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Effects of Ascorbic Acid and Analogs on the Activity of Testicular Hyaluronidase and Hyaluronan Lyase on Hyaluronan

ODILIA N. OKORUKWU and KOEN P. VERCRUYSSE*

Chemistry Department, Tennessee State University, 3500 John A. Merritt Blvd., Nashville, TN 37209, USA

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We have evaluated the inhibition of testicular hyaluronidase and hyaluronan lyase by L-ascorbic acid and chemical analogs. We observed that L-ascorbic acid, D-isoascorbic acid and dehydroascorbic acid inhibited both types of enzymes, but showed stronger effects towards hyaluronan lyase. But these compounds were observed to degrade the substrate, hyaluronan, by themselves. Of the other ascorbic acid analogs tested, saccharic acid inhibited hyaluronan lyase, while not affecting the enzymatic activity of testicular hyaluronidase, nor affecting the physic-chemical stability of hyaluronan. This is the first compound, to our knowledge, to be shown to possess such selective inhibition. Therefore, we propose that saccharic acid could serve as a lead compound for the development of potent and selective inhibitors of bacterial hyaluronan lyase or of polysaccharide lyase enzymes in general as we observed this compound to be capable of inhibiting chondroitinase ABC in addition to hyaluronan lyase.

Keywords: Hyaluronidase; Hyaluronan lyase; Ascorbic acid analogs; Inhibition

INTRODUCTION

Hyaluronan (HA), a naturally occurring linear polysaccharide comprising of β-(1 → 4)-linked D-glucuronic acid β-(1 → 3)-*N*-acetyl-D-glucosamine disaccharide units (see Figure 1), is ubiquitously present in the extracellular matrix (ECM) of all higher animals, especially in soft connective tissues.[1] HA serves many passive biological functions (providing structural support or hydration of tissues) but possesses also active cell-biological functions, e.g., in cancer development, cell motility or embryonic development.[1] Some of these cell-biological functions depend on the molecular mass of the HA molecule involved. Low-molecular mass (<200 kDa) fragments of HA have been shown to be angiogenic and pro-inflammatory, properties not attributed to the high molecular mass HA molecules (1–2 Mda).[1] Low molecular mass fragments of HA are generated through the action of HA degrading enzymes, the hyaluronidases (HAses). Just as for its substrate, HAses are ubiquitously found in nature.[2] A mechanistic distinction can be made between HAses produced by mammalian cells and by bacteria. Bacterial HAse (EC 4.2.2.1; hyaluronate lyase) is a so-called lyase as it hydrolyzes HA through an elimination process. The mammalian type of HAse, e.g., testicular HAse, (EC 3.2.1.35; hyaluronate-4-glycanohydrolase) acts upon its substrate through a hydrolysis mechanism and is considered a hydrolase.[3] This difference in enzyme mechanism yields mixtures of chemically distinct HA oligosaccharides generated from the HA parent molecule (see Figure 1). The oligosaccharides obtained following degradation with bacterial HAse contain Δ-4,5 unsaturated glucuronic acid residues at their non-reducing end. Apart from bacterial HAse, other bacterial polysaccharide degrading enzymes, e.g., chondroitinase or pectin lyase, act upon their substrates using the "lyase" mechanism.[4] Eukaryotic polysaccharide degrading enzymes generally act upon their substrates using the "hydrolase" mechanism.[3] Mio and Stern recently presented a survey of the literature

*Corresponding author. Tel.: +1-615-963-5340; Fax: +1-615-963-5326; E-mail: kvercruysse@tnstate.edu

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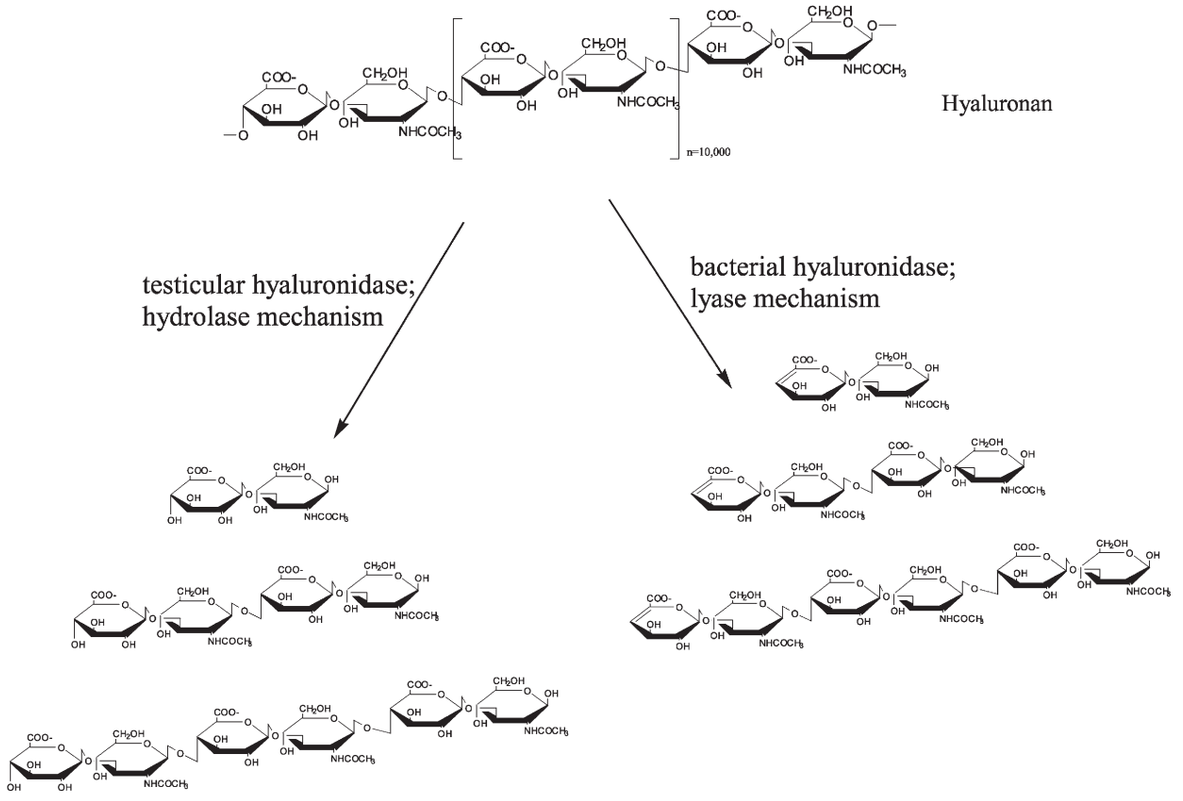

FIGURE 1 Chemical structures of hyaluronan polysaccharide and oligosaccharides. Chemical structures of hyaluronan oligosaccharides generated by testicular hyaluronidase using a hydrolase mechanism or generated by bacterial hyaluronidase using a lyase mechanism.

concerning HAse inhibitors.[5] Polyanionic substrate analogues like heparin or heparan sulfate, some cyclooxygenase inhibitors, certain antiallergic agents and various plant-derived compounds (flavonoids, hydrangenols, curcumins) all have been reported as HAse inhibitors.[5] However, these studies involved only testicular HAse and the methodology used to evaluate some of the inhibitory properties has been criticized for producing potential false positive results.[6] Li *et al.* reported the inhibition of HA lyase from *Streptococcus pneumoniae* by ascorbic acid but did not report on the potential inhibition of testicular HAse by this compound.[7] Furthermore, the evaluation of ascorbic acid as a potential HAse inhibitor is complicated by the fact that this compound is capable of degrading HA by itself.[8] Based upon these earlier reports and using established methodology,[9,10] we evaluated the effects of ascorbic acid, compound **(1)**, and commercially available analogs of **(1)** (see Figure 2) on the physico-chemical stability of HA and the enzymatic activity of both testicular HAse and HA lyase from *Streptomyces hyalurolyticus*. We observed that D-saccharic-1,4-lactone, compound **(7)** in Figure 2, inhibits HA lyase from *Streptomyces hyalurolyticus* in a concentration-dependent fashion but has little to no effect on the enzymatic activity of testicular HAse or the physico-chemical stability of HA. The significance of this finding is discussed.

MATERIALS AND METHODS

Materials

Hyaluronan (sodium salt from *Streptococcus zooepidemicus*), bovine testicular hyaluronidase (Type I-S; 359 Units/mg), hyaluronate lyase (from *Streptomyces hyalurolyticus*; 324 Units/amp), L-ascorbic acid, D-isoascorbic acid, dehydroascorbic acid, L-gulonic-γ-lactone, D-ribonic-γ-lactone, D-gulonic-γ-lactone, D-saccharic-1,4-lactone and α-D-glucoheptonic-γ-lactone were obtained from Sigma Chemical Co (St Louis, MO). All other chemicals and solvents used were of analytical grade.

Gel Permeation Chromatography (GPC)

GPC analyses were performed on a Varian Prostar chromatography system (Walnut Creek, CA) using a Waters Ultrahydrogel 2000 column (Milford, WA). Analyses were performed at room temperature with a mobile phase consisting of 50 mM NaCl:methanol

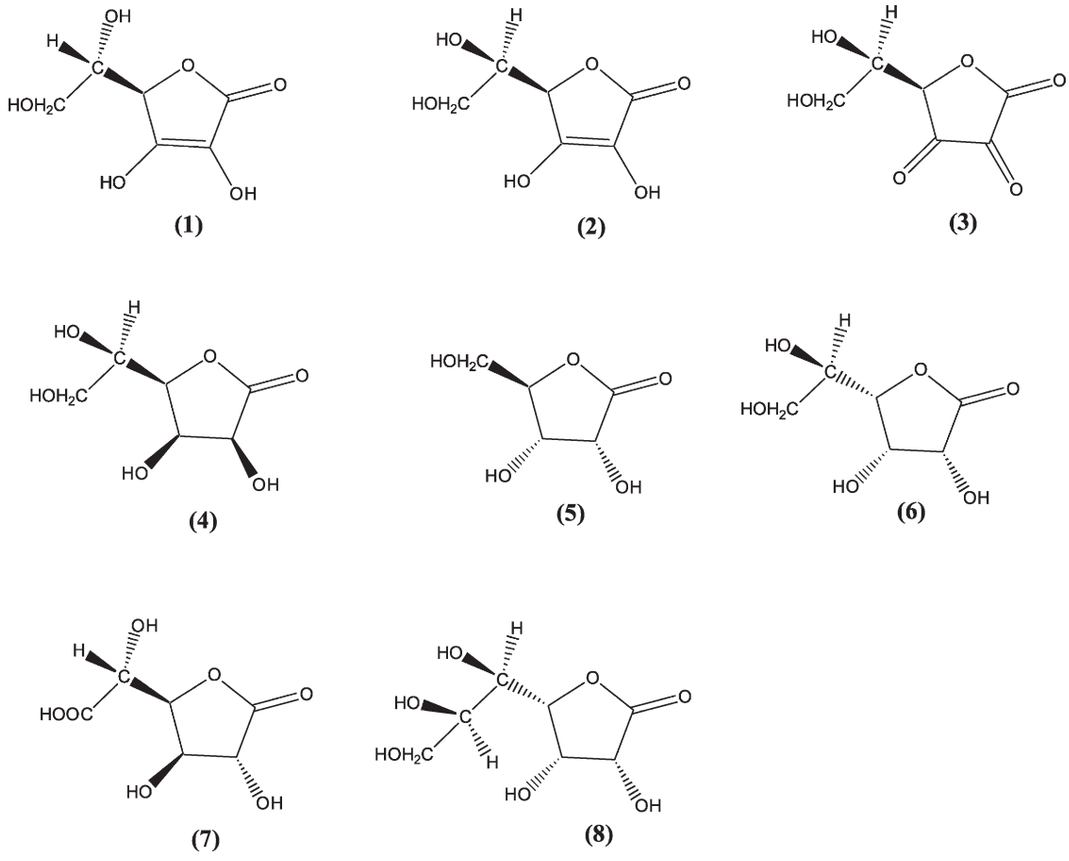

FIGURE 2 Chemical structures of the test compounds used in this study. **(1)** L-ascorbic acid (= Vitamin C), **(2)** D-isoascorbic acid, **(3)** dehydroascorbic acid, **(4)** L-gulonic-γ-lactone, **(5)** D-ribonic-γ-lactone, **(6)** D-gulonic-γ-lactone, **(7)** D-saccharic-1,4-lactone and **(8)** α-D-glucoheptonic-γ-lactone.

(9:1) at a flow rate of 0.75 mL/min and monitored using UV-detection at 210 nm. The sample injection volume was 20 μL.

Stability Studies

The physico-chemical stability of HA (final concentration 1 mg/mL) was studied in the enzyme assay buffer in the presence of various concentrations of the test compounds at room temperature for various periods of time. The extent of HA degradation by the test compounds was evaluated by comparing the GPC retention time of HA stored in the absence of any test compound (Tr,o) to the retention time of HA stored in the presence of the test compound (Tr,x).

Enzyme Assays

All enzyme assays were performed in a 5 mM Na-acetate buffer (pH = 6.5) containing 140 mM NaCl. HA mixtures (final concentration 1 mg/mL) were incubated with various concentrations of the test compound dissolved in the assay buffer. To these mixtures either testicular HAse (final concentration 3.5 Units/mL) or bacterial HA lyase (final concentration 2.5 Units/mL) was added. The samples were left at room temperature for 45 min and stored at −20°C till GPC analysis. The difference between the GPC retention time of undegraded HA (Tr,o) and the GPC retention time of HA following enzymatic treatment (Tr,x) was taken as a measure of the enzymatic activity.

RESULTS

Figure 3 compares the GPC retention times of HA following 48 hours exposure to 1 mM of compounds **(1)**–**(8)** at room temperature to the GPC retention time of HA kept at room temperature for 48 hours in the absence of any additional compound. The exposure to 1 mM compounds **(1)**–**(3)** increased the retention time of HA, indicating a reduction in the size of the HA molecule, while the exposure to 1 mM of compounds **(4)**–**(8)** had little to no effect on the GPC retention time of HA. Figure 4 presents the effects of compounds **(1)**–**(8)** on the enzymatic

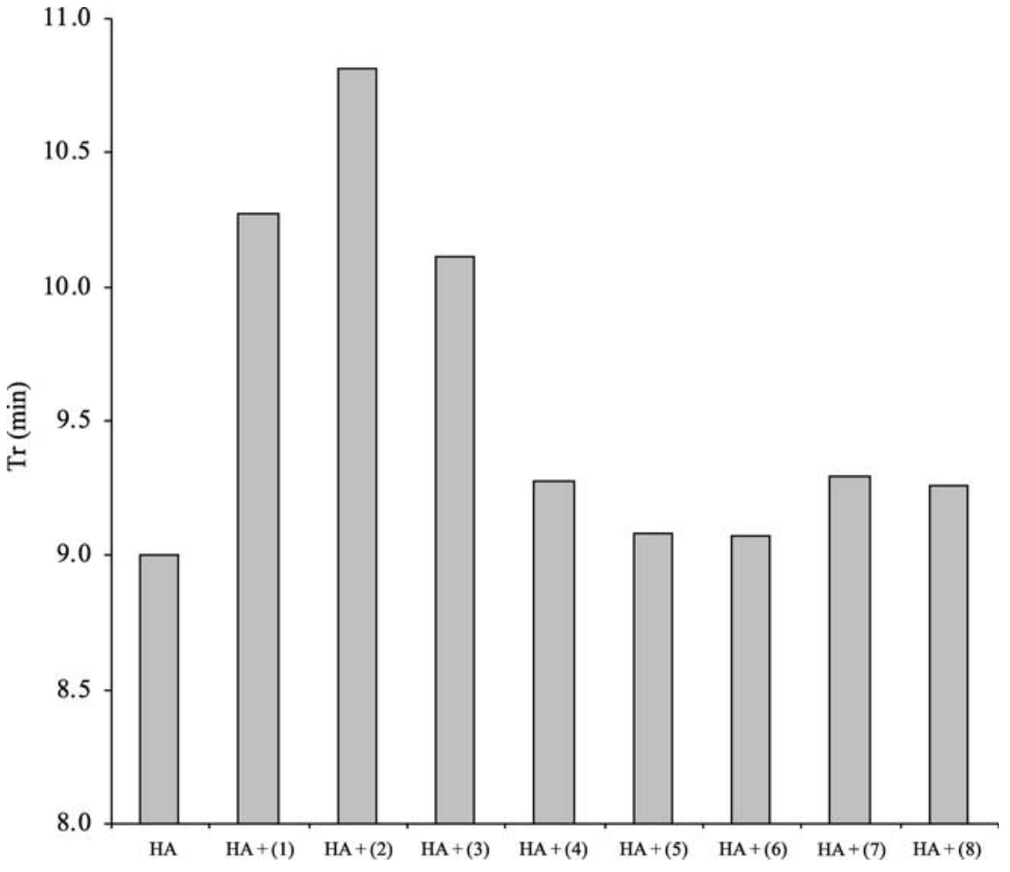

FIGURE 3 Effect of the test compounds on the physico-chemical stability of hyaluronan. Comparison of the GPC retention times (Tr in min) of an HA solution (1 mg/mL) prepared in 5 mM Na acetate buffer containing 140 mM NaCl stored at room temperature for 48 h and following exposure to1 mM compounds **(1)–(8)** at room temperature for 48 h.

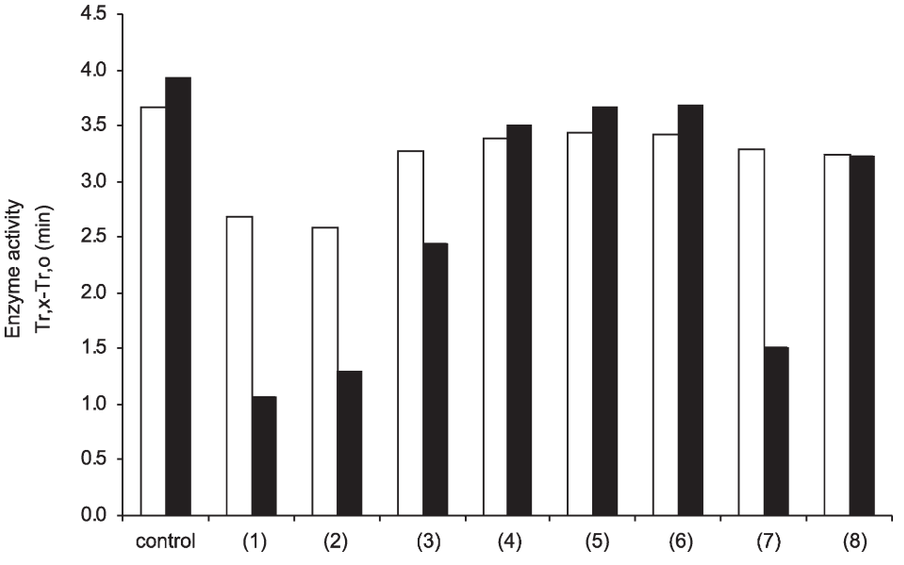

FIGURE 4 Effect of test compounds on the enzymatic activity of testicular hyaluronidase or hyaluronan lyase. Difference in GPC retention time of undegraded HA (Tr,o in min) and HA following enzymatic hydrolysis by testicular hyaluronidase (open bars) or hyaluronan lyase (closed bars) in the absence (control) or presence of 1 mM compounds **(1)–(8)** (Tr,x in min). The enzymatic reactions were performed and evaluated as described in Materials and Methods.

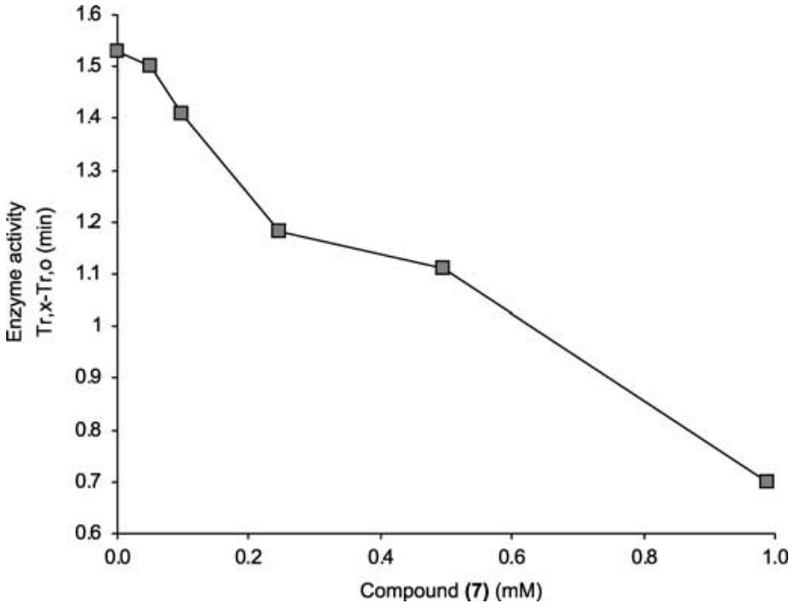

FIGURE 5 Concentration-dependent inhibition of hyaluronan lyase by saccharic acid. Difference in GPC retention time of undegraded HA (Tr,o in min) and HA following enzymatic hydrolysis by hyaluronan lyase in the absence or presence of various concentrations of compound **(7)** (Tr,x in min). The enzymatic reactions were performed and evaluated as described in Materials and Methods.

activity of both testicular and bacterial HAse. At 1 mM, compounds **(1)** and **(2)** inhibited both types of enzymes but showed stronger inhibitory capacity towards the bacterial type of enzyme. At 1 mM, compound **(3)** showed no inhibition towards testicular HAse and only weak inhibition towards bacterial HAse. At 1 mM, compound **(7)** did not inhibit testicular HAse but exhibited over 50% inhibition of the bacterial type of enzyme. At 1 mM compounds **(4)**, **(5)**, **(6)** or **(8)** exhibited no inhibition towards either enzyme. In addition, the activity of both enzymes was evaluated in the presence of 10 mM of compounds **(4)**, **(5)**, **(6)** or **(8)**. At this concentration, the compounds did not inhibit testicular HAse but exhibited weak inhibition towards bacterial HAse (results not shown). Figure 5 presents the concentration-dependent inhibition of bacterial HAse by compound **(7)**. This compound exhibited a concentration-dependent inhibition of the bacterial type of HAse in a concentration range between 0.1 and 1 mM. In addition, it was observed that at a concentration of 0.5 mM compound **(7)** exhibited over 50% inhibition of chondroitinase ABC acting upon chondroitin sulfate C as the substrate as evaluated by GPC (results not shown).

DISCUSSION

Compound **(7)** or saccharic acid is the first compound, to our knowledge, to be shown to possess selective inhibition towards bacterial HAse, while not affecting the enzymatic activity of testicular HAse. In addition, this compound does not affect the physico-chemical stability of the HA molecule as ascorbic acid does. We propose that saccharic acid might function as a model compound to develop potent and selective inhibitors of bacterial HA lyase. In addition, in view of the capacity of saccharic acid to inhibit bacterial chondroitinase, we propose this compound as a model inhibitor of lyase enzymes in general. Inhibitors of bacterial HA lyase might serve as novel antibacterial agents and, so, alternative to the antibiotics. Many bacteria, including various *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Propionibacterium*, *Streptomyces* or *Clostridium* species, have been demonstrated to produce HA lyase.[11] The production of HA lyase by these bacteria has been recognized as a spreading factor: enhancing the bacteria's potential to invade the tissues of its host or enhancing the spreading of the toxins produced by the bacteria.[11]

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