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Comparative study of non-porous anion-exchange chromatography, capillary gel electrophoresis and capillary electrophoresis in polymer solutions in the separation of DNA restriction fragments

Chinuyo Sumita, Yoshinobu Baba*, Kayoko Hide, Naomi Ishimaru, Kazuko Samata, Atsuko Tanaka and Mitsutomo Tsuhako

Kobe Women's College of Pharmacy, Kitamachi, Motoyama, Higashinada-ku, Kobe 658 (Japan)

ABSTRACT

HPLC using a 100-mm column packed with a non-porous anion exchanger gave high-resolution separations of DNA restriction fragments up to 8000 base pairs (bp). High-resolution separation of DNA restriction fragments up to 12 000 bp was achieved using capillary gel electrophoresis (CGE) and capillary electrophoresis (CE) in entangled polymer solutions. These methods were compared with respect to their performance and efficiencies in the resolution of DNA restriction fragments. The resolving power of CGE is higher than those of the other techniques. The 50–100 bp resolution of DNA fragments up to 500 bp was realized using HPLC, 50 bp resolution using CE in entangled polymer solutions and 10 bp resolution using CGE. The plate number that was achieved with CGE of $3 \cdot 10^6$ plates/m was higher than those of HPLC of $1 \cdot 10^6$ and CE in polymer solutions of $7 \cdot 10^5$ plates/m. The advantages and limitations of HPLC and capillary electrophoretic techniques are discussed.

INTRODUCTION

During the past decade, high-performance liquid chromatography (HPLC) has been developing rapidly as a technique for separating mixtures of DNA fragments [1], because the availability of numerous separation columns makes HPLC an attractive alternative to conventional methods. Among the several novel packing materials developed for the separation of DNA fragments, a non-porous microparticulate (<5 μm) anion exchanger [2] has shown a high resolving power for DNA restriction fragments [3–7] and oligonucleotides in single-base resolution [8–11]. Potentially, non-porous anion-exchange chromatography offers rapid separation and determination when combined with reliable area integration. More recently, capillary electrophoresis (CE) has become an important technique for high-resolution and high-speed separations of DNA fragments. DNA restriction fragments of up to 12 000 base pairs (bp) and polynucleotides of up to 500 bases can be separated with high efficiency by capillary gel electrophoresis (CGE) [7,11–14]. Additionally, CE in entangled polymer solutions is effective for the high-resolution separation of DNA restriction fragments up to 12 000 bp [7,15,16].

As HPLC, CGE and CE in polymer solutions have their own advantages and limitations, they should each have suitable specific applications. Previously, we compared HPLC with CGE with respect to their performance in the single-base resolution of oligonucleotides [11]. In this study, we examined the performance and efficiencies of non-porous anion-exchange chromatography in comparison with CGE and CE in polymer solu-

* Corresponding author.

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tions with respect to the resolving power and the speed of separation of DNA restriction fragments up to 12 000 bp. For this purpose, we investigated the suitability of a laboratory-made cross-linked polyacrylamide gel-filled capillary [12–14] for CGE, a laboratory-made linear polyacrylamide coated-capillary [7,15,16] for CE in entangled polymer solutions and a commercially available column for HPLC, namely a TSKgel DEAE-NPR non-porous anion-exchange column [2,5,8] in the separation of DNA restriction fragments.

EXPERIMENTAL

Chemicals

The DNA samples used were obtained as follows: a 1-kbp DNA ladder (1.0 μg/μl) from Gibco (Tokyo, Japan); a pBR322 DNA-*Hae III* digest (1 unit) from Sigma (St. Louis, MO, USA); and a ΦX174 DNA-*Hae III* digest (0.24 μg/μl) from Toyobo (Osaka, Japan). The DNA samples were diluted to *ca.* 0.1 μg/μl with water purified in a Milli-Q system (Millipore) and stored at −18°C until used. The 1-kbp DNA ladder contains 23 fragments of 75, 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, 1636, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10 180, 11 198 and 12 216 bp. The pBR322 DNA-*Hae III* digest contains 22 fragments of 8, 11, 18, 21, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540 and 587 bp. The ΦX174 DNA-*Hae III* digest contains eleven fragments of 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 bp. Methylcellulose (4000 cP) was purchased from Sigma. All other chemicals were of analytical-reagent or electrophoretic grade from Wako (Osaka, Japan).

HPLC equipment, column and eluents

An LC-800 HPLC system (Jasco, Tokyo, Japan) was used for the separation of DNA fragments. A TSKgel DEAE-NPR column (35 mm × 4.6 mm I.D.) purchased from Tosoh (Tokyo, Japan) contains an anion exchanger based on a non-porous polymer support, having a particle diameter of 2.5 μm. A 100-mm long TSKgel DEAE-NPR column was donated from Tosoh. The eluents were (A) 0.25 *M* sodium chloride in 20 m*M* tris(hydroxymethyl)-aminomethane (Tris)–HCl buffer (pH 9.0) and (B) 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0). Sample solution (10–20 μl) was injected into the column and chromatographed at a flow-rate of 1.0 ml/min (35-mm column) or 0.5 ml/min (100-mm column). The column temperature was kept constant (30, 40, 50 and 60°C) to within 0.2°C. DNA fragments were detected at 260 nm.

Capillary electrophoresis

CGE separations were carried out by using an Applied Biosystems Model 270A capillary electrophoresis system. Polyimide-coated fused-silica capillaries (375 μm O.D., 100 μm I.D.; GL Sciences, Tokyo, Japan) of 30 cm effective length and 50 cm total length were used. Gel-filled capillaries, in which polyacrylamide gel is chemically bonded to the capillary wall, were prepared according to the literature [14] with slight modifications as follows. The polyimide coating was removed at a distance of *ca.* 20 cm from the outlet end. The capillary was rinsed with 1 *M* NaOH solution for 15 min and with Milli-Q-purified water for 30 min by using a vacuum injection system [13]. A solution of 0.4% 3-methacryloxypropyltrimethoxysilane in 6 m*M* acetic acid was continuously passed through the capillary for 10 min. The capillary was left for 1 h at 40°C and then rinsed with Milli-Q-purified water for 10 min. The acrylamide solution (3%T, 0.5%C) was prepared in a buffer solution (100 m*M* Tris–borate–2 m*M* EDTA, pH 8.3) and carefully degassed in an ultrasonic bath for 5 min. Polymerization is initiated with the addition of both 80 μl of 10% N,N,N′,N′-tetramethylethylendiamine (TEMED) solution and 20 μl of 10% ammonium peroxodisulphate solution into 5 ml of acrylamide solution. The polymerizing solution was quickly introduced into the capillary for 5 min. Polymerization in the capillary was completed within 5 h at 40°C. Sample solution was introduced into the capillary electrophoretically at a negative polarity of 5 kV for 5 s. Gel-filled capillaries were run with the buffer solution described above at negative polarity of 13 kV (260 V/cm, 15–25 μA) at 25°C. DNA fragments were detected at 260 nm.

CE separations in entangled polymer solutions were carried out by using a Waters Quanta 4000 capillary electrophoresis system. Polyimide-coated fused-silica capillaries (375 μm O.D., 100 μm I.D.; GL Sciences) of 42 cm effective length and 50 cm total length were used. Linear polyacrylamide-coated capillaries were prepared according to the literature [16]. The buffer was 50 m*M* Tris–borate–2.5 m*M* EDTA–0.5% or 0.7% methylcellulose (pH 8.0). Sample solution was introduced into the capillary electrophoretically at negative polarity of 5 or 10 kV for 10 s. CE in entangled polymer solutions was performed using linear polyacrylamide-coated capillaries with the buffer solution at a negative polarity of 10 kV (200 V/cm, 18–20 μA) at room temperature (23–25°C). DNA fragments were detected at 254 nm.

RESULTS AND DISCUSSION

Non-porous anion-exchange chromatography of DNA restriction fragments

Fig. 1 shows a chromatogram of pBR 322 DNA-*Hae III* restriction fragments obtained by HPLC using a 35-mm TSKgel DEAE-NPR column. The separation was performed by gradient elution at 40°C. The gradient and column temperature were optimized by preliminary experiments with the aid of an HPLC computer simulation system [17–19]. Multi-linear gradient elution as shown in Fig. 1 gave a better solution of large DNA fragments than linear gradient elution, whereas both elution techniques separated completely shorter DNA fragments. When the column temperature was changed for optimization, some DNA fragments could not be separated at low temperature (30°C). Separation at high temperatures (50 and 60°C) required longer analysis time, whereas the resolution of DNA fragments was similar to that in Fig. 1. The DNA fragments were separated approximately in proportion to the chain length under the optimum conditions in Fig. 1 and identified according to the literature [4,5]. The fragments of 8 and 11 bp could not be identified owing to their very low detectability. The peaks of the 123, 192, 458 and 504 bp fragments are eluted slightly later than expected from their chain lengths, because they are A-T-rich fragments [5].

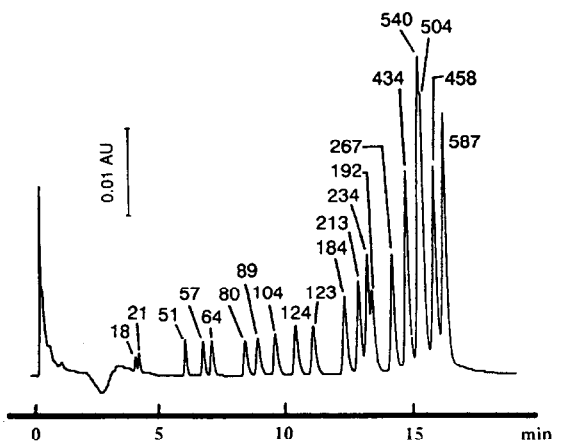

Fig. 1. HPLC separation of pBR322 DNA-*Hae III* digest. Column: TSKgel DEAE-NPR (35 mm × 4.6 mm I.D.). Eluents: (A) 0.25 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0) and (B) 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0). Gradient programme: 0–3 min from 27 to 34% B, 3–60 min from 34 to 100% B at 40°C. Flow-rate: 1.0 ml/min (70 kg/cm^2). Detection: 260 nm.

Fig. 1 illustrates that under optimum conditions eleven fragments ranging from 18 to 184 bp are baseline resolved and all the other components except for the fragments of 192 and 504 bp are almost completely separated. The separation of twenty kinds of the DNA fragments was completed within 17 min. The plate number of each peak was estimated to be $(4–6)\cdot 10^4$ $[(1.1–1.7)\cdot 10^6$ per metre]. The resolution, R_s, was calculated to be in the range 0.2–1.5. The reproducibility of the retention time was in the range 1–2% relative standard deviation (R.S.D.) ($n = 5$).

Fig. 2a shows chromatograms of the 1-kbp DNA ladder with the 35-mm column under the same conditions as in Fig. 1. The DNA fragments were identified according to the literature [5] and the peaks of 201 and 1018 bp fragments were eluted later than expected, for the same reason as described above. The mixture of DNA fragments ranging from 75 to 517 bp was separated almost completely. Some peaks of DNA fragments ranging from 2036 to 8144 bp appeared, but were poorly resolved. Large fragments around 10 kbp in length could not be resolved. The separation was completed within 20 min. We tried to improve the resolution of

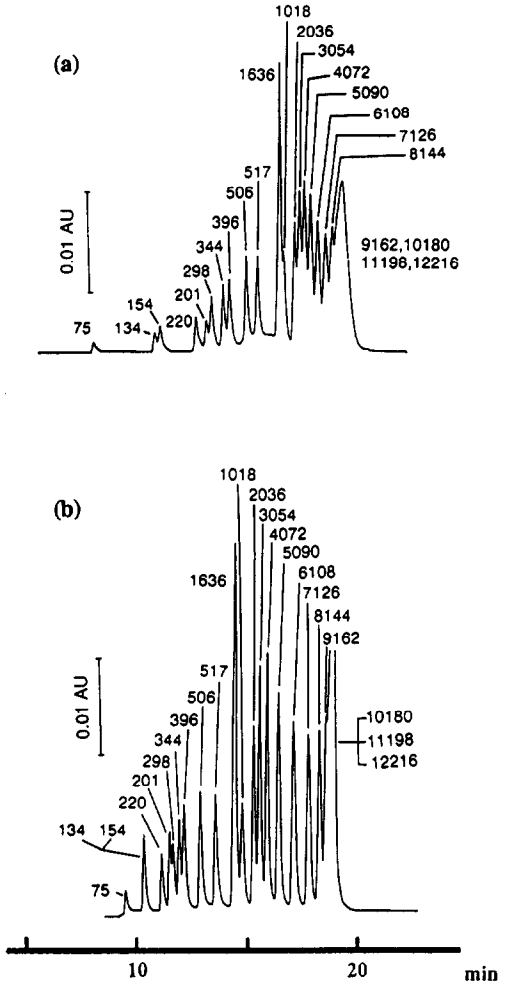

Fig. 2. (a) HPLC separation of a 1-kbp DNA ladder using the 35-mm column. Separation conditions as in Fig. 1. (b) HPLC separation of a 1-kbp DNA ladder, using the 100-mm column. Column: TSKgel DEAE-NPR (100 mm × 4.6 mm I.D.). Eluents A and B as in Fig. 1. Gradient programme: 0–47 min from 50 to 100% B at 50°C. Flow-rate: 0.5 ml/min (100 kg/cm^2). Detection: 260 nm.

large fragments of more than 2000 bp by varying the column temperature and the gradient elutions, but could not achieve a better resolution. The plate number, resolution and reproducibility were almost the same as those in Fig. 1.

We then used a longer column in order to obtain a better resolution of large fragments. The resolution of DNA fragments ranging from 1018 to 8144 bp was greatly improved by using a 100-mm TSKgel DEAE column under the same separation conditions as in Fig. 2a, but a very long time was required for the analysis of all fragments. The gradients were optimized to obtain the rapid and complete resolution of large fragments, instead of the loss of the resolution of short fragments up to 396 bp. A linear gradient with an increasing ratio of eluent B to A at the initial gradient stage was found to be effective in minimizing the analysis time and maximizing the resolution of large fragments, as shown in Fig. 2b. The large fragments ranging from 2036 to 8144 bp were almost completely resolved within less than 20 min, whereas the resolution of short fragments ranging from 134 to 396 bp was inferior to that in Fig. 2a. The plate number, which was $(0.4–1)\cdot 10^5$ [$(0.4–1)]\cdot 10^6$ per metre] using the 100-mm column, was slightly higher than that achieved with the 35-mm column, because the values of the plate number per metre were very similar to each other.

Capillary electrophoresis of DNA restriction fragments

Fig. 3 shows an electropherogram for the 1-kbp DNA ladder obtained with the linear polyacrylamide-coated capillary using 0.5% methylcellulose at 200 V/cm. The electrophoretic conditions were optimized according to the literature [16]. The resolving power of 0.5% methylcellulose solution was adequate for the rapid and

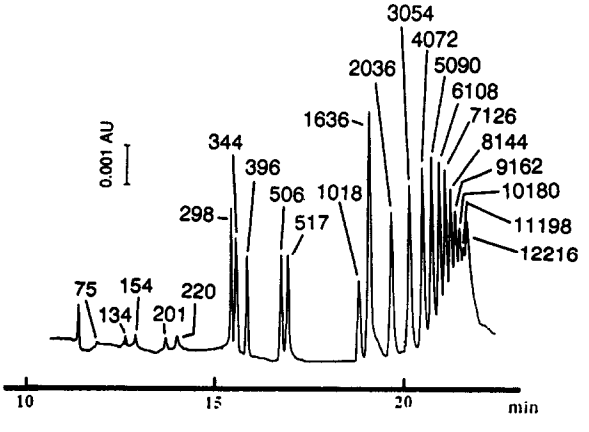

Fig. 3. Separation of a 1-kbp DNA ladder by CE in polymer solutions. Linear polyacrylamide-coated capillary, 100 μm I.D., 375 μm O.D., total length 50 cm, effective length 42 cm. Running buffer: 50 m*M* Tris–borate–2.5 m*M* EDTA–0.5% methylcellulose (pH 8.0). Field: 200 V/cm. Current: 20 μA. Injection: 5 kV for 10 s. Detection: 254 nm.

complete separation of these DNA fragments. The fragments were identified by their sizes in base pairs, and the assignments agreed with the reported separation of the 1-kbp DNA ladder [14,15]. Fig. 3 demonstrates that the separation of individual ladder fragments is excellent. DNA fragments ranging from 75 to 4072 bp are baseline resolved and larger fragments ranging from 5090 to 12 216 bp are almost resolved under the conditions given, yet the separation was completed in less than 24 min. The plate number of each peak was estimated to be $3 \cdot 10^5$ ($7 \cdot 10^5$ per metre). R_s was in the range 0.8–2.0. The reproducibility of the migration time was in the range 1–2% R.S.D. ($n = 5$).

Fig. 4 illustrates the separation of the 1-kbp DNA ladder sample by CGE with a cross-linked polyacrylamide gel (3%T and 0.5%C)-filled capillary at 260 V/cm. The separation conditions were optimized in several preliminary experiments with differing gel composition, electric field and capillary size. The fragments were identified by their sizes in base pairs, and the assignments agreed with the reported separation of a 1-kbp DNA ladder [14]. Fig. 4 clearly demonstrates that DNA fragments ranging from 75 to 4072 bp are baseline resolved and larger fragments ranging from 5090 to 12 216 bp are almost resolved, yet the separation was completed within 15 min. The plate number was $(4–6) \cdot 10^5$ [$(1.3–2.0) \cdot 10^6$ per metre]. R_s for each band was in the range 0.5–2.0. The reproducibility of the migration time was in the range 2–4% R.S.D. ($n = 5$).

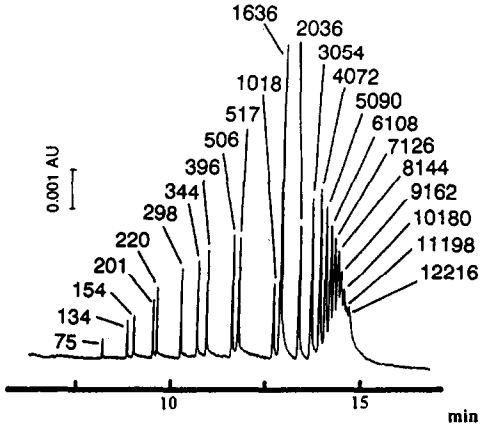

Fig. 4. CGE separation of a 1-kbp DNA ladder. Capillary: 100 μm I.D., 375 μm O.D., total length 50 cm, effective length 30 cm. Running buffer: 100 m*M* Tris–borate–2 m*M* EDTA (pH 8.3). The gel contained 3% T and 0.5% C. Field: 260 V/cm. Current: 20 μA. Injection: 5 kV for 5 s. Detection: 260 nm.

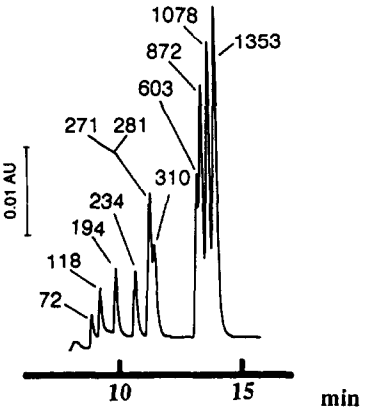

Fig. 5. HPLC separation of a ΦX174 DNA-*Hae III* digest. Separation conditions as in Fig. 2b.

Comparison of the resolving powers of the techniques

Figs. 5–7 illustrate comparisons of these techniques in the separation of a mixture of DNA restriction fragments (ΦX174 DNA-*Hae III* digest) including a critical pair of 271 and 281 bp fragments, which differ in length by only 10 bp.

HPLC separation (Fig. 5) with the 100-mm column gave a baseline resolution of short frag-

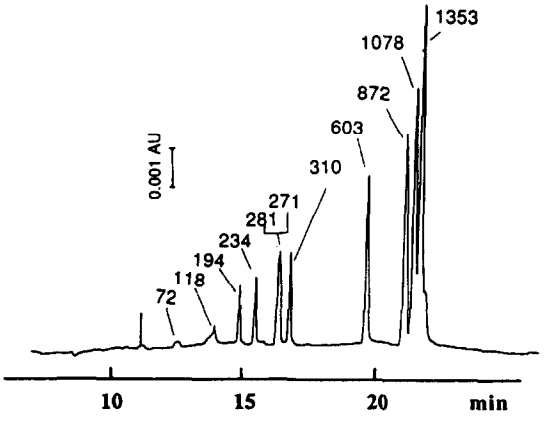

Fig. 6. Separation of a ΦX174 DNA-*Hae III* digest by CE in polymer solutions. Running buffer: 50 m*M* Tris–borate–2.5 m*M* EDTA–0.7% methylcellulose (pH 8.0). Injection: 10 kV for 10 s. Other conditions as in Fig. 3.

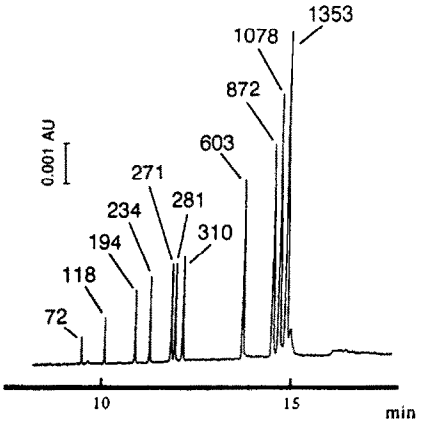

Fig. 7. CGE separation of a ΦX174 DNA-*Hae III* digest. Separation conditions as in Fig. 4.

ments ranging from 72 to 234 bp, but no separation between the 271 and 281 bp fragments was obtained. Some peaks of large fragments ranging from 603 to 1353 bp were separated. A further increase in the resolution was not achieved even by changing the separation conditions. CE separation (Fig. 6) in the entangled polymer solution achieved almost complete resolution of all fragments. No separation between the 271 and 281 bp fragments was obtained, however, in spite of the high resolution. A further improvement in resolution was reported to be achieved by the addition of ethidium bromide to the running buffer [10]. The CGE separation (Fig. 7) gave an excellent resolution of all fragments and two fragments of 271 and 281 bp, which differ by only 10 bp and were not separated by the other two techniques (Figs. 5 and 6), were baseline resolved. Overall, Figs. 5–7 demonstrate that the CGE technique gives a superior performance to the other techniques in the separation of the critical pair of 271 and 281 bp fragments.

HPLC offers a rapid separation of DNA fragments, as shown in Figs. 1, 2 and 5. Especially the 100-mm column appears very suitable for high-resolution separations of DNA fragments. For example, DNA fragments up to 500 bp are baseline resolved when two fragments differ by 50–100 bp. Larger fragments ranging from 2000 to 8000 bp can be separated completely when there is sufficient difference in their size in base pairs (1000 bp). However, HPLC cannot achieve a 10 bp resolution of the fragments of 271 and 281 bp and it is difficult to obtain complete 200–300 bp resolution of large fragments ranging from 600 to 1300 bp, as shown in Fig. 5. The resolving power of CE in polymer solution is slightly higher than that of HPLC. The 20–50 bp resolution of DNA fragments up to 500 bp was achieved, as shown in Figs. 3 and 6. Additionally, large fragments ranging from 1000 to 4000 bp are resolved almost completely, when two fragments differing by 150–1000 bp and larger fragments up to 12 000 bp are separated. CE in polymer solution and HPLC failed to resolve the critical pair of fragments of 271 and 281 bp, as shown in Fig. 6. Of these techniques, CGE offers the highest resolving power of DNA restriction fragments, as shown in Figs. 4 and 7. All fragments up to 5000 bp are baseline resolved when two fragments differ by 10–500 bp, and even two fragments of 271 and 281 bp were completely resolved from each other, as shown in Fig. 7. Larger fragments ranging 6000 to 12 000 bp were separated.

The resolving power of these techniques can be evaluated from their plate numbers. The plate number in HPLC with the 100-mm column is comparable to that of CE in polymer solutions, and that of CGE is higher than those of the other techniques. The plate number per metre of HPLC $[(0.4–1)\cdot 10^6]$ is higher than that of CE in polymer solution $[(5–7)\cdot 10^5]$. The use of a 1000-mm column, therefore, is expected to realize high-resolution separations of DNA fragments by HPLC, but leads to severe damage of the packing material, because a very high pressure drop (*ca.* 1000 kg/cm^2) may be applied in elution even at 50°C and a flow-rate of 0.5 ml/min. In contrast, longer capillary is easily available in CE techniques and will accomplish higher resolution of DNA fragments. These results clearly illustrate that the resolving power of CGE is the highest of these techniques.

We have found that CGE and CE in entangled polymer solutions were suitable for the rapid separation of a wide base pair range of DNA fragments and the resolving power of CGE is higher than that of CE in entangled polymer solutions. However, the use of linear polyacrylamide-coated capillaries and polymer solu-

tions as the molecular sieving medium avoids the difficulties associated with the preparation and use of gel-filled capillaries, such as bubble formation during polymerization and electrophoresis [12]. Additionally, the resolving power of CE in entangled polymer solutions can be improved by the combined use of polymer and ethidium bromide.

Although the resolving power of CGE cannot be matched by non-porous anion-exchange chromatography at present, HPLC has some advantages with respect to quantification and fractionation. HPLC offers accurate quantification when combined with reliable integration, whereas accurate quantification by CE techniques remains a problem. Additionally, HPLC is the only choice for preparative work on DNA fragments, because HPLC is suitable for large-scale preparative separations.

In conclusion, the results suggest that HPLC, CGE and CE in polymer solutions are equally sufficient for the high-resolution separation of DNA restriction fragments. These techniques may be a preferred alternative to slab gel electrophoresis and are applicable to the high-speed analysis of polymerase chain reaction (PCR) products and the rapid and high-precision restriction mapping of DNA fragments.

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