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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HYALURONIC ACID AND OLIGOSACCHARIDES PRODUCED BY BOVINE TESTES HYALURONIDASE

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SUMMARY

A high-performance liquid chromatographic method of analysing hyaluronic acid and oligosaccharides produced by bovine testes hyaluronidase is presented. Using silica gel packed columns and an aqueous mobile phase, it is possible to separate the mono-, di-, tetra- and hexasaccharides from higher oligosaccharides and hyaluronic acid and to follow the enzymatic conversion of polysaccharides to oligosaccharides. The analysis is performed within 30 min and only 20 μl of sample (containing 20–60 μg of saccharides) are needed.

INTRODUCTION

The glycosaminoglycan hyaluronic acid (HA) is a linear naturally occurring polysaccharide having the repeating structure -O-β-D-glycopyranuronosyl-(1 $\rightarrow$ 3)-O-(2-acetamido-2-desoxy-β-D-glycopyranosyl)-(1 $\rightarrow$ 4)-, known as N-acetylhyalobiuronic acid.

Enzymatic digestion of HA can be performed by *exo*-glycanohydrolases such as β-N-acetylglucosaminidase (E.C. 3.2.1.30) and β-gluruconidase (E.C. 3.2.1.31), cutting off monomer units from the end of the HA molecule, or by *endo*-glycanohydrolases (E.C. 3.2.35), cleaving the 1 $\rightarrow$ 3 bond leaving a reducing glucuronic acid end group, or cleaving the 1 $\rightarrow$ 4 bond leaving a reducing glucosamine end group. Bovine testes hyaluronidase is of the latter type, being an *endo*-β-N-acetylglucosaminidase.

For investigating the action of different hydrolases on HA, several techniques have been used *e.g.*, end group analysis, gel filtration and electrophoresis, paper chromatography, radial diffusion, viscometric and turbidimetric measurements.

In this paper we describe the application of high-performance liquid chromatography (HPLC) to the well known enzymatic digestion of HA with bovine testes hyaluronidase.

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MATERIALS AND METHODS

High-performance liquid chromatography

A 1010B liquid chromatograph from Hewlett-Packard (Böblingen, G.F.R.) was used with a differential refractometer (LD Refracto Monitor; Laboratory Data Control, Riviera Beach, Fla., U.S.A.) as detector. The detector response was recorded on a Hewlett-Packard 3380A integrator. Injections were made with a Rheodyne Model 7210 injector (Rheodyne, Berkeley, Calif., U.S.A.) with a 20-μl loop.

Separation was performed on a μBondagel E linear column, followed in series by two μPorasil GPC 60 Å columns (Waters Assoc., Milford, Mass., U.S.A.). Each had dimensions of column 300 × 4 mm I.D. The μBondagel column had a nominal molecular weight separation range of 2000–2,000,000, the μPorasil column a range of 100–10,000. The mobile phase was 20 m*M* sodium acetate buffer (pH 4.0) containing 1.5 mg/l of HA. The column compartment was heated to 40°, and a flow-rate of 0.40 ml/min was used. The pressure drop over the three columns was about 65 kp/cm^2. Injections were made with a 100-μl SGE syringe (SGE, North Melbourne, Australia).

Enzymatic digestion

HA as the potassium salt obtained from human umbilical cord (Grade I; Sigma, St. Louis, Mo., U.S.A.) was dissolved in 20 m*M* sodium acetate buffer (pH 4.0) at concentrations of 1.5–4.0 mg/ml, and this solution was used as a substrate for the digestion with chromatographically purified bovine testes hyaluronidase (Sigma). The enzyme was supplied in vials containing 15,000 NF units, which was dissolved in the acetate buffer to give an activity of 4000 NF units/ml. Digestion was started by the addition of 25 NF units of enzyme per milligram of HA. Similar additions of enzyme were repeated after 24, 48 and 72 h to give a total of 100 NF units added per milligram of HA.

Samples from the incubation mixture were drawn at different times, the enzyme activity being stopped by boiling in a water bath for 5 min, and stored at 4°.

Preparation of low-molecular-weight oligosaccharides

The 120-h digestion product was separated on a Sephadex G-25 Superfine (Pharmacia, Uppsala, Sweden) column (935 × 26 mm I.D.), equilibrated and eluted with 10% (v/v) of ethanol in 0.2 *M* sodium chloride solution according to Flodin *et al.*[1]. A constant flow of 10 ml/h was maintained with a peristaltic pump (P3; Pharmacia). The effluent was continuously monitored with a differential refractometer (LDC), and 5-ml fractions were collected.

The fractions containing oligosaccharides were pooled and desalted on Sephadex G-25 Superfine columns (80 × 30 mm I.D.) with 10% (v/v) of ethanol in water as the eluent. The desalted fractions were lyophilized and redissolved in water.

Determination of reducing N-acetylglucosamine

During digestion with bovine testes hyaluronidase, reducing N-acetylglucosamine end groups are liberated, and their analysis was performed according to Reissig *et al.*[2] with modifications. To 250 μl of sample, 50 μl of 1 *M* sodium hydroxide solution and 60 μl of 0.8 *M* dipotassium tetraborate solution were added in screw-

capped tubes to give a final pH of 9.1. The capped tubes were heated for 3 min at 100° in a heating block (BT 3; Grant, Cambridge, Great Britain). After cooling, 1.8 ml of DMAB reagent (5 g of *p*-dimethylaminobenzaldehyde dissolved in 5 ml of concentrated hydrochloric acid and 50 ml of glacial acetic acid, and diluted with 9 volumes of glacial acetic acid just before use) was added. After incubation for 20 min in a water-bath at 37°, the extinctions at 543 nm were measured in an LKB 2074 spectrophotometer (LKB, Bromma, Sweden), 0.250 m*M* N-acetylglucosamine being used as a standard.

Determination of total glucuronic acid

The analysis was performed according to Bitter and Muir[3]. To 250 μl of sample was added 1.5 ml of a 0.05 *M* solution of dipotassium tetraborate in concentrated sulphuric acid, carefully avoiding an increase in temperature above ambient. The capped tubes were heated for 10 min at 100° in a heating block (Grant). After cooling, 50 μl of a 0.25% (w/v) solution of carbazole in ethanol were added, followed by heating for 15 min at 100°. The tubes were cooled to ambient temperature and the extinctions at 514 nm were measured in the LKB 2074 spectrophotometer. A mixture of 0.100 m*M* glucuronic acid and 0.100 m*M* N-acetylglucosamine was used as a standard.

Determination of total N-acetylglucosamine

The analysis was performed according to Blix[4] with modifications. The sample (750 μl) was hydrolysed with 750 μl of concentrated hydrochloric acid in sealed glass ampoules for 3 h at 100° in a heating block (Grant). The mixture was cooled and transferred into 15-ml vials with about 0.5 ml of water, quickly frozen in acetone–carbon dioxide and lyophilized (Hetosicc; Heto, Birkerød, Denmark). The residue was dissolved in 1500 μl of water and 500 μl of this solution were added to 500 μl of acetylacetone reagent [10 ml of 0.2 *M* dipotassium tetraborate (pH 9.1), 220 μl of 5 *M* sodium hydroxide solution and 200 μl of acetylacetone] in a capped tube. After 30 min at 100° in a heating block (Grant), the tubes were cooled with ice and 200 μl were added to 1000 μl of DMAB reagent (see *Determination of reducing N-acetylglucosamine*) in LKB cuvettes. Colour development took place at 50° for 45 min in an LKB incubator, followed by measurement of extinctions in the LKB 2074 spectrophotometer at 543 nm. As a standard, a mixture of 0.5 m*M* N-acetylglucosamine and 0.5 m*M* glucuronic acid, treated in the same way as the samples, was used.

Paper chromatography

Descending paper chromatography was performed according to Partridge[5]. The solvent used was 1-butanol–acetic acid–water (44:16:40). Volumes of 20 μl of sample containing 10–40 μg of oligosaccharides were applied on Whatman No. 1 paper. The strips (180 × 420 mm) were irrigated for 40 h at room temperature. The formation of chromogen took place in a box containing saturated water vapour at 105° for 10 min. After spraying with DMAB reagent, the reducing N-acetylglucosamine-containing compounds gave a red colour.

Determination of exo-glycanohydrolases

β-N-Acetylglucosaminidase and β-glucuronidase activity was assayed accord-

ing to Khar and Anand[6] and Brot *et al.*[7]. The assays were modified for performance on an ABA-100 analyser (Abbott, Pasadena, Calif., U.S.A.) using a 415/450 nm filter set at 37° and *p*-nitrophenol substrates (Koch-Light, Colnbrook, Great Britain).

RESULTS

A 4 mg/ml solution of umbilical cord HA was incubated with bovine testes hyaluronidase as described under Materials and Methods. No β-glucuronidase or N-acetylglucosaminidase activity could be measured in the enzyme preparation. After incubation for 120 h the mixture was chromatographed on the Sephadex column. Part of the elution profile is shown in Fig. 1. The effluent was continuously monitored by the refractometer and 5-ml fractions were analysed for reducing N-acetylglucosamine content.

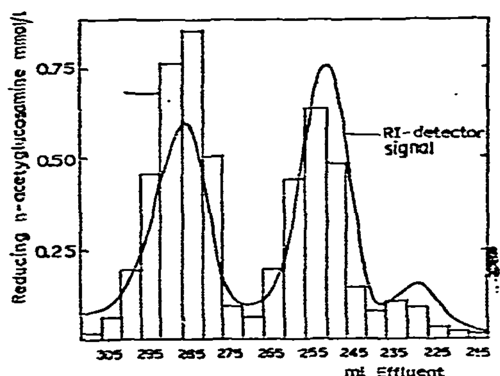

Fig. 1. Separation of oligosaccharides on Sephadex G-25 Superfine from the enzymatic digestion of HA. The peak with a maximum at about 250 ml of effluent was shown to contain hexasaccharide and the peak at 285 ml was shown to contain mainly tetrasaccharide.

The fractions eluted from 245 to 265 ml were pooled [pool (a)], and also the fractions eluted from 280 to 300 ml [pool (b)]. Each of the two pools was desalted, lyophilized and redissolved in 2 ml of water.

Pool (a) was found to give a total N-acetylglucosamine to glucuronic acid ratio of 1.04 and a total N-acetylglucosamine to reducing N-acetylglucosamine ratio of 2.95. Paperchromatography revealed a single spot with a mobility relative to N-acetylglucosamine of 0.41.

Pool (b) gave a total N-acetylglucosamine to glucuronic acid ratio of 1.00 and a total N-acetylglucosamine to reducing N-acetylglucosamine ratio of 1.77. Paper chromatography showed a heavily coloured spot with a relative mobility of 0.58, and a less coloured spot with a mobility of 0.77 relative to N-acetylglucosamine.

We conclude that pool (a) consisted of hexasaccharide and pool (b) of a mixture of tetrasaccharide and a small amount of disaccharide.

Fig. 2 shows the HPLC separation of a mixture of standards of approximately 1.5 mg/ml of each of the constituents of umbilical cord HA (elution time 13.79 min), hexasaccharide from pool (a) (17.84 min), tetrasaccharide from pool (b) (18.95 min), disaccharide from pool (b) (20.52 min), glucuronic acid (21.90 min) and N-acetyl-

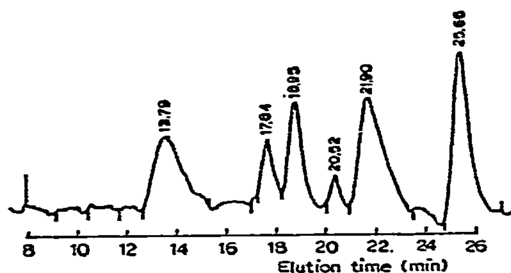

Fig. 2. HPLC of a mixture of six compounds. The refractometer response shows that HA is eluted at 13.79, hexasaccharide at 17.84, tetrasaccharide at 18.95, disaccharide at 20.52, glucuronic acid at 21.90 and N-acetylglucosamine at 25.66 min.

glucosamine (25.66 min). The void volume of the column system was about 3 × 1.8 ml, corresponding to an elution time of 13.5 min (0.40 ml/min) and a total volume of 3 × 3.4 ml, corresponding to 25.5 min, according to specifications from Waters Assoc.

In another experiment, a 1.6 mg/ml solution of HA was digested with bovine testes hyaluronidase. The HA had been desiccated over silica gel, and when analysed an N-acetylglucosamine to glucuronic acid ratio of 1.02 and a glucuronic acid content of 3.0 m*M* (theoretical value of pure potassium salt of HA, 3.8 m*M*) were found. Fig. 3 shows the recorder traces obtained in the HPLC analysis of the digest. After 120 h HA is degraded mainly to hexasaccharides and tetrasaccharides with only minor amounts of higher-molecular-weight oligosaccharides. Paper chromatography of the 120-h digestion product showed the same result.

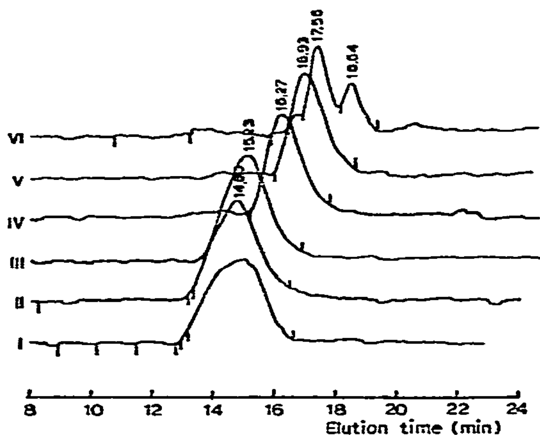

Fig. 3. HPLC of six samples (I–VI) drawn at different times from enzymatic digestion of HA. Data on the samples are given in Table I.

Table I shows the time course of the same digestion. Samples drawn from the incubation mixture at the times indicated were analysed for released end groups, and from these results the average molecular chain length could be calculated as the ratio of end groups to total monosaccharides. Using a molecular weight of 379 for the

TABLE I

DATA ON THE SIX SAMPLES FROM THE EXPERIMENT IN FIG. 3

Samples I–VI were drawn at times shown in the second column. The third column gives the concentration of liberated end groups, and the fourth and fifth columns show calculated values of the average number of disaccharides per chain and the number-average molecular weight.

Sample No.	*Time of digestion (h)*	*Reducing N-acetyl-glucosamine (mmol/l)*	*Average number of disaccharides per chain*	*Number-average molecular weight*
I	0	0	—	—
II	4	0.046	65.2	24700
III	12	0.101	29.7	11250
IV	48	0.405	7.4	2800
V	96	0.750	4.0	1520
VI	120	1.110	2.7	1050

repeating structure (N-acetylhyalobiuronic acid minus one molecule of water), the number-average molecular weights could be calculated.

DISCUSSION

Separation of the hyaluronic acid compounds in the system described is achieved by size exclusion in the three silica gel packed columns. Addition of trace amounts of HA to the mobile phase gave a better recovery and prevented adsorption to the columns. The μBondagel column has a packing material of varying porosity (125–1000 Å) with an ether-modified hydrophilic surface of the silica gel. The μPorasil columns have a narrow pore size range around 60 Å and a non-bonded surface. The packing material is not completely inert, however. The negatively charged HA and oligosaccharides seem to be partly excluded from the pores, *e.g.*, N-acetylglucosamine is eluted significantly later than glucuronic acid. Similarly, the Dextran T series (Pharmacia) is eluted much later than the glucuronic acid containing anions of the same molecular weight: Dextran T 10 (mol. wt. 10,000) at 18.7 min, Dextran T 20 (mol. wt. 20,000) at 17.8 min. Consequently, for our purpose the dextrans cannot be used as standards for molecular weight determinations.

The chosen column combination favours the separation of lower oligosaccharides. Fractionation must occur with elution times in the range 13.50 min (void volume) to 25.50 min (total volume) at a flow rate of 0.40 ml/min, but in this range the hexasaccharide already appears at 17.80 min, leaving the range 13.50–17.80 min to fractionation of higher oligosaccharides and polysaccharides. Umbilical cord HA is considered to have a very high molecular weight. Assuming (1) a molecular weight of several millions, corresponding to the peak of sample I in Fig. 3, and a molecular weight of 1150 corresponding to hexasaccharide in sample VI (17.56 min), and (2) gel filtration with a linear relationship between elution time and log (molecular weight), we would find weight-average molecular weights of the peaks in samples II–V up to ten times higher than the calculated number-average molecular weights shown in Table I. This means that the HA is degraded into a very polydisperse mixture by the bovine testes hyaluronidase, which fits well with the broad peaks in Fig. 3.

HA of other origin, such as pig skin (Seikagaku, Tokyo, Japan), vitreous

humour (EGA-Chemie, Steinheim/Albuch, G.F.R.) and bacteria (a gift from Dr. E. Kjems, Statens Seruminstitut, Copenhagen, Denmark), were also chromatographed; they were all eluted slightly later than the umbilical cord HA, and the peaks were narrower. Other charged glycosaminoglycanes such as the chondroitin sulphates and their disaccharide constituents (Seikagaku) were readily chromatographed under the same conditions.

The columns were stable for several months, but could be regenerated if necessary with 3% (w/v) of urea in the mobile phase.

We conclude that the proposed HPLC method for the characterization of HA and the enzymatic digestion products is advantageous because of the small sample volume, the speed of separation and the possibility of using a mobile phase with the same composition as the medium used for the enzymatic reaction.

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