Degradation of high-molecular-weight hyaluronan: a rotational viscometry study

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Abstract: The flow behavior of four hyaluronan (HA) samples was studied along with the influence of the material of the viscometer on the flow parameters investigated. The changes in viscosity were measured by a rotational viscometer equipped with metal or Teflon cups and coaxial cylindrical spindles. Within 5 hours of the experiment, a moderate increase of viscosity was observed in all samples. The shear flow measurements of HA samples indicated a non-Newtonian behavior. In another set of experiments, kinetics of HA degradation after addition of H_2O_2 was compared using both types of cups and spindles. The course of viscosity decrease due to oxidative degradation was observed to differ substantially for the two types of the material, from which the viscometer cups and spindles were built.

Key words: hyaluronan degradation, oxidants, rotational viscometry.

Abbreviations: HA, hyaluronan.

Introduction

High sensitivity of hyaluronan (HA) against oxidative species predetermine this biopolymer to be used as an *in vitro* probe for studying the effect of the oxidative species on the biopolymer, as well as for assessment of pro-oxidative or anti-oxidative properties of various compounds. A proper selection of the methods for monitoring the changes, occurring in HA affected by the oxidative species, is a necessary condition for obtaining the meaningful results. MIYAZAKI et al. (1998) observed HA degradation by means of rotational viscometer even when the sample solution was just left in the metallic cup of the viscometer. Other authors (JAHN et al., 1999) using a rotational viscometer also observed a moderate time-dependent decrease of viscosity of the HA solution.

In the present study we attempted to eliminate any contact of the high-molecular-weight HA samples with metal by replacing the metallic cup and spindle of the Brookfield rotational viscometer by a laboratory made Teflon equipment. In addition, we compared the kinetics of HA degradation that took place upon H_2O_2 addition on using either metal or Teflon cup/spindle equipped viscometers. We used 882 μM H_2O_2 since this concentration was approximately in the middle of the range (180–2800 μM) employed by other investigators (LINDVALL & RYDELL, 1994; HAWKINS & DAVIES, 1996; LI et al., 1997; PRAEST et al., 1997; AL-ASSAF et al., 1999; JAHN et al., 1999; YAMAZAKI et al., 2003).

The goal of this study was to prove a necessity of use of the inert material for preparation of the cup and spindle in a rotation viscometry experiment. Another goal was to demonstrate a non-Newtonian behavior of the high-molecular-weight HA.

Material and methods

Biopolymers

High-molecular-weight HA samples with molecular weight ranging from 90.2×10^3 to 1.553×10^6 Da were generous gifts of HA manufacturers: Fidia Farmaceutici S.p.A., Abano Terme, Padua, Italy; Genzyme Corp., Cambridge, MA, USA; Lifecore Biomedical Inc., Chaska, MN, USA; CPN Ltd., Ústí nad Orlicí, Czech Republic.

Chemicals

Analytical purity grade NaCl was from Slavus Ltd., Bratislava, Slovakia. Aqueous H_2O_2 solution (30%), analytical purity grade, was purchased from Chemapol, Prague, Czech Republic.

HA solutions for determination of flow characteristics

The 3 mg/mL HA stock solution was prepared in 0.15 M NaCl. For measurement 8 mL of this solution was used.

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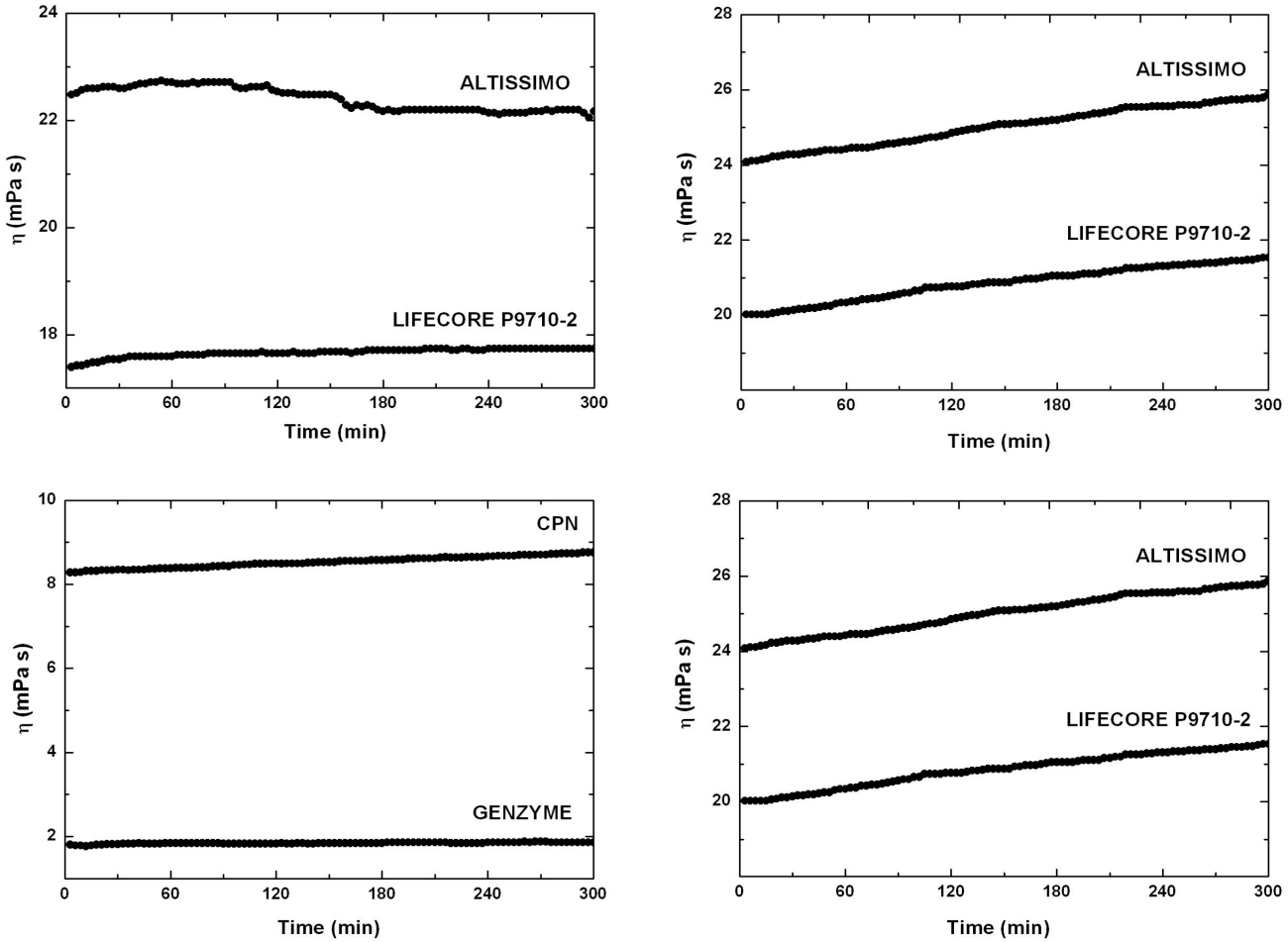

Fig. 1. Long-term measurements of viscosity of the HA solutions performed by using metal (left panels) and Teflon (right panels) cups/spindles.

HA solution for degradation study

The degradation was started by adding 800 μL of 3% H_2O_2 to the HA solution (sample Lifecore P9710–2). The mixture was subsequently stirred for 20 s. Immediately 8 mL of this mixture were transferred to the cup reservoir of the viscometer.

Methods

The measurements were carried out at 25 ± 0.1 °C by using a digital rotational viscometer Brookfield DV-II+ PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, USA). Two experimental arrangements were used: (i) the standard metal cup/spindle made by Brookfield Engineering Labs.; and (ii) the Teflon cup/spindle constructed in our laboratory.

Long-term viscosity measurements were performed during a period of over 5 h by using either the metal or the Teflon cups/spindles. Measurements of high-molecular-weight HA samples were performed at 105 rpm (rotational spindle speed), while those of low-molecular-weight HA at 200 rpm.

Shear flow measurements were performed by using the Teflon cup/spindle. The shear rate decreased from 211 s^{-1} (160 rpm) to 40 s^{-1} (30 rpm) and then increased again from 30 rpm to 160 rpm. The measurements were conducted at 30 s intervals.

The kinetics of HA degradation was investigated by means of both the metal (105 rpm) and the Teflon (160 rpm) cups/spindles during a period of over one hour.

Results

Long-term viscosity measurements

Figure 1 shows changes of viscosity that occurred during the 5-h measurements. In the case of sample Altissimo, long-term viscosity measurements involving the metal cup/spindle revealed an unsteady course of viscosity change, while a slight increase of viscosity occurred in the case of the other samples. Contrary to the high-molecular-weight HA, in the case of low-molecular-weight HA no difference was observed in the course of viscosity increase when metal or Teflon cups/spindles were used (Fig. 1). The viscosity values measured were in good agreement for the samples Genzyme and CPN.

Shear flow behavior measurements

Figure 2 illustrates shear flow behavior measurement of the HA sample Lifecore P9710–2. As can be

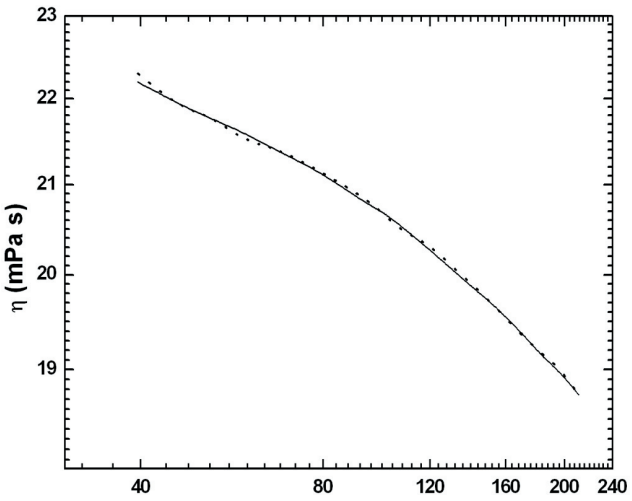

Fig. 2. Shear flow behavior of HA solution (Lifecore P9710–2; 3 mg/mL). Measurements of viscosity at different shear rates were attained by changing rotational spindle speed from 160 to 30 rpm (full line) and from 30 to 160 rpm (dotted line) and performed by using Teflon cup/spindle.

seen, the fluid displays a decreasing viscosity with an increasing shear rate. This represents a typical attribute of a non-Newtonian fluid. Similar results we obtained for all samples (data not shown).

Kinetics of HA degradation

The kinetics of HA degradation was investigated by determining the viscosity changes occurring during one hour. The effect of the addition of 3% H_2O_2 (i.e. 882 μM) on HA viscosity (Lifecore P9710–2) is shown in Figure 3. There is a significant difference between the two measurements conducted using metal or Teflon cups/spindles. The addition of H_2O_2 caused a dramatic decrease of viscosity within one hour, reaching 17% of the initial value (Fig. 3). Upon visual inspection of the solution in the cup reservoir, we observed the generation of bubbles and, at the end of treatment, a brown particulate material, rust, was found in the reservoir.

A parallel experiment using a Teflon cup/spindle failed to reveal either bubbles or rust formation. The viscosity decreased within one hour to 68% of the initial value (Fig. 3).

Discussion

There have been many reports indicating that HA degradation is mediated by free radicals produced by reaction between oxygen and trace amounts of metal (M) ions, such as iron and/or copper. Hydroxyl radicals are generated by the following reactions (Miyazaki et al., 1998):

$M + O_2 \rightarrow M^+ + O_2^{\bullet -}$

$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$

$M + H_2O_2 \rightarrow M^+ + {}^{\bullet}OH + OH^-$

Since the cup reservoir and spindle of the viscometer used are usually made of stainless steel, the influence of metal on HA degradation should be taken into account.

In our preceding paper (Stankovská et al., 2004), we observed a moderate time-dependent increase of viscosity of HA solutions during long-term measurements. We suggested that release of trace amount of metal cations into the HA solution took place, resulting in the formation of macrochelates. The only exception was the high-molecular-weight HA sample Altissimo, which revealed an irregular change of viscosity over the time course of the measurement.

In order to corroborate our observations, in the present study we applied an inert Teflon cup/spindle. We expected that due to elimination of the metal ion effect, we would observe a constant viscosity. However, the measurements performed with all HA samples revealed a uniform increase of viscosity in time. The most probable explanation of the observed phenomenon could be a consistent orientation of the

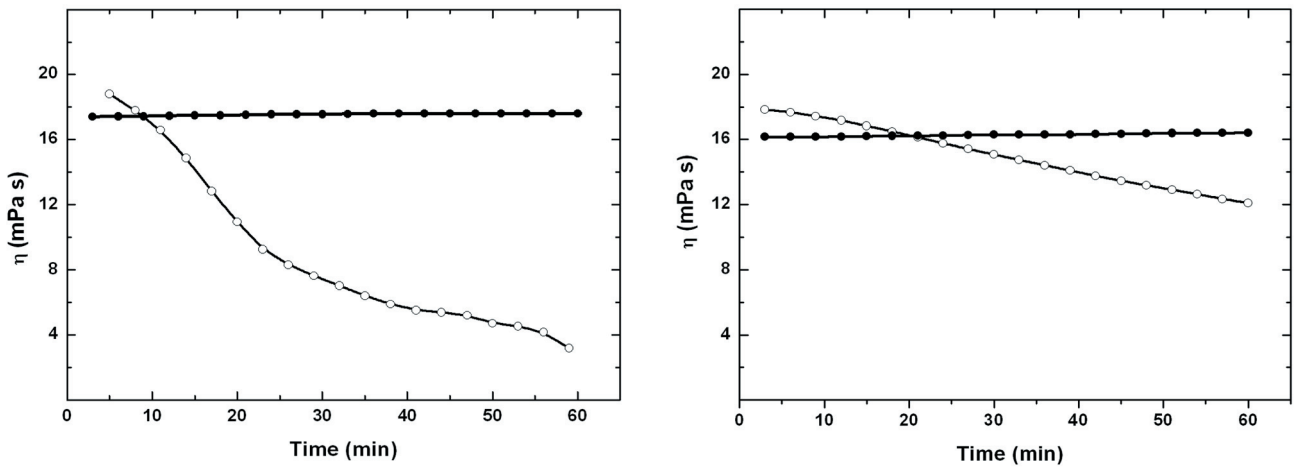

Fig. 3. Kinetics of viscosity changes of HA solutions Lifecore P9710–2. The measurements were performed by using metal (left) and Teflon (right) cups/spindles. Full circles correspond to the measurements without hydrogen peroxide addition. Open circles reflect the kinetics of HA degradation in the presence of 882 μM H_2O_2.

macromolecular chains of the biopolymer called rheopexy.

The influence of the material of the rotational viscometer on HA viscosity was investigated by several authors (JAHN et al., 1998; MIYAZAKI et al., 1998). Based on the results obtained in the presented study and on the discrepancy observed earlier (STANKOVSKÁ et al., 2004), we recommend the use of inert cup/spindle material, e.g. Teflon, when investigating HA degradation in the presence of oxidizing agents.

Hereby we have obtained a better insight into the rheological behavior of high-molecular-weight HA *in vitro* and suggested a method for obtaining the unequivocal results. Rheological behavior of this biopolymer *in vivo* will be a subject of the future study.

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