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Use of glucomannan for the separation of DNA fragments by capillary electrophoresis

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ABSTRACT

Glucomannan, a natural polysaccharide extracted from *Amorphophallus konjac*, was employed as a sieving additive for the separation of restriction DNA fragments in capillary electrophoresis. A stable solution of entangled glucomannan was successfully prepared under mild conditions. The separation capillary filled with 0.25% glucomannan exhibited excellent resolution and reproducibility on separation of DNA fragments over the range of *ca.* 1400 base pairs. The separation was dependent on the size of the fragments, which allowed the determination of the size of separated DNAs directly from the migration time. In addition to these separation characteristics, glucomannan has practical advantages in its chemical stability, non-toxicity and ease of handling. Because the method is applicable to the separation and characterization of the DNA fragments generated by digestion with restriction enzymes or produced by polymerase chain reactions, it is concluded that glucomannan is a good alternative to the conventional sieving additives for capillary electrophoresis, such as polyacrylamide and hydroxymethylcellulose.

INTRODUCTION

The separation of biological macromolecules such as DNA is an important technique in modern biological science. For instance, molecular cloning of a gene requires many experimental steps for the analysis and separation of DNA fragments generated by restriction enzyme digestion or produced by polymerase chain reaction (PCR). Because most of these procedures are currently performed manually by polyacrylamide or agarose slab gel electrophoresis, the separation and analysis of DNA fragments are laborious and often time-consuming steps in gene cloning studies.

Capillary electrophoresis has been developed for the determination of various substances, including biological macromolecules [1–7]. The advantages of this method are high resolution, rapid and sensitive analysis and easy operation and automation. The efficient separation of polynucleotides has been achieved using a capillary filled with polymer gel or entangled polymer

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solution such as cross-linked or non-cross-linked polyacrylamide gels [8], polyethylene glycol [9] and fibrous polysaccharide derivatives such as hydroxypropylmethylcellulose [9,10]. The advantages and disadvantages of these materials have been discussed previously [8–11]. In this mode of separation, polynucleotides are basically resolved according to their size. Thus the matrices of these materials are thought to serve as pores to sieve the polynucleotide molecules. As the separation depends on the pore size, a matrix with large pores is generally suitable for the separation of large DNA fragments such as phage DNA, and *vice versa*.

Glucomannan, a natural polysaccharide extracted from *Amorphophallus konjac*, is a linear polymer mainly consisting of D-glucose and D-mannose [12], and the gel form of this material is known as a Japanese traditional food, "konjac". We intended to use this material as an entangled polymer solution for the capillary electrophoresis of DNA fragments because it is chemically stable and non-toxic, and has a pore size larger than that of agarose [13]. In this work, we prepared entangled glucomannan solution and examined its separation characteristics in the separation of DNA fragments.

EXPERIMENTAL

Apparatus

A CE-800 capillary electrophoresis system (JASCO, Tokyo, Japan), equipped with a high-voltage d.c. power supply (Model 890-CE) providing up to 30 kV and a UV–Vis spectrophotometer (Model 875-CE) for on-column detection, was employed. For data acquisition and processing, a Model 807-IT intelligent integrator (JASCO) was used. Electrophoresis was performed in a fused-silica capillary with the inner surface coated with DB-Wax (J&W Scientific, Folsom, CA, USA) (50 cm × 100 μm I.D. × 375 μm O.D.).

Chemicals

Glucomannan, purified from konjac flour by ethanol precipitation [14], was obtained from Kurita Water Industries (Tokyo, Japan). The preparation used in this study has an average molecular mass of $1.3 \cdot 10^6$ as estimated from the viscosity of the aqueous solution. All other chemicals used were of analytical-reagent grade. ΦX174 DNA digested with restriction enzyme *Hae*III was obtained from Nippon Gene (Tokyo, Japan) and used at a sample concentration of 0.47 mg/ml in 10 m*M* Tris–HCl containing 20 m*M* NaCl and 1 m*M* EDTA (pH 7.9). Restriction fragments of plasmid DNA were prepared by digestion of Bluescript II SK(+) (Stratagene, La Jolla, CA, USA) with restriction enzyme *Dde*I (Toyobo, Tokyo, Japan). The digestion was performed under the conditions recommended by the supplier. The digest was precipitated with ethanol, redissolved in distilled water and then subjected to electrophoresis. Polymerase chain reaction (PCR) product of the mRNA encoding the η-chain of 14–3–3 protein [15] was prepared using two synthetic primers (primer 1,5′ - TCGAATTCATGGGTGACCGCGAGC-A-3′; primer 2,5′-GATAGGATCCGCACTGG-ATACTTAGT-3′).

Preparation of entangled glucomannan solution

Because the glucomannan forms an insoluble gel on boiling in an alkaline solution [16], the entangled glucomannan solution was carefully prepared under mild conditions. The particles of glucomannan were added to an electrolyte solution at a concentration of 0.25% (w/w) and swollen by heating at 60°C in a water-bath. The swelling was completed within 4 h. The partially solubilized glucomannan, in the form of a clear paste, was then mixed on a vortex mixer with occasionally heating in boiling water until the preparation became almost homogeneous. The preparation was allowed to come to room temperature, centrifuged to remove small insoluble material and degassed by aspiration. Alternatively, the entangled glucomannan solution could be prepared by continuous stirring of the mixture at 60°C for 6 h. The glucomannan solution thus obtained was stable at room temperature at least for several months. Excessive heating of glucomannan (100°C, more than 1 h) should be avoided because a white gel is irreversibly formed.

Capillary electrophoresis

As an electrolyte solution, 70 m*M* Tris–HCl (pH 8.2) containing 14 m*M* EDTA was used. Prior to each analysis, 20 μl of glucomannan stock solution were injected into the coated fused-silica capillary using a glass syringe with a joint of PTFE tubing. Samples were injected electrophoretically from the cathode end of the capillary at 5 kV for 1 s. Electrophoresis was run at a constant voltage of 5 kV (100 V/cm for a 50-cm capillary) and the UV absorbance at 260 nm was measured at 20 cm from the cathode end.

RESULTS AND DISCUSSION

Separation of HaeIII digest of ΦX174 DNA

To examine the separation performance of the glucomannan-filled capillary, we chose an *Hae*III restriction digest of ΦX174 plasmid DNA as a standard DNA sample. Based on the nucleotide sequence of the plasmid DNA and the known specificity of *Hae*III, the digest should contain eleven fragments ranging from 72 to 1353 base pairs [17]. Fig. 1 shows a separation pattern of the *Hae*III digest of ΦX174 DNA using a 0.25% glucomannan-filled capillary. The method resolved all eleven fragments in less than 30 min. The peaks were identified by co-electrophoresis of the digest with each fragment isolated by conventional 6% polyacrylamide slab gel electrophoresis (shown in Fig. 1a). The fragments in peaks 5 and 6, which differ in length by only ten base pairs, could be separated by the present method employing an electrolyte solution containing 14 m*M* EDTA. However, a similar electrolyte solution containing lower or higher concentrations of EDTA gave poor resolution and broadening of some peaks (Fig. 1b and c).

A calibration graph of migration time *versus* the size of the fragments separated on the glucomannan-filled capillary is shown in Fig. 2. The migration time of the fragments is correlated with the size of the fragments over the range of *ca.* 1400 base pairs, indicating the size-dependent migration of the DNA fragments in the glucomannan-filled capillary.

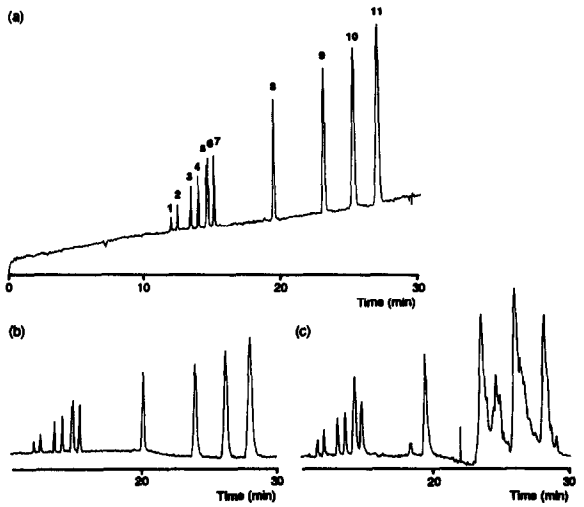

Fig. 1. Separation of *Hae*III digest of ΦX174 DNA using a 0.25% glucomannan-filled capillary. Peaks: 1 = 72; 2 = 118; 3 = 194; 4 = 234; 5 = 271; 6 = 281; 7 = 310; 8 = 603; 9 = 872; 10 = 1078; 11 = 1353 base pairs. Conditions: capillary, DB-WAX, was filled with 0.25% glucomannan dissolved in 70 m*M* Tris–HCl (pH 8.2) containing (a) 14, (b) 2 and (c) 26 m*M* EDTA. Effective length, 20 cm; total length, 50 cm; applied field, 100 V/cm; sample, injected electrophoretically at 5 kV for 1 s. Current generated: (a) 40; (b) 25; (c) 56 μA.

Ferguson plot analysis

The separation characteristics of the glucomannan-filled capillary were further examined by Ferguson plot analysis [18,19], where the logarithm of the mobility of the restriction fragments was plotted against the concentration of the glucomannan solution in the range 0.05–0.7% (Fig. 3). At glucomannan concentrations up to 0.25%, the DNA fragments ranging from 72 to 1353 base pairs exhibit a good linear relationship described by the equation

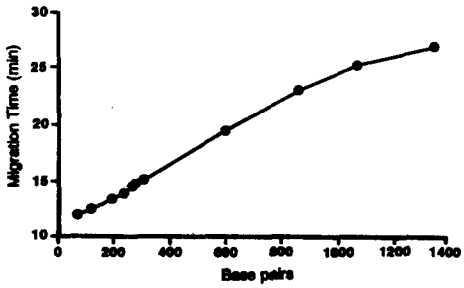

Fig. 2. Size calibration graph for *Hae*III digest of ΦX174 DNA in 0.25% glucomannan. Conditions as in Fig. 1a.

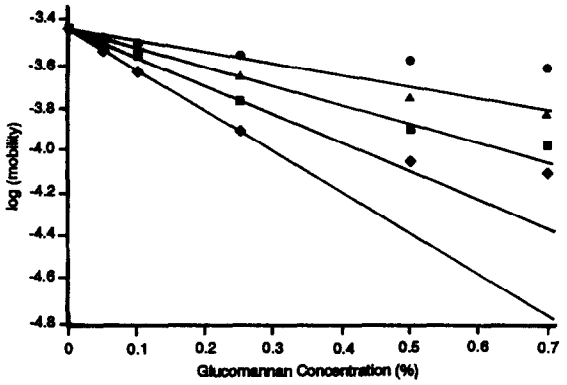

Fig. 3. Ferguson plots for entangled glucomannan solution. The lines represent the mobility of *Hae*III fragments of ΦX174 DNA as a function of glucomannan concentration (%, w/w). ● = 72; ▲ = 310; ■ = 603; ◆ = 1353 base pairs. Conditions as in Fig. 1a.

$$\log \mu = \log \mu_0 - k_R c \quad (1)$$

where μ is the measured mobility ($cm^2/V \cdot s$), μ_0 is the mobility in free solution, k_R is the slope (called the retardation coefficient) and c is the glucomannan concentration (%). The value of the retardation coefficient, k_R, is varied from −0.47 for the 72 base pair fragment to −1.9 for the 1353 base pair fragment. These values decrease with increasing size of the fragments, suggesting sieving of the DNA fragments in the entangled glucomannan solution. However, at glucomannan concentrations higher than 0.25%, these DNA fragments exhibit larger values of the mobility than those estimated from the above equation. Whether this is caused by changes in the conformation of DNA or in the structure of the glucomannan matrix awaits further investigation.

Reproducibility

The stability and reproducibility of the glucomannan-filled capillary were examined using the *Hae*III digest of ΦX174 DNA. In this experiment, electrophoresis was performed repeatedly with a single capillary without refilling the glucomannan solution. The capillary could be used for at least four analytical cycles without a detectable alteration in separation performance (Fig. 4a,b). The profile was similar even after ten consecutive analytical cycles, except that the separation between the two small fragments, of 271 and 281 base pairs, became insufficient (data not shown). A significant deterioration in peak resolution was observed after the fourteenth cycle, in which the fragments smaller than 281 base pairs were not separated (Fig. 4c). Fig. 4c also shows that the migration time of each fragment gradually decreased during the series of analytical cycles. However, the performance of the capillary was restored completely by refilling it with fresh glucomannan solution (Fig. 4d). The capillary exhibited a run-to-run reproducibility in migration time of 0.7% ($n = 5$) and a day-to-day reproducibility of 2.4% ($n = 5$) for the 1353 base pair peak. These results suggest that the glucomannan-filled capillary can be used for pattern analysis about ten times with satisfying performance of the separation without refilling and is also usable for further analysis such as the determination of the size of DNA frag-

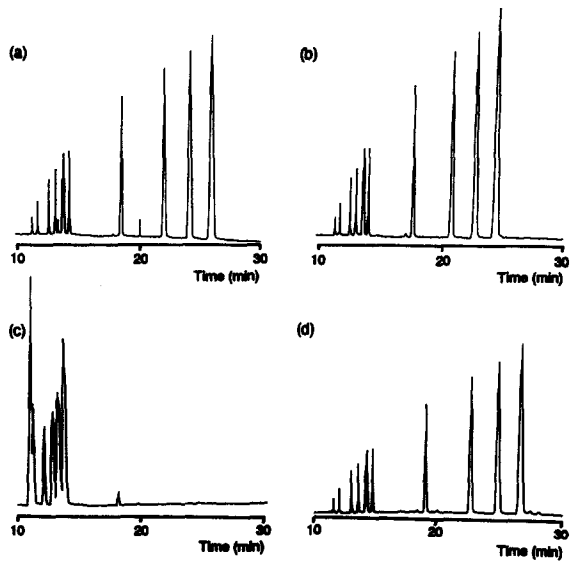

Fig. 4. Stability of the glucomannan-filled capillary. Sample: *Hae*III digest of ΦX174 DNA. The glucomannan-filled capillary was used repeatedly without refilling: (a) cycle 1; (b) cycle 4; (c) cycle 14. After the fourteenth analytical cycle, the capillary was refilled with the glucomannan solution, and the pattern of cycle 1 is shown in (d). All other conditions as in Fig. 1a.

ments directly from the migration time by replacing it.

Application to the analysis of plasmid DNA and PCR product

The glucomannan-filled capillary was applied to the analysis of plasmid DNA and its restriction fragments. Bluescript II SK(+) is a closed 2961 base pair DNA and produces four fragments of 166, 409, 540 and 1846 base pairs on digestion with *Dde*I. As shown in Fig. 5a, this 2961 base pair plasmid DNA appeared as a broad peak at 34.1 min, following some impurities at 14–15 min present in the commercial preparation. After digestion for 1 h with *Dde*I, the intact DNA disappeared and four fragments were generated (Fig. 5b). Based on the calibration graph in Fig. 2, the size of each fragment was estimated as 186 base pairs for peak 1, 440 base pairs for peak 2, 578 base pairs for peak 3 and 2000 base pairs for peak 4. These values are in good agreement with the size of the restriction fragments expected from the sequence of this plasmid DNA as described above, although the use of internal standards, such as the *Hae*III digest of ΦX174 DNA, allowed a more precise estimation of the size of the fragments. The rough estimation of the size of separated DNAs is even satisfactory for practical gene cloning studies.

The product of the polymerase chain reaction (PCR) of the η-chain of the brain 14–3–3 protein

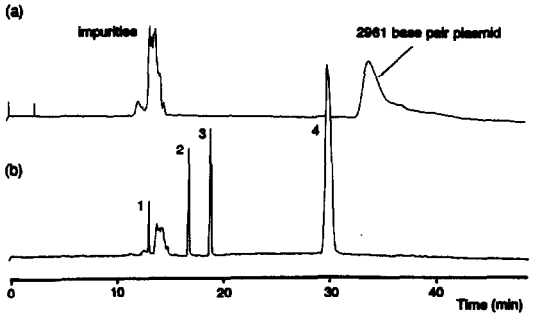

Fig. 5. Analysis of plasmid DNA and its restriction digest. Sample: Bluescript II SK(+), (a) before digestion and (b) after digestion with *Dde*I for 1 h. The digest was desalted and concentrated as described under Experimental and subjected to electrophoresis. Conditions as in Fig. 1a.

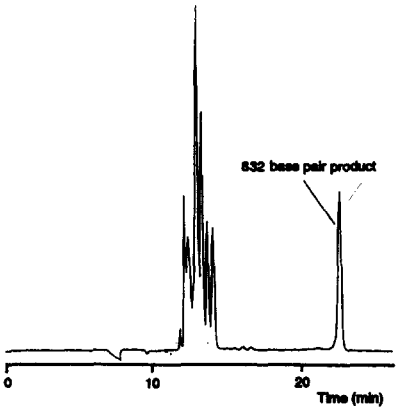

Fig. 6. Analysis of polymerase chain reaction (PCR) product. PCR product of η-chain of brain 14–3–3 protein prepared as described under Experimental was directly injected. Conditions as in Fig. 1a.

[15] was also analysed by the present method (Fig. 6). Following six peaks at 12–14 min due to NTPs and PCR primers, a single peak was detected at 22.5 min. From the migration time, the size of the product was estimated to be 822 base pairs. This value is close to the theoretical size of the fragment (832 base pairs), suggesting that the PCR procedure generated a single DNA as we expected.

CONCLUSIONS

This is the first report to describe the use of glucomannan in capillary electrophoresis. The entangled glucomannan solution, prepared easily under the mild conditions, was stable for at least several months and could be used as a sieving additive for capillary electrophoresis. The glucomannan-filled capillaries exhibited a size-dependent separation of DNA fragments. Because of its excellent resolution and reasonable reproducibility, the method should be applicable to the separation and analysis of a wide range of DNA samples such as plasmid DNA, restriction DNA fragments and PCR products. In addition, because glucomannan has two physical states, solution and gel, future studies may provide valuable information on the sieving mechanism of DNA fragments in electrophoresis.

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