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Journal of Chromatography A, 772 (1997) 129–135

JOURNAL OF CHROMATOGRAPHY A

Polyacrylamide gradient gel-filled capillaries with low detection background

Yi Chen[a,*], Feng-Ling Wang[a], Uli Schwarz[b]

[a]*Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, China*

[b]*Max-Planck-Institut für Entwicklungsbiologie, Abteilung Biochemie, Spemannstrasse 35, 72076 Tübingen, Germany*

Abstract

A method has been explored for the production of low background polyacrylamide gradient gel-filled capillaries. The main idea is to prepare the capillaries by filling them with short steps of gelling solutions, including a long section of buffer for detection. This method is demonstrated to be practicable by scanning the filled capillaries. Smooth gradients can be formed at a step-length of 1.5 cm if the relevant capillaries are filled with solutions in a step-down order. By using this method, the desired capillaries were prepared with a success rate of >90% and gradient position error of ±0.62 mm. As shown by capillary gradient gel electrophoresis, this new type of capillary can be used for fast and efficient separation of polyaspartate and murein oligosaccharides (from *E. coli*). They also allow the use of any available UV wavelength for high detection sensitivity. The unlabelled oligosaccharides at a total concentration of 1 mg ml^{-1} were directly detected at 200 nm.

Keywords: Capillary columns; Gradient gel-filled capillaries; Oligosaccharides; Polyamino acids

1. Introduction

Capillary gel electrophoresis (CGE) [1] can be considered as a miniaturized version of slab gel electrophoresis (SGE). Such miniaturization, once achieved, leads to a tremendous increase in the electrophoretic efficiency [2] as shown in the CGE separation of biopolymers [3] such as proteins [1,4–10], oligosaccharides [11,12], oligonucleotides [2,8,13–15] and DNA fragments [16,17], including DNA sequencing [18–20]. Considering that slab gradient gel electrophoresis (SGGE) is more efficient than SGE in the separation of closely-sized proteins [21] and in the identification of gene mutants [22–24], capillary gradient gel electrophoresis (CGGE) should logically be more effective than CGE. Nevertheless, although CGE has been explored for more than 10 years [1], CGGE remains undeveloped. The critical reason is the difficulty in preparing the gradient gel-filled capillaries.

We first tried to use the preparation method established for slab gradient gels but failed, mainly because of the difficulty in controlling the filling speed. Compared to a slab gel chamber, the bores of capillaries are relatively uneven and hence solution flowing speed may change within a tube and from tube to tube. For a tube having only microliters of volume, any minute speed variation will cause inaccurate location of gradients, resulting in irreproducible capillaries. We then explored a new method and a step filling procedure was established [9,10].

In this paper, we will discuss the production of linear gradient gel-filled capillaries with low de-

*Corresponding author.

PII S0021-9673(97)00111-8

tection background, using the step filling method. The main idea is to prepare the capillaries by filling them with stepwise gelling solutions at sufficiently short steps, including a long section of buffer used for detection. Owing to molecular diffusion and solution adhering-mixing mechanism, the very short solution steps can only be maintained for a while and a smooth gradient will be formed before polymerization occurs.

To follow the establishment of the gradients, a capillary UV-scanning system was established. With this system, capillaries filled with monomer solutions at different step-lengths were scanned at 285 nm. The results show that smooth gradients are formed at a step-length of about 1.5 cm if the capillaries are filled with the solutions in a step-down order (filled in from high to low concentrations, with short step-down filling). If the filling order is reversed, the step-length must be reduced to less than 0.8 cm which is not recommended.

By using the step-down filling method, gradient gel-filled capillaries were prepared with a success rate of >90% and a gradient location error of ±0.62 mm. These capillaries can be used for fast and efficient separation of polyaspartate and unlabelled murein oligosaccharides (from *E. coli*). They also allow the use of any available UV wavelength for sensitive detection of the separated compounds. The unlabelled oligosaccharides separated at a total concentration of 1 mg ml^{-1} were directly detected at 200 nm.

2. Experimental

2.1. Chemicals

Tris [tris(hydroxymethyl)aminomethane] and tricine [N-tris(hydroxymethyl)methylglycine] were from Fluka (Buchs, Switzerland) and Dojindo (Tokyo, Japan), respectively. EDTA, acetonitrile (HPLC grade) and γ-MPS (γ-methacryloxypropyltrimethoxysilane) came from Sigma (St. Louis, MO, USA). Acrylamide and Bis [N,N′-methylenebis-(acrylamide)] were research reagent grade from Beijing Purchasing Station (Chinese Pharmacy, Beijing, China). TEMED (N,N,N′,N′-tetraamethylethylenediamine) was analytical reagent grade from Xi'an Chemical Works (Xi'an, China). Boric acid, APS (ammonium persulfate) and other chemicals were all analytical reagent grade from Beijing Chemical Works (Beijing, China). Water used was triply distilled.

2.2. Samples

Poly(Asp) [poly-(α,β)-DL-aspartate$^-$ Na$^+$, M_r=5–10·10^4, from Sigma] was dissolved in water at 25 mg ml^{-1}. Poly(GlcNAc-Mur)$_{n>13}$ is a mixture of α(1→4) linked interpolymer of N-acetylglucosamine-2 (GlcNAc) and muramic acid (Mur), with more than 13 GlcNAc-Mur units. These oligosaccharides were prepared by enzymatic digestion of the murein peptidoglycan from the sacculi of *E. coli* KN126 (for detailed preparation see [25]) and desalted with the Beckman HPLC system (model 126 with model 166 UV detector): A 50-μl aliquot of sample was injected into a 20 cm×4 mm I.D. with 3 μm of C_{18} packing and eluted with water–HCl (pH 2.0) at 0.5 ml min^{-1} for 10 min at room temperature. The elution was followed by pure acetonitrile and the peak in front of acetonitrile collected. The collected solution was dried with nitrogen gas and stored at −20°C. Before use, it was dissolved in water at a total concentration of 1 mg ml^{-1}.

2.3. Preparation of capillaries [9]

The step-down filling procedure is recommended from our experience. By this method, the capillaries should be pre-constructed as follows:

$$L = L_b + L_g + 2L_c = L_{det} + L_{inj} + L_g - 3\ \text{cm}$$

$$L_{det} = (L_b + L_c + 1.5\ \text{cm}) \quad (> 10.5\ \text{cm for Beckman P/ACE system}) \qquad (1)$$

$$L_{inj} = (L_c + 1.5\ \text{cm}) < 6\ \text{cm}$$

where L is the total length of capillaries, L_g is the gel length and L_b the buffer length (about 9 cm for Beckman P/ACE system); L_c is the extra length at each tube end for easy manipulation and will be cut off after preparation; L_{det} and L_{inj} are the coating lengths at the detection and injection ends respective-

ly (Fig. 1A). This coating is required to suppress the electroosmotic effect.

In addition, the gelling solutions, which are the monomer solutions containing 0.05–0.1% (v/v) TEMED and 0.035–0.06% (w/v) APS added before filling, are prepared according to Eq. (2)

$$n = L_g/L_s$$
$$H_s = (C_n - C_1)/(n-1); \quad (C_n > C_1) \qquad (2)$$
$$C_i = (i-1)H_s + C_1 = C_n - (n-i)H_s; \quad (i = 1, 2, 3, ..., n)$$

where n is the number of gel steps with a step-length of L_s (=1.5 cm, see Section 3) and step-height of H_s (=concentration increment of each step); $(C_n - C_1)$ is the gradient range and C_i the monomer concentration of step i. The concentration is given by x%T+y%C where x%T (w/v) is the sum concentration of BIS and acrylamide and y%C the mass ratio of BIS over the total monomers. Eq. (2) is only suitable for the preparation of linear concentration gradients. For non-linear gradients, C_i and H_s should be changed. If $H_s = 0$, constant gels result.

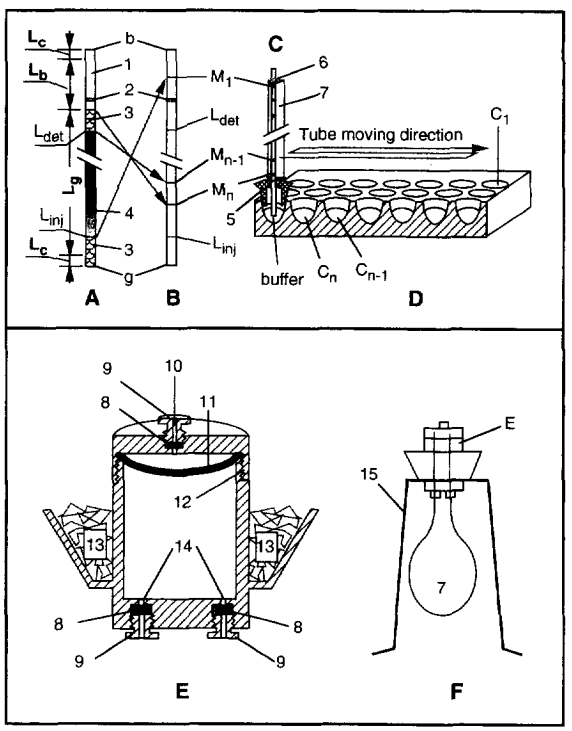

Fig. 1. Several critical steps and devices for filling gradient gel-filled capillaries. (A) Structure of a gradient gel-filled capillary; (B) Marking the capillary reversely; (C) Assembled capillary during filling; (D) PTFE plate with a cluster of 2-ml wells; (E) Longitudinal section of a pressure-tight cylinder made of stainless steel; (F) Polymerizing position. (1) buffer (2) detection window (3) immobilized gel plug (4) gradient gel without immobilization (5) rubber cap (6) plasticine (7) capillary (8) rubber septa (9) screw (10) diameter 1-mm hole for injection (11) sealing ring (12) screw-thread (13) ice (14) holes for plugging capillary (15) hanging shell (E) pressurized cylinder; L_b = buffer length, L_g = gel length, L_c = extra length, L_{det} and L_{inj} = coating length, C_i = concentration of gelling solution i.

2.3.1. Capillary coating procedure [9,10]

The end b of a new capillary (J&W) marked red at L_{det} and L_{inj} (Fig. 1A) is dipped into a methanol solution containing 0.5% γ-MPS and 0.5% acetic acid. When the solution flows up (by capillary force) to the mark L_{det}, the capillary is pulled out and laid vertically at room temperature for 15 min. This capillary is washed by sucking water from end g for 5 min and dried by an inrush of a hot air (>50°C) for 10 min. The L_{inj} wall is treated the same way but without drying. The capillary is then filled with a gelling solution of 1.5%T+5%C and kept at room temperature for 1 h. After washing with water for 5 min, it is dried with an air flow at room temperature for about 5 min.

2.3.2. Filling procedure [9]

A partially coated capillary is reversely marked black (Fig. 1B), at an interval of L_s (normally 1.5 cm) in between the M_1 and M_n marks. It is then assembled in a triangle-tube and fixed with plasticine. A rubber cap is mounted near the end g (Fig. 1C, No. 5).

Buffer and gelling solutions are prepared in $(n+1)$ adjacent wells drilled on a PTFE plate (Fig. 1D). The buffer is added to the first well followed by C_n, C_{n-1},...,C_2 and C_1 gelling solutions.

The end g of the capillary is dipped into the first well and the cap gently pressed into the well to pump up the buffer. Once the buffer reaches the mark M_n, the capillary is quickly transferred to the second well till the buffer reaches the mark M_{n-1}. This operation is repeated until C_1 solution is finally filled in.

The filled capillary is quickly pulled out from the triangle-tube and both of its ends are plugged into one pressure-tight cylinder (5 ml, Fig. 1E). The cylinder is then pressured by injection of about 4.5

ml ice-cooled water and further cooled by ice (Fig. 1E, No. 13). The capillary is hung up (Fig. 1F) for 5 h to accomplish the polymerization and finally stored at room temperature by dipping both its ends into a running buffer for more than 3 days. This storage increases the capillary shelf-life and resolution, for unknown reasons.

Before use, a detection window is created 2–3 cm from the gel–buffer boundary (Fig. 1A, No. 2) by manually removing the over-coating of the capillaries with a scalpel. The capillary with L_c cut off is equilibrated with running buffer at -150 V cm^{-1} and 25°C until the current becomes stable.

2.4. Scanning

The solution gradients were followed by passing the capillaries (with over-coating burned off) through a Beckman UV detector (model 166) modified by replacing its flow-cell with an aluminium block (Fig. 2). The moving speed of the capillaries was controlled at 0.5 mm s^{-1} with a common recorder (Pharmacia). All the capillaries were scanned 10 min after filling and the data acquired at 2 Hz using a computer with the SYSTEM GOLD software (version 8.0). Both of the data from the Shimadzu spectrophotometer (model UV-2100) and the laboratory-made scanning system (Fig. 2) show that the most sensitive scanning wavelength is 285 nm whereas the curves are not linear or parallel to the gradients (Fig. 3A and B). The linearity of the curves is improved at 290–310 nm but the scanning sensitivity is also reduced greatly.

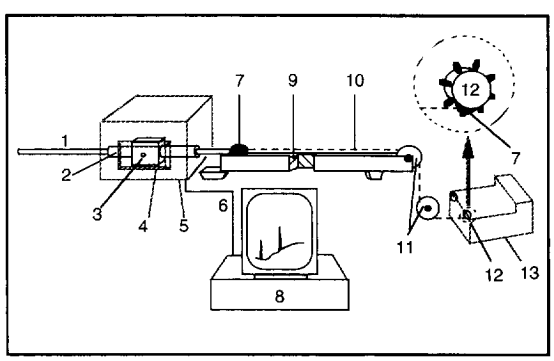

Fig. 2. Capillary UV-scanning system; (1) capillary, (2) 0.4-mm I.D. fused-silica tube, (3) aluminium block, (4) phototube block, (5) Beckman model 166 UV detector, (6) cable, (7) junction fixative (such as plasticine), (8) data acquisition unit, (9) capillary guide, (10) nylon wire, (11) guide wheel, (12) roller and (13) speed controller (a recorder).

2.5. Electrophoresis

All electrophoresis was performed by using the Beckman P/ACE system (model 2050) and the operation controlled with the SYSTEM GOLD software. All the gel-filled capillaries of 30/37 cm (effective/total length)×75-μm I.D. were run at -7.4 kV and 25°C for the separation of poly(Asp) or 35°C for the separation of poly(GlcNAc-Mur)$_{n>13}$. Poly(Asp) was

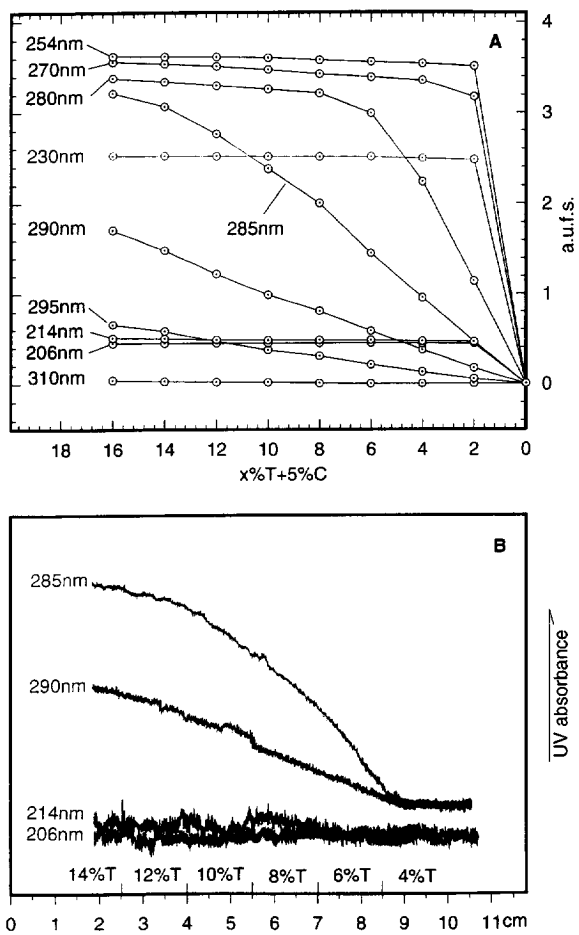

Fig. 3. Dependence of scanning sensitivity on wavelength, with buffer absorption as baseline. (A) measured from isolated solutions with spectrophotometer (B) scanned from a 75-μm I.D. capillary with linear gradient solutions. Solutions: x%T+y%C+0.1 *M* tricine+0.05 *M* Tris.

introduced into the C_1 end of the gradient gels by diffusion for 20 s [8–10] and poly(GlcNAc-Mur)$_{n>13}$ by electromigration at -5 kV cm^{-1} for 5 s. The separated bands were detected at 200 nm and 1 s rise-time while the peak data acquired at 1 Hz.

3. Results and discussion

3.1. Conditions for forming smooth gradients

Stepwise filling of a capillary will yield smooth gradients depending on the filling order and the step-length. Fig. 4 shows that, as the step-length decreases, the gradients become smoother and smoother (from line 1 to line 3). By step-down filling method, a smooth gradient is obtained at 1.5-cm L_s (Fig. 4B, line 3). If the filling order is reversed, a step gradient is still found at the same L_s (Fig. 4A, line 3). In this case, smooth gradients are formed only with steps shorter than 0.8 cm which is tedious and not recommended. Molecular diffusion and solution adhering–mixing effects are considered to be involved in this gradient formation. Since diffusion is independent on the filling direction, the solution adhering–mixing mechanism should be responsible for the influence of filling order. A solution adhered at the tube tip and on the tube wall will be washed off by the front of its following solution, leading to broadening of the boundary. This broadening effect can become significant if the latter solution has a lower concentration than the former one. Other factors such as the concentrations and concentration differences also affect the gradient formation but are not so evident (Fig. 4 line 1 and 2) as the filling direction.

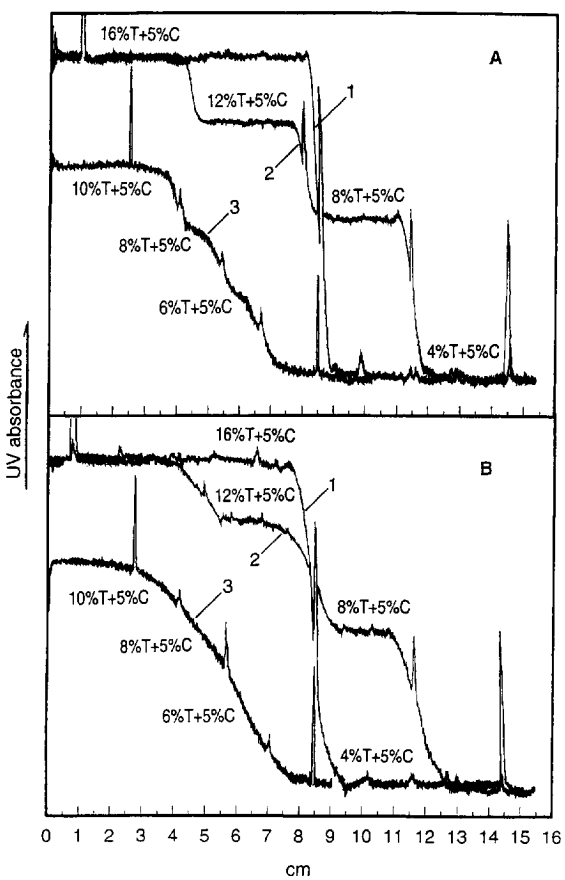

Fig. 4. Influences of step-length and filling direction on gradient formation scanned from 75-μm I.D. capillaries. The peaks show the filling marks, some of which have been partially washed off. The filling direction is $C_1 \rightarrow C_n$ (A) or $C_n \rightarrow C_1$ (B).

3.2. Reproducibility of the preparation

The reproducibility of the preparation depends on the accuracy of positioning the solutions inside a capillary while the positioning in turn depends on the observation of the solution flow. Clearly, the flow of the solutions is difficult to observe inside a very narrow capillary. For easy observation, the capillary is amplified with the assistance of a triangle-tube (Fig. 1C).

Another factor affecting reproducibilty is solution flow-rate or filling speed. High filling speed may cause the solutions to pass the required borders. However, if the speed is too slow, polymerization may happen before the filling is finished. Supposing that the polymerization is designed to start at 15 min after the gelling solutions are prepared, the filling speed should be controlled at 0.1–1.0 cm s^{-1}. We prefer to keep it at about 0.5 cm s^{-1}. For 30 cm gels, the total filling time is thus about 50–60 s.

Precise location of the solutions in the capillaries was thus achieved, with an average position error of ±0.62 mm. This was measured as follows: Five capillaries with the same gradient as in Fig. 4B, line 2 were scanned. The middle points of the three slopes were considered to be real boundary position,

the distance between the points and the peaks (peak center) was defined as the position error. The mean errors of the five measurements were ±0.71, 0.54 and 0.61 mm for the boundaries of 16–12%T, 12–8%T, 8–4%T, respectively. The overall average error was ±0.62 mm.

3.3. Elimination of voids

Voids are a common problem in the preparation of polyacrylamide gel-filled capillaries, resulting from the volumetric losses of the gelling solutions during polymerization [8]. Unexpectedly, the voids are reduced in gradient gels as compared with homo-gels but they still exist. To eliminate the void problem, a polymerization method suggested in our previous work [8–10] was employed: The capillary ends are cooled and slightly pressurized (Fig. 1E and F); To effectively inhibit the formation of voids, only the gel ends are immobilized (Fig. 1A) to prevent the gels from migration during electrophoresis. Void-free capillaries can thus be prepared with a success rate of >90% for gels below 16%T+5%C.

By scanning the gelling solution-filled capillaries, we find that the polymerization usually starts from the tube ends and the high concentration parts. The cooling of the tube ends is hence necessary to inhibit the end-polymerization. If the polymerization starts only from the high concentration parts, the volumetric losses of the gels can easily be compensated for by the not-yet-polymerized solutions with the assistance of a slight pressure.

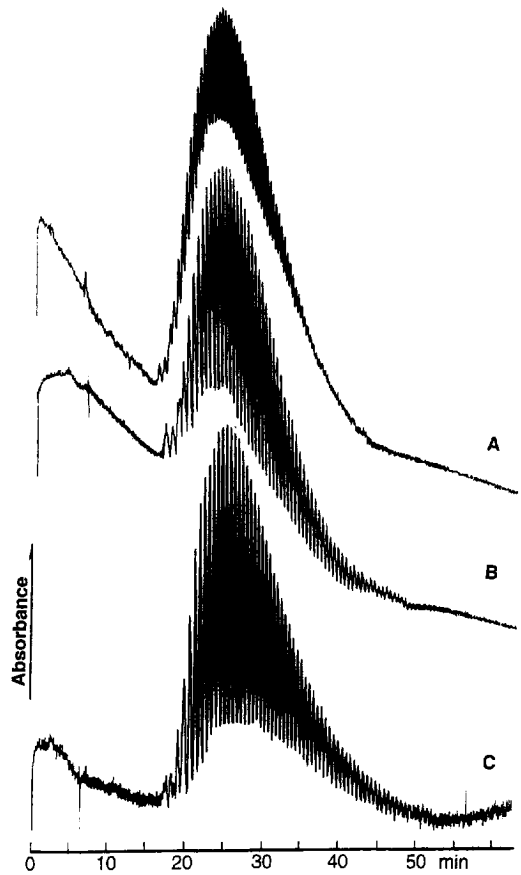

Fig. 5. CGGE (B) and CGE (A and B) of poly(Asp). Gel: 28 cm of 10%T+5%C (A), 10–15% T+5% C (B) and 15% T+5% C (C). Running buffer: 0.1 *M* tricine and 0.05 *M* Tris.

3.4. Resolution and detection sensitivity

The performance of the new capillaries was primarily tested by CGGE of poly(Asp) and poly(GlcNAc-Mur)$_{n>13}$. A significant feature of the capillaries is that they can yield high efficiency and high running speed. Figs. 5 and 6 shows that a (C_1–C_n) gradient gel (B) generates the faster running speed than a constant gel at a high concentration of C_n (C) and much higher efficiency than a gel at C_1 (A), even higher than the concentrated gel (compare Fig. 6B and C). Another feature is that they allow the use of any available wavelength for sensitive detection. The unlabelled poly(GlcNAc-Mur)$_{n>13}$, which does not have reducing ends [25] and cannot be labelled by common methods, can be directly detected at 200 nm even at the total concentration of 1 mg ml^{-1} or at an average concentration of about 0.03 mg ml^{-1} per peak. From the viewpoint of detection, the gradient gel-filled capillaries seem to be more sensitive than the homogenous gel-filled columns with also low detection background. From Fig. 5B and C we can find that the former capillaries yield about 4-fold higher peaks than the latter ones. The reason is that an ascending gel gradient sharpens the sample zones due to the solutes stacking at the migration fronts. This stacking effect is however not as significant as we expected. Possibly, the descending gradient between the gel and buffer (gel–buffer boundary) partially unties the stacked zones.

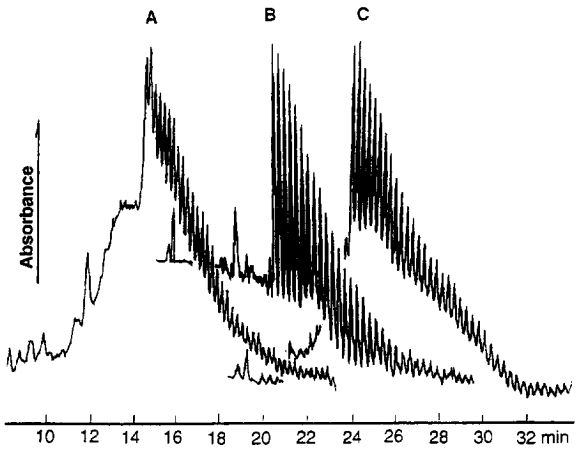

Fig. 6. CGGE (B) and CGE (A and B) of murein oligosaccharides from the succuli of *E. coli*. Gel: 27 cm of 3% T+3% C (A), 3–5% T+3% C (B) and 5% T+3% C (C). Running buffer: 0.25 *M* boric acid, 0.1 *M* Tris and 1.5 m*M* EDTA.

4. Conclusion

We have demonstrated that linear gradient gel-filled capillaries can be reproducibly prepared by a step filling procedure. Such capillaries have been tested by CGGE of oligosaccharides and poly(Asp), which shows that they are superior to homogenous gel-filled ones. The advantage of the discussed method is that it can be used for the preparation of not only linear but also non-linear gradient gel-filled capillaries. The main disadvantage is that the filling operation requires multiple steps.

Acknowledgments

We would like to think for the financial supports of this project from the Chinese National Divisions of Education and Personnel, the Chinese Foundation of Natural Sciences, and the Director Funds of the Institute of Chemistry, Chinese Academy of Sciences. The kind donation of the Beckman P/CE system from Alexander von Humboldt Foundation and a research fellowship offered by the Max-Planck Society are specially acknowledged by Y.C.

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