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Chemical Papers 70 (6) 820–827 (2016)
DOI: 10.1515/chempap-2016-0011

ORIGINAL PAPER

Radical-scavenging activity of glutathione, chitin derivatives and their combination‡

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Received 6 August 2015; Revised 13 November 2015; Accepted 19 November 2015

Since chitosan and its amino-, cinnamo- or cinnamo-amino- derivatives are acid-soluble, the effect of acetic acid on hyaluronan (HA) macromolecules degraded by Cu(II) ions and ascorbate was examined to produce reactive oxygen species (ROS). Further, the effects of glutathione (GSH), chitosan and its derivatives, added individually or in combination, on the quenching of ROS and $ABTS^{\bullet+}$ cation radical were examined using rotational viscometry and ABTS assay, respectively. The results of the rotational viscometry indicated a rapid degradation of HA by ROS after the addition of acetic acid. Chitosan and its derivatives moderately decreased the rate of HA degradation, while GSH decreased the rate of HA degradation more significantly. Moreover, GSH enhanced the protection of HA macromolecules against their degradation in the presence of chitosan or its derivatives. The results of the ABTS assay confirmed the results of the rotational viscometry. The GSH in the combination with chitosan and its derivatives reduced $ABTS^{\bullet+}$ more intensively than when added individually.
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Keywords: ABTS assay, glycosaminoglycans, polysaccharides, radical-scavenging activity, thiols, wound-healing

Introduction

Chitosan is a biocompatible, biodegradable, nontoxic renewable biopolymer produced by alkali treatment of chitin, which is the second most abundant natural polysaccharide after cellulose and is generally found in the composition of crustacean shells. Chitin consists of β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) as a repeating unit. The deacetylation of chitin yields chitosan, which is actually a copolymer of GlcNAc and β-(1→4)-2-amino-2-deoxy-D-glucopyranose with a deacetylation degree over 60 %. Chitosan is used in cosmetics, photography, food and nutrition, ophthalmology, wastewater treatment and as an artificial skin (Dodane & Vilivalam, 1998; Kumar et al., 2007; Jeon et al., 2000; Shahidi et al., 1999). The unique properties of chitosan over other polysaccharides are attributed to its free amino groups, which account for its basic character.

Chitosan has been used as a wound dressing in burn-healing, for the proliferation and activation of

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‡Presented at the 5th International Scientific Conference – Applied Natural Sciences 2015, Demänovská Dolina – Jasná, Slovakia, 30 September–2 October 2015.

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inflammatory cells in granulation tissue and, consequently, for accelerating wound-cleaning and re-epithelisation (Sezer et al., 2007). Further, chitosan derivatives such as amino-, cinnamo- and cinnamoamino chitosan with improved physical-chemical properties have been synthesised (Mohy Eldin et al., 2012, 2015).

Like chitosan, hyaluronan (HA) is a β-linked polysaccharide, which tends to have structural functions. It is a straight-chain glycosaminoglycan, composed of D-glucuronic acid and *N*-acetyl-D-glucosamine which is a major component of the extracellular matrix (ECM). It is particularly prominent during wound repair, embryogenesis, and whenever rapid tissue turnover and repair occur. HA can exist in a number of forms. It may be free, bound to HA-binding proteins known as hyaladherins or intercalated into complex structures such as in the ECM (Aya & Stern, 2014). HA is used in medicine for the treatment of osteoarthritis, in ophthalmology, cosmetics, drug delivery and wound-healing (Gigante & Callegari, 2011; Necas et al., 2008; Papakonstantinou et al., 2012; Rah, 2011; Reitinger & Lepperdinger, 2013; Stern & Maibach, 2008; van den Bekerom et al., 2006).

High-molar-mass HA is readily degraded into small fragments after tissue injury, primarily by increased levels of hyaluronidases and reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Xing et al., 2014). ROS and RNS-degrading HA are formed during the inflammatory response in sepsis, tissue inflammation and ischemia-reperfusion injury. The most direct evidence for this is accumulated in the synovial fluid, where inflammatory oxidation leads to degradation of the native high-molar-mass HA with a resulting decrease in synovial fluid viscosity and cartilage degeneration, and in the airways, where ROS can degrade luminal epithelial HA (Cyphert et al., 2015).

Thiols are characterised as having a thiol (SH) functional group. Biothiols (or biologically derived thiols) are the most important antioxidants, protecting cells from any kind of oxidative damage. One of the extensively studied biothiols, glutathione (L-glutamyl-L-cysteinyl-glycine; GSH), is an antioxidant that protects cells against oxidative stress. It is synthesised from γ-glutamyl cysteine by glutathione synthetase. GSH also plays a role in the reductive processes that are essential for the synthesis of proteins and DNA. Its other physiological roles include the storage and transport of cysteine, plus a coenzymatic role in several reactions with foreign compounds (Demirkol et al., 2004). GSH is mainly cytosolic in the concentration range of approximately 1–10 mM; however, in the plasma, the range is only 1–3 μM (Haddad & Harb, 2005; Rees et al., 2008). This monothiol is also found in most plants, microorganisms and all mammalian tissues (Hami et al., 2013).

There have been several publications concerning HA in combination with chitosan (Kujawa et al., 2005; Lim et al., 2001; Oyarzun-Ampueroa et al., 2009; Tan et al., 2009; Yamane et al., 2005); however the combination of HA, chitosan and thiol compounds such as GSH has not been studied to date. High-molar-mass HA is readily degraded by Cu(II) ions and ascorbate, a source of free radicals, which mimics both the acute phase of joint inflammation and damage to skin (Cyphert et al., 2015).

This study sought to oxidatively degrade hyaluronan and to assess the antioxidative effects of chitosan and its derivatives in the absence and presence of glutathione.

Experimental

Shrimp shells were collected from the waste of seafood restaurants in Alexandria, Egypt. Acetic acid (purity 99.8 % and 99 %), *p*-benzoquinone (purity 99 %), NaOH pellets (purity 99–100 %) and L-glutathione were obtained from Sigma–Aldrich, Steinheim, Germany. Ethylenediamine (purity 99 %) was obtained from Alfa Aesar, Karlsruhe, Germany. Ethanol was from Mikrochem, Pezinok, Slovakia. Cinnamaldehyde (purity 98 %) was purchased from Scharlau Chemie, Sentmenat, Spain. High-molar-mass hyaluronan Lifecore P0207-1A was purchased from Lifecore Biomedical, Chaska, USA (M_w = 970.4 kDa). The analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were from Slavus, Bratislava, Slovakia. L-Ascorbic acid and $K_2S_2O_8$ (p.a. purity, max 0.001 % of nitrogen) were from Merck, Darmstadt, Germany. 2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid diammonium salt] (ABTS; purum, > 99 %) was from Fluka, Seelze, Germany.

Extraction of chitin from shrimp shells

The extraction process was as follows: the shells were demineralised by dispersion in 5 % HCl (shells/HCl solution ratio of 1 : 14 (mass/volume)) at ambient temperature overnight. After 24 h, the shells were quite soft and were rinsed with water to remove acid and calcium chloride. The demineralised shells were then treated with aqueous 5 % NaOH solution at ambient temperature for 24 h (shells/NaOH solution ratio of 1 : 12 (mass/volume)). The residue was collected and washed to neutral reaction under running tap water and then distilled water to afford chitin (Islam et al., 2011).

Preparation and purification of chitosan from chitin

Chitosan was prepared by the simple deacetylation of chitin as reported by Rigby (1936) using 50 % aqueous NaOH solution with a chitosan/NaOH solution ratio of 1 : 50 (mass/volume) at 100–120 °C for 12 h. The resulting chitosan was washed to neutral

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reaction under running tap water and rinsed with distilled water.

Following the method reported by Signini and Campana Filho (1999), the chitosan sample was dissolved in 2 % acetic acid and left to stand overnight. The solution was then filtered through a cheesecloth to remove contaminants and undissolved particles. The chitosan was then precipitated with 5 % NaOH, collected and washed with distilled water to remove the excess of alkali.

Preparation of cinnamo-chitosan, amino-chitosan and cinnamo-amino chitosan

Cinnamo-chitosan was prepared by the method reported by Mohy Eldin et al. (2015). The previously purified chitosan (1 g) was dissolved in 50 mL of 2 % acetic acid and stirred at ambient temperature for 6 h. The resulting viscous solution was filtered through a cheesecloth to remove undissolved particles, then ethanol (10 mL) containing cinnamaldehyde (6.2 mM) was added to the solution under stirring to obtain a homogeneous solution. This mixture was stirred at 50 °C for 6 h to form the chitosan Schiff base as a dark yellow gel which was added to an excessive volume of 5 % NaOH solution. The resulting precipitate was filtered, washed several times with distilled water and ethanol to remove the unreacted cinnamaldehyde and dried in a vacuum oven at 60 °C overnight.

Amino-chitosan was prepared by the method reported by Mohy Eldin et al. (2012) as follows: the modification of chitosan was performed in three steps: chitin activation, amination and deacetylation. For chitin activation, the chitin (4 g) was dispersed in distilled water (50 mL), dissolved in *p*-benzoquinone and stirred for 6 h. The activated chitin was separated and washed well with distilled water. For chitin amination, the activated chitin was dispersed in ethylenediamine (50 mL), dissolved in distilled water and stirred for 6 h. The aminated modified chitin was separated and washed well with distilled water. Deacetylation of the aminated chitin was performed following the methods detailed by Rigby (1936) and Wolfrom et al. (1958). The aminated chitin derivative was treated with 40 % aqueous NaOH solution with a chitosan/NaOH solution ratio of 1 : 50 (mass/volume) at 120–150 °C for 6 h. The amino-chitosan thus obtained was separated and washed well with distilled water.

The previously prepared amino-chitosan (1 g) was dissolved in 50 mL of 2 % acetic acid and the solution was stirred at ambient temperature for 6 h. The resulting viscous solution was filtered through the cheesecloth to remove any undissolved particles and ethanol (10 mL) with cinnamaldehyde (6.2 mM) was added to the solution under stirring to obtain a viscous solution. This mixture was stirred at 50 °C for 6 h to form a corresponding Schiff base as a deep yellow gel which was added to the excess of the 5 % NaOH solution. The resulting precipitate was filtered, washed with water and ethanol several times to remove the unreacted cinnamaldehyde and dried in a vacuum oven at 60 °C overnight.

ABTS assay

The $ABTS^{\bullet+}$ cation radical was formed by the reaction of $K_2S_2O_8$ (3.3 mg) in H_2O (5 mL) with ABTS (17.2 mg) and stored overnight in the dark below 0 °C. The $ABTS^{\bullet+}$ solution (1 mL) was diluted with acetic acid (0.5 %) to a final volume of 60 mL. All the stock solutions of chitosan, its amino-, cinnamo- and cinnamo-amino-derivatives and GSH (4.92 mg mL^{-1}) were prepared in acetic acid (0.5 %). A modified ABTS assay (Rapta et al., 2009) was used to assess the radical-scavenging activity of GSH, chitosan and its derivatives and the combination of chitosan and its derivatives with GSH using a UV-1800 spectrophotometer (SHIMADZU, Japan). The UV/VIS spectra were recorded in defined times, in a 1 cm quartz cuvette after mixing the substance solution (50 μL) with the $ABTS^{\bullet+}$ cation radical solution (2 mL).

Preparation of stock and working solutions

Hyaluronan (20 mg) was dissolved in an 0.15 M aqueous NaCl solution for 24 h in the dark. The HA sample solutions were prepared in two steps: first, 4.0 mL and after 6 h, 3.90 mL or 3.85 mL of the 0.15 M NaCl solution were added when working in the absence or presence of the samples, respectively. The solutions of ascorbate and glutathione (16 mM), cupric chloride (160 μM) were prepared in 0.5 % acetic acid.

Uninhibited hyaluronan degradation

First, HA degradation was induced by an oxidative system comprising $CuCl_2$ (1.0 μM) and ascorbic acid (100 μM) as follows: 160 μM $CuCl_2$ solution (50 μL) was added to the HA solution (7.90 mL) and the mixture was stirred for 30 s, then left to stand at ambient temperature for 7.5 min. Next, 16 mM ascorbic acid solution (50 μL) was added and, after stirring for 30 s, the solution was immediately transferred into the viscometer Teflon cup reservoir.

Inhibited hyaluronan degradation

The effectiveness of the GSH, chitosan and its derivatives was investigated as follows: *i*) 160 μM $CuCl_2$ solution (50 μL) was added to the HA solution (7.85 mL or 7.80 mL), the mixture was stirred for 30 s and left to stand at ambient temperature for 7.5 min. Next, the GSH (4.92 mg mL^{-1} solution, 50 μL) or chitosan, amino-, cinnamo- and cinnamo-amino chitosan (4.92 mg mL^{-1}) was individually or consecutively added to the HA mixture, followed by

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stirring for 30 s. Finally, 16 mM ascorbic acid solution (50 μL) was added and the mixture was stirred for 30 s. The solution was then immediately transferred into the viscometer Teflon cup reservoir; *ii*) the second experimental regime was similar to that described in *i*), but 16 mM ascorbic acid solution (50 μL) was added after standing at ambient temperature for 7.5 min, followed by stirring for 30 s. After 1 h, the GSH (4.92 mg mL^{-1} solution, 50 μL) or the chitosan and its derivatives (4.92 mg mL^{-1}) were individually or in combination added to the reaction mixture, followed by stirring for 30 s after the addition of each component. The reaction mixture was then immediately transferred into the viscometer Teflon cup reservoir.

Viscosity measurements

The dynamic viscosity (η) of the reaction mixture (8 mL) containing HA (2.5 mg mL^{-1}), ascorbate (100 μM) and Cu(II) ions (1 μM) in the absence and presence of samples was monitored using a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs, Middleboro, MA, USA) 2 min after addition of all the reactants at (25.0 ± 0.1) ℃ and at a shear rate of 237.6 s^{-1} for 5 h in the Teflon cup reservoir (Valachová et al., 2011; Topoľská et al., 2015).

Results and discussion

To model inflammatory conditions in the joints or skin, where pH is approximately 5–6, acetic acid (0.5 vol. %) was used. First, the addition of acetic acid (0.5 vol. %) into Weissberger's biogenic oxidative system (WBOS), which led to HA degradation reaching the decrease in η of the HA solution by 5.45 mPa s after 5 h (Fig. 1, curve 0, the reference), was examined. Further, the influence of chitosan and its derivatives (4.92 mg mL^{-1}) on HA degradation was studied. As seen in Fig. 1a, the addition of substances prior to the start of HA degradation resulted in an attenuated HA degradation induced by $^{\bullet}OH$ radicals for chitosan (curve 1) and cinnamo-amino-derivative (curve 4) over 5 h and for amino- and cinnamo-derivatives over 3 h (curves 2 and 3, respectively). However, when added 1 h later, i.e. during the production of peroxy-type radicals (Baňasová et al., 2014), all the substances promoted HA degradation (Fig. 1b).

Chitosan and its derivatives are not sufficiently effective for the treatment of skin injuries, because they are not effective donors of $H^{\bullet}$. When skin cells are damaged, the concentration of GSH is lowered (released from cytosol), hence the aim was to investigate the influence of GSH itself on scavenging free radicals.

Fig. 2a shows that 100 μM of GSH (4.92 mg mL^{-1} solution) (curve 1) was effective in inhibiting HA degradation. The decrease in η of the HA solution was only 0.82 mPa s after 5 h. This indicates the

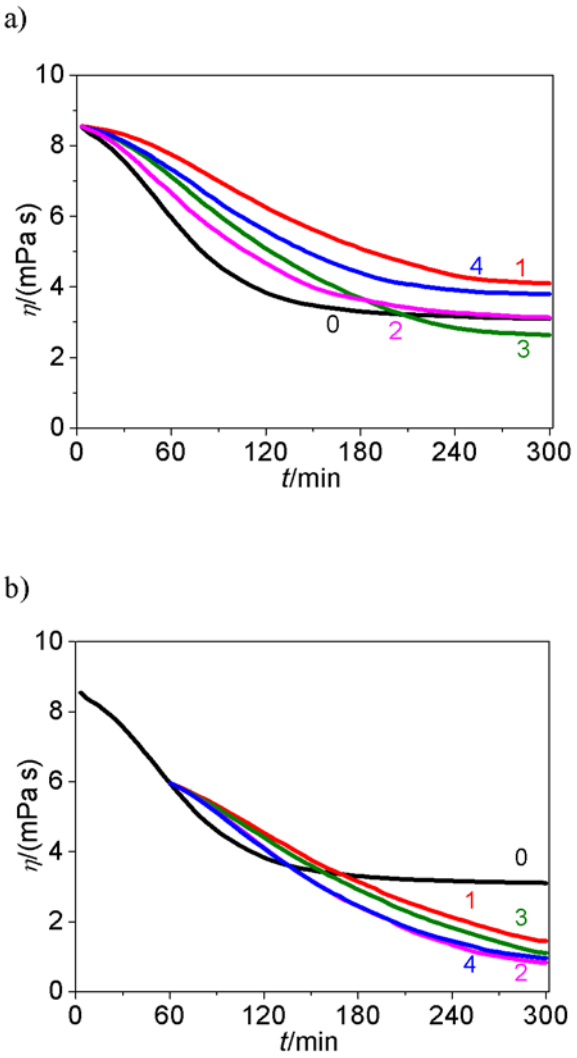

Fig. 1. Time-dependent changes in η of the HA solution exposed to WBOS (curve 0) and after addition of chitosan (curve 1), amino- (curve 2), cinnamo- (curve 3) and cinnamo-amino derivative (curve 4) at concentration of 4.92 mg mL^{-1} before HA degradation begins (a) and 1 h later (b).

good $H^{\bullet}$ donor properties of GSH. The addition of GSH (0.492 mg mL^{-1}) (curve 2) resulted in a more rapid HA degradation and was shown to be effective against $^{\bullet}OH$ radicals in part. GSH (4.92 mg mL^{-1}) added 1 h later decreased the rate of HA degradation and scavenged the forming peroxy-type radicals (Fig. 1b, curve 1). However, GSH (0.492 mg mL^{-1}) promoted HA degradation (Fig. 1b, curve 2).

Since none of the substances completely inhibit HA degradation, a combination of chitosan and its derivatives with GSH was examined.

Fig. 3 shows the results of the substances examined, both individually and in their combination. Of the all combinations, the most effective was cinnamo-chitosan and GSH. As revealed in Fig. 3a, cinnamo-chitosan alone (curve 1) moderately decreased the rate

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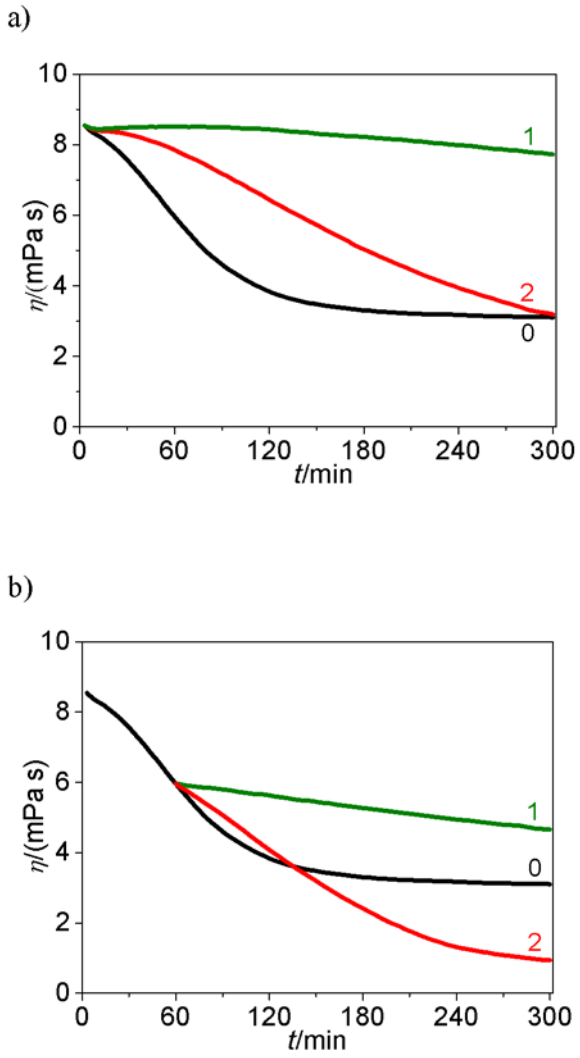

Fig. 2. Time-dependent changes in η of HA solution exposed to WBOS (curve 0) and after addition of GSH at concentration 4.92 mg mL^{-1} (curve 1) and 0.492 mg mL^{-1} (curve 2) before HA degradation begins (a) and 1 h later (b).

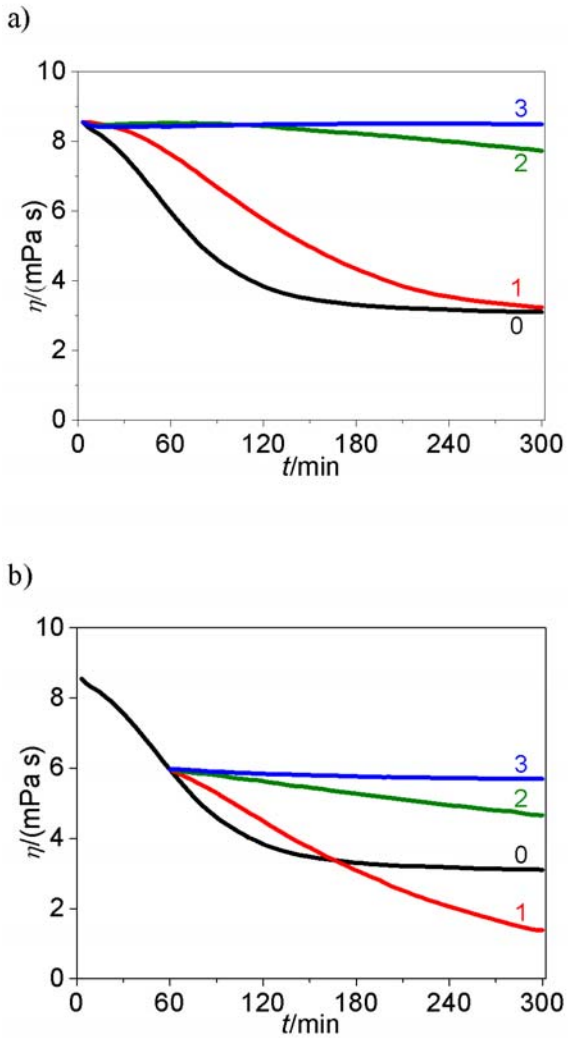

Fig. 3. Time-dependent changes in η of HA solution exposed to WBOS (curve 0) and after addition of cinnamochitosan (curve 1), GSH (curve 2) and cinnamochitosan with GSH (curve 3) before HA degradation begins (a) and 1 h later (b). Concentration of substances was 4.92 mg mL^{-1}.

of HA degradation compared against the reference (curve 0). On the other hand, the addition of cinnamochitosan to GSH (curve 3) promoted the inhibition of HA degradation more significantly than GSH itself (curve 2) achieving a decrease in η by 0.06 mPa s and 0.82 mPa s, respectively.

Similar results can be observed in Fig. 3b, where the chitosan, after its addition to GSH, almost completely inhibited the degradation of HA with a final value of decrease in η by 0.27 mPa s (curve 3) compared with 1.3 mPa s (curve 2), when only GSH was examined. The cinnamo-chitosan itself promoted the HA degradation (curve 1). The results for other substances are summarised in Table 1.

Table 1 displays a percent efficiency of chitosan derivatives (4.92 mg mL^{-1}) with GSH (4.92 mg mL^{-1}) in inhibiting HA degradation under both experimental regimes. Compared to the 100 % efficiency of cinnamochitosan + GSH, other combinations of substances exhibited a similar efficiency, which was slightly higher than in the experimental regime (*ii*) (Table 1). On the other hand, the GSH showed a slightly lower inhibitory activity.

The addition of the examined substances 1 h later was designed based on the results of electron paramagnetic resonance, which demonstrated the disappearance of $^{\bullet}$OH radicals within 1 h (Šoltés et al., 2006). Later, a propagation phase of the HA free-radical degradation occurs, which is illustrated by Eqs. (1)–(3). Polymers with CH groups, which also include HA, are readily degraded by $^{\bullet}$OH radicals. The hydroxyl radical abstracts H$^{\bullet}$ from the HA macromolecule to produce a C-derived macroradical, the so-called alkyl radical A$^{\bullet}$. Under aerobic conditions, during a phase

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Table 1. Inhibitory activity of substances against HA degradation[a]

Regime	Substance				
	GSH	Cinnamo-chitosan + GSH	Amino-chitosan + GSH	Cinnamo-amino-chitosan + GSH	Chitosan + GSH
	Inhibitory activity/%				
Experimental (*i*)	91	100	99.7	98.6	98
Experimental (*ii*)	78	100	98.0	90.0	96

a) Day-to-day reproducibility of three experiments was $\leq$ 1.7 %.

known as propagation, a dioxygen molecule reacts with the alkyl radical to produce peroxy-type radicals AOO$^{\bullet}$. The peroxy-type radicals, generated by the random trapping of H$^{\bullet}$ from adjacent HA macromolecules, form hydroperoxyl and novel HA-derived macroradicals.

$$HA + {}^{\bullet}OH \rightarrow A^{\bullet} + H_2O \quad (1)$$

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \quad (2)$$

$$HA + AOO^{\bullet} \rightarrow AOOH + A^{\bullet} \quad (3)$$

Due to a continual process of propagation reactions, biopolymer fragments of lower sizes are formed, which leads to the decrease in η in the HA solution. The radical process, involving the four steps comprising initiation, propagation, transfer and termination, can be stopped by the addition of a free-radical scavenger. When such a scavenger is mixed with the HA solution prior to adding WBOS, the scavenger may be examined as a preventative antioxidant (against the production of $^{\bullet}$OH radicals) while, on adding the substance during the propagation phase of the HA degradation, the substance is examined as a chain-breaking antioxidant (against the production of peroxy-type radicals AOO$^{\bullet}$).

Fig. 4 displays the percentage of ABTS$^{\bullet+}$ present after addition of the substances individually or in combination. It is evident that neither chitosan (curve 1) nor GSH (curve 2) effectively scavenged ABTS$^{\bullet+}$, which means that they are weak donors of electrons. The amounts of the non-scavenged ABTS$^{\bullet+}$ represented 75 % and 56 %, respectively, after 20 min. On the other hand, mixing the chitosan with GSH resulted in a complete reduction of ABTS$^{\bullet+}$ (curve 3). This indicates that both the substances act synergistically in donating electrons. Data on the ABTS$^{\bullet+}$ scavenging by chitosan and its derivatives without/with GSH are summarised in Table 2.

Table 2 displays the percentage of ABTS$^{\bullet+}$ present after the addition of chitosan and its derivatives (4.92 mg mL^{-1}) in the absence and presence of GSH (4.92 mg mL^{-1}). As revealed, the amounts of the non-scavenged ABTS$^{\bullet+}$ were 65–80 % for chitosan and its derivatives and 56 % for GSH. The addition of GSH

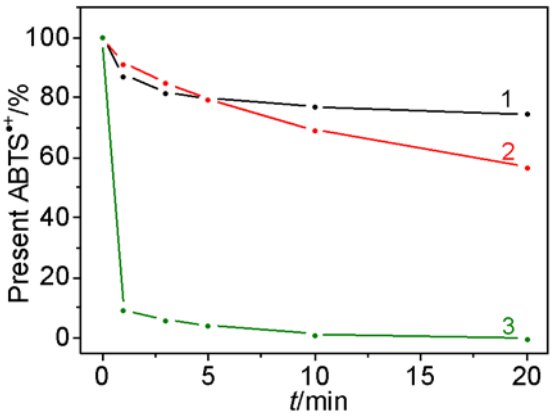

Fig. 4. Percentage of present ABTS$^{\bullet+}$ after addition of chitosan (curve 1), GSH (curve 2) and combination of chitosan and GSH (curve 3). Concentration of substances was 4.92 mg mL^{-1}.

Table 2. Percentage of ABTS$^{\bullet+}$ present after addition of chitosan and its derivatives in absence and presence of GSH[a]

Substance	Present ABTS$^{\bullet+}$/%
Chitosan	75
Amino-chitosan	80
Cinnamo-chitosan	71
Cinnamo-amino chitosan	65
Chitosan + GSH	0
Amino-chitosan + GSH	0
Cinnamo-chitosan + GSH	4
Cinnamo-amino chitosan + GSH	3

a) Measurements were performed at wavelength of 730 nm. Day-to-day reproducibility of three experiments was $\leq$ 1.9 %.

to chitosan and amino-chitosan resulted in a complete reduction of ABTS$^{\bullet+}$. For the other two derivatives, a minute amount of ABTS$^{\bullet+}$ (3 %, 4 %) remained non-scavenged.

Conclusions

This study proved that the derivatives investigated protected HA against oxidative degradation and they

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acted as donors of both $H^{\bullet}$ and electrons. A prominent observation is that chitosan and its derivatives in combination with glutathione enhanced a protective effect against reactive oxygen species. These combinations showed excellent, almost 100 % scavenging of $ABTS^{\bullet+}$ cation radicals. It may be concluded that chitosan and its derivatives are advantageous for the preparation of membranes for treating damaged skin. Membranes enriched with HA and an antioxidant such as glutathione may result in an enhanced protective efficiency, which was the subject of a recently patented study (Šoltés et al., 2015).

Acknowledgements. This study was supported by VEGA grant no. 2/0065/15.

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