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Note

Separation of hyaluronan oligosaccharides by the use of anion-exchange HPLC

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There has been a recent surge of interest in hyaluronan (HA), a glycosaminoglycan. Hyaluronan is a linear polymer composed of the repeating disaccharide unit [(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-*O*-β-D-glucopyranuronosyl]. Originally thought to serve only a structural function in connective tissue, HA has now been found to play important roles in such diverse functions as cell–cell recognition[1], limb, lung, and brain morphogenesis[1,2] and as a potential regulator of tumor angiogenesis[3]. It has also found biomedical applications in ophthalmic surgery and wound healing[4]. In the course of our NMR investigations of the solution conformation of hyaluronan as a function of chain length, we have developed a rapid, simple method for the preparation and analysis of monodisperse hyaluronan oligosaccharides. With this method we are able to separate HA oligomers of between 2 and 20 disaccharide units at a moderate pH so that chemical modification of the HA is unnecessary.

Several methods for the separation of hyaluronan oligosaccharides already exist. Preparation of HA fragments has traditionally been accomplished by gel-permeation chromatography coupled with uronic acid analysis for peak identification[5,6]. Although this method is capable of separating HA oligomers up to 23 disaccharide units, it is tedious and time consuming, especially when used to prepare relatively large (> 10 mg) quantities of each fragment. HPLC methods including normal-phase[7], reversed-phase ion-pair[8], and weak-anion exchange[9] have also been used for the analysis and separation of HA oligosaccharides of varying length. Similar methods[10–16] have been used to separate HA oligosaccharides from other glycosaminoglycans. However, the largest fragment that any of the published HPLC methods has been able to separate is six disaccharide units[8]. The development of a gel electrophoresis method allowed the analysis of oligosaccharides between 7 and 250 repeating disaccharide units[17,18]. A capillary zone electrophoresis method has

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also been published which is capable of separating oligomers up to seven disaccharide units[19].

Recently, neutral and acidic mono- and oligo-saccharides have been separated by high-performance anion-exchange chromatography (HPAEC) with pellicular anion-exchange resin[20,21]. We have used this stationary phase to separate HA oligosaccharides produced by enzymatic digestion. Oligomers of up to 20 repeating disaccharide units have been separated. With UV detection at 230 nm, we found this method to be suitable on both an analytical and a semi-preparative scale.

EXPERIMENTAL

Materials.—Purified high molecular weight (1.0–1.8 MDa) sodium hyaluronate from *Streptococcus zooepidemicus* was purchased as Hyalumed from Genzyme Corporation (Boston, MA). Bovine testicular hyaluronidase was purchased from Worthington Biochemicals (Freehold, NJ). For the HPLC mobile phases, mono and dibasic sodium phosphate (99.95%) and sodium acetate (99.999%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Bio-Gel P-30 and P-2 polyacrylamide beads were purchased from Bio-Rad Laboratories (Richmond, CA). All water used was filtered through a Millipore Milli-Q water system (Waters Associates, Milford, MA). All other chemicals were reagent grade.

Enzymatic digestion of HA.—The digestion procedure was similar to that described by Cowman et al.[6]. First, 40 mg of high molecular weight HA were dissolved in 10 mL of 100 mM sodium acetate buffer, pH 5.0. After incubation at 37°C for 30 min, 3000 U of hyaluronidase in 1.2 mL of the same buffer were added. After 30 min, half of the solution was removed, and the digestion was stopped by boiling the solution for 5 min. The remaining digestion was stopped after 90 min. The longer digestion time was used to produce a larger proportion of the shorter fragments. The digested HA was filtered through a 0.45-μm filter to remove precipitated protein. These solutions were injected directly onto the ion-exchange column.

The digestion procedure for the semi-preparative work was essentially the same with the exception that the concentration of the sodium acetate buffer was 10 mM in order to keep the final salt concentration of the sample similar to the initial salt concentration of the gradient. The digestion was allowed to proceed for 3 h. After the digested HA was filtered, it was lyophilized to dryness and redissolved in Milli-Q water to a final HA concentration of 28.5 mg/mL.

Preparation of standards.—Standards were prepared by the digestion of 40 mg of high molecular weight HA for 4 h in 100 mM sodium acetate as described above. The HA oligomers were then separated on a 2.6 × 200-cm column that had been packed with Bio-Gel P-30 (−400 mesh) polyacrylamide beads. The procedure was similar to that described by Cowman et al.[6] except that 0.1 M potassium phosphate (pH 7.0) was used as the mobile phase. Fractions were collected and analyzed for uronic acid content by the method of Bitter and Muir[22]. The center fractions from each peak were then pooled and lyophilized. The oligomers were

redissolved in 20 mL of Milli-Q water and desalted on a 1.5 × 30-cm Bio-Gel P-2 column. The length of each oligosaccharide was then verified by examination of the NMR spectrum of the amide protons[23].

HPLC conditions.—A Dionex 4 mm × 250-mm CarboPac PA100 column was used with two Shimadzu LC-6A pumps and a Shimadzu SCL-6A system controller. A Shimadzu SIL-6A automatic injector was used. Detection was accomplished with a Shimadzu SPD-6AV UV-vis spectrophotometric detector operating at 230 nm.

For separation by the application of the phosphate gradient, mobile phase A was Milli-Q water. Mobile phase *B* was a 1.0 M solution of sodium phosphate, pH 6.3. Samples were eluted by a gradient which consisted of 5% *B* for the first 5 min, then from 5 to 35% *B* over the next 50 min. For separation with the acetate gradient, mobile phase *A* was also Milli-Q water. Mobile phase *B* was 1.0 M sodium acetate, pH 5.0. The gradient consisted of 10% *B* for the first 5 min, then from 10 to 80% *B* over the next 40 min. The flow rate was 1.0 mL/min.

DISCUSSION

Separation of neutral carbohydrates with a pellicular anion-exchange resin is usually accomplished at high pH so that the ring hydroxyl groups are deprotonated to the oxyanion, which interacts with the resin. Hyaluronan, however, is known to be unstable under alkaline conditions[24]. Therefore, the separation was done at pH 6.3 and 5.0 by making use of the carboxylate group, which has pK 3.45 (ref. 25).

Fig. 1 shows the separation of the 90-min digestion using the linear phosphate gradient described in the Methods section. Bovine testicular hyaluronidase is an endoglycanohydrolase which cleaves hyaluronan at the β-(1 → 4) linkage, leaving a homologous series of even-numbered oligosaccharides with glucuronic acid at the terminal nonreducing end. The end product of digestion is the tetrasaccharide (HA2), which eluted slightly after the solvent peak and was not fully resolved from the hexamer (HA3). The largest oligomer separated was one of 20 disaccharide units.

Standards were used to verify the retention times of the HA oligomers. Fig. 2 shows the HA standards of 3, 4, and 5 repeating units. Their retention times correspond to the retention times of the digestion peaks labelled HA3, HA4, and HA5 in Fig. 1. The cause of skewing observed in Fig. 2c is unknown. It is not caused by overloading of the column, or by a contaminant detectable by NMR.

Better separation of the short oligomers was achieved with the sodium acetate gradient. Fig. 3 shows the separation of the 30-min digestion. Due to strong background absorption from the acetate, UV detection could not used. Therefore, fractions were collected every 30 s and tested for uronic acid content by the method of Bitter and Muir[22]. Again, standards were used to verify retention times of the oligomers.

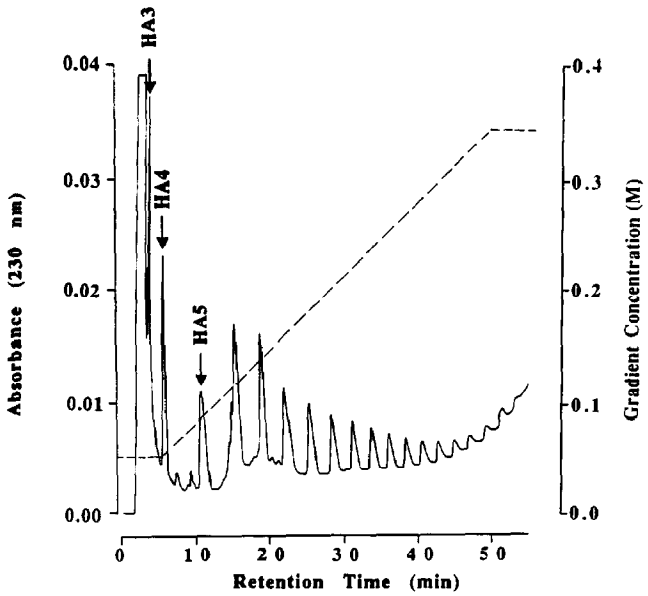

Fig. 1. Separation of HA oligomers up to 20 repeating disaccharide units (HA20) on a PA100 column with a pH 6.3 phosphate buffer gradient. Peaks indicated were tested with standards (see Fig. 2); 0.8 mg of the 90-min digestion was loaded onto the column.

The detection limit of the phosphate gradient separation was tested with the HA4, and was found to be 1 μg. This limit is comparable to other methods that detect HA by UV[11,14,15]. The detection limit of the acetate gradient separation is limited by the sensitivity of the uronic acid assay, which is reported to be 1μg/mL uronic acid[22].

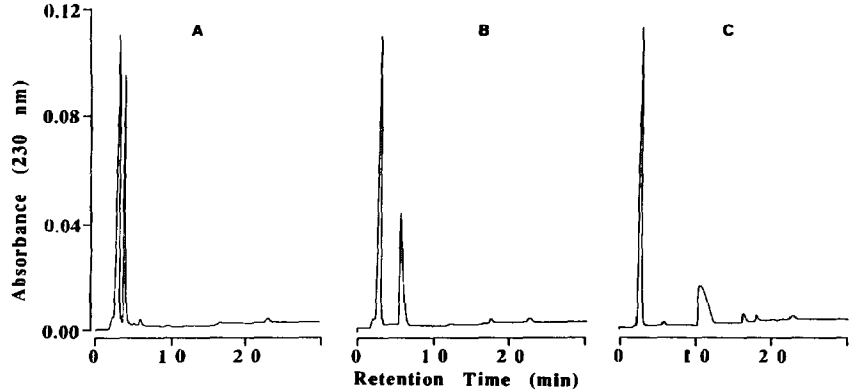

Fig. 2. Separation of HA standards using the same conditions as in Fig. 1. (A) 0.2 mg of HA3, (B) 0.2 mg HA4, and (C) 0.2 mg HA5.

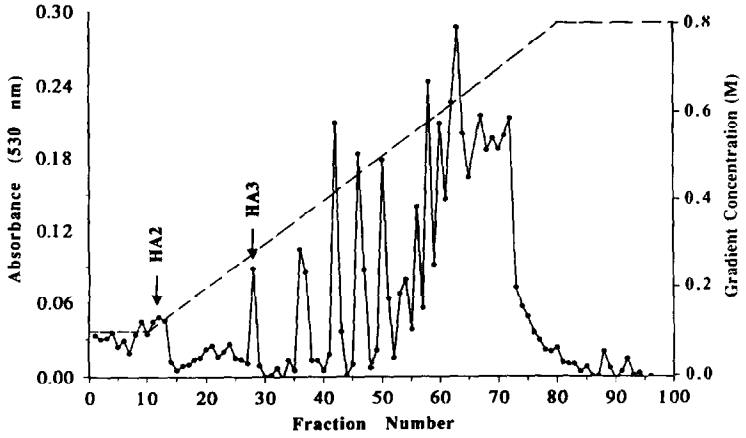

Fig. 3. Separation of HA oligomers on a PA100 column with a pH 5.0 sodium acetate buffer gradient. Improved separation of the shorter fragments was obtained; 2.0 mg of the 30-min digestion were loaded onto the column. Fractions were collected every 0.5 mL. Detection was accomplished with the uronic acid assay of Bitter and Muir[22].

For preparative-scale work we were able to load up to 14 mg of the 3-h digestion onto the column without significant loss of resolution. Recovery from the column was nearly quantitative.

This is the first time, to our knowledge, that HA oligosaccharides larger than six disaccharide units have been separated by HPLC. The conditions are relatively mild and require no chemical modification of HA. For semipreparative work this method offers a distinct advantage over traditional liquid chromatography in that it is much more rapid. For analysis, it is also a simple technique for assessing sample integrity and purity.

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