Chemical Change Involved in the Oxidative Reductive Depolymerization of Hyaluronic Acid*

(Received for publication, October 12, 1989)

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The oxidative reductive depolymerization (ORD) of hyaluronic acid has been investigated. A solution of hyaluronate \((M = 4.07 \times 10^5)\) in phosphate buffer (pH 7.2) was incubated in the presence of \(\text{Fe}^{2+}\) for 24 h at 37 °C under an oxygen atmosphere to yield depolymerized hyaluronate (ORD fragments; an average \(M_r\) of 2,600). The ORD fragments contain 21 and 24% less hexosamine and uronic acid, respectively. The ORD reaction proceeds essentially by random destruction of unit monosaccharides due to oxygen-derived free radicals, followed by secondary hydrolytic cleavage of the resulting unstable glycosidic linkages.

In vitro, such substances as L-ascorbic acid, cysteine, and ferrous salt, in the absence or presence of hydrogen peroxide, have been shown to degrade hyaluronate, a glycosaminoglycan of high molecular weight which is responsible for the viscosity of synovial fluids (Greenwald and Moy, 1980; McNeil et al., 1985). Since the depolymerization by antioxidants takes place only when oxygen is present in the system, the phenomenon has been termed oxidative reductive depolymerization, or the ORD reaction (Pigman and Rizvi, 1959). Because of its immediate biological interest, hyaluronate has been the substrate most extensively studied with respect to this reaction (Sundblad and Balazs, 1966; Herp, 1980). It is generally accepted that free radicals, especially the highly reactive hydroxyl radical, may play an important role in the degradation process of hyaluronate (Herp, 1980). However, the precise mechanism of polysaccharide depolymerization has not been elucidated, and the reaction products have not been completely characterized. A major difficulty in the analysis of this reaction is the separation and characterization of newly formed end groups, which are generated only in minute amounts.

In this study, a procedure for obtaining highly depolymerized hyaluronate (ORD fragments; an average \(M_r\) of 2,600) has been established for the first time; and the ORD fragments were digested with chondroitinase AC-II to give the smallest fragments participating in the cleavage of hyaluronate, each of which was separated, characterized, and quantitatively evaluated. The results are reported herein.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

**Time Course of ORD Reaction of Hyaluronate** The depolymerization of hyaluronate on an analytical scale was conducted under the conditions described under "Experimental Procedures," and the time course showed a steady fragmentation of the polysaccharide chain (Fig. 1). After a 20-h reaction, the starting hyaluronate \((M_r 8.7 \times 10^5)\) was converted to hyaluronate fragments (ORD fragments) with \(M_r\) values ranging from 1,000 to 10,000, indicating that the polysaccharide underwent cleavage at about 300 positions in the chain. In this experiment, either exclusion of oxygen from the system (Pigman and Rizvi, 1959; Kennedy and Cho Tun, 1972) or addition of radical scavengers, such as D-mannitol, to the system (Greenwald and Moy, 1980; Wong et al., 1981) completely prevented the progress of the reaction. The accelerating effect of phosphate ions (Pigman and Rizvi, 1959) and the role of L-ascorbic acid (Harris et al., 1972; Wong et al., 1981) were also confirmed.

**Preparation of ORD Fragments** To obtain the ORD fragments \((M, 2600; \text{dodecasaccharide on an average})\) in high yield, hyaluronate \((M, 4.07 \times 10^5)\) with a total of 1.75 g was subjected to the ORD reaction as described under "Experimental Procedures." The ORD fragments remained unchanged in their \(N\)-acetyl content (10.9%), but contained 21 and 24% less hexosamine and uronic acid, respectively. The formation of the 4,5-unsaturated uronic acid component in...
Oxidative Reductive Depolymerization of Hyaluronate

The ORD reaction of hyaluronate has been reported by Kennedy and Cho Tun (1972). The ORD fragments obtained in this work were analyzed both by proton NMR and by the periodate-thiobarbiturate method (Weissbach and Hurwitz, 1959). The spectrum showed no sign of the existence of olefinic structure in the ORD fragments (Fig. 2), and no color reaction was indicated by the method (data not shown). The reason for the conflicting observations described above is not clear at present.

Digestion of ORD Fragments with Chondroitinase AC-II and Fractionation of Digestion Products—The ORD fragments are expected to be chemically modified at the reducing and/or nonreducing ends as previously described (Cleland et al., 1969; Kennedy and Cho Tun, 1972). The internal saccharide sequence of the fragments seems intact, as seen from the data of hexosamine and uronic acid determination. The observation that there was no olefinic linkage in the ORD fragments enabled us to use chondroitin sulfate lyases to depolymerize the fragments, resulting in the formation of the smallest oligosaccharides bearing modified terminal groups in addition to the unsaturated hyaluronate disaccharide (HAA2) formed from the internal sequence of the fragment. Hyaluronate is known to be the best substrate for chondroitinase AC-II among the glycosaminoglycans tested (Hiyama and Okada, 1975). In this study (data not shown), the ORD fragments were separately digested with chondroitinase AC-I, AC-II, or ABC; and each digestion product was analyzed by gel filtration. The results indicated that chondroitinase AC-II gave the highest yield of HAA2 as shown by the peak areas (\( A_{\text{peak}} \)) of the disaccharide fraction (see Fraction III in Fig. 3 for reference). The products, obtained by preparative digestion of the ORD fragments with chondroitinase AC-II, were fractionated on a Bio-Gel P-6 column into tri- to hexasaccharide fractions (Fraction I and II), a monosaccharide fraction (Fraction IV), and a disaccharide fraction (Fraction III) as shown in Fig. 3. We also observed that repeated digestion...
with chondroitinase AC-II converted most Fraction I to Fraction III material (data not shown). This finding suggests that Fraction I consisted mainly of higher homologs of Fraction III material. Fraction IV, which contained a single component positive to both the Morgan-Elson and Park-Johnson (1949) reactions, was identified as N-acetyl-d-glucosamine by TLC positive to both the Morgan-Elson and Park-Johnson (1949) reactions. Fraction I consisted mainly of higher homologs of Fraction I and II-2. An elution profile similar to that shown in Fig. 4 was obtained by monitoring with a differential refractometer as well as by manual uronic acid assays (data not shown). Analytical data of Fraction II-1 and II-2 materials and those of related oligosaccharide references are summarized in Table I. The FAB/MS spectrum of Fraction II-1 revealed a molecular ion peak at m/z 526 and a fragment ion peak at m/z 362 (FAB/MS spectrum not shown). The mass number of the latter was identical to that of the HA∆2 moiety, which may originate from oligosaccharide Fraction II-1 undergoing A-type cleavage as described by Dell (1987). These FAB/MS data were consistent with the carbon-13 NMR data of Fraction II-1, indicating the presence of an HA∆2 unit at the nonreducing end of Fraction II-1. On the other hand, the behavior of Fraction II-1 in anion-exchange HPLC and the difference in the M₆ values of Fraction II-1 and HA∆2 suggested the presence of an aldopentauronic acid residue (M₆ 147). The proton NMR spectrum of the methyl ester of Fraction II-1 showed an intensity of the methyl ester signal (δ 3.79) corresponding to about 2 mol/mol of olefinic signal (δ 6.18), indicating the presence of two carboxyl groups in Fraction II-1. Since it was difficult to characterize these substituents in the M₆ 147 fragment by carbon-13 NMR because of marked reduction in signal intensity (see Footnote b to Table II), the monosaccharide moieties of Fraction II-1 were reduced to the corresponding alditols as previously described (Taylor et al., 1976; Iinoue and Nagasawa, 1985) and analyzed by GLC after trimethylsilylation. The retention times for the two peaks obtained by GLC were identical to those of standard d-arabinitol (3.94 min) and 3,4-dideoxy-4-enehexitol (9.25 min). It is known that trimethylsilylated N-acetyl-d-glucosaminol derived from the N-acetyl-d-glucosamine unit in Fraction II-1 is not eluted from the GLC column under the conditions used. Accordingly, the structure of Fraction II-1 was established as 4,5-unsaturated GlcA(β1→3)GlcNAc(β1→9)-d-arabo-pentaerotic acid (Fig. 6a).

Oligosaccharide Fraction II was resolved into oligosaccharide Fractions II-1 and II-2. An elution profile similar to that shown in Fig. 4 was obtained by monitoring with a differential refractometer as well as by manual uronic acid assays (data not shown). Analytical data of Fraction II-1 and II-2 materials and those of related oligosaccharide references are summarized in Table I. The FAB/MS spectrum of Fraction II-1 revealed a molecular ion peak at m/z 526 and a fragment ion peak at m/z 362 (FAB/MS spectrum not shown). The mass number of the latter was identical to that of the HA∆2 moiety, which may originate from oligosaccharide Fraction II-1 undergoing A-type cleavage as described by Dell (1987). These FAB/MS data were consistent with the carbon-13 NMR data of Fraction II-1, indicating the presence of an aldopentauronic acid residue (M₆ 147). The proton NMR spectrum of the methyl ester of Fraction II-1 showed an intensity of the methyl ester signal (δ 3.79) corresponding to about 2 mol/mol of olefinic signal (δ 6.18), indicating the presence of two carboxyl groups in Fraction II-1. Since it was difficult to characterize these substituents in the M₆ 147 fragment by carbon-13 NMR because of marked reduction in signal intensity (see Footnote b to Table II), the monosaccharide moieties of Fraction II-1 were reduced to the corresponding alditols as previously described (Taylor et al., 1976; Iinoue and Nagasawa, 1985) and analyzed by GLC after trimethylsilylation. The retention times for the two peaks obtained by GLC were identical to those of standard d-arabinitol (3.94 min) and 3,4-dideoxy-4-enehexitol (9.25 min). It is known that trimethylsilylated N-acetyl-d-glucosaminol derived from the N-acetyl-d-glucosamine unit in Fraction II-1 is not eluted from the GLC column under the conditions used. Accordingly, the structure of Fraction II-1 was established as 4,5-unsaturated GlcA(β1→3)GlcNAc(β1→9)-d-arabo-pentaerotic acid (Fig. 6a).

Separation and Characterization of ORD Oligosaccharides in Fraction II—As shown in the elution diagram (A₃₆₅) of Fig. 4, ORD oligosaccharide Fraction II was resolved into oligosaccharide Fractions II-1 and II-2. An elution profile similar to that shown in Fig. 4 was obtained by monitoring with a differential refractometer as well as by manual uronic acid assays (data not shown). Analytical data of Fraction II-1 and II-2 materials and those of related oligosaccharide references are summarized in Table I. The FAB/MS spectrum of Fraction II-1 revealed a molecular ion peak at m/z 526 and a fragment ion peak at m/z 362 (FAB/MS spectrum not shown). The mass number of the latter was identical to that of the HA∆2 moiety, which may originate from oligosaccharide Fraction II-1 undergoing A-type cleavage as described by Dell (1987). These FAB/MS data were consistent with the carbon-13 NMR data of Fraction II-1, indicating the presence of an aldopentauronic acid residue (M₆ 147). The proton NMR spectrum of the methyl ester of Fraction II-1 showed an intensity of the methyl ester signal (δ 3.79) corresponding to about 2 mol/mol of olefinic signal (δ 6.18), indicating the presence of two carboxyl groups in Fraction II-1. Since it was difficult to characterize these substituents in the M₆ 147 fragment by carbon-13 NMR because of marked reduction in signal intensity (see Footnote b to Table II), the monosaccharide moieties of Fraction II-1 were reduced to the corresponding alditols as previously described (Taylor et al., 1976; Iinoue and Nagasawa, 1985) and analyzed by GLC after trimethylsilylation. The retention times for the two peaks obtained by GLC were identical to those of standard d-arabinitol (3.94 min) and 3,4-dideoxy-4-enehexitol (9.25 min). It is known that trimethylsilylated N-acetyl-d-glucosaminol derived from the N-acetyl-d-glucosamine unit in Fraction II-1 is not eluted from the GLC column under the conditions used. Accordingly, the structure of Fraction II-1 was established as 4,5-unsaturated GlcA(β1→3)GlcNAc(β1→9)-d-arabo-pentaerotic acid (Fig. 6a).

Separation and Characterization of ORD Oligosaccharides in Fraction III—It was suggested by paper electrophoresis that Fraction III contained a neutral component besides a large amount of HA∆2 originating from the internal sequence of the ORD fragments. On the other hand, it was noted that the ratio of the peak area of A₃₆₅ to that of A₅₂₀ in the elution diagram of Fraction III (Fig. 3) was approximately twice that of HA∆2 itself, indicating the presence of some oligosaccharide(s) containing saturated uronic acid residue(s). To separate these minor constituents, Fraction III was subjected to merciric acetate treatment (Ludwigs et al., 1987), which is known to cleave HA∆2 into N-acetyl-d-glucosamine and 4-deoxy-5-keto-1-threo-hexuronic acid units. The unreacted minor constituents were recovered as an oligosaccharide fraction (Fraction III-1 in Fig. 5), which was shown to contain acidic (Fraction III-1A) and neutral (Fraction III-1N) components by paper electrophoresis. Each component was isolated by

Fig. 4. Separation of ORD oligosaccharide Fraction II into Fractions II-1 and II-2 by anion-exchange HPLC. A solution of the ORD oligosaccharide (Fraction II in Fig. 3) in water was applied to a SAX-4201-N column, and the column was eluted with 0.15 M CH₃COONH₄ (pH 5.0). The eluent was monitored by both a UV detector and a differential refractometer and assayed manually for uronic acid. The arrows indicate the elution positions of unsaturated hyaluronate di- to hexa saccharide standards and of Ag₂O-oxidized HA∆2. For additional information, see "Experimental Procedures" and the legend to Table I.
preparative ion-exchange chromatography, and Fraction III-1N material was identified as N-acetylhyalobiuronic acid by TLC and paper electrophoresis. The development of Fraction III-1N material on a TLC plate for 2 h at 33°C gave a minor spot near the position of HA2Δ2 in addition to major spots of N-acetyl-d-glucosamine and of the chromogen which presumably resulted from the Morgan-Elson reaction of Fraction III-1N. However, the development of the same material on a filter paper for 15 h at 23°C gave only the spots of N-acetyl-d-glucosaminide and of the chromogen (data not shown). The FAB/MS spectrum of Fraction III-1N did not give the molecular ion peak like Fraction III-1N as observed in the case of extremely unstable materials. Nevertheless, two distinct fragment ion peaks at m/z 204 and 126 were obtained. The former was identical to N-acetyl-d-glucosamine, which probably came from the reducing N-acetyl-d-glucosamine residue in Fraction III-1N; and the latter was assumed to be due to a 3- or 4-carbon fragment of Fraction III-1N (spectral data not shown). Fraction III-1N material was hydrolyzed, and the product was reduced with NaBH₄ to give the corresponding alditols. These were analyzed by GLC after trimethylsilylation as the Me₃Si derivatives of glycerol, meso-erythritol, and d-arabinitol (retention times of 1.59, 3.40, and 9.15 min, respectively). The retention time (3.18 min) of the only peak obtained was close to that of the Me₃Si derivative of a tetritol, meso-erythritol. It was suggested that the carbon skeleton of the tetritol may originate from C(1)-(C(4)) of the D-glucuronic acid residue bonded to the 3-HO group of the reducing N-acetyl-d-glucosamine residue in Fraction III-1N via glycosidic linkage. The original form of the C-4 carbinol group in the assumed tetritol seems to be an aldehyde group because it is known that the most prominent fragment in the oxidation of d-glucose induced by a hydroxyl radical is l-threo-tetritaldose (see Structure 1) (Schuchmann and von Sonntag, 1977). Consequently, the structure of Fraction III-1N was tentatively assigned as l-threo-tetritaldoxy-(1→3)GlcNAc (Fig. 6). The ratio of the Fraction III-1N residue at the nonreducing ends to the total nonreducing ends of the ORD fragments was calculated to be 8% from the yield of Fraction III-1N and Mₜ of the ORD fragments. Since Fraction III-1N was extremely unstable, it was suggested that most of the nonreducing N-acetyl-d-glucosamine ends of the fragments, which amounted to 45% of the total nonreducing ends as described below, may be formed by secondary hydrolytic cleavage of the Fraction III-1N-type residues located at the nonreducing ends.

**Determinations of N-Acetyl-d-Glucosamine and d-Glucuronic Acid Residues at Reducing or Nonreducing Ends of ORD Fragments**—The nonreducing N-acetyl-d-glucosamine ends were released by digestion of the ORD fragments with chondroitinase AC-II (Fraction IV in Fig. 3). The ratio of the nonreducing N-acetyl-d-glucosamine ends to the total nonreducing ends was calculated to be 45% from the yield of the N-acetyl-d-glucosamine residue and Mₜ of the fragments. The amount of the nonreducing N-acetyl-d-glucosamine residues was also determined by digestion with exo-N-acetyl-β-d-glucosaminidase. It was found that the content of the N-acetyl-d-glucosamine residue was nearly identical to that obtained by chondroitinase AC-II digestion. On the other hand, the ratio of the N-acetylhyalobiuronic acid residues (Fraction III-1A) at the nonreducing ends to the total nonreducing ends of the ORD fragments was calculated to be 20% from the yield of Fraction III-1A and Mₜ. This content agreed approximately with that of the nonreducing d-glucuronic acid ends (22%), which was calculated from the yield of the d-glucuronic acid residue released by digestion with exo-β-d-glucuronidase. The presence of the component monosaccharides of hyaluronic acid at the terminal position of the fragments strongly suggested, as discussed above, that the original ORD fragments (primary product in the ORD reaction of hyaluronate) would undergo secondary decomposition (probably by hydrolysis) of some unstable glycosidic substituents in the fragments during the ORD reaction and the following fractionation process. This would result in the formation of the nonreducing N-acetyl-d-glucosamine (Fraction IV) and N-acetylhyalobiuronic acid (Fraction III-1A) ends.

A similar discussion as above may be applied to the reducing terminal position of the ORD fragments. The NaBH₄-reduced ORD fragments were digested with chondroitinase AC-II, and the products were fractionated by gel filtration to give an elution diagram nearly identical to that of Fig. 3 except with a slightly higher proportion of Fractions I plus II (elution diagram not shown). Since the reducing N-acetyl-d-glucosamine end is reduced with NaBH₄, to give the corresponding alditol, chondroitinase AC-II digestion of the NaBHI-reduced Fraction III was difficult (see “Experimental Procedures”), the fraction was subjected to alkaline treatment prior to analysis, as described below. Upon heating in 0.15 M Na₂CO₃ for 6 h at 40°C (Inoue and Nagasawa, 1985), HA2Δ2 reduced Fraction III was difficult (see “Experimental Procedures”), the fraction was subjected to alkaline treatment prior to analysis, as described below. Upon heating in 0.15 M Na₂CO₃ for 6 h at 40°C (Inoue and Nagasawa, 1985), HA2Δ2 decomposed to give the chromogen of the Morgan-Elson reaction and 4-deoxy-5-keto-l-threo-hexuronic acid, whereas reduced HA2Δ2 remained unchanged. Reduced HA2Δ2 in the fraction was quantitatively determined by anion-exchange HPLC. The ratio of the reducing N-acetyl-d-glucosamine ends to the total reducing ends of the ORD fragments was calculated to be 51%, which roughly corresponded to the sum of both ratios of the N-acetyl-d-glucosamine (Fraction IV) and 3-O-substituted N-acetyl-d-glucosaminide (Fraction III-1N) residues at the nonreducing ends of the fragments (45 and 8%, respectively; Fig. 6).

We have similarly examined the presence of the reducing d-glucuronic acid ends in the ORD fragments. If the d-glucuronic acid residues located at the reducing ends of the fragments, NaBH₄-reduced ORD oligosaccharide Fraction II, which corresponds to ORD oligosaccharide Fraction II in Fig. 3, should contain NaBH₄-reduced HA2Δ2-(1→3)-l-gulonic acid in addition to NaBH₄-reduced HA2Δ2-(1→3)-d-lyxonic acid (the reduced product of Fraction II-1) and 4,5-unsaturated 3-deoxy-GlcA-(1→3)-N-acetyl-d-glucosaminide (the reduced product of Fraction II-2) as components. The carboxyl groups in these partially reduced oligosaccharides were converted to the corresponding carboxil groups. The fully reduced oligosaccharides were hydrolyzed, and the products were treated with Dowex 1 (H⁺ form) anion-exchange resin according to the procedure described previously (Yamaguchi et al., 1976) to separate an alditol fraction, which was analyzed by GLC after trimethylsilylation. The retention time (3.94 min) of the only peak observed was not identical

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\[ \text{NaBH}_4 \text{-reduced HA2Δ2}, \text{4,5-ununsaturated 3-deoxy-GlcA(1→3)-GlcNAc} \] 
\[ \text{Linker et al., 1956}. \]
FIG. 6. Sequences at reducing and nonreducing ends of ORD fragments formed by ORD reaction of hyaluronic acid and structures of isolated ORD oligosaccharides. a, Fraction II-1; b, Fraction II-2; c, Fraction III-1N. The arrows indicate the sites cleaved by chondroitinase AC-II digestion. The percentages in parentheses indicate the molar proportions of each ORD fragment having the indicated structure at the reducing or nonreducing ends to the total reducing or nonreducing ends. For additional information, see the text.

Oxidative Reductive Depolymerization of Hyaluronate

Reducing terminal residue

COOH

GlcNAc-GlcA-GlcNAc-0

Fr. II-1 (24%)

Fr. IV (45%)

Non-reducing terminal residue

Fr. III-1A (20%)

Fr. III-1N (8%)

Reducing GlcNAc (51%)

(a)

(b)

(c)

REFERENCES


1985) that oxygen-derived free radicals may play an important role in the degradation process of hyaluronate. The information on the reaction mechanism and products of the ORD reaction of hyaluronate obtained by this study will contribute to the investigation of the chemical change of hyaluronate molecules in living bodies.

The uronic acid and hexosamine content in the ORD fragments (24 and 21%, respectively), which unexpectedly coincided with the proportions obtained for Fractions II-1 and II-2 (see Fig. 6), the degree of degradation of the D-glucuronic acid unit in hyaluronate was suggested to be much higher than that of the N-acetyl-D-glucosamine unit, as discussed above. On the other hand, the only N-acetyl-D-glucosaminic acid was isolated as a chemically modified N-acetyl-D-glucosamine unit, in agreement with the data of N-acetyl content. In this study, the D-arabo-penta-uronic acid (as Fraction II-1) and L-threo-tetrodialdose (as Fraction III-1N) residues were confirmed as chemically modified D-glucuronic acid units. Nevertheless, the existence of other types of residues originating from the D-glucuronic acid unit is also likely, as suggested by the presence of abundant reducing and non-reducing N-acetyl-D-glucosamine ends.

It has been shown (Balazs et al., 1967) that in synovial fluid of inflamed joints, the hyaluronate concentration is reduced and the distribution of the Mₐ of hyaluronate is also shifted to lower values. It has been reported (McCord, 1974; Greenwald and Hoy, 1980; Del Maestro et al., 1980; McNeil et al.,


Proton and carbon 13 NMR spectra were recorded with a Varian XL-400 spectrometer using samples pre-equilibrated with D2O at a concentration of 5-12 mmol/l in D2O at 27°C. Chemical shifts were referenced to deuterodimethyl sulfoxide (1.37 in D2O for proton NMR and 64.1 in D2O for carbon 13 NMR) as an internal standard.

DSS-400 spectra were recorded with a JEOL DRX-300 spectrometer equipped with a JNM-ALX-300 MHz spectrometer. The samples (1.0 mmol/dl) were dissolved in water (<0.5 ml) and aliquots of these solutions containing 50-100 mM sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 were added and attached to a direct-insertion probe. The probe was then inserted into the inner bore of the NMR tube filled with deuterium oxide (99.8%). The sample volume was 6 ml and references were used for measuring proton chemical shifts: water, 5.28 ppm; DSS (dioxane-3, 2.49 ppm) and N,N-Dimethylformamide (95.45 ppm).

Chemical shift values are given in ppm downfield from the DSS reference.

Characterization of OXG Glycopeptides [in Fig. 1. (3)]

Separation by Anion-Exchange Chromatography

A solution of OXG glycopeptides (1.5 mg) was applied to a C18 high performance liquid chromatography (HPLC) column (Hitachi, Japan) and was washed with 0.1 M sodium acetate buffer (pH 4.5). The elution was monitored by monitoring the absorbance at 214 nm. The column effluent was eluted with a linear gradient of methanol (0-80%) in water over 10 min. The fractions were monitored at 214 nm. The absorbance was measured at 214 nm. The samples were lyophilized and used for further analysis.

Characterization of OXG Glycopeptides

The OXG glycopeptides were characterized by mass spectrometry (MS), NMR spectroscopy, and HPLC analysis. The MS analysis was performed on a Q-TOF mass spectrometer (Micromass, Wincheser, UK). The NMR spectra were recorded on a Varian Inova 400 MHz spectrometer (Varian, Palo Alto, CA) in D2O. The HPLC analysis was carried out on a Shimadzu LC-10ATVP system (Shimadzu, Japan) using a C18 reversed-phase column. The elution was monitored at 214 nm.
Oxidative Reductive Depolymerization of Hyaluronate

Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>13C NRR values</th>
<th>Retention time (min)</th>
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<tr>
<td>OHD</td>
<td>0.31</td>
<td>19.0</td>
<td>525</td>
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<tr>
<td>HAM</td>
<td>0.32</td>
<td>26.7</td>
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<tr>
<td>H.4A6</td>
<td>0.20</td>
<td>33.6</td>
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<tr>
<td>A$-oxidized</td>
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Table II

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<td>HAM</td>
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<td>33.6</td>
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Carbon 13 NRR data for synthetic N-acetyl-D-glucosaminic acid and 4,5-unsaturated D-glucuronic acid as well as 4,5-unsaturated D-glucuronic acid

Hyaluronate

Table I

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Carbon 13 NRR data for synthetic N-acetyl-D-glucosaminic acid and 4,5-unsaturated D-glucuronic acid as well as 4,5-unsaturated D-glucuronic acid
Chemical change involved in the oxidative reductive depolymerization of hyaluronic acid.
H Uchiyama, Y Dobashi, K Ohkouchi and K Nagasawa


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