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Control of enzymatic degradation of hyaluronan by divalent cations

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Abstract

Enzymatic degradation of hyaluronan (HA) by testicular hyaluronidase (HAase, hyaluronate 4-glucanohydrolase) requires inclusion of mono- or divalent cations in the reaction mixture. Most divalent cations activated HAase with equal potency; however, Cu^{2+} suppressed degradation, and Ca^{2+} showed a concentration-dependent regulation of size of the oligosaccharide products. Careful selection of HAase assay parameters is critical for discovery of novel HAase inhibitors and for preparation of controlled-size oligosaccharide fragments.

Keywords: Hyaluronidase; Hyaluronan; Gel-permeation chromatography; Perfusion anion-exchange chromatography

1. Introduction

Hyaluronan (HA), a naturally occurring linear polysaccharide comprising β-(1→4)-linked D-glucuronic acid β-(1→3)-*N*-acetyl-D-glucosamine disaccharide units (Fig. 1), is found in the extracellular matrix (ECM) of all higher animals, especially in soft connective tissues. This polyanionic polymer has a range of naturally occurring molecular sizes from several hundred to over 10 million daltons (MDa), with an average size of 1–2 MDa [1]. By binding to the HA-binding proteins (HABP) present in the ECM, HA participates in defining the interaction and the organization of the complex proteoglycan-based aggregates [2]. HA and its chemically modified derivatives have recently begun to occupy center stage in areas ranging from drug delivery to tissue engineering [3].

Cell surfaces possess HA receptors, e.g., CD44 and RHAMM [4], that play important roles in normal physiology and disease states [5,6]. Relatively small HA fragments (oligosaccharides) exhibit different and specific cell-biological properties when compared with the megadalton-sized biopolymer. Oligosaccharide fragments of HA (starting with six disaccharide units, i.e., HA_{12}) have angiogenic potential by stimulating the proliferation and migration of endothelial cells [7–9]. The interaction of HA fragments with CD44 receptors on macrophages has important effects in the early stages of the inflammatory response [10]. The activation of CD44 by HA fragments has been shown to stimulate the gene expression of IL-1β, TNF-α, and IGF-1 [11] of several members of the chemokine gene family [12] and of nitric oxide synthase [13]. This gene induction seems to

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occur in a nuclear factor (NF) κB-dependent fashion [13,14]. Thus, CD44 is an important component of the signal transduction pathway leading to inflammatory leukocyte recruitment and activation [15].

Small fragments of HA, with their angiogenic and pro-inflammatory potential, are generated through an enzymatic digestion of the high-molecular-mass parent HA molecule. The enzyme responsible for this degradation of HA is called hyaluronidase (HAase; hyaluronate 4-glucanohydrolase, EC 3.2.1.35). HAases comprise a class of enzymes that are ubiquitously distributed in prokaryotes and eukaryotes, performing a broad range of biochemical and biological functions. The properties and functions of these enzymes have been extensively reviewed in recent years [16–18].

HAase is found in the testicular tissues of most mammals and is located on the acrosomal region of spermatozoa. The enzyme plays a major role in the passage of the spermatozoa towards the oocyte by digesting the HA that is part of the ECM of the oocyte [19]. The lysosomes of all tissues of most mammals contain HAase, where it is involved in the catabolism of HA [18]. A large range of animal venoms (snake, bee, lizard, scorpion, spider, ant) contain HAase activity, a property that facilitates the spreading of the venom through the ECM; in venoms, HAases are often referred to as spreading factors [16]. Bacterial species such as pneumococci, staphylococci, and streptococci, as well as hookworm larvae, produce HAase (hyaluronate lyase, EC 4.2.2.1.), and the production of this enzyme is often correlated with their virulence [16,20]. Species of the fungus *Candida* produce HAases although their roles have not been established [21].

Tumors are often enriched in HAase relative to normal tissues, as has been demonstrated for breast [22], prostate [23], gynecological [24], and other tumors [25]. This production of HAase by those tumors can affect the further development of the tumor. By acting on HA present in the ECM, HAase

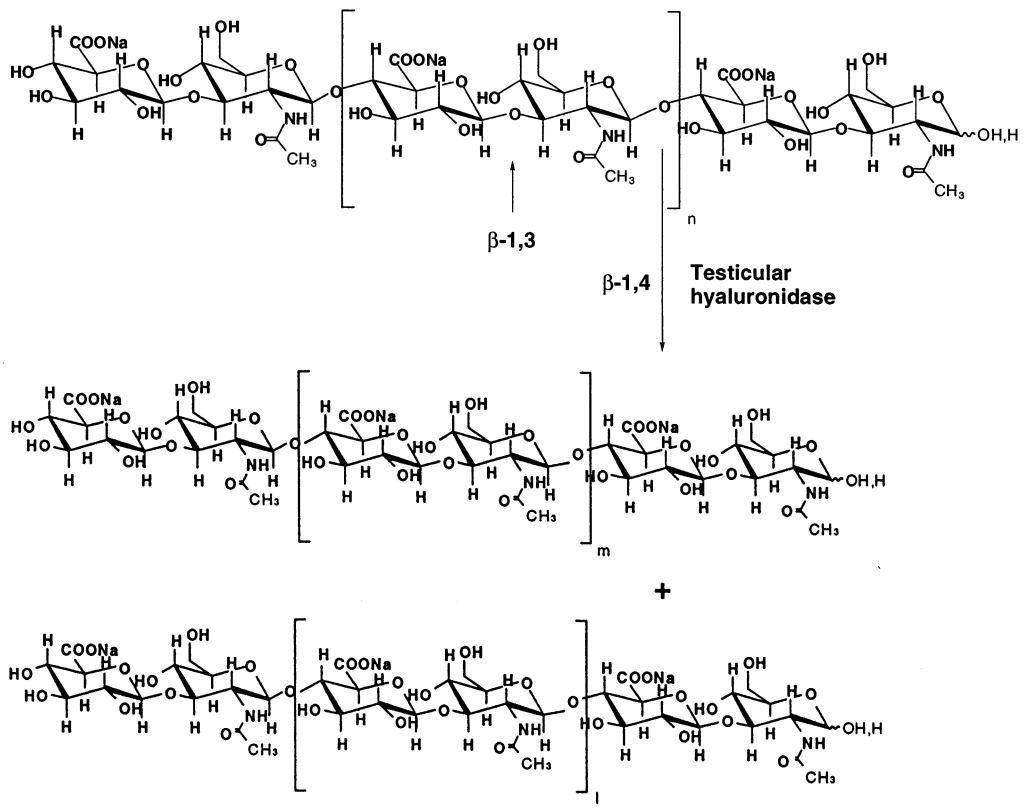

Fig. 1. Chemical structure of HA and the mode of hydrolysis by testicular HAase.

generates small oligosaccharide fragments of HA, which have been shown to possess angiogenic potential [26] and thus to enhance the metastatic potential of cancers [27–29].

HAase has a direct function on the fertilization process and an indirect effect, through the generation of HA oligosaccharides, on the angiogenic development of some tumors or the development of an inflammatory response. Thus, inhibitors of this enzyme could have potential as non-hormonal contraceptive agents [30–32] or as novel anti-angiogenic or anti-inflammatory compounds. A correlation between the activation or inhibition of HAase and the degranulation of mast cells has been reported [33–38]. Inhibitors of the enzyme prevented this degranulation and the activators of the enzyme stimulated the degranulation of the mast cells. Based on these studies, it was postulated that inhibitors of HAase could act as lead compounds for novel antiallergy drugs [33].

One aim of this study was to develop methods and techniques to generate HA oligosaccharides or mixtures of HA species of intermediate size (10^3–10^4 Da). These oligosaccharides and their chemical derivatives [39] would be valuable tools to probe the cell-biological and cell-physiological functions of HA in normal and disease states. Earlier investigations had shown that the total digestion of HA by HAase in the presence of varying amounts of $CaCl_2$ could lead to digestion endproducts with different average molecular mass [40]. Depending on the reaction conditions, e.g., $CaCl_2$ concentration, there was a limit to the average molecular mass of HA suitable for further digestion by HAase. We have extended these studies by investigating the effects of a broad range of other metal salts on the digestion of HA by HAase. We report herein an optimized approach to HA digestion to give oligosaccharides in a defined size range; our results also reveal aspects of HAase activity that are important in the discovery or design of inhibitors of this enzyme.

2. Results

Preincubation of HA with salts.—First, the effect of a broad range of metal chloride salts on the digestion of HA by HAase was evaluated.

Table 1
Enzymatic digestion of HA by HAase and the effect of the preincubation of HA with various chloride salts [a]

Preincubation of HA	Digestion after 24 h (%)	Digestion after 48 h (%)
Buffer	12	54
Divalent salts		
Ca^{2+}	76	96
Mg^{2+}	74	94
Mn^{2+}	75	96
Zn^{2+}	74	95
Ba^{2+}	69	95
Sr^{2+}	68	94
Co^{2+}	64	96
Ni^{2+}	66	97
Cu^{2+}	44	55
Monovalent salts		
Na^+	64	88
NH_4^+	68	90
Li^+	68	90
K^+	67	90

[a] HA (3.3 mg/mL) was preincubated with buffer or with 133 mM of the tested chloride salts. HAase was added to these mixtures such that the final concentrations were 2.5 mg/mL HA, 100 mM salt and 12.5 U/mL HAase (addition of 25 U). After 24 h of digestion, a sample was taken and 25 U of freshly-dissolved HAase was added. A second sample was taken 24 h after the second addition of enzyme (48 h total digestion time). The progress of the enzymatic digestions was evaluated using GPC as outlined in Section 4.

HA (3.3 mg/mL) was preincubated with buffer or with 133 mM of $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$, $SrCl_2$, $CoCl_2$ $NiCl_2$, $CuCl_2$, NaCl, NH_4Cl, LiCl, or KCl. HAase was dissolved in buffer and added to these HA/salt preincubation mixtures such that the final concentrations were 2.5 mg/mL HA, 100 mM salt, and 12.5 U/mL HAase (addition of 25 U). After 24 h of digestion, a sample was taken and treated for gel-permeation chromatography (GPC) analysis and 25 U of freshly dissolved HAase was added. After another 24 h a second sample was taken and treated for GPC analysis. The progress of these enzymatic digestions as determined by GPC analysis is shown in Table 1. No degradation of HA could be observed in the presence of the tested chloride salts when HAase was omitted from the reaction mixture.

Preincubation of HAase with selected salts.—To test for a direct effect of the salts on HAase, the enzyme was preincubated with selected salts and subsequently added to HA. In the first set

of experiments, HA (3.3 mg/mL) was preincubated for 20 min with 200 mM NaCl, and the enzyme (40 U; 50 U/mL) was preincubated with buffer without any added salts or with 100 or 400 mM NaCl, $CaCl_2$, $CoCl_2$, $ZnCl_2$, or $CuCl_2$. This HAase/salt preincubation mixture was then added to the HA/NaCl mixture such that the final reaction conditions were 2.5 mg/mL HA, 150 mM NaCl, 12.5 U/mL HAase (25 U), and 25 or 100 mM of the other salts. These reaction mixtures were incubated at 37 °C, and a sample was taken after 2 h. In the second set of experiments, the same reactions were performed but without the prior preincubation of HA with NaCl. The results of these two sets of experiments are presented in Fig. 2.

Total digestion of HA in the presence of selected salts.—Total enzymatic digestions of HA were performed in the presence of selected metal salts by continuous addition of freshly dissolved HAase until no shift in the GPC peak retention time of HA could be observed. HA (2.5 mg/mL) was preincubated for 6 h with 100 or 500 mM $CaCl_2$, $NiCl_2$, $CoCl_2$, or $CuCl_2$. HAase (25 U) was added such that the final reaction conditions were 2.5 mg/mL HA, 100 or 500 mM $CaCl_2$, $NiCl_2$, $CoCl_2$ or $CuCl_2$ and 5 U/mL HAase. The mixtures were kept at 37 °C, and every 12 h a sample was taken for GPC analysis; after aliquot removal, an additional 25 U of freshly dissolved HAase was added. Fig. 3 presents the time profiles of these enzymatic digestions. Table 2 presents an overview of the molecular masses of the end products obtained after these enzymatic digestions, as estimated by GPC analysis and by calibrating the columns with HA standards of known molecular mass. The intermediate or end products of the total digestion of HA in the presence of 500 mM $CuCl_2$ could not be determined as the dilution of the samples from this reaction mixture with the phosphate buffer for GPC analysis resulted in a co-precipitation of the HA with the insoluble Cu-phosphate salts. Partial co-precipitation of HA was also observed in some of the samples taken from the enzymatic reactions in the

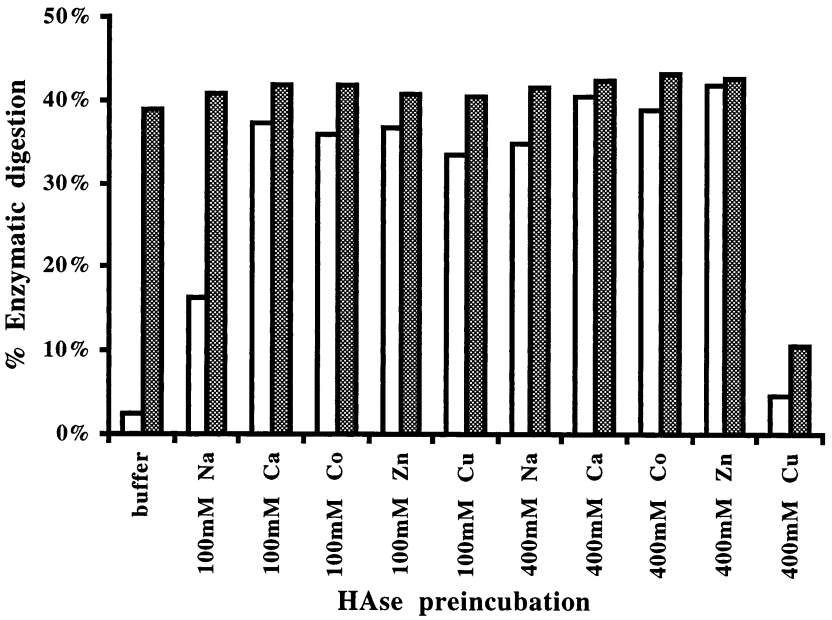

Fig. 2. Enzymatic digestion of HA by HAase and the effect of the preincubation of HAase with various chloride salts. HA (3.3 mg/mL) was preincubated with either buffer (open bars) or 200 mM NaCl (closed bars) and the enzyme (40 U; 50 U/mL) was preincubated with 0, 100, or 400 mM of the tested chloride salts. This HAase/salt preincubation mixture was added to the HA preincubation mixture such that the final reaction conditions were 2.5 mg/mL HA, 0 or 150 mM NaCl, 12.5 U/mL HAase (25 U) and 0, 25 mM or 100 mM of the other salts. The reaction mixtures were incubated at 37 °C, and a sample was taken after 2 h. The progress of the enzymatic digestion was evaluated by GPC as outlined in Section 4.

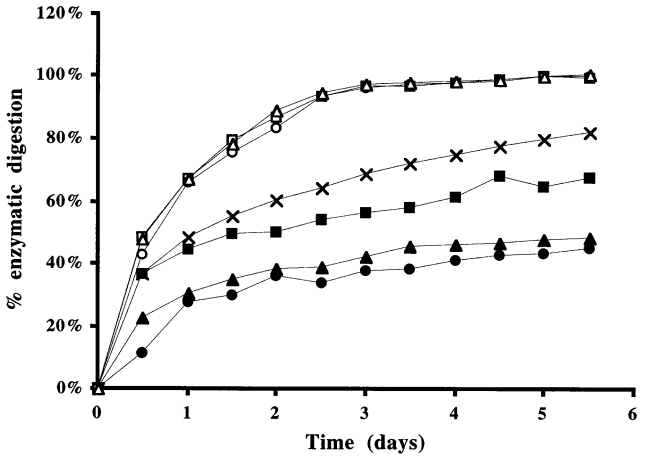

Fig. 3. Effect of the preincubation of HA with some selected salts on the total digestion of the polymer by HAase. HA (2.5 mg/mL) was preincubated with 100 mM $CaCl_2$ (□), $NiCl_2$ (○), $CoCl_2$ (△), $CuCl_2$ (×) or 500 mM $CaCl_2$ (■), $NiCl_2$ (●), $CoCl_2$ (▲). 25 U of HAase were added such that the final reaction conditions were 2.5 mg/mL HA, 100 or 500 mM $CaCl_2$, $NiCl_2$, $CoCl_2$ or $CuCl_2$ and 5 U/mL HAase. The mixtures were kept at 37 °C, and every 12 h a sample was taken for GPC analysis and 25 U of freshly dissolved HAase were added. Time profiles of the progression of the enzymatic reactions were evaluated by GPC and outlined in Section 4.

presence of 500 mM $NiCl_2$ or $CoCl_2$, especially in the initial stages of the degradation process.

Total digestion of HA in the presence of $CaCl_2$ and the production of HA oligosaccharides.—The total digestion of HA in the presence of $CaCl_2$ was investigated more extensively in order to obtain end products with different molecular mass distributions. HA was preincubated with $CaCl_2$ at varying ratios, and HAase was added until no changes in the peak retention times of the GPC profiles could be observed. The molecular mass distribution of the end products obtained was found to be dependent on the HA and $CaCl_2$ concentrations present in the enzymatic digestion mixtures. In these experiments, the ratio of the $CaCl_2$ concentration to the concentration of carboxylic functions on HA was correlated with the percentage of enzymatic digestion estimated from the GPC analyses (see Fig. 4). The concentration of carboxylic functions of HA was estimated by dividing the concentration of HA in mg/mL by 400, the molecular mass of the repeating unit of HA (see Fig. 1). A linear relationship could be observed ($r^2 = 0.914$), indicating that the end product of the total digestion of HA can be modulated by varying the ratio of the concentration of $CaCl_2$ to the HA concentration. Total digestion of HA by testicular HAase yields a mixture of oligosaccharides (HA_4, HA_6, and HA_8) as has been previously established [41]. At the lower ratios of $CaCl_2$ to HA carboxylic functions, the enzymatic digestions yielded similar profiles. The generation of HA oligosaccharides was evaluated by the analysis of these species using perfusion anion-exchange chromatography.

A typical chromatographic profile of a digested HA sample is shown in Fig. 5. By comparing the sample run with the blank run, it can be observed that multiple peaks were baseline separated. Fractions of peaks 2 and 3 were collected, desalted, and subjected to MALDI-MS analysis. Fig. 6(a, b) shows typical MALDI-MS profiles thus obtained. The main cluster of peaks in the MALDI-MS profile (Fig. 6(a)) obtained from peak '2' in Fig. 5 correspond to HA_6 as different glucuronate salts. A molecular mass of 1154.6 corresponds to HA_6 with three protonated glucuronic acids, 1176.8 corresponds to HA_6

Table 2
Total enzymatic digestion of HA by HAase in the presence of selected divalent cations and the average molecular mass of the endproducts [a]

Enzymatic digestion conditions	Average molecular mass of the end products
100 mM $CaCl_2$	mixture of oligosaccharides (≤4.0 kDa)
100 mM $NiCl_2$	mixture of oligosaccharides (≤4.0 kDa)
100 mM $CoCl_2$	mixture of oligosaccharides (≤4.0 kDa)
100 mM $CuCl_2$	6.0 kDa
500 mM $CaCl_2$	17.3 kDa
500 mM $NiCl_2$	80.0 kDa
500 mM $CoCl_2$	65.0 kDa
500 mM $CuCl_2$	n.d. [b]

[a] HA (2.5 mg/mL) was totally digested by repeated addition of 25 U HAase every 12 h (5 U/mL) for 5 days at 37 °C in the presence of 100 or 500 mM $CaCl_2$, $NiCl_2$, $CoCl_2$, or $CuCl_2$. The average molecular mass of the endproducts thus obtained was estimated using GPC analysis after calibration with HA standards of known molecular mass.

[b] Not determined: end products co-precipitated with Cu-phosphate during sample preparation for GPC analysis.

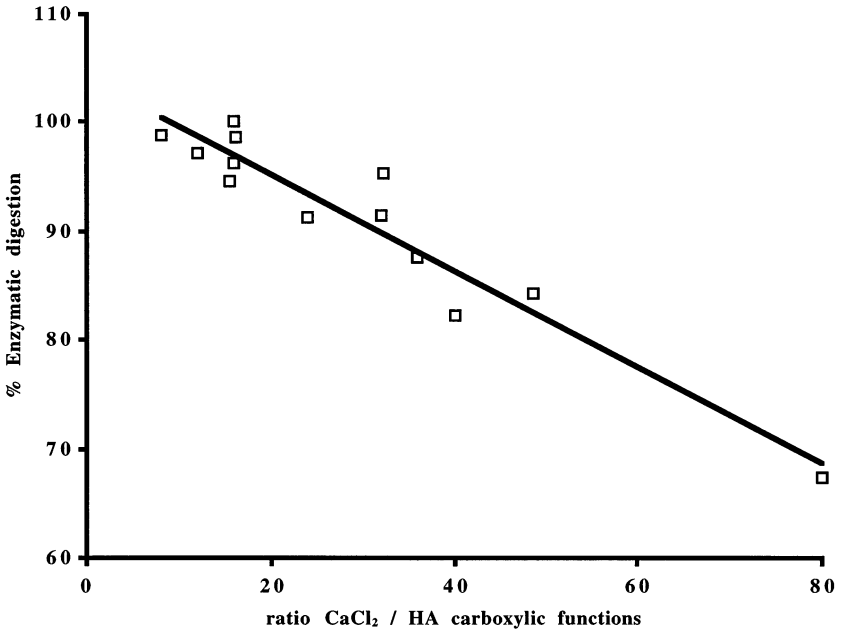

Fig. 4. Relationship between the end products of the total enzymatic digestion of HA by HAase and the ratio of the concentration of $CaCl_2$ to the concentration of carboxylic functions of HA present in the reaction mixture. HA, dissolved at various concentrations, was preincubated with $CaCl_2$ at various ratios, and HAase was added to these mixtures until no shifts in the peak retention times of the GPC profiles could be observed. The concentration of carboxylic functions on HA was estimated by dividing the concentration of HA in mg/mL by 400, the molecular mass of the repeating unit of HA. The progress of the enzymatic digestions was evaluated using GPC as outlined in Section 4.

with two protons and one sodium atom, and 1198.7 corresponds to HA_6 with one proton and two sodium atoms. Similarly, the main peaks in the MALDI-MS profile (Fig. 6(b)) obtained from peak '3' in Fig. 5 correspond to HA octasaccharide or HA_8. A molecular mass of 1533.8 corresponds to HA_8 with four protons, 1555.8 corresponds to HA_8 with three protons and one sodium atom, 1577.8 corresponds to HA_8 with two sodium atoms, and 1198.7 corresponds to HA_8 with three sodium atoms. Only minor impurities of other oligosaccharide fractions could be observed. By extrapolation of these results, it follows that the sequence of peaks observed in Fig. 5 corresponds to a well-resolved series of HA oligosaccharides consisting of HA_4 (peak 1), HA_6 (peak 2), HA_8 (peak 3), HA_{10} (peak 4), HA_{12} (peak 5), HA_{14} (peak 6), HA_{16} (peak 7), HA_{18} (peak 8), and so on.

Inhibition studies with EGTA.—HA (1 mg/mL) was dissolved in acetate buffer containing 25 mM $CaCl_2$ and preincubated with 0, 10, 30, or 60 mM EGTA-Na_4. After the addition of HAase (3.8 U/mL) the reactions were incubated at 37 °C for 2 h. The enzymatic activity was evaluated using GPC analyses and compared with similar experiments in which the HA/$CaCl_2$ mixtures were preincubated with 40, 120, or 240 mM NaCl. The addition of NaCl to the reaction mixtures increased the enzymatic activity by 4–5% relative to reactions containing only HA and $CaCl_2$. The presence of 10 mM EGTA-Na_4 increased the enzymatic activity by 13%. The addition of 30 or 60 mM EGTA-Na_4 decreased the enzymatic activity by 63% and 100%, respectively. A similar pattern, i.e., activation at concentrations *below* the added [$CaCl_2$] and inhibition at concentrations *above* the added [$CaCl_2$], was observed with EDTA-Na_4 (results not shown); however, inhibition by EDTA-Na_4 was always lower than that observed for EGTA-Na_4. Preincubation of HAase with similar concentrations of EGTA-Na_4 did not affect the enzymatic activity (results not shown).

3. Discussion

Earlier studies on the degradation of HA by HAase have shown that this process has an absolute requirement for cations, but the ef-

fects of these added cations are always described in terms of an activation of HAase [16,33]. We tested the effects of a broad range of metal salts by preincubating them with either HA or with HAase prior to the enzymatic reaction. Our results confirm that the degradation of HA by HAase is enhanced by the presence of mono- and divalent cations. When no extra salt is added to the reaction mixture, the digestion appears to proceed very slowly (see Table 1). Only after the addition of a second aliquot of enzyme could significant digestion of the polymer be detected. This might indicate that in the presence of only the buffer salts the digestion of HA proceeds at different velocities with an acceleration of the digestion after some initial degradation has occurred.

NaCl and $CaCl_2$ are most frequently used as the activators of the enzyme. However, it was surprising that of the other monovalent and divalent salts tested (see Table 1), only $CuCl_2$ showed significant inhibition and none showed higher activation. The digestion seemed to proceed somewhat slower in the presence of monovalent cations compared with the group of enzymatic reactions in the presence of divalent cations. When compared with the digestion in the presence of the other divalent salts, $CuCl_2$ seems to inhibit the digestion of HA by HAase, but when compared with the digestion of HA in the absence of any salt added, $CuCl_2$ still seems able to activate the digestion of HA by HAase.

When HA was preincubated with NaCl (Fig. 2, closed bars) the preincubation of the enzyme with 100 or 400 mM NaCl, $CaCl_2$, $CoCl_2$, $ZnCl_2$, or $CuCl_2$ had no effect on the activity of the enzyme, except when the enzyme was preincubated with 400 mM $CuCl_2$. Surprisingly, the enzyme was not affected by the presence of these salts ($CuCl_2$ being an exception). Similarly, the enzyme could be mixed with Me_2SO up to a concentration of 20% v/v without any effect on the enzymatic activity (results not shown). When HA was not preincubated with any salt (Fig. 2, open bars) and the only salt added to the digestion mixtures was the salt preincubated with HAase, the enzymatic reactions consistently proceeded slower than in the previous case. In this context, it is worth mentioning that the reproducibility of the assay, expressed by the relative standard deviation, was found to be

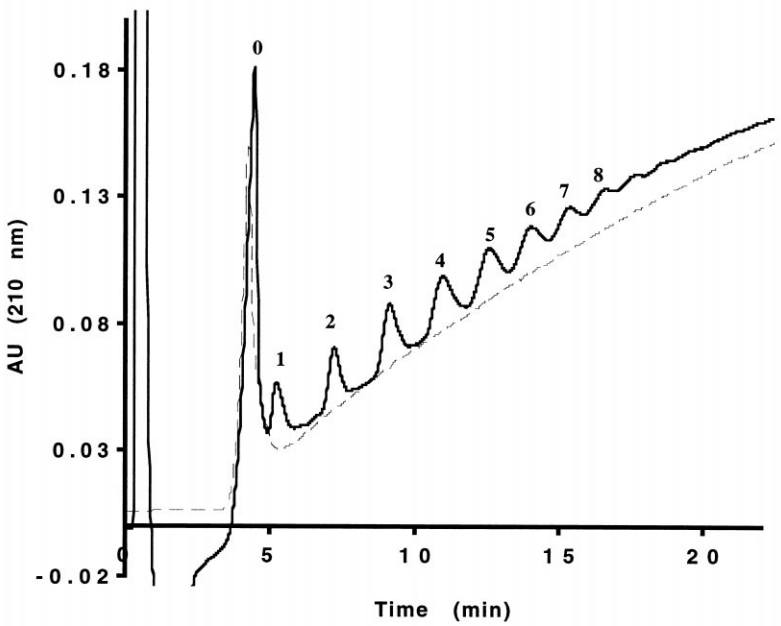

Fig. 5. Perfusion anion-exchange chromatography of HA oligosaccharides as outlined in Section 4 (dashed line = blank; solid line = sample). The drifting baseline seen in all our blank and sample analyses is a reflection of the NaCl gradient as observed by monitoring the UV absorbance at 210 nm. All profiles, blanks and samples, contained a peak, designated as '0', believed to be NaOAc. By extrapolation of the results from Fig. 6(a, b), the sequence of peaks corresponds to HA_4 (peak 1), HA_6 (peak 2), HA_8 (peak 3), HA_{10} (peak 4), HA_{12} (peak 5), HA_{14} (peak 6), HA_{16} (peak 7), HA_{18} (peak 8).

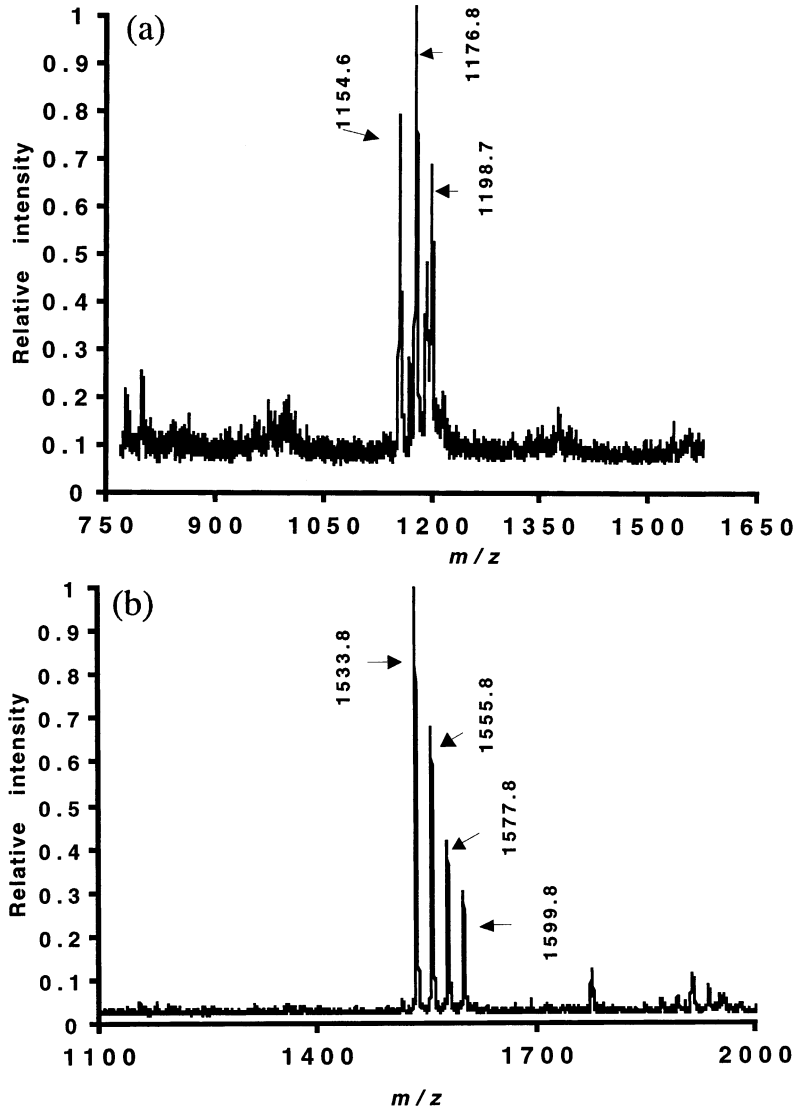

Fig. 6. MALDI-TOF spectra of HA oligosaccharides purified by perfusion anion-exchange chromatography (peaks 2 and 3 in Fig. 5, respectively) as outlined in Section 4. (a) The peaks obtained from peak '2' in Fig. 5 correspond to HA hexasaccharide or HA_3 (1154.6 corresponds to HA_3 with three glucuronic acids, 1176.8 corresponds to HA_3 with two glucuronic acids and one Na-glucuronate and 1198.7 corresponds to HA_3 with one glucuronic acid and two Na-glucuronates). (b) The peaks obtained from peak '3' in Fig. 5 correspond to HA octasaccharide or HA_4 (1533.8 corresponds to HA_4 with four glucuronic acids, 1555.8 corresponds to HA_4 with three glucuronic acids and one Na-glucuronate, 1577.8 corresponds to HA_4 with two glucuronic acids and two Na-glucuronates and 1198.7 corresponds to HA_4 with one glucuronic acid and three Na-glucuronates).

2.3% ($n = 4$). These observations suggest that the activating influence of the cations on the degradation of HA by HAase occurs through an activation of HA rather than of HAase. The addition of the cations to HA may change the conformation of the polymer such that more endoglucanase sites are exposed, thus facilitating hydrolysis by the enzyme. Studies have shown that cations such as Na^+, K^+, or Ca^{2+} can change the conformation of HA in solution, leading to a variety of possible conformations the polymer can adopt [42–44]. The observed uniformity of the enzymatic reactions when HA was preincubated with NaCl prior to digestion with HAase (Fig. 2, closed bars) is probably due to the dominating effect of this preincubation of HA with NaCl. The variations observed when HA was not preincubated with any salt (Fig. 2, open bars) are likely due to the fact that some equilibration between HA and the salt added from the HAase preincubation needed to be reached, and that this equilibration process depended on the type and amount of cation added.

At 100 mM, $CuCl_2$ seems to have a dual effect on the hydrolysis of HA by HAase (Table 1). No direct inhibiting effect of this metal on the enzyme could be observed at a concentration of 100 mM (Fig. 2). With 100 mM $CuCl_2$, the average molecular mass of the end product of the total digestion of HA (Fig. 3 and Table 2) was higher compared to the end products obtained with 100 mM $CaCl_2$, $NiCl_2$, or $CoCl_2$, suggesting that $CuCl_2$ limits the degradation of HA by HAase. These combined observations lead us to conclude that the mixed effect of $CuCl_2$ on the hydrolysis of HA by HAase is due to a mixed effect on HA. We propose that after partial hydrolytic cleavage of the polymer, the resulting intermediate-sized HA complexes with divalent cations in such a way as to affect its further digestion by HAase. This could depend on the type and amount of salt present, since different end products were obtained from the total digestion of HA in 500 mM $CaCl_2$, $NiCl_2$, or $CoCl_2$ compared with the end products obtained in 100 mM $CaCl_2$, $NiCl_2$, or $CoCl_2$ (Fig. 3 and Table 2). It is reasonable to propose that the type and the concentration of cations present could permit HA to adopt conformations that facilitate or prevent its hydrolysis by HAase. For example, Payan and co-workers have studied the digestion of HA by *Streptomyces* HAase as a function of temperature and source of HA and suggested that the enzymatic degradation depended on the conformation of HA [41]. Fibroblasts derived from human lung or human uterine cervix tissue have been shown to degrade HA in a limited fashion, stopping at a molecular mass of 40 kDa rather than proceeding to the usual oligosaccharides [45,46]. Recently, Lepperdinger et al. reported on the existence of a lysosomal HAase incapable of digesting HA fragments with a molecular mass of 20 kDa [47]. They reasoned that there are domains present in HA that are resistant to this type of HAase. The data herein support the notion that the occurrence of such domains could depend on the amount or type of cation present in the environment of HA.

The total digestion of HA in the presence of 100 mM $CaCl_2$, $NiCl_2$, or $CoCl_2$ yielded identical time profiles and end products (Fig. 3 and Table 2). Different end products were obtained from the enzymatic digestions in 100 mM $CuCl_2$ or 500 mM $CaCl_2$, $NiCl_2$, or $CoCl_2$ (Fig. 3 and Table 2). Thus, the molecular mass distribution of the HA species present after total digestion by HAase could be modulated depending on the amount and type of salt present. Similar interpretations can be made from the digestion results obtained by varying the ratio of $CaCl_2$ to the amount of HA in the digestion mixture (Fig. 4). The average molecular mass of the end products obtained by total digestion of the polymer by HAase clearly depended on this ratio. This observation could be used to facilitate the controlled production of HA species with intermediate average molecular mass (10–100 kDa). In addition, use of perfusion anion-exchange chromatography allows the fractionation of individual oligosaccharide species of HA from a digestion mixture. Our fractionation of HA oligosaccharides and their characterization by MALDI-TOF analysis are comparable to earlier studies. Price et al. studied the digestion of HA by bacterial HAase. The oligosaccharides were fractionated using high-performance anion-exchange chromatography and analyzed using electrospray-ionization mass spectrometry [48]. Monodisperse HA oligosaccharides and their derivatives would be valuable tools in the study of the cell-biological and cell-physiological properties of this biopolymer.

The observation that HA can be partially or totally co-precipitated with Ni^{2+}, Co^{2+}, or Cu^{2+} when phosphate salts are added is an indication that HA has metal-chelating properties. Recently, the complexes formed between HA and Cu^{2+} or Zn^{2+} have been investigated using EXAFS and XANES measurements [49]. Both metals were found to be coordinated via carboxylate groups and oxygen atoms on HA. With Cu^{2+}, the complex adopted a distorted octahedral structure, while the Zn^{2+} atoms showed tetrahedral coordination. The metal-chelating properties of HA could have important implications in the search for inhibitors of HAase. The inhibition of this enzyme is often investigated using $CaCl_2$ as an activating agent [33–37,50–52]. From our experiments it follows that any compound with Ca^{2+}-chelating properties might act as an inhibitor of the degradation of HA by HAase in the presence

of $CaCl_2$ as we have shown for EGTA-Na_4. It is therefore important, when screening for potential HAase inhibitors, to be cognizant that metal chelators may reduce apparent enzyme activity via this effect on the substrate itself.

Although the concentrations of HA and salts used in our experiments are nonphysiological, it is tempting to speculate about any physiological functions of HA as a metal-chelator. Upon binding to its cell-surface receptor and consequent internalization, HA could transport cell-biologically relevant divalent cations such as Ca^{2+}, Mg^{2+}, or Zn^{2+} inside the cell. Cell-surface HA receptors such as CD44 or RHAMM have been found to be linked to signal transduction pathways. The binding of oligosaccharide fragments of HA to CD44 receptors on endothelial cells induces protein tyrosine kinase activity leading to the activation of a cytoplasmic cascade [53]. In human peripheral blood lymphocytes, CD44 was shown to be associated with Src family kinases such as Lck and Fyn [54]. Similarly, in T-lymphocytes CD44 signaling was mediated through tyrosine kinases [55]. RHAMM isoforms present on the cell surface and in the cytoplasm are involved in regulating extracellularly regulated kinase (ERK) activity, and the intracellular isoform was found to act downstream of Ras [56]. In addition, RHAMM has been found to be involved in focal adhesion turnover [57,58]. Several studies have shown that low-molecular-mass HA induces the expression of multiple genes (inflammatory genes, nitric oxide synthase, ICAM-1, and VCAM-1) involving the activation of NF κB [11–14,59]. The cytoplasmic tail of CD44 is linked to cytoskeleton proteins such as ankyrin, and this interaction mediates the effects of binding with HA on the adhesion to the ECM, migration or transformation of cells [60–62]. It is tempting to speculate that the cell-biological activities induced by the interaction of HA with its cell-surface receptor is in part due to the accompanying influx of Ca^{2+} with the internalization of HA.

4. Experimental

Materials. — Fermentation-derived HA (Crystalhyal) was provided by Clear Solutions Biotechnology, Inc., a member of The Collaborative Group (Stony Brook, NY). Bovine testicular HAase type IV-S (EC 3.2.1.35; partially purified to a specific activity of 1100 U/mg) and EGTA tetrasodium salt (EGTA-Na_4) were obtained from Sigma Chemical (St. Louis, MO). HA standards of known molecular mass were kindly donated by Dr Ove Wik (Pharmacia & Upjohn, Uppsala, Sweden). All other chemicals used were of analytical grade.

Gel-permeation chromatography.—GPC was performed on two Ultrahydrogel columns 2000 and 250 (Waters, Milford, MA) connected in series to a chromatography system (Waters, Milford, MA) consisting of a pump (model 515), a differential refractometer (model 410), and a tunable UV detector (model 486) set at 210 nm. The mobile phase consisted of a mixture of phosphate buffer (100 mM NaH_2PO_4–50 mM Na_2HPO_4; pH 6.4) and methanol at a ratio of 80:20 v/v. The flow rate was 0.5 mL/min. All samples were diluted to a concentration of 0.3 mg/mL.

Enzymatic reactions.—All solutions (HA, salts, HAase) were dissolved in acetate buffer (5 mM NaOAc, 70 mM acetic acid; pH adjusted to 3.75). The preincubations of HA with any salts were kept at rt for 6 h, and the preincubations of the enzyme with any salts were kept at rt for 20 min. All enzymatic reactions were performed at 37 °C. Samples of the enzymatic reaction mixtures were diluted with GPC mobile phase to a final HA concentration of 0.3 mg/mL and kept at −30 °C until GPC analysis. Before GPC analysis the samples were filtered to remove the precipitated phosphate salts present. All enzymatic digestions were evaluated based on the peak retention time of the UV signal of HA during the various stages of the enzymatic reaction. The time difference between the peak retention time of undigested HA ($T_{r,0}$) and the peak retention time of totally digested HA ($T_{r,\infty}$) was taken as 100% enzymatic digestion. Totally digested HA was generated by addition of fresh enzyme until the GPC retention time of HA did not shift further. The time difference between $T_{r,0}$ and any intermediate HA peak retention time ($T_{r,x}$) was taken as a measure of the intermediate progress of the enzymatic digestion reaction. This is illustrated in the following equation:

$$\% \text{ Enzymatic digestion} = \frac{T_{r,x} - T_{r,0}}{T_{r,\infty} - T_{r,0}}$$

Anion-exchange perfusion chromatography.—Perfusion chromatography was performed using the BioCAD™ Sprint™ Perfusion Chromatography® System (Perseptive Biosystems, Framingham, MA) on an HQ/M Poros strong anion-exchange column (Perseptive Biosystems) with a flow rate of 5 mL/min and using UV detection at 210 nm. The column was equilibrated for 3 min with a 4:1 mixture of water–20 mM NaOAc (v/v) and, after injection of the sample, eluted with the same solvent mixture for an additional 2 min. HA oligosaccharides were eluted from the column with a 20 min NaCl gradient to a final 4:1 solvent mixture containing 250 mM NaCl–20 mM NaOAc (v/v). This solvent mixture was held for an additional 3 min, at which time the solvent mixture was restored to its initial conditions.

Mass spectrometry analyses.—All MALDI-TOF spectra were recorded at The University of Utah Mass Spectrometry Facility on a Voyager DE-STR instrument (Perseptive Biosystems) fitted with a 337 nm nitrogen laser and a Tektronix (Beaverton, OR) TDS-540C 2 GHz digitizing oscilloscope. The matrix was 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI), 10 mg/mL in water. Samples were dissolved in water at approximately 10 μM, and 1 μL of sample was mixed with 2 μL of matrix. Aliquots of the mixed sample and matrix (0.5–1.0 mL) were spotted on flat gold-plated sample holders using the co-crystallization method and allowed to evaporate under ambient conditions. Externally calibrated negative ion mass spectra were acquired in the linear mode of operation at 1 ns resolution, and more than 100 individual laser shots were summed. The accelerating potential was 20 kV, and the extraction delay was 200 ns.

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