Short Communication

Effect of Gluco-Monosaccharides and Different Conditions on Digestion of Hyaluronan by Testicular Hyaluronidase

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Abstract. The changes of molecular size of hyaluronan during enzymatic reaction of bovine testicular hyaluronidase at different conditions are monitored by size exclusion high performance liquid chromatography. The effect of glucuronate, galacturonate, glucosamines and pyridoxin as potential inhibitors of hydrolysis is evaluated. The most effective of all tested inhibitors was the presence of glucuronate which not only inhibited the hydrolysis, but also initiated enzymatic reconstruction by transglycosylation reaction at pH 7.0 and absence of any buffer or salt. That effect was not found in the presence of a salt or with any other of the compounds tested.

Key words: Hyaluronan — Hyaluronidase — Inhibition — GlcUA — Transglycosylation

Hyaluronan (HA) constitutes of 1→4 linked disaccharides β-D-glucuronic acid-(1→3)-β-D-N-acetyl-β-D-glucosamine. HA is widely distributed in extracellular matrix and is found in every mammalian tissue. It serves diverse biological functions such as organization of cartilage proteoglycans, provides viscoelastic properties in joint synovial fluid, maintains tissue hydration and it also participates in mechanisms controlling cellular activities (Hascall 1981). Small fragments of HA have pro-inflammatory and angiogenic potential by stimulating the macrophages and endothelial cells (West et al. 1985). The turnover rates of high molecular HA and presence of small HA fragments are regulated by enzymes called hyaluronidases. The enzymes are divided into three distinct classes according to their occurrence and mode of action (Kreil 1995). One of these enzymes, bovine testicular hyaluronidase (Hyase, EC 3.2.1.35) acts as endohexosaminidase splitting β-(1→4) glycosidic bonds of N-acetylglucosamine (GlcNAc) yielding even-numbered oligosaccharides with glucuronic acid (GlcUA) at the non-reducing end. Transglycosylation reaction

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was also observed with the testicular hyaluronidase (Weissman 1955). Its pH optimum was observed at 7.0. The smallest substrate, HA-hexasaccharide with GlcUA at non-reducing end, serves also as the acceptor of transglycosylation and was elongated under optimal conditions up to docosaccharide (Saitoh et al. 1995). However, the precise mechanism of enzymatic reaction is not yet understood.

Continuing great clinical interest in HA and hyaluronidases evokes the interest in potential inhibitors or activators of enzyme reactions. Inhibitors could have potential as angiogenic and anti-inflammatory agents or as novel non-hormonal contraceptives.

In this work we present analysis of the effect of monosaccharide HA building analogs, namely glucuronate(s) and glucosamines, on the aforementioned enzymatic reactions.

Carboxyl group of GlcUA residue was found to be essential for activation of enzymatic reaction (Zhong et al. 1994) and the chondroitines containing GlcUA serve also as the substrates for the hyase. In mammals, GlcUA is degraded further by the normal metabolic pathway (e.g. pentose phosphate pathway). In contrast to GlcUA, the GlcNAc is not degraded, but it is reutilized in biosynthesis of the glycosaminoglycans (Roden et al. 1989). This fact evocates that structurally similar glucosamine sulfate (available as dietary supplement) is recommended for treatment of cartilage disorders, such as osteoarthritis.

Monosaccharides are tested for inhibition/activation of enzymatic reaction of hyase at different conditions. Size-exclusion chromatography is used to evaluate the change of hyaluronic acid molecular size during the time course of enzymatic reaction.

HA (molecular mass ∼400 kDa) was obtained from Contipro Ltd. (Ústí nad Orlicí, Czech Republic), testicular hyaluronidase (hyase) was a pharmaceutical product of Sevapharma Ltd. (Prague, Czech Republic). Tris-HCl was from Serva (Heidelberg, Germany); $NaNO_3$, NaCl and phosphates were from Merck (Darmstad, Germany). Pullulans P-5, P-50, P-100, P-200, P-400 and P-800 (Shodex Standard P-82 set) were from Macherey-Nagel GmbH (Düren, Germany). GlcUA, GlcNAc, glucosamine chloride were purchased from Sigma (St. Louis, MO, U.S.A.). Glucosamine sulfate was prepared from glucosamine chloride by exchange with anion-exchange resin.

Stock solution of HA was prepared as 5 mg HA/ml in appropriate aqueous solvents (in mol/l): 0.1 $NaNO_3$, 0.1 NaCl, 0.1 Tris-HCl (pH 7.0 and 8.0), 0.1 phosphate buffer (pH 7.0) as homogeneous clear solutions. The solutions were stirred overnight. Hyase was dissolved in these same media before the tests. The tested system solutions were prepared in plastic vials by addition of effectors (or inhibitors) in concentration of 5 mg/ml to HA solution. There were no changes in solubility of HA after addition of effectors or inhibitors. The enzymatic reaction was started by addition of hyase solution (7–10 turbidity reducing units (TRU)/mg HA). The reaction mixtures were incubated at 37 ℃. At 10, 20, 40, 60, 80, 120, 160 min from the start of reaction 0.5 ml of the sample in triplicate was removed for analysis. Enzymatic reaction was stopped immediately by boiling the sample for 5 min in

boiling water bath. Denatured enzyme (protein) was removed by centrifugation (10,000 rpm, 5 min) and the supernatant was analyzed on HPLC.

The changes of HA molecular size at different time during the course of enzymatic reaction were examined by size-exclusion HPLC. HPLC system from Laboratorní přístroje (Prague, Czech Republic) consisted of two columns (250×8 mm) connected in series and packed with Biospher GM 300 and Biospher GM 1000 sorbents from Labio (Prague, Czech Republic). The mobile phase used was 0.1 mol/l $NaNO_3$ solution. Eluates were monitored by a differential refractometric detector. Pullulan standards were used as reference material for calculation of HA average molecular weight.

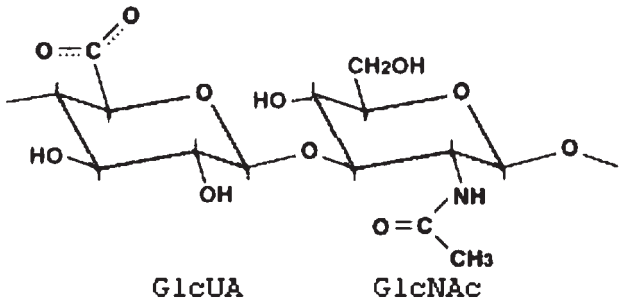

Figure 1. Molecular structure of repeating unit of hyaluronic acid (HA): →4)-β-D-GlcUA-(1→3)β-D-GlcNAc-(1→ .

The effects of structurally HA-related monosaccharides: GlcUA, GlcNAc and over-the-counter drugs: glucosamine sulfate and pyridoxin on the enzymatic reaction of hyaluronidase were examined. The chemical structure of HA repeating unit is shown in Figure 1. The changes of relative molecular size of HA during the enzymatic reaction were monitored by HPLC. The kinetic profiles of the degradation of hyaluronan polysaccharide in the presence of different effectors (inhibitors) at neutral pH are presented in Figure 2. The optimal condition for degradation following up was found at ratio of 10 TRU hyase/1 mg HA. At this concentration a 90% decrease in molecular size of HA was observed in 20 minutes. GlcUA and GlcNAc, the HA constituent monosaccharides, have different competitive inhibition effect (Fig. 2).

As can be seen from Fig. 2, GlcUA has more profound influence compared to GlcNAc. Interestingly, the galacturonate (GalUA) also shows inhibition properties, even more pronounced than constituent GlcNAc. The uronates with carboxylic group are evidently most tightly bound structures to hyaluronidase (Bystrický et al. 2001). The exceptional role of uronate has been confirmed recently when iduronate was successfully used in transglycosylation reaction of hyaluronidase (Takagaki et al. 2000). The shapes of kinetic profiles of HA degradation in the presence of GlcUA

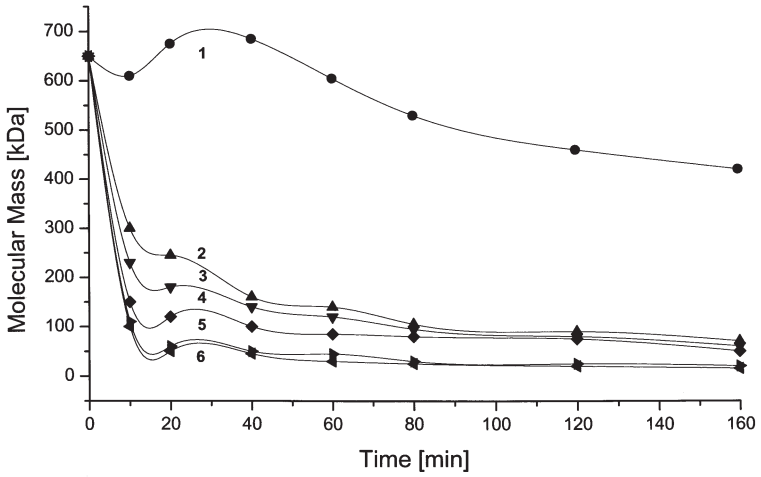

Figure 2. Kinetics of enzymatic reaction of bovine testicular hyaluronidase at pH 7.0 and presence of: 1, GlcUA; 2, GalUA; 3, pyridoxin; 4, GlcNAc; 5, glucosamine hydrochloride; 6, glucosamine sulfate. Apparent average molecular mass of HA is determined from HPLC calibrated with pullulans.

are unusual (Fig. 2). After the first few minutes of degradation the slope is changed to take into account the synthesis of HA macromolecular chains.

This unexpected effect has not been observed with HA polysaccharide yet. The enzymatic reconstruction of glycosaminoglycan by transglycosylation reaction of bovine testicular hyaluronidase was presented only with oligosaccharides (Takagaki et al. 2000). The presence of GlcUA is undoubtedly necessary for elongation of polysaccharide chain here, although its role is difficult to specify exactly. One can speculate that the presence of GlcUA supports the ligand concentration, and as the ligand, it can also be transported by hyase and attached to polysaccharide acceptor. Another idea is that GlcUA just inhibits hydrolysis at certain point of degradation more effectively and in this way it favors the oposite direction of enzymatic reaction. To better understand this phenomenon, it is necessary to collect more data on transglycosylation of HA polysaccharides.

As opposed to GlcUA, glucosamines do not exhibit any pronounced competitive inhibitory effect on hyaluronidase reaction at neutral pH (Fig. 2). Pyridoxin (vitamin B6), slightly inhibited HA digestion (i.e. inhibited reduction of molecular mass). Structurally, pyridoxin posseses three hydroxyl substituents on the pyridine ring. It was reported that flavonoids, which have three hydroxyl groups on aromatic rings, are potent inhibitors of HA digestion (Kuppusamy and Das 1991). Pyridoxin did not show any comparable effect.

Representative curves in Figure 3 indicate the effect of different pH values of unbuffered solution on kinetics of HA digestion by hyase (with GlcUA present).

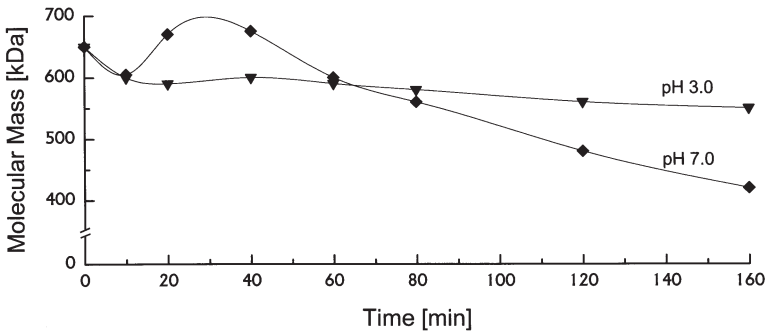

Figure 3. Kinetics of unbuffered enzymatic reaction of bovine testicular hyaluronidase in the presence of GlcUA at pH 3.0 and pH 7.0.

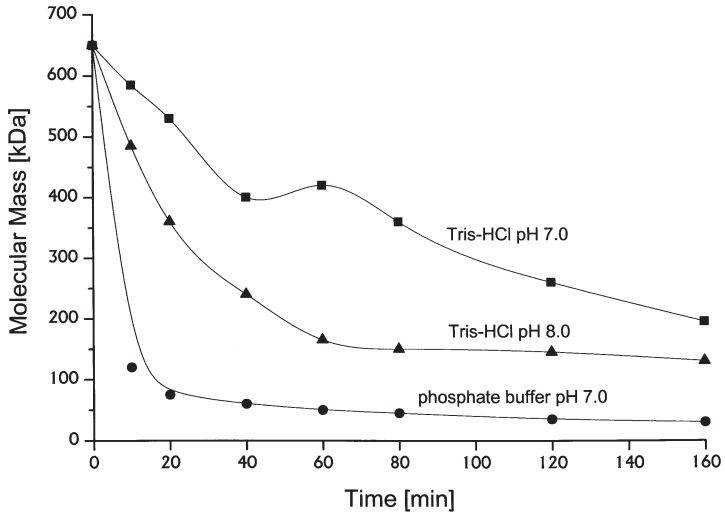

Figure 4. Kinetics of buffered enzymatic reaction of bovine testicular hyaluronidase in the presence of GlcUA in Tris-HCl at pH 7.0, in Tris-HCl at pH 8.0 and in phosphate buffer at pH 7.0.

In Figure 4 there are representative curves that indicate effect of different buffers. As shown in Figure 5, representative curves indicate the effect of different salts present in the solution. Hyase reaction is sensitive to pH of solution. When GlcUA was present in acidic form, hyase did not react; pH was obviously out of activity range of hyase enzyme (pH = 3.0). The optimal pH for hydrolysis by hyase was observed at about 5.0 (Highsmith et al. 1975). Interestingly, in Tris-HCl buffer the hydrolysis of HA at pH 7.0 was less effective than at pH 8.0. The time course of

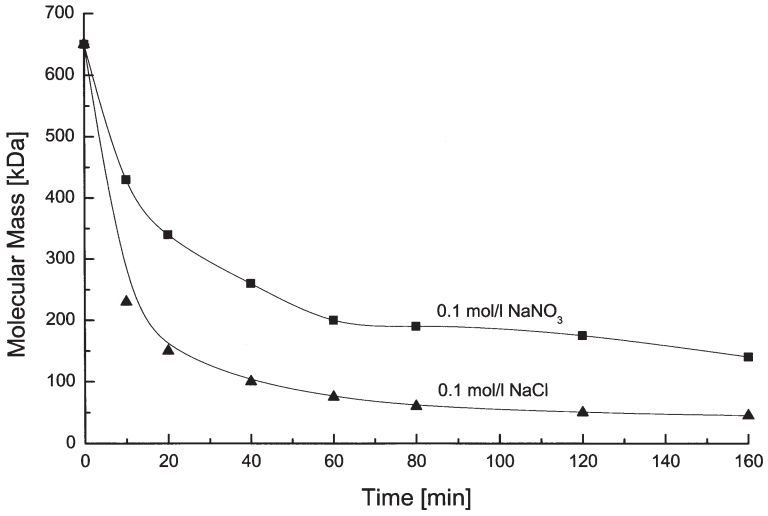

Figure 5. Kinetics of enzymatic reaction of bovine testicular hyaluronidase in the presence of GlcUA and 0.1 mol/l $NaNO_3$ or 0.1 mol/l NaCl.

enzymatic reaction at pH 7.0 was not monotonous; it can indicate that hydrolysis is not the only type of reaction here. This condition may be favorable for transglycosylation reaction. Saitoh et al. (1995) suggested that the pH optimum for such transglycosylation is 7.0. From Fig. 4 and 5 it is clear, however, that the presence of NaCl or phosphate buffer (at pH 7.0) in the solution seems to suppress that effect. The kinetics of digestion in phosphate buffer at pH 7.0 in the presence of GlcUA and without GlcUA (control experiment) was the same. The effect of salts was observed at pH 5.2. The different effect of different anions (Cl^-, NO_3^-) on HA digestion in the presence of GlcUA is shown in Fig. 5. The effect of salts on the reaction is evidently observed here. Other studies have claimed that the presence of cations is required for activation of the enzyme (Kakegawa et al. 1985). Sensitivity of hydrolysis in the presence of different cations in solution has been presented recently (Vercruysse et al. 1999). Mainly divalent ions can change the conformation of HA. The availability of exposed sites for the hydrolysis reaction can be altered by this way. Our observations show that ionic environment as well as the presence of an organic buffer (for example Tris-HCl) trigger the effect of GlcUA, thus supporting the hypothesis that the reaction is highly sensitive to ionic environment.

In summary, our results showed that among other potential monosaccharides, the presence of GlcUA in the system influenced the course of hyase catalysed enzymatic reaction most remarkably. It seems that GlcUA-competitive inhibition of hydrolysis is not the only effect, but under certain conditions can also invoke synthesis of HA polysaccharide chain by transglycosylation reaction. The effect is extremely sensitive to pH and the presence of other ionic substances, e.g. salts. More detailed

studies based on presented data may lead to better understanding of reconstruction and degradation of HA polysaccharide under physiological conditions.

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