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Degradation of high-molecular-weight hyaluronan by hydrogen peroxide in the presence of cupric ions

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Abstract—Dynamic viscosity (η) of the high-molecular-weight hyaluronan (HA) solution was measured by a Brookfield rotational viscometer equipped with a Teflon cup and spindle of coaxial cylindrical geometry. The decrease of η of the HA solution, indicating degradation of the biopolymer, was induced by a system containing H_2O_2 alone or H_2O_2 plus $CuCl_2$. The reaction system H_2O_2 plus $CuCl_2$ as investigated by EPR spin-trapping technique revealed the formation of a four-line EPR signal characteristic of a ·DMPO–OH spin adduct. Thus, hydroxyl radicals are implicated in degradation of high-molecular-weight HA by the system containing H_2O_2 and $CuCl_2$.

Keywords: Hyaluronan degradation; Rotational viscometry; EPR spectroscopy; Spin-trapping technique; DMPO

1. Introduction

Hyaluronan (HA; hyaluronic acid, hyaluronate) is a polysaccharide constituent of almost all tissues in vertebrate organisms. High-molecular-weight HA is one of the functionally most essential components of synovial fluid (SF). The physiological concentration of HA in the SF of humans is 2–3 mg/mL. The average molecular weight of the HA biopolymer in the SF of healthy subjects is several million daltons. The degradation of high-molecular-weight HA, occurring under oxidative stress and/or inflammation, is accompanied with loss of viscoelastic properties of SF.[1]

A system containing hydrogen peroxide and ferrous cation, known as the Fenton generator of ·OH radicals, has been often applied for studies of oxidative injury of various biomolecules, for example, nucleic acids, proteins, and lipids.[2] High-molecular-weight HA is also a very suitable object for investigation of the degradative action of ·OH radicals.[3–8] Previously, the attack of hydroxyl radicals on HA and its model compounds was intensively studied by EPR using rapid-flow and spin-trapping techniques.[5,7,9,10]

Along with iron, copper is generally present in HA samples recovered from various biological sources. It has already been demonstrated that copper ions in their lower oxidation state (Cu^+) are several times more 'redox active' than Fe^{2+} ions.[11]

The goals of the present study were as follows: (1) to investigate the degradative action of H_2O_2 alone on high-molecular-weight HA; (2) to prove the ability of $CuCl_2$ added to the reaction system containing hydrogen peroxide to accelerate the rate of HA degradation; and (3) to prove generation of ·OH radicals in the system containing H_2O_2 plus $CuCl_2$ by spin-trapping EPR technique.

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2. Experimental

2.1. Biopolymers

The high-molecular-weight HA sample, coded 'LIFECORE P9710-2', used throughout the study was the product of Lifecore Biomedical, Inc., Chaska, MN, USA. The weight-average of the molecular weights of the HA sample was 1.2 MDa.[12]

2.2. Chemicals

Analytical purity grade NaCl and $CuCl_2{\cdot}2H_2O$ were from Slavus Ltd., Bratislava, Slovak Republic. Aqueous H_2O_2 solution (30%) was purchased from Chemapol, Prague, Czech Republic. Water used was of redistilled deionized quality grade. DMSO–dimethyl sulfoxide (UV purity grade), spin trapping agent DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) and selective singlet oxygen trap TMP (4-hydroxy-2,2,6,6-piperidine) (*p.a.*) were from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO, USA). DMPO was distilled before application and stored under argon at −18 °C.

2.3. Stock/working solutions

The actual concentration of the aq H_2O_2 solution was determined by titration with a standardized permanganate solution[13] and by a spectrophotometric method.[14] The stock H_2O_2 solution was prepared by dissolving NaCl in H_2O_2 solution to a salt concentration of 0.15 M. Similarly, the stock cupric chloride solution (400 μM) was prepared in 0.15 M NaCl. Both stock solutions were diluted appropriately with aq NaCl (0.15 M). The resulting working solutions were freshly prepared each day.

2.4. Degradation studies

The LIFECORE P9710-2 sample (20.0 mg) was dissolved overnight in the dark at room temperature in 0.15 M aq NaCl, in two steps. First, 4.0 mL of solvent was added in the morning. The next 3.2 mL-portion of the solvent was added after 6 h. On the next morning, 800 μL of the working H_2O_2 solution was mixed to the formed gel-like solution at 30 s moderate stirring. The resulting solution was immediately transferred into the Teflon cup reservoir of the rotational viscometer. For investigating the system comprising H_2O_2 plus $CuCl_2$, an appropriate amount of the cupric chloride working solution was admixed into the gel-like HA solution 9 min before application of hydrogen peroxide.

2.5. Rotational viscometry

The recording of the viscometer output parameters started 2 min after the experiment onset. The changes of η and torque were monitored at 25 ± 0.1 °C by using a digital rotational viscometer Brookfield DV-II+ PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, USA) equipped with a cup-spindle pair built of Teflon® at our laboratory.[15,16] At the spindle rotational speed of 180 rpm, the shear rate equaled 237.6 s^{-1}.

2.6. Spin-trapping EPR spectroscopy

The solutions for EPR spin-trapping experiments were prepared under air immediately before measurements and were filled into a 50-μL boron capillary. Its reproducible position in the EPR cavity was maintained by a defined tight fitting with silicon rings in a broad EPR tube. The EPR measurements at the X-band were performed with a Bruker EMX EPR spectrometer equipped with a standard TE_{102} (ER 4102 ST) rectangular cavity. The EPR spectra were recorded at 37 °C. Temperature control was achieved using a Bruker temperature control unit ER 4111 VT. The EPR spectrometer settings were as follows: microwave frequency, 9.43 GHz; microwave power, 10.034 mW; center field, 335.3 mT; sweep width, 8 mT; gain, 5×10^5; modulation amplitude, 0.1 mT; modulation frequency, 100 kHz; scan, 21 s; time constant, 81.92 ms, number of scans, 10. The *g*-values were determined with uncertainty of ±0.0001 by simultaneous measurement of a reference sample containing DPPH (1,1-diphenyl-2-picrylhydrazyl) fixed on the EPR cell. The experimental EPR spectra acquisition and simulation were carried out using WIN EPR and SimFonia standard programs (Bruker).

3. Results

Addition of H_2O_2 to the HA solution resulted in a decrease of the η values (Fig. 1). The higher the H_2O_2 concentration, the faster was the decrease of η values. However, at lower H_2O_2 content (110 mM or 55 mM), an induction period lasting approximately 15 or 30 min, respectively, could be recognized in the time dependencies of dynamic viscosity (△ and ▽ in Fig. 1). The LIFECORE P9710-2 sample degradation was monitored either on attaining the nominal η value 5.8 mPa·s or 300 min after the reaction onset. Within the monitored interval of η values, the torque ranged between 72% and 36%.

The results presented in Figure 2 unambiguously prove that any $CuCl_2$ amount added to the system containing 55 mM H_2O_2 accelerated the HA degradation. Even the lowest $CuCl_2$ addition (0.1 μM) resulted in a dramatic increase of the degradation rate. It should be pointed out, however, that in the absence of H_2O_2 the addition of cupric chloride alone, at the tested concen-

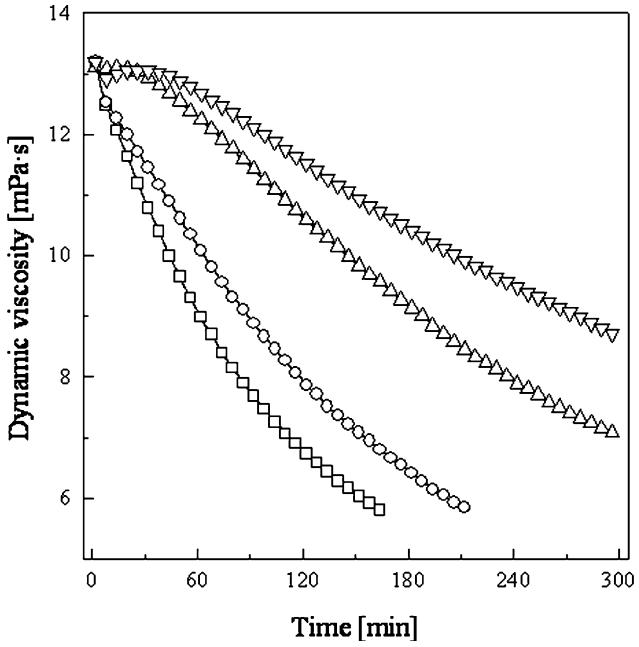

Figure 1. Effect of H_2O_2 addition on dynamic viscosity of the hyaluronan (HA) solution (LIFECORE P9710-2). H_2O_2 initial concentrations (in mM): (□) 882; (○) 441; (△) 110; (▽) 55.

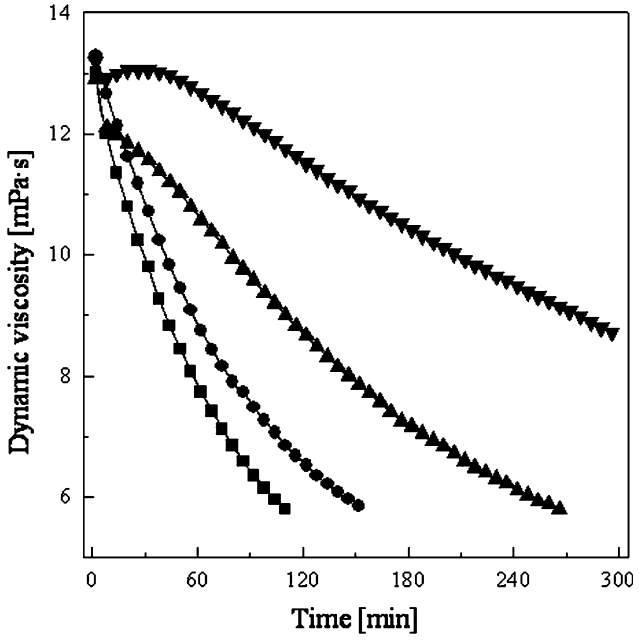

Figure 2. Effect of $CuCl_2$ addition on dynamic viscosity of hyaluronan solution (LIFECORE P9710-2) in the presence of 55 mM H_2O_2. $CuCl_2$ initial concentrations (in μM): (■) 2.5; (●) 1.25; (▲) 0.1; (▼) 0.0.

trations 2.5, 10.0, and 40.0 μM, did not cause any degradation of the biopolymer.[15]

The time course of 30 EPR spectra monitored at 37 °C in the system containing 55 μM H_2O_2 in the presence of DMPO (8 mM) is shown in Figure 3a. A low EPR intensity of ·DMPO adducts was measured in this solution.

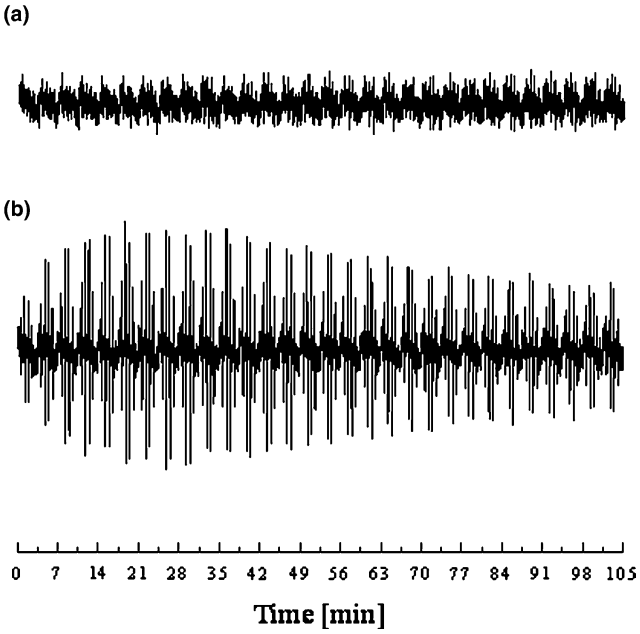

Figure 3. Time course of 30 EPR spectra (magnetic field width of 8 mT) measured at 37 °C in 55 μM aq H_2O_2 containing DMPO spin-trapping agent (initial DMPO concentration 8 mM): (a) $c(CuCl_2) = 0$ μM; (b) $c(CuCl_2) = 2.5$ μM.

The signal intensities found were comparable with the results obtained in reference (blank) experiments using DMPO without addition of hydrogen peroxide. However, analogous experiments carried out in the presence of 2.5 μM $CuCl_2$ plus 55 μM H_2O_2 resulted in the formation of a four-line EPR signal (Fig. 3b), characteristic of the ·DMPO–OH adduct ($a_N = 1.493$ mT, $a_H^{\beta} = 1.465$ mT; $g = 2.0059$), which is in good accordance with catalog data.[17] Figure 4 demonstrates dependence of the changes of ·DMPO–OH integral EPR signal intensity on reaction time, evaluated by the double integration of experimental EPR spectra sets. These data reveal the essential role of cupric ions on hydroxyl radical generation in the presence of H_2O_2. The experimental and simulated EPR spectra obtained in the solutions containing 2.5 μM $CuCl_2$ plus 55 μM H_2O_2 are depicted in the inset of Figure 4. However, the EPR spectra corresponding to the addition of $O_2^{\cdot -}$ or ·OOH to DMPO molecules were not observed. This is in agreement with the literature data reporting very low stability of ·DMPO–O_2^-/OOH spin adducts in aqueous media and their conversion to ·DMPO–OH.[18,19] The experiments performed in mixed DMSO–water solvent (5:1; v/v) in the presence of Cu^{2+} ions and H_2O_2 evidenced the generation of three ·DMPO adducts attributed to ·DMPO–OOH ($a_N = 1.403$ mT, $a_H^{\beta} = 1.228$ mT, $a_H^{\gamma} = 0.10$ mT; $g = 2.0058$), ·DMPO–OCH_3 ($a_N = 1.340$ mT, $a_H^{\beta} = 0.900$ mT, $a_H^{\gamma} = 0.16$ mT; $g = 2.0058$), and ·DMPO–CH_3 ($a_N = 1.484$ mT, $a_H^{\beta} = 2.165$ mT; $g = 2.0056$), in accordance with reaction mechanisms generating $O_2^{\cdot -}$/·OOH, ·OH, ·CH_3 and CH_3OO·

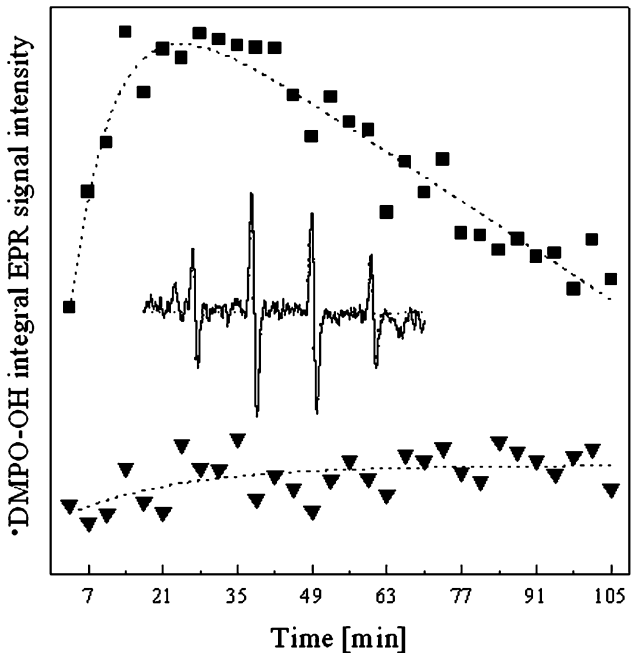

Figure 4. Time dependencies of the integral EPR signal intensity of ·DMPO–OH adduct monitored at 37 °C in 55 μM aq H_2O_2 containing DMPO spin-trapping agent (initial DMPO concentration 8 mM): ▼ $c(CuCl_2) = 0$ μM; ■ $c(CuCl_2) = 2.5$ μM. Inset represents the experimental (solid line) and simulated (dotted line) EPR spectra of ·DMPO–OH.

radicals, which are subsequently trapped by the DMPO molecule.[20]

4. Discussion

4.1. Reaction of H_2O_2 with $CuCl_2$

Upon mixing an aqueous $CuCl_2$ solution with H_2O_2, one can observe the intense formation of gas bubbles (due to release of O_2). Since H_2O_2 plus Cu^{2+} is an especially efficient system for degradation of high-molecular-weight polysaccharides including HA,[21–24] and since the presence of both hydrogen peroxide and copper ions have been claimed in the rheumatic joint,[25–27] the reaction of H_2O_2 with $CuCl_2$ could be of (pathophysiological) importance.

Previously, H_2O_2 was applied as the analytical reagent for the determination of trace transition metal ions using their catalytic effect on hydrogen peroxide decomposition (Eq. 1). The reaction mechanism has been suggested to form peroxo–metal complexes, which self-decomposition results in oxygen evolution.[2,28,29]

$$2H_2O_2 \xrightarrow{\text{transition metal ions}} 2H_2O + O_2 \quad (1)$$

The oxidation and reduction processes of hydrogen peroxide in acidic and alkaline aqueous media can be summarized in the following reactions[30,31]

$$H_2O_2 + 2H^+ + 2e \rightleftarrows 2H_2O \quad E^\theta = 1.78\ V \quad (2a)$$

$$HO_2^- + H_2O + 2e \rightleftarrows 3OH^- \quad E^\theta = 0.88\ V \quad (2b)$$

$$O_2 + 2H^+ + 2e \rightleftarrows H_2O_2 \quad E^\theta = 0.68\ V \quad (3a)$$

$$O_2 + H_2O + 2e \rightleftarrows HO_2^- + OH^- \quad E^\theta = -0.08\ V \quad (3b)$$

A possible primary hypothetical oxidation reaction

$$Cu^{2+} + H_2O_2 \rightarrow Cu^{3+} + {\cdot}OH + OH^- \quad (4)$$

analogous to the Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\cdot}OH + HO^-$, involves formation of ·OH radicals, that is, reactive species capable of degrading HA. However, it does not explain the formation of O_2 bubbles and, moreover, operates with copper in a hypervalent oxidation state, that is, Cu^{3+}.[32]

On studying HA degradation, Al-Assaf et al.[33] formulated the reaction of H_2O_2 with Cu^{2+} as follows:

$$2Cu^{2+} + H_2O_2 \rightarrow 2Cu^+ + O_2 + 2H^+ \quad (5)$$

In this case, O_2 is a product of the reaction (Eq. 5) and here hydrogen peroxide plays the role of a reductant. The reduced copper ions Cu^+ subsequently react with H_2O_2 in a Fenton-like reaction (Eq. 6):

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {\cdot}OH + OH^- \quad (6)$$

The cuprous ions formed can be re-oxidized by molecular oxygen producing super-oxide anion (Eq. 7) or hydroperoxyl radicals (Eq. 8), and additionally Cu^+ can be stabilized by chloride ions present in the experimental system (Eq. 9).

$$Cu^+ + O_2 \rightarrow Cu^{2+} + O_2^{\bullet -} \quad (7)$$

$$O_2^{\bullet -} + H^+ \rightleftarrows {\cdot}OOH \quad (8)$$

$$Cu^+ + 2Cl^- \rightarrow [CuCl_2]^- \quad (9)$$

The combination of Eqs. 5–8 demonstrates the decomposition of hydrogen peroxide and the formation of reactive radical species (Eq. 10):

$$2H_2O_2 \rightarrow {\cdot}OH + {\cdot}OOH + H_2O \quad (10)$$

Singlet oxygen ($^1O_2$) is a highly reactive form of oxygen capable of rapidly oxidizing many biologically active molecules. Its formation in $O_2^{\cdot -}$ generating systems has often been proposed but a clear-cut evidence for a damaging role of singlet $^1O_2$ in such systems has not been obtained.[34]

According to Hanaoka et al.,[29] the reactive intermediates $O_2^{\cdot -}$ and ·OH might be supplemented by singlet oxygen generation: $Cu^{2+} + O_2^{\cdot -} \rightarrow Cu^+ + {}^1O_2$. Contrary to EPR spin-trapping experiments evidencing the production of ·OH and ·OOH free radicals in aqueous or DMSO–H_2O Cu^{2+}–H_2O_2 solutions, our studies do not corroborate the generation of $^1O_2$ in DMSO solutions using EPR spectroscopy, monitoring the singlet oxygen-induced oxidation of TMP agent to nitroxide

free radical, TEMPOL.[35,36] Hence it can be summarized that the system H_2O_2 plus $CuCl_2$, along with molecular oxygen, generates hydroperoxyl and hydroxyl radicals, the latter species being involved in degradation of high-molecular-weight HA.

4.2. Degradation of high-molecular-weight HA by H_2O_2

The molecule of H_2O_2 itself is classifiable as a weak, oxidative species. Yet traces of transition metals usually present in HA samples facilitate the biopolymer degradation by H_2O_2. Particularly in the case of the LIFECORE P9710-2 sample, a 4 ppm concentration of copper ions has been claimed ['Certificate of Analysis' (Lifecore Biomedical Inc., Chaska, MN, U.S.A.)]. Thus in the HA solution used (2.5 mg/mL), the Cu^{2+} concentration equaled 0.1 μM.

Addition of the working H_2O_2 solution to the LIFECORE P9710-2 sample containing traces of copper ions (0.1 μM) revealed rapid decline of the dynamic viscosity of the tested solutions (cf. Fig. 1). As follows from the law of mass action, a higher H_2O_2 concentration is more efficient as concerns HA degradation. The results presented in Figure 1, however, indicate that on applying the concentration of 110 mM H_2O_2 (△ in Fig. 1), an induction period of HA degradation appears in the kinetic curve, and such a period is visibly more pronounced when a lower H_2O_2 concentration (55 mM) is used (▽ in Fig. 1).

It should be noted here that the viscometric data shown in Figure 1 reflect the changes in HA degree of polymerization. However, the break of the HA polymer chain represents a complex mechanism including different intermediate species.[9,10] The redox-active copper ions have to be generated from the inactive copper species chelated or liganded by the HA macromolecule. The lower H_2O_2 concentrations applied (110 mM; 55 mM) and the less effective H_2O_2 decomposition in the presence of copper ions in trace amount (0.1 μM) resulted in a slow generation of a low, albeit gradually growing concentration of hydroxyl radicals. This fact together with the progressive multistep rearrangement and strand-breaking of the formed HA macroradicals result in appearance of an induction period in a process represented in Figure 1 (△ and ▽).

4.3. Degradation of high-molecular-weight HA by H_2O_2 plus $CuCl_2$

Addition of 0.1 μM $CuCl_2$ to the HA solution containing 55 mM H_2O_2 resulted in a dramatic increase in the rate of HA degradation (▲ in Fig. 2). The decline of the dynamic viscosity of the solution, beginning after a certain lag period, takes place continually during the whole time period of the measurements. On the basis of the results presented in Figure 2, one can conclude that an additional excess amount of cupric ions (1.25 or 2.5 μM $CuCl_2$) would act 'catalytically' in the manner outlined in reactions 5–8.

5. Conclusions

- The low resistance of HA against oxidative species predestines this biopolymer to be utilized as an *in vitro probe* for investigating the damaging action of oxidants and/or to evaluate the efficiency of various substances to act as pro-oxidants or antioxidants. In principle, changes of the sample dynamic viscosity can be monitored easily by a rotational viscometer.
- H_2O_2 added to the HA solution caused degradation of the biopolymer, while transition metals present in the original HA sample and the added $CuCl_2$ acted catalytically as concerns the decrease in sample viscosity.
- In aqueous media, the results of EPR experiments demonstrated the essential role of cupric ions in hydroxyl radical generation in the presence of H_2O_2. In aerated DMSO–water mixed solvent (5:1; v/v), on the other hand, the EPR spin-trapping experiments performed in the presence of hydrogen peroxide plus Cu^{2+} ions evidenced the generation of three DMPO-adducts attributed to ·DMPO–OOH, ·DMPO–OCH_3, and ·DMPO–CH_3.

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