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Determination of hyaluronic acid by high-performance liquid chromatography of the oligosaccharides derived therefrom as 1-(4-methoxy)phenyl-3-methyl-5-pyrazolone derivatives

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ABSTRACT

Hyaluronic acid (HA) was digested with various kinds of depolymerizing enzymes and the products were analysed by high-performance liquid chromatography (HPLC) after derivatization with 1-(4-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP). As hyaluronate 4-glycanohydrolase (EC 3.2.1.35) from sheep testis showed a high efficiency for depolymerization, giving the tetra- and hexasaccharides abundantly, and is inexpensive, a method for the specific determination of HA was established, based on digestion by this enzyme followed by determination of the tetra- or hexasaccharide derived therefrom as the PMPMP derivatives by HPLC with UV detection. This method allowed the determination of HA in the range 0.5–50 μg with high reproducibility.

INTRODUCTION

Hyaluronic acid (HA) is widely distributed among connective tissues of mammalian bodies and microorganisms, and is important for the treatment of geriatric arthritis and as an additive in cosmetics. HA is a macromolecular compound having a relative molecular mass of more than 10^6. It is composed of glucuronic acid and N-acetylglucosamine linked alternatively through the $\beta 1 \rightarrow 3$ and the $\beta 1 \rightarrow 4$ linkages, respectively, as shown in Fig. 1.

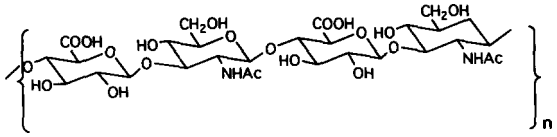

Fig. 1. Structure of hyaluronic acid.

In clinical analysis, HA is determined exclusively by electrophoresis on a cellulose acetate membrane. It can be also determined by size-exclusion chromatography with UV photometric detection at a low wavelength [1], but sensitivity and selectivity are not high. HA can be depolymerized by hydrolases to give oligosaccharides having various degrees of polymerization (DP). Hyaluronate 4-glycanohydrolase (hyaluronoglucosaminidase, EC 3.2.1.35) from animal testes [2] and hyaluronate 3-glycanohydrolase (hyaluronoglucuronidase, EC 3.2.1.26) from leech [3] cleave the glucosaminide and glucuronide linkages, respectively. The resultant oligosaccharides can be measured by high-performance liquid chromatography (HPLC) after derivatization with 2-aminopyridine [4]. There is another type of depolymerizing enzyme, hyaluronate lyase (EC 4.2.2. 1), which cleaves specifically the hexosaminide linkage, giving oligosaccharides having one double

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bond each on the glucuronic acid residue at the non-reducing ends. The resultant oligosaccharides show weak absorption at 232 nm due to the double bond [5]. Modification of the reducing ends of the resultant oligosaccharides with 2-aminopyridine enhances UV absorption and allows fluorescence detection with high sensitivity [4]. The accurate HPLC determination of individual oligosaccharides derived with these enzymes will make it possible to elucidate the mechanisms of enzymic reactions and also determine the content of HA.

In a previous study, we developed a method for the determination of urinary chondroitin sulphates based on the capillary electrophoresis of unsaturated disaccharides derived therefrom by digestion with chondroitinase ABC, as 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives [6]. This method allows the simultaneous sensitive determination of chondroitin, chondroitin sulphate A and C and related glycosaminoglycans with UV absorption, and is suitable for clinical analysis because of the simplicity of the procedure. In continuation of this work, we compared the capabilities of PMP analogues, and found that 1-(4-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP) derivatives of oligosaccharides showed a higher molar absorptivity than PMP derivatives.

In this paper, we propose a method for the determination of HA, based on HPLC of the oligosaccharides derived therefrom as PMPMP derivatives.

EXPERIMENTAI

Materials

A sample of HA from pig skin, obtained from Sigma (St. Louis, MO, USA) was used throughout. Hyaluronate 4-glycanohydrolase (sheep testis, Type V) was also obtained from Sigma. Samples of hyaluronate 3-glycanohydrolase *(Hirudo medicinalis)* and hyaluronate lyase *(Streptomyces hyalurolytics)* were purchased from Seikagaku Kogyo (Tokyo, Japan). PMPMP reagent was prepared according to the method described previously [7]. All other chemicals were of the highest grade commercially available.

Instruments

The HPLC system consisted of a Hitachi 655A-12 liquid chromatographic pump, a Shimadzu SPD-6A UV detector and a Shimadzu C-R6A chromate-recorder for measurements of peak area and elution time. A column (150 mm × *6* mm I.D.) packed with Cosmosil 5C18-AR (average particle diameter 5 μm) (Nacalai Tesque, Kyoto, Japan) was used as the stationary phase. Elution was performed with acetonitrile-100 m*M* phosphate buffer (pH 7.0) (15:85, v/v) at a flow-rate of 0.8 ml/min, and PMPMP derivatives of HA-derived oligosaccharides were monitored at 249 nm.

Fast atom bombardment mass spectrometry (FAB-MS) of HA-derived oligosaccharides was performed on a JEOL SX102 apparatus in the negative-ion mode (glycerol as matrix) using JEOL standard software.

Enzymic digestion

HA (50 mg) was dissolved in 60 m*M* citrate buffer (pH 5.2, 5.0 ml), hyaluronate 4-glycanohydrolase from sheep testis (0.5 mg) was added and the mixture was incubated for 24 h at 37°C. The enzymic reaction was terminated by keeping the mixture on a boiling water-bath for 3 min and the mixture was centrifuged at 10 000 g for 10 min. The supernatant solution was applied to a column of Sephadex G- 15 (70 cm × 2.5 cm I.D.) equilibrated with 0.1% acetic acid, and the column was eluted with the same solvent. Fractions of 7 ml were collected, with UV monitoring at 220 nm. A 300-μl portion of each fraction was subjected to carbazoleesulphuric acid assay for glucuronic acid [8]. Each of the fractions that showed a positive reaction to the assay was lyophilized and a 100-μg portion was examined by FAB-MS. Another portion was derivatized with PMPMP and the elution volume of the product was compared with those of the peaks from the derivatized digestion mixture obtained on an analytical scale (see Fig. 3a).

Comparative study of depolymerizing enzymes

In the comparative study of various depolymerizing enzymes, HA (50 μg) was dissolved in water (10 μl) and an aqueous solution (1 U, 10 μl) of hyaluronidase and 100 m*M* citrate-phosphate buffer (pH 5.0 for hyaluronate 3-glycanohydrolase from *Hirudo medicinalis,* pH 5.2 for hyaluronate 4-glycanohydrolase from sheep testis and pH 6.0 for hyaluronate lysase from *Streptomyces hyalurolytics) (40* μl) were added. Each mixture was incubated for 24

h at 37°C. After terminating the enzymic reaction by heating the mixture on a boiling water-bath for 3 min, the mixture was evaporated to dryness in a centrifugal concentrator (CC-10; Tomy, Tokyo, Japan), derivatized with PMPMP and the product analysed by HPLC.

Time course study

HA (1 mg) was dissolved in 2 ml of water. An aqueous solution (20 U, 200 μl) of hyaluronate 4-glycanohydrolase (sheep testis) and 100 m*M* citrate-phosphate buffer (pH 5.2) (800 μl) were added and the solution was incubated at 37°C. After specified periods, aliquots (150 μl each) were removed and evaporated to dryness in a centrifugal evaporator. The residues were derivatized with PMPMP and the products analysed by HPLC.

Determination of HA

An aqueous solution (100 μl) containing HA (0.1-50 μg) was added to citrate-phosphate buffer (pH 5.2) (40 μl). Hyalyronate 4-glycanohydrolase from sheep testis (5 U per 10 μl in water) was added to the mixture, which was incubated for 5 h at 37°C. The enzymic reaction was terminated by heating the mixture on a boiling water-bath for 3 min and the mixture was evaporated to dryness in a centrifugal evaporator. The residue was directly subjected to derivatization with PMPMP (see below).

Derivatization with PMPMP

The procedure was essentially the same as that reported for the derivatization of oligosaccharides from glycoproteins [7]. An 0.3 *M* solution of sodium hydroxide (20 μl) was added to a lyophilized sample of HA-derived oligosaccharides or the residue obtained by the enzymic digestion described above. To the mixture was added a methanolic 0.5 *M* solution of PMPMP (20 μl) was added and the solution was kept at 70°C for 20 min. After addition of 0.3 *M* HCl (20 μl) for neutralization, water (200 μl) and ethyl acetate (200 μl) saturated with water were added. The mixture was shaken vigorously and the organic phase was carefully removed. Ethyl acetate (200 μl) was added and the procedure was repeated twice more. The aqueous phase finally obtained was evaporated to dryness in a centrifugal evaporator, the residue was dissolved in 15% aqueous acetonitrile (200 μl) and an aliquot (20 μl) of the solution was injected onto the HPLC column.

RESULTS AND DISCUSSION

The reducing ends of reducing oligosaccharides are modified with PMPMP in such a manner that the hemiacetal bond is cleaved and two molecules of PMPMP are attached to C-l' of the sugar moiety through C-4 of the pyrazolone ring (Fig. 2).

The derivatization is quantitative under weakly alkaline conditions and the remaining reagent can be easily removed by extraction with ethyl acetate. The derivatives have a high molar absorptivity around 249 nm, and a negative charge due to enolization of the keto group at the C-3 position.

The PMPMP derivatives of HA-derived oligosaccharides were efficiently separated in the reversed-phase partition mode using an ODS column and a neutral phosphate buffer containing acetonitrile.

Fig. 3 compares the elution profiles of the PMPMP-derivatized products of HA digestion by (a) hyaluronate 4-glycanohydrolase from sheep testis, (b) hyaluronate 3-glycanohydrolase from *Hirudo medicinalis* and (c) hyaluronate lyase from *Streptomyces hyalurolytics*. All of the enzymic reactions were carried out under conditions ensuring completion.

The product of digestion by hyaluronate 4-glycanohydrolase was derivatized with PMPMP. The chromatographic profile (Fig. 3a) shows a relatively simple pattern composed of a few peaks of PMPMP oligosaccharides. It also shows a peak of the remaining reagent (peak 3) and a few minor peaks. The digestion product similarly obtained was fractionated on a column of Sephadex G-50. Each fraction was monitored for uronic acid, and each uronic acid-containing fraction was examined by FAB-MS. The fraction giving the most intense colour in the carbazole-sulphuric acid assay gave a peak of

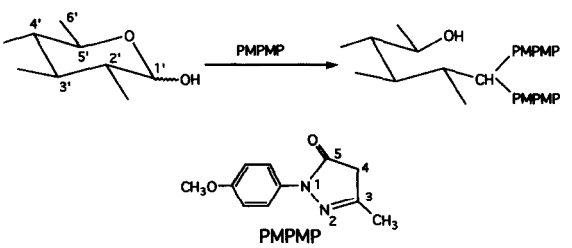

Fig. 2. Reaction of cabohydrates with PMPMP reagent.

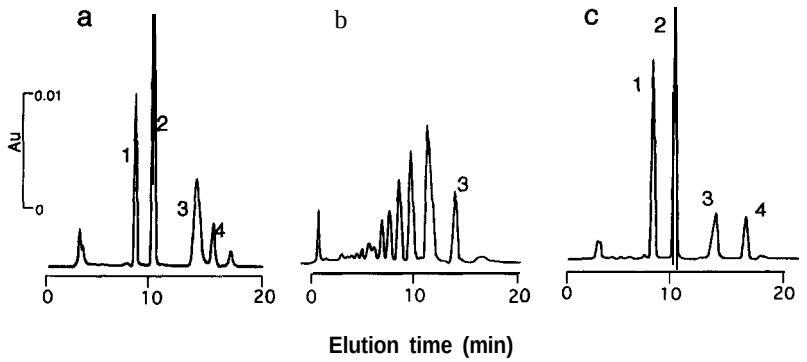

Fig. 3. Comparison of the elution profiles of the PMPMP-derivatized digestion mixture of HA. The digestion by all these enzymes was almost complete under the conditions employed. Digestion with (a) hyaluronate 4-glycanohydrolase from sheep testis, (b) hyaluronate 3-glycanohydrolase from *Hirudo medicinalis* and (c) hyaluronate lyase from *Streptomyces hyalurolytics*. The procedures for enzymic digestion and derivatization with PMPMP are described under Experimental. Column, Cosmosil 5C18-AR (150 mm × 6 mm I.D.); column temperature, ambient; eluent, 100 m*M* phosphate buffer (pH 7.0)–acetonitrile (15:85, v/v); flow-rate, 0.8 ml/min; detection UV absorption at 249 nm. Peaks: 1 = hexasaccharide: 2 = tetrasaccharide; 3 = reagent (PMPMP); 4 = disaccharide.

m/z 797 (Fig. 4), which corresponds to $[M-1]^-$ ion from the HA tetrasaccharide. Another portion of this fraction was derivatized with PMPMP and examined by HPLC under the conditions as in Fig. 3. The derivative from this fraction gave a peak identical with peak 2 in Fig. 3a. Hence peak 2 was identified as the tetrasaccharide GlcUAβ1→3GlcNAcβ1→4GlcUAβ1→3GlcNAc. In a similar manner, peaks 1 and 4 were assigned to the hexa- and disaccharides, respectively. The PMPMP derivatives of this series of oligosaccharides were eluted in order of decreasing DP in this separation mode.

The digestion product with hyaluronate 3-glycanohydrolase (Fig. 3b) gave multiple peaks of oligo-

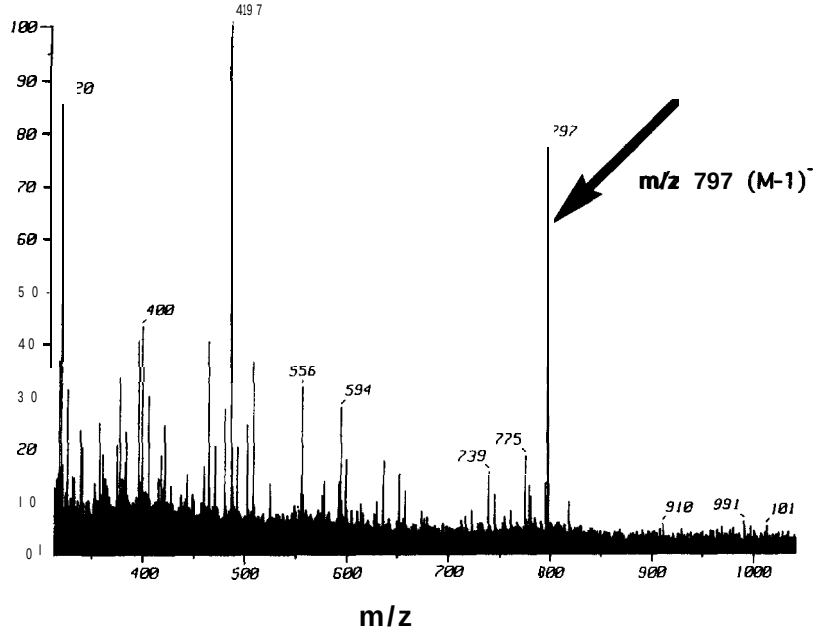

Fig. 4. Negative-ion FAB mass spectrum of the hyaluronate 4-glycanohydrolase-derived tetrasaccharide.

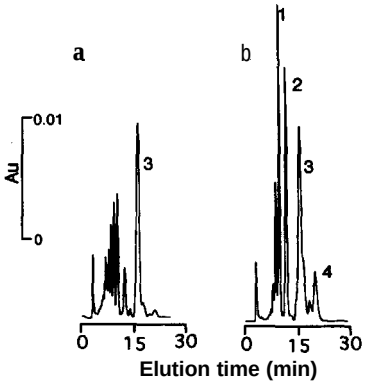

Fig. 5. Elution profiles of the PMPMP-derivatized incomplete digestion mixture of HA. HA (50 μg) was digested by hyaluronate 4-glycanohydrolase from sheep testis. Digestion period: (a) 20 min; (b) 5 h. Peak assignment and analytical conditions as in Fig. 3.

saccharides having various DP values. Although HA was digested for an ample period using a large amount of the enzyme, oligosaccharides having high DP values were resistant to further hydrolysis.

The digestion of HA with hyaluronate lyase from ***Streptomyces hyalurolytics*** (Fig. 3c) gave a similar pattern as that in Fig. 3a, although the structures of the resultant oligosaccharides are slightly different from those of the corresponding oligosaccharides in Fig. 3a in that they have a double bond per molecule.

Comparison of these three profiles led to the conclusion that the use of either hyaluronate 3-glycanohydrolase or hyaluronate lyase is advantageous for the determination of HA because of the higher depolymerizing efficiencies. Of these two enzymes, hyaluronate 3-glyconohydrolase is to be preferred because it is cheaper.

Fig. 5 shows the chromatographic patterns obtained with a smaller amount of hyaluronate 3-glycanohydrolase to establish the time course of digestion. The profile for a 20-min digestion (Fig. 5a) shows weak multiple peaks of higher oligosaccharides, whereas that for a 5-h digestion (Fig. 5b) shows large peaks of lower oligosaccharides (mainly the tetra- and hexasaccharides). However, oligosaccharides having DP values higher than 8 still remained with 5-h digestion. These observations suggest that this enzyme randomly cleaves interglycosidic linkages.

We optimized the conditions for the production of the tetra- and hexasaccharides from HA by digestion with hyaluronate 3-glycanohydrolase from sheep testis. Fig. 6a shows the pH dependence of the production of these saccharides. At *ca.* pH 5 the yields of both oligosaccharides became maximum.

Fig. 6b shows the effect of incubation period on the yields of both oligosaccharides. Under the conditions employed, the yields of both oligosaccharides became almost constant after 5 h of incubation. The production of the hexasaccharide was

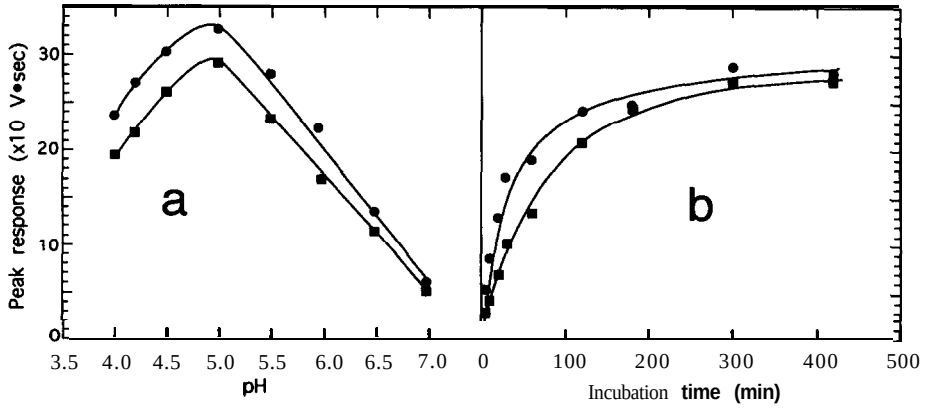

Fig. 6. Optimization of the enzyme digestion of HA by hyaluronate 4-glycanohydrolase from sheep testis. (a) pH dependence; (b) effect of incubation period. The conditions for the enzymic digestion are described under Experimental. Analytical conditions as in Fig. 3. ■ = PMPMP derivative of the tetrasaccharide; ● = PMPMP derivative of the hexasaccharide.

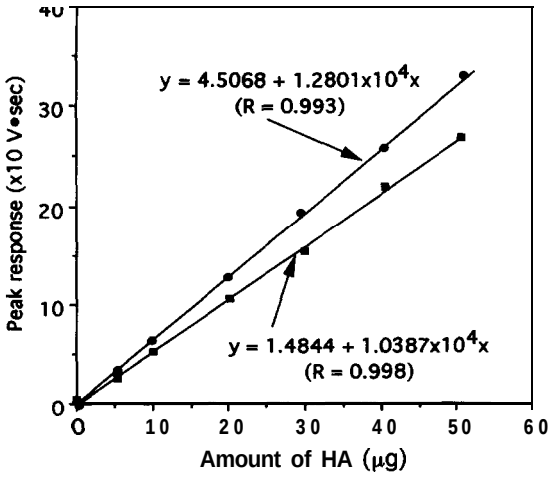

Fig. 7. Calibration graphs for HA, as observed from the yields of the tetra- or hexasaccharide derived therefrom. The conditions for the enzymic digestion are described under Experimental. Analytical conditions as in Fig. 3. ■ = Observed from the yield of the PMPMP derivative of the tetrasaccharide; ● = observed from the yield of the PMPMP derivative of the hexasaccharide.

much greater than that of the tetrasaccharide in the initial stages, but it was almost the same as the latter when the incubation time was longer than 7 h. It seems that large molecules of HA and HA-derived oligosaccharides are easily cleaved by hyaluronate 3-glycanohydrolase but the smaller oligosaccharides below hexasaccharides are resistant to this enzyme. This enzyme might require at least two binding sites to exert its enzymic affect. One of the binding sites is obviously the point of scission, and the other(s) are at point(s) sufficiently apart from the point of scission. HA-derived oligosaccharides smaller than the hexasaccharides cannot have these additional binding sites, and therefore cannot be cleaved. Hence hyaluronate 4-glycanohydrolase randomly cleaves the glucuronide bond, provided that the DP is larger than 8, but is does not work on smaller oligosaccharides.

Under the conditions established above (incubation for 5 h at 37°C at pH 5.2), various amounts of HA were digested and the PMPMP derivatives of the tetra- and hexasaccharides were analysed by HPLC. The calibration graphs based on the peak response were linear from 0.5 to 50 μg of HA (Fig. 7), and the relative standard deviations of peak response at the l.O-, lO.O- and 50-μg levels of HA were 2.3, 1.5 and 1.8%, respectively, for the derivative of the hexasaccharide. The detection limit was 0.1 μg as HA (10 ng as amount injected). Use of the calibration graph for the tetrasaccharide gave similar results.

The established method is sensitive. The specificity depends on that of hyaluronate 4-glycanohydrolase. The process of derivatization also excluded substances that could possibly interfere in the determination. Therefore, it may be useful for the determination of HA in drugs and cosmetics in addition to clinical samples. The results of its application to such samples will appear elsewhere.

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