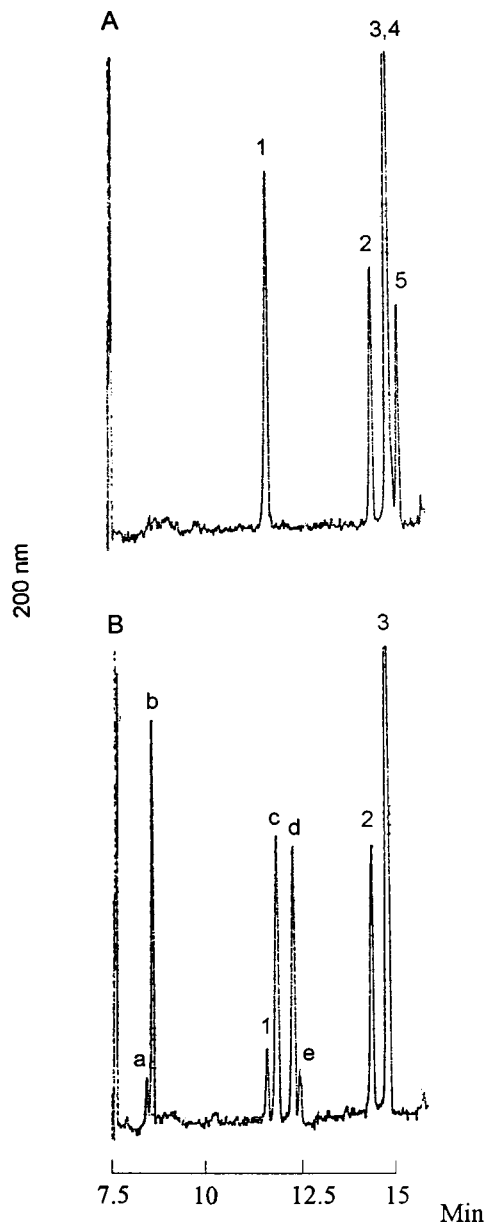


Figure 4 Analysis by CE of CS tetrasaccharides

(A) Pool 5, obtained by fractionation of a testicular hyase digest of CS by gel chromatography on Sephadex G-25 (see the Experimental section), was analysed by CE at pH 9.8. Observed is a peak at 12.17 min, numbered 1, and a cluster of three peaks at 15.3–16.3 min, numbered 2, 3 and 5. A shoulder, suggestive of a fourth peak and numbered 4, is seen on the down slope of peak 3. (B) Pool 5, after digestion using GBS hyase, was also analysed by CE at pH 9.8. Peaks 4 and 5, but not 2 and 3, disappeared as a result of the digestion. Peak 1 was largely digested. The disaccharides produced by the digestion were identified by their mobilities relative to standards as: a, DiOS; b, Δ DiOS; c, Di6S and/or Di4S; d, Δ Di6S; and e, Δ Di4S.

digested using GBS hyase prior to separation by CE (Figure 4B). Of the clustered peaks numbered 2–5, only 4 and 5 were susceptible to digestion by the enzyme.

To separate the tetrasaccharides and allow for their identification, pool 5 was fractionated by anion-exchange HPLC on a SAX-Partisil column using a gradient of KH_2PO_4 in aq. 10% (v/v) methanol (Figure 5). Four peaks were separated, and aliquots from each were analysed by CE. Then the peaks of Figure 5 were numbered 1–5 to match, by electrophoretic

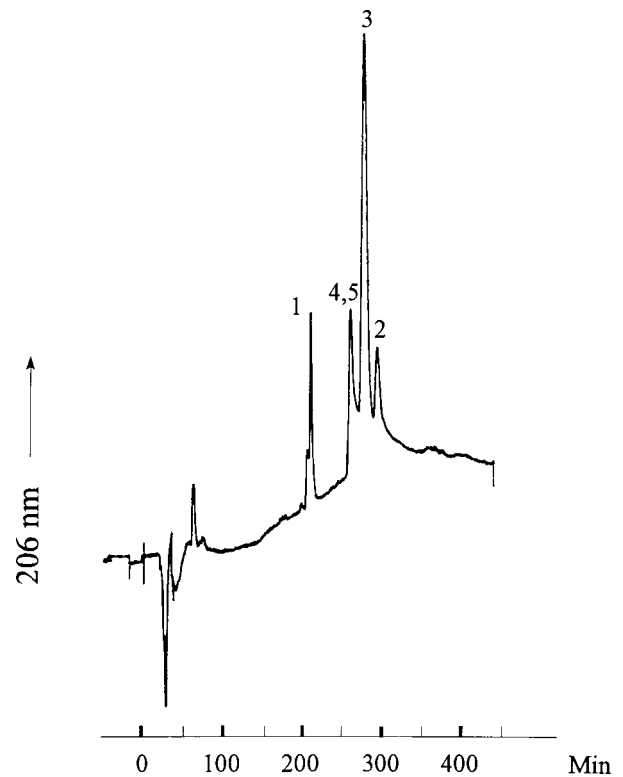


Figure 5 Fractionation of CS tetrasaccharides by anion-exchange HPLC

CS tetrasaccharides (pool 5) were applied to a Partisil-SAX column (250 mm \times 4.6 mm, 10 μm particle size) and fractionated in a gradient of KH_2PO_4 (30–300 mM) in aq. 10% (v/v) methanol. The flow rate was 0.2 ml/min, and the elution of saccharides was detected at 206 nm. Fractions constituting the four peaks eluted between 200 and 300 ml were separately pooled, desalted on a column of Sephadex G-10 developed with aq. 20% (v/v) methanol [4] and dried using a Sorvall SpeedVac apparatus. CE of aliquots from each peak permitted their identification (Table 2). Each peak number represents the same tetrasaccharide as in Figure 4.

Table 2 Analyses by CE of CS tetrasaccharides separated by anion-exchange HPLC

The peak numbers are identified in Figures 4 and 5. Peak 1 was seen to contain monosulphated tetrasaccharides, but was not further analysed. Abbreviation: tr, a trace component.

Peak	Disaccharides identified in a chondroitinase ACII digest			Identity of tetrasaccharide
	At 232 nm	At 200 nm		
2	Δ Di4S	Δ Di4S	Di6S	Di6S-Di4S
3	Δ Di4S	Δ Di4S	Di4S	Di4S-Di4S
4, 5	Δ Di6S (tr)	Δ Di6S (tr)	Di6S (tr)	Di6S-Di6S
	Δ Di6S	Δ Di6S	Di4S	Di4S-Di6S

mobility, the similarly numbered peaks of Figure 4. The first peak was found to contain monosulphated tetrasaccharide (Table 2) and was not further analysed. Aliquots from the other peaks were digested with chondroitinase ACII and the disaccharide products analysed by CE. Identification of the characteristic disaccharides from each tetrasaccharide permitted identification of each tetrasaccharide (Table 2). It was found that peak 2 is Di6S-Di4S, the major peak (peak 3) is Di4S-Di4S, a minor

Table 3 Unsaturated disaccharides released from bovine nasal-cartilage (BNC) and basking-shark (BS) CSs by GBS hyase

A CS (20 μg in 100 μl) was digested exhaustively with GBS hyase (12 μg) for 24 h at 30 °C or with chondroitinase AC II (12 m-units) for 2 h at 30 °C. Digests, diluted five-fold with HPLC-grade water, were analysed by CE at pH 9.8. Peaks were detected at 232 nm and integrated. Data given are from the average of triplicate analyses.

Enzyme digestion	CS (0.2 mg/ml)	$\Delta\text{Di}0\text{S}$		$\Delta\text{Di}6\text{S}$		$\Delta\text{Di}4\text{S}$	
		($\mu\text{g}/\text{ml}$)	%*	($\mu\text{g}/\text{ml}$)	%	($\mu\text{g}/\text{ml}$)	%
GBS hyase	BNC	0.74	100.7	0.77	40.7	0	0
	BS	0.58	99.1	11.44	83.3	0	0
Chondroitinase AC II	BNC	0.73		1.89		11.21	
	BS	0.58		3.73		1.55	

* The disaccharide released by GBS hyase is expressed as a percentage of the total present (i.e. that released by digestion with chondroitinase AC II).

component (peak/shoulder 4) is of Di6S-Di6S and peak 5 contains Di4S-Di6S. Therefore, as peaks 4 and 5, but not peaks 2 and 3, were absent following incubation with GBS hyase (Figure 4), it is evident that Di6S-Di6S and Di4S-Di6S are cleaved by GBS hyase, whereas Di6S-Di4S and Di4S-Di4S are not. To verify these findings, aliquots from the separately isolated peaks 2, 3 and 4/5 were incubated with GBS hyase and the digests analysed by CE. Only the constituents of peaks 4 and 5 were digested by the enzyme. Peaks 2 and 3 remained intact (results not shown). The further significance of these results is discussed below.

Two CSs from different sources and content of 6-sulphated repeats (bovine nasal cartilage and basking-shark gill cartilage, with 13.7% and 86.6% Di6S respectively) were digested with the GBS hyase in order to determine the proportion of their total Di6S and Di0S repeats which could be released as $\Delta\text{Di}6\text{S}$ and $\Delta\text{Di}0\text{S}$ respectively. The digestions were allowed to go to completion (i.e. the incubation time at which no further increase in A_{232} occurred). The unsaturated disaccharides released were identified and quantified by CE analyses and were compared with total disaccharide compositions of these two CSs (Table 3). A much higher proportion (83.3%) of the Di6S content of the shark Di6S can be released by GBS hyase (cf. 40.7% from the bovine nasal CS). Di0S was completely released, as $\Delta\text{Di}0\text{S}$, by GBS hyase from both CS preparations.

Treatment of both GBS hyase digests by oxymercuration [4,11] resulted in the loss of all remaining 6-sulphated residues (results not shown). It is evident that, after hyase digestion of the CSs, any 6-sulphated residues not released as $\Delta\text{Di}6\text{S}$ occupy non-reducing terminal positions, making them susceptible to destruction by oxymercuration. The significance of these results is discussed in greater detail below with reference to a proposed model of GBS hyase specificity towards CS.

GENERAL DISCUSSION

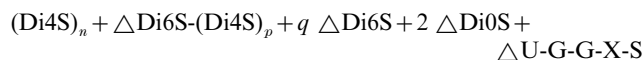
Previous results indicated that the initial attack on HA by GBS hyase may be random endolytic followed by processive cleavage [5]. Thus a digest of HA taken to only 5% of completion by slow enzymic action contained not only $\Delta\text{Di}i\text{HA}$, but also a broad range of very large HA oligosaccharides, as determined by gel chromatography. In the present study, an HA oligosaccharide, HA_{12} , was shown to be cleaved endolytically by GBS hyase. It is reasonable to expect that GBS hyase cannot act processively on a small HA oligosaccharide. Indeed, it was found that, in digests, at 60–90% of completion, ΔHA_4 and ΔHA_6 tend to accumulate.

It would appear that cleavage by GBS hyase is initially and finally non-processive. Nevertheless, GBS hyase can rapidly cleave HA, its principal substrate, to yield unsaturated disaccharide, $\Delta\text{Di}i\text{HA}$, as the sole product of complete digestion. Even in partial digests, the predominant product is $\Delta\text{Di}i\text{HA}$, which indicates in agreement with earlier findings [5] that the enzyme's mode of action is primarily processive. Other glycosaminoglycan lyases, for example heparinase I [12], also act processively. Random endolytic cleavage followed by a series of processive cleavages provides a rapid and efficient means for the complete digestion of HA. Evidence has been presented for processive cleavage proceeding from the non-reducing to the reducing end of HA or HA oligosaccharides. This finding will aid computer-assisted studies, which are presently underway, to properly dock HA in the active-site cleft of the modelled GBS hyase molecule [13].

Our results indicate that the GBS hyase will cleave a 4-/6-sulphated chondroitin chain as illustrated below:



↓ GBS hyase



(where the sequence U-G-G-X-S represents the linkage region of CS)

An essential feature of this model is that no Di6S repeat remains in an internal position along a CS chain following exhaustive digestion with GBS hyase. Cleavage will occur at a Di6S bounded by Di4S repeats, but the resulting residue of $\Delta\text{Di}6\text{S}$ will remain glycosidically linked in a non-reducing terminal position. In support of this aspect of the model, it has been found that, after exhaustive digestion of a CS with GBS hyase, although some 6-sulphated residues are not released as $\Delta\text{Di}6\text{S}$, they invariably occupy non-reducing terminal positions (i.e. they are removed by oxymercuration). Where a cluster of Di6S repeats, $(\text{Di}6\text{S})_q$, occurs within the chain of CS, cleavage takes place with the release of $q \Delta\text{Di}6\text{S}$. The activity of GBS hyase towards 6-sulphated disaccharide repeats within a CS chain is reminiscent of cleavage by this enzyme at unsulphated sites [4].

In support of the model, although only 41% of the Di6S content of bovine nasal-cartilage CS was released as $\Delta\text{Di}6\text{S}$ by GBS hyase, the remainder could be completely destroyed by oxymercuration (a procedure which removes non-reducing terminal unsaturated uronic acid residues). Of the total Di6S content of a CS, that part released by GBS hyase digestion is derived from 'clusters' of two or more Di6S repeats. Thus much of the Di6S of this CS is distributed in short segments throughout the chain. This result is consistent with the findings of Cheng and others [14], who reported a heterogeneous distribution of 6-sulphated repeats in the CS from bovine nasal cartilage, although in CSs from other sources (e.g. tracheal cartilage) 6-sulphated residues were clustered within a certain segment of the chain. By contrast, 81% of the Di6S of basking-shark CS is released by GBS hyase. The Di6S of this CS is organized into longer segments, uninterrupted by Di4S repeats. Interestingly, the GBS hyase-catalysed release of all Di0S as $\Delta\text{Di}0\text{S}$ from the CSs suggests that the Di0S repeats are clustered and/or linked to Di6S repeats.

The glycosaminoglycan chains, whether they be chondroitin, dermatan or heparan sulphates, which are attached to certain proteoglycans, are now known to perform essential and quite specific functions. For example, the heparan sulphate chains of the basement-membrane proteoglycan perlecan act as receptors

for the basic fibroblast growth factor [15]. Glycosaminoglycans have a characteristic disaccharide repeat pattern, but the extent and positions of substitutions with ester sulphate groups may vary considerably. A particular sulphation pattern may relate to a CS's specific biological activity [16], so methods for the determination of sequence of sulphated disaccharide repeats are assuming more importance. Recently, the utility of MS methods for sequencing glycosaminoglycans has gained attention [17,18]. Nevertheless, limited specific enzymic cleavage of the glycosaminoglycan, to provide oligosaccharides amenable to MS study, is a prerequisite. Detailed knowledge of the substrate specificities of glycosaminoglycan-degrading enzymes, such as the GBS hyase, is essential for their use in this important application, as well as in other studies of this ubiquitous family of biopolymers.

Support for this work from the National Institutes of Health (grant AI-41596) is gratefully acknowledged. The MS Shared Facility of the Comprehensive Cancer Center at the University of Alabama at Birmingham, supported in part by National Institutes of Health grant P30 CA-13148, provided ESI-MS services. We thank Ms Anne Burrows for expert secretarial assistance.

REFERENCES

- 1 Zangwill, K., Schuchat, A. and Wenger, J. (1992) *Morbid. Mortal. Wkly. Rep.* **41**, 25–32
- 2 Hauge, M., Jespersgaard, K., Poulsen, K. and Kilian, M. (1996) *Infect. Immun.* **64**, 919–925
- 3 Reference deleted
- 4 Baker, J., Yu, H., Morrison, K., Averett, W. and Pritchard, D. (1997) *Biochem. J.* **327**, 65–71
- 5 Pritchard, D., Lin, B., Willingham, T. and Baker, J. (1994) *Arch. Biochem. Biophys.* **315**, 431–437
- 6 delCardayre, S. and Raines, R. (1994) *Biochemistry* **33**, 6031–6037
- 7 Jandik, K., Gu, K. and Linhardt, R. (1994) *Glycobiology* **4**, 289–296
- 8 Park, Y., Cho, S. and Linhardt, R. (1997) *Biochim. Biophys. Acta* **1337**, 217–226
- 9 Rodén, L., Baker, J., Cifonelli, J. and Mathews, M. (1972) *Methods Enzymol.* **28**, 73–140
- 10 Carney, S. and Osborne, D. (1991) *Anal. Biochem.* **195**, 132–140
- 11 Chai, W., Kogelberg, H. and Lawson, A. (1996) *Anal. Biochem.* **237**, 88–102
- 12 Ernst, S., Rhomberg, A., Biemann, K. and Sasisekharan, R. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4182–4187
- 13 Pritchard, D., Trent, J., Li, X., Zhang, P., Egan, M. and Baker, J. (2000) *Proteins*, in the press
- 14 Cheng, F., Yoshida, K., Heinegård, D. and Fransson, L.-Å. (1992) *Glycobiology* **2**, 553–561
- 15 Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G. and Yayon, A. (1994) *Cell* **79**, 1005–1013
- 16 Lander, A. (1999) in *Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins* (Kreis, T. and Vale, R., eds.), pp. 351–356, Oxford University Press, New York
- 17 Rhomberg, A., Ernst, S., Sasisekharan, R. and Biemann, K. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4176–4181
- 18 Venkataraman, G., Shriver, Z., Raman, R. and Sasisekharan, R. (1999) *Science* **286**, 537–542

Received 10 January 2000; accepted 3 April 2000