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Analysis of Double-Stranded DNA Fragments by Capillary Electrophoresis Using Entangle Polymer Solutions in Uncoated Fused Silica Capillary Columns

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DNA fragments (51–587 bp) were separated by capillary electrophoresis using entangled polymer, hydroxyethylcellulose, in uncoated fused silica capillary columns. The factors affecting the separation of DNA fragments with hydroxyethylcellulose media were evaluated, i.e., the concentration of buffer and entangled polymer, effects of additives (methanol, ethidium bromide, EDTA), temperature, and injection methods. Maximum performance was obtained by adding 5% methanol in 0.5% hydroxyethylcellulose solution at 30°C. Addition of methanol in polymer media increased the resolution of small size DNA fragments (< 100 bp). On the other hand, addition of ethidium bromide and EDTA, which are commonly used in conventional DNA separation, reduced the resolution of DNA fragments in the polymer solution. It turns out that the separation behavior of DNA in entangled polymer is more sensitive to the running condition compared to that in polyacrylamide gel-filled capillary, but the reproducibility of DNA separation in entangled polymer is reliable.

Keywords: Capillary electrophoresis, DNA separation, Entangled polymer, Hydroxyethylcellulose, Uncoated capillary.

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Introduction

The separation of double-stranded DNA fragments by capillary electrophoresis (CE) has received significant attention during the past decade because of its sensitivity, rapidity, and the possibility of automation. Capillary gel electrophoresis (CGE) is an attractive alternative to slab gel electrophoresis for DNA sequencing (Cohen *et al.*, 1987; Drossman *et al.*, 1990; Bianchi *et al.*, 1993; Lu *et al.*, 1994) and analysis of DNA fragments including polymerase chain reaction (PCR) products (Marino *et al.*, 1994; Williams *et al.*, 1994). However, the preparation of gel-filled capillary is delicate because of the formation of air bubbles and inhomogeneities during polymerization inside a capillary and the fragile nature of the medium. The reproducibility of CGE is dramatically diminished during a few runs. Numerous efforts to solve these problems have been presented by changing the gel matrix, agarose, cross-linked polyamylamide, and linear polyacrylamide. Despite the distinct advantage of high resolution for wide-range DNA fragments in cross-linked polyamylamide, alternative sieving materials are required for separating samples based on molecular sizes.

The use of low- to moderate-viscosity entangled polymer solution, instead of gels filled in capillary, as a sieving medium avoids these problems and permits the experimental procedures to be simplified. Unlike gel-filled capillary, CE using entangled polymer in running buffer is more stable and reproducible at higher electric field. Various entangled polymers were employed for DNA separations. Mixed poly(ethylene oxide) solution in uncoated capillary column has been used for DNA sequencing (Cheng and Yeung, 1995; Kim and Yeung, 1997) and poly(acryloylaminoethoxyethanol)-coated capillary was adopted for the separation of double stranded DNA fragments in the 100–4000 base-pair size range (Talmadge *et al.*, 1997). Of the various polymers in sieving

buffers, derivatized cellulose appears to be attractive because the low viscosities, water solubility, and nontoxicity of cellulose-containing buffers allow simple and high-speed separations. Without detailed studies on the differences between cellulose derivatives, several materials were employed for DNA separation. Methylcellulose (MC) has been used for the separation of small DNA fragments (20–587 bp) in siloxane-bonded polyacrylamide coated capillary (Crehan *et al.*, 1992) and for the separation of multikilobase length DNA in dilute MC solution with pulsed-field CE (Kim and Morris, 1994b). Hydroxypropylmethylcellulose (HPMC) was adopted for the separation of restriction fragments (Kim and Morris, 1994a) and for heteroduplex DNA polymorphism analysis (Cheng *et al.*, 1994). The poor resolutions with HPMC were observed because of high viscosity of the polymer solution. A systematic study of the separation of double-stranded DNA in hydroxypropylcellulose (HPC) was undertaken and a new mechanism of DNA migration in unentangled polymer was suggested (Mintnik *et al.*, 1995). Hydroxyethylcellulose (HEC) solution was employed as a separating matrix in capillary electrophoresis and the DNA separation mechanism in dilute and semi-dilute HEC solutions was studied, and it turns out that the interaction between DNA molecule and polymer below the entanglement threshold is an important factor for the separation (Grossman and Soane, 1991; Barron *et al.*, 1993). The DNA conformational change caused by the interaction between DNA and polymer molecule in HEC solution, both above and below the entanglement limit, was visualized with video microscopy (Shi *et al.*, 1995). The rapid sizings of the short tandem repeat alleles were presented by employing the HEC solution (Burtler *et al.*, 1994; Wang *et al.*, 1995).

Another problem is the internally bonded polyacrylamide coating of fused silica capillary tubes for DNA separation. This coated capillary decreases the reproducibility of separation because of variations of a bonded layer and the degradation of coating over several runs. It is another choice to use untreated fused silica capillary for DNA separation in entangled polymer instead of coated capillary.

In this study, we are presenting the separation of DNA fragments in HEC solution in uncoated capillary. The factors affecting the separation of DNA fragments in HEC solution were evaluated.

Materials and Methods

Chemicals Hydroxyethylcellulose (HEC) was purchased from Merck (Darmstadt, Germany) and DNA molecular marker V (pBR322/*Hae*III) from Boehringer Mannheim (Mannheim, Germany). Methyl cellulose (MC), hydroxypropylmethylcellulose (HPMC), ethidium bromide (EtBr), trizma base, and boric acid were obtained from Sigma (St. Louis, USA). Organic solvents including methanol were of HPLC grade and solutions for CE were prepared in triply distilled water.

Instrumentation Analysis of the DNA fragments was accomplished using a Model 270A capillary eletrophoresis system (Applied Biosystems, Foster City, USA). For the experiments, an untreated fused silica capillary (Polymicro Technologies, Phoenix, USA), 62.6-cm long (40 cm to the detector) × 50 μm I. D., was used as a separation column. On-column UV detection was measured at 260 nm, and the temperature of the column chambers was kept constant at 30°C unless otherwise specified. Prior to each run, the uncoated capillary was rinsed with 1 M sodium hydroxide and running buffer by the built-in vacuum system at 508 mmHg for 10 min. The sample was introduced at the anodic end of the capillary by the electrokinetic (5 kV, 30 s) or the vacuum system at 127 mmHg for 10 s. After the sample injection, the anodic end of the capillary was put into the running buffer with the anodic electrode and the electrophoretic voltage was applied to 25 kV (400 V/cm). The running buffer was TB (89 mM Tris, 89 mM boric acid, pH 8.3) containing various concentrations of HEC (0.1 ~ 0.5%) and additives. Negatively-charged DNA would remain at the anodic end of the capillary and strong electroosmotic mobility toward the cathode pushes DNA to the UV detector at the cathode end. Thus, the largest DNA fragment, which has the smallest electrophoretic mobility toward the anode, will pass the detector first, followed by the smaller ones in order of size.

Sample preparations The DNA samples used were DNA molecular weight marker, pBR322/*Hae*III digest at concentrations ranging from 10 to 100 mg/ml. PCR products (532 bp) were obtained by amplification of hepatitis B virus (HBV) surface region and analyzed with CE without any treatment except dilution with triply distilled water.

Results and Discussion

The separation of DNA fragments using HEC polymers instead of cross-linked polyamylamide gel matrix is a simple and convenient procedure. However, the optimization of DNA separation has to be done because the separation mechanism in a HEC solution is different from that in cross-linked gel matrix. The factors affecting the separation of pBR322/*Hae*III DNA fragments were examined by changing the polymer concentration, additives, and running conditions.

Effects of entangled polymer In order to separate DNA fragments with derivatized cellulose, various polymers including HPMC, MC, and HEC were examined. Of these, HEC was employed for this purpose because of its low viscosity, ease of handling, and good reproducibility. Figure 1 shows the separation of the *Hae*III digest of pBR322 DNA (51–587 bp) in uncoated fused silica capillary with 0.2%, 0.3%, 0.4%, and 0.5% HEC in TB running buffer. The peaks were identified by their decreasing peak area. When samples were introduced at

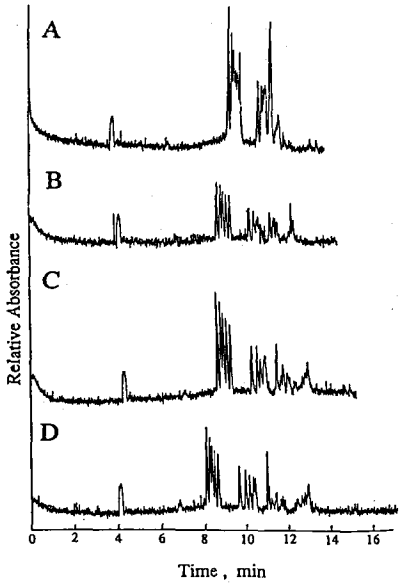

Fig.1. Capillary electrophoresis of pBR322/*Hae*III DNA fragments from 51 to 547 bp at different HEC concentrations: A, 0.2%; B, 0.3%; C, 0.4%; and D, 0.5% in 89 mM Tris-89 mM boric acid (1.0× TB, pH 8.3). CE condition: capillary, fused silica capillary (62.6 × 50 μm, to detector 40 cm); applied voltage, +25kV; temperature, 30°C; detection, 260 nm; peaks from front, 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124/123, 104, 89, 80, 64, 57 and 51 bp.

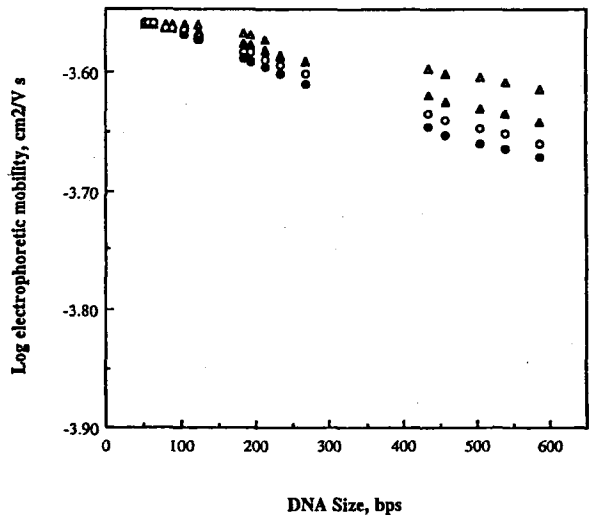

Fig. 2. Logarithmic plot of the electrophoretic mobility vs DNA sizes (bp) for various HEC concentrations based on the results obtained from Fig. 1. HEC concentrations are 0.2%, △; 0.3%, ▲; 0.4%, ○; 0.5%, ●.

the anodic end of the capillary, negatively-charged DNA would remain at the anodic end and strong electroosmotic flow toward the cathode makes the DNA molecule mobilize to the UV detector at the cathode. The largest DNA fragment, which has the smallest electrophoretic mobility to the anode, will pass the detector first, followed by the smaller ones in order of size. The electrophoretic mobility toward the anode was decreased by raising the HEC concentration, even though the electroosmotic mobility was constant regardless of HEC concentration. The sieving ability of HEC for various sizes of DNA fragments was dependent upon the polymer concentration. Below the entangle threshold (Φ^* of HEC is 0.21%) (Barron *et al.*, 1993), the poor resolution for medium size DNA fragments (51–587 bp) was obtained, and raising the HEC concentration by 0.5% provided better resolution for the small fragments (< 124 bp). Therefore, the migration behavior of DNA fragments is usually adjusted by changing the polymer concentration. In 0.2% HEC, small fragments less than 124 bp were not resolved as shown in Fig. 2, because the electrophoretic mobility of these fragments are the same. When increasing the HEC concentration, the resolution of small fragments (< 124 bp) is rapidly improved because a proper size of small sieves for short DNA separation is formed (Fig. 2). Although 0.5% HEC was best for resolution for this range of DNA, 0.4% HEC was used mainly for further studies without any additives because high viscosity of 0.5% HEC solution makes it difficult to inject the samples and precondition the capillary with buffer containing polymer.

Effects of injection method The method of sample introduction was important for the better resolution as shown in Fig. 3. It is known that electrokinetic injection at the cathode in coated capillary is appropriate for negatively-charged DNA. With uncoated capillary, it is thought that hydrodynamic injection at the anode is necessary because electrokinetic injection of DNA at the anode is difficult owing to the negative electrophoretic mobility. However, hydrodynamic injection using viscous HEC solution as running buffer introduced the small amount of samples as shown in Fig. 3A. Contrary to the prediction, electrokinetic injection at the anode was possible because of the strong electroosmotic mobility (Fig. 3B). The sample introduced by electrokinetic injection was stacked by following hydrodynamic H_2O injection, and the sharp peaks were achieved (Fig. 3C). The combined electrokinetic and hydrodynamic injection was used for further studies.

Effects of buffer and additives In contrast to the rigid cross-linked polyacrylamide gel matrix, the resolution of DNA fragments in entangled polymer solution is easily affected by buffer composition or additives. The effect of TB buffer concentration on the separation of DNA fragments in HEC solution is shown in Fig. 4. As the buffer concentration was increased, the migration time and the resolution were increased. By lowering the buffer concentration to 0.5×, the resolution completely disappeared. At 1.0×, the resolutions of DNA fragments

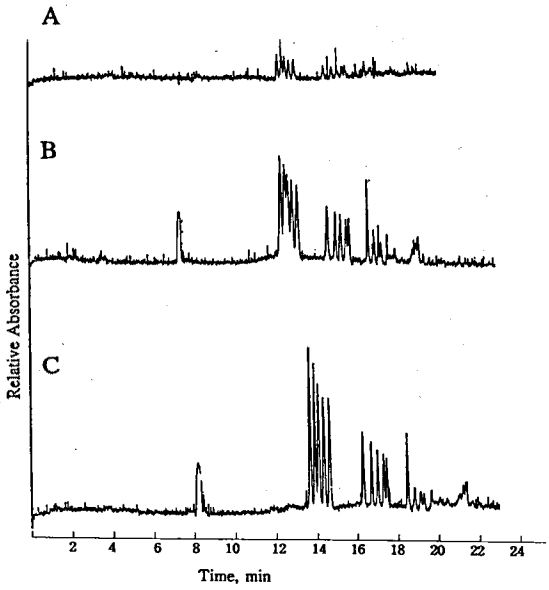

Fig. 3. Effect of the injection method on the separation of DNA fragments. Running buffer was 1.0× TB (pH 8.3) containing 0.5% HEC; A, hydrodynamic injection with built-in vacuum system at 127 mmHg for 15 s; B, electrokinetic injection in anode at +5 kV applied voltage for 30 s; C, electrokinetic injection (same as B) was followed by hydrodynamic injection of H_2O at 5 mmHg for 15 s. Other conditions were as in Fig. 1 except for the capillary length (86 cm × 50 μm, to detector 60 cm).

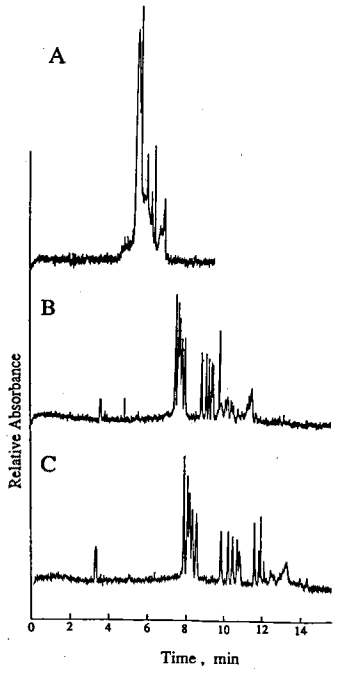

Fig. 4. Effect of TB buffer (pH 8.3) concentration on the separation of DNA fragments : A, 0.5× TB; B, 1.0× TB; C, 1.5× TB buffer containing 0.4% HEC. Other experimental conditions were as in Fig.1.

smaller than 100 bp were improved, and the resolution was abolished again for smaller fragments at 1.5×. Thus, 1.0× TB was used for further study. Alteration of electroosmotic flow resulted from changing the ionic strength of running buffer affects the resolution of DNA fragments in entangled polymer. It could be explained that increase of electroosmotic flow by lowering ionic strength of the buffer might abolish the small electrophoretic differences depending on the DNA size in the polymer solution.

The separations of DNA have been performed on conventional gel electrophoresis using TB or TBE buffer indistinguishably. However, EDTA in TB buffer diminished the resolution of DNA fragments as shown in Fig. 5. The migration times were increased and the bands of small fragments (<260 bp) were dramatically broadened upon raising the EDTA concentrations in running buffer. This phenomenon can be explained by the fact that electrophoretic mobility of DNA fragments toward the cathode is rapidly increased by chelating Mg^{++} or cations in DNA with EDTA. Chelation of divalent cations with EDTA affects the electrophoretic mobility of small DNA fragments (<250 bp) further. Therefore, TB running buffer containing 0.4% HEC without EDTA addition was employed for further studies.

The dependence of DNA intercalating agent, EtBr, on the migration behavior of DNA fragments is shown in Fig. 6 for 0.4% HEC. It is known that the addition of DNA intercalating agent raises the detection sensitivity of DNA in UV or fluorescence detector and alters the migration pattern (Kim and Morris, 1994). In this experiment, as EtBr concentration was raised, the migration time decreased and the dramatic decay of the resolution occurred for five large DNA fragments (587, 540, 504, 458, 434 bps), while improvements for short fragments (<124 bp) was obtained. These changes might be originated from decreases in electrophoretic mobility of EtBr-intercalating DNA fragment, because the electroosmotic mobility over the various EtBr concentrations was nearly constant. EtBr inserted between stacked DNA base pairs causes the unwinding of the helix and shielding of the negative changes of DNA. The mobility difference of EtBr-intercalated large DNA was diminished by the structural changes of DNA fragments. These decrease the electrophoretic mobility of EtBr-intercalated DNA fragments and the resolution of the large DNA fragment was almost completely lost. In contrast to the prediction, optimum separation could be achieved without the DNA intercalating agent EtBr.

In order to resolve the small DNA fragments (<100 bp) in entangled HEC solution, several attempts have been made by changing additives (urea, methanol) and the running temperature. The addition of methanol to 0.5% HEC polymer solution improved, the resolution of small fragments (<124 bp) as shown in Fig. 7. The better

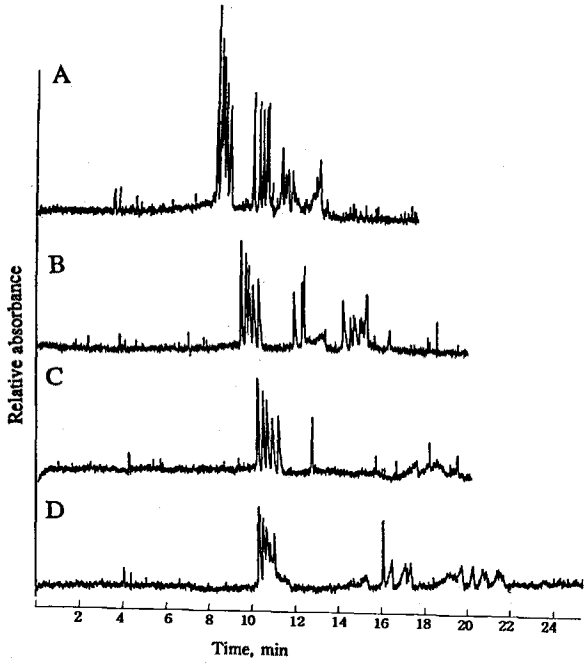

Fig. 5. Effect of EDTA in running buffer (1.0× TB containing 0.4% HEC) on the separation of DNA fragments: A, no EDTA; B, 1 mM EDTA; C, 2 mM EDTA; D, 3 mM EDTA in running buffer. Other experimental conditions were as in Fig. 1.

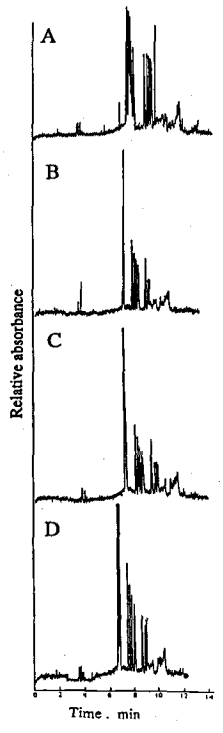

Fig. 6. Effect of EtBr on DNA fragment separation in 1.0× TB containing 0.4% HEC. The concentration of EtBr was varied from 0 to 3 μM: A, no EtBr; B, 1 μM EtBr; C, 2 μM EtBr; D, 3 μM EtBr. Other experimental conditions were as in Fig. 1.

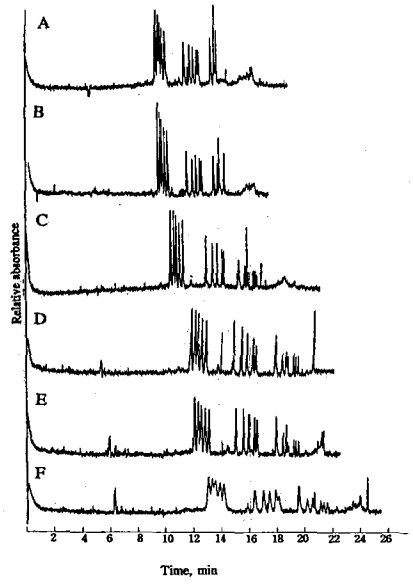

Fig. 7. Capillary electrophoresis of pBR322/*Hae*III DNA fragments on 0.5% HEC entangled polymer solution containing various concentration of methanol: A, 0%; B, 2.5%; C, 5.0%; D, 7.5%; E, 10.0%; F, 12.5%. Other experimental conditions were as in Fig. 1.

resolution was demonstrated by raising the methanol concentration to 7.5%, but the resolution decayed beyond 7.5% methanol. This arises from a decrease of electroosmotic flow and an increase of electrophoretic mobility of DNA fragments by adding methanol in the buffer. The decrease in electroosmotic flow following the addition of methanol has been previously discussed (Lee *et al.*, 1993) and is associated with the change in the zeta potential that arises from the changes in viscosity and the dielectric constant of the buffer. The reduction in the thickness of the compact layer next to the interface might be another factor responsible for the decrease in the electroosmotic flow. On the other hand, the electrophoretic mobilities toward the anode end, which is the reverse flow of the electroosmotic flow, (presented in Fig. 8) depended on the added methanol concentration. A part of the electrophoretic mobility decrease could arise from the increase of viscosity in the presence of methanol. Another factor reducing the electrophoretic flow was the decreasing dielectric permittivity of the water–methanol as the percentage of methanol increased. A lower dielectric permittivity would favor the protonated neutral DNA in the equilibrium between the negatively-charged DNA and protonated neutral DNA. Such a decrease in the concentration of negative-charged DNA would possibly reduce the electrophoretic mobility toward the anode. A high resolution of DNA fragments was obtained by the reduction of electroosmotic and electrophoretic flow with up to 7.5% methanol; however, the separation efficiencies were dramatically decreased with further addition of

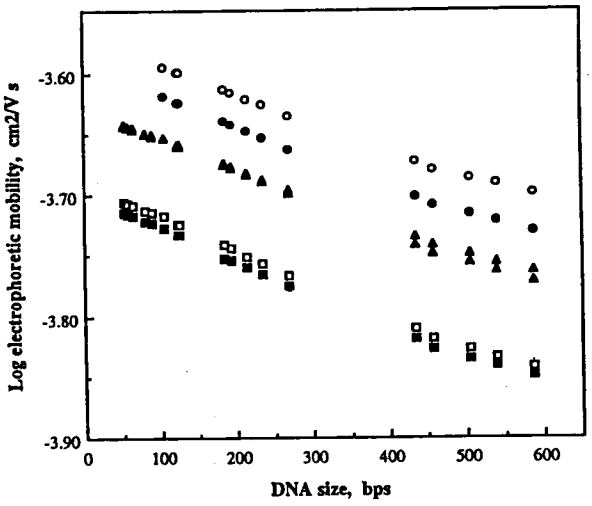

Fig. 8. Logarithmic plot of the electrophoretic mobility vs. DNA molecular size (bp) for different methanol concentrations based on the results obtained from Fig.7. Methanol concentrations were varied: ○, 0%; ●, .2.5%; △, 5.0%; ▲, 7.5%; □, 10.0%; ■, 12.5%.

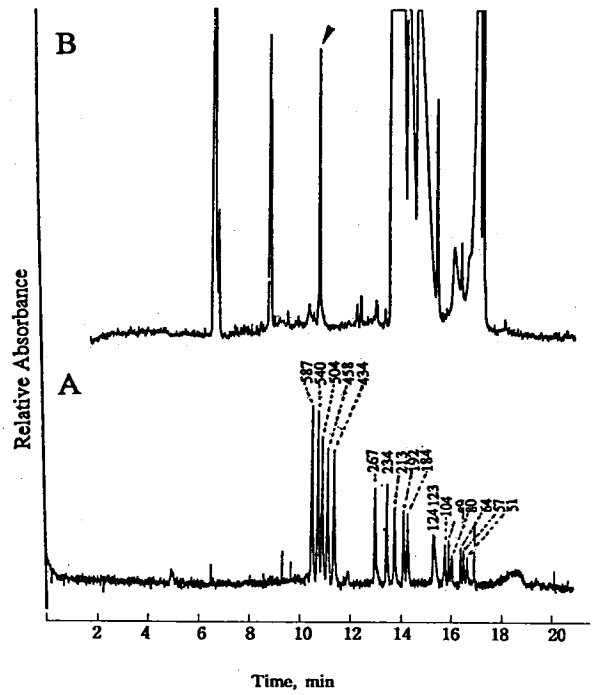

Fig. 9. A. Capillary electrophoresis of pBR 322/*Hae*III DNA fragments at the most optimum separation. Capillary and experimental conditions were the same as in Fig. 1 with running buffer (1.0× TB) containing 0.5% HEC and 5.0% methanol, with combined electrokinetic and hydrodynamic injections as in Fig. 3. B. Electropherogram of PCR product (532 bp) obtained from the amplification of HBV surface region. Experimental conditions are the same as in Fig. 9A. The arrow head indicates the PCR product (532 bp).

methanol. As shown in Fig. 8, the electrophoretic mobility based on DNA sizes were nearly parallel depending on the methanol concentration. The explanation could be that the sieve size of HEC entangled polymer was not changed by the addition of methanol. The optimum separation of DNA fragments ranging in size from 51 bp to 587 bp with the best resolution and the separation efficiency was achieved with 1.0× TB buffer (pH 8.3) containing 0.5% HEC polymer and 5% methanol at an applied voltage of +25 kV. Under these condition, the 89- and 80-bp fragments, and 64-, 57-, 51-bp fragments are completely resolved, although it was impossible to separate single base differences between 123- and 124-bp fragments with this method (Fig. 9).

An important factor affecting the reproducibility and the resolution of DNA fragments was the temperature, which could alter the viscosity of the solution and other parameters. At lower than 20°C, the viscosity of the polymer solution was too high to handle, and at 45°C and 60°C, the resolution of DNA fragments was completely lost and the baseline became unstable (data not shown). This could arise from the change of pore size and viscosity in HEC entangled polymer solution by raising the temperature. However, the reliable separations were obtained at 30°C, therefore maintaining the exact temperature of capillary and sample vials was critical for obtaining the precise results. We assessed the precision of the method by repeated analysis of DNA fragments. As shown in Table 1, the coefficient of variations (C.V.) of the migration time were less than 1.0% for the within-run precision. In this optimum condition, the real sample obtained by PCR amplification of hepatitis B virus (HBV) surface region was analyzed without any treatment except

Table 1. Intraday reproducibility of migration time of DNA fragments ranging in size from 587 to 51 bp.

DNA size, bp	migration time (min) (mean ± SD., n = 5)	C.V.[a] (%)
587	9.472 ± 0.046	0.486
540	9.643 ± 0.049	0.511
504	9.777 ± 0.049	0.501
458	9.955 ± 0.050	0.498
434	10.152 ± 0.049	0.485
267	11.419 ± 0.056	0.487
234	11.778 ± 0.061	0.515
213	12.013 ± 0.062	0.518
92	12.260 ± 0.066	0.536
184	12.377 ± 0.067	0.542
124/123	13.167 ± 0.084	0.640
104	13.435 ± 0.093	0.696
89	13.571 ± 0.111	0.821
80	13.601 ± 0.131	0.964
64	13.922 ± 0.123	0.886
57	14.018 ± 0.128	0.913
51	14.120 ± 0.095	0.674

[a]C.V.: coefficiency of variation.

dilution with triply distilled water. The peak of PCR product (532 bp), was detected in the predicted position, even though the unreacted primers and dNTPs appeared as huge sizes of bands. If the smaller fragments (<200 bp) is needed to be separated, the pretreatment for removing the unreactants should be done by ultrafiltration or dialysis.

To optimize the separation of DNA fragments (51–587 bp) using low-viscosity HEC entangled polymer in uncoated capillary column, factors affecting the separation were systematically examined by changing buffer and polymer concentrations, adding additives in the running buffer (EDTA, EtBr, and methanol), altering the sample injection procedure, and raising the running temperature. It appears that the resolutions of certain DNA fragments are dependent on the entangled polymer concentrations; raising the polymer concentration increases the resolution of small DNA fragments. The injection method is critical for the sample introduction because of the high viscosity of running buffer containing the polymer. Combined electrokinetic injection with the hydrodynamic method provided the better resolution. Addition of methanol raised the separation of small DNA fragments because methanol in buffer decreases the electroosmotic and electrophoretic mobility. EDTA and EtBr in running buffer diminished the separation efficiency and the resolution. Running temperature was more critical for the reproducibility of the separation in the entangled polymer than the CE only in the buffer solution. Maximum performance for the separation of DNA fragment size ranging from 51 bp to 587 bp was achieved by adding 5% methanol in 0.5% HEC solution at 30°C in uncoated capillary column. The possibility of using this protocol for analyzing the PCR products was confirmed by examining the reproducibility of the method and applying it for analyzing the PCR product of the HBV surface region.

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