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High-molar-mass hyaluronan degradation by Weissberger's system: Pro- and anti-oxidative effects of some thiol compounds

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ABSTRACT

Pro- and anti-oxidative effects of two thiol compounds, D-penicillamine and reduced L-glutathione, on the kinetics of degradation of high-molar-mass hyaluronan samples were monitored *via* rotational viscometry. The degradation was induced under aerobic condition by the so-called *Weissberger's* system [ascorbate plus Cu(II)]. Electron paramagnetic resonance spectroscopy was used to confirm the presence of free radicals generated during the biopolymer degradation. Infrared spectroscopy and non-isothermal chemiluminometry were used to characterize the biopolymers after processing.

1. Introduction

The most attractive representative of glycosaminoglycans, the polyelectrolyte hyaluronic acid, (HA; hyaluronan, Scheme 1) is an important component in synovial fluid forming highly viscoelastic solutions at a concentration range of *ca.* 2.0–4.0 mg/mL with excellent lubricating and shock-absorbing properties. Since 1980s, hyaluronan and its derivatives have been intensively explored for use as topical, injectable, and implantable vehicles for the controlled delivery of a variety of drugs [1]. HA is a linear, unbranched biopolymer (molar mass ranging between 10^4 and 10^7 Da), composed of a repeating disaccharide unit (molar mass ≈ 400 Da) consisting of D-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc) linked by a β 1–4 glycosidic bond; the disaccharides are linked by β 1–3 bonds. At higher concentrations HA solutions exhibit the phenomenon of non-Newtonian flow behaviour due to the formation of a micro-heterogeneous network [2]. In the presence of a reductant along with transition metal ions, in particular, copper ions, HA degradation results from metal-mediated production of reactive free radicals, mostly hydroxyl.

It is well known that the degradation of HA macromolecules affected by trace amount of copper(II) ions is promoted by L-ascorbic acid. Under aerobic conditions the system of ascorbate and copper(II) ions provides hydrogen peroxide, which turns into •OH radicals by a Fenton-like reaction [3,4].

L-Ascorbic acid ($AscH_2$), in solutions, has to be present as ascorbate (99.95% $AscH^-$) at pH 7.4 (Scheme 2). L-Ascorbic acid alone is a potent, highly effective essential antioxidant in living organisms [5], which, in the absence of transition metal ions, is capable of scavenging various types of reactive oxygen and nitrogen radical species [3].

In the role of antioxidant in the human body, ascorbate always functions in concert with L-glutathione (GSH) and other thiol compounds, as well as with α-tocopherol and several further low-molar-mass antioxidants preventing highly oxygenated tissues from oxidative damage [6].

Among various thiol compounds such as L-glutathione, D-penicillamine is also of interest. The non-naturally occurring α-amino acid, D-penicillamine (D-pen; β,β-dimethyl cysteine or 3-mercapto-D-valine, Scheme 3), is a pharmaceutical of the chelator class marketed under the trade name Cuprimine. As shown by X-ray

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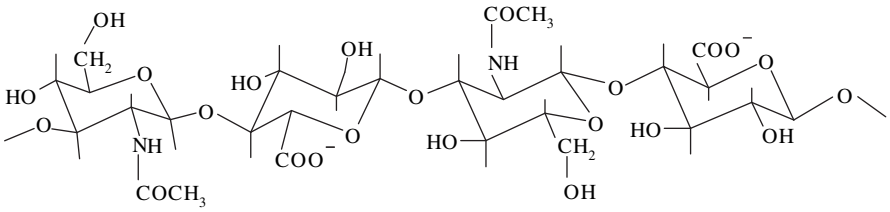


Scheme 1. Hyaluronan polyanion.

photoelectron spectroscopy, the D-pen molecule when dissolved forms a zwitterion [7].

The chief metabolic *in vivo* transformation of D-pen in patients with RA is the oxidation of this species to D-pen–protein disulfides [8–11]. D-pen, a strong chelating agent, reduces Cu(II) to Cu(I) (Scheme 4) while at the same time being oxidized to D-penicillamine disulfide, generating *in vitro* hydrogen peroxide (H_2O_2), in an oxygen-dependent manner. It can also react with the generated H_2O_2 [12]. *In vitro* studies have shown that D-pen/Cu(I) complexes are poorly water-soluble in the pH range of 1.90–7.60 [13], whereas, in the human body, D-pen forms mixed complexes with Cu(I) and dioxygen, whose stability is really high [14].

A tripeptide, reduced L-glutathione – composed of L-glutamate (*glu*), L-cysteine (*cys*) and glycine (*gly*) moieties (Scheme 5) – is commonly named the "mother" of all antioxidants. Along with thioredoxin, they rank among the major thiol antioxidants. GSH is the most ubiquitous low-molar-mass thiol in cells, present in roughly millimolar concentrations. Intracellular L-glutathione exists in a reduced (GSH) and oxidized (GSSG) form. The thiol group of a reduced GSH might serve as a donor of a reducing equivalent ($H^+ + e^-$). The GSH molecule disposes of four exchangeable protons: two –COOH (pK_a 2.50 and 3.70), one–SH (pK_a 9.20) and one $-NH_3^+$ (pK_a 9.50) [15].

The present work examines the role of the above thiol compounds, D-pen and GSH in the time- and concentration-dependent degradation of high-molar-mass hyaluronan initiated by the so-called *Weissberger's* system [ascorbate plus Cu(II)]. One should notice that in the case of D-pen, the thiol group is bound to a tertiary carbon atom while in the case of GSH this group is bound to a primary carbon atom.

Along with the method of rotational viscometry, the undergoing processes and the final hyaluronan fragments were analyzed by using electron paramagnetic resonance and attenuated total reflectance infrared spectroscopies as well as non-isothermal chemiluminometry.

2. Experimental

2.1. Biopolymers

Two high-molar-mass hyaluronan samples B22157 (M_w = 1.34 MDa) and P9710-2A (M_w = 808.7 kDa) were kindly donated by the manufacturers: Genzyme Corporation, Cambridge, MA, USA (B22157) and Lifecore Biomedical Inc., Chaska, MN, U.S.A. While the producer of the HA sample B22157 declared the content of all heavy metal contaminants equal to 2 ppm, the declared content of transition metals in the second HA sample contains, as given by Certificate of Analysis, 4 ppm Cu and 13 ppm Fe.

2.2. Chemicals

Analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were purchased from Slavus Ltd., Bratislava, Slovakia; D-penicillamine, reduced L-glutathione, and DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany; L-ascorbic acid was the product of Merck KGaA, Darmstadt, Germany. Dimethylsulfoxide (0.05% of water content) was purchased from Merck KGaA.

Redistilled deionised high quality grade water, with conductivity of $\leq$0.055 μS/cm, was produced by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

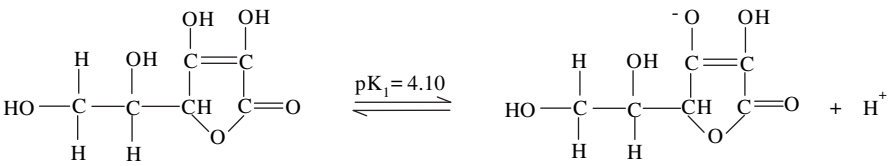
2.3. Preparation of stock and working solutions

The working solutions of the HA samples (2.5 mg/mL) were prepared in dark at room temperature in 0.15 M aqueous NaCl in two steps: at the beginning the first, 4.0 mL of the solvent was added, and 3.85 mL of the solvent was added after 6 h. Stock solutions of L-ascorbic acid (4 and 16 mM), D-pen and GSH (8, 16 and 32 mM), cupric chloride (16 mM diluted to a 160 μM solution), were also prepared in 0.15 M aqueous NaCl.

2.4. Study of uninhibited/inhibited hyaluronan degradation

Anti- and pro-oxidative effects of the two chosen thiols, D-pen and GSH, on the kinetics of degradation of high-molar-mass HA samples were studied by using the oxidative system comprising ascorbate plus Cu(II). The thiol compound was loaded into the reaction mixture either before the reaction onset or within 1 h.

The first experimental set was carried out by adding the thiol compound at the beginning of the HA treatment: The solution mixture was stirred for 30 s prior to each processing. A volume of 50 μL of 160 μM $CuCl_2$ solution was added and stirred for 30 s and



L(+)-Ascorbic acid Ascorbate

Scheme 2. L(+)-Ascorbic acid and ascorbate.

(1) (2)

Scheme 3. D-penicillamine: normal/unionized (1) and zwitterionic form (2).

left to stand for 7 min 30 s at room temperature. Then 50 μL of D-pen or GSH (8, 16 and 32 mM) were added to the examined solution and stirred again for 30 s. Finally, 50 μL of L-ascorbic acid (16 mM) added was stirred for 30 s. Then the assayed mixture was immediately loaded into the viscometer cup reservoir. D-pen initial concentration in the degradative system was 50, 100 and 200 μM while in the case of GSH it was 1.0, 10, 50, 100 and 200 μM.

The second experimental set involved the same concentrations of the components, each stirred for 30 s, however, L-ascorbic acid was added 1 h after the addition of D-pen or GSH.

2.5. Rotational viscometry

The resulting reaction mixture (8.0 mL) was transferred into the Teflon® cup reservoir of a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). Recording of the viscometer output parameters started 2 min after the experiment onset. Changes of the dynamic viscosity values of the system were measured at 25.0 ± 0.1 °C in 3 min intervals for up to 5 h. The viscometer Teflon® spindle rotated at 180 rpm, i.e. at a shear rate of 237.6 s^{-1}.

To determine the reaction products, the polymer was recovered by standard precipitation/drying procedures. The average product yield was 66%.

2.6. FT-IR spectroscopy

Fourier-transform infrared (FT-IR) spectra were measured with a Nicolet Magna - IR 750 spectrometer (Thermo Electron Corp., Madison, WI, USA) with DTGS detector and OMNIC 7.1 software. 128 scans at a resolution of 4 cm^{-1} were averaged. Attenuated total reflectance infrared (ATR) spectra were measured in the solid state with a Pike Technologies MIRacle™ single reflection horizontal attenuated reflectance accessory with a ZnSe crystal. Fragmented biopolymers in solid form, related to the intact HAs (P9710-2A and B22157), were chosen to test a possible incorporation of a thiol residue into the biopolymer structure during processing.

2.7. Non-isothermal chemiluminometry

Non-isothermal chemiluminescence method-based measurement of the fragmented HA (P9710-2A) sample in solid form was carried out using a photon-counting instrument Lumipol 3 manufactured at the Polymer Institute of the Slovak Academy of Sciences [16].

2.8. EPR spectroscopy

The generation of free radicals during hyaluronan degradation was examined by spin trapping technique in an EPR X-band EMX spectrometer (Bruker, Rheinstetten, Germany) at ambient temperature [17]. A representative HA sample (B22157; 2.5 mg/mL), was chosen using reaction conditions as follows: 1. 1.0 μM Cu(II) + 200 μM D-pen addition before the reaction onset; 2. 1.0 μM Cu(II) + 200 μM D-pen addition before the reaction onset + 100 μM L-ascorbic acid. The spectra were recorded at the 1st, 20th, 60th, 90th and 120th min after the addition of L-ascorbic acid. A 250 μL of each sample solution was thoroughly stirred with 5.0 μL of DMPO spin trap prior to each experimental set carried out in a thin flat EPR quartz cell. The operational parameters of the equipment [17] were adjusted as follows: centre field 3354 G, sweep width 100 G, time constant 81.92 ms, conversion time 20.48 ms, receiver gain 5e+5, microwave power 10 mW, and modulation amplitude 2 G.

3. Results and discussion

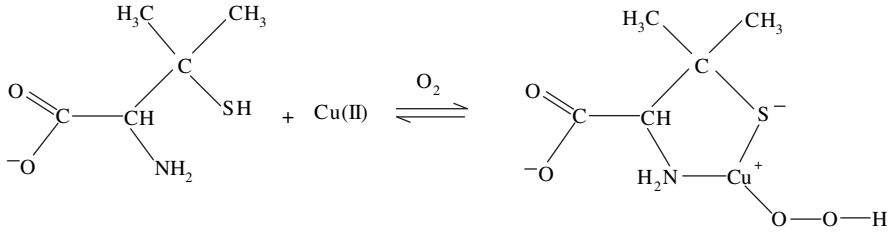
3.1. Rotational viscometry; effects of time- and concentration-dependent addition of thiols

Fig. 1A and B illustrates the dynamic viscosity versus time profiles of the P9710-2A sample solution (2.5 mg/mL) with the addition of 1.0 μM Cu(II) plus 100 μM L-ascorbic acid, to which either 1.0, 10, 50, 100, 200 μM of L-glutathione (A) or 50, 100, 200 μM of D-penicillamine (B) was added. As evident, addition of GSH (10, 50, 100, or 200 μM) resulted in a marked protection of the HA macromolecules against degradation. The greater the GSH concentration used, the longer was the observed stationary interval in the sample η values. Within the time interval examined (5 h), concentrations of GSH 50, 100 and 200 μM led to the total inhibition of the viscosity decay. However, the inhibitory effect of D-pen demonstrated another kinetic course.

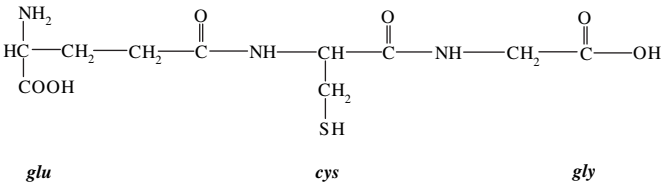
However, two characteristic features should be pointed out in the results represented in Fig. 1:

(i) at the lowest GSH concentration used, i.e. 1.0 μM (Fig. 1A, line 1), the time-dependent course of the HA degradation was more rapid than that of the reference experiment with the nil thiol concentration. Thus, one could classify GSH traces as functioning as a pro-oxidant;
(ii) D-pen, dose dependently, acts as a scavenger of •OH radicals within the first 30–90 min (Fig. 1, panel B, lines 1, 2, and 3) then, however, its inhibition activity is lost and degradation of hyaluronan takes place.

Fig. 2 depicts the effect of the addition of 50 μM of GSH (A) or D-pen (B) before the reaction onset (line 1) or 1 h after the



Scheme 4. Chelation complex formation of D-pen and copper ion in an aqueous milieu under aerobic conditions.



Scheme 5. Reduced L-glutathione.

degradation had started (line 2). The application of the 1 h-delayed addition of the thiol compound has been designed supposing that the depletion of formed •OH radicals, initiating the HA degradation processes, was almost accomplished within this time period as confirmed by the recent EPR study [18]. It means that the scavenging effectiveness of the thiol compound in the propagation phase of the HA degradation may be evaluated. By taking into account the above tenets, it can be stated again that while GSH demonstrated a total inhibition of the degradation of HA macromolecules (Fig. 2A, line 2), D-pen indicated much less efficiency (Fig. 2B, line 2).

3.2. FT-IR spectroscopy

Analyses of the FT-IR spectra of fragmented HA samples, differing in their ultimate molar mass, treated by the oxidative system – composed of ascorbate plus cupric ions – in the presence of a thiol compound, revealed changes in their typical spectral bands when compared to those achieved for intact HA samples (P9710-2A and B22157) and for the corresponding thiol spectrum.

Considering the P9710-2A and B22157 samples, treated with D-pen addition (Fig. 3A,B), one can observe that by the biopolymer modification with this thiol, changes are seen in infrared spectra in the region of stretching vibrations of C–H bonds at 2919 cm^{-1} and 2856 cm^{-1} for the HA (P9710-2A) sample or at 2925 cm^{-1} and 2858 cm^{-1} for the HA (B22157) sample, respectively. The formation of a new peak of low intensity at 1741 cm^{-1} (A) or 1742 cm^{-1} (B), respectively, characteristic for stretching vibrations of C═O bond of esters, might be the consequence of such HA modification. The region between 1200 cm^{-1} and 1000 cm^{-1} shows the bands typical for the polysaccharide chain [19]. Modification of the HA samples (P9710-2A and B22157) is shown by the shift of a specific band maximum from 1038 cm^{-1} or 1039 cm^{-1} to 1027 cm^{-1}. Regarding both HA samples, peaks of low intensities can be seen at 2595, 2525 or 2463 cm^{-1}, respectively, by magnifying the corresponding region [data on files with the authors]. These peaks, which are characteristic for D-pen alone, could serve as indicators of the HA modification by this thiol compound.

In the case of the HA sample (P9710-2A) treated with L-glutathione addition (Fig. 3 C), the changes in infrared spectrum are found in the region of stretching vibrations of C–H bonds at 2928 cm^{-1} and 2853 cm^{-1}. The peak of a low intensity at 1712 cm^{-1} (*record 2*), might indicate L-glutathione incorporation into the biopolymer. L-glutathione alone is represented by a peak which is related to the stretching vibration of C═O bonds at 1709 cm^{-1} (Fig. 3, C3*). The changes at 1537 cm^{-1} and 1652 cm^{-1} (*record 2*), in which L-glutathione shows the shoulders of absorption peaks, might be caused by the L-glutathione incorporation. The shift of the specific band maximum from 1038 cm^{-1} to 1026 cm^{-1} in the region of ring vibrations of the polysaccharide also indicates the modification of the hyaluronan.

On comparing the results of the FT-IR spectroscopic analyses, some indications of possible chemical modification of the HA chain by a thiol-related residue have been revealed. As far as we know, such a novel observation could have a really high impact in the given research scope. Namely, it is well established that the –SH group of cysteine and methionine residues of proteins are readily susceptible to oxidation by the action of ROS [20]. When a low-molar-mass thiol, such as glutathione is present, the oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups and the low-molar-mass thiols. In this case we speak of S-glutathiolation [20,21]. The proof/disproof of the hyaluronan glutathiolation or "penicillaminothiolation" calls for further investigative work.

3.3. Non-isothermal chemiluminescence

Non-isothermal chemiluminescence runs in oxygen and nitrogen for intact hyaluronan and that processed with ascorbate plus cupric ions with the addition of L-glutathione or D-penicillamine are shown in Fig. 4. The curves give the maximum of the light emission at temperatures above 200 °C (Table 1). The temperature of maximum chemiluminescence intensity in nitrogen is lower than that in oxygen. Alkyl radicals, formed from different initiating routes (direct scission of the main chain bonds, decomposition of hydroperoxides) are converted in oxygen to peroxyl radicals which emit light from excited carbonyl groups formed in mutual disproportionation. However, one fact is worth mentioning. The peak maximum of chemiluminescence from the intact hyaluronan in

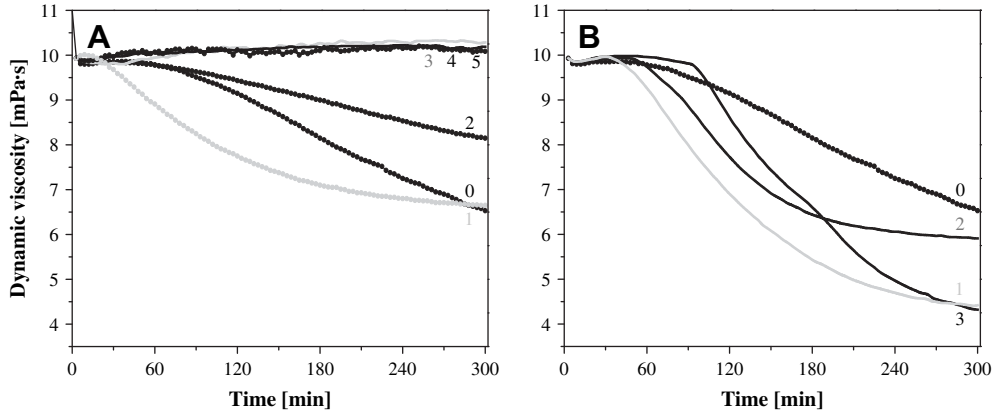


Fig. 1. Comparison of the effect of L-glutathione (panel A) and D-penicillamine (panel B) on the P9710-2A sample degradation induced by the system containing 1.0 μM $CuCl_2$ plus 100 μM L-ascorbic acid. The thiol was added before the reaction onset. Concentration of L-glutathione in μM: 1–1.0; 2–10; 3, 4, 5–50, 100, and 200, respectively. Concentration of D-penicillamine in μM: 1–50; 2–100 and 3–200. Reference experiment: 0–nil thiol concentration.

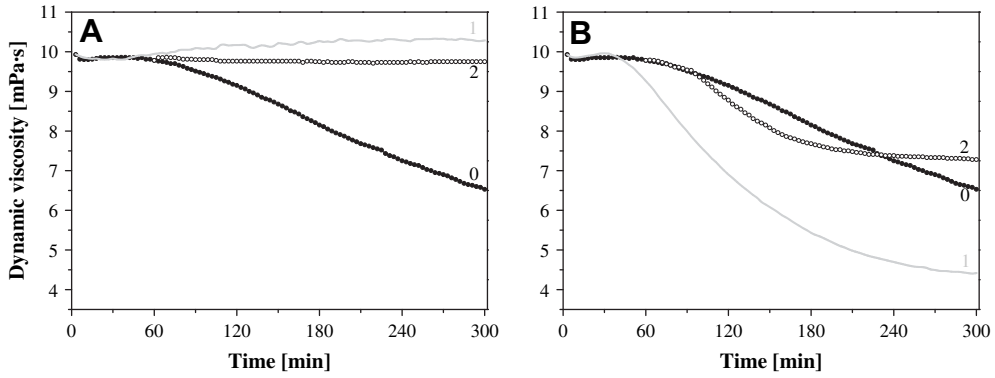


Fig. 2. Comparison of the effect of L-glutathione (panel A) and D-penicillamine (panel B) on the P9710-2A sample degradation induced by the system containing 1.0 μM $CuCl_2$ plus 100 μM L-ascorbic acid. The thiol was added before the reaction onset (1) or after 1 h (2). The concentrations of L-glutathione and D-penicillamine added in μM: 50. Reference experiment: 0–nil thiol concentration.

nitrogen is situated at 210 °C, approximately (Table 1) while the peak in polypropylene corresponding to hydroperoxides is at 160 °C [22].

While in oxygen we may follow partially the oxidation of intact hyaluronan sample occurring *via* hydroperoxides, in nitrogen the initiating process appears to be only the scission of the bonds forming the backbone of the hyaluronan macromolecule. The area below the curve indicated that the processed samples contain a considerably lower amount of potentially reactive sites for both routes of initiation than intact samples.

The kinetic model which we propose here for hyaluronan degradation was first used by Ekenstam [23] and developed by Emsley [24] for degradation of cellulose. The degree of polymerization (DP) was defined there as the ratio of concentration of monomer units (N) and polymer molecules (i) as follows:

$$\mathrm{DP} = \frac{N}{i} \tag{1}$$

The concentration of hyaluronan molecules increases over time with increasing extent of degradation. The process of chain scissions is assumed to be of zero order ($\mathrm{d}i/\mathrm{d}t = k$) and the concentration i of macromolecules changes in time as a linear function of time.

$$i = i_0 + kt \tag{2}$$

where k is the rate constant of main chain scissions and i_0 is the initial concentration of macromolecules in the system, respectively. For DP we thus have:

$$\mathrm{DP} = \frac{N}{i_0 + kt} \tag{3}$$

$$\text{or } \mathrm{DP} = \frac{\mathrm{DP}_0}{1 + \frac{k}{i_0}t}$$

where $\mathrm{DP}_0 = N/i_0$

After transformation we obtain

$$\frac{1}{\mathrm{DP}} - \frac{1}{\mathrm{DP}_0} = \frac{k}{i_0 \mathrm{DP}_0}\ t \tag{4}$$

$$\text{or } \frac{\mathrm{DP}_0}{\mathrm{DP}} = 1 + \frac{k}{i_0}\ t \tag{5}$$

Eq. (4) is the Ekenstam equation [23] known from the literature for isothermal degradation of linear macromolecules. However, as eq. (5), gives the rate constant k/i_0 of the magnitude s^{-1}, it is better to work with it. This allows for a direct comparison between samples of different initial DP.

The chemiluminescence intensity I was assumed to be proportional to the rate of change of the degree of polymerization (DP) of the solid polymer [25–27].

$$I = \Phi\left[-\frac{\mathrm{dDP}}{\mathrm{d}t}\right] \tag{6}$$

where Φ is a proportionality constant involving quantum yield of chemiluminescence reaction. The above case corresponds to the randomly reacting sites on hyaluronan backbone giving rise eventually to hydroperoxides which are cleaved *via* alkoxyl radicals and reduction of the molar mass takes place.

The evaluation of non-isothermal kinetics may be somewhat problematic. However, if we assume that the isothermal equation (5) is valid, then the first derivative in time of the degree of polymerization (DP) is:

$$-\frac{\mathrm{dDP}}{\mathrm{d}t} = \frac{k}{i_0 \mathrm{DP}_0}\ \mathrm{DP}^2 \tag{7}$$

This equation may be converted to non-isothermal conditions by the simple introduction of the rate of heating as follows:

$$-\frac{\mathrm{dDP}}{\mathrm{d}T}\frac{\mathrm{d}T}{\mathrm{d}t} = \frac{A\exp(-E/RT)}{i_0 \mathrm{DP}_0}\ \mathrm{DP}^2 \tag{8}$$

Here T is temperature, A and E are pre-exponential factor and activation energy, respectively, and $\mathrm{d}T/\mathrm{d}t = \beta$ is the linear rate of sample heating.

After integration of eq. (8) and back substitution into eq. (6) we obtain:

$$I = \Phi\ \frac{A\exp(-E/RT)}{i_0}\ \frac{\mathrm{DP}_0}{\left[1 + \frac{A}{\beta i_0}\int_{T_{\mathrm{room}}}^{T}\exp(-E/RT)\mathrm{d}T\right]^2} \tag{9}$$

It was already assumed that the light emission comes from several runs corresponding to several independent initiating events. Provided that the process of light emission corresponds to two such events ($i = 2$) which are attributed to parallelly occurring main chain scissions and free radical degradation of hyaluronan via hydroperoxides, we have:

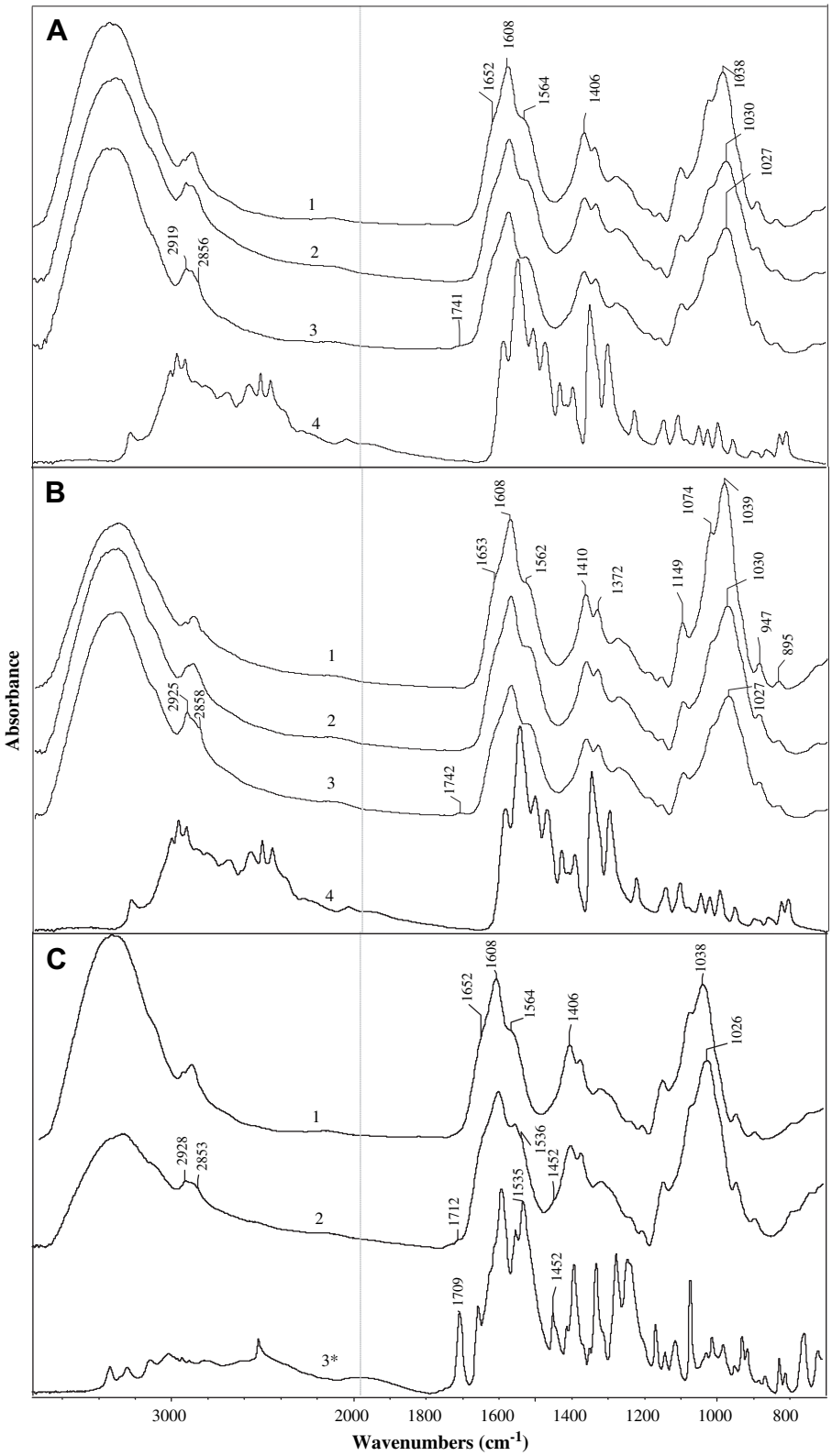


Fig. 3. Comparison of the FT-IR spectra of modified and unmodified HA samples: Panels A and C: HA sample (P9710-2A); B: HA sample (B22157). Panels A and B: Treatment with D-penicillamine addition; C: Treatment with addition of L-glutathione. Intact HA sample (1). Fragmented HA sample on the thiol addition before the reaction onset (2) or within 1 h (3) on the thiol addition. D-Pen (4). GSH (3*). (The treatment conditions: HA sample treated 5 h with 1.0 μM Cu(II) + 100 μM L-ascorbic acid, + 50 μM D-pen or GSH.).

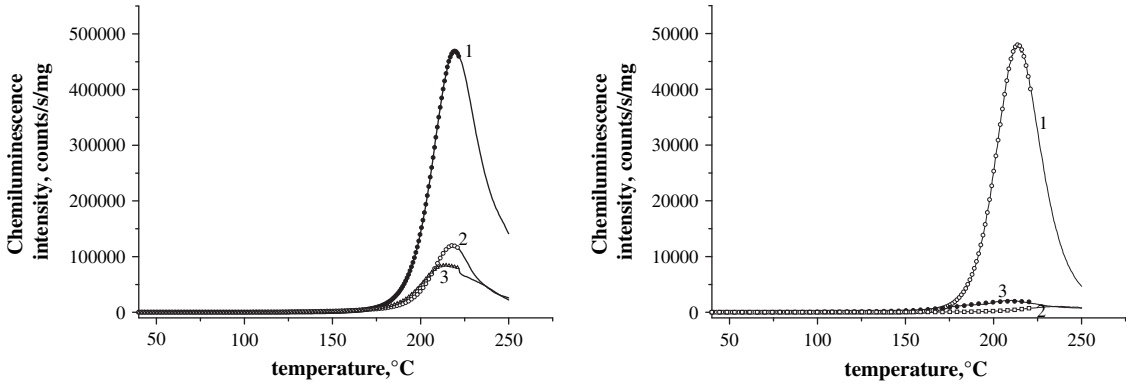


Fig. 4. Effect of L-glutathione and D-penicillamine addition to a HA sample on hydroperoxide elimination determined by a non-isothermal chemiluminescence method: in the presence of oxygen (left) and nitrogen (right): intact P9710-2A sample (1); HA sample treated 5 h with 1.0 μM Cu(II) plus 100 μM L-ascorbic acid with addition of 50 μM of L-glutathione (2) or D-penicillamine (3). The points on lines 1, 2 and 3 represent the theoretical fit by the sum of 2 s order temperature dependent functions (Equation 10).

$$I = \sum_{i=1}^{2} \frac{P_i}{\left[1 + \frac{A_i}{\beta i_0} \int_{T_{room}}^{T} \exp(-E_i/RT)\mathrm{d}T\right]^2} \quad (10)$$

Here P_i is the proportionality constant including the corresponding terms from eq. (8). This constant may be ascribed to the area below the corresponding component process. A_i and E_i are the pre-exponential factor and activation energy of respective component of the initiation reaction (Table 2). In a computation procedure we determine the corresponding parameters for any of the two initiating events by non-linear regression analysis taking into account the experimental runs normalized to one by dividing the data by maximum chemiluminescence intensity.

In Table 2 we show the activation energy and pre-exponential factor for two-component oxygen process and one-component nitrogen process. The activation energy below 100 kJ/mol and above 240 kJ/mol was found for the respective faster and slower component of the process. The latter value is typical for direct scission of bonds linking the monomer units while the former corresponds more to the process occurring *via* hydroperoxides.

The ratio of the faster and slower component of the oxidation process is P_1/P_2 which characterizes the extent of hydroperoxidation changes with the sample processing. While for the intact sample it is 1:7, for the D-pen-processed sample it is 1:3 and for the GSH-processed sample it is almost 6:1 (Table 2).

The light intensity emitted from the intact and processed hyaluronans may be understood as a fingerprint of the extent of oxygenated structures in hyaluronan macromolecule. Alkyl radicals and/or peroxyl radicals being formed preferentially from direct scission of main chain bonds and after the reaction with oxygen in their recombination provide the energy which may be used for excitation of e.g. carbonyl groups. These groups, which may be formed by oxidation from hydroxyls of glucopyranosyl units in positions 2 or 3, during the hyaluronan storage were reported that they may weaken the C–O–C bonds linking the monomer units in hyaluronan. Provided that L-glutathione and/or D-penicillamine are added to the system tested, the chemiluminescence intensity is significantly reduced. This fact corresponds with an effect of this species on decomposition of hydroperoxides and on removal of oxygenated structures (Fig. 4).

Table 1
The area S below chemiluminescence-temperature runs and temperature T_{max} of the maximum chemiluminescence intensity for samples from Fig. 4.

Sample	*S*, counts/g	T_{max}, °C
Oxygen		
Intact HA sample	3.98×10^{11}	219.7
GSH-processed HA sample	8.64×10^{10}	214.1
D-Pen-processed HA sample	9.77×10^{10}	217.7
Nitrogen		
Intact HA sample	3.86×10^{10}	213.6
GSH-processed HA sample	3.02×10^{9}	208.7
D-Pen-processed HA sample	1.02×10^{9}	238.7

3.4. EPR spectroscopy

EPR experiments show that in the absence of D-pen addition of cupric ions and the DMPO spin trap into the HA solution leads to the generation of •OH radicals. This situation might indicate that DMPO alone or in cooperation with the polysaccharide can act as "a reducing medium" for cupric ions [17]. Even more hydroxyl radicals are generated in the presence of D-pen and cupric ions. Addition of D-pen gives rise to hydroxyl radical formation immediately at the beginning of the process [see inset (2) in Fig. 5]. However, we can conclude that the more hydroxyl radicals are generated in the presence of D-pen, the later is the pulse-rise-time of hydroxyl radicals at the period after addition of L-ascorbic acid (dashed line in Fig. 5). We propose that by this action D-pen inhibits other processes, which are later responsible for a rapid degradation of the biopolymer. This might be due to the copper chelation where it figures in the form of Cu(I) possibly forming hydroxyl radicals

Table 2
The parameters of eq. (10) for non-isothermal chemiluminescence runs – the rate of heating 2.5 °C/min.

Parameter	Intact HA sample	D-Pen-processed HA sample	GSH-processed HA sample
Oxygen			
P_1, counts/g	7.09×10^{10}	3.65×10^{10}	1.93×10^{11}
P_2, counts/g	3.51×10^{11}	7.61×10^{10}	3.16×10^{10}
A_1, s^{-1}	3.66×10^{4}	4.75×10^{3}	1.17×10^{7}
A_2, s^{-1}	1.55×10^{23}	1.14×10^{26}	1.66×10^{27}
E_1, kJ/mol	84.2	63.8	99.4
E_2, kJ/mol	240.5	266.5	272.9
Nitrogen			
P_1, counts/g	–	–	–
P_2, counts/g	3.8×10^{10}	2.4×10^{9}	3.7×10^{9}
A_1, s^{-1}	–	–	–
A_2, s^{-1}	2.7×10^{22}	5.7×10^{11}	1.6×10^{7}
E_1, kJ/mol	–	–	–
E_2, kJ/mol	230.7	143.7	92.2

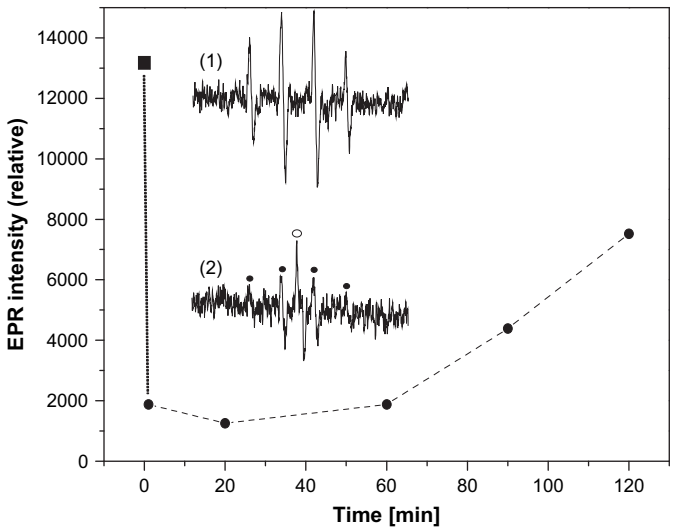


Fig. 5. Time dependence of EPR intensity of •DMPO–OH adduct observed for the system containing HA sample (B22157), $CuCl_2$ (1.0 μM), D-pen (200 μM), and L-ascorbic acid (100 μM). The EPR spectra were measured before addition of L-ascorbic acid [HA + $CuCl_2$ + D-pen; see inset (1) and EPR intensity marked with full square] and in the 1st, 20th, 60th, 90th, and 120th min after addition of L-ascorbic acid. 250 μL of the corresponding sample was mixed with 5 μL of DMPO spin trap and the EPR spectra were immediately measured. EPR signal of the ascorbyl anion radical is marked with an empty circle and •DMPO–OH adduct with full circles in inset (2).

similar as proposed for system ascorbate-Cu(II) ions. However D-pen forms stable mixed complexes with Cu(I) and dioxygen and consequently the copper is mostly blocked by this complexation in further activities in the presence of L-ascorbic acid. As most of Cu(II) ions are "blocked" with D-pen at the beginning of the process, ascorbate acts as an effective scavenger of hydroxyl radicals generated under formation of ascorbyl anion radical [see inset (2) in Fig. 5].

3.5. Outline of the radical formation responsible for hyaluronan degradation

The so-called Weissberger's system [ascorbate plus Cu(II)] is a well-known generator of hydrogen peroxide (Scheme 6). Depending on the reaction conditions, the hydrogen peroxide may play a dual role in the next stages: it acts either as a reductant or an oxidant of copper ions:

$$2Cu(II) + H_2O_2 \rightarrow 2Cu(I) + O_2 + 2H^+$$

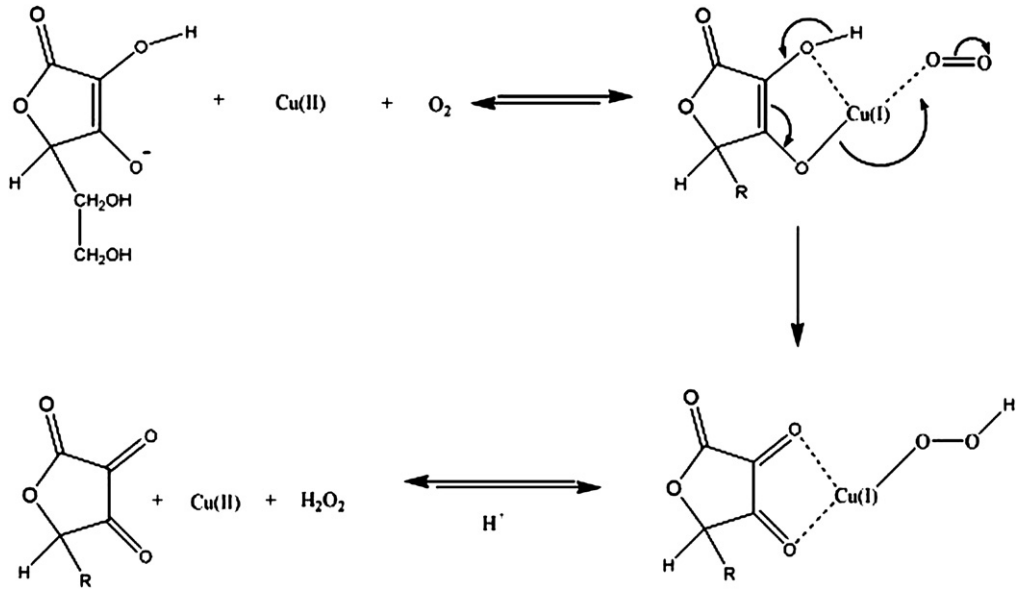
$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^- \text{ (Fenton – like reaction)}$$

The second reaction is, however, dominating, resulting in the formation of deleterious •OH radicals. Whereas a strong reducing agent, ascorbate, is gradually consumed in the Weissberger's cycle, the generated hydrogen peroxide is immediately decomposed by the action of cuprous ions.

The pro-degradation effect of low concentrations of GSH (1.0 μM) which are close to stoichiometric ratio with copper ions represents a challenge for interpretation. Probably an interplay between the reducing and complexation efficiencies of L-ascorbic acid and GSH changing the ratio between Cu(I)/Cu(II) concentrations in favour of Cu(I) leading to the promotion in formation of H_2O_2 and thus to a larger extent of free radical formation may be of significance. At the same time GSH seems to be depleted by its reaction with Cu(II) and is not able to scavenge reactive hydroxyl radicals efficiently [29]. When increasing the concentration of GSH, it starts to scavenge the reactive free radicals and inhibitory effect appears which is more or less related to the reactivity of GSH in free radical transfer. L-Ascorbic acid which is in surplus when compared with Cu ions appears not to be as efficient free radical scavenger as GSH.

The standard reduction potential for the redox couple $AscH^{\bullet -}$/$AscH^-$ is 0.282 V and for the redox pair Cu(II)/Cu(I) it is 0.16 V in an aqueous solution at pH 7.0 [30]. As a result of HA chelating activity of Cu(II), the actual reduction potential value may be, however, different. A 100-fold concentration excess of L-ascorbic acid, over copper ions in our oxidative system tested give cyclic rises of the reduction of cupric ions to cuprous ones, which subsequently decompose hydrogen peroxide, thus generating highly reactive hydroxyl radicals – the initiators of the HA degradation.

The action of D-pen (100 μM) was first self-tested in the system of hyaluronan and cupric ions in the absence of L-ascorbic acid. It was observed that D-pen in the role of a reducing agent of cupric ions caused no biopolymer degradation during the time interval



Scheme 6. : Proposed mechanism of hydrogen peroxide formation by the Weissberger's system [ascorbate plus Cu(II)]; adapted from Fisher and Naughton [28].

given of 5 h [31]. The same results were confirmed in the case of GSH self-testing (50, 100, and 200 μM). The presence of L-ascorbic acid in the system mentioned, vide supra, however, exhibited a markedly different action. The whole process, on running the reference experiment with a nil concentration of D-pen/GSH, upon a 100-fold concentration ratio of L-ascorbic acid, and Cu(II) [31] is characterized by HA biopolymer degradation. The interaction of ascorbate and Cu(II) ions appears to be crucial step in hydrogen peroxide formation (Scheme 6). Briefly, the sequence of the reactions could be described as follows: Abstraction of H• from the hyaluronan chain lead to A• i.e. a *C*-centred (macro)radical species ($HA + {}^{\bullet}OH \rightarrow A^{\bullet} + H_2O$). In the presence of oxygen, the A• (macro)radical is converted into peroxyl radical, which can further react with HA yielding hydroperoxides ($A^{\bullet} + O_2 \rightarrow AOO^{\bullet}$; $AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$). The AOOH could subsequently decompose in the reaction catalyzed by cuprous ions ($AOOH + Cu(I) \rightarrow AO^{\bullet} + Cu(II) + OH^{-}$) yielding AO• i.e. alkoxy (macro)radicals. Then, the oxygen-centred AOO• and AO• radicals may continue the further propagation reactions finally resulting in the biopolymer degradation evidenced by a decrease of the dynamic viscosity values of the reaction mixture. As shown in our previous paper, the longer the chain of the hyaluronan sample, the more it is susceptible to degradative action of radicals generated [31].

A strong inhibitory effect of the GSH molecule, within the concentration interval of 50–200 μM, might partially be the result of the formation of bulky L-glutathionyl tripeptide radicals (GS•), which recombine quickly giving a stable GSSG molecule.

4. Conclusions

L-glutathione is significantly more efficient in stabilization of the system hyaluronan + 1.0 μM of Cu(II) + 100 μM of L-ascorbic acid than D-penicillamine. An ascorbate plus Cu(II) system acts as fragmentation agent leading to the reduction of the molar mass of hyaluronan. Provided that the ratio of GSH and Cu(II) approaches to the stoichiometry 1:1, this thiol is no longer efficient and displays a pro-degradation effect. Probably an interplay between the reducing and complexation efficiencies of ascorbate and GSH with respect to Cu(II) ions changing the ratio between Cu(I)/Cu(II) concentrations in favour of Cu(I) may be of importance. Chemiluminescence measurements indicated that intact hyaluronan at elevated temperatures degrades by the direct scission of bonds linking monomer units in hyaluronan macromolecule. The processing leads to a significant reduction of oxygenated structures and according to the values of activation energy the role of hydroperoxides in the main chain scission becomes significant. The formation of hydroxyl radicals in the system of ascorbate plus Cu(II) has been demonstrated by EPR spectroscopy by using a radical trap. Despite certain changes in biopolymer structure after processing, tested by the FT-IR method, incorporation of a thiol compound into hyaluronan network may be expected.

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