Journal of Chromatography A, 653 (1993) 329–335
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 404

High-resolution separation of DNA restriction fragments by capillary electrophoresis in cellulose derivative solutions

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(First received March 16th, 1993; revised manuscript received June 17th, 1993)

ABSTRACT

The performance and the efficiency of several cellulose derivatives as a molecular sieving agent for the capillary electrophoretic separation of DNA restriction fragments were investigated. All fragments up to 12 000 base pairs (bp) in the 1-kbp DNA ladder were resolved using linear polyacrylamide-coated capillaries filled with a buffer solution containing 0.5% cellulose derivative and the separation was completed within 17 min. High-concentration (0.7%) cellulose derivative solutions are effective for the complete separation of small fragments (50–1000 bp) of a *Hin*cII and a *Hae*III digest of ΦX174 DNA. A plate number of $0.5–1 \cdot 10^6$ plates per metre was achieved. The migration time and the resolution of DNA fragments were manipulated by varying several parameters, such as the size (viscosity) and the concentration of cellulose derivatives and the applied field strength. Some guidelines are presented for choosing these parameters, depending on the size of the DNA fragments being separated.

INTRODUCTION

Slab gel electrophoresis is the standard method used to separate, identify and purify DNA fragments and is successfully applied to the mapping of double-stranded DNA restriction fragments, polymerase chain reaction (PCR) analysis, restriction fragment length polymorphism (RFLP) analysis and Southern blotting [1,2]. Although slab gel electrophoresis is simple and capable of resolving a broader molecular weight range of DNA fragments, the technique is time-consuming, labour-intensive and non-quantitative.

Capillary electrophoresis (CE) is an automated technique offering the benefits of rapid separation and high resolution of DNA restriction fragments [3,4]. DNA restriction fragments of 100–120 000 base pairs (bp) are separated within 20 min by capillary gel electrophoresis (CGE) using capillaries filled with cross-linked polyacrylamide gel [5–7] and linear polyacrylamide [5]. High-speed and high-resolution separation of DNA restriction fragments can be performed by using CE in entangled polymer solutions [6–16]. The performance of a few hydrophilic polymers has been tested in the separation of DNA fragments [6–16]. Very little, however, is known about the effect of changes in the structure and physical properties of the hydrophilic polymers on the electrophoretic behaviour of DNA fragments in the capillary.

In this paper, we investigate the use of several types of cellulose derivatives, whose basic structure is shown in Fig. 1 and features are listed in Table I, as entangled polymers for the CE separation of double-stranded DNA restriction fragments in the size range 100–12 000 bp. We examined the effect of the structure and the physical properties of cellulose derivatives on the resolving power of CE in cellulose derivative

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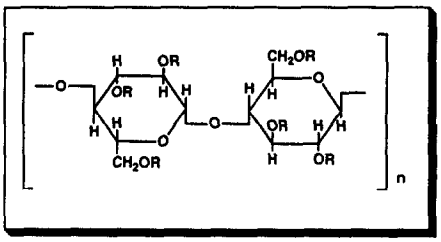

Cellulose Derivative

Methyl Cellulose	—H	—CH_3	
Hydroxypropyl Methyl Cellulose	—H	—CH_3	—$CH_2CH(OH)CH_3$
Hydroxypropyl Cellulose	—H	—$CH_2CH(OH)CH_3$	

Fig. 1. Structure of cellulose derivatives.

solutions, *i.e.*, how wide a range of molecular mass of DNA fragments can be separated with high resolution. For this purpose, we prepared and used capillaries in which linear polyacrylamide was chemically bound to the capillary inner surface by using a bifunctional reagent such as 3-methacryloxypropyltrimethoxysilane [17]. The reduction in the electroosmotic flow by capillary wall deactivation was effective for high-performance separation of a complex mixture of DNA fragments due to improved resolution and reproducibility [8,10,11,15].

EXPERIMENTAL

Chemicals

The DNA restriction fragments of a ΦX174 DNA/*Hae*III digest (0.24 μg/μl) and a ΦX174 DNA/*Hin*cII (0.29 μg/μl) digest were purchased from Toyobo (Osaka, Japan). A 1-kilo base pair (kbp) DNA ladder (1.0 μg/μl) was obtained from Gibco BRL (Tokyo, Japan). The ΦX174 DNA/*Hae*III digest contained eleven fragments of 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1357 bp. The ΦX174 DNA/*Hin*cII digest contained thirteen fragments of 79, 162, 210, 291, 297, 335, 341, 345, 392, 495, 612, 770 and 1057 bp. The 1-kbp DNA ladder contained 23 fragments of 75, 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, 1636, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198 and 12216 bp. The DNA samples were diluted ten-fold with Milli-Q water and stored at −18°C until use. Cellulose derivatives (Fig. 1) listed in Table I were a gift from ShinEtsu Chemicals (Tokyo, Japan). 3-Methacryloxypropyltrimethoxysilane was purchased from ShinEtsu Chemicals. All other chemicals were of analytical-reagent or electrophoretic grade from Wako (Osaka, Japan).

Preparation of linear polyacrylamide-coated capillary and running buffer

Polyimide-coated fused-silica capillaries (375 μm O.D., 100 μm I.D., Polymicro Technologies, Phoenix, AZ, USA) of 42.5 cm effective length and 50 cm total length were used. Linear polyacrylamide-coated capillaries were prepared as previously described [17] with slight modification. The capillary was leached by passing 1 *M* NaOH solution for 15 min by using a vacuum injection system [18], and subsequently rinsed with Milli-Q water for 15 min and acetonitrile for 15 min. A solution of 0.4% 3-methacryloxypropyltrimethoxysilane and 0.4% acetic

TABLE I

CELLULOSE DERIVATIVES USED FOR THE ENTANGLED POLYMER IN CAPILLARY ELECTROPHORESIS

Cellulose	Abbreviation	Viscosity[a] (cP)	Methyl group (%)	Hydroxypropyl group (%)
Methyl cellulose	MC-4000	4290	30.0	0
	MC-8000	7980	29.8	0
Hydroxypropyl cellulose	HPC-11000	11000	0	63.3
Hydroxypropyl methyl cellulose	HPMC-4000a	4550	27.8	5.6
	HPMC-4000b	4390	29.0	9.3
	HPMC-15000	15900	28.7	5.8

[a] Viscosity of a 2% cellulose aqueous solution measured at 20°C.

acid in acetonitrile was continuously passed through the capillary for 1 h. The capillary was then rinsed with acetonitrile for 15 min and Milli-Q water for 15 min. The acrylamide solution (3% T and 0% C[a]) was prepared in a buffer solution [50 m*M* tris(hydroxymethyl)-aminomethane (Tris), 50 m*M* boric acid and 2.5 m*M* EDTA] and carefully degassed in an ultrasonic bath for 10 min. Solutions of 10% (v/v) N,N,N′,N′-tetramethylethylenediamine (TEMED) and 10% (w/v) ammonium peroxydisulphate were prepared freshly. Polymerization was initiated by the addition of 20 μl of ammonium peroxydisulphate solution and 80 μl of TEMED solution into 5 ml of acrylamide solution. The polymerizing solution was quickly passed through the capillary for 10 min and left to polymerize for 30 min. The capillary was then rinsed with Milli-Q water for 15 min to remove polyacrylamide that had not reacted with the silanol group on the capillary inner surface.

Running buffer used in this study was 50 m*M* Tris–borate, 2.5 m*M* EDTA and an appropriate amount of cellulose derivative (pH 8.3) and prepared as follows (because it took a very long time to dissolve the cellulose derivative when it was added to water directly). Cellulose derivative (0.5 g) was added to *ca.* 40 ml of hot Milli-Q water (>70°C) with stirring. After the mixed solution became a hot aqueous slurry, 20 ml of a buffer solution (250 m*M* tris–borate and 12.5 m*M* EDTA) were added to the hot aqueous slurry. The aqueous slurry was cooled to below 20°C, which resulted in an aqueous solution. The resultant solution was diluted to 100 ml with Milli-Q water.

Apparatus

CE separations in cellulose derivative solutions were carried out by using a Waters Quanta 4000 capillary electrophoresis system. The DNA fragment mixture was introduced electrophoretically at negative polarity of 10 kV for 10 s into the capillary and separated by a linear polyacrylamide-coated capillary filled with running buffer at negative polarity of 10–20 kV (200–400 V/cm, 18–22 μA) at room temperature (24–27°C). Electrokinetic injection was used for the separation because this technique yielded a more efficient separation than pressure injection [11]. DNA fragments were detected at 254 nm.

[a] C = g N,N′-methylenebisacrylamide (Bis)/% T; T = (g acrylamide + g Bis)/100 ml solution.

RESULTS AND DISCUSSION

Fig. 2 shows examples of the separations of the 1-kbp DNA ladder using the linear polyacrylamide-coated capillary filled with buffer solution including 0.5% cellulose derivatives (MC-4000, HPMC-4000a and HPMC-4000b) which have similar viscosity but are different in the structure and composition of the alkyl

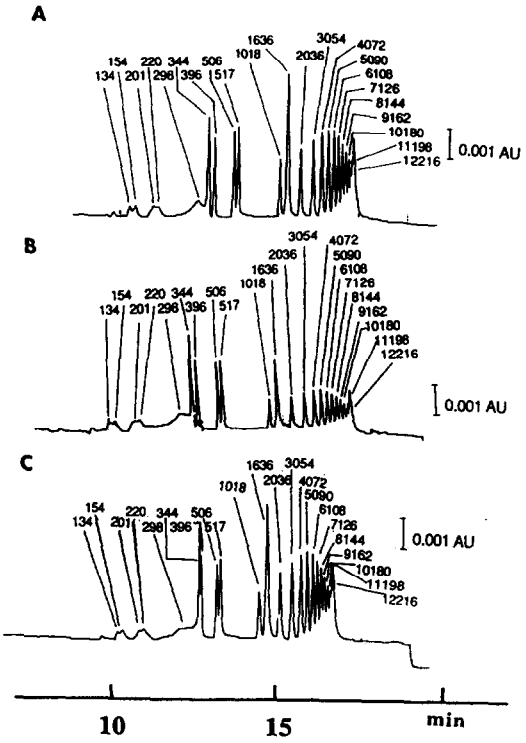

Fig. 2. CE separation of the 1-kbp DNA ladder. Linear polyacrylamide-coated capillary: 100 μm I.D., 375 μm O.D., total length 50 cm; effective length 42.5 cm. Running buffer: 50 m*M* Tris–borate, 2.5 m*M* EDTA and 0.5% cellulose derivative (pH 8.3). Cellulose derivative: (A) MC-4000, (B) HPMC-4000a and (C) HPMC-4000b. Field: 200 V/cm. Current: 18 μA. Injection: 10 kV for 10 s. Temperature: room temperature. Detection: 254 nm. Resolved fragments are labelled by their size in base pairs.

groups, as listed in Table I. The 1-kbp DNA ladder provides a good reference for the examination of the resolving power of the polymer solutions because it covers a broader molecular weight range of DNA fragments. The fragments were identified by their sizes in base pairs, the assignments agreeing with the reported separation of the 1-kbp DNA ladder obtained in a polyacrylamide gel-filled capillary, where peaks were assigned by spiking with slab gel isolated fragments [5]. The peak for DNA of 75 bp could not be assigned owing to very low detectability.

It can be seen in Fig. 2 that all separations of individual ladder fragments, obtained at 200 V/cm, are excellent and provide results very similar to those previously reported using cross-linked gel-filled capillaries [5–7], capillaries filled with a methylcellulose solution [6,7,10,15] and capillaries filled with an agarose solution [14]. The mixture of DNA fragments ranging from 134 to 8144 bp is baseline resolved; larger fragments ranging from 9162 to 12216 bp are almost completely resolved, and yet the separation was completed within only 17 min. Additionally, the 506- and 517-bp pair, differing by only 11 bp, which are usually not separated on the slab gel electrophoresis [5,10], was completely resolved. The plate number of each peak in each separation was estimated to be in the range $0.5–1 \cdot 10^6$ plates per metre. The results in Fig. 2 illustrate that a change in the structure and the composition of alkyl groups on cellulose derivatives does not affect the performance and the efficiency of the separation of DNA fragments.

Fig. 3 illustrates the separations of the 1-kbp DNA ladder fragments using cellulose derivatives (MC-8000, HPC-11000 and HPMC-15000) with different viscosities. The efficiency of DNA separations under the conditions used can be compared with that in other media, as shown in Fig. 2. The representative cases of Fig. 3A and B show that the separation is equivalent to that obtained using cellulose derivatives of relatively low viscosity. However, poor resolution of DNA fragments was obtained using HPMC-15000, as shown in Fig. 3C. We tried to improve the resolution of DNA fragments by changing the concentration of HPMC-15000, but did not achieve a better resolution. In addition, HPMC-15000 buffer is inadequate for the entangled polymer solution, because it is too viscous to be easily loaded into the capillary.

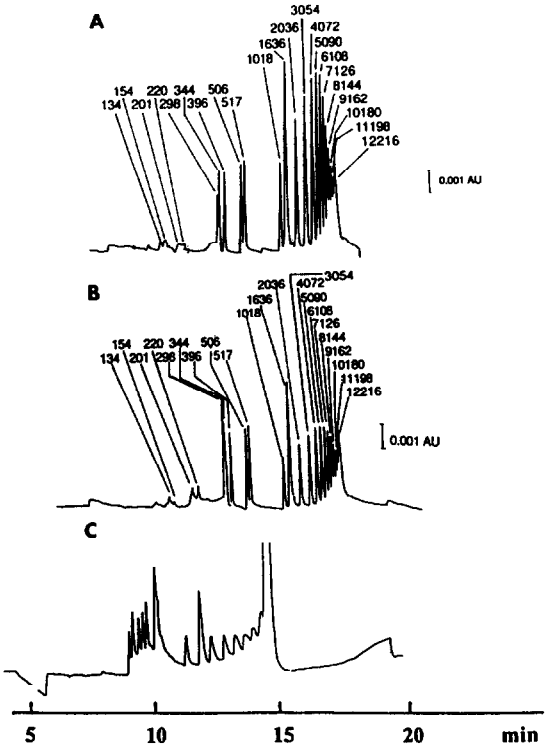

Fig. 3. Effect of the viscosity of cellulose derivative on the CE separation of the 1-kbp DNA ladder. Cellulose derivative: (A) MC-8000, (B) HPC-11000 and (C) HPMC-15000. Other conditions as in Fig. 2.

The influences of the concentration of cellulose derivatives on the separation of the 1-kbp DNA ladder fragments are shown in Figs. 4 (HPMC-4000b) and 5 (HPC-11000). CE in 0.1% HPMC-4000b cellulose solution failed to resolve each fragment of the 1-kbp DNA ladder (Fig. 4A), but some peaks of DNA fragments were separated using 0.3% HPMC-4000b, as shown in Fig. 4B. The 0.7% HPMC-4000b cellulose solution (Fig. 4C) gave a better resolution of small fragments up to 517 bp than the 0.5% solution (Fig., 2C), but fragments larger than 6108 bp were not separated using the 0.7% solution. The separation time (24 min) of Fig. 4C is longer than that (17 min) of Fig. 2C. In the CE separation using 0.7% HPC-11000 (Fig. 5C) as well as 0.7% HPMC-4000b (Fig. 4C) cellulose solution, small DNA fragments up to 517 bp

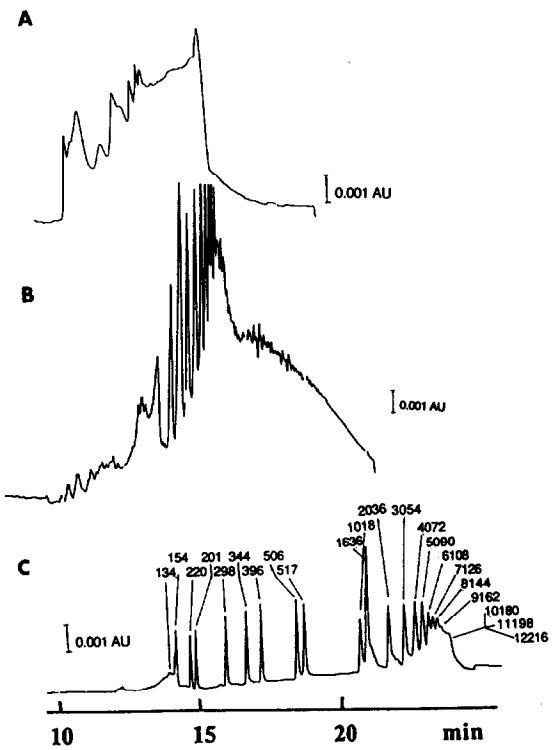

Fig. 4. Effect of the concentration of HPMC-4000b on the CE separation of the 1-kbp DNA ladder. Concentration of HPMC-4000b: (A) 0.1%, (B) 0.3% and (C) 0.7%. Other conditions as in Fig. 2.

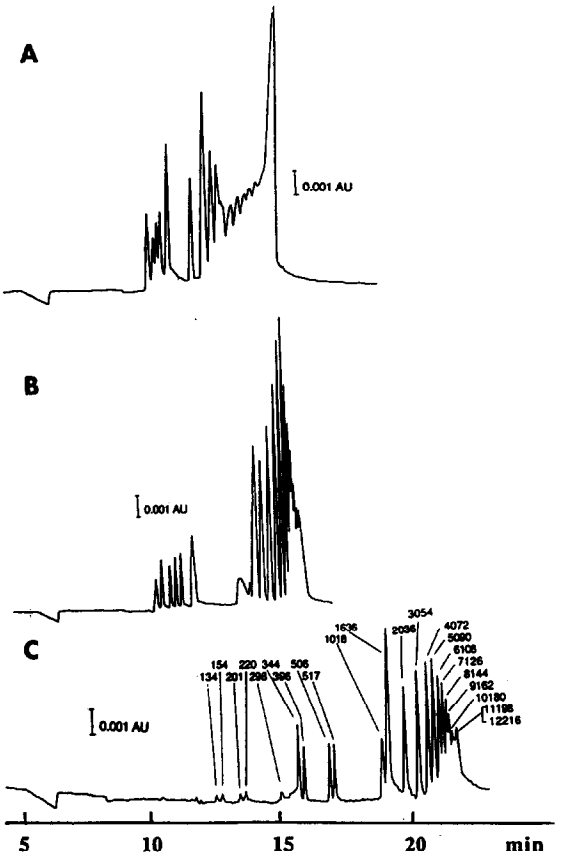

Fig. 5. Effect of the concentration of HPC-11000 on the CE separation of the 1-kbp DNA ladder. Concentration of HPC-11000: (A) 0.1%, (B) 0.3% and (C) 0.7%. Other conditions as in Fig. 2.

were baseline resolved, but the resolution of fragments larger than 8144 bp was poor. In addition, a longer separation time was required. The resolving power of HPC-11000 at lower concentration (Fig. 5A and B) seems to be higher than that of HPMC-4000b (Fig. 4A and B). These results show that an individual cellulose derivative solution has a characteristic limiting polymer concentration to exhibit the molecular sieving effect, and that a high-viscous cellulose derivative, which has longer chain length of polymer, can be an effective sieving agent even at a lower concentration, as will be discussed below.

The separation mechanism for DNA fragments using hydrophilic polymer solutions in CE has been investigated by Grossman and Soane [12,13]. The transient pores are formed by the entanglement of the polymer chains when the polymer volume fraction, Φ, is higher than the polymer volume fraction at which the polymer chains begin to interact with one another. Such a polymer volume fraction, Φ^*, is called the overlap threshold and expressed as:

$$\Phi^* = N^{-0.8} \quad (1)$$

where N is the number of segments in the cellulose polymer chain. Eqn. 1 predicts that the overlap threshold will decrease with an increase in the chain length of the cellulose derivative, and this can be proved by the results, as shown in the comparison of HPC-11000 (Fig. 5) with HPMC-4000b (Fig. 4). The overlap threshold of hydroxyethyl cellulose, whose molecular mass is 191 800 and N value is 1026, is calculated to be 0.39% [12,13]. The overlap thresholds of HPMC-4000b and HPC-11000 are estimated to

be 0.6 and 0.3%, respectively, because, according to the manufacturer, HPMC-4000b and HPC-11000 have average molecular masses of 90 000 and 250 000, respectively. These values agree roughly with the experimental values of 0.5% for HPMC-4000b and 0.3% for HPC-11000.

Transient pores with a broader distribution of size than the permanent pores in the gels must be present in the polymer network. The entangled polymer solution is therefore characterized by an average pore size, $\xi(\Phi)$, which is expressed as follows [12,13]:

$$\xi(\Phi) = a\Phi^{-0.75} \quad (2)$$

where Φ is the polymer volume fraction and a is the length of a polymer segment. As expected from eqn. 2, the effective pore diameters should decrease with increasing concentration of cellulose derivative, providing improved separation of small fragments. The small DNA fragments in the 1-kbp ladder exhibit significant improvement in resolution when the concentration of cellulose derivative is increased from 0.1 to 0.7%, as shown in Figs. 4 and 5.

Fig. 6 illustrates the effect of the applied field on the separation of the 1-kbp DNA ladder using 0.5% HPMC-4000b. The migration time of each fragment decreases with an increase in the applied field. The efficiency of this separation increases, as expected, with fields up to about 300 V/cm. However, at a field of 400 V/cm, the resolving power of cellulose derivative solution decreases, because band broadening results from the stretching of DNA.

Fig. 7 demonstrates the use of 0.7% MC-4000 polymer solution for the separation of small DNA fragments in the range 72–1353 bp. This range of the *Hinc*II and *Hae*III restriction fragments of ΦX174 DNA is crucial to DNA fragment typing using the PCR technique. Resolution is excellent and efficiency exceeded $1 \cdot 10^6$ plates/m. It can be seen from Fig. 7 that, in spite of the high resolution, no separation between the 335-, 341- and 345-bp fragments (Fig. 7a), or between the 271- and 281-bp fragments (Fig. 7b), was obtained. The same situation was also reported by other research groups [11]. However, these unseparated pairs can be completely baseline resolved by adding ethidium bromide to the buffer solution [11,16].

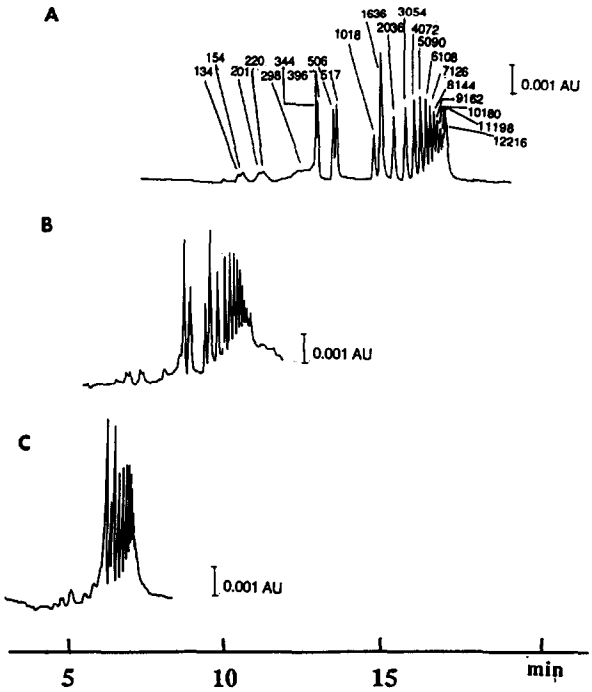

Fig. 6. Effect of the applied field on the CE separation of the 1-kbp DNA ladder. Cellulose derivative: 0.5% HPMC-4000b. Field: (A) 200 V/cm, (B) 300 V/cm and (C) 400 V/cm. Other conditions as in Fig. 2.

All cellulose derivatives tested here, except for HPMC-15000, can be used in the separation of double-stranded DNA restriction fragments. The size (viscosity) and the concentration of cellulose derivative are the most important parameters to manipulate the migration time and resolution. The choices within these parameters depend primarily on the size of the DNA fragments being separated. High-concentration (0.7–0.9%) cellulose derivative solutions are most effective for separating small fragments of DNA (50–100 bp). Their resolving power is extremely high, and fragments of DNA that differ in size by as little as 5–10 bp can be separated from one another. Although they can be run with high resolution, high-concentration polymer solutions have the disadvantage of being more difficult to load into the capillary than low-concentration polymer solutions owing to very high viscosity.

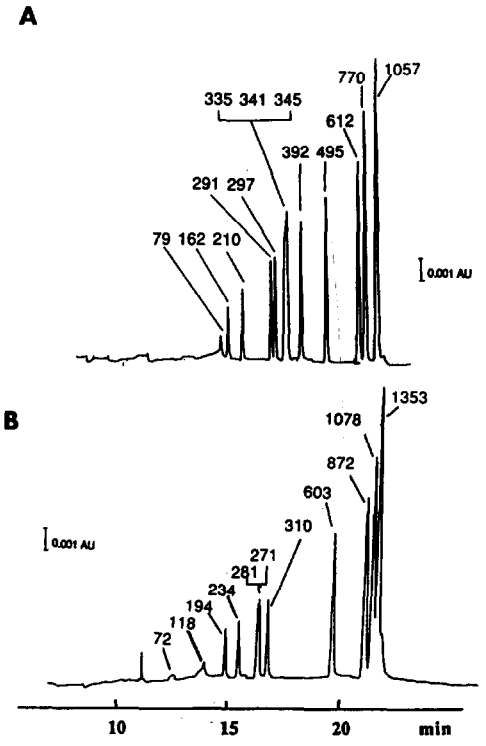

Fig. 7. CE separation of the DNA restriction fragment mixtures of (A) a *Hin*cII restriction digest of ΦX174 DNA and (B) the *Hae*III restriction digest of ΦX174 DNA. Running buffer: 50 m*M* Tris–borate, 2.5 m*M* EDTA and 0.7% MC-4000 (pH 8.3). Other conditions as in Fig. 2. Resolved fragments are labelled by their size in base pairs.

Low-concentration polymer solutions have a slightly lower resolving power than high-concentration polymer solutions but have a wider range of separation. DNAs from 100 bp to approximately 10 kbp in length can be separated with high resolution using 0.4–0.5% cellulose derivative solutions.

In this study, we demonstrate that the use of several cellulose derivatives as molecular sieving agents for CE represents an excellent approach for high-resolution separation of a broader molecular weight range of DNA restriction fragments. CE with cellulose derivative solutions has real potential as a powerful tool for gene diagnosis, such as RFLP analysis and single-strand conformation polymorphism (SSCP) analysis.

ACKNOWLEDGEMENTS

We acknowledge Associate Professor Makoto Otsuka at Kobe Women's College of Pharmacy for his helpful discussions on the nature of cellulose derivatives. We are indebted to Mr. Fumio Kobayashi at ShinEtsu Chemicals for his gift of cellulose derivatives. This work was partially supported by a Grant-in-Aid for Creative Basic Research (Human Genome Program), a Grant-in-Aid for Cancer Research and a Grant-in-Aid for Scientific Research from the Japan Ministry of Education, Science, and Culture.

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