Simultaneous Analysis of Genes by Capillary Electrophoresis with a Laser-Induced Fluorescence Detector Using a Stepwise Field Strength Gradient

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A mixture of polymerase chain reaction (PCR) products, 100, 105, 300, 310, 485, and 500 base pair (bp) DNA fragments, was analyzed by capillary electrophoresis equipped with a laser-induced fluorescence detector (CE-LIF) using a stepwise gradient of electric field strength. The optimum condition for the analysis of PCR products was 0.5% methylcellulose and 160 V/cm from 0 to 10 min and 270 V/cm from 10 to 17 min.

The length (bp) of DNA could be estimated from the relationship between the relative migration time and bp length. The relative standard deviation (R.S.D.) of DNA size (bp) was less than 3.5% and the difference from the true value was only 2.4 bp.

Key words capillary electrophoresis; fluorescence detector; PCR product; stepwise voltage gradient

Capillary electrophoresis (CE) is an excellent tool for the analysis of DNA fragments from the standpoint of resolution, speed, and automation. Accordingly, DNA sequencing[1] and human gene analysis[2—5] have been carried out using the CE technique. In the analysis of DNA fragments by CE, buffer solution, electric field, temperature, capillary length, the method of detection, and fluorescent dyes have been investigated in detail.[6—14] DNA fragments shorter than 250 base pair (bp) are able to be resolved by the constant electric field method,[13] but DNA fragments longer than 300 bp can not be resolved. In practical gene diagnosis, mixtures of longer DNA fragments of up to 500 bp must be analyzed. If DNA samples up to 500 bp can be simultaneously analyzed by CE with high accuracy and high speed, the technique could be applied to the diagnosis of disease. Thus, we tried to analyze simultaneously the PCR products of 100 to 500 bp DNA fragments by capillary electrophoresis with a laser-induced fluorescence detector (CE-LIF) using a stepwise electric field strength gradient, and we were able to establish conditions for simultaneous analysis of DNA fragments.

Additionally, the reproducibility in the analysis of DNA fragments employing a stepwise gradient of electric field strength was also investigated in the present study. The determination of exact DNA size and excellent reproducibility is essential for the practical application of CE-LIF to the analysis of disease-causing genes and gene diagnosis.

Experimental

Samples and Materials GeneAmp PCR reagent kit for 500 bp DNA fragment was purchased from Takara (Kyoto, Japan). This kit contains AmpliTaq DNA polymerase, four deoxyribonucleotide triphosphates (dNTPs), control template from λDNA, PCR buffer, control primer #1 (5′-GATGAGTTCGTGTCCGTACAACTGG-3′) and control primer #2 (5′-GGTTATCGAAATCAGCCACAGCGCC-3′). Using these primers, a 500 bp DNA fragment was obtained as a PCR product. Other primers (25 base nucleotides), instead of the primer #2 for 100, 105, 300, 310, and 485 bp DNA fragments, were supplied from Genset K. K. (Tokyo, Japan). The sequences of primers for 100, 105, 300, 310, and 485 bp fragments are 5′-AGACGGGCAATCAGTTCATCTTTCG-3′, 5′-AGCGGAGACGGGCAATCAGTTCATC-3′, 5′-GAATCACGGTATCCGGCTGCGCTGA-3′, 5′-GACGTATCCAGAATCACGGTATCCG-3′, and 5′-CCACAGCGCCTCCCGTTATTGCATT-3′, respectively (Fig. 1). The PCR was performed on a thermal cycler (GeneAmp PCR system 2400, Perkin Elmer Applied Biosystems Division, Foster City, CA, U.S.A.) with 25 cycles of amplification (15 s at 94 °C and 30 s at 68 °C). Excess dNTPs and primers were removed from the PCR product using a tube with a filter (Suprec-02, Takara). Methylcellulose (4000 cp) was purchased from Sigma (St. Louis, MO, U.S.A.). A fluorescent dye, (1-(4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-quinolinium)-3-trimethylammonium propane diiodide (YO-PRO-1), was obtained from Molecular Probes(Eugene, OR, U.S.A.). All other chemicals were of analytical-reagent or electrophoretic grade from Wako (Osaka, Japan).

Capillary Electrophoresis Capillary electrophoretic apparatus with a laser-induced fluorescence detector developed by us was used. A fused-silica capillary of 100 μm i.d. and 38 cm effective length (55 cm total length) purchased from Polymicro Technologies (Phoenix, AZ, U.S.A.) was used. The inner wall of capillary was coated with polyacrylamide as described.[15] The running buffer solution was 50 mM Tris–borate, 0.5 or 0.7% methylcellulose and 0.2 or 0.5 μM YO-PRO-1. The addition of YO-PRO-1 into the running buffer solution remarkably improved the sensitivity of detection. Further, because DNA samples are partially neutralized by the intercalation of YO-PRO-1 with positive charge into the double helix of DNA molecule, the separation of DNA fragments was improved.

The capillary was rinsed for 5 min with HPLC-grade methanol prior to CE analysis and the buffer solution was introduced into the capillary using a vacuum injection system.[15] Sample solution was injected electrophoretically at 5 or 10 kV for 5 or 10 s. Electrophoresis was carried out by the stepwise electric field strength gradient method at room temperature. DNA fragments were detected at 550 nm (Ex.; 488 nm).

Results and Discussion

Analysis of PCR Products The PCR products of 100—105, 300—310, and 485—500 bp are a mixture of 100, 300, and 500 bp DNA fragments and fragments (105, 310, and

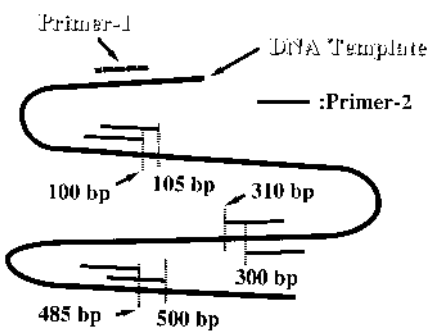

Fig. 1. The Method for Amplifying Several PCR Products

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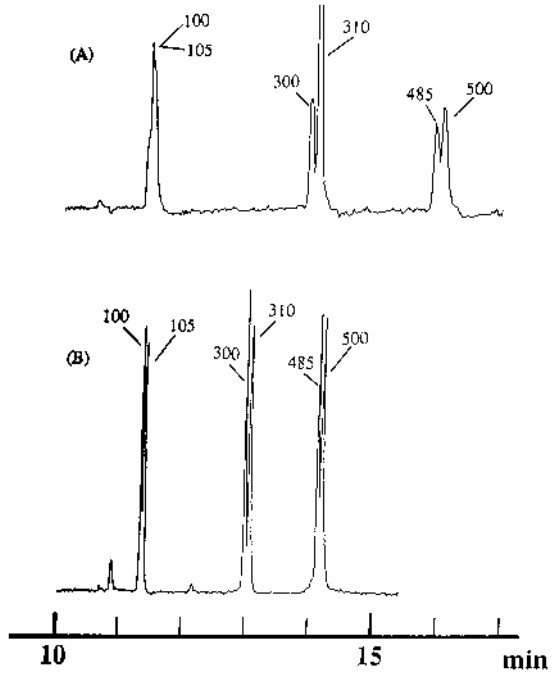

Fig. 2. CE Separation of 100, 105, 300, 310, 485, and 500 bp DNA Fragments

Capillary; 100 μm i.d., 375 μm o.d., total length 55 cm, effective length 38 cm. Running buffer; 50 mM Tris–boric acid, 0.5% methylcellulose and 0.5 μM YO-PRO-1. Field; (A) 180 V/cm (11 μA), (B) 160 V/cm 10 min (10 μA) and 270 V/cm 7 min (17 μA). Injection; (A) 5 kV, 10 s, (B) 10 kV, 5 s. Detection; 550 nm.

485 bp) which differ by 3—5% from the 100, 300, and 500 bp species, respectively. Figure 2(A) shows the electropherogram of the PCR products (the mixture of 100—105, 300—310, and 485—500 bp DNA fragments) performed using 0.5% methylcellulose at the constant electric field of 180 V/cm. The 300—310 and 485—500 bp DNA fragments were resolved, but the 100 and 105 bp DNA fragments were not at all. Satisfactory results could not be obtained at constant electric fields between 120 and 270 V/cm. Thus, the stepwise electric field strength gradient method was investigated to improve the resolving power. The method consisted of two consecutive steps, a low electric field after an initial high electric field and a high electric field after an initial low electric field. The latter step was suitable for the simultaneous separation of all samples.

Figure 2(B) shows the electropherogram of the 100—105, 300—310, and 485—500 bp DNA fragments run from 0 to 10 min at 160 V/cm and then for 7 min at 270 V/cm. The fragments which differ by about 200 bp were completely separated at the lower electric field, and the fragments which differ by only 5—15 bp were finely separated at the higher electric field. The plate number of each peak was estimated to be $(0.3—1.0)\times10^6$ [$(0.6—1.8)\times10^6$ per meter]. This result indicated that the lower electric field is effective for the separation of the longer DNA fragments and the higher electric field for the shorter DNA fragments. The resolving power for the DNA fragments was much higher than those obtained using the constant electric field of 120—270 V/cm as shown in Fig. 2(A) and (B). The analysis time is also shortened by employing the stepwise gradient. The reason why the mixture of the shorter and longer DNA fragments is successfully separated by the stepwise electric field strength gradient method has been described in our previous paper[16] using a theoretical formulation.

Table 1. Reproducibility (n=10) of Migration Time and Relative Migration Time for 300 bp

DNA size (bp)	Migration time (min)	R.S.D. (%)	Relative migration time	R.S.D. (%)
100	11.74	0.48	0.875	0.26
105	11.78	0.48	0.878	0.29
300	13.41	0.65	1.000	—
310	13.48	0.63	1.005	0.10
485	14.58	0.65	1.087	0.08
500	14.65	0.65	1.092	0.09

Table 2. Reproducibility of DNA Size

DNA size (bp)		R.S.D. (%)	Difference from the true value
True	Experimental[a)]		
100	100.0	2.97	0.01
105	103.8	3.54	1.20
300	300.0	—	—
310	311.1	0.67	1.14
485	487.4	0.45	2.40
500	500.0	0.47	0.00

a) Average DNA size is calculated by Eq. (1).

Reproducibility in Analysis of PCR Products To investigate the reproducibility of migration time, we analyzed the 100—105, 300—310, and 485—500 bp DNA samples 10 times using a stepwise gradient of electric field strength. The average of migration time and relative migration time for the 300 bp DNA fragment, and the relative standard deviation (R.S.D.) values are summarized in Table 1. The R.S.D. values of migration time are in the range of 0.4—0.7%, showing high reproducibility. Further, the R.S.D. values of relative migration time for the 300 bp fragment are excellent, below 0.29%. The R.S.D. values for greater than 310 bp fragments are less than 0.1%. These results indicate that the mixtures of DNA fragments which differ by 200 bp and DNA fragments which differ by only 3—5% from 100, 300, and 500 bp, can be completely resolved by the stepwise electric field strength gradient method with high reproducibility. Further, the analysis was achieved within 15 min. Thus, this CE-LIF technique using a stepwise gradient of electric field was proved to be useful for the analysis of several genes at a time.

Additionally, the length of DNA fragments estimated from the relative migration time for the 300 bp fragment is shown in Table 2. The R.S.D. values were in the range of 0.45 and 3.54%. For 100 and 105 bp DNA, the R.S.D. values were large (2.97—3.54%), but for the greater than 300 bp DNA they were very small (0.45—0.67%). The difference from the true values was less than 2.4 bp.

Conclusion

The CE-LIF technique using a stepwise electric field strength gradient has excellent resolving power, and provides rapid separation and striking reproducibility in the analysis of PCR-amplified 100—105 bp, 300—310 bp, and 485—500 bp DNA fragments. Several gene samples can be rapidly and precisely analyzed at the same time by the stepwise elec-

tric field strength gradient method, making the technique applicable to the analysis of disease-causing genes and DNA diagnosis.

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