

Exploration of the action pattern of *Streptomyces* hyaluronate lyase using high-resolution capillary electrophoresis

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Abstract

Hyaluronic acid was treated exhaustively with a hyaluronate lyase (hyaluronidase, EC 4.2.2.1) from *Streptomyces hyalurolyticus* to obtain a tetrasaccharide and a hexasaccharide product in a molar ratio of 1 to 1.2. The tetrasaccharide product was fluorescently labeled at the reducing end by reductive amination with 7-amino 1,3-naphthalene disulfonic acid (AGA) and the structure of the conjugate was determined spectroscopically. Partial treatments of hyaluronic acid with hyaluronate lyase afforded complex mixtures of oligosaccharides that were similarly fluorescently labeled. These labeled oligosaccharide mixtures were analyzed using high-resolution capillary electrophoresis. The resulting electropherograms showed the content of each hyaluronic acid derived oligosaccharide, having a degree of polymerization (dp) from 4 to 50, throughout the enzymatic reaction. Computer simulation studies gave comparable kinetic profiles suggesting that hyaluronate lyase exhibits a random endolytic action pattern. Interestingly, oligosaccharides of certain size (dp) were under-represented in these oligosaccharide mixtures suggesting that linkages at spacings of 10 to 12 saccharide units are somewhat resistant to this enzyme. The cause of this resistance might be the result of secondary or higher order structural features present in the hyaluronic acid polymer.

Keywords: Glycosaminoglycan; Hyaluronic acid; Hyaluronate lyase; Action pattern; Capillary electrophoresis, high resolution; (*Streptomyces*)

1. Introduction

Hyaluronic acid (hyaluronan), a high molecular weight, linear polysaccharide is a glycosaminoglycan

found in the extracellular matrix and is a prominent component of soft connective tissues [1]. Hyaluronic acid is biosynthesized in these tissues by addition of nucleotide sugars to the reducing end of the growing polysaccharide chain, while the nonreducing end is extruded through the plasma membrane of the cell into the pericellular space. Hyaluronic acid has been assigned a variety of important physiological functions. As a component of the extracellular matrix it maintains water balance and is important in cellular interactions. Hyaluronic acid also seems to play a crucial role during development and differentiation

Abbreviations: AGA, 7-amino 1,3-naphthalene disulfonic acid; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; FAB MS, fast atom bombardment mass spectrometry; CE, capillary electrophoresis; GlcNpAc, *N*-acetyl-D-glucosamine; GlcAp, D-glucuronic acid; ΔUAp, 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid; dp, degree of polymerization.

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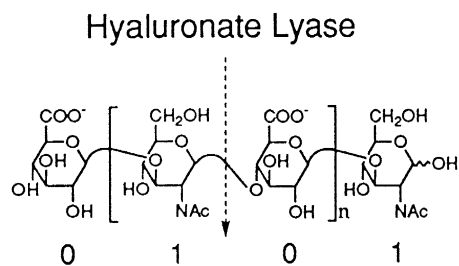


Fig. 1. Primary structure of hyaluronic acid. The number 1 corresponds to $\rightarrow 3\text{-}\beta\text{-D-GlcNpAc}(1 \rightarrow$ and the number 0 corresponds to $\rightarrow 4\text{-}\beta\text{-D-GlcAp}(1 \rightarrow$ and n is 250 to 2500.

and has many other important cell regulatory activities [1].

Hyaluronic acid has alternating repeating unit of the structure $\rightarrow 3\text{-}\beta\text{-D-GlcNpAc}(1 \rightarrow 4\text{-}\beta\text{-D-GlcAp}(1 \rightarrow$ (Fig. 1). It is a large polydisperse polysaccharide having an average molecular weight of up to 4 million [1]. Hyaluronic acid is an extended, flexible, helical rod shaped molecule [2,3]. It exhibits a number of secondary structures maintained by intramolecular hydrogen bonding. In aqueous solution it exists primarily as a two-fold helix. The amphipathicity of this helix supports intermolecular hydrophobic interactions resulting in its self-association [4].

Hyaluronic acid can be broken down enzymatically into oligosaccharides. Two distinct types of hyaluronidase are known, one being a hydrolase from animal origin (e.g. testis, sperm, skin, snake venom and leech) and the other an eliminase from bacterial sources (*Propionibacterium*, *Peptostreptococcus*, *Staphylococcus*, *Sterptococcus*, and *Streptomyces* genera) [5–7]. These enzymes have been used to study the structure of hyaluronic acid and to prepare hyaluronic acid-derived oligosaccharides for the evaluation of biological activity [8,9]. The hyaluronidase from *Streptomyces hyalurolyticus* (EC 4.2.2.1) was first found by Ohya and Kaneko in 1970 [10]. This enzyme is specific for hyaluronic acid and does not act on the related galactosaminoglycans, chondroitin and chondroitin sulfate. *Streptomyces* hyaluronate lyase cleaves $\rightarrow 3\text{-}\beta\text{-D-GlcNpAc}(\beta 1 \rightarrow 4)\beta\text{-D-GlcAp}(1 \rightarrow$ linkages through an elimination reaction, yielding 4,5-unsaturated tetrasaccharide and hexasaccharide as final products [5,11]. Octasaccharides were shown to be substrates of minimum size and tetrasac-

charides and hexasaccharides are not degraded by the enzyme [11,12].

The action pattern of other polysaccharide lyases acting on various glycosaminoglycans has recently been reported [13]. This study examines the action pattern of *Streptomyces* hyaluronidase. Hyaluronic acid was treated with *Streptomyces* hyaluronidase, samples representing kinetic time points were recovered, fluorescently labeled by reductive amination and analyzed by a newly developed method of high resolution capillary electrophoresis (CE). The experimentally observed distribution of oligosaccharide products was compared to computer-simulated product distributions. The results of these studies suggest that the observed product distribution is consistent with the influence of the higher order structure of hyaluronic acid on the action pattern of this enzyme.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

The sodium salt of hyaluronic acid (from human umbilical cord) was purchased from Sigma (St. Louis, MO, USA). Hyaluronidase (from *Streptomyces hyalurolyticus*, EC 4.2.2.1) was from Seikagaku (Tokyo, Japan) and monopotassium salt 7-amino 1,3-naphthalene disulfonic acid (AGA) was from Aldrich (Milwaukee, WI, USA) as a 85% purity. Gel permeation chromatography was performed on Sephadex G-50 (superfine) from Pharmacia (Piscataway, NJ, USA) and on Bio-Gel P-2 (fine) from Bio-Rad (Richmond, CA, USA). All other reagents used were analytical grade.

2.1.2. Equipment

$^1\text{H-NMR}$ was obtained using a UNITY-500 spectrometer at the operating frequency of 500 MHz equipped with a VXR 5000 computer system from Varian Instruments at 25°C. CE was performed using a Dionex Capillary Electrophoresis System with advanced computer interface, model I, equipped with high voltage power supply capable of constant or gradient voltage control (Sunnyvale, CA, USA). CElect™-N capillary column was from Supelco (Bellefonte, PA, USA). Mass spectroscopy was per-

formed with a VGZAB-HF instrument in the fast atom bombardment (FAB) ionization mode. The FAB ion source used was a standard VG Analytical system, equipped with a saddle-field atom gun. Xenon was used for the bombarding fast-atom beam.

2.2. Methods

2.2.1. Computer simulation model

A number chain (01010101..., representing the hyaluronic acid substrate) was constructed composed of 3000 number pairs of ones and zeros, where 1 represents $\rightarrow 3)\beta\text{-D-GlcNpAc}(1 \rightarrow$ and 0 represents $\rightarrow 4)\beta\text{-D-GlcAp}(1 \rightarrow$ (Fig. 1). These number pairs correspond to both hyaluronate lyase cleavable sites (10, $\rightarrow 3)\beta\text{-D-GlcNpAc}(1 \rightarrow 4)\beta\text{-D-GlcAp}(1 \rightarrow$) and hyaluronate lyase uncleavable sites (01, $\rightarrow 4)\beta\text{-D-GlcAp}(1 \rightarrow 3)\beta\text{-D-GlcNpAc}(1 \rightarrow$). The action of hyaluronate lyase on substrate was simulated using an algorithm consisting of 3 steps: 1. cut a selected number chain, 2. select a number chain among the remaining cleavable ones, and 3. print a time history output for each number chain. In the first step a random number generator is used to select the cleavable site at which the number chain is to be cut. In the second step a number chain is either selected (model A) with a priority proportional to the number of cleavable sites remaining in the chain or (model B) randomly. The time history of each number chain is printed out after each process cycle. Each of the three steps are iterated until all the cleavable sites within all the number chains have been cut. One final constraint is that number chains corresponding to tetrasaccharide (0101) or hexasaccharide (010101) are not considered cleavable.

2.2.2. Preparation and characterization of hyaluronic acid tetrasaccharide-AGA standard

Hyaluronic acid (100 mg) was treated with 100 TRU (Turbidity reducing unit) of *Streptomyces* hyaluronidase (EC 4.2.2.1, 100 TRU/ampoule) in 200 ml of 0.02 M sodium acetate-acetic acid buffer, at pH 6.0, containing 0.15 M NaCl. After incubation for 6 h at 60°C, another 100 TRU of the same enzyme was added to the reaction mixture followed by further digestion at 60°C for 12 h. After that another 100 TRU enzyme was added and incubated until the absorbance at 232 nm was constant. At

reaction completion, the mixture was lyophilized, re-dissolved in a small volume of water, and was fractionated with 0.2 M NaCl on a column (4.8 \times 100 cm) of Sephadex G-50. Two major peaks, corresponding to a tetrasaccharide and hexasaccharide, were lyophilized, desalted on Bio-Gel P-2 (6 \times 42 cm) and then lyophilized. The tetrasaccharide fraction from the Sephadex G-50 column was analyzed by CE using absorbance detection at 232 nm. This tetrasaccharide was then fluorescently labeled by reductive amination with AGA and following the removal of excess reagents (see below), its purity was again assessed by CE using fluorescence detection ($\lambda_{\text{ex}} = 250$ nm, $\lambda_{\text{em}} = 420$ nm) and its structure characterized by FAB-MS and $^1\text{H-NMR}$. Sample (1 mg) was prepared for NMR analysis by exchanging three times with $^2\text{H}_2\text{O}$ (99.96%) followed by in vacuo desiccation over P_2O_5 .

2.2.3. Partial degradation of hyaluronic acid with *Streptomyces* hyaluronidase

Hyaluronic acid (2.5 mg) was digested with 10 TRU of *Streptomyces* hyaluronidase in 1 ml of 0.02 M sodium acetate-acetic acid buffer (pH 6.0), containing 0.15 M NaCl at 60°C. At selected time intervals 500 μg of the reaction mixture was taken out and boiled at 100°C for 3 min to inactivate the enzyme and the absorbance at 232 nm was measured. The five time points were desalted on a Bio-Gel P-2 column (1 \times 42 cm) and lyophilized. Each fraction was labeled by reductive amination with AGA (see below). All labeled fractions were then analyzed by CE. The CE system was operated in the reverse polarity mode by applying the sample at the cathode and using 20 mM phosphoric acid adjusted to pH 2.5 with 1 M dibasic sodium phosphate [14]. The capillary (50 μm inner diameter, 360 μm outer diameter, 70 cm long and 65.5 cm effective length) was washed manually before use with 0.5 ml of distilled water and then 0.5 ml of running buffer. Samples were applied using either by gravity injection (20 sec) with hydrostatic pressure (45 nm) or by pressure injection (5 psi, 2 sec), resulting in sample volumes of 9.2 nl and 69.9 nl, respectively. Each analysis was performed at 18 kV and utilized fluorescence detection ($\lambda_{\text{ex}} 250$ nm, $\lambda_{\text{em}} 420$ nm). AGA was used as an internal standard and the tetrasaccharide component

of each mixture was identified by comigration with tetrasaccharide-AGA standard.

2.2.4. Fluorescent labeling with AGA

The oligosaccharides were labeled at their reducing ends with the AGA fluorophore (Fig. 3) by reductive amination [15]. AGA was used only after repeated recrystallization from deionized water [16]. Dry oligosaccharides (125 μg) were added 50 μl of 0.15 M AGA solution in acetic acid/water (3:17, v/v) and 50 μl of 1.0 M NaBH_3CN solution in dimethylsulfoxide (DMSO). The solution was vortex-mixed, and incubated at 45°C for 6 h. After the reaction was complete, the products were desalted on a Bio-Gel P-2 column (1 \times 42 cm) that was eluted with distilled water. Each fraction was monitored by absorbance at 239 nm. The pooled fractions were lyophilized.

3. Results and discussion

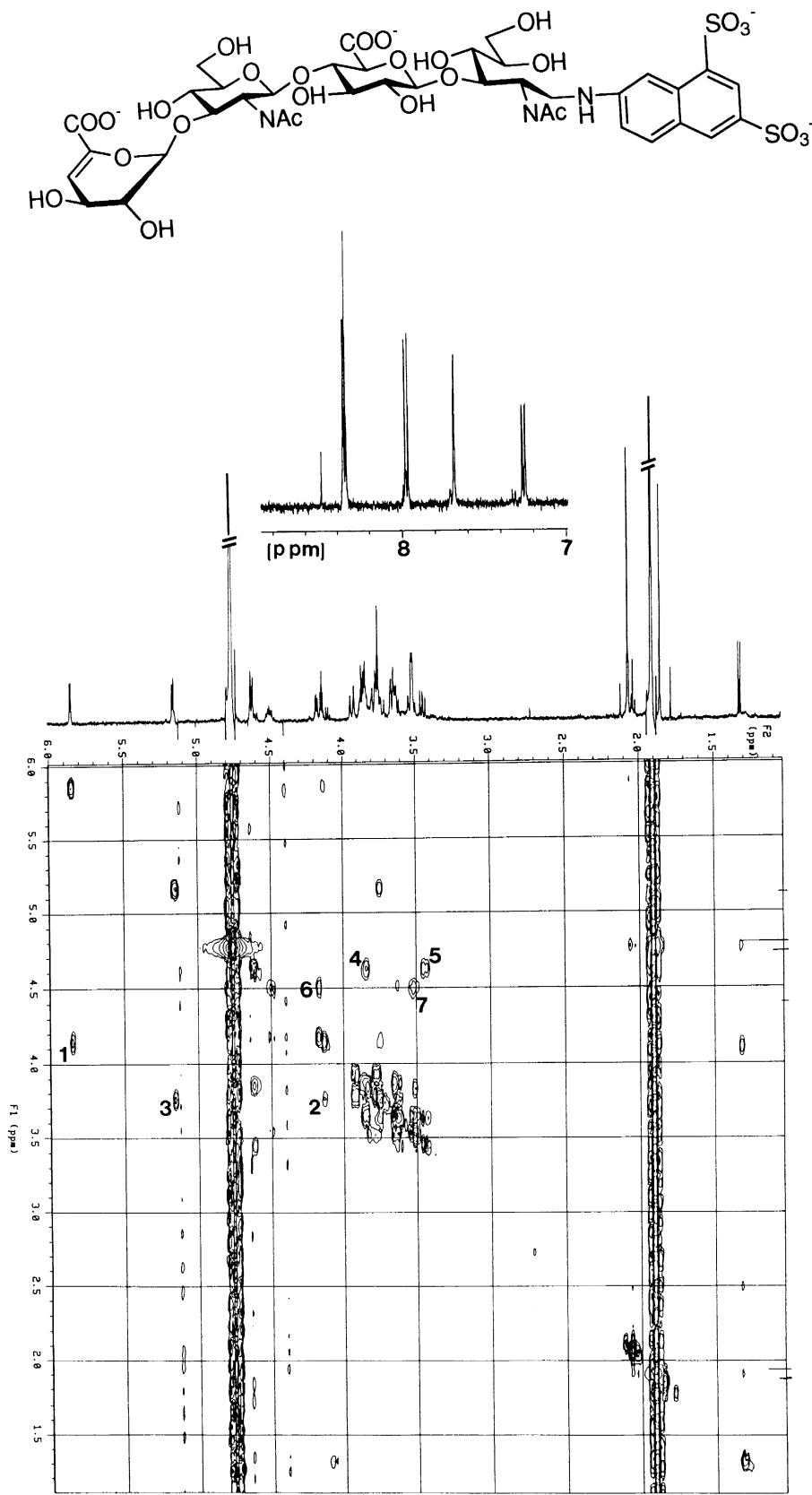
The hyaluronic acid used in this study was from human umbilical cord and had an intermediate molecular weight with an intrinsic viscosity of ≈ 60 $\text{mPa} \cdot \text{s}$, permitting the preparation of up to 2.5 mg/ml solutions with viscosities that could be mixed efficiently during enzymatic depolymerization. Exhaustive depolymerization of hyaluronic acid with *Streptomyces* hyaluronate lyase (Fig. 1) followed by size fractionation on Sephadex G-50 afforded an expected mixture of two major oligosaccharide products in a ratio of 1:1.2 as measured by both gel permeation chromatography (GPC) and CE analysis. Following desalting on Bio-Gel P2, these two oligosaccharide products were each of >90% purity as determined using CE with absorbance detection at 232 nm. $^1\text{H-NMR}$ analysis of these oligosaccharides at 500 MHz afforded spectra consistent with the structure of both unsaturated tetrasaccharide $\Delta\text{UAp}(1 \rightarrow 3)\text{GlcNpAc}(\beta 1 \rightarrow 4)\text{GlcAp}(\beta 1 \rightarrow 3)\text{GlcNpAc}\alpha,\beta$ in 38% yield and unsaturated hexasaccharide $\Delta\text{UAp}(1 \rightarrow 3)\text{GlcNpAc}(\beta 1 \rightarrow 4)\text{GlcAp}(\beta 1 \rightarrow 3)-$

$\text{GlcNpAc}(\beta 1 \rightarrow 4)\text{GlcAp}(\beta 1 \rightarrow 3)\text{GlcNpAc}\alpha,\beta$ in 47% yield. The unsaturated tetrasaccharide was selected for the labeling of its reducing end by reductive amination with AGA using NaBH_3CN in 15% acetic acid/DMSO. When formamide was used as a solvent for labeling [17] additional, minor satellite peaks were observed using CE fluorescence detection. The slightly basic conditions in formamide may result in C-2 epimerization of the reducing end GlcNAc to ManNAc or limited peeling reactions [18]. While these additional minor peaks do not adversely effect the analysis of the mixture of tetrasaccharide and hexasaccharide obtained in the exhaustive enzymatic digestion of hyaluronic acid, these additional peaks unnecessarily complicate the electropherograms of the partially digested hyaluronic acid making peak assignments difficult.

The HA tetrasaccharide-AGA conjugate was obtained at >90% purity (by CE) following desalting to remove excess AGA, organic solvent and salts. This tetrasaccharide-AGA conjugate was characterized by one dimensional $^1\text{H-NMR}$ and two dimensional COSY-45 spectroscopy (Fig. 2), as well as negative-ion FAB mass spectrometry to unequivocally establish its structure prior to its use as a standard. The molecular weight of fully protonated tetrasaccharide-AGA conjugate is 1045 (M). Molecular ion peaks observed in the FAB mass spectrum are assignable to $[\text{M} - \text{H}_{x+1} + \text{Na}_x]^-$ ($x = 2, 3$ and 4): $[\text{M} - 4\text{H} + 3\text{Na}]^- = m/z$ 1110, $[\text{M} - 3\text{H} + 2\text{Na}]^- = m/z$ 1088, and $[\text{M} - 5\text{H} + 4\text{Na}]^- = m/z$ 1132. Characteristic base peak was also observed at m/z 543 corresponding to $[\text{M} - (\Delta\text{UA-HexNAc}) - 3\text{H} + 3\text{Na} - 2\text{NaSO}_3]^-$. Fragment ions are observed at $m/z = 779$ and 907 corresponding to $[\text{M} - (\text{naphthalene ring system}) - 2\text{H} + \text{Na}]^-$ and $[\text{M} - \Delta\text{UA} - 2\text{H} + \text{Na}]^-$ respectively. The $^1\text{H-NMR}$ of the tetrasaccharide-AGA conjugate was fully assignable through the cross-peaks observed in the COSY-45 spectrum (Fig. 2).

The action pattern of *Streptomyces* hyaluronate lyase on hyaluronic acid was previously suggested to be endolytic [11]. Our laboratory has also recently

Fig. 2. Two dimensional COSY-45 spectrum of tetrasaccharide-AGA conjugate. The inset shows the aromatic region from 9.0 to 7.0 ppm in which the protons of the naphthalene ring system appear. The labeled cross-peaks are: 1, H-3/H-4 of ΔUAp ; 2, H-2/H-3 of ΔUAp ; 3, H-1/H-2 of ΔUAp ; 4, H-1/H-2 of GlcNpAc ; 5, H-1/H-2 of GlcAp ; 6, H-2/H-3 of GlcNpAc-AGA ; 7, H-1/H-2 of GlcNpAc-AGA .



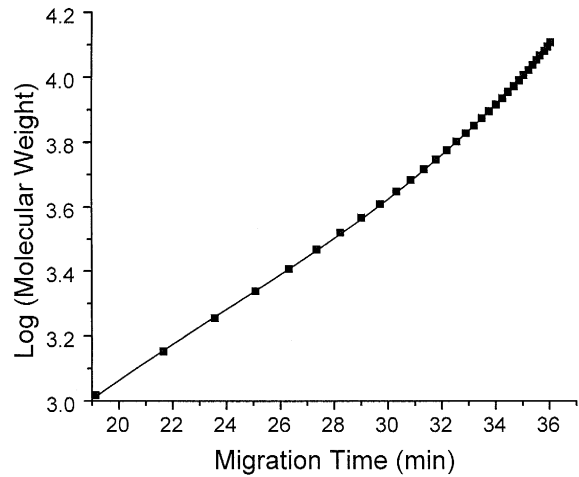
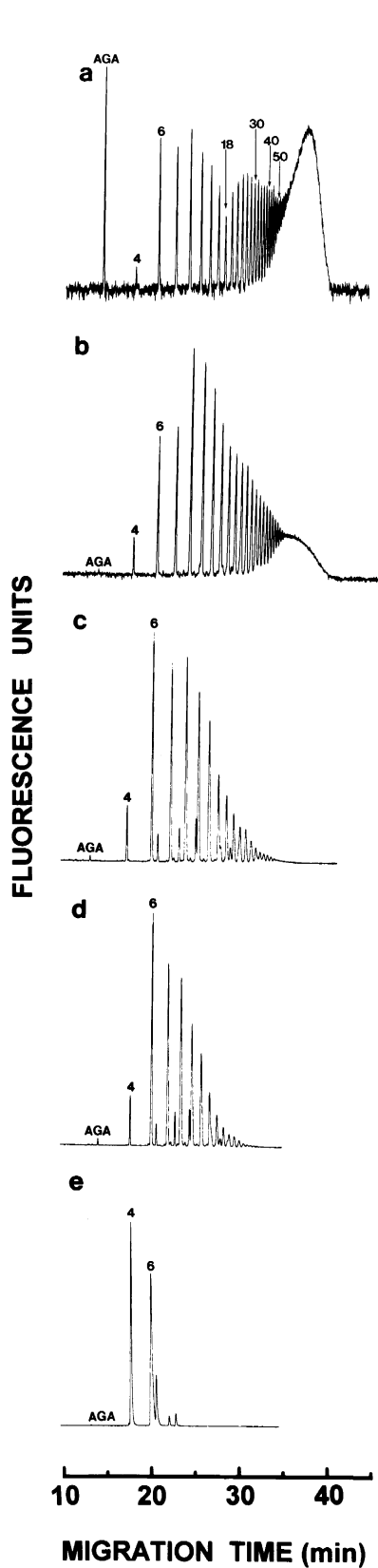


Fig. 4. Log molecular weight of oligosaccharide-AGA conjugate as a function of migration time on capillary electrophoresis. These data are taken from Fig. 3, panel a.

completed a detailed study on the action patterns of several other polysaccharide lyases acting on their glycosaminoglycan substrates [13]. The study of the action pattern of these enzymes on their polymeric substrates is complicated by two factors. First, with the exception of hyaluronic acid, all glycosaminoglycans are microheterogeneous containing variable disaccharide sequences with differential sensitivity to a given polysaccharide lyase. This results in complicated, non-ideal action patterns influenced by the primary structure or sequence of the glycosaminoglycan substrate [13,19–21]. Second, there are a limited number of available methods for monitoring the kinetics of polysaccharide depolymerization. The eliminase action of polysaccharide lyase on glycosaminoglycan substrate affords an unsaturated uronic acid residue [6] at the cleavage site, the appearance of

Fig. 3. Capillary electropherograms of AGA labeled oligosaccharide mixtures prepared through controlled depolymerization of hyaluronic acid with hyaluronate lyase. Panels a, b, c, d and e correspond to 16.9, 22.5, 43.7, 60.9 and 100% reaction completion. Each electropherogram contains AGA as an internal standard. The structure of the tetrasaccharide and hexasaccharide in the final product mixture (panel e) were established by $^1\text{H-NMR}$ analysis. Tetrasaccharide-AGA conjugate standard (Fig. 2) co-migrated with the peak marked 4 in each electropherogram. The peaks labeled dp 18, 30, 40, 50 were assigned by counting from the tetrasaccharide-AGA standard and correspond to peaks of unexpectedly low intensity.

which can be monitored throughout the enzymatic reaction to afford the percent of reaction completion. This assay is often coupled to viscosimetric measurement of the average molecular weight of the products to afford information on the action pattern of the enzyme [13]. It is much more informative to directly monitor the formation and/or disappearance of individual substrate and/or product chains throughout the enzymatic reaction. Such detailed measurement provides substantially more information on the enzymes action pattern. Both gel permeation chromatography and strong-anion-exchange HPLC have been used to provide some of this additional information [19,20]. Unfortunately, these methods are generally not of sufficiently high resolution for the analysis of larger oligosaccharides (> hexasaccharides) and

thus, provide only a limited amount of additional data.

Capillary electrophoresis is a very high resolution separation method that has recently been applied in our laboratory for the analysis of glycosamino-glycan-derived oligosaccharides [14]. Migration in an open capillary (CZE) is primarily a function of both the charge and size of an oligosaccharide [22]. Since the oligosaccharides generated from hyaluronic acid using hyaluronate lyase have a uniform charge density (i.e., one carboxylate group per disaccharide repeat) poor separation between oligosaccharides of differing size was anticipated [22]. This problem can be circumvented if single dominant charge group is introduced into each oligosaccharide chain. Reductive amination with AGA, a disulfonated naphthalamine,

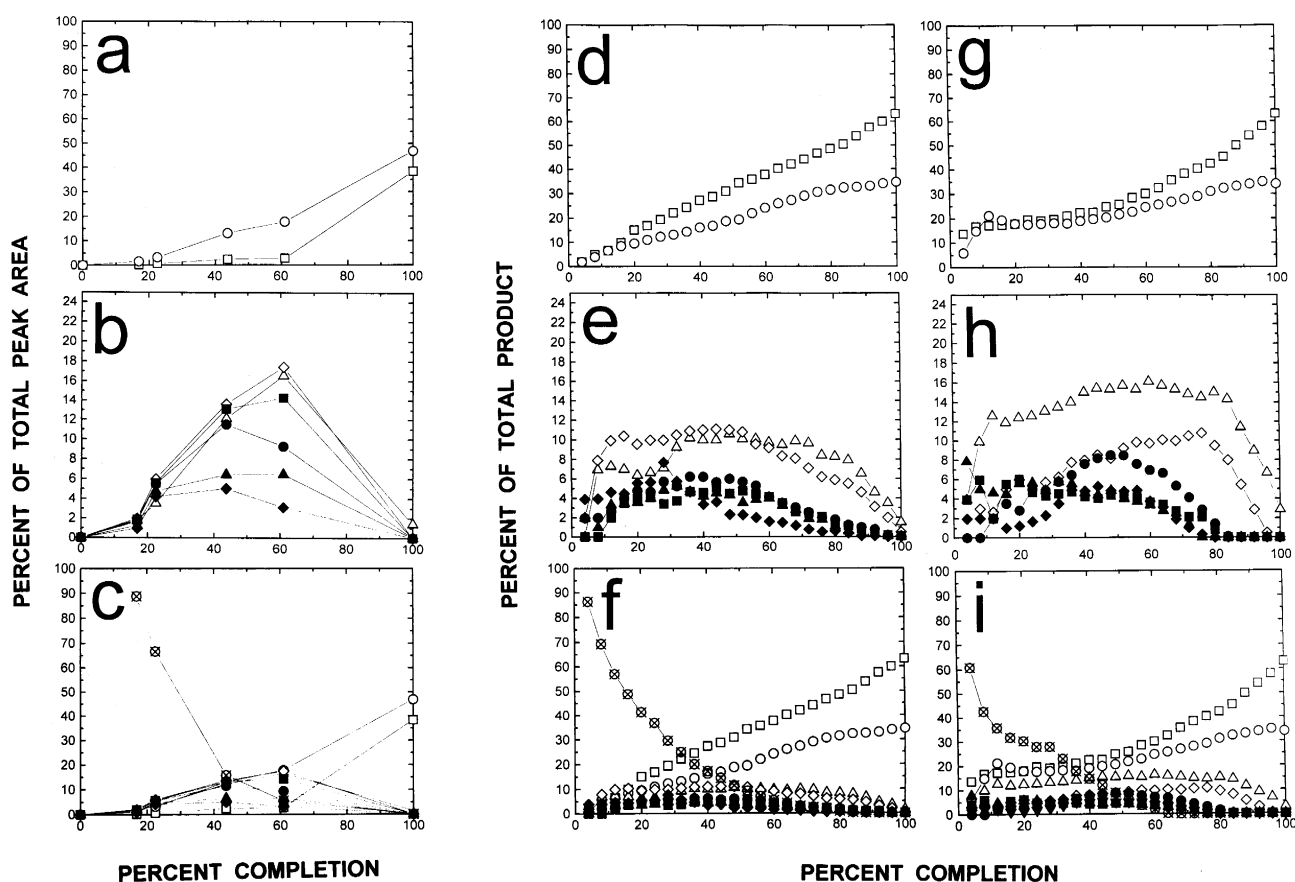


Fig. 5. Percent of total peak area (experimental) or percent of total product (simulated) as a function of percent of completion. Panels a, b and c are experimental data obtained from peak areas measured in Fig. 3 and absorbance measurement at 232 nm during the hyaluronate lyase catalyzed depolymerization of hyaluronic acid. Panels d, e and f are simulated data obtained using model A and g, h and i using model B (see Section 2). The symbols in all panels correspond to oligosaccharide products of dp: \square , 4; \circ , 6; \triangle , 8; \diamond , 10; \blacksquare , 12; \bullet , 14; \blacktriangle , 16; \blacklozenge , 18; \otimes , > 18.

labels each oligosaccharide at its reducing end. This reaction is both quantitative and clean affording a single fluorescently labeled conjugate from each oligosaccharide present in the reaction mixture. The sulfonate groups carried by AGA are much more acidic than the carboxylate groups (having a $pK_a < 1$ compared to a $pK \approx 3.5$ [23] of hyaluronic acid. In low pH (pH 2.5) CE separations, only the sulfonate groups of AGA remain charged giving each oligosaccharide a net charge of -2 . With molecular charge held constant, oligosaccharide size becomes the dominant feature of controlling migration rate through the capillary.

The depolymerization of hyaluronic acid was followed kinetically by both absorbance at 232 nm and CE analysis (Fig. 3). The electropherograms show a complicated mixture of oligosaccharides early in the reaction time course that is simplified on completion of the reaction to two major products (Fig. 3, panels a and e). The chemistry used to introduce the fluorescent label affords no measurable minor products that would complicate peak assignments. AGA was used as an internal standard in each electropherogram to ensure consistency of migration time and the tetrasaccharide-AGA conjugate was used to identify the peak corresponding to it in each electropherogram. The remaining peaks were identified by counting the number of peaks between each and the tetrasaccharide-AGA standard. Peaks could be clearly assigned up to a degree of polymerization (dp) of > 50 corresponding to a molecular weight of 9737 (Fig. 3, panel a). A plot of migration time as a function of the log molecular weight gave a nearly linear relationship (Fig. 4) suggesting that the separation was behaving ideally and that the assignment of dp based on peak counting was correct.

The area corresponding to each peak in all of the electropherograms (Fig. 3) was determined. When these areas were divided by the total moles of product formed (measured by absorbance at 232 nm prior to fluorescent labeling), the mole percent of each product could be calculated. This experimentally determined mole percentage was plotted as a function of percent reaction completion (absorbance at 232 nm for each kinetic time point divided by absorbance at 232 nm at reaction completion) (Fig. 5a–c). Using computer simulation studies, two random endolytic action patterns were examined. In the first (model A)

substrate chains that have more cleavable portion were preferentially selected by the enzyme while in the second (model B) substrate chains were chosen at random by the enzyme. Models A and B (Fig. 5d–f and g–i) both models give simulated product profiles similar (model A matches the experimental data slightly better than model B) to those experimentally observed (Fig. 5a–c) thus confirming that hyaluronate lyase acts on hyaluronic acid through a random endolytic action pattern. Interestingly, both models predict a final tetrasaccharide to hexasaccharide ratio of 2:1 compared to an experimentally observed ratio of 1:1.2. This experimental data of the final product composition matches that reported by Shimada and Matsumura [11].

Close examination of several of the electropherograms (Fig. 3, panel a) shows that in several instances, the size (area) of a particular peak, corresponding to the amount of a particular oligosaccharide in the reaction mixture, is less than that expected based on the surrounding peaks. For example, the peaks corresponding to oligosaccharides having a dp of 18, 30, 40, 50 are found in significantly reduced amounts. This suggests the presence of resistant (less susceptible to enzyme cleavage) site repeating unit, corresponding to 10–12 saccharide residues. Since hyaluronic acid has a uniform primary sequence, these resistant sites might be ascribed to restricted enzyme access based on secondary or higher order structural motifs.

Hyaluronic acid is both less highly charged and considerably larger than the other GAGs, having chain lengths from 500 to several thousand disaccharides (molecular weight 100 to 1000 kDa). HA flexibility has been examined by electric birefringence in dilute aqueous solutions and results suggest it may exist as the weakly bending rod or a worm-like chain with a persistence length of 20 nm or 20 disaccharide units [2]. Such a length corresponds to approximately twice the distance between lyase-resistant sites observed in this study.

Rotary-shadowing-electron microscopic analysis and computer simulation of hyaluronic acid structure in vacuo and in water have reproduced important X-ray diffraction [24] and NMR [25] findings on the higher order structures of hyaluronic acid. In vacuo, two-fold, three-fold, and four-fold helices were found, whereas, in water only the two-fold helix was stable.

The two-fold helix — i.e., two disaccharide units per complete helical turn — seen in water is presumably stabilized by hydrogen-bonding networks in the water, the water making a bridge between uronate carboxylate and acetamido NH groups [3]. Surface tension, due to the polar solvent pulling away from the hydrophobic patches, repeated along opposite sides of the molecule contributes to the stiffness of hyaluronic acid [4]. These hydrophobic patches may be involved in interactions with proteins including hyaluronate lyase. Counterions (monovalent or divalent) seem to have a greater effect on a local conformation than on chain conformation [26]. Computer simulation showed that duplex formation is sterically possible and energetically likely [3]. The duplex formation could occur at physiological pH if the chains are of sufficient length (> 7 disaccharides, preferably > 20 disaccharides), the hyaluronic acid concentration is sufficiently high (≈ 2 mg/ml), and the salt concentration is adequate (0.15 M NaCl) [27]. Thus, duplex formation is likely under the experimental conditions used in this study and may suggest a second reason for a periodic distribution of hyaluronate lyase-resistant sites.

Hyaluronic acid binds to chondrocytes derived from the Swarm rat chondrosarcoma with high affinity [9]. These studies showed that chondrocyte binding site recognizes five disaccharide repeats of hyaluronic acid. Interestingly, this also corresponds the same length as the spacing of lyase resistant sites. Thus, motifs of five or six disaccharide units may represent biologically relevant protein binding sites in hyaluronic acid.

In conclusion, hyaluronic acid has been depolymerized with *Streptomyces* hyaluronate lyase and the oligosaccharide products labeled with a charged fluorescent tag. High-resolution CE analysis was used to follow the kinetics of enzyme catalyzed depolymerization of hyaluronic acid. The results of this study confirmed the random endolytic action pattern of this enzyme and also suggests some secondary or higher order structural motif occurring approximately every 10–12 saccharide residues impart chain resistance to enzymolysis. The use of hyaluronate lyase in conjunction with high resolution capillary electrophoresis might offer a new approach to understanding the secondary and higher order structures of hyaluronic acid under physiologically relevant conditions.

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References

- [1] Laurent, T.C. and Fraser, J.R. (1992) *FASEB J.* 6, 2397–2404.
- [2] Trimm, H.H. and Jennings, B.R. (1983) *Biochem. J.* 213, 671–677.
- [3] Scott, J.E., Cummings, C., Brass, A. and Chen, Y. (1991) *Biochem. J.* 27, 699–705.
- [4] Scott, J.E. (1992) *FASEB J.* 6, 2639–2645.
- [5] Linhardt, R.J., Galliher, P.M. and Cooney, C.L. (1986) *Appl. Biochem. Biotechnol.* 12, 135–175.
- [6] Linhardt, R.J. (1994) in *Current Protocols in Molecular Biology, Analysis of Glycoconjugates* (Varki, A., ed.), pp 17.13.17–17.13.32, Wiley Interscience, Boston, MA.
- [7] Ernst, S., Langer, R., Cooney, C.L. and Sasisekharan, R. (1995) *Crit. Rev. Biochem. Mol. Biol.* 30, 387–444.
- [8] Nemeč, R.E., Toole, B.P. and Knudson, W. (1987) *Biochem. Biophys. Res. Commun.* 149, 249–257.
- [9] McCarthy, M.T. and Toole, B.P. (1989) *J. Cell. Physiol.* 141, 191–202.
- [10] Ohya, T. and Kaneko, Y. (1970) *Biochim. Biophys. Acta* 198, 607–609.
- [11] Shimada, E. and Matsumura, G. (1980) *J. Biochem.* 88, 1015–1023.
- [12] Takazono, I. and Tanaka, Y. (1984) *J. Chromatogr.* 288, 167–176.
- [13] Jandik, K.A., Gu, K. and Linhardt, R.J. (1994) *Glycobiology* 4, 289–296.
- [14] Pervin, A., Al-Hakim, A. and Linhardt, R.J. (1994) *Anal. Biochem.* 221, 182–188.
- [15] Jackson, P. (1990) *Biochem. J.* 270, 705–713.
- [16] Lee, K.B., Al-Hakim, A., Loganathan, D. and Linhardt, R.J. (1991) *Carbohydr. Res.* 214, 155–168.
- [17] Liu, J., Desai, U.R., Han, X.J., Toida, T. and Linhardt, R.J. (1995) *Glycobiology* 5, 765–774.
- [18] Toida, T., Vlahov, I.R., Smith, A.E., Hileman, R.E. and Linhardt, R.J. (1996) *J. Carbohydr. Chem.* 15, 351–360.
- [19] Linhardt, R.J., Merchant, Z.M., Rice, K.G., Kim, Y.S., Fitzgerald, G.L., Grant, A.C. and Langer, R. (1985) *Biochemistry* 24, 7805–7810.
- [20] Linhardt, R.J., Cohen, D.M. and Rice, K.G. (1989) *Biochemistry* 28, 2888–2894.
- [21] Cohen, D.M. and Linhardt, R.J. (1990) *Biopolymers* 30, 733–741.

- [22] Linhardt, R.J. and Pervin, A. (1996) *J. Chromatogr. A* 720, 323–335.
- [23] Wang, H.M., Loganathan, D. and Linhardt, R.J. (1991) *Biochem. J.* 278, 689–695.
- [24] Winter, W.T., Smith, P.J.C. and Arnott, S. (1975) *J. Mol. Biol.* 99, 219–235.
- [25] Scott, J.E., Heatley, F. and Hull, W.E. (1984) *Biochem. J.* 220, 197–205.
- [26] Chakrabarti, B. (1977) *Arch. Biochem. Biophys.* 180, 146–150.
- [27] Turner, R.E., Lin, P. and Cowman, M.K. (1988) *Arch. Biochem. Biophys.* 265, 484–495.