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CAPILLARY ELECTROPHORESIS OF NUCLEIC ACIDS WITH A FULLY AUTOMATED APPARATUS

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SUMMARY

Nucleic acids with a wide range of molecular size, from mononucleotides to calf thymus DNAs, were subjected to capillary electrophoresis employing the conditions for isotachophoretic analysis of proteins; Ampholine was mixed with the sample solution and the electrolyte solutions for isotachophoresis were used. For reproducible analyis, electrophoresis was performed with a fully automated apparatus. Nucleotides were separated from bases owing to their high electrophoretic mobility. The chain length of oligonucleotides was not the major factor with regard to their electrophoretic mobility. Polynucleotides (above 10^3 bases) appeared as sharp UV peaks in the zone of the hydrogencarbonate ion and their UV peak width was increased by DNase digestion, suggesting heterogeneity of the product. The applicability of capillary electrophoresis to the separation of large-molecular-sized polynucleotides has been demonstrated.

INTRODUCTION

Gel electrophoresis has been widely used for the analysis of nucleic acids, for the determination of DNA sequences[1] and for the analysis of the reaction products of restriction enzymes. The disadvantages of gel electrophoretic techniques in the analysis of nucleic acids are that DNA molecules of very large size are trapped on the gel top and the procedures involved, including the process of gel preparation, are very complicated. From the point of view of simple operation, high-performance liquid chromatography (HPLC) is preferable, but only the analysis of small-sized nucleic acids has been reported. Hence separation methods that require no supporting materials such as gel or resin particles and that involve simple procedures are desirable.

Capillary electrophoresis is a technique that employs no supporting material. We have previously applied capillary electrophoresis to the qualitative and quantitative analysis of high-molecular-weight proteins[2–5]. Further, the procedures can be very simple and we were able to construct a fully automated apparatus[5]. We report here the separation of nucleic acids by automated capillary electrophoresis. Conditions for protein analysis were employed without modification; Ampholine was mixed with the samples and electrolyte solutions for isotachophoresis were used. Analyses of nucleic acids with a wide range of molecular size, from mononucleotides to calf thymus DNA, are reported.

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EXPERIMENTAL

Materials

Bases (adenine, guanine, uracil, xanthine, hypoxanthine) and adenosine-5′-monophosphate (from yeast) were obtained from Wako (Osaka, Japan). Adenosine-5′-diphosphate (from equine muscle, cat. No. 8146), adenosine-5′-triphosphate (from equine muscle, cat. No. 5394), calf thymus DNAs (cat. No. D-1501), calf liver RNAs (cat. No. 7250) and deoxyribonuclease I (from bovine pancreas, cat. No. DN-25) were purchased from Sigma (St. Louis, MO, U.S.A.). Plasmid DNAs pNO1523 (*ca.* 5200 base pairs) and lambda phage DNAs (50 000 base pairs, cat. No. 27-4111-01) were obtained from Pharmacia (Uppsala, Sweden). The above materials were used without further purification. Single-strand oligodeoxyribonucleotides (18–62 bases) were synthesized with a Applied Biosystems (Foster City, CA, U.S.A.) 380B DNA synthesizer.

An ampholytes mixture was obtained from LKB (Bromma, Sweden) (Ampholine 3.5–10). 2-Amino-2-methyl-1-propanol was purchased from Nakarai Chemicals (Kyoto, Japan), tranexamic acid from Daiichi Seiyaku (Tokyo, Japan), hydrochloric acid (1 *M*, special grade for amino acid sequence analysis), potassium hydroxide and hexane from Wako (Osaka, Japan), hydroxypropylmethylcellulose (HPMC) from Aldrich (Milwaukee, WI, U.S.A.) and sodium azide from Kanto Chemical (Tokyo, Japan). Reagents for preparation of electrode solutions were used without further purification.

Apparatus

Capillary electrophoresis was performed with a fully automated apparatus. Details of the construction of the apparatus have been described elsewhere[5]. A commercial capillary electrophoresis apparatus (IP-2A; Shimadzu, Kyoto, Japan), equipped with a high-voltage d.c. power supply, a potential gradient (PG) detector, a UV detector (254 nm) and a two-pen recorder, was modified in the electrolyte pumping system and in the flow lines[3]. For automatic sample loading, a sampler (an AutoAnalyzer component; Technicon Instruments, New York, U.S.A.) which can equip 40 sample cups and a micro-peristaltic pump (Chromato Research, Yokohama, Japan) were employed. This equipment, peristaltic pumps for loading the electrolyte solutions, the micro-peristaltic pump for sample loading, the sampler, the high-voltage d.c. power supply and the recorder were controlled by a system controller, consisting of the following components: an 8-bit microcomputer (NEC PC-8001; Nippon Electric, Tokyo, Japan) equipped with an FGU-8000 640 × 200 dot graphic unit, an input–output (I/O) unit (NEC PC-8013; Nippon Electric), a relay interface boad, a 5-in. floppy disk unit (NEC PC-8031; Nippon Electric), and a dot-matrix printer (MP-82; Epson, Nagano, Japan). The signal from the UV detector and the PG detector were amplified and digitized by an 8-bit analogue-to-digital converter (MB4052; Fuzitsu, Tokyo, Japan) and stored in the microcomputer for data processing. Details of the data acquisition and data processing will be described elsewhere.

Capillary electrophoresis

Sample solution was prepared as follows. A stock solution of 4% Ampholine (pH 3.5–10)–0.1% sodium azide was prepared and stored at 4°C. The Ampholine

stock solution (25 μl) was mixed with distilled, deionized water (375 μl) and a nucleic acid solution (20 μl), then the mixture was poured into the sample cup of the sampler. With DNA samples of large molecular size, the samples tend to precipitate when mixed with the Ampholine solution. In these cases, 1 *M* sodium hydroxide solution (5 μl) was further added to the sample mixture and the precipitates were dissolved. Capillary electrophoresis was carried out with the automated apparatus described above. The leading electrolyte solution was 5 m*M* hydrochloric acid–9.3 m*M* 2-amino-2-methyl-1-propanol (pH 9.9). The terminating electrolyte solution was 50 m*M* tranexamic acid–potassium hydroxide (pH 10.8). The solutions were kept in amber-glass bottles and overlayed with 1-cm layer of hexane to minimize the dissolution of carbon dioxide. A PFEP tube (230 mm × 0.5 m I.D.) was used as the separation tube, the inner surface of which had been coated with HPMC[5]. Electrophoresis was run at a constant current of 150 μA for 4.8 min (initial voltage about 6 kV) and then a constant current of 50 μA for 11 min. The time needed for one cycle of analysis was 26 min, including the time for buffer exchange and sample loading.

RESULTS AND DISCUSSION

Mononucleotides

The results of the analysis of mononucleotides and bases are shown in Fig. 1. The sample ions were separated as sharp UV peaks after the zone of azide ion (lower trace). The peaks were detected 5–10 min after electrophoresis was started. However, the time of detection cannot be used for the identification of the samples, as it will be affected by

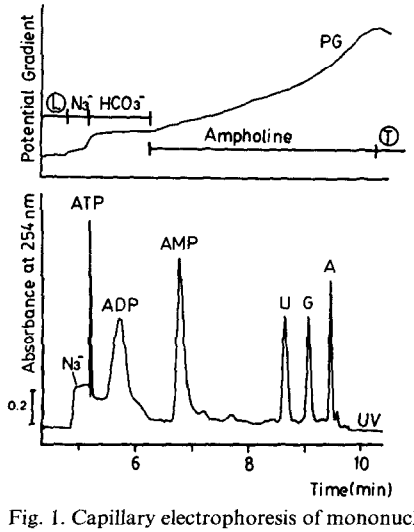

Fig. 1. Capillary electrophoresis of mononucleotides and bases. 5′-ATP (1 mg/ml, 2 μl), 5′-ADP (1 mg/ml, 2 μl), 5′-AMP (1 mg/ml, 1 μl), uracil (250 μg/ml, 2 μl), guanine (250 μg/ml, 2 μl) and adenine (250 μg/ml, 1 μl) were mixed with the Ampholine stock solution (25 μl) and diluted to 420 μl with distilled, deionized water, and an aliquot (5 μl) was introduced into the capillary with the automatic sampler. The upper and the lower curves show the potential gradient (PG) and UV absorbance, respectively. The zone lengths are indicated on the PG curve; L, leading ion (chloride); N_3^-, azide ion; HCO_3^-, hydrogencarbonate ion; T, terminating ion (tranexamic acid); A, adenine; G, guanine; U, uracil.

the amount of ions in the preceding zones. We then referred to the potential gradient (PG) values of the migrating zones. The upper trace in Fig. 1 shows the PG values. The zones of chloride, azide, hydrogencarbonate, Ampholine and tranexamic acid were identified and the zone widths are indicated. A smaller PG value represents a larger electrophoretic mobility. ATP migrated at the boundary of the azide and HCO_3^- zones, ADP was in the zone of HCO_3^- and AMP was in the zone of Ampholine. Bases, which have no phosphate group, migrated in the zone of Ampholine and showed a smaller electrophoretic mobility than AMP. These results suggest that the charge on the phosphate group is the major factor affecting the large electrophoretic mobility of nucleotides. The sensitivity of mononucleotides and bases at 254 nm on this apparatus was several picomoles.

Oligonucleotides

Some examples of the analysis of oligodeoxyribonucleotides are shown in Fig. 2. The chain lengths ranged from 18 to 62 bases. Most of the oligonucleotide samples

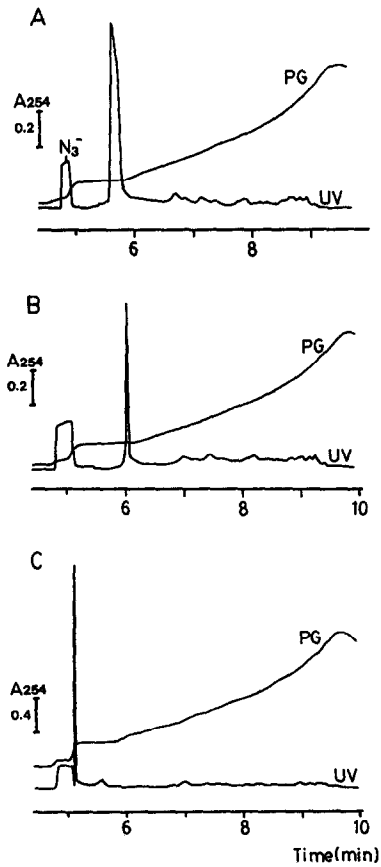

Fig. 2. Examples of the capillary electrophoretic patterns of oligodeoxyribonucleotides. (A) 25 bases, dATTCATTCTGTTCTAAGCCTGTTCC; (B) 62 bases, dTCGACTTATTACAGGTACCGACCTTC-GTCGTCAGAACCGAAACGAACCGGGTGGTTTTTGGT; (C) 18 bases, dCAGCTGGAATTCCA-GCTG.

examined (twelve out of thirteen) migrated around the boundary of the HCO_3^- and Ampholine zones, as shown in Fig. 2A and B. Hence the difference in chain length in this range cannot be a major factor affecting the electrophoretic mobility of oligonucleotides. On the other hand, one of the samples (18 bases) migrated around the boundary of the azide and HCO_3^- zones (Fig. 2C). The reason for the large electrophoretic mobility of this sample is unclear. However, the results suggest the possibility that the base composition of oligonucleotides might affect their electrophoretic mobility.

Polynucleotides

Several samples of commercially available polynucleotides were analysed with the automated apparatus. Fig. 3A shows the pattern of calf liver RNAs. In the UV pattern, two UV peaks appeared. The UV peak with smaller mobility was detected around the boundary of the HCO_3^- and Ampholine zones, with almost the same mobility as most of the oligonucleotide samples. Plasmid DNAs (cloning vector pNO1523, *ca.* 5200 base pairs) and calf thymus DNAs (*ca.* 10^4 base pairs) both showed UV peaks in the zone of HCO_3^- (Fig. 3B and C), although the sample of calf thymus

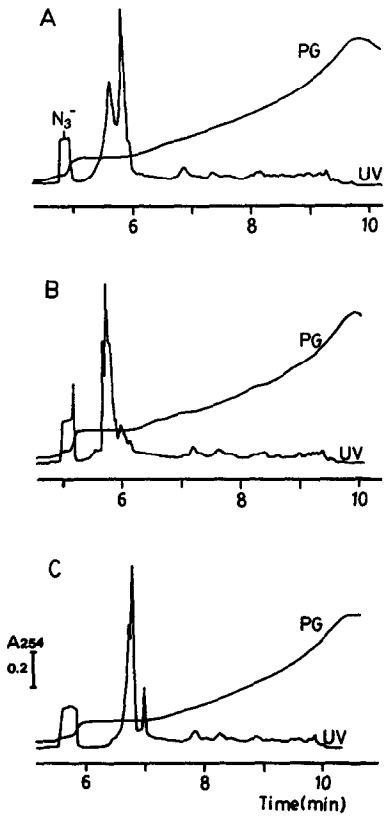

Fig. 3. Capillary electrophoresis of polynucleotides. (A) Calf liver RNAs (62 ng); (B) pN01523 plasmid DNAs (58 ng); (C) calf thymus DNAs (93 ng).

DNAs showed a small UV peak at the PG position corresponding to the boundary of the HCO_3^- and Ampholine zones.

The effect of enzyme digestion on the mobility of polynucleotides was examined. The results of digestion of calf thymus DNA are shown in Fig. 4. Without the enzyme, the DNA sample showed two sharp UV peaks, as shown in Fig. 4A. As the digestion time proceeded, the peak with larger electrohoretic mobility decreased in height (Fig. 4B) and finally disappeared (Fig. 4C and D). Although the UV pattern of the digested DNA sample showed one peak, the peak ranged throughout the HCO_3^- zone, indicating heterogeneity of the digest. In the overnight digest, several UV peaks appeared within the Ampholine zone (Fig. 4D, arrows). From the mobility of the peaks, they were tentatively identified as bases (*cf.*, Fig. 1).

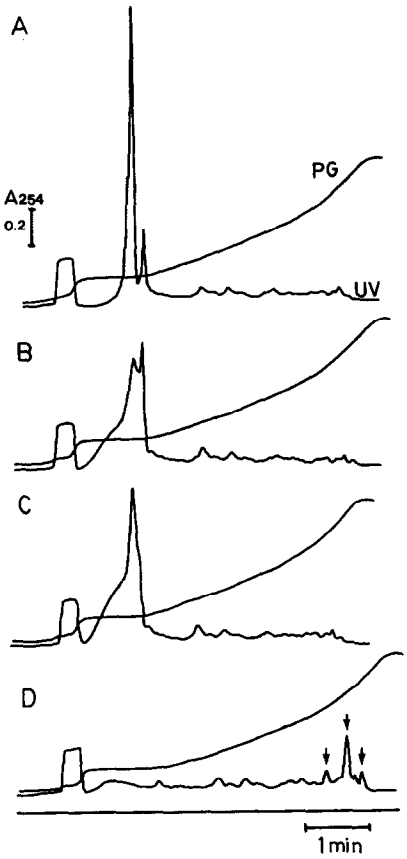

Fig. 4. Capillary electrophoresis of the enzymatic digest of calf thymus DNAs. Calf thymus DNAs solution (50 μg of DNAs in 220 μl of 4.5 m*M* Tris–HCl–5 m*M* $MgSO_4$–0.02 *M* NaCl, pH 7.5) was mixed with deoxyribonuclease I (DNase) solution (16 units in 32 μl of 5 m*M* Tris–HCl–5 m*M* $MgSO_4$, pH 7.5). The mixture was incubated (31°C) and analysed after appropriate time intervals. (A) Reference sample (without DNase) incubated overnight; (B) with DNase, 30-min incubation; (C) with DNase, 60-min incubation; (D) with DNase, incubated overnight.

Effect of the zone length of hydrogencarbonate ion

In the course of the analysis of polynucleotides, we noted that their UV zone widths were affected by the zone length of HCO_3^-. As shown in Fig. 5A, when the zone length of HCO_3^- was increased by adding sodium hydrogencarbonate to the sample solution, calf thymus DNAs showed multiple UV peaks ranging in wide UV zones (*cf.*, Fig. 3C). Lambda phage DNAs also showed multiple UV peaks, as shown in Fig. 5B. These results suggest that the polynucleotide zone is migrating in the HCO_3^- zone with almost the same mobility as HCO_3^-, and that the zone electrophoresis of polynucleotides in HCO_3^- ion might offer a high resolution for these samples.

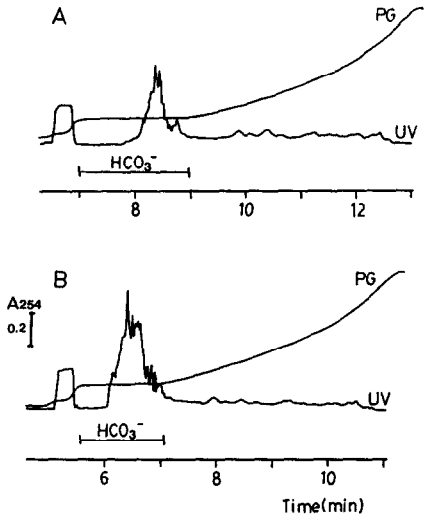

Fig. 5. Capillary electrophoresis of polynucleotides with increased concentration of hydrogencarbonate ion. The hydrogencarbonate ion zone length was increased by adding sodium hydrogencarbonate to the sample solution. (A) Calf thymus DNAs (93 ng); (B) lambda phage DNAs (93 ng).

Capillary electrophoresis for the analysis of polynucleotides

Capillary electrophoresis, which employs no support materials, has been applied previously to the analysis of bases, nucleosides and oligonucleotides[6–11]. However, few attempts at the analysis of polynucleotides (above 10^3 bases) have been reported. As shown above, polynucleotides in addition to oligo- and mononucleotides could be analysed with the capillary electrophoresis apparatus employig the electrolytes conditions for protein analysis. Further, the bases could be separated under the same conditions. Automation of the sample loading steps contributed to an improved reproducibility of the nucleotides analysis. In some instances, we noted precipitation of polynucleotides when the sample was mixed with the Ampholine solution; we then raised the pH of the solution and were able to dissolve the samples. For the analysis of polynucleotides, care must be taken to dissolve the samples and to load them quantitatively. In order to improve the resolution of polynucleotides, the use of capillary zone electrophoresis employing HCO_3^- ion as the electrolyte seems to be promising.

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