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Note

Carboxyl-reduced hyaluronan: preparation and enzymatic assay

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Hyaluronan is a linear polysaccharide having a disaccharide repeating unit [(1 → 3)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-β-D-glucopyranosyluronic acid] (Scheme 1). This natural glycosaminoglycan is widely distributed in such animal tissues as synovial fluid, vitreous body of the eye, connective and skeletal tissue, umbilical cord, and dermis [1]. Hyaluronan is also found as an extracellular product of many bacteria [2].

At physiological pH, due to its conformational features and polyanionic character, hyaluronan in aqueous systems exhibits considerable stiffness and consequently large hydrodynamic volume [3]. The combination of such characteristics enhances chain–chain interaction and hydrogen-bonding networks and assigns peculiar viscoelastic properties to solutions of high-molecular-weight hyaluronan [4].

Considering these features of hyaluronan, chemical modification of the monomer structure is a powerful perturbative approach both for obtaining new derivatives with applicative potential and to throw light on the conformational and structural behaviour of the biopolymer. Hyaluronan has been subjected to many chemical modifications, most of which involve crosslinking and coupling [5–7]. *N*-Deacetylation of hyaluronan by hydrazinolysis causes severe reduction of molecular weight [8]. Esterification of carboxylic groups by means of various organic halides was accomplished without degradation of the hyaluronan chain [9].

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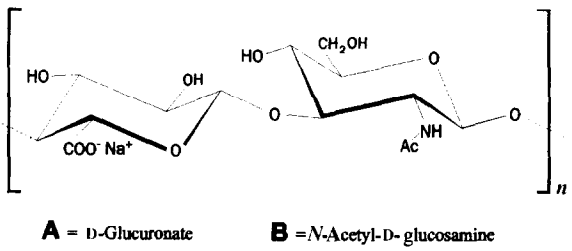

Scheme 1. The repeating unit of hyaluronan.

We report here on a simple chemical modification of hyaluronan, performed in aqueous medium, under mild conditions and without apparent reduction of molecular weight. The reaction involves reduction of the carboxylic functions to primary alcoholic functions by the method of Taylor and Conrad [10]. This method is widely used in analytical determination of the primary structure of glycuronans, in order to eliminate the unusual stability to acid hydrolysis of glycosidic bonds involving glycuronate residues. The procedure is also standard in the linkage-sequence determination of acidic sugars by GC–MS analysis of partially methylated alditol acetates [11]. An application of carbodiimides with hyaluronan has already been described in the literature, dealing with the coupling of this polysaccharide with several primary amines [12].

1. Experimental

Materials and apparatus.—Sodium hyaluronate, biotechnological grade, was kindly provided by FAB, Fidia Advanced Biopolymers, S.r.l., Abano Terme, Italy. The molecular weight of the sample, determined by GPC, was 150 Kd. Biogel P10 (200–400 mesh) was obtained from Bio-Rad Laboratories (Richmond, CA). Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) was purchased from Sigma Chemical Co. (St. Louis, MO). The pH-stat apparatus was assembled with the following Radiometer units: PHM82, TTT80, ABU80, REA270, REC80 (Radiometer Analytical A/S, Copenhagen, Denmark). Diafiltration was performed with an ultrafiltration cell equipped with a Diaflo membrane YM100 (nominal exclusion limit: 100 Kd) (W.R. Grace Amicon Division, Danvers, MA). A differential refractometer (model R-401, Waters Associates, Milford, MA) was employed to monitor the gel-chromatography effluent. Analysis of monosaccharide composition of reduced hyaluronan was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) on a Dionex DX-300 apparatus fitted with a Carbopak PA-1 anion-exchange column (Dionex, Sunnyvale, CA).

Reduction.—The chemical reduction of hyaluronan was obtained by means of water-soluble carbodiimide EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] (E. Merck, Darmstadt, Germany) as described by Taylor and Conrad [10]. Variable amounts of solid EDC ranging from 2.1 to 2.8 mol were added to 100 mL of aq soln containing 760 mg of sodium hyaluronate (1.9 meq). The pH was maintained at 4.75 by a pH-stat

in order to minimize the rearrangement of the active ester to the *N*-acylurea derivative. When lactonization was complete (~ 2 h), 50 mL of 1.5 M cold $NaBH_4$ was slowly added using a peristaltic pump (36 mL/h). Reduced hyaluronan was purified by diafiltration against 7 vol of bidistilled water, frozen and lyophilized.

Hydrolysis.—The extent of reduction of each sample was determined by analysis of component monosaccharides. Samples of reduced hyaluronan (~ 5 mg) were hydrolyzed by 2 M CF_3CO_2H (2 mL) overnight at 105 °C; these conditions have been verified to optimize stability and recovery of the constituent monosaccharides. CF_3CO_2H was evaporated under vacuum. Dry samples were solubilized in deionized water (2 mL) and filtered on a 0.45-μm Millex unit (Millipore Co., Bedford, MA).

HPAE chromatography.—Quantitative monosaccharide analysis was performed by HPAE-PAD as described by Townsend et al. [13]. The eluents used were: (A) 100 mM NaOH, (B) 1 M CH_3CO_2Na in 100 mM NaOH, (C) deionized water, at a flow rate of 1.0 mL/min, combined in a multiple gradient constructed as follows: 15 mM NaOH isocratically for 26 min, a linear gradient of NaOH to 100 mM over 20 min, a linear gradient of CH_3CO_2Na to 1 M over 40 min, and a return to the initial condition (15 mM NaOH) during 10 min. PAD electrode potentials were set as $E1 = 0.1$ V, $E2 = 0.6$ V, $E3 = -0.6$ V with applied durations of 490, 90, 60 ms, respectively.

Enzymatic digestions.—Unmodified and reduced hyaluronan preparations were subjected to hyaluronidase action by a procedure similar to that described by Cowman et al. [14].

Gel chromatography.—The lyophilized sample digest (~ 80 mg) was solubilized in 1 mL of 0.25 M ammonium acetate buffer, pH 7.0, and filtered on a 0.45-μm Millex unit. A sample solution (250 μL) was applied to a 142 × 1.8 cm column of Biogel P-10, equilibrated in 0.25 M NH_4OAc buffer, pH 7.0. The column effluent, at a flow rate of 10 mL/h, was detected by means of a differential refractometer.

2. Results and discussion

Reduction and characterization.—Our approach to the controlled reduction of carboxylate groups started with the observation of Hoffman et al. [15], according to which the biological activity of some chemically modified glycuronans was strictly related to their content of carboxylate groups. Compared to other glycuronans, hyaluronan proved quite difficult to reduce. This resistance is believed to be a consequence of the hydrogen bonds involving carboxylate groups, since carboxylate and acetamido groups are quite close to each other. The same reasons have been alleged to explain the unusual resistance of hyaluronan to periodate oxidation [16]. The reduction process occurs through two consecutive reactions: lactonization between carboxyl and hydroxyl groups, and subsequent reduction of the resulting ester groups.

The extent of lactonization can be evaluated by measuring the proton uptake and the dissociation degree of the polymer under the experimental conditions.

Using increasing amounts of EDC, we observed a trend in proton uptake as shown in Fig. 1. The four samples of reduced hyaluronan (HR2, HR3, HR4, HR5) exhibited a degree of reduction (DR) in good agreement with the proton uptake when the

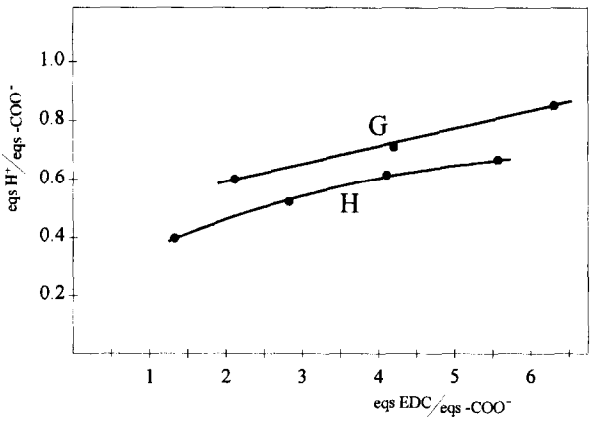

Fig. 1. H^+ uptake during lactonization of hyaluronan (H) and gellan (G) at pH 4.75.

eqs EDC/eqs-COO^- (eqs = equivalents) ratio is kept below 2 (samples HR2 and HR3), whereas, as shown in Table 1, the proton uptake and DR diverge above that ratio (samples HR4 and HR5).

For comparative purposes, it may be worth reporting that, under similar experimental conditions, we prepared three reduced samples of gellan, a glycuronan whose carboxylate groups are also shielded by hydrogen bonds [17], although considerably less so than hyaluronan. Fig. 1 reports the resulting proton uptake data. Surprisingly enough, as shown in Table 1, in gellan samples (GR1, GR2, GR3) we observed a DR exceeding the proton uptake, which may be related to the higher apparent pK_a of the gellan.

The recovery of reduced hyaluronan was almost complete and the product loss in the diafiltered solution, as tested by hexosamine colorimetric assay, proved negligible.

Enzymatic assay.—As an integral part of this work we investigated whether a partially reduced hyaluronan sample (HR3) was a substrate still recognized by hyaluronidase (hyaluronate 4-glycanohydrolase). Bovine testicular hyaluronidase acts as an endoglycanohydrolase, cleaving hyaluronan at the β-(1 → 4) linkage, leaving as end products a tetrasaccharide $(AB)_2$ and hexasaccharide $(AB)_3$ with traces of octasaccharide $(AB)_4$, all with *N*-acetylglucosamine moiety as terminal reducing end.

Fig. 2 shows the separation of an exhaustive digest of original hyaluronan as reported

Table 1
EDC amounts, H^+ uptake and degree of reduction (DR) of hyaluronan samples (HR*x*) and gellan samples (GR*x*) [a]

	HR2	HR3	HR4	HR5	GR1	GR2	GR3
eqs EDC/eqs -COO^-	1.1	2.2	3.3	4.4	2.1	4.2	6.3
eqs H^+/eqs -COO^-	0.41	0.53	0.62	0.67	0.60	0.70	0.82
DR	0.42	0.52	0.54	0.56	0.75	0.87	0.95

[a] eqs = equivalents.

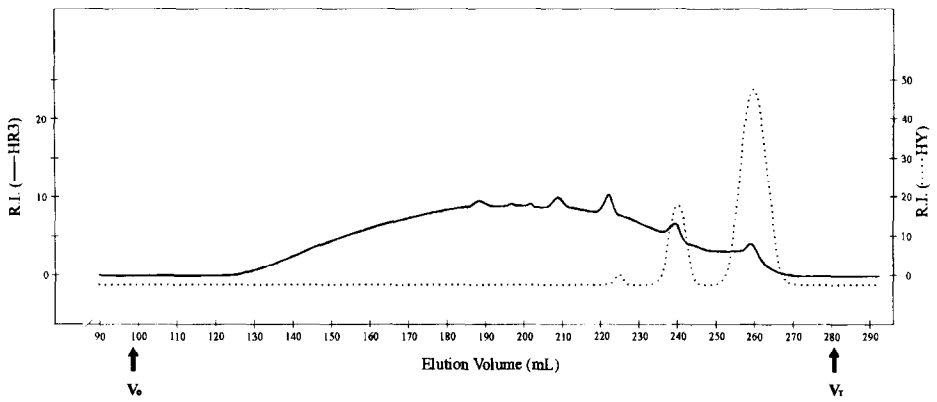

Fig. 2. Differential refractive index (% shift) of exhaustive digest gel filtration of partially reduced hyaluronan (— HR3) by bovine testicular hyaluronidase, compared with exhaustive digest of original hyaluronan (··· HY).

by Cowman et al. [18]; in the same figure appears the elution profile derived from the sample HR3 (DR 0.52) after digestion with hyaluronidase. A clear breakdown of molecular weight of the partially reduced hyaluronan occurs after exposure to hyaluronidase, but the resulting fragments are not further recognized by the enzyme after losing carboxylate groups. These fragments have molecular weights between 20 and 1.5 Kd, as suggested by the gel-filtration resin employed and the calibration performed with dextran standards. The elution profile suggests that the reduction involves consecutive sites of variable length, separated from each other by segments where the carboxylate groups have been somehow protected. The analysis of oligosaccharides derived from the exhaustive digestion of hyaluronan shows clearly that the smallest chain portion recognized by hyaluronidase cannot be smaller than eight monosaccharidic units [19]. Therefore the significant presence of the peaks $(AB)_2$ and $(AB)_3$ in the reduced hyaluronan digest implies that the length of any chain portion untouched by the reduction must exceed eight monosaccharide units.

In general, the overall behaviour of reduced hyaluronan against hyaluronidase is quite similar to the one described for partially esterified hyaluronan derivatives [20].

The question as to whether the reaction provides a statistical reduction or follows some rules (giving alternate or block modification) can be solved only by detailed spectroscopic studies combined with statistics. As a preliminary discussion, a DR of 0.52 homogeneously distributed along the chain would leave only few sites for the enzyme to work on, and thus the chain would maintain its initial length after digestion. The preponderant presence of shorter fragments proves that the reduction process unhomogeneously spared many blocks. This suggest a far-from-random mechanism, which can be reasonably ascribed to stable entanglements and the chain–chain interactions that characterize hyaluronan in aqueous solutions.

Chemical and physico–chemical characterization of reduced hyaluronan as well as its ester derivatives [21] is in progress.

Acknowledgements

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