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Hyaluronan degradation by copper(II) chloride and ascorbate: rotational viscometric, EPR spin-trapping, and MALDI–TOF mass spectrometric investigations

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Abstract—The degradation of high-molar-mass hyaluronan (HA) by copper(II) chloride and ascorbate was studied by means of rotational viscometry. It was found that even small amounts of $CuCl_2$ present in the oxidative system led to the pronounced degradation of HA, reflected in a rapid decrease of the dynamic viscosity of the biopolymer solution. Such degradation was induced by free radicals generated in elevated amounts in the presence of copper ions. Electron paramagnetic resonance investigations performed on a model oxidative system containing Cu(II) and ascorbic acid proved the formation of relatively stable ascorbate anion radicals resulting from the reaction of ascorbic acid with hydroxyl radicals. In this way, by scavenging the hydroxyl radicals, ascorbic acid protected HA from their degradative action. Matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry was applied to analyze the degraded HA. The results showed that only regular fragmentation of hyaluronan occurred using the mentioned oxidative system that led to the formation of HA oligomers with unaffected primary chemical structure.

Keywords: Hyaluronan degradation; Rotational viscometry; EPR spectroscopy; Spin-trapping technique; MALDI–TOF mass spectrometry; Ascorbate; $CuCl_2$; Hydroxyl radicals

1. Introduction

Biomacromolecules, such as nucleic acids and proteins, have often been employed to study the in vitro degradative actions of various oxidants. In these studies, both the biopolymers and the applied oxidative conditions are adjusted to mimic 'pathological' events, such as, arteriosclerosis and rheumatic diseases. The key task is to detect chemical and/or physical changes occurring in the target biomacromolecule.[1] In the case of an enzyme, reduction of its activity has been employed as a simple marker of the extent of functional impairment.[2]

Under arthritic inflammatory conditions of the affected joint, synovial fluid (SF) undergoes a significant reduction of its viscoelastic properties. Thus, changes of the kinematic and/or dynamic viscosity have been subjected to extensive investigations to disclose the agent(s) primarily responsible for the observed decrease of the SF viscoelasticity. In order to simplify the system studied, that is, to exclude effects of further molecules, investigators concentrated mainly on high-molar-mass

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hyaluronan (HA) since the degradation of this biopolymer is accompanied by a significant loss of viscoelastic properties of its solutions.[3]

The applied oxidative systems usually involved redox reactions of transition metal ions, for example, iron, copper, and titanium, although application of the latter does not have any physiological relevance. A serious drawback in employing iron(II) salts is, however, the spontaneous oxidation of Fe^{2+} cations to the Fe^{3+} under aerobic conditions. For example, iron(II) chloride is very unstable when dissolved in aqueous solutions. It is oxidized by the action of atmospheric oxygen, and the solution gradually darkens, yielding a brownish-colored liquid. Therefore, common generators of free hydroxyl radicals (·OH) based on the use of Fe^{2+} salts usually avoid the Fe^{3+} precipitation [e.g., in the form of FeO(OH)] by complexing Fe^{2+} ions with ethylenediamine tetraacetate (EDTA) and, simultaneously, recycle the oxidized Fe^{3+} ions by adding ascorbate (vitamin C). A typical example of such an ·OH generating system is Udenfriend's reagent consisting of 142 μM ascorbic acid, 80 μM EDTA, and 15 μM $FeSO_4$ in 0.1 M phosphate buffer, pH 5.5.[4] As shown by Deguine et al.,[5] a modified Udenfriend's reagent containing 48 mM ascorbic acid, 28 mM EDTA, and 5 mM $FeCl_2$ even at high dilutions (up to 12,000-fold) efficiently degraded high-molar-mass sodium hyaluronate.

Copper is another transition metal of high pathophysiological relevance, which exists in two distinct redox states, with Cu^{2+} being much more stable than Cu^{+}. Recently, we have demonstrated that even traces of $CuCl_2$ in the presence of hydrogen peroxide are effective generators of ·OH radicals:[6] In the systems comprising $CuCl_2$ and H_2O_2, at the initial stage Cu^{2+} ions are reduced by hydrogen peroxide, and subsequently the produced Cu^{+} ions react with the excess H_2O_2 yielding ·OH radicals.

The aim of this communication is to present further results on the efficiency of ·OH radical generating systems by employing only traces of $CuCl_2$ as the source of transition metal ions. Further components investigated as ·OH radical generators were ascorbic acid, hydrogen peroxide, as well as a combination of these compounds. As a marker of the degradative action of the generated ·OH radicals, the changes in the dynamic viscosity of a high-molar-mass hyaluronan solution were monitored using a rotational viscometer. In order to obtain more information on the reaction pathways, the ·OH radical generating system(s) were also investigated by electron paramagnetic resonance (EPR) spectroscopy. The chemical changes that occurred in the hyaluronan macromolecule were assessed by applying enzymatic degradation of the biopolymer, followed by matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometric analysis of the HA oligosaccharides that were formed.

2. Experimental

2.1. Biopolymers

The two samples of intact high-molar-mass hyaluronan used throughout the study were provided by Dr. K. Thacker from Lifecore Biomedical Inc., Chaska, MN, USA (sample coded LIFECORE P9710-2; $M_w = 1215$ kDa, $M_w/M_n = 1.79$) and by the company CPN Ltd, Ústí nad Orlicí, Czech Republic (sample coded CPN; $M_w = 659.4$ kDa; $M_w/M_n = 1.88$).[7] In the LIFECORE P9710-2 sample, the presence of 13 ppm of iron and 4 ppm of copper ions has been claimed ['Certificate of Analysis' (Lifecore Biomedical Inc.]. Bovine testicular hyaluronate lyase (product No. 53718 with an activity of about 0.25 U/mg) purchased as lyophylizate from Fluka Chemie AG (Buchs, Switzerland) was used without further purification.

2.2. Chemicals

Analytical purity grade NaCl and $CuCl_2{\cdot}2H_2O$ were from Slavus Ltd, Bratislava, Slovakia. Methanol and ascorbic acid were from E. Merck KGaA, Darmstadt, Germany. Ethanol (~96%, v/v) and an aqueous solution of H_2O_2 (~30%) were purchased from Chemapol, Prague, Czech Republic. Spin-trapping agents, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Sigma Chemical Co., St. Louis, MO, USA) and 5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO; Radical Vision, Marseille, France), were stored under argon at −18 °C. DMPO was distilled before application. The MALDI matrix (2,5-dihydroxybenzoic acid, DHB), obtained from Fluka, was used as supplied. The water used for all solutions was of redistilled deionized quality grade.

2.3. Preparation of the working solutions

The stock hydrogen peroxide solution (8.82 M) was prepared by dissolving NaCl in commercial H_2O_2 to a salt concentration of 0.15 M. The stock $CuCl_2$ solution (16.0 μM) and that of ascorbic acid (16.0 mM) were prepared in 0.15 M NaCl. These solutions, freshly prepared each day, were appropriately diluted with 0.15 M NaCl. The actual concentrations of aqueous H_2O_2 solutions were determined by a spectrophotometric method.[8]

2.4. Degradation studies by rotational viscometry

For the degradation studies, 20.0 mg of the high-molar-mass HA sample (LIFECORE P9710-2) was dissolved in 0.15 M aq NaCl overnight in the dark at room temperature in two steps: First, 4.0 mL solvent was added in the morning, the next 4.0 mL portion of the solvent was added after 6 h. [A similar procedure was applied