Carbohydrate Polymers 148 (2016) 189–193

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Hydrogen peroxide generation by the Weissberger biogenic oxidative system during hyaluronan degradation

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ARTICLE INFO

Article history:
Received 6 January 2016
Received in revised form 11 April 2016
Accepted 14 April 2016

Keywords:
Catalase
Glycosaminoglycans
Reactive oxygen species

ABSTRACT

By applying the enzyme catalase, our study on hyaluronan degradation confirms the generation of hydrogen peroxide using the Weissberger biogenic oxidative system (WBOS), which is composed of ascorbate and cupric ions. Dynamic viscosities of hyaluronan (HA) solutions influenced by WBOS in the absence and presence of catalase were analysed by rotational viscometry. Molar masses of HAs were determined by size-exclusion chromatography with multi-angle laser-light scattering. Our results show that catalase dose-dependently inhibited the degradation of HA macromolecules, which presumably confirms the generation of H_2O_2 in the reaction system. This has implications in range of biomedical applications such as arthritic joint treatment, tissue engineering, ocular and cosmetic surgery.

1. Introduction

Recently, we have published a study on the influence of thiol compounds on oxidative degradation of a high-molar-mass hyaluronan. We used the Weissberger biogenic oxidative system (WBOS, Scheme 1), which is believed to be a source of hydrogen peroxide and subsequently hydroxyl radicals, which degrade HA macromolecules (Valachová et al., 2015). However, the direct proof of the formation of H_2O_2, the precursor of OH radicals, is missing from the literature (Weissberger, LuValle, & Thomas Jr., 1943).

Catalase (Fig. 1), a common enzyme found in nearly all living organisms, consists of four identical subunits, each containing a single heme for H_2O_2 decomposition to molecules of water and oxygen (Sharma, Jha, Dubey, & Pessarakli, 2012).

Catalase has also been reported to decompose hydroperoxides (Campanella, Spuri Capesciotti, Vincenzo Russo, & Tomassetti, 2008; Radi, Sims, Cassina, & Turrens, 1993). The catalase C-terminus 'Ala-Asn-Leu' binds Pex—the peroxisome-targeting transporter localized in cell peroxisomes, which acts as an effective *locus* for fixation followed by a fast decomposition of H_2O_2 molecules. It has long been assumed that catalase functions exclusively at the peroxisome and not at other subcellular organelles. Nonetheless, (Yano, Arroyo, & Yano, 2004) detected discernible amounts of catalase in cellular cytoplasm, and at endoplasmic reticulum, nucleus, and cytoplasmic membrane.

Hyaluronan (HA) is a nonsulfated glycoaminoglycan, present in all vertebrates, whose macromolecules are composed of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by β-(1→4) and β-(1→3) linkages. In mammals, HA, which is an important component of the extracellular matrix involved in the structure of connective tissues Kudva and Patterson (2014), can modulate a variety of cellular and tissue functions. Differently sized HAs trigger different signal transduction pathways. For example, tetrasaccharides are antiapoptotic and act as inducers of heat shock proteins; HA oligomers with 8–16 repeating units, stimulated angiogenesis *in vivo*, and endothelial proliferation *in vitro*. HAs having the molar mass lower than 1000 kDa induce proinflammatory responses in macrophages, while larger HA molecules (1000–5000 kDa) suppress angiogenesis, immune responses, and inflammation (Collins & Birkinshaw 2013a; Duan & Kasper, 2011) Vrentzos et al. (2014)

Reactive oxygen and nitrogen species (ROS and RNS) that degrade HA macromolecules are generated during the inflammatory response in sepsis, tissue inflammation, and ischemia-reperfusion injury. The most direct evidence for this is in synovial

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http://dx.doi.org/10.1016/j.carbpol.2016.04.063
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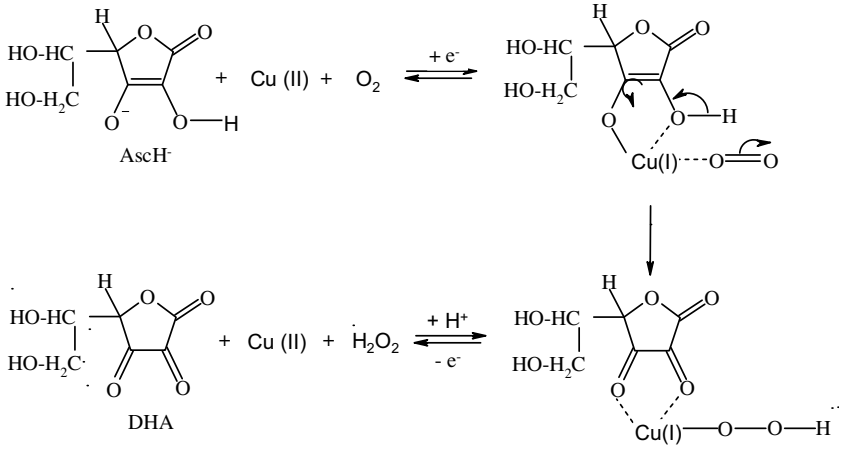

$$H_2O_2 + [Cu(I)\ \text{---}\ complex] \longrightarrow {}^{\bullet}OH + HO^- + [Cu(II)\ \text{---}\ complex]$$

Scheme 1. The modified Weissberger biogenic oxidative system (Hrabárová, 2012).

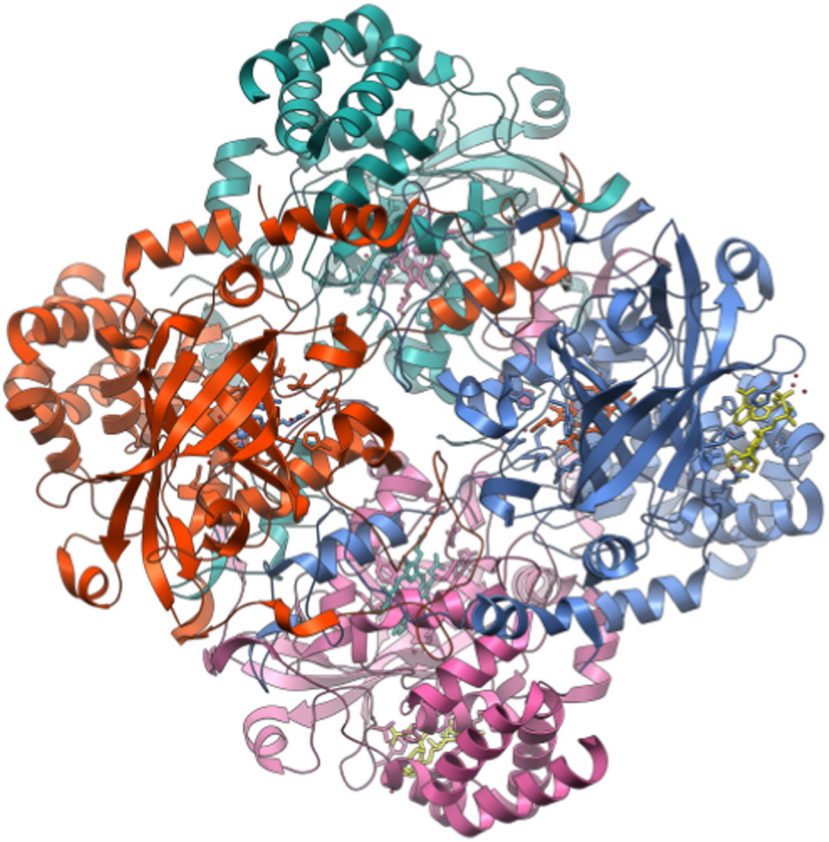

Fig. 1. Catalase, https://en.wikipedia.org/wiki/Catalase.

fluid (SF), where inflammation leads to degradation of high-molar-mass HA with resulting decrease in viscosity of SF. The OH radical has been found to be one of the most efficient initiators of free-radical HA chain degradation (Parsons, 2015).

The aim of the current study is to confirm the generation of hydrogen peroxide as the primary reaction product when using the Weissberger biogenic oxidative system to degrade HA.

2. Materials and methods

2.1. Chemicals

Hyaluronan sample (sodium salt) of the M_w molar-mass average 662.4 kDa coded P0207-1B was sourced from Lifecore Biomedical, Chaska, MN, USA. Analytical purity grade NaCl, $CuCl_2{\cdot}2H_2O$, 96% ethanol were purchased from Slavus, Bratislava, Slovakia. Ascorbic acid, K_2HPO_4 and KH_2PO_4 were the products of Sigma Aldrich,

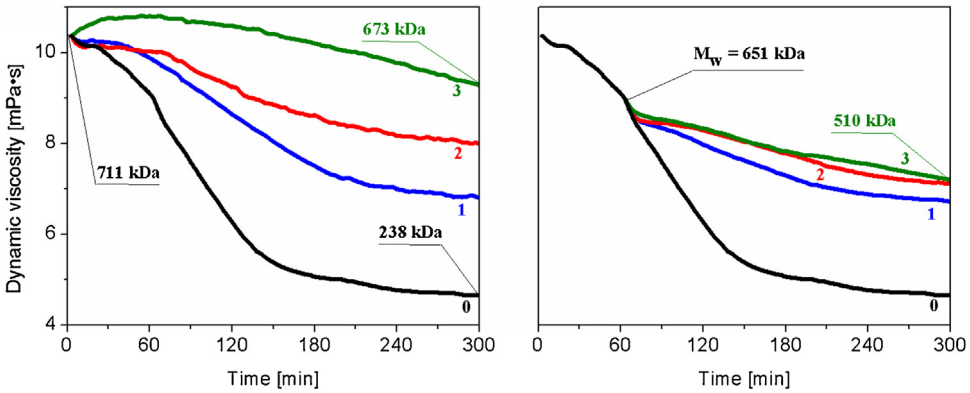

Fig. 2. Effects of catalase on the HA degradation induced by the oxidative system (curve 0). Catalase added to the oxidative system before HA degradation begins (left panel) and 1 h after the reaction onset (right panel) expressed in μg/ml: 6.2 (1), 31.3 (2), 62.5 (3). Values of the so-called polymer peak molar mass (M_p) and extrapolated value of M_w of HA exposed to oxidative degradation for one hour are indicated within the figure.

Table 1
SEC-MALS results of the relative molecular mass of HA samples.

Sample	M_p [kDa]	M_w [kDa]	M_z [kDa]	M_w/M_n	Rg_z [nm]
A—(Control HA)	711.4	662.4	951.8	1.80	80.8
B—(62.5 μg/ml—Catalase)	673.4	649.4	917.2	1.75	80.5
C—(62.5 μg/ml—Catalase added 1 h after start of the reaction)	509.9	486.1	686.9	1.66	65.9
D—(no Catalase)	238.1	281.0	407.1	1.59	46.2

Bratislava. Catalase sourced from bovine liver (2000–5000 U/mg protein) was purchased from Sigma Aldrich, Bratislava. Redistilled deionized high purity-grade water, with conductivity of <0.055 μS/cm, was produced using the TKA water purification system from Water Purification Systems, Niederelbert, Germany.

2.2. Preparation of stock and working solutions

Hyaluronan samples (24 mg) were dissolved in aqueous NaCl solution for 24 h in the dark. HA sample solutions were prepared in two steps: first, 4.0 ml and after 6 h 3.85, 3.65 or 3.4 ml of 0.15 M NaCl were added when working in the absence or presence of the catalase. Solutions of ascorbate (16 mM), and cupric chloride solution (320 μM solution) were prepared also in 0.15 M aqueous NaCl. Catalase (EC 1.11.1.6) of the concentration of 1 mg/ml was prepared in potassium phosphate buffer (50 mM; pH 7.4).

2.3. Rotational viscometry (uninhibited/inhibited hyaluronan degradation)

The procedure was performed as described in Valachová et al. (2015), however the reference system Cu(II) ions (1.0 μM) and ascorbate (100 μM) was enriched in the addition of potassium phosphate buffer (50 mM, 1 ml). The addition of 50, 250 and 500 μl of catalase solution corresponds to 100–250 U (6.2 μg/ml), 500–1250 U (31.3 μg/ml) and 1000–2500 U (62.5 μg/ml) of catalase in the reaction system, respectively. After finishing the measurement, HA samples were precipitated in 96% ethanol, and after 24 h the precipitate was centrifuged for 10 min at 3000 rpm and dried in a dessicator.

2.4. Size-exclusion chromatographic analyses with multi-angle light scattering (SEC-MALS)

The molar mass distribution (MMD) of HA samples was determined by a modular multi-detector SEC system. This system consisted of an Alliance 2695 separation module from Waters (Milford, MA, USA) equipped with two on-line detectors: A multi-angle light scattering photometer (MALS Dawn DSP-F) from Wyatt (Santa Barbara, CA, USA) and a 2414 differential refractometer (DRI) from Waters (Milford, MA, USA); the latter was used as a polymer concentration detector. The setup of this multi-detector SEC system was serial in the following order: Alliance-MALS-DRI. The wavelength of the MALS laser was 632.8 nm. The light scattering signal was detected simultaneously at fifteen scattering angles ranging from 14.5° to 151.3°. The calibration constant was calculated using toluene as standard, assuming a Rayleigh factor of 1.406×10^{-5} cm^{-1}. The angular normalization was performed by measuring the scattering intensity of a concentrated solution of BSA (bovine serum albumin) globular protein in the mobile phase, assumed to act as an isotropic scatterer. A flow rate of 0.5 ml/min, was used to avoid shear-degradation of the polymer within the two Shodex (KB806 and KB805) columns from Tosoh Bioscience (Stuttgart, Germany); the mobile phase consisted of 0.20 M aqueous NaCl at 35 °C. The dn/dc value used was 0.150 ml/g. The molecular weight measurements were performed on carefully prepared solutions, to avoid degradation, in the presence and absence of catalase. For further reading on the importance of solution preparation the reader is directed to (Collins & Birkinshaw, 2013b).

2.5. Fourier-transformed infrared spectroscopy (FT-IR)

FT-IR spectra of the precipitated samples of the native HA, HA exposed to WBOS in the absence and presence of catalase were measured with Nicolet 6700 (Thermo Fisher Scientific, USA) spectrometer equipped with DTGS detector and Omnic 8.0 software. The spectra was collected in the region from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}, the number of scans was 128. Diamond Smart Orbit ATR accessory was used for measurement in solid state.

3. Results and discussion

The HA solution was exposed to oxidative degradation by Cu(II) ions (2.0 μM) and ascorbate (100 μM) in the presence of potassium phosphate buffer (6.25 mM; pH 7.4). The reduction in dynamic viscosity of the HA solution within 300 min was 5.1 mPa s as shown in Fig. 2, curve coded 0.

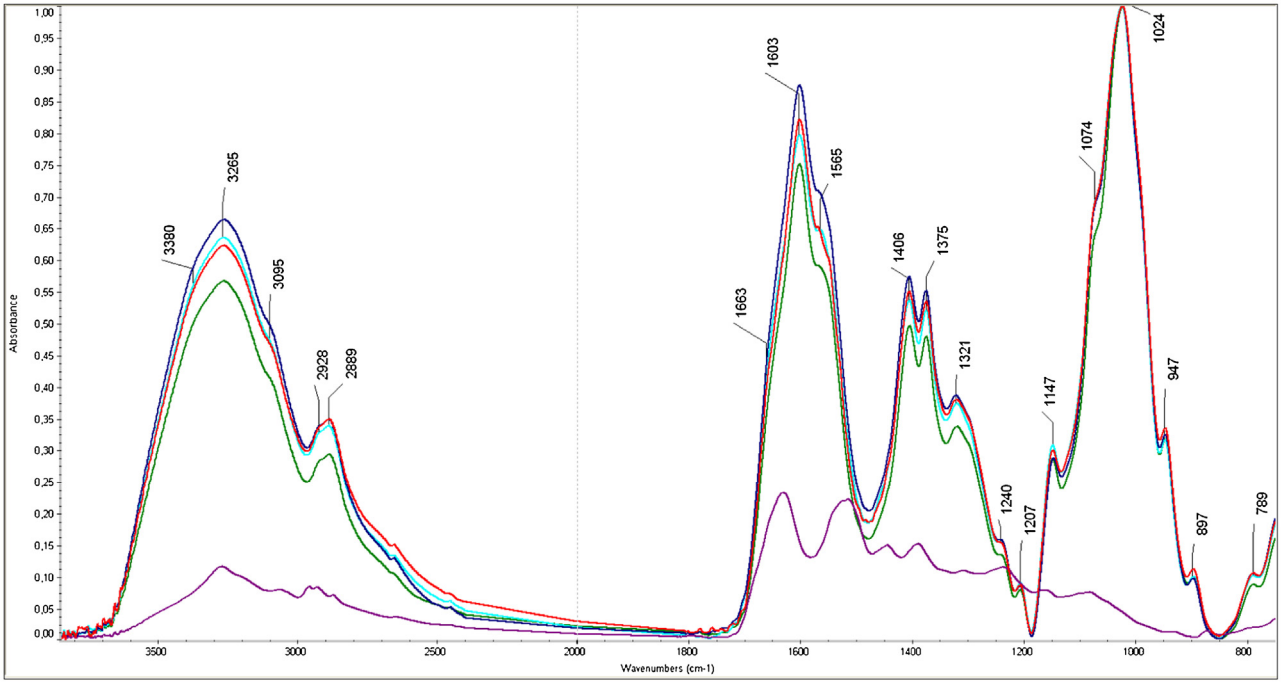

Fig. 3. The FT-IR spectrum of catalase (purple), HA samples: A (red), B (dark blue) and C (light blue), D (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2 shows time and dose- dependent changes in dynamic viscosity of the HA solutions after addition of different amount of catalase. The left panel in Fig. 2 demonstrates that catalase addition significantly inhibits HA degradation when incorporated into the reaction vessel at time zero. This observation is an indication that the *in situ* generated H_2O_2 molecules are decomposed and therefore the subsequent production of OH radicals is prevented.

Right panel in Fig. 2 shows that catalase addition 1 h after the reaction onset particularly also suppressed HA degradation. However, there is practically no catalase dose-dependence in the viscosity of the polymer solution. As already proved, 1 h after the reaction onset no ascorbate and thus no H_2O_2 or OH radicals are present within the reaction vessel (Šoltés et al., 2006). The continuation of HA degradation when OH radicals are no longer produced (after 1 h of the reaction onset) is attributed to the presence of peroxy- and alkoxy-type macroradicals formed from the polymer, which macroradicals by themselves maintain the propagation phase of HA degradation (Valachová et al., 2015).

To ensure that the kinetic curves in Fig. 2 represent a decrease in the molar masses of HA, the molar-mass parameters of the precipitants were measured by the SEC-MALS method. The results of the chromatographic analysis are summarised in Table 1. As evident, the defined average values of molar masses of the samples point out that the oxidatively degraded HA without addition of catalase (sample D) was extensively degraded compared to the originally applied HA sample (A). Results further show that the addition of catalase (the enzyme concentration in the reaction vessel = 62.5 μg/ml) significantly prevented reductions in HA molar masses (sample B). On the other hand, the molar masses of the oxidatively degraded HA containing catalase (at the highest concentration levels) added 1 h after the reaction onset (sample C) were partly decreased. The molar mass (M_w) of the HA exposed to oxidative degradation for one hour was calculated by a graphical interpolation resulting in M_w value of 651 kDa (cf. Fig. 2).

Catalase has been shown to inhibit the degradation of HA initiated by OH radicals through the decomposition of hydrogen peroxide, whose amount is immediately generated according to the amount of cupric ions present in the reaction vessel. During the first cycle, decomposed ascorbate equals to the applied $CuCl_2$ moles (it means 2 μM) generating thus 2 μM of H_2O_2. However, the informed reader understands that due to the presence of the formed [Cu(I)—complex], the hydrogen peroxide is immediately decomposed. The initiator, the OH radicals begin HA degradation, and simultaneously a new reaction cycle between ascorbate and regenerated cupric ions begins. The novel 2 μM of H_2O_2 is decomposed and a new 2 μM of OH radicals are generated. The initiation reaction, *i.e.* the generation of OH radicals is ended when the whole amount of ascorbate is used for production of hydrogen peroxide or more precisely in generating OH radicals. Since catalase decomposes the generated molecules of H_2O_2, it is obvious that the enzyme addition into the reaction vessel before starting HA degradation has to retard or even totally stop the free-radical reaction leading to HA fragments (cf. Fig. 2. left panel, curves 1–3). Our results clearly indicate that catalase dose-dependently inhibited the degradation of HA macromolecules, which indirectly supports our assumption on the *in situ* generation of H_2O_2 in the WBOS reaction system.

The FT-IR spectral data obtained demonstrate no structural changes, when compared to the native HA (Fig. 3, red curve) indicating that catalase, either in a native or degraded form, if any present, is not determinable in the analysed precipitants.

Although, the poor residence time *in vivo* of HA can be attributed to multiple factors the ability of catalase to retard the free-radical degradation of HA initiated by the action of hydrogen peroxide is of interest to the scientific community.

4. Conclusions

Weissberger's biogenic oxidative system is a generator of H_2O_2 molecules. This tenet is supported by the chemistry of ascorbate oxidation catalyzed by Cu(II) ions, which can be described as follows: the dioxygen molecule O=O plus two electrons resulted

in $^{-}O—O^{-}$, *i.e.* the dianion of H_2O_2, which in aqueous solution reacts with two H_3O^{+} cations yielding $H_2O_2 + 2H_2O$. The addition of catalase, the enzyme which decomposes (hydro-)peroxides, should result in inhibition of H_2O_2 generation: this presumption is unambiguously proved by the reported results, which show a dose-dependent inhibitory action of catalase on the degradation of high-molar-mass HA. FTIR results show that hyaluronan macroradicals do not react with catalase since there is no evidence of products originating from such a reaction. These results have implications in the prevention of H_2O_2 induced calcium overload in cardiac myocytes leading to myocardial infraction as well as disease associated with H_2O_2 induced cell and DNA damage.

Acknowledgement

The study was supported by the grant VEGA 2/0065/15.

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