The hyaluronan lyase of *Streptococcus pyogenes* bacteriophage H4489A

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Many pathogenic streptococci produce extracellular hyaluronan lyases which are thought to aid the spread of the organism in host tissues. In addition, several phages of group A streptococci are known to synthesize a bound form of hyaluronidase. It has been suggested that the function of this hyaluronidase is to facilitate penetration of the hyaluronan capsule by phage and thus to gain access for the phage to the cell surface of the host streptococcus [Hynes, Hancock and Ferretti (1995) Infect. Immun. **63**, 3015–3020]. In the present work, the hyaluronidase of *Streptococcus pyogenes* bacteriophage H4489A, expressed in *E. coli*, has been purified and characterized. The enzyme was shown to be a lyase with a distributive action pathway. Unlike most bacterial hyaluronidases that have been characterized, the phage enzyme was found to specifically cleave hyaluronan, which adds credence to the view that its function is to digest the hyaluronan capsule of the host organism. This bacteriophage lyase may provide a practical alternative to the lyase from *Streptomyces hyalurolyticus* as a reagent for the specific cleavage of hyaluronan.

Key words: action pathway, hyaluronidase, substrate specificity.

INTRODUCTION

Hyaluronan (HA) lyases are produced by many different genera of bacteria. In Gram-positive organisms, the HA lyase is often secreted and is thought to have a role in pathogenesis. It has been noted [1] that all Gram-positive bacteria which produce HA lyases appear to be capable of causing infections in animals. The degradation of HA and other glycosaminoglycans by these enzymes may facilitate the invasion of the tissues of an animal host by bacteria [2]. Possession of HA-degrading activities may also facilitate bacterial adhesion and colonization [3]. Several bacteriophages from HA-encapsulated group A streptococci are known to produce hyaluronidases [4,5] and the nucleotide sequences of the hyaluronidase genes *hylP* and *hylP2* from two bacteriophages of *Streptococcus pyogenes*, H4489A [6] and 10403 [4], respectively, have been reported. The sequences of *hylP* and *hylP2* are similar, but showed no sequence similarity with the HA lyase genes from *Streptococcus agalactiae* [7] or *Streptococcus pneumoniae* [8]. It has been suggested that the biological function of bacteriophage hyaluronidase is to degrade the HA capsule of the bacterial host cell, thereby facilitating attachment of phage to the cell wall [4]. Bacterial HA lyases cleave *N*-acetylglucosaminidic bonds of HA by an elimination mechanism. The products are unsaturated oligosaccharides, often disaccharides, with a Δ-4,5-uronate residue at nonreducing termini [9]. Some HA lyases also have a limited ability to cleave chondroitin sulphates in a similar manner [10]. In the present work, we examine the substrate specificity of the *hylP*-derived hyaluronidase to gain further insight into its biological role. Additionally, this knowledge allows us to add the enzyme to the growing list of lyases of defined specificities. These lyases have application in structural studies of connective tissue glycosaminoglycans.

EXPERIMENTAL

Materials and reagents

Plasmid pSF49, containing the *hylP* gene of *S. pyogenes* bacteriophage H4489A, was kindly supplied by Dr Wayne L. Hynes (Department of Biological Sciences, Old Dominion University, Norfolk, VA, U.S.A.). Its construction was as described previously [6]. HA from rooster comb was a gift from Dr Jim Christner (Environmental Test Systems Inc., Elkhart, IN, U.S.A.). Oligosaccharides (ΔHA_4, ΔHA_6, ΔHA_8 and ΔHA_{10}) were isolated from HA which had been partially digested using the HA lyase from *Streptomyces hyalurolyticus* and fractionated by chromatography on a Biogel P-4 column [11]. Chondroitin sulphate was isolated from bovine nasal cartilage, chondroitin 4-sulphate from the Swarm rat chondrosarcoma aggrecan and chondroitin 6-sulphate from gill cartilage of the basking shark following exhaustive proteolytic digestion [12]. Chondroitin was prepared by the solvolytic desulphation of chondroitin sulphate. The pyridinium salt of bovine nasal cartilage chondroitin sulphate was prepared [13] and then desulphation was carried out in dimethylsulphoxide/methanol (9:1, v/v) at 80 °C for 1 h [14].

The preparation and purification of the recombinant group B streptococcus (GBS) HA lyase have been described previously [15,16]. The working solution of pure enzyme (8.28 mg/ml in 50 mM aqueous ammonium acetate) was stored at 4 °C. An HA lyase from *S. hyalurolyticus* was purchased from Sigma, dissolved in 50 mM ammonium acetate, pH 6.5/0.05% BSA to give a concentration of 500 units/ml and stored at 4 °C. Chondroitin AC II lyase from *Arthrobacter aurescens* was purchased from Sigma, dissolved in 0.1 M Tris, 0.03 M sodium acetate, and 0.01 M EDTA, pH 7.4 to give a concentration of 10 units/ml and stored at 4 °C.

Abbreviations used: HA, hyaluronan; DS, dermatan sulphate; GBS, group B streptococcus; LB, Luria–Bertani broth; ΔDiHA, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔDi0S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic 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; ΔHA_4–ΔHA_{10}, unsaturated oligosaccharides with degrees of polymerization = 4–10, respectively, from the digestion of HA with streptomyces HA lyase.

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Expression and purification of phage HA lyase

The complete *hylP* gene was amplified from a plasmid miniprep using a forward primer containing an *Nhe*I site and a reverse primer containing an *Xho*I site. The forward primer (5′-GCG CTA GCA TGA CTG AAA ATA TAC CAT TAA GAG TCC-3′) and the reverse primer (5′-CGC TCG AGC TAT TTT TTT AGT ATG AGT TTT TTT AAC-3′) generated a product 1.08 kb in length. PCR conditions were as follows: The reaction mixture was composed of 4 μl plasmid template (25 μg/ml), 1.5 μl each of the two primers (10 μM), and 93 μl of Gibco/BRL High Fidelity PCR mix. The mixture was heated to 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 45 s and 72 °C for 3 min. The product was gel-purified, cloned into a pET21a vector and cut with *Nhe*I and *Xho*I using standard procedures. The product (pSD102) was transformed into competent INVαF′ *E. coli* and transformants were selected on Luria–Bertani (LB) medium plates containing 50 μg/ml ampicillin. A plasmid miniprep was sequenced to confirm that the desired product had been obtained. The plasmid was then transformed into BL21(DE3)-competent *E. coli* cells.

BL21(DE3)/pSD102 was grown overnight at 37 °C in LB medium (200 ml) containing 50 μg/ml ampicillin. Aliquots (40 ml) of this starter culture were added to 800 ml of the same medium in each of five flasks. The cultures were incubated at 30 °C, with vigorous shaking, until the absorbance at 600 nm reached 0.5. Enzyme expression was induced by adding isopropyl thiogalactoside to give a final concentration of 1 mM. After 7 h, the cells were harvested by centrifugation and the pellets were washed twice with 100 ml of 50 mM Hepes, 10 mM EDTA, pH 7.5. The cells were resuspended in the same buffer and lysed using a Model 300 Sonic Dismembrator (Fisher Scientific, Fair Lawn, NJ, U.S.A.) operated at 60 % of maximum power. Sonication was carried out in short bursts in order to avoid overheating the mixture. The lysate was centrifuged at 11000 ***g*** for 1 h and the supernatant was loaded on to a column (2.5 cm × 15 cm) of CM-Sepharose which had been equilibrated with 50 mM Hepes, 10 mM EDTA, pH 7.5. The column was washed with 200 ml of the same buffer and bound material was eluted using a gradient of NaCl (0 to 1.0 M) in the same buffer. Fractions (3 ml) were assayed for enzyme activity and protein content. The peak of enzyme activity was pooled and concentrated using a Centriprep-30 device (Amicon, Danvers, MA, U.S.A.). Finally, the buffer was exchanged with 50 mM ammonium acetate, pH 6.8. The final product gave a single band, with a molecular mass of 39.5 kDa, after SDS/PAGE on an 8 % gel.

Assay of HA lyase activity

Routine assays for HA lyase activity were carried out by adding sample (10 μl) to a cuvette containing HA (0.4 mg) in 0.4 ml of 50 mM ammonium acetate, 10 mM calcium chloride, pH 6.8. The rate of increase in absorbance at 232 nm was measured. *E. coli* mutant clones were screened for hyaluronidase production by growth on T-soy agar plates containing HA (0.4 mg/ml) and BSA (10 mg/ml), essentially as described [17]. After overnight growth at 37 °C, the plates were flooded with 2 M acetic acid, which caused precipitation of a HA/BSA complex in the gel. HA lyase-producing colonies were surrounded by clear zones on a turbid background. The diameter of a clear zone gave an indication of the amount of enzyme activity present.

Separation and quantification of the products of digestion of HA, chondroitin and chondroitin sulphate by HA lyases

Digestion products were separated using an HP^{3D} Capillary Electrophoresis System (Hewlett-Packard Co., 3495 Deer Creek Rd, Palo Alto, California, U.S.A.). Samples were injected hydrostatically (20 s at 50 mbar) on to a straight capillary (50 μm × 72 cm). Electrophoresis was at 20 kV in a phosphate/borate/SDS buffer, pH 9.3 at 40 °C [18] for 15 min. Preconditioning of the capillary was in 0.1 M sodium hydroxide for 2 min and in phosphate/borate/SDS buffer, pH 9.3 for 5 min. Separations were monitored at 200 nm and at 232 nm. Migration times were determined relative to that of an internal standard, benzoic acid (M_{IS}). The peak for benzoic acid was seen at approximately 12.5 min.

RESULTS

Properties of the enzyme

Some properties of the cloned enzyme are given in Table 1. Compared with the molecular masses of other bacterial HA lyases, which range from 77 kDa to 121 kDa [1], that of the phage enzyme, 39.5 kDa, is notably low. The high pI of the phage enzyme, 9.28, and the GBS HA lyase, 8.84, have been exploited for the purification of both enzymes [16].

Cleavage of HA

When HA at 0.5 mg/ml was digested by the phage enzyme (8.28 μg/ml) an increase in absorbance at 232 nm was noted. Cleavage of the substrate by elimination, in contrast to hydrolysis, results in increased absorbance at 232 nm as unsaturated uronosyl residues, which terminate newly formed oligosaccharides, accumulate. Thus, this enzyme acts through eliminative cleavage.

Table 1 Some properties of phage HA lyase

Property	Value
Molecular mass (kDa)	39.5
Molar absorption coefficient at 280 nm ($M^{-1} \cdot cm^{-1}$)	17210
pI	9.28
Optimal pH	6.0
Turnover number (bonds cleaved/enzyme molecule per s at 30 °C)	4.9
Stability (at 10 mg/ml in 20 mM Hepes, pH 7.0)	> 6 months at 4 °C

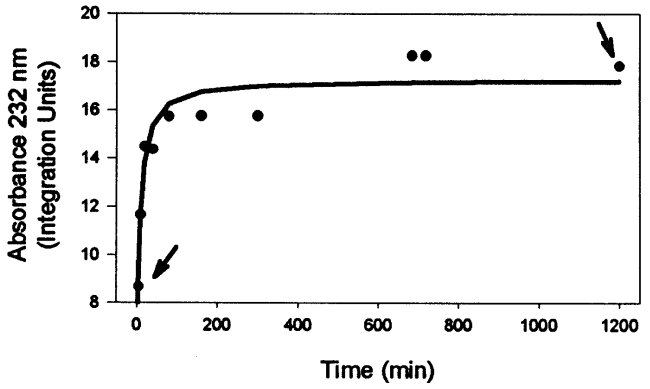

Figure 1 Digestion of HA by phage enzyme: the Progress Curve

HA (5 μg) was incubated in 0.05 M ammonium acetate/10 mM calcium chloride, pH 6.5 with 83 ng of phage HA lyase in a final volume of 10 μl at 30 °C for up to 20 h. Digests were diluted with water (40 μl) and analysed by capillary electrophoresis. Plotted at each time point is the combined absorbance at 232 nm for the unsaturated oligosaccharide products. The oligosaccharide products generated after 5 min and 1200 min (marked by arrows) are illustrated in Figure 2.

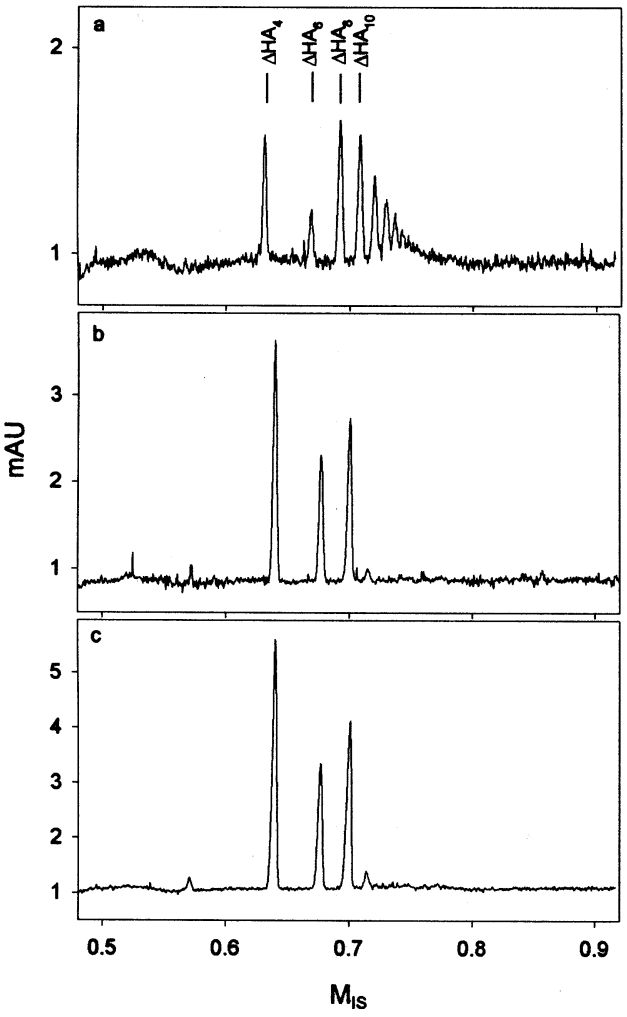

Figure 2 The unsaturated oligosaccharide products of digestion of HA by phage enzyme

HA digests were prepared (see legend to Figure 1) and the HA oligosaccharides produced were separated by capillary electrophoresis as described in the Experimental section. Digests were incubated at 30 °C for (**a**) 5 min, (**b**) 20 h, and (**c**) 20 h with addition of a further 5 μg of HA and reincubation for 20 h. M_{IS} = migration times relative to an internal standard, benzoic acid. mAU, milli-absorbance units.

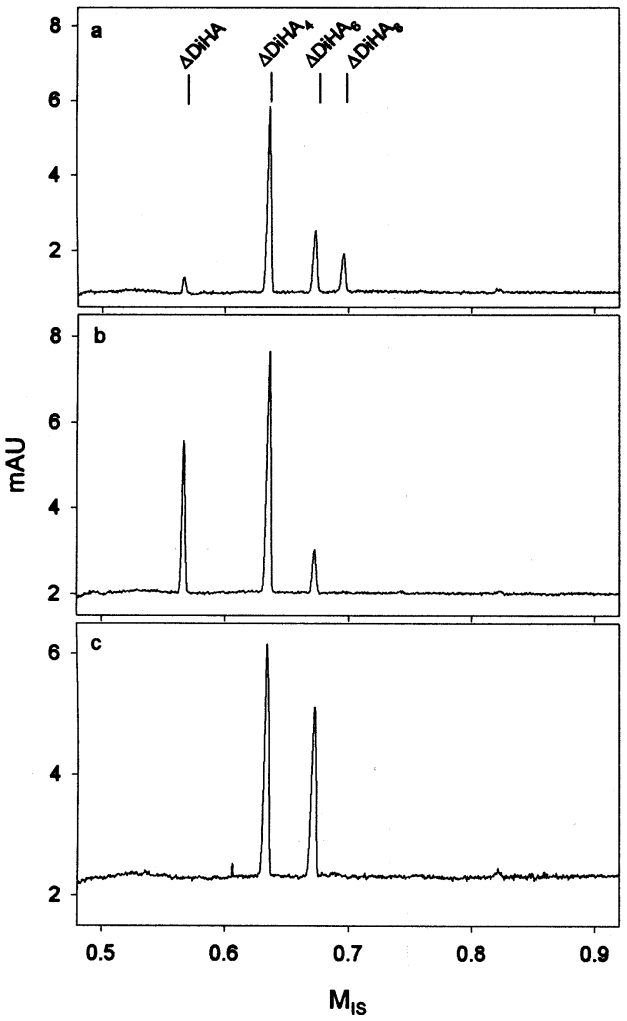

Figure 3 The unsaturated oligosaccharide products of digestion of HA, employing high concentrations of phage enzyme

HA (5 μg) was incubated in 0.05 M ammonium acetate/10 mM calcium chloride, pH 6.5 with (**a**) 830 ng of phage HA lyase, (**b**) 8.3 μg of phage HA lyase, and (**c**) 4.5 units of streptomyces HA lyase in a final volume of 10 μl at 30 °C for 20 h. Digests were diluted with water (40 μl) and analysed by capillary electrophoresis. mAU, milli-absorbance units.

The rate of the reaction, even in its initial stages, was not linear with time. From the apparent initial rate of reaction of 50 milli-absorbance units (mAU)/min, a turnover number of 4.9 (bonds cleaved/enzyme molecule per s at 30 °C) was calculated. Clearly, this enzyme cleaves HA at a much lower rate than the HA lyase from group B streptococci, with a turnover number of at least 8600 [11]. The relatively low rate of HA cleavage by the phage enzyme suggested that it might be acting distributively rather than processively. When reaction catalysed by the phage enzyme was complete, the absorbance reading was only 36.5 % of that attained under similar conditions employing the GBS HA lyase. This simple finding further suggested that the phage enzyme was not cleaving all *N*-acetylglucosaminidic linkages to yield 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (ΔDiHA) as the sole product, but was yielding higher oligosaccharide(s) (i.e., it was acting distributively).

To determine the oligosaccharide composition of the phage lyase-digested HA, samples of the digest were analysed by capillary electrophoresis. Samples were taken at a time when digestion appeared to be complete (20 h, Figure 1) and at a much earlier stage (5 min). At 5 min, low levels of the HA oligosaccharides ΔHA_4–ΔHA_{20} were detected (Figure 2a), whereas at 20 h, ΔHA_4, ΔHA_6 and ΔHA_8 were prominent (Figure 2b). No additional peaks, which could be detected at 200 nm and not at 232 nm, were observed, indicating that no hydrolytic cleavage of the substrate occurred during enzymic digestion. The absence of a single small oligosaccharide produced throughout the digestion, as happens with a processive enzyme (e.g., GBS HA lyase), provides clear proof of the distributive or non-processive action pattern of this enzyme. When digestion is complete at this enzyme level, three oligosaccharide products remain: ΔHA_4, ΔHA_6 and ΔHA_8. Further HA substrate was added and the digestion continued for an additional 20 h. More ΔHA_4, ΔHA_6 and ΔHA_8, with traces of ΔDiHA and ΔHA_{10}, were generated (Figure 2c). Therefore, enzyme had remained active in the digest and the zero increase in absorbance at 10–20 h (Figure 1)

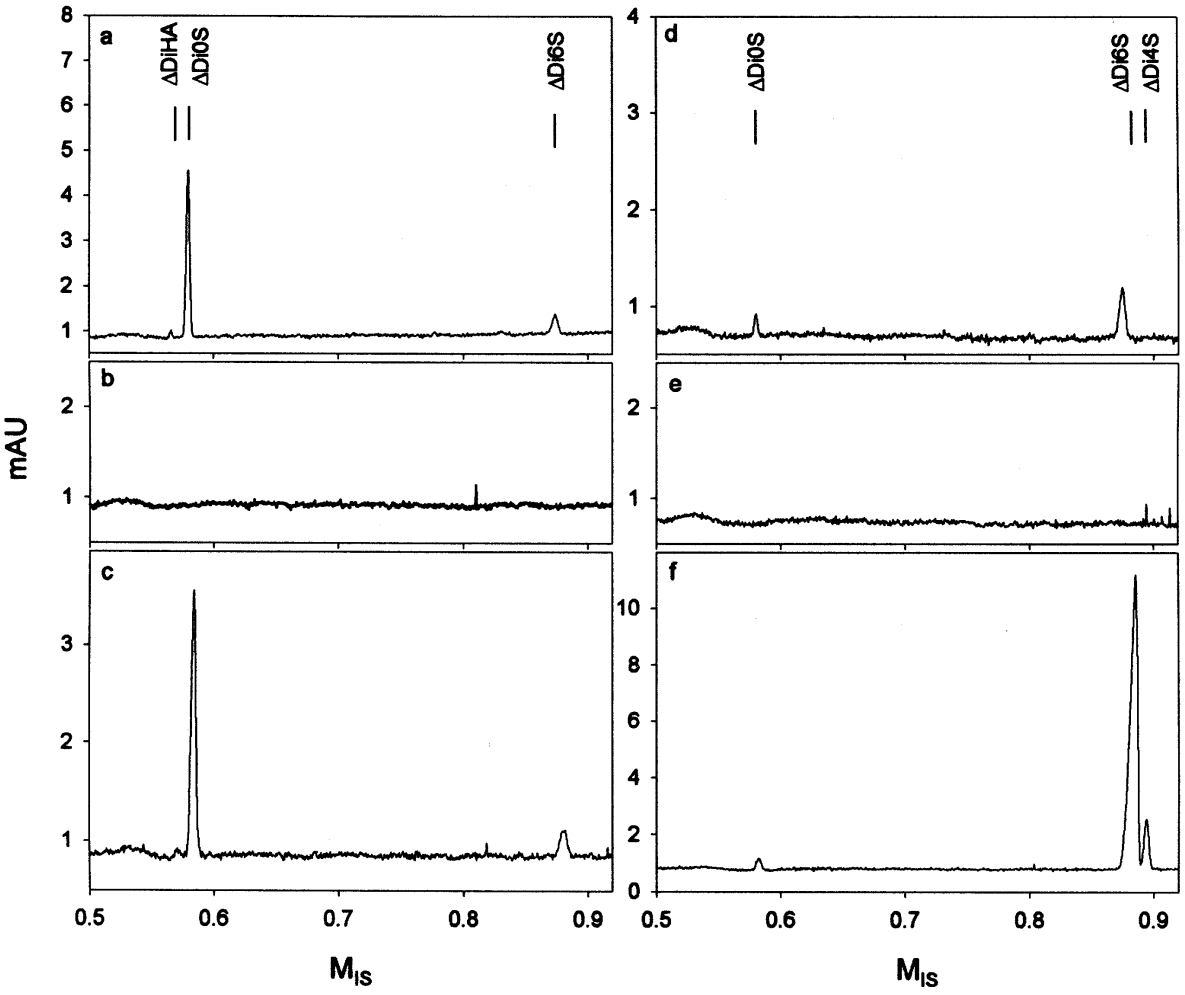

Figure 4 Analysis by capillary electrophoresis of the enzymic digestion products of chondroitin and chondroitin 6-sulphate

5 μg of chondroitin (**a**), (**b**) and (**c**) or 5 μg of chondroitin 6-sulphate (**d**), (**e**) and (**f**) were incubated with 11.7 ng of the GBS HA lyase (**a**) and (**d**), 82.8 ng of the phage HA lyase (**b**) and (**e**) or with 1 milli-unit of chondroitin AC lyase (**c**) and (**f**) in a final volume of 10 μl for 20 h at 30 °C. Digests were diluted with water (40 μl) and analysed by capillary electrophoresis. mAU, milli-absorbance units.

Table 2 Specificity of hyaluronan/chondroitin lyases

C0S, chondroitin; C4S, chondroitin 4-sulphate; C6S, chondroitin 6-sulphate.

Source	Lyase	Specificity	Reference
Streptomyces hyalurolyticus	hyaluronan	HA	[19]
Streptococcus agalactiae	hyaluronan	HA, C0S, C6S	[10,21]
Streptococcus dysgalactiae	hyaluronan	HA, C0S	[22,28]
Streptococcus intermedius	hyaluronan	HA, C0S (C4S, C6S)	[23]
Proteus vulgaris	chondroitin ABC	(HA, C0S), C4S, C6S, DS	[9]
Flavobacterium heparinum	chondroitin AC I	HA, C0S, C4S, C6S	[9]
Arthrobacter aurescens	chondroitin AC II	HA, C0S, C4S, C6S	[29]
Flavobacterium heparinum	chondroitin B	DS	[30]
Flavobacterium heparinum	chondroitin C	HA, C0S, C6S	[31]

resulted from exhaustion of substrate, not from lack of active enzyme.

HA was also digested using a higher concentration of the phage enzyme (Figures 3a and 3b). With the phage enzyme at 82.8 μg/ml (Figure 3a), digestion was more complete (cf. Figure 2b). ΔHA_8 and possibly ΔHA_6 were cleaved further and ΔHA_4 became the predominant peak. At a very high enzyme concentration, 0.828 mg/ml, the ability of the enzyme to generate ΔDiHA was well demonstrated (Figure 3c). This pattern of cleavage differs from that of the streptomyces HA lyase; the only

known lyase which specifically cleaves HA. At a high level of the streptomyces enzyme (450 units/ml), digests of HA contained no ΔDiHA (Figure 3c). ΔHA_4 and ΔHA_6 were the sole products, which is in agreement with an earlier published report [19].

Substrate specificity of the phage HA lyase

Chondroitin and HA are homologues, which differ only by the configuration of the C4 hydroxyl group on the repeating *N*-acetylhexosamine moiety. Limited segments of chondroitin sequence are found in many chondroitin sulphate chains. As shown (Figure 4b), chondroitin was not cleaved by the phage enzyme, since no 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi0S) or higher oligomer was detected. Neither was any cleavage product detected at 232 nm when enzyme at a 100-fold greater concentration was employed. Under similar conditions, chondroitin was cleaved by the GBS HA lyase (Figure 4a) and by chondroitin AC II lyase. In both cases, the product, ΔDi0S, is seen at 7.2 min. The small peak at 10.9 min is of 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi6S) and was formed from the residual chondroitin 6-sulphate in this preparation of chondroitin.

Chondroitin 6-sulphate was not cleaved by the phage enzyme (Figure 4e), since no ΔDi6S or higher oligomer was detected in the digest. No cleavage products were detected at 232 nm when enzyme at a 100-fold greater concentration was employed. At the same time, chondroitin 6-sulphate was cleaved by GBS HA lyase (Figure 4d) and by chondroitin AC II lyase (Figure 4f). Additionally, small peaks of ΔDi0S (Figures 4d and 4f) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi4S) at 11.1 min (Figure 4f) are seen.

Chondroitin 4-sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin were not cleaved by the phage enzyme (results not shown). Therefore, from among the glycosaminoglycans, only HA is cleaved by the phage HA lyase.

DISCUSSION

All bacterial hyaluronidases (that have been studied) cleave HA using an elimination mechanism. Therefore, they are properly termed hyaluronan lyases, to distinguish them from mammalian hyaluronidases, which are hydrolases. There are several examples of bacterial HA lyases that act distributively or processively, or exhibit partial processive cleavage. Often, their specificities are not limited to the cleavage of HA (Table 2). It has been suggested that an ability to cleave the chondroitin sulphates of connective tissue proteoglycans may enable pathogenic bacteria to invade tissues more completely [5]. If the biological function of the phage enzyme is to facilitate bacterial invasion of tissues, there would be a need for the phage to produce an enzyme which can also cleave chondroitin sulphates. However, it has been suggested that the function of the phage enzyme is to aid bacteriophage infection of the bacterial cell by degrading its HA capsule [4]. Clearly for this purpose, exclusive specificity for HA, as demonstrated for the phage HA lyase in the present study, is adequate.

The phage enzyme may also contribute to cell damage during human group A streptococcal infections. This possibility is supported by a recent report [20] in which it was demonstrated that lysogenic phage in group A streptococci were induced when the bacteria were co-cultured with their normal target, human pharyngeal cells. This induction resulted in the release of toxins from both the phage and the lysed streptococci. Cell exposure to these toxic products is then likely to be considerably enhanced by the degradation of HA in the extracellular matrix caused by the released phage HA lyase.

In the present work, we have shown that the phage 'hyaluronidase' is an eliminase and should properly be called an HA lyase. Also, unlike most bacterial HA lyases, it was found to be specific for HA. It was also found to be a distributive or non-processive enzyme. These findings are consistent with the suggestion that digestion of the HA capsule of the host bacterial cell is a physiological function of the phage HA lyase. The only other GAG lyase known to be specific for HA is that from *S. hyalurolyticus* (Table 2). At similar protein concentrations, the phage enzyme cleaved HA at a 30-fold faster rate than the streptomyces HA lyase. The phage HA lyase may be a useful alternative choice in applications when rapid selective degradation of HA is required.

Most HA lyases have been reported to possess chondroitin lyase activity (Table 2). The substrate specificity of the cloned HA lyase from *S. agalactiae* [10,21] is well established. However, the ability of HA lyases, purified to apparent homogeneity from cultures of *Streptococcus dysgalactiae* [22] and *Streptococcus intermedius* [23], to degrade chondroitin sulphate may be caused, at least in part, by a contaminating chondroitin lyase. Chondroitin lyases usually have some capacity to cleave HA. The chondroitin AC I and ABC I lyases have been cloned [24,25], but the commercially available, non-recombinant enzymes, which may possess some low-level, contaminating HA-degrading activity, are still widely used. Within the two categories of glycosaminoglycan lyases, HA and chondroitin lyases, there are many differences in specificity (Table 2). Table 2 lists only specificities for different glycosaminoglycans. A full understanding of specificity also involves a precise determination of the types of glycosidic bond cleaved [5]. As the list of lyases of well-defined specificities grows, these enzymes are likely to find application in structural studies of chondroitin sulphates. Cleavage of a chondroitin sulphate with specific lyases, followed by identification of the oligosaccharide fragments using MS [26], is potentially a powerful tool for studying its sequence. As with heparan sulphates, there are chondroitin sulphates (e.g., the chondroitin sulphate E of thrombomodulin [27]) with structures which are well-adapted to perform certain functions. The need for satisfactory methods for the sequencing of such chondroitin sulphates, which have demonstrable and specific biological activities, is likely to become more urgent in the near future.

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