Study of pro- and anti-oxidative properties of D-penicillamine in a system comprising high-molar-mass hyaluronan, ascorbate, and cupric ions

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Abstract

OBJECTIVES: The study presents results of pro- and anti-oxidative effects of D-penicillamine on hyaluronan degradation by ascorbate plus cupric ions.
METHODS: The well established degradative system comprising high-molar-mass hyaluronan and ascorbate plus Cu(II) ions was used. Primarily, the effects of replacement of ascorbic acid in this system by D-penicillamine were investigated. Then, D-penicillamine was added into the above degradative system before reaction onset or 1h after the reaction had started. To monitor hyaluronan degradation kinetics, rotational viscometry was applied.
RESULTS: No hyaluronan degradation occurred when ascorbate was replaced by D-penicillamine. The drug addition into the complete degradative system at the reaction onset caused a marked inhibition of hyaluronan degradation. However, the inhibitory effect turned to a pro-oxidative one within appr. 1 h.
CONCLUSION: The dual behavior of D-penicillamine on hyaluronan degradation can relate to: (i) the drug completely traps •OH radicals generated from ascorbate plus Cu(II) ions under aerobic conditions; (ii) thiyl radicals generated from D-penicillamine react with D-penicillamine anions resulting in novel radical-reactive species, which e.g. by reducing dioxygen molecules can generate further •OH radicals.

Abbreviations

MDa	- megagram/mol
Mw	- weight-molar-mass average
HA	- hyaluronan
RA	- rheumatoid arthritis
rpm	- rotational speed per minute
SF	- synovial fluid

ORIGINAL ARTICLE

INTRODUCTION

In high amounts, copper can be poisonous and even fatal to organisms. This biogenic transition metal has been implicated in the pathogenesis of neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Wilson's disease, as well as amyotrophic lateral sclerosis. In Wilson's disease (an autosomal recessive disease) body tissues, mostly the liver and brain, retain too much copper. D-Penicillamine, due to its high capacity for chelation of copper, has been used for treating patients with Wilson's disease [1].

D-Penicillamine is also used to treat patients with severe active rheumatoid arthritis (RA) unresponsive to conventional therapy. This compound functions as an immunomodulating/third-line disease-modifying anti-rheumatoid drug [2]. One of the reasons for its use has also been the observation that in the synovial fluid (SF) from RA patients, compared to SF from healthy subjects, the mean concentration of copper increases by a factor of three [3].

D-Penicillamine is a 3-mercapto-D-valine (Figure 1). In the presence of copper ions, however, the positive/"therapeutic" action of D-penicillamine can also be related to the potency of this drug to produce hydrogen peroxide. On the other hand, D-penicillamine has been grouped among the scavengers of H_2O_2. The opposing properties of D-penicillamine, namely its ability to produce as well as to scavenge hydrogen peroxide may be relevant to its toxic or therapeutic actions in rheumatoid diseases [4].

The main components of SF include the filtrate of blood plasma and a high-molar-mass hyaluronan (HA). It is a linear polysaccharide formed of two disaccharide units containing *N*-acetyl-D-glucosamine and D-glucuronic acid (Figure 1). In an aqueous milieu, HA is represented by negatively charged hyaluronate macromolecules with extended conformations, which impart high viscosity/viscoelasticity to its solution [5]. The observed decrease of the mean molar mass of HA during joint inflammation in RA patients have sometimes be used as an indirect marker of the disease development.

As reviewed recently [6], HA macromolecules effectively demonstrate a high sensitivity to damaging action of various oxidants, among others to hydrogen peroxide damaging action or more specifically to hydroxyl (•OH) radicals generated according to the reaction H_2O_2 + Cu(I)/Fe(II) → •OH + Cu(II)/Fe(III) + HO^-. The •OH radical, due to its extremely high reactivity, extracts a proton ($H^\bullet$) from the HA macromolecule resulting in the production of a *C*-type macroradical. Under aerobic conditions, the latter is reformed into a peroxyl-type radical species, which subsequently participates in the propagation of HA chain breaking [7].

The aim of this study was to exploit the high sensitivity of HA macromolecules in testing the ability of D-penicillamine *plus* Cu(II) ions to generate oxidative/degradative species, namely hydrogen peroxide or more specifically •OH radicals. The study presented here was compared to our previous results dealing with the application of the so-called Weissberger's system, consisting of ascorbate *plus* Cu(II) ions for the production of H_2O_2 [8]. The pro- or anti-oxidative properties of D-penicillamine were also tested by applying a three-component mixture comprising high-molar-mass hyaluronan (marker of oxidative reactions) and ascorbic acid *plus* $CuCl_2$ (generator of •OH radicals). The two recently established experimental designs [9] enabled us to prove/disprove the D-penicillamine scavenging ability not only against hydrogen peroxide and more specifically •OH radicals, but also against peroxyl- and possibly alkoxyl-type radical species.

MATERIAL AND METHODS

Biopolymers

Five hyaluronan samples, differing by their weight-molar-mass averages (M_w), ranging from 0.43 to 1.34 MDa, were kindly donated or purchased from the following HA manufacturers: Genzyme Corporation, Cambridge, MA, U.S.A; Lifecore Biomedical Inc., Chaska, MN, U.S.A.; Sigma Chemicals Co., St. Louis, MO, U.S.A.; and CPN Ltd., Ústí nad Orlicí, Czech Republic [10]. While the producer of the HA sample (B22157; Genzyme Corporation) declared the content of all heavy metal contaminants 2 ppm, the content of trace transition metals in the original HA sample (P9710-2; Lifecore Biomedical Inc.) was only stated: Fe=13, Pb=7 and Cu=4 ppm. The content of contaminating metals in the HA sample (P9710-2A) was considered identical to that of the original HA (P9710-2) sample [11]. The remaining three HA samples lacked any specification as to the content of contaminating metals.

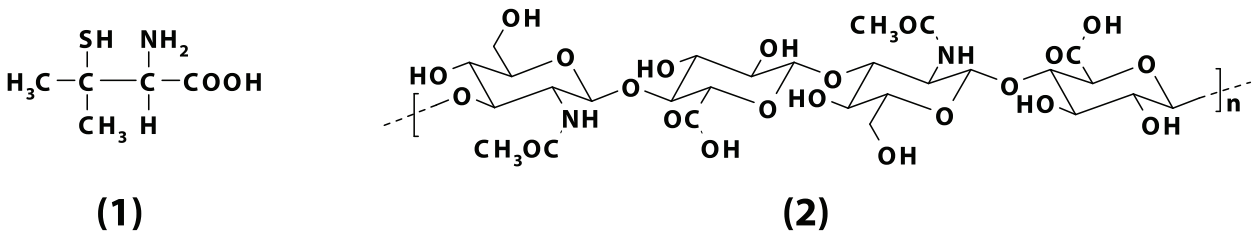

Figure 1. Structural formula of D-penicillamine (1), and hyaluronan (2) – the acid form.

Chemicals and drugs

The analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were from Slavus Ltd., Bratislava, Slovakia; D-penicillamine was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany; L-ascorbic acid was purchased from Merck KGaA, Darmstadt, Germany. Redistilled deionized high quality grade water, with conductivity of ≤ 0.055 μS/cm, was produced by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

Study of hyaluronan degradation

The HA sample (20 mg) was dissolved overnight in the dark in 0.15 M aqueous NaCl in the two steps: First, 4.0 mL of the solvent was added in the morning. Next 3.90 mL (3.85 mL when added Cu(II), ascorbic acid and D-penicillamine) of the solvent was added within 6 hrs The stock solutions were also prepared in 0.15 M aqueous NaCl as follows: ascorbic acid, D-penicillamine, $CuCl_2$, all of the same concentration 16.0 mM. The next day, 50.0 μL of 16.0 mM ascorbic acid was added to the HA solution and slowly stirred for 30 s. Then 50.0 μL of 160 μM $CuCl_2$ solution was added and stirred for 30 s. The final solution tested (8 mL) containing HA (2.5 mg/mL), $CuCl_2$ (1.0 μM), and ascorbate (100 μM) underwent measurement of dynamic viscosity of the HA sample during 5 hrs.

Time dependence of dynamic viscosity of HA solutions was tested as a consequence of the effect of D-penicillamine *plus* copper(II) ion by adding $CuCl_2$ prior to the drug. Namely, 50.0 μL of 160 μM $CuCl_2$ solution was added to the HA solution and stirred for 30 s. After a 9 min equilibration, 50.0 μL of 16.0 mM D-penicillamine was added and the sample was stirred for 30 s. The 8 mL final sample solution underwent measurement of dynamic viscosity during 5 hrs.

Study of the inhibition of hyaluronan degradation

Inhibitory studies of the degradation of high-molar-mass HA samples B22157 and P9710-2A were carried out by using two different systems composed of $CuCl_2$ (1.0 μM), ascorbic acid (100 μM), and D-penicillamine (100 μM), added either before the reaction onset or 1 h after it.

Rotational viscometry

The solution (8 mL) containing HA (2.5 mg/mL), $CuCl_2$ (1.0 μM), ascorbic acid (100 μM), and/or D-penicillamine (100 μM) was transferred into the Teflon® cup reservoir of the Brookfield LVDV-II+PRO rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). The recording of the viscometer output parameters started 2 min after the experiment onset. Dynamic viscosity of the system was 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 the shear rate equaling 237.6 s^{-1} [12]. Under the above specified experimental settings the torque values ranged in the interval between 84 and 18 %.

RESULTS AND DISCUSSION

Degradation of high-molar-mass hyaluronan samples by ascorbate plus copper(II)

Generally, cuprous ions Cu(I) reduce dissolved molecules of dioxygen to $O_2^{\bullet -}$ [13]. Cu(I) ions are oxidized to cupric ones within the reaction cycle and the reduction of O_2 molecules does not further continue. Yet due to the presence of ascorbate, the redox reaction cycles as well as the $O_2^{\bullet -}$ production are continued until the entire consumption of the reductant – the ascorbate molecules. The superoxide anion radicals

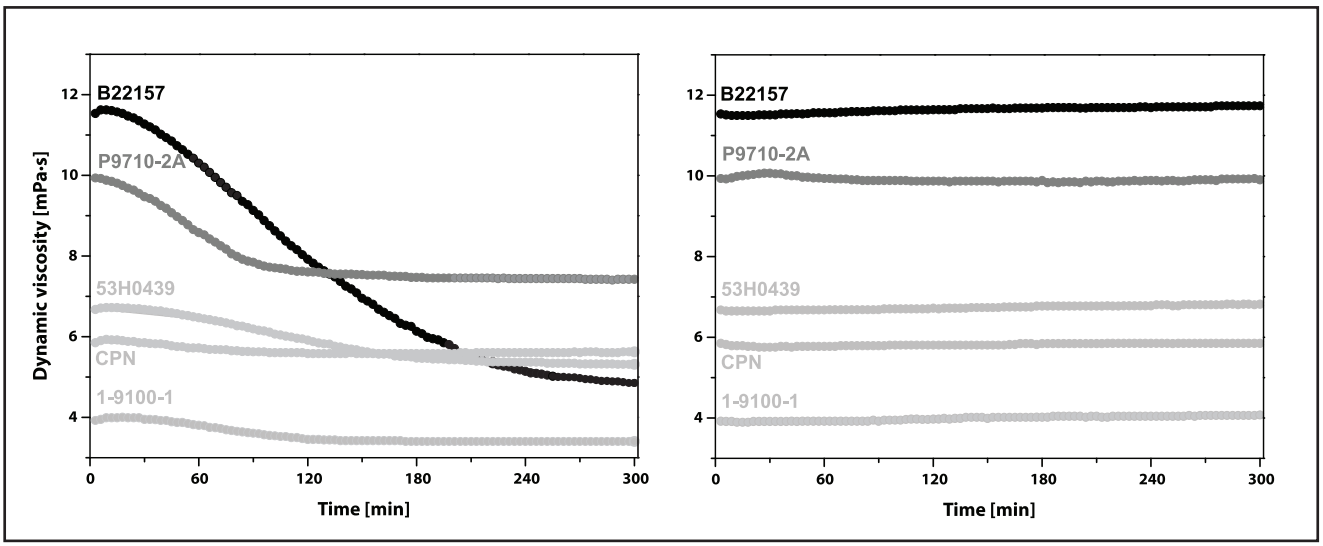

Figure 2. Time dependence of dynamic viscosity of hyaluronan solutions.
Left panel: HA samples of various Mw with addition of 100 μM ascorbic acid and 1.0 μM $CuCl_2$.
Right panel: HA samples of various Mw with addition of 100 μM D-penicillamine and 1.0 μM $CuCl_2$.

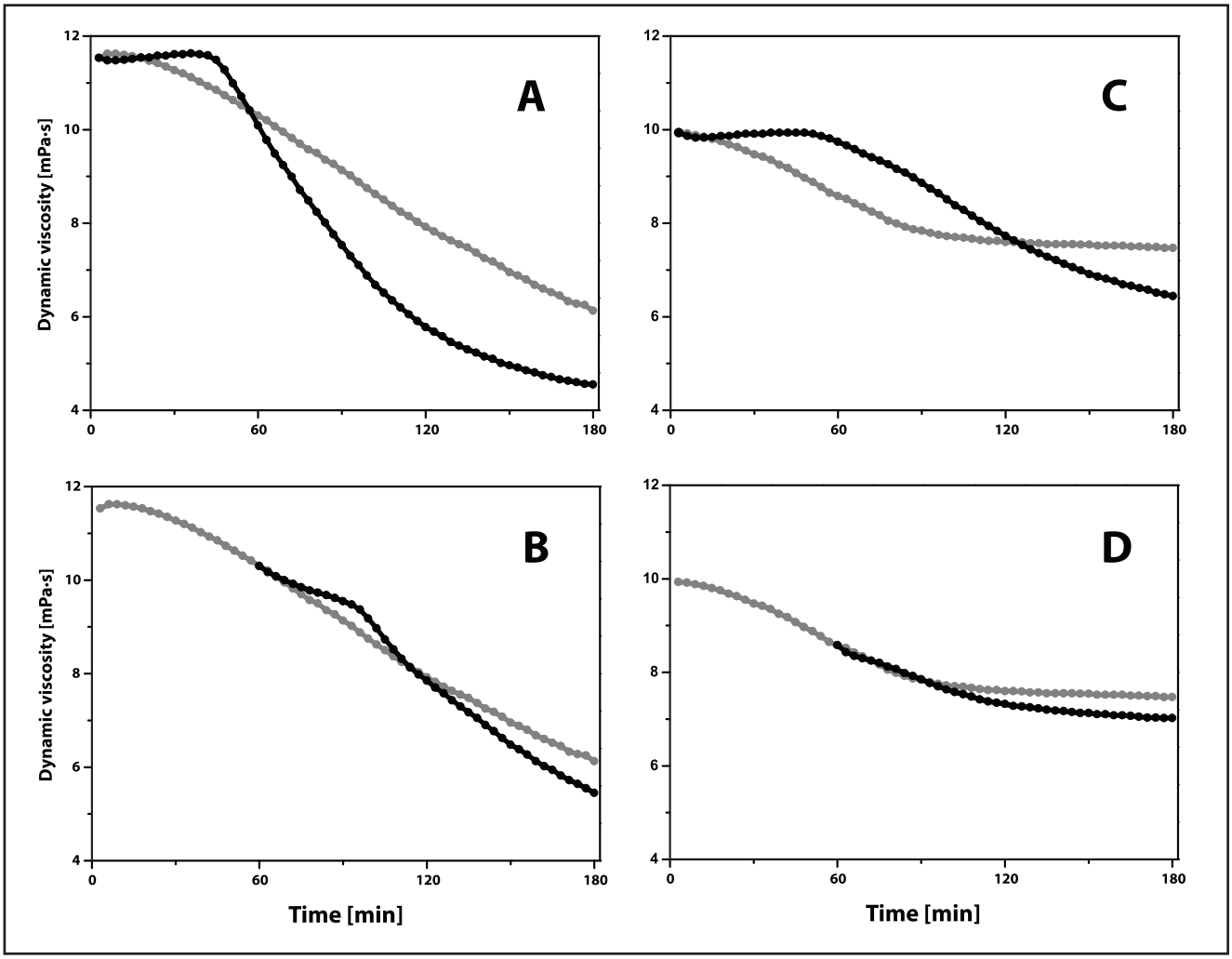

Figure 3. Time-dependence of dynamic viscosity of hyaluronan solutions.
HA sample (B22157)
Panel A: Addition of $CuCl_2$, D-penicillamine and ascorbic acid (black lines) vs. addition of ascorbic acid and $CuCl_2$ (gray lines);
Panel B: Addition of $CuCl_2$, ascorbic acid and D-penicillamine added after 1h (black lines) vs. addition of ascorbic acid and $CuCl_2$ (gray lines).
HA sample (P9710-2A)
Panel C, D: The same conditions as given for panel A and B, respectively. Concentrations used: 100 μM ascorbic acid, 1.0 μM $CuCl_2$, and 100 μM D-penicillamine.

dismutate spontaneously and in this way a continuous flux of H_2O_2 can be anticipated. Hydrogen peroxide, however, is decomposed by the present cuprous ions yielding •OH radicals, which act as initiating species in HA degradation. *C*-macroradicals (hereafter denoted as A•) generated by the reaction HA + •OH → A• + H_2O under aerobic conditions turn to AOO• type radicals. It is these macro-peroxyl species that propagate the HA degradation. Figure 2 (left panel) documents this process. As evident, within a very short time, the viscosity interval of the HA sample solutions gradually declines as the consequence of the reduction of HA molar mass. It should be emphasized that, as shown in Figure 2 (left panel), HA macromolecules of a greater size/longer chain (B22157 and P9710-2A) are much more sensitive to the degradative action of •OH radicals than the three residual HA samples.

The testing D-penicillamine in the function of a reducing agent of cupric ions revealed (cf. Figure 2, right panel) that during the time interval investigated (5 hrs) no degradation of any HA sample occurred. Thus it can be stated that the participation of ascorbate and D-penicillamine in copper redox cycling differs markedly.

Action of D-penicillamine on the degradation of high-molar-mass hyaluronan samples initiated by ascorbate plus copper(II)

Figure 3 (panels A and C) show the situation when D-penicillamine is added to the system comprising HA and ascorbate *plus* Cu(II) at the reaction onset. The results (black lines) up to nearly 60 min support a tenet that D-penicillamine totally inhibits HA degradation. Thus during this time interval, D-penicillamine plausibly traps the generated •OH radicals, yielding,

most probably an R-S• radical intermediate, which may react with R-S$^-$ yielding an intermediate R-S-S-R•$^-$ radical type compound. It should be emphasized that similar reactions were unambiguously established for glutathione (G-SH) participation in scavenging •OH radicals yielding G-S-S-G•$^-$ species. The glutathione action could be a suitable exemplary model for studying D-penicillamine behavior. When D-penicillamine was added to the system 1 h after the reaction onset (cf. Figure 3, panels B and D, black lines), its reaction inhibitory action was not so marked. Contrary to the situation represented in panels A and C, after 1 h of the reaction presumably no significant amount of H_2O_2 (or more specifically •OH radicals) remained present in the system studied. Moreover, as indicated especially by the results shown in panel D, the drug was not able to scavenge/quench the AOO• type radicals.

The most interesting observation is, that the addition of D-penicillamine to the system studied starts to promote HA degradation after a given inhibitory time period. As evident from the graphical plotting, the slopes of the black lines exhibit a much steeper course compared to those recorded for D-penicillamine free systems (gray lines). To explain this phenomenon, the action of G-SH could be again considered. It is well established that G-S-S-G•$^-$ species effectively reduce dioxygen molecules generating thus O_2•$^-$, H_2O_2, and hence further •OH radicals. Analogously, the oxidized D-penicillamine (R-S•) may be converted to R-S-S-R•$^-$ radical type species, which finally promotes the generation of an excess of •OH radicals. The formation, kinetics, and fate of R-S-S-R•$^-$ radical type species in the system comprising HA and ascorbate *plus* Cu(II) remain as yet unraveled.

We may conclude that the observations of this study will stimulate further efforts to prove/disprove the pro-oxidative participation of R-S-S-R•$^-$ type of radical anion substances in HA degradation initiated by the ascorbate *plus* Cu(II) system. In addition with R-S-S-R•$^-$ anion radicals, several further radicals may be implicated. The opposing pro- and anti-oxidative properties of D-penicillamine may be relevant to its toxic or therapeutic actions not only in RA diseases but also in other disorders where an excess of copper ions plays an important role.

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