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High-performance capillary electrophoresis of hyaluronic acid: determination of its amount and molecular mass

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Abstract

The amount and the molecular mass of hyaluronic acid (HA) were determined by high-performance capillary electrophoresis. HA was observed at around 15 min by using an untreated fused-silica capillary (75 μm I.D.) of 58 cm length (effective length, 50 cm) at 20 kV in 50 m*M* phosphate buffer (pH 4.0). Calibration curves showed good linearity from 0.01 mg/ml to 3.3 mg/ml for all HA samples examined. The lower limit of detection by monitoring the absorbance at 185 nm was 1.0 μg/ml at the signal-to-noise ratio of 5. HA samples were examined in a buffer containing pullulan (PU) as an additive for the matrix formation material. The HA samples showed marked peak-broadening when analyzed in the buffer solution containing PU with a specified molecular mass. The peak broadening was based on the dispersion of the molecular mass of the HA sample analyzed.

Keywords: Hyaluronic acid; Pullulan

1. Introduction

Hyaluronic acid (HA) is a major component of the extracellular matrix and is known to influence cell behavior and to play a role in angiogenesis, morphogenesis and tissue remodelling [1]. HA is also a diagnostic marker in a myriad of disorders such as neoplastic, liver, rheumatoid and lung disease [2]. HA is a linear polymer, having a regularly alternating disaccharide unit of *N*-acetylglucosamine and glucuronic acid (Fig. 1), and it is used for various therapeutic purposes such as maintaining viscoelastic properties in synovial fluid [3].

The biological functions of HA are closely related to the molecular mass, which may be as high as 10^7 [4]. The purpose of the present study is to determine the amount and molecular mass of HA.

It has been reported that unsaturated disaccharides produced by the action of eliminase were determined by high-performance liquid chromatography (HPLC) or capillary electrophoresis at UV 232 nm [5]. The oligosaccharides, produced by an endo-type glycosidase, were also determined by HPLC after being labeled at the reducing end with a chromophoric reagent, 1-(4-methoxy)phenyl-3-

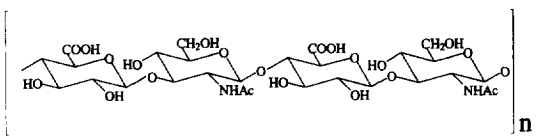

Fig. 1. Structure of hyaluronic acid.

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methyl-5-pyrazolone [6]. These methods can be applied to the determination of the absolute amount of each glycosaminoglycan. The estimation of the molecular mass of HA, however, is impossible by these methods. The method employing size exclusion chromatography makes it possible to determine both molecular mass and the amount simultaneously [7]. Detection of the peaks is usually accomplished by monitoring the absorbance at 210 nm or by using a differential refractometer, though the sensitivity is not high. Grimshaw et al. recently reported a direct quantitative analysis of hyaluronic acid in vitreous humor by capillary electrophoresis [8]. However, electrophoretic behaviors of HA samples having different molecular masses were not described.

At pH values where carboxyl groups in HA molecules are present as carboxylate anions, the charge to the molecular-mass ratio of HA samples having different molecular mass becomes constant due to the uniform structure of the molecules (see Fig. 1). Therefore, the determination of the amount of HA can be easily performed by using the simple zone electrophoresis mode, because HA should show a single peak even though it contains various molecular species having different molecular masses.

Alternatively, in analysis using an entangled polymer solution, the mobility of a substance of a high molecular mass species becomes small due to high resistance caused by passing through the matrix formed by the polymer. A substance of a lower molecular mass species migrates through the polymer matrix faster than the one of a high molecular mass species. Attempts to separate DNA or protein molecules according to their molecular masses in such a polymer network have been reported by several groups [9,10].

However, HA, as is well known, is not a single compound but a mixture of molecular species having different numbers of disaccharide units. For example, the molecular species of an HA preparation obtained from human umbilical cord range from 800 000 to 1 200 000 in molecular mass. The analysis of such a large range at a high molecular mass was another aim of the present study. To estimate the molecular mass distributions of HA samples, we chose some pullulan preparations with different molecular mass values as the additive in the running buffer. We found that the concentrations and the molecular mass of pullulan, used as the additive, affected the migration times of the HA samples and caused a peak broadening of the HA samples, based on the range of their molecular mass values.

2. Experimental

2.1. Reagents and materials

A standard sample of HA (HA-L) from pig skin having the distribution of molecular mass of 40 000–60 000 was obtained from Seikagaku Kogyo (Nihonbashi, Chuo-ku, Tokyo). An HA sample (HA-M) from human umbilical cord with the molecular mass range of 800 000–1 200 000 was also obtained from Seikagaku Kogyo. A pharmaceutical preparation of HA (HA-H, commercial name Opelead, a product from Shisei-do, Yokohama, Japan) was obtained from a drugstore, and its molecular mass distribution was estimated to be 1 500 000 to 2 100 000. A sample of pullulan (PU) with an estimated molecular mass of 91 700 was obtained from Seikagaku Kogyo, and other preparations of PU having estimated molecular mass values of 48 000, 212 000, 380 000 and 1 600 000 were obtained from Showadenko (Shibadaimon, Minato-ku, Tokyo). A sample of dextran (DX) with the molecular mass of 60 000–90 000 was obtained from Wako Pure Chemicals (Dosho-machi, Osaka). These polysaccharide samples are used as markers for size exclusion chromatography. Naphthalene-1,3,6-trisulfonic acid trisodium salt (internal standard) was also obtained from Wako Pure Chemicals, and used without purification. All aqueous solutions were prepared using water purified with a Mili-Q purified system (Millipore). Other reagents and solvents used were of the highest grade commercially available.

2.2. Apparatus

A Waters Quanta 4000E apparatus (Waters Japan Co., Shinagawa, Tokyo) for high-performance capillary electrophoresis was used for all experiments. The operation temperature was maintained at 25°C to standardize reproducibility in repeated analyses. A fused-silica capillary (58 cm×75 μm I.D.) was also obtained from Waters. The polyimide coating at the

8-cm position from the end of the capillary was removed by burning the coating for a detection window. Detection was performed by monitoring the UV absorbance at 185 nm. The sample solution was introduced to the capillary by the hydrostatic method (10 cm height, 30 s). The analysis was performed by applying the potential of 20 kV. The untreated capillary used in the present work was conditioned prior to use with a sequential 5 min rinse of 0.1 *M* NaOH, a 3 min rinse with water and a 5 min rinse with the buffer.

2.3. Preparation of the running buffer containing a sample of neutral polysaccharide

The dissolution of PU or DX in the running buffer caused an increase in viscosity. Therefore, the running buffer containing a polysaccharide sample was kept for at least 12 h at room temperature after dissolution of the polysaccharide to form a homogeneous solution. Since the polysaccharide samples are available as highly purified products for size exclusion chromatography, the resultant solution was used without filtration after degassing the solution at reduced pressure for several min. An aqueous phosphate buffer (50 m*M*, pH 4.0) was used as the running buffer. The sample solutions were introduced from the cathodic end of the capillary.

2.4. Sample preparation

A sample of HA (250 μg) was dissolved in water (1.0 ml), and an aliquot (50 μl) was mixed with an aqueous solution (0.1%, 25 μl) of the internal standard (naphthalene trisulfonic acid trisodium salt; NTS). The mixture was kept in the refrigerator (4°C) until use.

2.5. Separation of HA-L on a column of Sephadex G-50

A sample of HA-L (15 mg) was dissolved in a small volume of an aqueous solution of sodium chloride (50 m*M*, 1 ml). The solution was applied on a column of Sephadex G-50 (100 cm length×1 cm I.D.) equilibrated with 50 m*M* NaCl solution. Each 2 ml fraction was collected while the absorbance was monitored at 230 nm. Fractions containing HA in the high molecular mass range (40–44 ml), those containing medium molecular mass range HA (45–56 ml) and those containing low molecular mass range HA (58–68 ml) were collected. Each fraction was dialyzed overnight against water and then lyophilized. Each fraction was numbered in the order of its molecular mass (see the inset in Fig. 6). The sample solution of each fraction for capillary electrophoresis was prepared as described in Section 2.4.

3. Results and discussion

3.1. Selection of the buffer for electrophoresis

Both acidic and basic buffer solutions are available as the running buffer for quantitative analysis of HA by capillary electrophoresis. Under basic conditions, HA introduced from the anode migrates toward the cathode with electroosmotic flow, overwhelming the movement by electrophoresis. Resolution among HA samples having different molecular mass values does not occur because all HA molecules have almost the same charge-to-molecular mass ratios. However, HA introduced from the cathode migrates toward the anode under acidic conditions in which carboxyl groups in the molecule are present as carboxylate anions. Neutral polymers in the running buffer do not move in the capillary at such acidic conditions, because the electroosmotic flow is very low. In the present paper, we describe our analysis of both the amount and the molecular mass of the HA samples used. To standardize the methods for these analyses, we employed an acidic buffer as the running buffer.

Fig. 2 shows the relationship between the relative migration velocity of the HA samples to that of the marker, NTS and the pH values of the running buffer.

Under acidic conditions, the samples should be introduced from the cathodic end due to the small electroosmotic flow. A direct determination of mobility is difficult under such acidic conditions. Therefore, we employed the relative migration velocity of the HA samples to that of NTS obtained by the following equation:

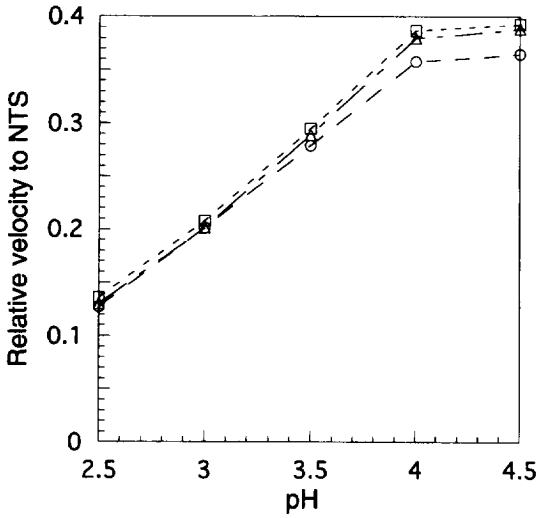

Fig. 2. Effect of pH of the running buffer on migration times of HA samples. (○) HA-L; (□) HA-M; (△) HA-H. Analytical conditions: capillary, fused-silica (58 cm×75 μm I.D.); running buffer, 50 m*M* phosphate buffer; applied voltage, 20 kV: detection, UV absorbance at 185 nm. NTS: naphthalene trisulfonic acid.

$$v = \frac{L}{T}$$

where v (cm/min) is the migration velocity, L is the effective length of the capillary (50 cm) and T is the migration time (min). Relative velocity was obtained by dividing the velocity of an HA sample by that of NTS. NTS (the internal marker) is a strong acidic compound. Its migration times were 4.72 min at pH 2.5 and 4.70 min at pH 4.0, respectively. The similar migration times at the pH values examined showed that NTS was fully ionized even at the low pH of 2.5. However, as shown in Fig. 2, a part of carboxyl groups of an HA sample was present as carboxylate anions at the low pH of 2.5, and its velocity relative to that of NTS was low. The relative velocities for HA-H and HA-M became almost constant ($\cong$0.38) at pH 4.0, and that for HA-L also became constant ($\cong$0.37) at pH 4.0. Therefore, we chose 50 m*M* phosphate buffer (pH 4.0) as the running buffer in the present study. A discussion of the reason for the slightly slower velocity of HA-L is presented later.

3.2. Calibration curves and reproducibility in quantitative determination of HA samples

Under the conditions described in Section 3.1, calibration curves showed good linearity in the range of 0.01 mg–3.3 mg/ml for HA-L and HA-M, and also in the range of 0.01 mg–1.6 mg/ml for HA-H. The linearity for each of these HA samples was expressed as $y=14.90x+0.029$ ($r=0.999$), $y=14.42x-0.024$ ($r=0.999$) and $y=18.09x+0.116$ ($r=0.995$), respectively; x is the concentration of an HA sample (w/v, %). The lower limit of detection was 1.0 μg/ml at the signal-to-noise ratio of 5. The sensitivity was about five times higher than that detected at 200 nm [8]. It was reported that with detection of the absorbance at 185 nm, sensitivity could be improved 10-fold [11]. Examples of the electropherograms at the lower limit of calibration are shown in Fig. 3.

Repeated analysis ($n=5$) of HA-L at the concentrations of 0.01, 1.7, and 3.3 mg/ml gave relative standard deviation values of 5.5%, 0.59% and 0.26%, respectively. The reproducibility of relative migration times was also excellent, with relative standard deviation less than 0.71%. Similar results were obtained in the analysis of HA-M. The slopes of the calibration curves for HA-L and HA-M were almost the same (14.90 and 14.92, respectively). The calibration curve for HA-H showed a slightly larger slope (18.09). Since the HA-H sample was provided as an aqueous solution for injection to the eye, the

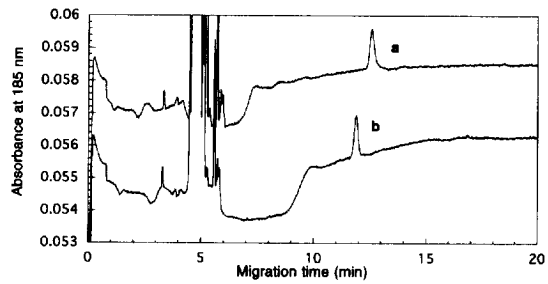

Fig. 3. Examples of electropherograms at the lower limit of calibration curves. (a) HA-L; (b) HA-H. Both are 10 μg/ml concentrations. The large peaks at 4.7 min are those of the internal standard (NTS).

suppliers definition of the concentration may be different from ours. Due to the extremely high sample viscosity, sample solutions of HA-H at concentrations of more than 1.5 mg/ml were not introduced quantitatively into the capillary.

3.3. Determination of the molecular mass of hyaluronic acid

3.3.1. Selection of a neutral polysaccharide for the formation of the entangled matrix

It is well known that the separation of double-stranded DNAs is based on their molecular sizes in the running buffer containing non-cross linked polymers such as hydroxyethyl cellulose [10,12]. Non-cross linked polymer molecules in the running buffer form an entangled matrix. When the solute molecules in the sample solution migrate through the matrix, they are fractionated according to their molecular mass.

In the present study, two kinds of neutral polysaccharides [dextran (DX) and pullulan (PU)] were examined as the neutral polymer, at 0.25% concentration. The standard samples of these polysaccharides are commercially available with a pre-determined molecular mass in a small range of molecular masses for size exclusion chromatography. Grossman and Soan reported that the concentration necessary to reach the entanglement state (the entanglement threshold) was about 0.3% for the solution of hydroxyethyl cellulose [13]. We therefore used a solution of 0.25% concentration. When DX (molecular mass 60 000–90 000) was examined as a matrix material, HA-H with the highest molecular mass, 1 500 000–2 100 000 was observed at 21.0 min, as shown in Fig. 4Aa.

HA-M was observed at almost the same migration times as those observed for HA-H. HA-L (molecular mass 40 000–60 000) migrated at a slower rate and was seen at 21.75 min (Fig. 4Ac). Conversely, the migration order was reversed when PU (molecular mass 48 000) was employed as the matrix material. HA-H migrated at a slower rate and was observed at 15.9 min (Fig. 4Ba). HA-L, however, was observed earlier (at 15.2 min) as a broad peak. These results are summarized in Table 1.

In the present analytical conditions where the electroosmotic flow is negligible, the total charge of a HA molecule, i.e., the sum of the numbers of carboxylate anions, is the driving force. The larger molecules of HA have greater numbers of carboxylate anions, and hence migrated faster in the absence of the matrix (see Fig. 2). Therefore, the faster migration velocity of HA-H at 0.25% of the DX concentration indicated that the size exclusion

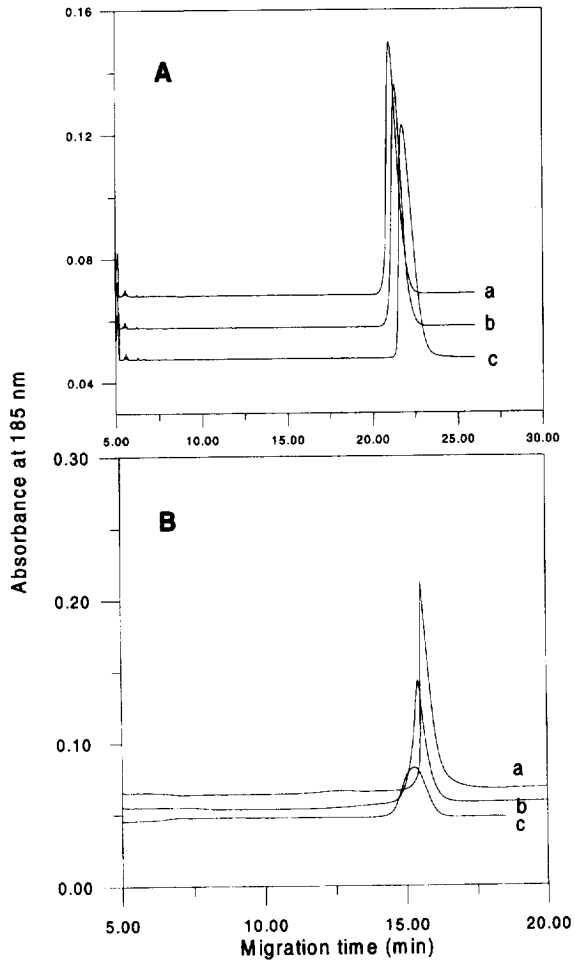

Fig. 4. Effects of DX (A) and PU (B) in the running buffer on migrations of HA samples. (A) DX (molecular mass 60 000–90 000) at 0.25% concentration; (B) PU (molecular mass 48 000) at 0.25% concentration. (a) HA-H; (b) HA-M; (c) HA-L for both (A) and (B). Other analytical conditions were the same as those in Fig. 2.

Table 1
Relative migration velocity and the number of the theoretical plates of hyaluronic acid samples in the presence of pullulan or dextran

Sample	Dextran (molecular mass, 60 000–90 000)		Pullulan (molecular mass, 48 000)	
	Relative velocity	NTP[a]	Relative velocity	NTP[a]
HA-L	0.237	3530	0.303	1400
HA-M	0.239	6130	0.294	5190
HA-H	0.245	5530	0.295	6960

[a] NTP: the number of theoretical plates. NTP was obtained from the following equation: $N=25(R_t/W)2$, where R_t is the migration time and W is the peak width at the 4.4% position of the peak height.

effect was slight. When PU (molecular mass 48 000) was used as a matrix substance at the same concentration, the three HA samples clearly migrated according to their molecular mass. Furthermore, the peak of HA-L, with a low molecular mass (40 000–60 000) became broad, as indicated by the number of theoretical plates (1400).

To achieve the size exclusion effect by using a buffer containing neutral polysaccharides, the concentration of the polysaccharide should be higher than the entanglement threshold concentration [13]. Though we did not measure the entanglement threshold of each polysaccharide solution in the present study, we started to examine the size exclusion effect of each polymer solution on the formation of the entangled matrix at 0.25% concentration, which is similar to that of the reported concentration of 0.3% for hydroxyethyl cellulose [13]. We found that the PU solution had a clear size exclusion effect on HA samples at this concentration, and that there was little effect when the DX solution was used. DX is a polymer with uniform α1→6 linked glycosidic linkages, and this linkage is flexible. In contrast, the maltotriose unit in a PU molecule has α1→4 glycosidic linkages which are more rigid than the α1→6 linkage [14]. It is likely that the structure composed of these alternating rigid and flexible linkages in a PU molecule is more favorable to form the entanglement matrix at a lower concentration. Because of the flexibility of DX molecules, higher concentrations may be required to reach the entanglement threshold. Based on these considerations, we employed PU as the polymer in the present work.

3.3.2. Effect of the molecular mass of PU on migrations of HA

The effects of the molecular mass of PU on the

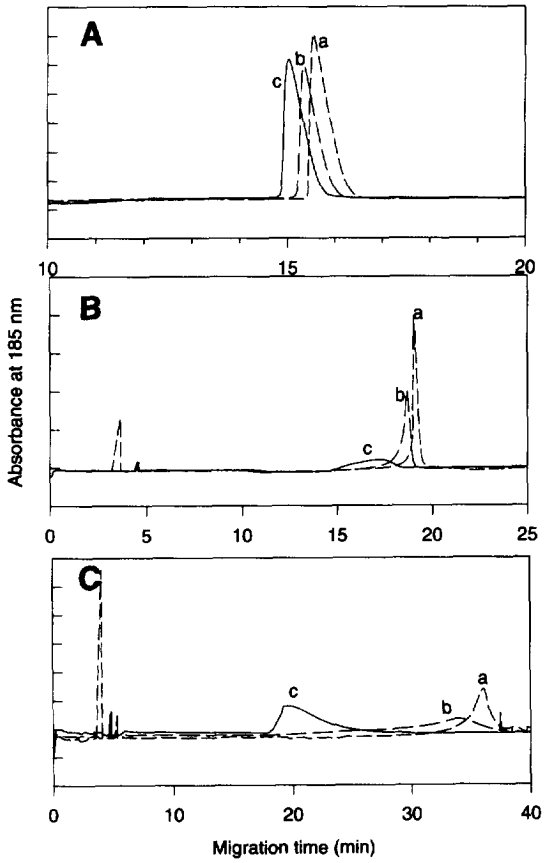

Fig. 5. Effect of the molecular mass of PU in the running buffer on migration of HA samples. (A) PU (molecular mass 92 000); (B) PU (molecular mass 380 000); (C) PU (molecular mass 1 600 000). The concentrations of all PU solutions were 0.50%. Other analytical conditions were the same as in Fig. 2. (a) HA-H; (b) HA-M; (c) HA-L for (A), (B) and (C). HA-H is a pharmaceutical preparation and contains an additive observed around 4 min. The small peak observed following the large peak is due to NTS (internal standard).

migrations of HA samples were examined by using several standard samples of PU of a specified molecular mass at 0.50% concentration. A few examples are shown in Fig. 5.

In the presence of PU with a small molecular mass (92 000), all of the HA samples gave symmetrical peaks around 15.5 min as shown in Fig. 5A. When PU (molecular mass 380 000) was used, the peak of HA-L became broad (Fig. 5Bc). HA-M and HA-H were observed at 18.6 min and 19.1 min, respectively, and were easily assigned from their relative migration times. However, they did not show any obvious peak broadening effect. In a solution of PU with the highest molecular mass (1 600 000), the peak of HA-M clearly broadened as shown in Fig. 5Cb. The peak broadening effect for HA-L and HA-H in this matrix was not as clear as that observed for HA-M.

Fig. 6 illustrates the peak broadening based on the size exclusion effect. HA-L was separated on a column of Sephadex G-50.

The elution profile is shown in the inset of Fig. 6. The molecular species with higher molecular mass values were eluted earlier and those with lower molecular mass were observed later by chromatography on a column of Sephadex G-50. The migration order was reversed when analyzed by capillary electrophoresis in the buffer with PU (molecular mass of 380 000) at 0.50% concentration (Fig. 6). These results indicated that the size exclusion effect was the major reason for the peak broadening, and also suggested that the selection of an appropriate PU preparation can allow the estimation of the distribution of the molecular mass.

3.3.3. Effect of the concentration of PU on migrations of HA

We also examined the effect of the concentration of PU on the migration of HA samples. Fig. 7 shows the effects of three concentrations of PU (molecular mass 380 000).

Even at a low concentration (0.25%) of PU, HA-L was observed as a broad peak (Fig. 7Ac). At 0.75% of PU, the peak of HA-L collapsed completely (Fig.

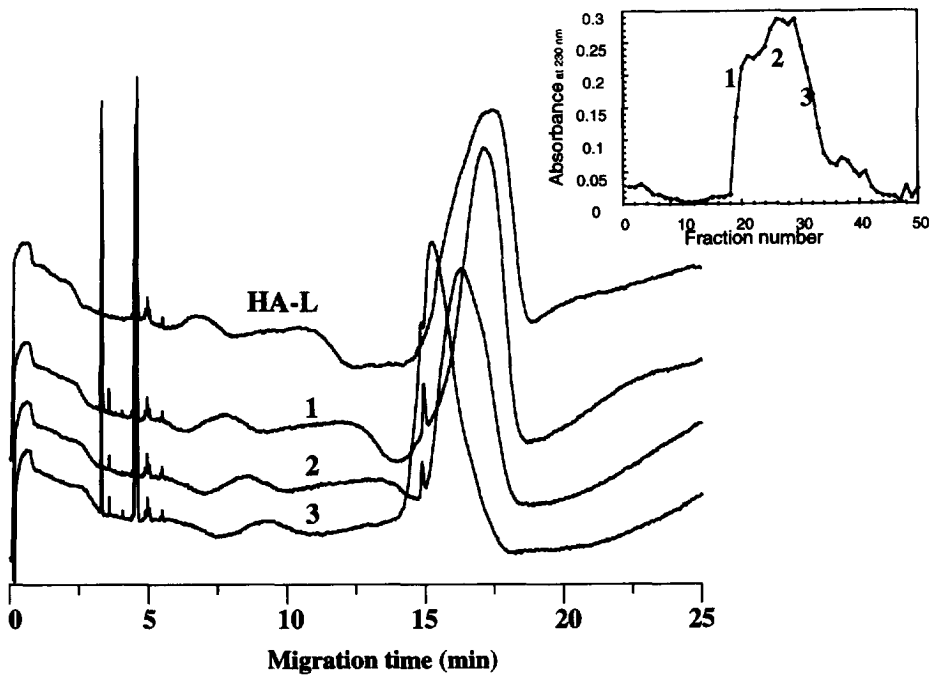

Fig. 6. Electropherograms of the fractions obtained from HA-L by chromatography on a column of Sephadex G-50. The inset shows the elution profile. Details were described in Section 2.5. Conditions for capillary electrophoresis: running buffer, 50 m*M* phosphate buffer (pH 4.0) containing PU (molecular mass 380 000) at 0.50% concentration.

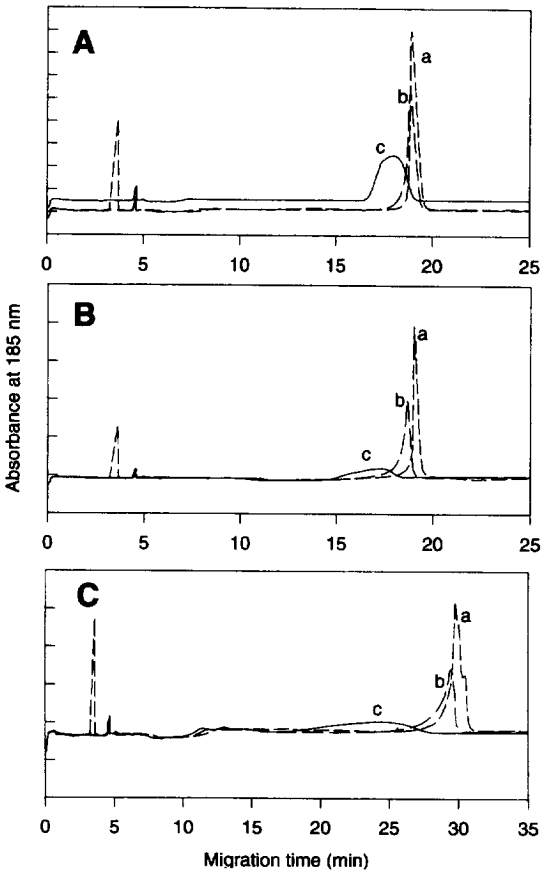

Fig. 7. Effect of the concentration of pullulan (PU) (molecular mass 380 000) in the running buffer. The concentrations of each PU solution are (A) 0.25%, (B) 0.50% and (C) 0.75%. Other analytical conditions were the same as in Fig. 2. (a) HA-H; (b) HA-M; (c) HA-L for (A), (B) and (C).

7Cc). The HA samples (HA-M and HA-H) with larger molecular mass values were observed at almost the same migration time (around 19 min) at 0.25% PU. However, HA-M migrated faster than HA-H at 0.50% PU, as shown in Fig. 7Bb. The peak broadening effect was not strong for HA-M and HA-H.

These results indicated that HA-L, which has a molecular mass 40 000 to 60 000, is in the fractionation range of the entangled matrix formed by PU (molecular mass 380 000) at the concentrations examined. However, the entangled matrix formed by PU (molecular mass 380 000) did not cause an obvious peak broadening effect in the analysis of HA-M and HA-H. This indicated that these HA samples could not be included in the matrix due to the dense structure of the matrix, and also suggested that a solution of an appropriate concentration of PU with a specified molecular mass might allow the size separation of an HA sample with a particular range of molecular mass.

This speculation was confirmed by observing the migration of HA samples using another preparation of PU (molecular mass 1 600 000). Fig. 8 shows the effect of the concentrations of PU at this molecular mass on the migration of HA samples.

The size exclusion effect was clearly seen in the more dilute solutions of PU. At the 0.050% concentration, the migration times of HA-L, HA-M and HA-H were 12.0 min, 13.8 min and 15.6 min, respectively. Each of the HA samples migrated according to its molecular mass and was easily identified from its migration time. Interestingly, peak broadening was not observed for HA-L, which had shown an obvious peak broadening in the buffer containing PU with the molecular mass of 380 000. The increase of the PU concentration also did not cause any marked peak broadening of HA-L. However, the peak shapes of HA-M were changed dramatically by the use of PU with the molecular mass of 1 600 000. Even at a solution of 0.050% of PU, the peak of HA-M became broad. At the 0.10% concentration solution, the peak of HA-M indicated that the sample contained two major molecular species of low molecular mass (a minor component) and high molecular mass (a major component), as shown in Fig. 8B. HA-H did not show any peak broadening at the 0.050% and 0.10% concentrations of PU. At the 0.20% concentration, HA-H showed an intense tailing peak. These results also supported the above discussion based on Fig. 7.

3.3.4. Relationship between migration velocities of HA samples and the concentrations of PU preparations

A plot describing the relationship between the logarithm of the relative migration velocities of the HA samples to that of NTS and the concentration of PU (molecular mass 380 000), is shown in Fig. 9. The plot is not a Ferguson plot, however, suggests that PU is working as entangled matrix.

HA-L, HA-M and HA-H showed good linearity

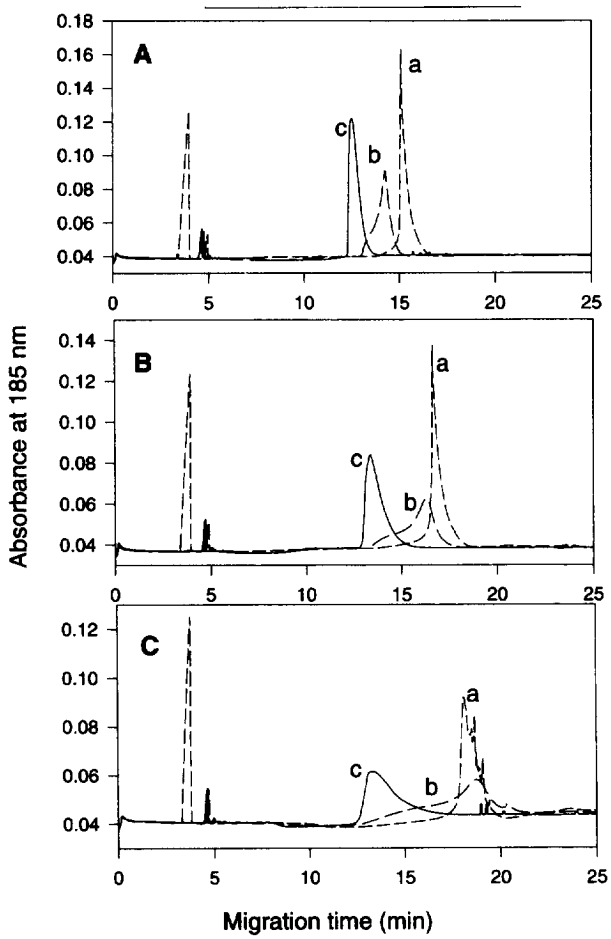

Fig. 8. Effect of the concentration of PU (molecular mass 1 600 000) in the running buffer. The concentration of PU is 0.050% in A, 0.10% in B and 0.20% in C. Other analytical conditions were the same as in Fig. 2. (a) HA-H; (b) HA-M and (c) HA-L for all panels.

(r=0.997, 0.995 and 0.983, respectively) to a concentration of 1.0% of PU.

The relationship between the logarithm of the relative migration velocities of the HA samples to that of NTS and the concentration of PU (molecular mass 1 600 000) is shown in Fig. 10.

Good linearity was observed for HA-H and HA-M in the relationship between the logarithm of the relative migration velocities to that of NTS and the concentrations of the PU solution. However, the relative velocities were almost constant for HA-L in the range of PU concentrations examined. These results indicated that the pore size of the entangled matrix formed by PU with high molecular mass (1 600 000) was very large. The molecular species of HA-L passed through the matrix independently with the increase of the concentration of PU.

4. Conclusions

HA samples with different molecular mass values were quantitatively determined by high-performance capillary electrophoresis in a bare fused-silica capillary using a slightly acidic buffer (pH 4.0) as the running buffer. Calibration curves showed good linearity with similar slopes from 0.01 mg/ml to 3.3 mg/ml for HA samples with different molecular mass distributions. The lower limit of detection was about 1 μg/ml.

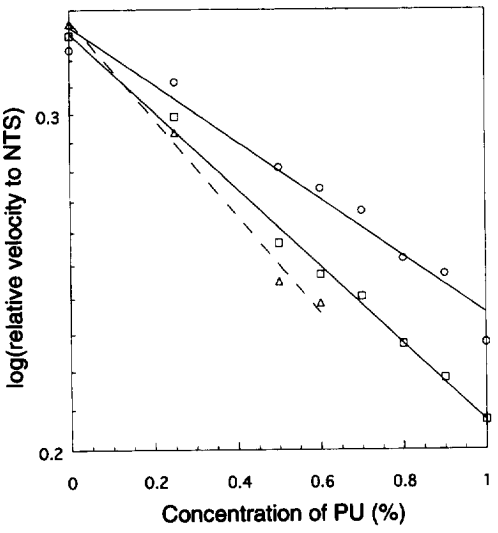

Fig. 9. Plot of log relative velocities of HA vs. PU (molecular mass 380 000). (○) HA-L; (□) HA-M; (△) HA-H. Relative velocity was calculated as described in Section 3.1.

In a buffer solution containing a PU preparation with a pre-determined molecular mass, the HA samples migrated based on their molecular mass. The relationship between the logarithm of the relative velocities of the HA samples and the concentrations of PU generally showed good linearity.

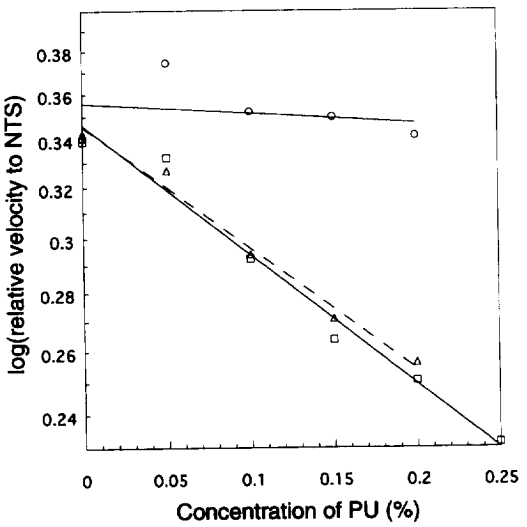

Fig. 10. Plot of log relative velocities of HA vs. PU (molecular mass 1 600 000). (○) HA-L; (□) HA-M; (△) HA-H. Relative velocity was calculated as described in Section 3.1.

Furthermore, the peak shapes of the HA samples were broadened markedly when a PU preparation with a pre-determined molecular mass at particular concentrations was used. The peak broadening was based on the size exclusion effect of the entanglement matrix of PU, as exemplified by using the HA sample from pig skin which was fractionated to three fractions on a column of Sephadex G-50. These results indicated that the effects of two kinds of size exclusion were observed in the capillary electrophoresis using an entangled polymer matrix: one is the regular size exclusion effect, e.g., the migration of HA-L in the solution of PU (molecular mass 380 000) as shown in Fig. 5b. In this case, the molecular species of HA-L can pass through the pore of the matrix. While passing through the matrix, the molecular species migrate at different velocities according to their molecular mass. We have to consider another mechanism for the migration of HA-H, which cannot be included in the matrix. Grossman and Soan suggested that a reptation model should be considered [13]. The reptation model assumes that large molecules behave like a snake; the head of the molecule "snakes" through the network. The migration of the HA-H molecules observed in the present study does not support such a model, because HA-H did not show an apparent molecular mass distribution under the present experimental conditions. The molecules seemed to be aggregated and to behave as a bulk of negative charges. However, there is as yet inadequate information about the molecular structure of HA in aqueous solution, and further studies are necessary to elucidate the mechanism of HA-H migration.

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