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Preparation of Polysaccharide Molecular Weight Markers from Hyaluronidase-digested Hyaluronic Acid

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Summary

Four polysaccharide molecular weight markers were prepared from hyaluronic acid digested partially with hyaluronidase.

The digestion products were fractionated and purified by gel filtration on Sephadex G–50 and G–25, and four fractions were isolated for markers. The markers were reduced with borohydride to convert their terminal reducing N-acetylglucosamine residues into N-acetylglucosaminitol, and then N-acetylglucosamine and N-acetylglucosaminitol of the reduced markers were analyzed by gas chromatography. From the molar ratio of N-acetylglucosamine to N-acetylglucosaminitol, the molecular weights of the markers were calculated to be 4900, 3000, 1900 and 1150. A linear relationship was held between logarithm of molecular weights and elution volumes of these markers in gel filtration on a Sephadex G–50 column.

Gel filtration on Sephadex has been found very useful for estimation of molecular weight when appropriate molecular weight markers are available. However, it is very difficult to find appropriate markers for estimation of molecular weights of oligo- and polysaccharides, and glycopeptides. Therefore, we attempted to prepare some markers useful for polysaccharides and glycopeptides having molecular weight of 1000 to 6000.

This paper describes the preparation of some polysaccharide markers from hyaluronic acid digested partially with hyaluronidase.

Materials and Methods

Hyaluronic acid. Crude hyaluronic acid from human umbilical cords was a gift from Sanshō Seiyaku Co. It was purified by precipitation with cetylpyridinium chloride[1]. Crude hyaluronic acid (0.5 g) was dissolved in 100 ml of 0.01 M sodium sulfate, and 1 g of Celite 545 and 5 m*l* of 10% cetylpyridinium chloride were added with stirring. The resulting precipitate collected by centrifugation was stirred in 50 m*l* of 0.1 M NaCl at 37°C for 2 hr, and the suspension was centrifuged. The precipitate was extracted with 50 m*l* of 0.4 M NaCl at 37°C for 2 hr, and the extracted hyaluronic acid was precipitated by addition of 3 volumes of ethanol. The hyaluronic acid was dissolved in 25 m*l* of 0.3 M sodium acetate and reprecipitated by addition of 3 volumes of ethanol. The last step was repeated twice for complete removal of cetylpyridinium chloride. The purified hyaluronic acid was washed and dried with 70% ethanol, absolute ethanol and ethylether successively. Analyses: $[\alpha]_D^{21}$–68.0, glucuronic acid 43.7%, glucosamine 46.5%.

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Hyaluronidase. Hyaluronidase from bovine testes was a product of Sigma. Its specific activity was 230 TRU[2]/mg protein.

N-Acetylglucosaminitol. This was prepared from N-acetylglucosamine as follows. N-Acetylglucosamine (0.3 g) was dissolved in 30 m*l* of water, and reduced with 0.5 g of sodium borohydride at 5°C for 3 hr. The reaction mixture was adjusted to pH 5 with 1 N acetic acid, and passed through a column (1.4×8 cm) of Dowex 50–X8 (200–400 mesh, H^+). The effluent was evaporated to dryness under reduced pressure, and the boric acid was removed as trimethylborate by repeated evaporation with methanol. The residue was crystallized from an aqueous ethanol solution. m.p. 135°C.

Analyses of sugars. Uronic acid was determined by the carbazole method of Bitter and Muir[3]. Hexosamine was determined by the method of Svennerholm[4] after hydrolysis of samples in 4 N HCl at 100°C for 12 hr.

Digestion of hyaluronic acid with hyaluronidase. To a solution of 100 mg of hyaluronic acid in 10 m*l* of 0.1 M acetate buffer, pH 5.0, containing 1.5 mmol of NaCl was added 5 mg of hyaluronidase. After being incubated at 37°C for appropriate time, the mixture was heated for 3 min in a boiling water bath, and filtered.

Fractional determination of the terminal reducing N-acetylglucosamine and the other N-acetylglucosamine of polysaccharide markers. The polysaccharide marker was taken so as to contain 0.1–1 μmol of the terminal reducing N-acetylglucosamine, dissolved in 1 m*l* of water, and reduced with 7 mg of sodium borohydride at 5°C for 5 hr. The reaction mixture was adjusted to pH 5 with 1 N acetic acid, and passed through a column (0.5×2.6 cm) of Dowex 50–X8 (200–400 mesh, H^+) to remove sodium ion. The effluent and washings were combined, and evaporated to dryness under reduced pressure, and the boric acid was removed by repeated evaporation with methanol. The residue was hydrolyzed in 1 m*l* of 2 N HCl at 100°C for 20 hr. To the hydrolyzate was added 50 μg of glucitol as internal standard, the mixture was evaporated to dryness, and N-reacetylated according to the following procedure of Levvy et al.[5] The residue was dissolved in 1.5 m*l* of water, and the solution was thoroughly mixed with 1.5 m*l* of 0.6% (v/v) acetic anhydride in acetone. The mixture was transferred to a column (0.8×6 cm) of Dowex 1–X8 (200–400 mesh, CO_3^{--}) equilibrated with aqueous acetone (1 : 1, v/v), and the column was washed with 5 m*l* of aqueous acetone. The effluent and washings were combined, and evaporated to dryness. The residue, after being left overnight over P_2O_5 and KOH in a vacuum desiccator, was trimethylsilylated with 30 μl of hexamethyldisilazane and 15 μl of trimethylchlorosilane in 100 μl of pyridine. An aliquot (0.5–2 μl) of the reaction mixture was injected to a Shimazu 4APF gas chromatograph equipped with a hydrogen flame ionization detector. The conditions used were as follows: column, glass column (0.4×200 cm) packed with 5% SE–30 on Shimalite W (60–80 mesh); carrier gas, N_2 50 m*l*/min; column temperature, 190°C.

Results

Digestion of hyaluronic acid with hyaluronidase. A study for estimating an appropriate digestion period was carried out. Hyaluronic acid was digested with hyaluronidase at 37°C for 1, 3, and 6 hr, and the digest was chromatographed on a Sephadex G–50 column. From the elution patterns of the digestion products shown in Fig. 1, a 2-hr period, which seemed to give an intermediate pattern between those of 1-hr and 3-hr digests, was considered to be appropriate to obtain polysaccharides suitable for calibration of Sephadex G–50.

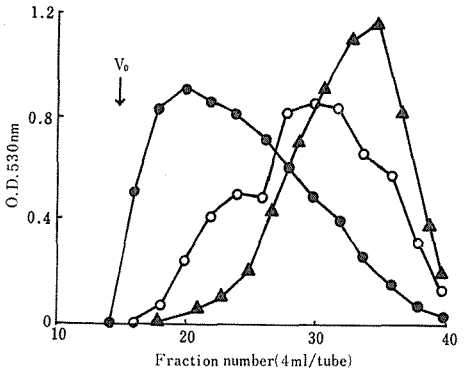

Fig. 1. Behavior of hyaluronidase digests of hyaluronic acid on Sephadex G–50. Three 10-m*l* portions of hyaluronic acid were digested with hyaluronidase at 37°C for 1, 3 and 6 hr. The digest was applied to a Sephadex G–50 column (1.25×143 cm), and eluted with 0.1 N acetic acid. The effluent was analyzed by the carbazole method. V_0, void volume peak measured with Blue Dextran 2000. ●——●, 1 hr; ○——○, 3 hr; ▲——▲, 6 hr.

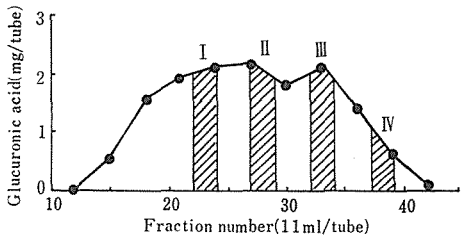

Fig. 2. Fractionation of a hyaluronidase digest of hyaluronic acid on Sephadex G–50. Hyaluronic acid (0.1 g) was digested with hyaluronidase at 37°C for 2 hr. The digest was concentrated to 5 m*l*, applied to a Sephadex G–50 column (2.2×140 cm), and eluted with 0.1 N acetic acid. The fractions corresponding to the shaded areas were selected for markers.

Fractionation and purification of 2-hr digestion products of hyaluronic acid. Hyaluronic acid was digested with hyaluronidase at 37°C for 2 hr, and the digest was fractionated on a Sephadex G–50 column. The elution pattern is shown in Fig. 2. The four fractions corresponding to the shaded area were separately pooled, and rechromatographed for purification on a Sephadex G–50 column. Four elution patterns obtained separately are shown in Fig. 3. In fractions I and II, the middle portions of the peaks

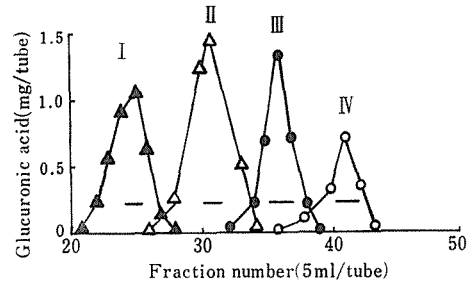

Fig. 3. Purification of fractions I, II, III and IV on Sephadex G–50. Fractions I, II, III and IV indicated in Fig. 2 were separately evaporated to dryness under reduced pressure, dissolved in 2 m*l* of water, and applied to a Sephadex G–50 column (1.6×146 cm). Elution was carried out with 0.1 N acetic acid.

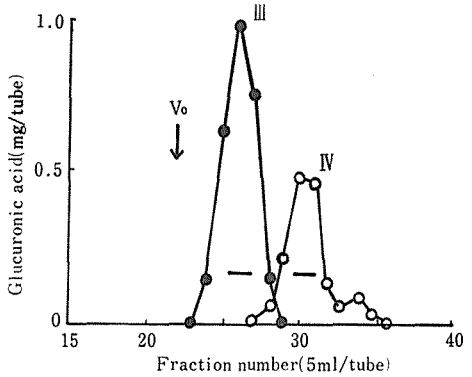

Fig. 4. Purification of fractions III and IV on Sephadex G–25. The middle portions of fractions III and IV indicated by bars in Fig. 3 were separately evaporated to dryness, dissolved in 1 m*l* of water, and applied to a Sephadex G–25 column (1.6×146 cm). Elution was carried out with 0.1 N acetic acid.

indicated by bars were pooled, and evaporated to dryness under reduced pressure. They are referred to as markers I and II. In fractions III and IV, the portions indicated by bars were pooled, and chromatographed on a Sephadex G–25 column for further purification. As shown together in Fig. 4, fraction III gave a homogeneous peak, and fraction IV gave a homogeneous main peak followed by a small peak. The middle portions of the peaks indicated by bars were pooled, and evaporated to dryness. They are referred to as markers III and IV.

Analyses of markers I, II, III and IV. Glucosamine and glucuronic acid contents of the markers were determined by the Elson-Morgan and carbazole reactions, respectively. These markers consisted of equimolar amounts of glucosamine and glucuronic acid, indicating that glucuronic acid and N-acetylglucosamine residues arrange alternately with the latter at the reducing end.

Estimation of molecular weights of markers I, II, III and IV. The molecular weight of the marker was calculated from the molar ratio of nonreducing N-acetylglucosamine residues to a terminal reducing N-acetylglucosamine residue in the carbohydrate chain. The marker was reduced with sodium borohydride, and N-acetylglucosaminitol derived from the terminal reducing N-acetylglucosamine and N-acetylglucosamine were analyzed by gas chromatography. A typical gas chromatogram obtained in the analysis of marker III is shown in Fig. 5, and the results are summarized in Table 1. The molecular weights of markers I, II, III and IV were calculated to be 4900, 3000, 1900 and 1150.

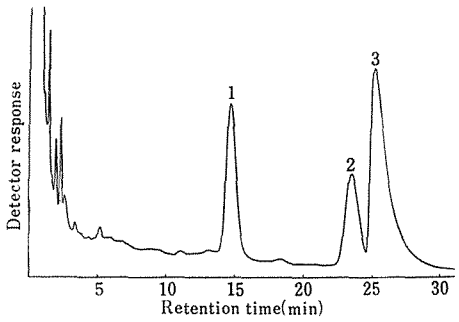

Fig. 5. Gas chromatogram of trimethylsilyl derivatives of N-acetylglucosamine and N-acetylglucosaminitol from borohydride-reduced marker III. 1. Glucitol (internal standard), 2. N-acetylglucosaminitol, 3. N-acetylglucosamine.

Table 1 Estimation of molecular weights of markers I, II, III and IV

Marker	Molar ratio		Chain length[a)]	Molecular weight[b)]
	N-Acetyl-glucosaminitol	N-Acetyl-glucosamine		
I	1.00	12.2	26 saccharides	4900
II	1.00	6.82	16	3000
III	1.00	4.18	10	1900
IV	1.00	2.07	6	1150

a) Estimated from the molar ratio of N-acetylglucosamine to N-acetylglucosaminitol.

b) Calculated from chain length and the molecular weights of glucuronic acid and N-acetylglucosamine.

Calibration of a Sephadex G–50 column with markers I, II, III and IV. A mixture of markers I, II, III and IV was chromatographed on a Sephadex G–50 column (Fig. 6). A linear relationship between the logarithm of molecular weights and elution volumes of these markers was observed as shown in Fig. 7. Molecular weight of a keratan sulfate fraction isolated from whale cartilage[6] was estimated to be 3000 on the calibrated column.

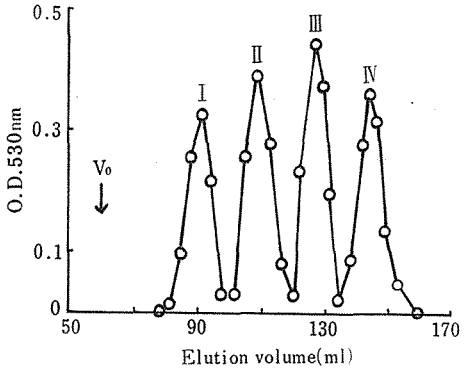

Fig. 6. Elution patterns of markers I, II, III and IV from a Sephadex G–50 column. A 1-*ml* mixture of markers I, II, III and IV, containing about 0.6 mg of each, was applied to a Sephadex G–50 column (1.25 × 144 cm), and eluted with 0.2 M NaCl at a flow rate of 3 *ml*/hr. Fractions of 1.9 *ml* were collected, and analyzed by the carbazole method.

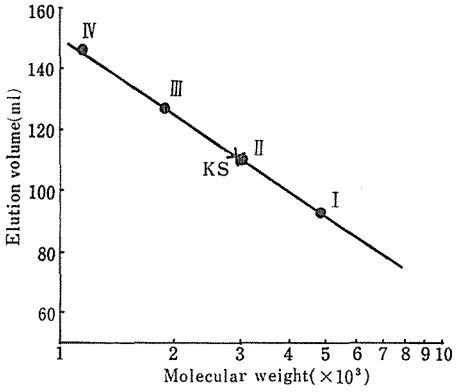

Fig. 7. Calibration curve for a Sephadex G–50 column. KS: A keratan sulfate preparation from whale cartilage.

Discussion

Hyaluronic acid from human umbilical cord is a linear macromolecule in which glucuronic acid alternates with N-acetylglucosamine[7]. No hybrid structure in the macromolecule has been reported. It is well known that testicular hyaluronidase acts on hyaluronic acid as endohexosaminidase[8]. Accordingly, all the polysaccharide fragments formed in testicular hyaluronidase digestion of hyaluronic acid have a terminal reducing N-acetylglucosamine residue. The digestion products are mainly tetrasaccharide concomitant with some disaccharide and higher oligosaccharides[9]. In the present work, brief digestion of hyaluronic acid was carried out to preserve appreciable amounts of polysaccharides.

The fractionation of the digestion products has been achieved successfully by Dowex 1–X10 column chromatography using dilute formic acid as eluant[9]. However, this ion exchange chromatography was not applicable to the fractionation of the present products composed mainly of polysaccharides. Gel filtration was found to be suitable for their fractionation.

The chain lengths of oligosaccharides can be estimated from the analysis of the terminal reducing sugar by the Park-Johnson or Nelson-Somogyi method[10]. However, these methods could not be applied to the estimation of the chain lengths of the polysaccharides isolated in this study since the reactivity of the terminal reducing N-acetylglucosamine

toward these colorimetric reagents tends to decrease with increase of chain length. Our method may permit satisfactory estimation of the chain length of 26 saccharides or more.

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摘　　要

ヒアルロン酸限定加水分解物からの多糖分子量マーカーの調製

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昭和54年5月30日　受理

多糖ないし少糖および糖ペプチドのゲルろ過による分子量測定用として分子量マーカーを調製した.

ヒアルロン酸のヒアルロニダーゼによる限定加水分解物から，4つの画分を分離した．これらを還元して還元末端 N-アセチルグルコサミンをN-アセチルグルコサミニトールに変えたのち，アミノ糖をガスクロマトグラフィーで分析し，N-アセチルグルコサミンと N-アセチルグルコサミニトールとのモル比から，これらの分子量を4900，3000，1900および1150と決定した．これらのマーカーは，セファデックス G-50 ゲルろ過において溶出容積と分子量（対数）との間に直線関係を示した.