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Stable homogeneous gel for molecular-sieving of DNA fragments in capillary electrophoresis

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Abstract

A polyacrylamide gel crosslinked with allyl-β-cyclodextrin can be used repeatedly for several weeks for the separation of DNA fragments, since bubbles are not generated during a run. Allyl-β-cyclodextrin can easily be synthesized in one step from allylglycidylether and β-cyclodextrin. The plate numbers for DNA fragments, up to about 1500 bp, are high: for the separation of pBR322/*Hae*III fragments they were in the range 450 000–1 600 000 m^{-1}. The resolution was almost independent of the concentration of the crosslinker (allyl-β-cyclodextrin) — in sharp contrast to gels crosslinked with *N,N′*-methylenebisacrylamide. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Capillary electrophoresis; DNA; Polyacrylamide; Allyl-β-cyclodextrin

1. Introduction

Analytical gel electrophoresis has probably had a greater impact on the progress of life sciences than any other technique. The first gel electrophoresis experiments were most likely those performed by Tiselius in 1927, when he "separated red phycoerythrin from blue phycocyanin in a slab of gelatin, obtaining beautiful narrow migrating zones" [1]. These experiments were never published. Wieme in collaboration with Rabaey described agar gel electrophoresis in the 1950s [2]. Immunoelectrophoresis, as proposed by Scheidegger and developed by Grabar, was based on this technique as described by Wieme [3]. The first molecular-sieving electrophoresis medium, starch gel, was described and published in 1955 by Smithies [4]. Interestingly, any gel, which can be used for molecular-sieve electrophoresis, can also be employed for molecular-sieve chromatography [5], because any electrophoretic method has its chromatographic counterpart [6].

The use of polyacrylamide matrices, introduced as anticonvection media for electrophoresis of proteins by Raymond and Weintraub [7], Ornstein and Davis [8], and Hjertén [9] (and as a stationary phase in chromatography by Hjertén and Mosbach [10]), has grown enormously during the last 30 years. It was soon recognized that these gels have the additional benefit of size discrimination when proteins and viruses move through the porous gel network [11,12]. Crosslinked polyacrylamide gels are being used today not only for analysis of proteins but also of nucleic acids, including DNA sequencing with single-base resolution from 20 to 600 bases [13–15]. Agarose gels, introduced by Hjertén for electrophoresis [16], are the sieving media of choice for DNA samples ranging from 300 to 50 000 base pairs (bp) in the non-pulsed mode. He also introduced these gels for immunoelectrophoresis [17] and chromatography [18] of proteins.

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The high resolving power of capillary gel electrophoresis (CGE), first used by Hjertén [19], has been demonstrated in the single-base resolution of DNA polynucleotides by Karger and co-workers [13–15]. The recently finished human genome project [20,21] is based on the development of rapid and accurate DNA sequencing technologies using capillary electrophoresis.

The drawbacks of crosslinked polyacrylamide gels, such as bubble formation and hydrolytic instability [22,23], have led to a predominant use of polymer solutions as sieving media in DNA separations during the last decade [24–33]. Although some authors have described the preparation of stable bubble-free polyacrylamide gels for capillary gel electrophoresis [23,34–37], these have not become widely used. Other types of crosslinked gel (mostly modified acrylamide matrices) have been introduced [38–40]. Additional information on crosslinked gels as sieving materials can be found in recent review articles, specifically dedicated to sieving media [41–44].

During the development of a charged polyacrylamide matrix crosslinked with *N,N′*-methylenebisacrylamide and 2-hydroxy-3-allyloxy-propyl-β-cyclodextrin (allyl-β-CD) for electrochromatographic separation of enantiomers [45], we observed that the gels were very stable. This finding prompted us to investigate whether this stability was retained when the hydrolytically labile *N,N′*-methylenebisacrylamide was omitted from the monomer solution, which appeared to be the case. The objective of this study was to investigate the application of this gel for molecular-sieve capillary electrophoresis of DNA fragments.

2. Experimental

2.1. Materials

Fused-silica tubing was purchased from MicroQuartz (Munich, Germany). Acrylamide, ammonium peroxysulfate (APS), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), DNA samples (AmpliSize™ molecular ruler 50–2000 bp ladder and 20 bp molecular ruler) were from Bio-Rad (Hercules, CA, USA). pBR322/*Hae*III was from Boehringer (Mannheim, Germany); sodium borohydride ($NaBH_4$) from Merck (Darmstadt, Germany); 3-(trimethoxysilyl)-propyl methacrylate (Bind-Silane) from Fluka (Buchs, Switzerland); β-cyclodextrin (β-CD) from Sigma (St. Louis, MO, USA); and allylglycidylether (AGE) from Aldrich (Steinheim, Germany).

2.2. Equipment

A laboratory-built capillary electrophoresis apparatus based on a Linear Model 200 UV detector (Linear Instruments, Reno, NV, USA) was used in all experiments.

2.3. Synthesis of allyl-β-CD

The entire procedure was carried out in a well-ventilated fume cupboard to avoid inhalation of the lachrymatory allylglycidylether. The synthesis method was similar to that already published [46,47] with some modifications to increase the yield [45]. It is based on the reaction between hydroxyl and epoxide groups at high pH. Briefly, 1.11 g of β-CD was dissolved in 6 ml of double-distilled water. About 3 ml of 1.5 *M* NaOH was added to increase the pH to 10–11. Then, 0.95 ml of AGE in small aliquots was dropped into the solution, which was stirred vigorously for 48 h at room temperature. The molar ratio of β-CD to AGE was 1:8. $NaBH_4$ (0.08 g) was added and the solution was stirred for an additional 2 h. This solution was stored at 4°C and used in all experiments without further treatment. The synthesis of β-cyclodextrin was thus very simple. The concentration of reacted and unreacted β-CD in this solution is close to 100 m*M*. However, we denote formally the solution obtained following the derivatization as '100 m*M*' allyl-β-CD solution, since it is difficult to estimate the correct concentration because we do not know the degree of substitution and how efficiently the reaction proceeds. As a consequence of this, we cannot express the total monomer concentration and the degree of crosslinking in terms of the parameters T and C, respectively — as is usually done [48]. Instead, we use the new parameters AA and CD for the concentration of acrylamide and allyl-β-CD, respectively, defined as

$$\mathrm{AA} = \frac{a \text{ g of acrylamide}}{100 \text{ ml of monomer solution}} \cdot 100 = a\% \text{ (w/v)}$$

$$\mathrm{CD} = \frac{b \text{ ml of allyl-}\beta\text{-CD solution}}{100 \text{ ml of monomer solution}} \cdot 100 = b\% \text{ (v/v)}$$

An AA3–CD9 gel was thus prepared by adding 0.03 g of acrylamide and 0.09 ml of the '100 m*M*' allyl-β-CD solution to 0.91 ml of the buffer, the volume of the catalyst system being ignored.

The '100 m*M*' allyl-β-CD solution did not change its composition upon storage, to judge from NMR analyses. This finding was supported by the observation that the performance of the gels did not change when the same allyl-β-CD solution was used during a period of 5–6 weeks.

2.4. Preparation of gel

Acrylamide (25–100 mg) was dissolved in 1 ml of 100 m*M* Tris–150 m*M* boric acid buffer, pH 8.2, containing different volumes (20–200 μl) of '100 m*M*' allyl-β-CD. These acrylamide–allyl-β-CD solutions (150 μl of each) were degassed using a water pump and 10 μl of 5% (w/v) APS and 10 μl of 5% (v/v) TEMED were added. The solutions were then sucked immediately into the Bind-Silane-treated fused-silica capillary (I.D., 25 or 50 μm) [49]. The polymerization started within a few minutes and was almost complete within 30 min, but the capillaries were usually not used until later. The gel-filled capillaries could be used repeatedly for long periods of time, often 1 month, without bubble formation or loss of resolution.

3. Results and discussion

Polyacrylamide gels, synthesized from acrylamide and *N,N′*-methylenebisacrylamide, are known to give a very high resolution in molecular-sieve electrophoresis of proteins and nucleic acids. However, bubbles are usually generated when the gels are used in capillary electrophoresis. Therefore, they are often replaced by polymer solutions, which, however, give a lower resolution than do gels [50,51]. The molar ratio between β-cyclodextrin and allylglycidylether in the synthesis mixture was 1:8, i.e. each D-glucopyranosyl unit in the β-cyclodextrin is, on an average, substituted with around one allyl group, provided that the reaction yield is close to 100% (a β-cyclodextrin molecule consists of seven D-glucopyranosyl units). An important difference between allyl-β-CD and *N,N′*-methylenebisacrylamide as crosslinker is, that the former is multifunctional whereas the latter is bifunctional. Therefore, the tendency of the gel to shrink during the polymerization or the electrophoresis should be less when allyl-β-CD is used as crosslinker, which may explain why bubbles seldom form in these gels. The multifunctionality may also account for the finding that the gel pores are small.

The treatment of the capillaries with Bind-Silane to attach the gel covalently to the wall was essential to obtain a stable gel. Sometimes the capillary became clogged by tiny particles during the silanization procedure. Following removal of the particles by an HPLC pump the capillaries could be filled with a gel, which, however, gave broad electrophoretic peaks (and sometimes bubbles). Therefore, one should discard all capillaries which following the silanization step contain particulate material.

The electrophoretic properties of the gels, including resolution and plate numbers, were tested at AA values in the range 1–10% (w/v) and for CD values between 2 and 10% (v/v). At AA values below 2.0–2.5% no gel formed, not even for CD values as high as 10–20%. Interestingly, gels with AA values around 10% (CD value: 5%) had pores so small that DNA fragments consisting of only 50 bp were too large to penetrate the gel. Gels synthesized at acrylamide concentrations between 2.5 and 8.0% were particularly studied because they afforded high resolution of DNA molecular ladders, such as 50–2000 bp and fragments with 20-bp increments. Typical separations are presented in Fig. 1a and b, respectively. The resolution is high, but decreases abruptly for DNA fragments larger than 1500 bp. To decrease the analysis times by increasing the voltage is not feasible for the larger DNA fragments (see Fig. 2). The gel concentrations employed in this report are, accordingly, appropriate for the analysis of PCR products and fragments of that size.

The resolution of DNA fragments at different concentrations of acrylamide at constant allyl-β-CD concentration was studied (Fig. 3a), as was also the

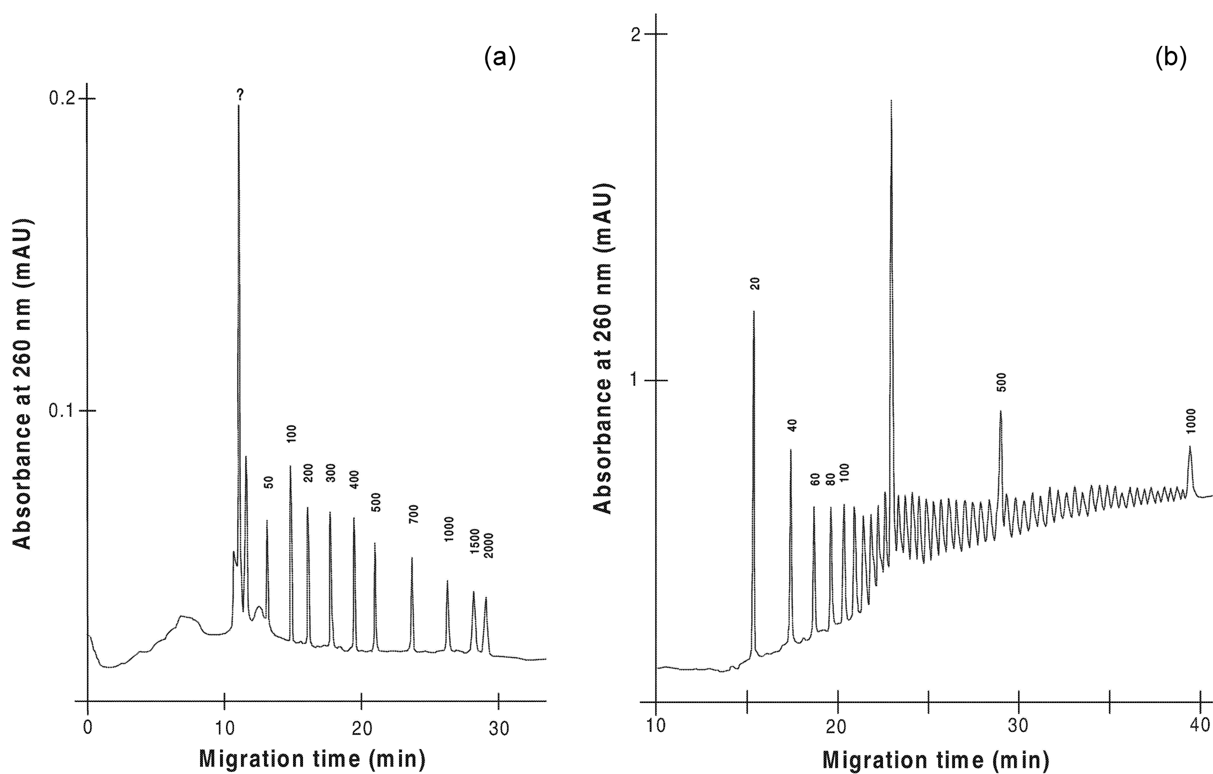


Fig. 1. Typical electropherograms of double stranded DNA molecular ladders: (a) 10 fragments (50, 100, 200, 300, 400, 500, 700, 1000, 1500 and 2000 bp) and (b) 50 fragments in exact 20-bp increments. Capillary: 20(17) cm×25 μm, treated with Bind-Silane and filled with (a) AA3–CD9 and (b) AA7–CD3 gels. Buffer: 0.1 *M* Tris–0.25 *M* boric acid, pH 8.5, containing 7 *M* urea. Injection: (a) 2 kV for 2 min and (b) 1 kV for 1 min; voltage: 2 kV.

effect of different allyl-β-CD concentrations at constant acrylamide concentration (Fig. 3b). Contrary to the behavior of polyacrylamide gels crosslinked with *N,N'*-methylenebisacrylamide, the same gels crosslinked with allyl-β-CD exhibit a resolution which is not significantly dependent on the concentration of the crosslinker (Fig. 3b). The advantage of synthesizing gels with low allyl-β-CD concentration is that the peaks are higher, i.e. the limit of detection is lower — an observation of practical importance.

Characteristic of gels crosslinked with allyl-β-CD is that bubbles seldom form, which means that the gels have a long lifetime. However, for high reproducibility the volumes of the electrode vessels should not be less than 2 ml to avoid changes in concentration and pH and the buffer should be changed after every run. A 5-min prerun with fresh buffer is also recommended.

Plots of current against field strength are shown in Fig. 4. A deviation from linearity occurs at field strengths above 125 V cm^{-1}. However, a calculation made using the formula:

$$H = 2 \cdot \frac{L}{\mathrm{v}} \cdot \frac{R^2 u_o^2}{192D}$$

[52,53], shows that the attendant thermal zone broadening is negligible.

The reproducibility of the resolution (R_s) observed in seven runs done in an AA7–CD3 gel over a period of the 3 weeks is presented in Fig. 5.

The separation of the pBR322 fragments cleaved with *Hae*III (Fig. 6) resulted in baseline resolution of all the components except the 123- and 124-bp fragments, which were poorly resolved (see insert in Fig. 6). The plate numbers were in the range

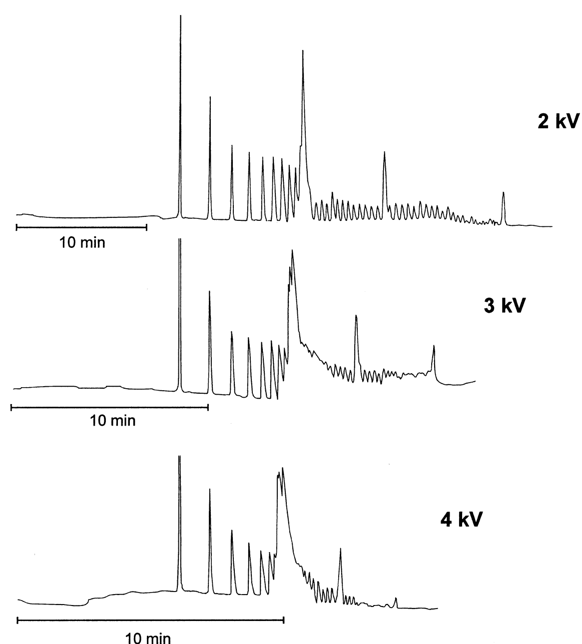
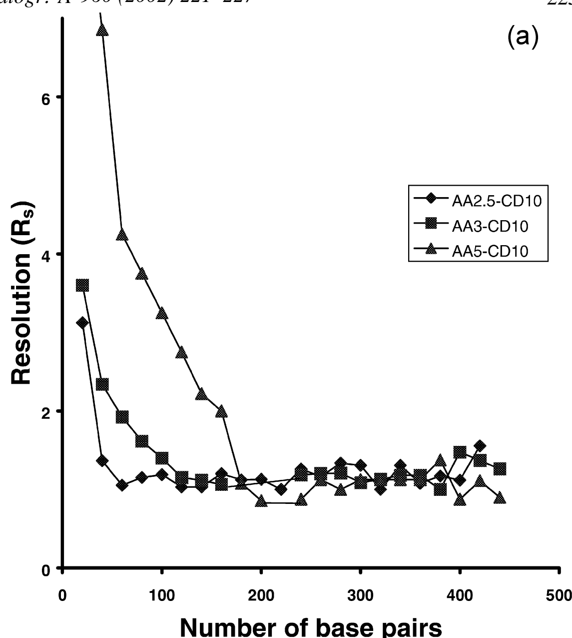


Fig. 2. Influence of the voltage on the resolution, using the 20-bp molecular ruler as sample. An increase in voltage resulted in faster separation, but the resolution for the larger DNA fragments decreased. However, baseline separation of the first five fragments and a recognizable pattern remained at 4 kV. Capillary: 20(17) cm×50 μm filled with AA5–CD10 gel. Buffer: 0.1 *M* Tris–0.25 *M* boric acid, pH 8.5, containing 7 *M* urea. Injection: 1 kV for 1 min. Observe that the time scales are different for the three electropherograms and that they are normalized in the sense that the distance between the first two peaks are the same.

450 000–1 600 000 m^{-1}, i.e. the acrylamide–allyl-β-CD gels compete favorably with other gels and with polymer solutions regarding efficiency. They have the additional advantage of long lifetimes, i.e. shorter analysis times compared to runs in polymer solutions, since there is no need for a time-consuming replacement of the sieving medium between experiments.

The new gels described have a broad application range, since they can be used for the separation of polynucleotides, peptides (not shown here) and enantiomers [45]. Different applications require gels of different composition — for instance, separation of enantiomers require higher allyl-β-CD concentrations (CD values 20–50) [45] than that used for molecular-sieving of DNA fragments. These gels are still more universal if charged groups are introduced,



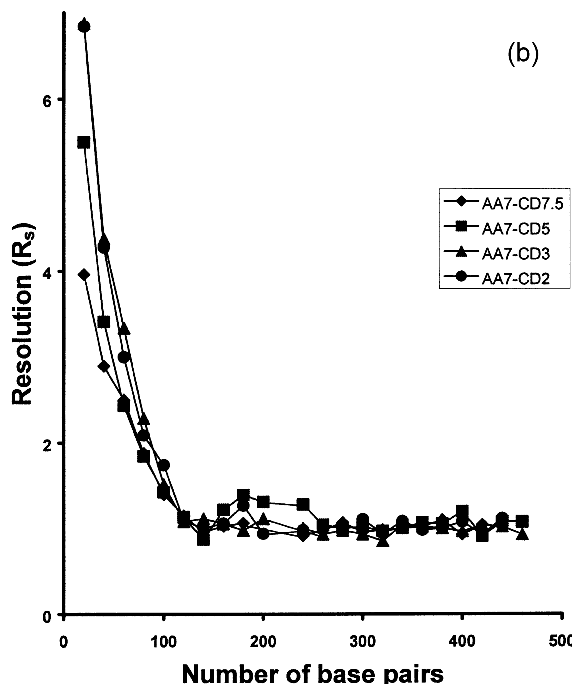


Fig. 3. Resolution as a function of the size of the DNA fragments obtained with 20-bp molecular ruler as sample. (a) The acrylamide concentration (the AA value) was systematically increased while the crosslinker concentration was kept constant (CD=10%, v/v). (b) The crosslinker concentration (the CD value) was systematically increased while the acrylamide concentration was held constant (AA=7%, w/v). The running conditions were the same as in Fig. 1b.

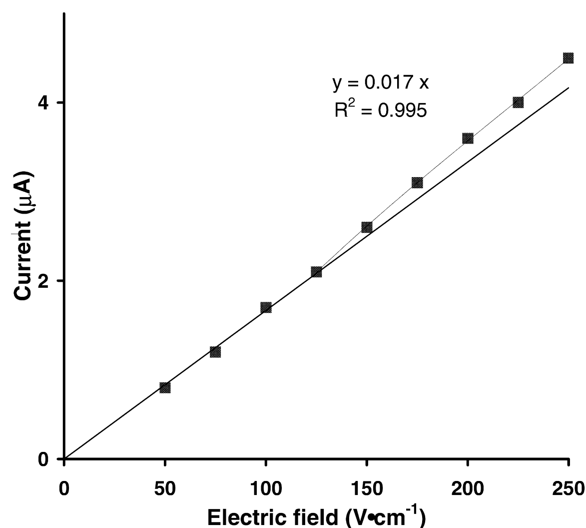


Fig. 4. Plot of current against the electric field strength. The capillary was filled with AA7–CD3 gel and installed in the home-built CE equipment (without active cooling). A deviation from linearity occurs above 125 V cm^{-1}. Most of the DNA analyses were performed at a field strength of 125 V cm^{-1}.

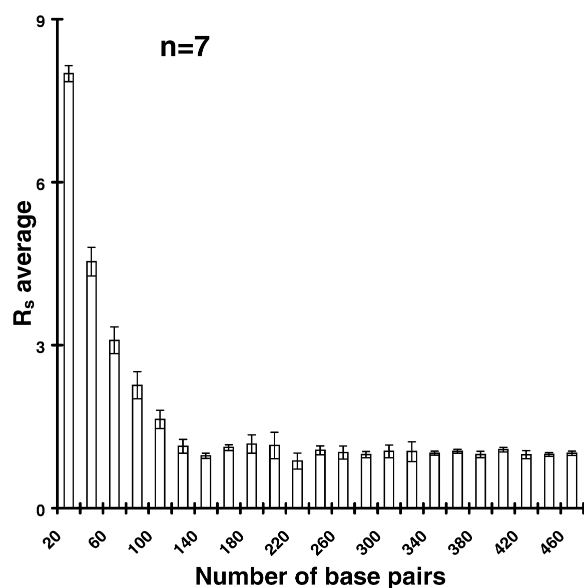


Fig. 5. Average resolution (R_s) in consecutive runs ($n=7$) performed with a 20-bp molecular ruler as sample in a capillary filled with an AA7–CD3 gel. The error bars show the standard deviation of the resolution; running conditions as in Fig. 1b.

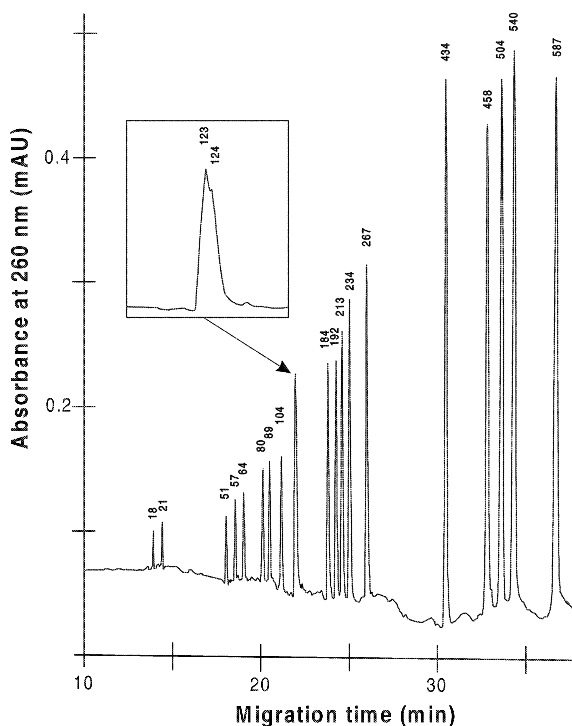


Fig. 6. Separation of pBR322–*Hae*III fragments in a capillary filled with an AA7–CD3 gel. The insert shows a partial separation of the two fragments of 123 and 124 bp. The two smallest fragments (8 and 11 bp) were not detected. Capillary: 15(12) cm×50 μm. Buffer: 0.1 *M* Tris–0.25 *M* boric acid, pH 8.5, containing 7 *M* urea. Injection: 0.5 kV for 7 s; voltage: 2 kV. The plate numbers are very high (450 000–1 600 000).

which makes them usable for electrochromatography [45]. Such experiments have been performed with enantiomers (not shown here). We have not yet explored whether they can be employed also for the separation of proteins, i.e. if the pores can be enlarged without risk of bubble formation.

4. Conclusion

A polyacrylamide gel synthesized from acrylamide using allyl-β-cyclodextrin as cross-linker permits (1) very high resolution when used in capillary electrophoresis as a molecular sieving medium for the separation of relatively short DNA fragments and (2) repeated runs on the same gel without bubble formation, which minimizes the total time for a

series of analyses and increases the reproducibility. The gel should be useful also in slab gel electrophoresis.

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