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COMPARISON OF FREE-RADICAL SCAVENGING PROPERTIES OF GLUTATHIONE UNDER NEUTRAL AND ACIDIC CONDITIONS

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ABSTRACT

The influence of an acid solvent – the diluted acetic acid – was examined in purpose to monitor high-molar-mass hyaluronan (HA) degradation induced by cupric ions and ascorbate. Further, protective effects of glutathione (GSH), dissolved either in saline or diluted acetic acid, against oxidative degradation of HA were monitored by rotational viscometry. Electron donating properties of GSH to the pre-formed $ABTS^{\bullet+}$ cation radical were examined by ABTS – the standard decolorization assay. Results of rotational viscometry demonstrated accelerated HA degradation in acidic rather than in neutral conditions. The same effect was demonstrated by examining GSH in the role of scavenging $ABTS^{\bullet+}$ cation radicals.

Keywords: antioxidative activity, thiols, radical scavenging capacity, dynamic viscosity

INTRODUCTION

Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Damage to cells caused by free radicals is believed to play a central role in the aging process and in disease progression. Antioxidants are the first line of defense against free radical damage, and are critical for maintaining optimum health and wellbeing. The need for antioxidants becomes even more critical with increased exposure to free radicals. Pollution, cigarette smoke, drugs, illness, stress, sunlight, and even exercise can increase free radical exposure. Because so many factors can contribute to oxidative stress,

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individual assessment of susceptibility becomes important [1]. Potentially effective antioxidants involve thiol-containing compounds. These compounds are central in many biochemical and pharmacological reactions. Disulfide bonds have an important role in determining the tertiary structure of proteins, and in many drugs the cysteine moiety is an important reactive center that determines their effects. Molecules containing cysteine residues are among the most easily modifiable compounds, being easily oxidized by transition metals or participating in thiol-disulfide exchange [2].

Glutathione (Figure 1, GSH) is a tri-peptide molecule composed of glutamic acid, cysteine and glycine. It is a ubiquitous endogenous thiol, maintains the intracellular reduction/oxidation (redox) balance and regulates signaling pathways during oxidative stress/conditions. It has been referred to as the body's "master antioxidant".

Glutathione is mainly cytosolic (90%) in the concentration range of ca. 1–10 mM; however, in blood plasma, the range is only 1–3 μM. About 10–15% of cellular GSH is located in mitochondria and a small percentage of GSH is located in the endoplasmatic reticulum. Since mitochondria have a very small volume, the local GSH concentration in these organelles is usually higher than that in the cytosol. This monothiol is found also in most plants, microorganisms, and all mammalian tissues [3–6]. Although GSH does not react directly with hydroperoxides, its use as a substrate for glutathione peroxidase has been recognized for almost 40 years as the predominant mechanism for reduction of H_2O_2 and lipid hydroperoxides [2].

While GSH does not react nonenzymatically with H_2O_2, another role for glutathione in antioxidant defense, that depends upon its stability to react with carbon centered radicals, has been proposed by Winterbourn [7]. GSH acts in concert with superoxide dismutase to prevent oxidative damage and exists in two forms the thiol-reduced and disulfide oxidized [2, 6].

Under conditions of moderate oxidative stress, oxidation of "cys" aminoacid residues can lead to the reversible formation of mixed disulfides between protein thiol groups and low-molar-mass thiols (S-thionylation), particularly with glutathione (S-glutathionylation). Protein S-glutathionylation can directly alter or regulate protein function (redox regulation) and may also have a role in protection of proteins from irreversible (terminal) oxidation. S-glutathionylation of protein cysteine residues protects against higher oxidation states of the protein thiol, thereby preserving the reversibility of this type of modification.

GSH participates in many cellular reactions: 1) it effectively scavenges free radicals and other reactive oxygen species (e.g., hydroxyl radicals, lipid peroxyl radicals, peroxynitrite, and H_2O_2) directly, and indirectly through enzymatic reactions. In such reactions, GSH is oxidized to form glutathione disulfide (GSSG), which is then reduced to glutathione by the NADPH-dependent glutathione reductase. 2) GSH reacts with various electrophiles, physiological metabolites (e.g., estrogen, melanins, prostaglandins, and leukotrienes) and xenobiotics to form mercapturates. 3) GSH conjugates with $NO^{\bullet}$ radical to form an *S*-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release glutathione and $NO^{\bullet}$. 4) GSH is required for the conversion of prostaglandin H2 (a metabolite of arachidonic acid) into prostaglandins D2 and E2 by endoperoxide isomerase. Moreover, S-glutathionylation of proteins (e.g., thioredoxin, ubiquitin-conjugating enzyme, and cytochrome c oxidase) plays an important role in cell physiology. Physiological functions are summarized in detail in Table 1 [8].

Table 1. Roles of glutathione in animals

Antioxidant defense
Scavenging free radicals and other reactive species
Removing hydrogen- and lipid-peroxides
Preventing oxidation of biomolecules
Metabolism
Synthesis of leukotrienes and prostaglandins
Conversion of formaldehyde to formate
Production of D-lactate from methylglyoxal
Formation of mercapturates from electrophiles
Formation of glutathione-NO adduct
Storage and transport of cysteine
Regulation
Intracellular redox status
Signal transduction and gene expression
DNA and protein synthesis, and proteolysis
Cell proliferation and apoptosis
Cytokine production and immune response
Protein S-glutathionylation
Mitochondrial function and integrity

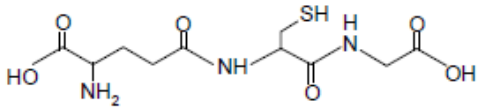


Figure 1. Structure of glutathione.

Depletion of GSH results in an increased vulnerability of the cells to oxidative stress [9]. In most cells and tissues, the estimated redox potential for the GSH/GSSG couple ranges from −260mV to −150mV.

GSH plays a more specific and well-documented role in the metabolism of copper and iron. It is believed to be responsible for the mobilization and delivery of copper ions for the biosynthesis of copper-containing proteins. In this case, (i) GSH is involved in reduction of Cu(II) to Cu(I), (ii) mobilization of copper ions from stores, and in delivery of copper ions during the formation of "mature" proteins. For the last function, Cu(II) must be reduced to Cu(I) before it can be incorporated into apoproteins, and GSH provides the reducing power. Interestingly, GSH is not only the carrier for Cu(I), but is also involved in copper mobilization from metallothioneins in a reversible manner [5].

Hyaluronan (HA, Figure 2) is a linear unbranched polysaccharide consisting of repeating disaccharide units of β-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine [10]. In the body, HA occurs in the salt form, and is omnipresent in the vertebrate connective tissues, particularly in the umbilical cord, synovial fluid, vitreous humor, dermis, cartilage. Significant amounts of HA are also found in lung, kidney, brain, and muscle tissues. Its molecular size can reach the values of up to 10^7 Da [10, 11]. Increased evidence has been gathered that low-molar-mass HA fragments have different activities than the native polymer.

Large matrix polymers of HA are spacefilling, anti-angiogenic, and immunosuppressive, whereas the intermediate-sized polymers comprising 25–50 disaccharides are inflammatory, immunostimulatory, and highly angiogenic [12]. In addition to its function as a structural molecule, HA also acts as a signaling molecule by interacting with cell surface receptors and regulating cell proliferation, migration, and differentiation. The unique viscoelastic nature of HA solutions/gels along with their biocompatibility and non-immunogenicity has led to their use in a number of clinical applications, including the supplementation of joint fluids in arthritis, usage as a surgical aid in eye surgery, to facilitate the healing and regeneration of surgical wounds, and as a drug delivery agent [11].

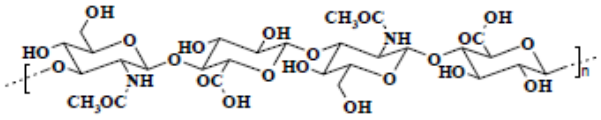


Figure 2. Structure of hyaluronan (acid form).

During inflammation, when HA is degraded, the conditions are slightly acidic. Therefore, the aim of this study was to compare the protective effects of GSH under neutral, i.e. normal/physiologic conditions, and acidic conditions occurring in inflammatory diseases against HA degradation induced by cupric ions and ascorbate and against pre-formed $ABTS^{\bullet+}$ cation radicals.

Materials and Methods

Materials

The high-molar-mass HA sample Lifecore P0207-1A was purchased from Lifecore Biomedical Inc., Chaska, MN, USA (M_w = 970.4 kDa). The analytical purity grade NaCl and $CuCl_2 \times 2H_2O$ were purchased from Slavus Ltd., Bratislava, Slovakia. L-Ascorbic acid and $K_2S_2O_8$ (p.a. purity, max 0.001% nitrogen) were the products of Merck KGaA, Darmstadt, Germany. 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; purum, >99%), GSH and acetic acid were purchased from Sigma-Aldrich, Steinheim, Germany. Deionized high-purity grade H_2O, with conductivity of ≤0.055 mS/cm, was produced by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

Methods

Preparation of Stock Solutions

The HA solution (2.5 mg/ml) was prepared in aqueous NaCl solution (0.15 M) in the dark in two steps: first, the solvent (4.0 ml) was added to HA (20 mg), and after 6 h of its

swelling, the same solvent (3.90 or 3.85 ml) was added. The stock solutions of ascorbic acid, GSH (16 mM) and $CuCl_2$ (160 μM) were dissolved also in aqueous NaCl solution (0.15 M).

Study of Uninhibited/Inhibited Hyaluronan Degradation

The procedure for examining HA degradation by the Weissberger biogenic oxidative system (WBOS) was as follows: a volume of 50 μl of 160 μM $CuCl_2$ solution was added to the HA solution (7.90 ml) and the reaction mixture after a 30-s stirring was left to stand for 7 min 30 s at room temperature. Then, 50 μl of ascorbic acid solution (16 mM) was added to the HA solution, and stirred again for 30 s.

The final reaction mixture (8.0 ml) was then immediately transferred into the viscometer Teflon® cup reservoir.

The procedures to investigate the pro- and antioxidative effects of acetic acid and GSH were as follows:

a) A volume of 50 μl of 160 μl $CuCl_2$ solution was added to the HA solution (7.85 ml), and the mixture, after a 30-s stirring, was left to stand for 7 min 30 s at room temperature. Then, 50 μl of 0.5% acetic acid or 50 μl of GSH (16 mM) dissolved both in saline or 0.5% acetic acid were added to the solution followed by stirring again for 30 s. Finally, 50 μl of ascorbic acid solution (16 mM) was added to the reaction mixture, stirred for 30 s and immediately transferred into the viscometer Teflon® cup reservoir.

b) In the second experimental setting, a procedure similar to that described in a) was applied; however, after standing for 7 min 30 s at room temperature, 50 μl of ascorbic acid solution (16 mM) was added to the reaction mixture and a 30-s stirring followed. After 1 h, finally 50 μl of 0.5% acetic acid or GSH (16 mM) was added to the reaction mixture, followed by 30-s stirring and immediate transfer into the viscometer Teflon® cup reservoir.

The resulting reaction mixture (8.0 ml) was transferred into the Teflon® cup reservoir of the Brookfield LVDV-II-PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, USA). Recording of the viscometer output parameters started 2 min after the onset of the experiment.

The changes of dynamic viscosity 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 the shear rate equaling 237.6 s^{-1} [13].

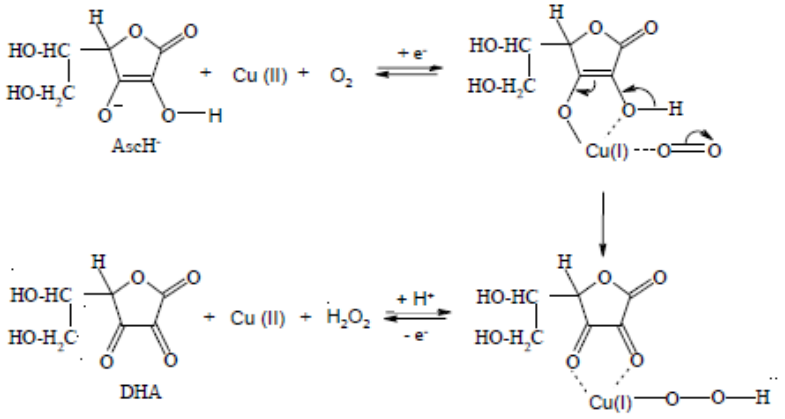
For the ABTS decolorization assay, the $ABTS^{\bullet+}$ radical cations were pre-formed by the reaction of an aqueous solution of $K_2S_2O_8$ (3.3 mg) in H_2O (5 ml) with ABTS (17.2 mg). The resulting bluish green radical cation solution was stored overnight in the dark below 0°C. Before the experiment, the solution (1 ml) was diluted into a final volume (60 ml) with H_2O or acetic acid solution (0.5%).

The GSH solution (1.0 mM) was prepared both in distilled water and in acetic acid solution (0.5%).

A modified ABTS assay [13] was used to test the radical-scavenging efficiency applying a UV-1800 spectrophotometer (SHIMADZU, Japan). The UV/VIS spectra were recorded in defined times, in 1-cm quartz cuvette after mixing the solution of the antioxidant (50 μl) with the $ABTS^{\bullet+}$ solution (2 ml).

RESULTS AND DISCUSSION

The scheme displaying the formation of H_2O_2 by reacting ascorbate with Cu(II) ions was suggested by Weissberger in 1943 [15]. Since then many papers have been published [16–27].



Scheme 1. Weissberger's biogenic oxidation system (adapted from [28]).

Scheme 1 illustrates the statement that, e.g., at the ratio of the reactants [Cu(II)]:[ascorbate] = 0.1/100 the reaction cycle will be repeated 1000-times and if all elementary reaction steps are performed at 100% the products will be dehydroascorbate and H_2O_2 – both in 100 μM concentrations. This proposition is virtually incorrect since the product generated, i.e., H_2O_2 is decomposed yielding $^{\bullet}OH$ radicals due to the presence of the reactant Cu(II) reduced to Cu(I)-intermediate [29].

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

Degradative action of Cu(II) ions and ascorbate on the molecule of HA was demonstrated also by Matsumura and Pigman [30] and Harris et al. [31].

In purpose to scavenge $^{\bullet}OH$ radicals, a well-known endogenous antioxidant – glutathione – was selected.

Figure 3 illustrates the results of a potential pro-oxidative effect of acetic acid itself in both experimental settings (a, b) on the oxidative degradation of HA macromolecules induced by WBOS (the reference).

The decline of dynamic viscosity (η) of the reference (black curve) represents the value 4.77 mPa·s after a 5-h treatment. However, the addition of acetic acid before initiating HA oxidative damage accelerated degradation of HA reaching the declines of η value by 5.11 (red curve). In case of adding acetic acid 1 h later this decline was somewhat greater and represents 5.53 mPa·s (green curve).

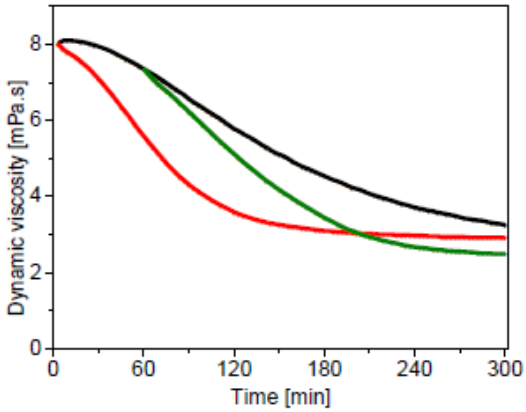


Figure 3. Effect of acetic acid (0.5%) on HA degradation induced by WBOS (black). Acetic acid was added to WBOS before initiating HA degradation (red) or after 1 h (green).

Hyaluronan itself was recorded to be slightly degraded in acidic conditions with pH below 1.6 and in basic medium with pH above 12.6 by using molar-mass-distribution analysis, however its rheological behavior was relatively not influenced by pH [32]. Since the pH in our reaction mixture was about 4, a rapid HA degradation may be attributed to the influence of acid medium in WBOS.

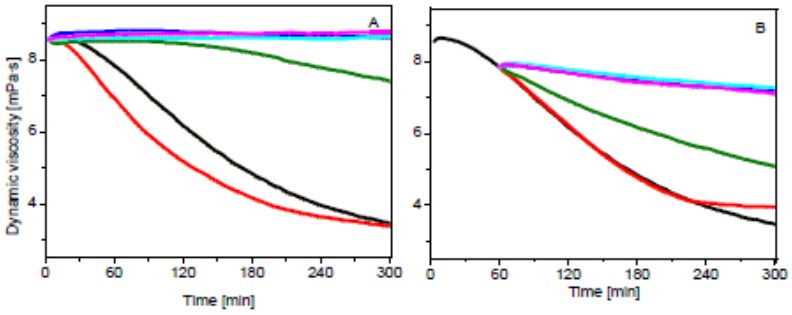


Figure 4. Effect of GSH (dissolved in saline) on HA degradation induced by the system composed of 1.0 μM $CuCl_2$ and 100 μM ascorbate. GSH was added to the reaction system before initiating HA degradation (panel A) and 1 h after the reaction onset (panel B). The concentrations of GSH in μM: 1 (red), 10 (green), 50 (blue), 100 (cyan) and 200 (magenta).

Results of investigating GSH (dissolved in saline) in the function of a potential antioxidant against HA degradation are reported in Figure 4. As evident, within the time interval examined (5 h), application of the GSH concentrations (50 and 100 μM) resulted in a marked protection of the HA macromolecules against degradation, leading to the total

inhibition of the solution viscosity decrease. The higher the GSH concentration used, the longer the observed stationary interval in the sample η values. However, the concentration of GSH – 10 μM – was not sufficient enough to inhibit HA degradation completely. At the lowest concentration, i.e. 1 μM GSH, a pro-oxidative effect can be observed. The function of GSH in a low concentration was examined also by the authors Nappi and Vass [33], who demonstrated a pro-oxidative action of GSH in 6.0 μM concentration generating thus $^{\bullet}OH$ radicals.

The pro-oxidative effect of GSH can be ascribed to the formation of an intermediate $[GSSH]^{\bullet-}$, which can convert molecular oxygen to hydrogen peroxide under aerobic conditions as follows [34]:

$$GSH + HO^{\bullet} \rightarrow GS^{\bullet} + H_2O$$
$$GSH \rightarrow GS^{-} + H^{+}$$
$$GS^{\bullet} + GS^{-} \rightarrow [GSSH]^{\bullet-}$$
$$[GSSH]^{\bullet-} + O_2 \rightarrow GSSH + O_2^{\bullet-}$$
$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^{+} \rightarrow H_2O_2 + O_2$$

GSH added to the reaction mixture 1 h later (Figure 4, panel B), i.e. already in a process of performing degradation, demonstrated similar efficacy as illustrated in panel A. At higher concentrations (50, 100 and 200 μM) a decrease of η was only around 1 mPa·s. Concentration of GSH 10 μM was sufficient to a mild protection of HA. However, GSH in 1 μM concentration was ineffective and its corresponding curve was identical to the reference one up to 240 min. The application of 1 h-delayed addition of the GSH solution was designed based on the results of EPR, which demonstrated disappearance of producing $^{\bullet}OH$ radicals up to 1 h (Figures 5a, 5b) by using the aqueous system composed of $CuCl_2$ (0.1 μM), ascorbic acid (100 μM), and the spin-trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; 250 mM) [29].

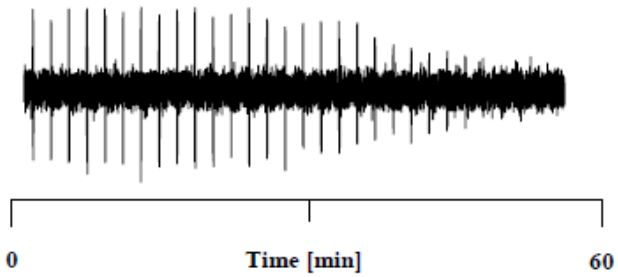


Figure 5a. Time course of EPR spectra of the aqueous mixture containing $CuCl_2$ (0.1 μM), ascorbic acid (100 μM), and spin trapper DMPO (250 mM) at room temperature – adapted from Šoltés et al. [29].

As seen during the first approximately 60 min of the reaction of WBOS components, the EPR signal detected was typical for ascorbyl anion radical ($Asc^{\bullet-}$; Figure 5a). The $^{\bullet}$DMPO-OH adduct was detectable as late as 1 h after initiating the reaction, i.e., after disappearance

of the EPR signal of ascorbyl anion radical, pointing to the depletion of ascorbate in the reaction mixture monitored. Figure 4b shows an explanatory chart of the time courses of the integral EPR signals of $Asc^{\bullet-}$ anion radical and the $^{\bullet}$DMPO-OH adduct.

The record illustrates the scans of the $Asc^{\bullet-}$ anion radical evidenced in time from 0.5 to 56 min.

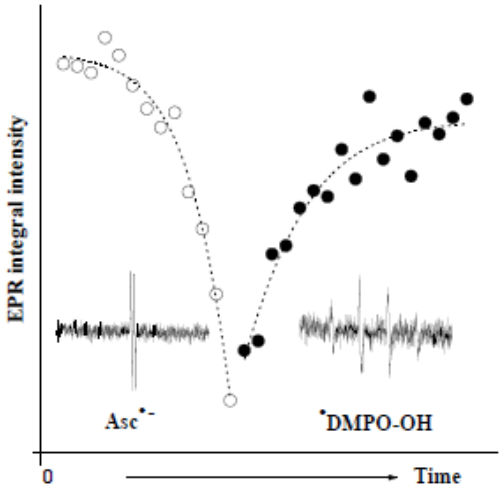


Figure 5b. Illustrative representation of the time dependences of the integral EPR signals of $Asc^{\bullet-}$ anion radical (○) and the $^{\bullet}$DMPO-OH adduct (●) – adapted from Šoltés et al. [29].

In the figure, both the EPR spectrum of the ascorbyl anion radical $Asc^{\bullet-}$ and that of the $^{\bullet}$DMPO-OH adduct are depicted.

The mechanism of HA degradation by $^{\bullet}$OH radicals followed by formation of peroxyl radicals and hydroperoxides is mentioned in reactions below

$$HA + {}^{\bullet}OH \rightarrow A^{\bullet} + H_2O$$

Polymers with –CH groups, such as HA, are readily degraded by $^{\bullet}$OH radicals. The $^{\bullet}$OH radical abstracts $H^{\bullet}$ radical from the HA macromolecule to produce a C-macroradical – the so called alkyl radical ($A^{\bullet}$). Under aerobic conditions, during a phase known as propagation a dioxygen molecule reacts with the alkyl radical to form peroxy-type radicals ($AOO^{\bullet}$).

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet}$$

which may followed by the reaction

$$HA + AOO^{\bullet} \rightarrow A^{\bullet} + AOOH$$

i.e. peroxyl radicals form hydroperoxyls and a novel C-macroradical by random trapping of the H• radical from adjacent the HA macromolecule.

Due to a continual process of propagation reactions the low-molar-mass fragments of the biopolymer are formed, which directs to the decrease of the HA solution dynamic viscosity. The radical process involving the four steps such as initiation, propagation, transfer, and termination can be stopped by addition of a free-radical scavenger. When such a scavenger is admixed into the HA solution before applying WBOS, the scavenger may be tested in a function of a preventive antioxidant (against production of •OH radicals) while, on adding the substance during the propagation phase of the HA degradation, the substance is examined in a function of a chain-breaking antioxidant (against production of peroxy-type radicals AOO•).

There exist only a few publications concerning to the activeity of thiol compounds in the reaction system ascorbate and Cu(II). One of them is the paper by the author Winkler [35], who demonstrated inhibition of ascorbate (1 mM) oxidation by GSH (100 and 1000 μM) in the presence of Cu(II) (10 μM). Similar results were obtained by Ohta et al. [36], who demonstrated that ascorbate inhibited GSH autoxidation in the environment of Cu(II) ions.

Applying acetic acid (pH 4) as a solvent of GSH instead of saline led to a new knowledge, i.e. a more intensive degradation of HA. To suppress HA degradation, it was necessary to apply GSH in 5-times higher concentration (1000 μM) compared to the experiments, where saline was used. Somewhat less protective effect of GSH against •OH radicals was observed at 100 μM concentration. No protective effects of GSH were demonstrated in low concentrations, i.e. 10 and 1 μM (Figure 6, panel A) reaching the values of η decrease by 5.36 and 5.87 mPa·s, respectively. A similar effect of GSH was observed in the reaction system generating predominantly peroxyl radicals (Figure 6, panel B).

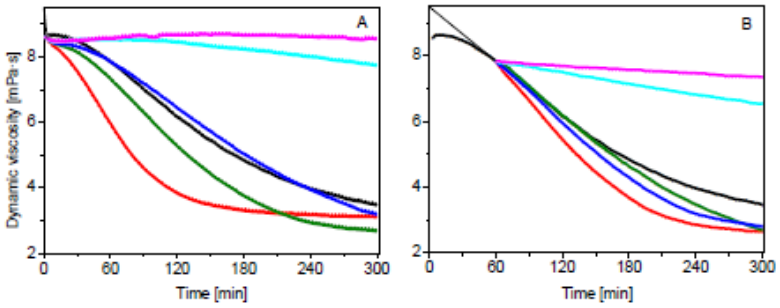


Figure 6. Effect of 0.5% acetic acid itself (red) and the effect of GSH dissolved in acetic acid (0.5%) on HA degradation induced by WBOS (black). GSH and acetic acid were added to the reaction system before initiating the degradation of HA (panel A) or after 1 h (panel B). The concentrations of GSH in μM: 1 (green), 10 (blue), 100 (cyan) and 1000 (magenta).

All principal viscometric/rheometric methods fall into one of two classes: (1) involving a moving fluid or (2) involving a moving element. The first class is characterized by a liquid moving through a definite channel/capillary – the variable measured is the time, which relates to the kinematic viscosity of the fluid. Capillary viscometers, being the simplest and most

widely used devices, are however not "true" rheological instruments. Capillary tube viscometers, characterized by shear rates in the range of hundreds up to thousands of reciprocal seconds, are suitable only for use with Newtonian fluids. The second class comprises either a linearly moving element, such as the falling ball, or a rotationally moving element.

In the latter group of instruments, either the stress is controlled and the resulting rotational speed is measured, or the rotational speed is controlled and the stress is measured. Those instruments in which the rotational speed is controlled and stress is measured can certainly indicate η changes with time.

Rotational rheometers, characterized by a very low shear rate, are addressed to characterize the rheological parameters of non-Newtonian fluids, including beyond controversy the HA solutions. Moreover, oscillatory (rotational) rheometers allow assessment of the storage (G') as well as loss (G'') moduli – the parameters, which provide information on polymer structure and might be related to the polymer molar mass distribution, cross-linking, and so forth [37].

The method of rotational viscometry determines hydrogen atom donating properties unlike the ABTS assay, by which electron donor properties are determined. Viscometry is a well-established method, whose results can be documented by many publications [38–50].

Figure 7 illustrates the results of decolorization of $ABTS^{\bullet+}$ in the presence of GSH of different concentrations (25, 12.5 and 2.5 μM) in acidic and neutral conditions 20 min after admixing GSH with $ABTS^{\bullet+}$ solution. It is evident that GSH demonstrated higher activity in scavenging $ABTS^{\bullet+}$ cation radical, i.e. better electron donor properties in neutral rather than in acidic conditions.

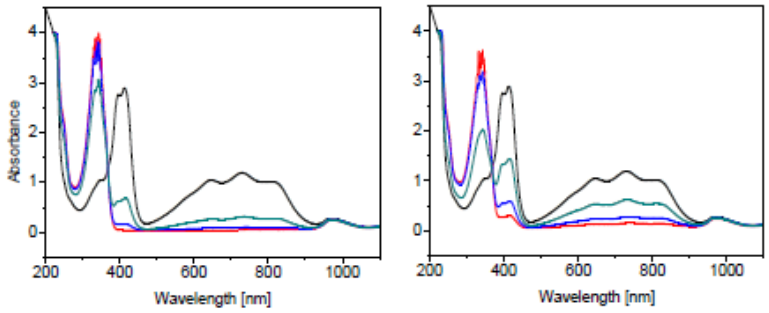


Figure 7. Effect of GSH dissolved in H_2O (left panel) or in acetic acid (right panel) on reducing $ABTS^{\bullet+}$ cation radical measured 20 min after the reaction onset. GSH concentrations in the $ABTS^{\bullet+}$ solution were in μM: 2.5 (green); 12.5 (blue) and 25 (red).

This result can correspond to the results observed by Ikebuchi et al. [51], who found that the glutathione redox cycle in cultured endothelial cells decreased by 20% at pH 6 and by 51% at pH 4 compared to that one at pH 7.4.

The same influence of GSH in neutral and acidic conditions is expressed in Figure 8, which depicts the kinetics of scavenging ABTS$^{\bullet+}$ cation radical by GSH after elapsing 1, 2, 5, 10 and 20 min at the wavelength 730 nm under the identical conditions as mentioned above.

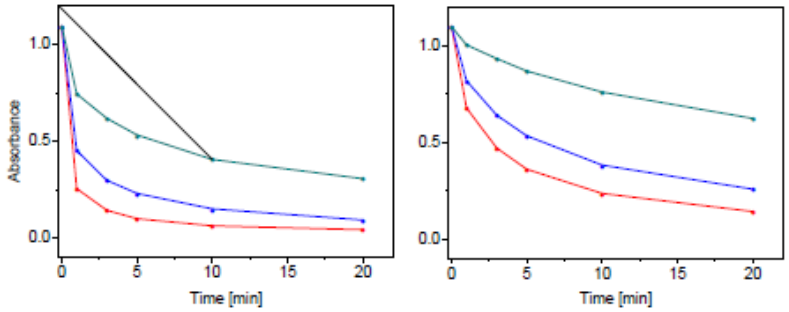


Figure 8. Time dependence of absorbance at 730 nm measured after addition of GSH into the ABTS$^{\bullet+}$ solution under the same experimental conditions than in Figure 7. Concentrations of GSH dissolved in H_2O (left panel) or in acetic acid (right panel) were in μM: 2.5 (green); 12.5 (blue) and 25 (red).

Scavenging of ABTS$^{\bullet+}$ cation radicals was measured in presence of GSH in neutral and acidic conditions. The assay uses intensively colored cation radicals of ABTS to test the ability of antioxidants to scavenge radicals. The original assay developed by Miller et al. [52] and Rice-Evans [53] utilized metmyoglobin-H_2O_2 to generate $^{\bullet}OH$, which then reacted with ABTS to produce the ABTS$^{\bullet+}$. However, quantitating antioxidant effects were equivocal because antioxidants could react with the original radical oxidant as well as the ABTS$^{\bullet+}$, causing an overestimation of antioxidant activity [54]. Thus, the assay has been revised to clearly generate ABTS$^{\bullet+}$ by using oxidizing agents such as potassium persulfate and manganese dioxide [55–57], then adding antioxidants and measuring direct reaction with an electron:

$$ABTS^{\bullet+} + e^{-} \rightarrow ABTS$$

bluish-green colorless

ABTS$^{\bullet+}$ exhibits a bluish-green color with maximum absorbance values at 645, 730, and 815 nm, which rapidly decreased after addition of GSH.

Overall, the ABTS assay offers many advantages that contribute to its widespread popularity in screening antioxidant activities of a wide range of materials. The assay is operationally simple, reactions are rapid (most methods take 30 min or less) and run over a wide range of pH. ABTS$^{\bullet+}$, being a singly positively charged cation radical, is soluble in both aqueous and organic solvents and is not affected by ionic strength, so it has been used in multiple media to determine both hydrophilic and lipophilic antioxidative capacities. Reactions can be automated and adapted to microplates [58] as well as to flow-injection and stopped-flow methods [59].

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