Ascorbate and Cu(II)-induced oxidative degradation of high-molar-mass hyaluronan. Pro- and antioxidative effects of some thiols

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Abstract

OBJECTIVE: This study presents the results of antioxidative and pro-oxidative efficacy of cysteamine and D-penicillamine (D-pen) in comparison to L-glutathione (L-GSH) on high-molar-mass hyaluronan (HA) degradation by cupric ions plus ascorbic acid.

METHODS: The substance tested was applied in the degradative system cupric ions plus ascorbate: (i) before the reaction onset or also (ii) 1 h after the reaction started. The results obtained were compared with that one recorded by using the degradative system in the absence of the substance tested. To monitor HA degradation kinetics, rotational viscometry was applied. Moreover, the standard ABTS and DPPH assays were used.

RESULTS: By using the method of rotational viscometry, D-pen showed dual effect: initial inhibitory effect on •OH radicals was changed to a pro-oxidative one in the dose and time dependent manner. Both L-GSH and cysteamine were recorded to be more effective scavengers of •OH radicals than D-pen. Cysteamine demonstated to be an excellent scavenger also of alkoxyl- and peroxyl- type radicals. Based on IC_{50} values, gained by ABTS assay, it is evident that D-pen showed higher radical scavenging capacity compared to cysteamine. Similar results were observed also in DPPH assay, although in this assay less effective radical scavenging capacities of both substances tested were recorded.

CONCLUSIONS: On the basis of the results obtained, it can be stated that D-pen can produce hydrogen peroxide or •OH radicals and can inhibit the production of these oxidants. Our results showed that both L-GSH and cysteamine are similarly effective in inhibiting of HA degradation. Moreover, cysteamine demonstrated to be a significant inhibitor of alkoxyl- and peroxyl- type radicals generated from C-type macroradical of HA.

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ORIGINAL ARTICLE

Abbreviations:

D-pen	- D-penicillamine
L-GSH	- L-glutathione
HA	- hyaluronan
Mw	- molecular weight
rpm	- rotational speed per minutes
DPPH	- 2,2-diphenyl-1-picrylhydrazyl
ABTS	- 2,2´-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid

INTRODUCTION

Hyaluronan (HA, Figure 1), a high-molar-mass polysaccharide, composed of *N*-acetyl-D-glucosamine and D-glucuronic acid, is present in skin, umbilical cord, vitreous body, and in synovial fluid. HA is readily degraded to fragments of lower molar mass by reactive oxygen species (Stankovská *et al.* 2006; Valachová *et al.* 2008; Šoltés *et al.* 2009; Valachová *et al.* 2010). As reviewed recently (Šoltés *et al.* 2006), HA macromolecules demonstrate a really high sensitivity to damaging action of •OH radicals generated according to e.g. the reaction: H_2O_2 + Cu(I)/Fe(II) → •OH + Cu(II)/Fe(III) + HO^-. The •OH radical, due to its extremely high reactivity, abstracts a proton (H•) from the HA macromolecule – which reaction produces a *C*-type macroradical, which under aerobic conditions is reformed into a peroxyl-type species participating in the propagation of the HA chain breaking (Rychlý *et al.* 2006).

D-Penicillamine (D-pen) is used to treat patients with severe active rheumatoid arthritis unresponsive to conventional therapy. This compound functions as an immunomodulating/third-line disease-modifying anti-rheumatic drug (Williams 1990) most probably by chelating Cu(II) ions. However, D-pen in the presence of copper ions can produce hydrogen peroxide. The opposing properties of D-pen may be relevant to its therapeutic or toxic actions in rheumatic diseases (Staite, 2005).

L-Glutathione (L-GSH), one of the main endogenous free radical scavenger was unequivocally demonstrated to protect the HA degradation against •OH radicals (Hrabárová *et al.* 2010).

Cysteamine, similarly to some other endogenous thiols, was claimed to be an effective protective substance under oxidative stress conditions (Haenen *et al.* 1989).

The aim of this study was to investigate the anti- and/or pro-oxidative properties of some thiol compounds, namely D-pen, L-GSH, and cysteamine, on using the high-molar-mass HA in function of a probe sensitive to degradative action of •OH radicals generated by the oxidative system consisting of cupric ions *plus* ascorbate. The recently established experimental designs enabled us to prove/disprove the tested substance to scavenge •OH radicals, and peroxyl-/alkoxyl-type species (Šoltés *et al.* 2009).

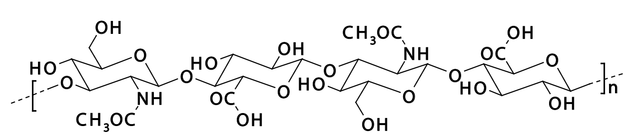

Fig. 1. Structural formula of hyaluronan – the acid form.

MATERIALS AND METHODS

Biopolymer, Chemicals and Drugs

Hyaluronan sample (P9710-2A, M_w = 808.7 kDa) was purchased from Lifecore Biomedical Inc., Chaska, MN, U.S.A. The analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were from Slavus Ltd., Bratislava, Slovakia; cysteamine; D-pen, L-GSH, 2,2´-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany; L-ascorbic acid and $K_2S_2O_8$ were from Merck KGaA, Darmstadt, Germany; ethanol and methanol were from Mikrochem, Pezinok, Slovakia. Redistilled de-ionized quality grade water, which conductivity has been ≤ 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 NaCl in two steps: first, 4.0 mL of the solvent was added in the morning. The next 3.90 mL of the solvent was added after 6 h. On the following morning, 50.0 μL of 160 μM $CuCl_2$ solution was added to the HA solution, stirred for 30 s and left to stand for 7.5 min. Then 50.0 μL of 16.0 mM ascorbic acid was added to HA solution and stirred again for 30 s. Next, the assayed solution (8 mL) containing HA (2.5 mg/mL), $CuCl_2$ (1.0 μM), and ascorbate (100 μM) underwent the measurement of the sample dynamic viscosity as specified below. The solutions of ascorbic acid and $CuCl_2$ were also prepared in 0.15 M NaCl.

Study of inhibited hyaluronan degradation

Inhibitory studies of the degradation of high-molar-mass HA sample P9710-2A were carried out by using two different oxidative systems composed of $CuCl_2$ (1.0 μM), ascorbic acid (100 μM), and substance (of the final concentrations 50 or 100 μM) added either before the reaction onset or 1 h after the reaction onset.

Rotational viscometry

The solution (8 mL) containing HA (2.5 mg/mL), $CuCl_2$ (1.0 μM), ascorbic acid (100 μM), and substance (50 or 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. The solution dynamic viscosity 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}.

ABTS and DPPH assays

Standard experimental procedures were used as published (Re *et al.* 1999; Cheng *et al.* 2006). Briefly: at *ABTS assay* 250 µl of $ABTS^{\bullet +}$ (prepared from ABTS and $K_2S_2O_8$) is added to 2.5 µl of D-pen or cysteamine solution and in the 6th min the absorbance of the sample mixture is measured at 734 nm; at *DPPH assay* 225 µl of $DPPH^{\bullet}$ is added to 25 µl of D-pen or cysteamine solution and in the 30th min the absorbance of the sample is measured at 517 nm. All samples (D-pen, cysteamine and quercetin) were measured quadruplicate in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) with Tecan Infinite M 200 reader (Tecan AG, Austria). IC_{50} values were calculated with CompuSyn 1.0.1 software (ComboSyn Inc., Paramus, USA).

Ionic fractions and log D values of D-penicillamine and cysteamine were calculated by free webservice at http://www.pharma-algorithms.com/webboxes/.

RESULTS AND DISCUSSION

Figure 2 curve marked 0 demonstrates that addition of Cu(II) ions followed by ascorbate resulted in a gradual dynamic viscosity decrease of the HA sample solution. As seen in Figure 2 (curves marked 50, 100), addition of D-pen at the reaction onset dose dependently prolonged the inhibition phase of •OH radical generation. However, after a given time period – namely 30 min at 50 µM, 40 min at 100 µM of D-pen, respectively – a really fast continual reduction of the sample dynamic viscosity was observed. On the contrary, L-GSH added at the reaction onset totally inhibited •OH radical generation (Figure 3).

Results in Figure 4, panel A, show that cysteamine is a significant scavenger of •OH radicals only at the highest concentration applied (100 µM), while somewhat less protective effect was obtained by using it at the concentration of 50 µM. However, cysteamine was recorded to totally inhibit generation of alkoxyl-, peroxyl- type radicals at both concentrations tested, i.e. 50 and 100 µM (Figure 4, panel B).

In the ABTS assay antirheumatic drug D-pen showed approx. 35-times higher scavenging activity (IC_{50} = 5.26 µM) than cysteamine (IC_{50} = 178.54 M). These values (Table 1) can be positively correlated to: the higher lipophilicity (and better solubility) of D-pen (log D = –1.40) and 0% fraction of its cationic form compared to log D = –2.29 for cysteamine and more than 80% fraction of cations in tested diluted ethanol solution at pH 7.4. For comparison, well-known antioxidant quercetin IC_{50} was 2.86 µM.

In the DPPH assay, both substances showed lower $DPPH^{\bullet}$ radical scavenging activity compared to the results obtained by the ABTS assay. IC_{50} value for D-pen (35.75 µM) was approx. 7-times lower than that one of cysteamine (248.49 µM), but much higher as published for quercetin (4.36 µM), as seen in Table 1.

Tab. 1. IC_{50} values of tested substances by using ABTS and DPPH assays.

Substance	IC_{50} [µM] ABTS assay	IC_{50} [µM] DPPH assay
D-Penicillamine	5.26	35.75
Cysteamine	178.54	248.49
L-Glutathione (Ho *et al.* 2007)	–	74.8
Quercetin[a]	2.86	4.36

[a]Nagy et al., unpublished data.

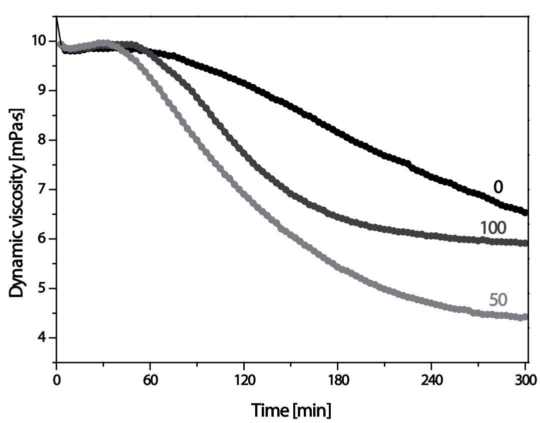

Fig. 2. Time dependent HA degradation by the oxidative system 1.0 µM $CuCl_2$ and 100 µM ascorbate (0). Effect of the addition of D-pen at the concentrations 50 or 100 µM before the reaction onset.

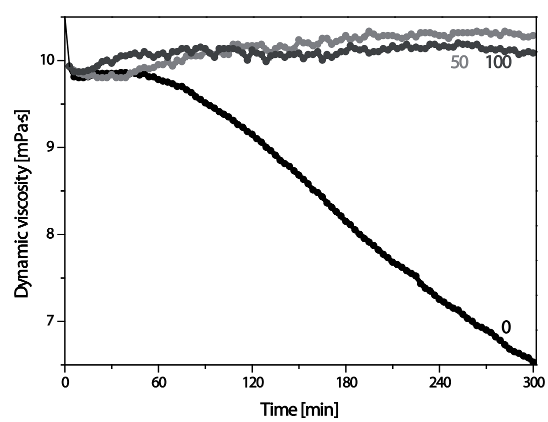

Fig. 3. Time dependent HA degradation by the oxidative system 1.0 µM $CuCl_2$ and 100 µM ascorbate (0). Effect of the addition of L-GSH at the concentrations 50 or 100 µM before the reaction onset.

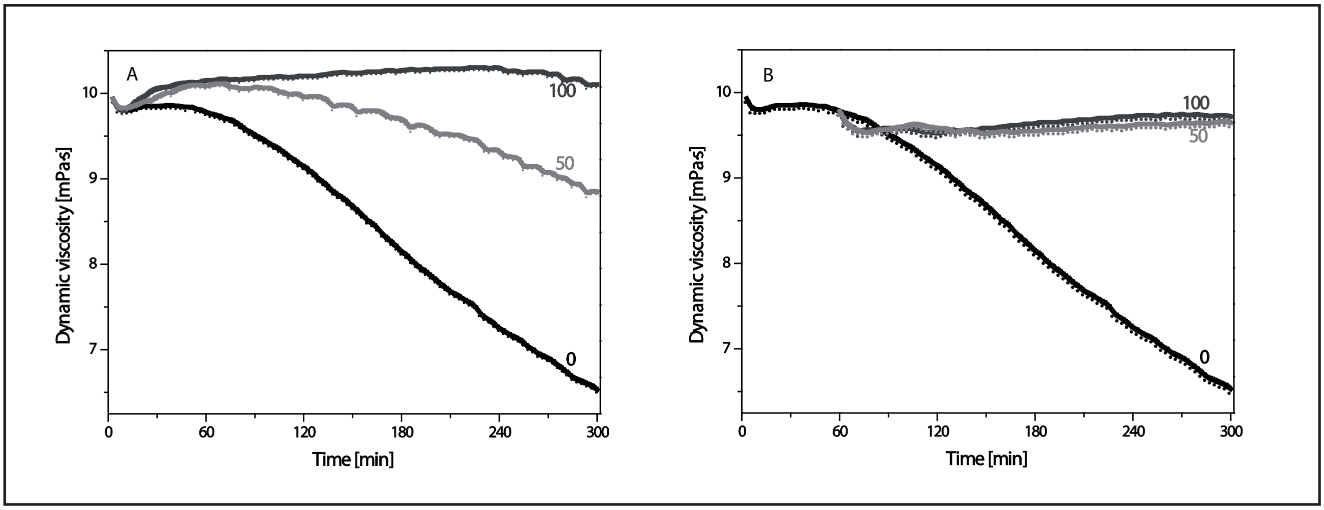

Fig. 4. Time dependent HA degradation by the oxidative system 1.0 µM $CuCl_2$ and 100 µM ascorbate (0). Effect of the addition of cysteamine (50, 100 µM) before the reaction onset (A) or 1 h after the reaction onset (B).

D-Pen again did not consist of cationic ions, while cysteamine cationic fraction equaled 91%. D-Pen was present as a zwitterionic molecule to 95–98% in both assays, which apparently favored its reaction ability with tested $ABTS^{\bullet+}$ cation radical and $DPPH^{\bullet}$ radical. On the contrary, a high ratio of cysteamine cationic form led to the decrease of its antiradical activity.

CONCLUSIONS

Our results showed that both L-GSH and cysteamine were similarly effective in inhibiting the degradation of high-molar-mass hyaluronan. Moreover, cysteamine demonstrated a significant inhibitory effects against alkoxyl- and peroxyl- type radicals generated from *C*-type hyaluronan macroradical. Contrary to the above observations, it can be stated that D-pen can produce hydrogen peroxide and/or $^{\bullet}OH$ radicals and by that way it can act as a pro-oxidative substance.

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