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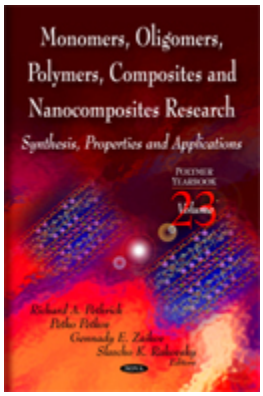

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Chapter 6

EFFECT OF L-GLUTATHIONE ON HIGH-MOLAR-MASS HYALURONAN DEGRADATION BY OXIDATIVE SYSTEM CU(II) *PLUS* ASCORBATE

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ABSTRACT

The antioxidative effect of L-glutathione (GSH) on the degradation of high-molar-mass hyaluronan was studied in an oxidative system composed of Cu(II) *plus* ascorbic acid by using rotational viscometry. GSH added to the oxidative system in sufficient amount resulted in total inhibition of hyaluronan degradation. Contrary to this result, GSH tested in a trace concentration was shown to act as a pro-oxidant. By combining the method of size-exclusion chromatography and that of rotational viscometry, the molar masses as well as the radius of gyrations of the biopolymer recovered from the oxidative system was determined.

Keywords: Free radicals, Hyaluronan degradation, L-Glutathione, Rotational viscometry, Size exclusion chromatography, Synovial fluid

INTRODUCTION

Bioanalysis is usually understood as a determination of one or more specific analytes within a biological matrix. A typical bioanalytical assay represents e.g., the determination of a xenobiotic/drug in blood plasma. Various methods have been available for analysis of low-molar-mass xenobiotics. Frequently, novel approaches are being published. The goals to

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establish novel analytical methods for the determination of a given xenobiotic are governed on the one hand by sophistification of analytical tools, on the other hand by selectivity, sensitivity, etc., necessary to fulfill the requirements for deeper insight into the complexity of biosamples themselves.

Let us assume, however, that the xenobiotic tested is a macromolecular compound. A biological sample is really a very complex mixture of high- and low-molar-mass compounds. Of these analytes, hyaluronan (formerly hyaluronic acid or hyaluronate) determination has attained a high interest both by physicians and analysts.

Generally, in the case of a homopolymeric analyte, such as that of hyaluronan (HA), the knowledge of its average molar mass can provide relevant information about the presence or absence of an inflammation within the synovial fluid (SF) of a joint. The mean molar mass of HA in SF of healthy individuals is 6–10 megaDaltons. This value, however, diminishes significantly in arthritic diseases, e.g., in osteoarthritis and rheumatoid arthritis, where the mean HA molar mass could reach only hundreds of kiloDaltons.

The primary determinant of the HA molar mass within a SF sample could be estimated from the dynamic viscosity of this body fluid. As demonstrated and advocated by Conrad et al. [1], currently SF samples of the volume ≤ 20 μL could completely fulfill the requirement of a quick and precise sample analysis. Figure 1 shows an example [1, 2]:

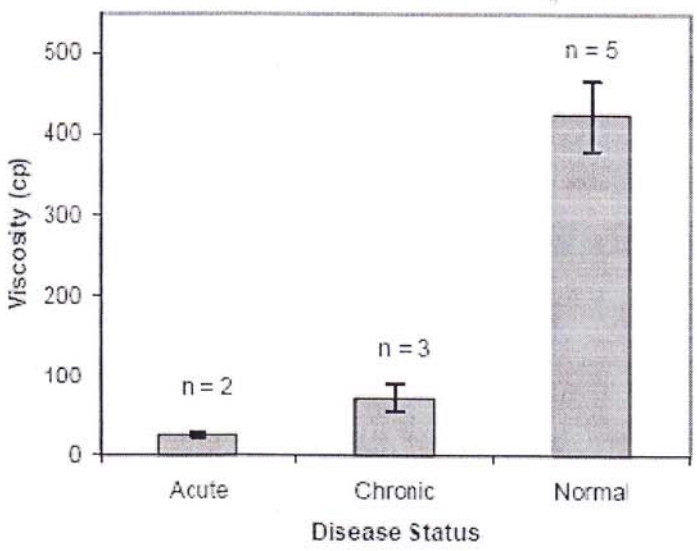

Figure 1. Synovial fluid viscosity measured in dogs with different stages of disease.

Viscosity is found to be an extremely good marker since there is a difference of about one order of magnitude between the viscosity of SF of healthy and affected subjects (cf. Figure 1). Analysts, however, show rather a reluctance to introduce routinely viscometry as a proper device for SF viscosity determination and to accept sample viscosity values as a diagnostic tool. Why? The primary reason is the observed instability of the SF viscosity itself. In the very course of measurements, it exhibits a rapid reduction.

Numerous papers have appeared concerning the degradation of high-molar-mass HA samples by the action of free radicals [3–11]. In light of these findings, the trace transition

metals along with ascorbate within the withdrawn SF sample may be considered the potential determinant of the decay of HA macromolecules. As shown, these two endogenic ingredients, in contact with oxygen either present in SF or absorbed/dissolved into the body fluid, start a significant HA degradation even under anoxic conditions [12].

To protect biosamples from e.g., oxidation, several "antioxidants" could be primarily supplied into the sample to be assayed. L-Glutathione (GSH), an endogenic antioxidant, belongs among the most efficient substances protecting the cells against reactive oxygen species (ROS) escaping from mitochondria. We did attempt to involve this xenobiotic in our model situation; i.e., we exposed high-molar-mass HA under aerobic conditions to the action of Cu(II) *plus* ascorbate. The degradation of biopolymer macromolecules was monitored by the method of rotational viscometry. The protective effect of GSH was then assayed by working with the antioxidant concentration between 10–200 μM. GSH was added to the reaction system at the beginning of HA degradation. Along with rotational viscometry, a size exclusion chromatographic (SEC) device equipped with an on-line multi-angle light-scattering (MALS) detector was employed.

EXPERIMENTAL

Biopolymers

The high-molar-mass hyaluronan sample P9710-2A (for specific characteristics, see Table 1) used was donated by the Lifecore Biomedical Inc., Chaska, MN, U.S.A.

Chemicals

NaCl and $CuCl_2 \cdot 2H_2O$ with analytical purity grade were purchased from Slavus Ltd., Bratislava, Slovakia; L-ascorbic acid was purchased from Merck KGaA, Darmstadt, Germany; L-glutathione was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Redistilled deionized 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).

Preparation of Stock and Working Solutions

The working solutions of the HA samples (2.5 mg/mL) were prepared overnight in the dark at room temperature in 0.15 M aqueous NaCl in two steps: first, 4.0 mL of the solvent was added in the morning. Then, 3.85 mL of the solvent was added after six hours. The stock solutions of ascorbic acid (16.0 mM), GSH (8.0, 16.0 and 32.0 mM), cupric chloride (16.0 mM, diluted to a 160 μM solution), were also prepared in 0.15 M aqueous NaCl.

Study of Uninhibited/inhibited Hyaluronan Degradation

Antioxidative effects of the chosen thiol, GSH, on the kinetics of degradation of high-molar-mass HA sample (20 mg) were tested in the oxidative system comprising Cu(II) *plus* ascorbic acid by adding the drug before the reaction onset. The experimental set (Figure 2) was carried out by adding the thiol at the beginning of degradation: HA solution was stirred for 30 seconds, then the solution of 50.0 μL of 160 μM $CuCl_2$ solution was added and stirred for 30 seconds and left to stand for 7.5 minutes at ambient temperature. GSH in the volume of 50.0 μL (1.60, 8.0, 16.0, and 32.0 mM) was added to the solution, examined, and stirred again for 30 seconds. Finally, 50.0 μL of ascorbic acid (16.0 mM) was added and stirred for 30 seconds. GSH concentrations in the degradative system tested were 10, 50, 100, and 200 μM. The results of the settings were compared with the reference experiments in the absence of GSH.

Rotational Viscometry

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, U.S.A.). Recording of the viscometer output parameters started two minutes after the experiment onset. The changes of dynamic viscosity of the system were measured at 25.0 ± 0.1 °C in three-minute intervals for up to five hours. The viscometer Teflon® spindle rotated at 180 rpm, i.e., at the shear rate equaling 237.6 s^{-1}. To determine the reaction products, the reservoir content was transferred into 20 mL of 96% ethanol, giving rise to precipitation of the polymer. The following day, the polymer precipitate was thoroughly rinsed with 20 mL of 96% ethanol, centrifuged, and dried in a desiccator. The dried polymeric product was obtained in the form of a firm pellet. The recovered product yield was 75%.

Chromatographic System

The molar mass distribution (MMD) of HA samples was obtained by a modular multi-detector size exclusion chromatographic system. The SEC system consisted of an Alliance 2695 separation module from Waters (Milford, MA, USA) equipped with two on-line detectors: a MALS Dawn DSP-F photometer from Wyatt (Santa Barbara, CA, USA) and a 2414 differential refractometer (DRI) from Waters, used as concentration detector. The setup of this multi-detector SEC system was serial in the following order: Alliance-MALS-DRI. Considering the high-molar mass of the HA samples analyzed, a relatively low flow rate, 0.4 mL/min, was used to avoid the shear-degradation of the polymer in the SEC columns. The experimental methodology for a reliable use of the MALS photometer was described in detail in the literature [13–15].

The experimental conditions of the SEC-MALS system were the following: Two TSKgel columns (G6000-G5000) from Tosoh Bioscience (Stuttgart, Germany); mobile phase: 0.15M NaCl; temperature: 35 °C; degassing: vacuum; injection volume: 150 μL; polymer concentration: ≈0.1–0.3 mg/mL on the basis of the HA molar mass.

Multi-Angle Light Scattering detector uses a vertically polarized He-Ne laser, λ=632.8 nm, and simultaneously measures the intensity of the scattered light at 18 fixed angular locations ranging in aqueous solvent from 14.5° to 158.3°. It is well known that an on-line MALS detector coupled to a concentration detector allows to obtain the true molar mass (M) and the size, i.e., the root mean square radius ($<s^2>^{1/2}$), hereafter denoted as radius of gyration - Rg, of each fraction of the eluting polymer. The MALS calibration constant was calculated using toluene as standard assuming a Rayleigh factor $R(\theta) = 1.406\times10^{-5}$ cm^{-1}. The normalization of the photodiodes was performed by measuring the scattering intensity in the mobile phase of bovine serum albumin (M = 66.4 kDa, Rg = 2.9 nm), the globular protein was assumed to act as an isotropic scatterer.

Specific refractive index increment (dn/dc) of HA with respect to the mobile phase was obtained from the literature [14]. The dn/dc value for HA in 0.15M NaCl is 0.15 mL/g.

RESULTS AND DISCUSSION

Rotational Viscometry

As seen in Figure 2, the addition of the two actors of HA oxidative damage, i.e., Cu(II) *plus* ascorbate (1.0 μM *plus* 100 μM), led within five hours to a significant reduction of the solution dynamic viscosity value from 9.93 mPa·s to 6.53 mPa·s. It is a well-known fact that the combination of ascorbate *plus* Cu(II) under aerobic conditions is an efficient $^{\bullet}OH$ radical generating system called the Weissberger system [16]: since ascorbate acts as a powerful reducing agent with a standard reduction potential of +0.282 V at pH 7 for the redox couple $AscH^{\bullet}/AscH^{-}$, it should reduce Cu(II) to Cu(I). Thus, taking into account that the reduction potential of the pair Cu(II)/Cu(I) = +0.16 V, Cu(I) should be able to reduce O_2 molecules to yield directly H_2O_2 [17–19]. The generated Cu(I) ions immediately yield $^{\bullet}OH$ radicals according to the Fenton-type reaction:

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

Due to its extremely high affinity for hydrogen, the generated $^{\bullet}OH$ radical abstracts from the HA macromolecule the radical $H^{\bullet}$, giving rise to a radical termed below $A^{\bullet}$ for the sake of simplicity. After this phase, called initiation, a phase called propagation follows. Within this phase under aerobic conditions, the radical $A^{\bullet}$ reacts with atmospheric oxygen yielding $AOO^{\bullet}$, which can naturally participate in the reactions leading to further production of $A^{\bullet}$ ($AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$). By continuing the latter propagation reaction, along with the reaction with atmospheric oxygen, still more generated $AOO^{\bullet}$ radicals can undergo fragmentation reactions yielding polymer fragments with a reduced molar mass [20]. Both reactions $A^{\bullet} + HA \rightarrow HA + A^{\bullet}$ and $AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$ can be classified as reactions by which the active radical site is transferred to another macromolecule. However as a rule, such a transfer reaction does not have any significant effect with regard to the potential retardation or termination of the radical degradation/fragmentation of the HA macromolecules. As demonstrated by many studies (see reviews [21, 22]), specific

scavengers/drugs are required to ensure retardation/inhibition of the propagation phase of HA degradation. It can be thus legitimately assumed that drug molecules most likely provide the radicals $A^{\bullet}$ with their own $H^{\bullet}$ radical, by which step drug molecules are converted to radicals, which can recombine in the reaction medium. The simultaneous decrease or the complete elimination of $A^{\bullet}$ and $AOO^{\bullet}$ radicals may lead to a pronounced retardation or even to cessation of HA degradation.

The above assumption has been confirmed by the above given results (cf. Figure 2). It is evident that the addition of 10 μM GSH retarded significantly the degradation of HA macromolecules. However since the concentration of $^{\bullet}OH$ radicals was higher than that of GSH, the transfer and the following termination steps of the radical reactions were not complete. Yet the increase of the applied concentration of GSH to 50 μM (curve coded 2) stopped any decrease of the HA solution dynamic viscosity. From this observation, one could conclude that under the given experimental conditions the efficiency of the generation of $^{\bullet}OH$ radicals is somewhere below 50%. Namely, at the total efficiency of the ascorbate (100 μM) conversion to H_2O_2, the yield of the $^{\bullet}OH$ radicals should be 100 μM. However, this is not true since already 50 μM GSH inhibited completely the degradation of HA solution. It should be pointed out here that the addition of GSH in greater excess to ascorbate (up to 2:1) does not generate the well-known glutathyil radicals:

$$GS^{\bullet} + GSH \rightarrow [GSSGH]^{\bullet}$$

which under aerobic conditions could yield further $^{\bullet}OH$ radicals by the sequence of the following reactions

$$[GSSGH]^{\bullet} + O_2 \rightarrow GSSG + HO_2^{\bullet}$$
$$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2$$
$$H_2O_2 \text{ metal catalyzed decomposition} \rightarrow {}^{\bullet}OH \text{ radicals}$$

To follow thoroughly the reaction mixture with the content of GSH (50 μM) efficient enough to inhibit fully the degradation of HA macromolecules after five-hour treatment, the solution was precipitated by its insertion into ethanol. The precipitated biopolymer was subsequently analyzed by the chromatographic method concerning the polymer molar mass distribution.

SEC-MALS

Table 1 summarizes the more important results obtained by using the native HA sample (P9710-2A) and that recovered after the above presented sample treatment, which is marked as P9710-$2A_{prec.}$ using the SEC-MALS system. Table 1 reports the usual averages of the molar masses (numeric- M_n, weight- M_w, and z- M_z), the molar mass of the peak of the chromatogram M_p, the dispersity indexes (M_w/M_n and M_z/M_w), and the dimension Rg of the macromolecules.

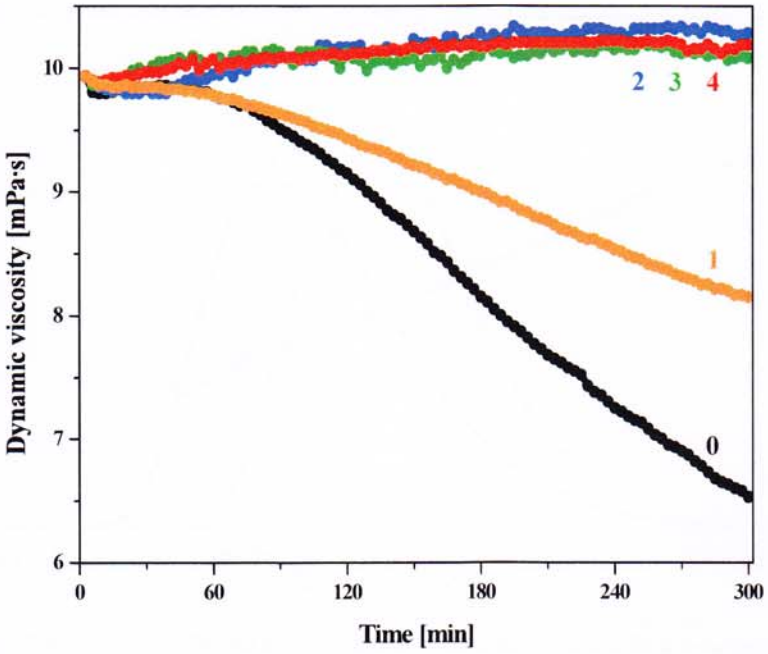

Figure 2. Time dependence of dynamic viscosity of the HA P9710-2A solution in the presence of 1.0 μM $CuCl_2$ and 100 μM ascorbate (0). Effect of 10 (1), 50 (2), 100 (3), and 200 (4) μM GSH.

Table 1. Summarized SEC-MALS results of two HA samples

Sample	M_p kDa	M_n kDa	M_w kDa	M_z kDa	M_w/M_n -/-	M_z/M_w -/-	R_g nm
P9710-2A	966.7	347.0	841.5	1,305.8	2.4	1.6	93.5
P9710-2A$_{prec.}$	399.6	215.6	411.0	606.5	1.9	1.5	61.0

Figure 3 represents the differential MMD of the two samples, i.e., the native HA sample P9710-2A and that recovered after the above presented treatment – P9710-2A$_{prec.}$. Despite the justified expectation, the average molar masses determined for the sample P9710-2A$_{prec.}$ were significantly lower than those valid for its parent P9710-2A biopolymer.

As can be assumed from Figure 4, the conformation, i.e., the molecular dimensions R_g as a function of the molar mass, of the two biopolymers do not differ to such a degree that one had to admit a significant change in shape or stiffness in the case of the dissolved HA macromolecules of the sample P9710-2A$_{prec.}$.

One of the reasons why the molar masses of the sample P9710-2A$_{prec.}$ differ from those which can be expected on the basis of the results represented in Figure 1 is that the biopolymer precipitate degraded during a long-time (one-day) dissolution before the SEC-MALS analysis. To prove or disprove this conclusion, we re-analyzed the P9710-2A$_{prec.}$ solution by using the rotational viscometric device. As observed, really the value of the solution dynamic viscosity was significantly reduced. Its value 5.30 mPa·s was far below that value valid for the intact sample P9710-2A, i.e. 9.93 mPa·s.

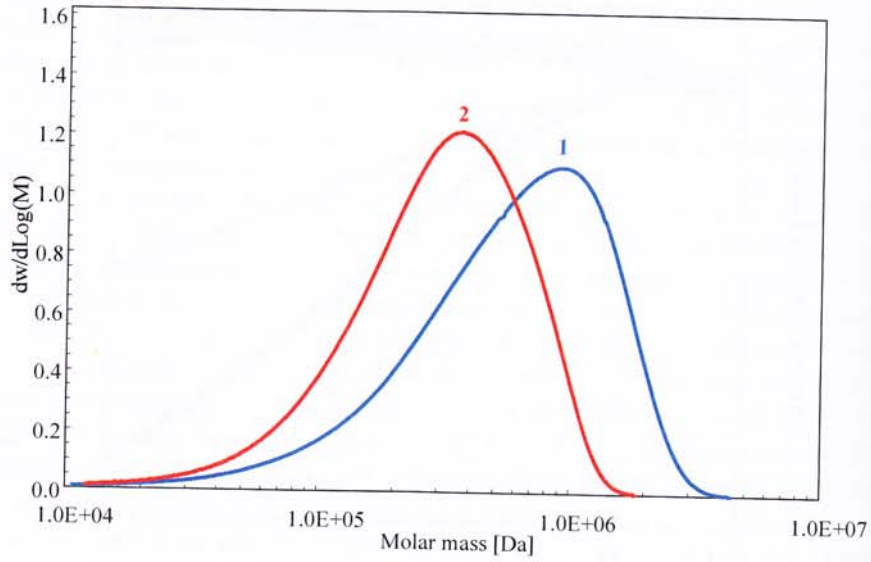

Figure 3. Comparison of differential molar mass distribution of intact P9710-2A (1) and P9710-2A$_{prec}$ (2) samples.

To estimate the value of the average molar mass of P9710-2A$_{prec.}$, we attempted also to use an empirical plot constructed from the values of dynamic viscosities of some previously run samples [23]. By taking into account that at the second minute of the determination the dynamic viscosity value of the P9710-2A$_{prec.}$ solution equaled 5.30 mPa·s, one can estimate the molar mass parameter for this sample to be approx. 502 kDa. This calculated molar mass value actually relates quite nicely to that determined by SEC-MALS, i.e. 411 kDa (cf. M_w value in Table 1).

The assumed reason for the significant molar mass decrease of the sample P9710-2A$_{prec.}$ compared to that of intact/native biopolymer P9710-2A is that by precipitating the polymer from the solution to ethanol, the polymer along with Cu(II) adsorbed also a certain amount of unreacted GSH (and/or GSSG). A possible unfavorable effect of trace amounts of GSH in the precipitate was therefore tested as follows: the measurement of dynamic viscosity of the sample P9710-2A was performed using the method of rotational viscometry, where besides two reactants Cu(II) *plus* ascorbate (1.0 μM *plus* 100 μM), a trace amount of GSH, namely 1.0 μM was added.

As shown in Figure 5, the addition of GSH at the last tested concentration 1.0 μM evidently potentiated the degradation of HA macromolecules in such a way that the rate of dynamic viscosity decrease can be considered to be pro-oxidative.

On balance then (cf. Figure 2), one can assume that GSH exhibits excellent antioxidative properties when added to the system in a proper amount. Yet when it is not present in a high enough concentration, there is a real risk that this substance will promote the degradation of the biopolymer (cf. Figure 5).

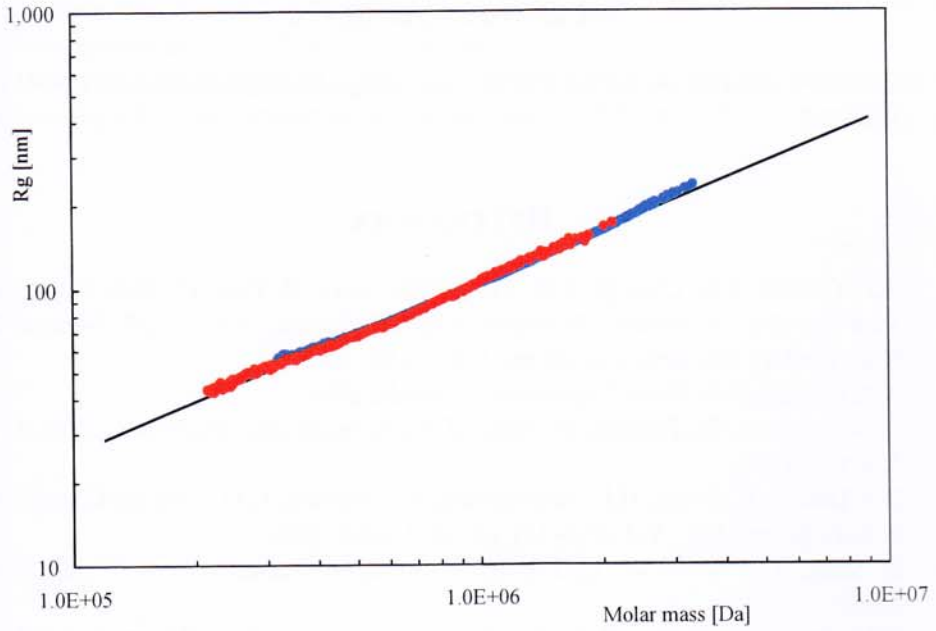

Figure 4. Comparison of experimental conformation plot, Rg = f(M), of intact P9710-2A (blue) and P9710-2A$_{prec}$ (red) samples.

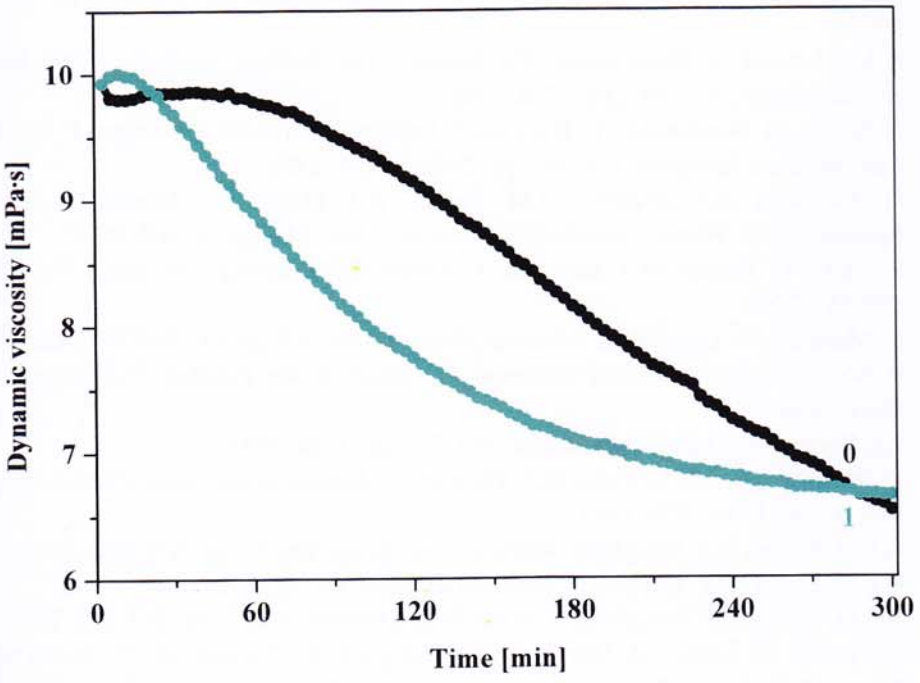

Figure 5. Time dependence of dynamic viscosity of the HA P9710-2A solution in the presence of 1.0 μM $CuCl_2$ and 100 μM ascorbate (0). Effect of 1.0 μM (1) GSH on HA degradation.

ACKNOWLEDGMENTS

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REFERENCES

[1] B.P. Conrad, S.O. Canapp, A.R. Cross, C.E. Levy, B. Galecki, M.B. Horodyski, R. Tran-Son-Tay, *3 Summer Bioengineering Conference*, June 25-29, Sonesta Beach Resort in Key Biscayne, Florida, pp. 1157-1158, 2003.

[2] B.P. Conrad, *PhD Thesis*, University of Florida, 2001.

[3] G. Matsumura, W. Pigman, *Archives of Biochemistry and Biophysics.*, Vol. 110, pp. 526-533, 1965.

[4] D.J. Deeble, E. Bothe, H.P. Schuchmann, B.J. Parsons, G.O. Phillips, C.von Sonntag, *Z. Naturforsch. [C].*, Vol. 45(9-10), pp. 1031-1043, 1990.

[5] H. Saari, Y.T. Konttinen, C. Friman, T. Sorsa, *Inflammation*, Vol. 17, pp. 403-415, 1993.

[6] B.M. Praest, H. Greiling, R. Kock, *Clinica Chimica Acta*, Vol. 266, pp. 117-128, 1997.

[7] V. Deguine, M. Menasche, L. Fraisse, P. Ferrari, Y. Pouliquen, L. Robert, *Clinica Chimica Acta*, Vol. 262, pp. 147-152, 1997.

[8] M. Jahn, J.W. Baynes, G. Spiteller, *Carbohydrate Research*, Vol. 321, pp. 228-234, 1999.

[9] S.A. Al-Assaf, S. Navaratnam, B.J. Parson, G.O. Phillips, *Archives of Biochemistry and Biophysics,* Vol. 441, pp. 73-82, 2003.

[10] L. Šoltés, M. Stankovská, V. Brezová, J. Schiller, J. Arnhold, G. Kogan, P. Gemeiner, *Carbohydrate Research*, Vol. 341, pp. 2826-28534, 2006.

[11] G. Mendoza, A.I. Álvarez, M.M. Pulido, A.J. Molina, G. Merino, R. Real, P. Fernandes, J.G. Prieto, *Carbohydrate Research,* Vol. 342, pp. 96-102, 2007.

[12] R. Stern, G. Kogan, M.J. Jedrzejas, L. Šoltés, *Biotechnology Advances*, Vol. 25, pp. 537-557, 2007.

[13] R. Mendichi, A. Giacometti Schieroni, *Polymer*, Vol. 43, pp. 6115-6121, 2002.

[14] R. Mendichi, A. Giacometti Schieroni, C. Grassi, A. Re, *Polymer,* Vol. 39, pp. 6611-6620, 1998.

[15] P.J. Wyatt, *Analytica Chimica Acta*, Vol. 272, pp. 1-40, 1993.

[16] A. Weissberger, J.E. LuValle, D.S. Thomas Jr., *Journal of American Chemical Society*, Vol. 65, pp. 1934-1939, 1943.

[17] A.E.O. Fisher, D.P. Naughton, *Medical Hypotheses*, Vol. 61, pp. 657-660, 2003.

[18] A.E.O. Fisher, D.P. Naughton, *Nutrition Journal*, Vol. 3, pp. 2, 2004.

[19] A.E.O. Fisher, D.P. Naughton, *Current Drug Delivery,* Vol. 2, pp. 261-268, 2005.

[20] J. Rychlý, L. Šoltés, M. Stankovská, I. Janigová, K. Csomorová, V. Sasinková, G. Kogan, P. Gemeiner *Polymer Degradation and Stability,* Vol. 91, pp. 3174-3184, 2006.

[21] J. Schiller, B. Fuchs, J. Arnhold, K. Arnold, *Current Medicinal Chemistry,* Vol. 10, pp. 2123-2145, 2003.

[22] L. Šoltés, R. Mendichi, G. Kogan, J. Schiller, M. Stankovská, J. Arnhold, *Biomacromolecules,* Vol. 7, pp. 659-668, 2006.

[23] K. Valachová, Application of models of high-molar mass hyaluronan degradation for evaluating antioxidant properties of new substances. *PhD Thesis*, Bratislava, 2009.