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Preparation of highly condensed polyacrylamide gel-filled capillaries

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Abstract

A fairly quick method has been established for the production of highly condensed polyacrylamide gel-filled capillaries. Void-free capillaries with inner diameters as small as 25 μm and monomer concentrations of up to over 30% T + 5% C can successfully be prepared within 5 h. These capillaries can be used for over 20 injections of poly-(α,β)-D,L-aspartate at 200 V/cm and 25°C, with gels immobilized at the capillary tips after polymerization, and for more than 70 injections with gels immobilized, during polymerization, over a longer section of at least 0.5 cm. The important polymerization conditions in this method are the application of a slight pressure, controlled polymerization directions and the selections of buffer components and/or the concentrations of radicals and catalyst.

1. Introduction

Highly condensed polyacrylamide gel-filled capillaries ($\geq$15% T + x% C*, $x > 0$) have been shown to be one of the basic conditions in capillary gel electrophoresis (CGE) of polyamino acids and oligosaccharides [1–4]. However, the preparation of these capillaries, which are not commercially available, is much more difficult than that of low-concentration gel-filled capillaries. Voids [5] or vacuum bubbles [6,7], formed inside the gels because of the volumetric losses of the resulting gels [5], increase dramatically with monomer concentration. The reason is not clear. A possible explanation might be the increase in gelatinization speed or more fragile gels formed.

To overcome the void problem, we tried several existing methods, such as pressurized (20 atm; 1 atm = 101 325 Pa) polymerizations [8,9] and programmed temperature polymerization [5]. Unfortunately, the success rate was less than 30%. We then turned to isotachophoretic polymerization [4,5]. In preparing 20 tubes of 50 cm × 50 μm I.D. with gel of 20% T + 5% C, there were 12 usable capillaries, having at least a 27 cm void-free length after equilibration. This is an excellent method, with a success rate of >60%. However, the preparation time is fairly long. In a routine procedure, the time for producing a 50-cm capillary with a 20% T + 5% C gel is about 80 h, including 50 h of polymerization and 30 h of equilibration [4]. It seemed desirable to establish a faster polymerization procedure.

In this paper, we present a 5-h method developed from the pressurized polymerizations [8,9] and programmed-temperature polymeriza-

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* %T = g acrylamide + g bisacrylamide per 100 ml of solution, %C = % bisacrylamide in T.

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tion [5]. The gels are chemically immobilized at the capillary tips by co-polymerization [10,11] and/or after polymerization. The stability of the prepared capillaries was examined with poly-aspartate as a testing sample and the important polymerization conditions were studied and are discussed.

2. Experimental

2.1. Materials

N-Tris(hydroxymethyl)methylglycine (Tricine), boric acid and urea (electrophoretically pure); γ-methacryloxypropyltrimethoxysilane; nuclease P1, polyadenylic acid [poly(A)], hydroxypropylmethylcellulose (HPMC), poly-(α,β)-D,L-aspartate Na^+, M_r (the average molecular mass detected by viscosity) = 5400 [$poly(Asp)_{5400}$] were purchased from Sigma (St. Louis, MO, USA). Oligoadenylic acid of 12- to 18mers [$poly(A)_{12-18}$] was supplied by Pharmacia LKB (Uppsala, Sweden). Acrylamide and N,N'-methylenebis(acrylamide) (bis), electrophoretically pure, were from Bio-Rad Labs. (Richmond, CA, USA). Tris(hydroxymethyl)aminomethane (Tris), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS) and other chemicals were all reagent grade from E. Merck (Darmstadt, Germany). Fused-silica capillaries with an outer diameter of 375 μm were from Composite Metal Services (Worcestershire, UK). The water used was purified with the Millipore Super Q system.

2.2. Preparation of samples

$Poly(Asp)_{5400}$ (50 mg/ml) and $poly(A)_{12-18}$ (1.7 units/μl) were dissolved in water. A partial hydrolysate of poly(A) was prepared by hydrolyzing poly(A) with nuclease P1: a 50-μl solution of 1.25% (w/v) poly(A) in water–0.3 *M* acetate, pH 6 (1:1) was digested with 1 μl Nuclease P1 (1 μg/1 μl water) at 40°C for 8 min and then stored at −20°C before use.

2.3. Preparation of gel-filled capillaries

Filling device

Sealed glass vials are used as "micro-pumps". A 4.7-ml (measured volume) threaded sample vial from Beckman (part No. 358807) is sealed by a modified screw-cap (Beckman, 360004), a rubber septum (3 mm thickness, cut from a rubber stopper) and a PTFE septum from Millipore (73005) as shown in Fig. 1A. The vial is evacuated or pressurized with a plastic syringe after plugging in the capillaries (Fig. 1B and C).

Preparation of one-end-modified capillaries [10,11,14]

One end of a new capillary is dipped into a 0.5% (v/v) modification solution (MS) of γ-methacryloxypropyltrimethoxysilane in water–acetic acid (1:1) until the solution reaches a height of 5–10 cm from the dipped end (checked against a light). This part is considered to be completely

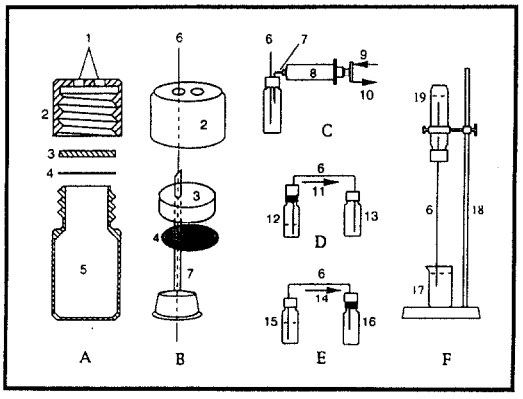

Fig. 1. Sealed vial and its use. (A) Construction of the sealed vial: 1 = 1.5-mm-diameter holes (one for plugging capillary and the other for syringe needle); 2 = plastic screw-cap; 3 = rubber pad; 4 = PTFE septum; 5 = 4.7-ml glass vial. (B) Plugging a capillary (6) through the rubber pad with the help of a syringe needle (7). (C) The sealed vial is being pressurized (9) or evacuated (10) with a 60-ml plastic syringe (8). (D) Evacuation filling: 11 = flow direction; 12 = unsealed vial with solution; 13 = evacuated vial. (E) Pressurization filling: 14 = flow direction; 15 = pressurized vial with solution; 16 = unsealed vial. (F) Polymerization position: 6 = capillary filled with acrylamide solution; 17 = beaker with 25–35°C water; 18 = hanging shelf; 19 = vial pressurized by injection of *ca.* 4 ml ice-cooled water.

modified. The capillary is kept at room temperature for 20 min, neglecting the natural evaporation of MS, and then washed for 5 min by pumping water into the non-modified end.

Preparation of full-length-modified capillaries

A new capillary is completely filled with MS, kept at room temperature for 20 min and then washed with water for 5 min.

Polymerization process

A 30–60-cm capillary, with or without modification, is rinsed for 2–5 min with a 0.5% solution of HPMC or a radical-free monomer solution and then filled with a polymerizing solution by an evacuated vial (Fig. 1D). Once the polymerizing solution reaches the outlet, the filling is stopped, and one tip of the tube, or the modified end in the case of filling a one-end-modified capillary, is immediately dipped into 50–70°C water for 1 min to start the polymerization from this end. The other end is then mounted tightly to a vial and about 4 ml of ice-cooled water are injected into the vial to cool this end and to build up pressure (the pressure calculated according to the law of the ideal gas is *ca.* 6 atm at 0°C). To finish polymerization, the capillary is hung vertically for 4 h in a shockless and windless place (20–30°C), with the pressurized end up and the polymerized end in 25–35°C water (Fig. 1F). After polymerization, the pressurized end (*ca.* 2–4 cm) is cut off to remove empty and/or liquid parts. The other end is checked under a microscope for possible voids.

The polymerizing solution mentioned above is a degassed monomer solution, containing 0.05% (v/v) TEMED and 0.03–0.05% (w/v) APS. It is prepared from a stock solution of 40% T + 5% C with buffer and water, ignoring the changes of final volumes [12,13]. For example, a polymerizing solution of 15% T + 5% C/TT15 (0.1 *M* Tricine + 0.05 *M* Tris) is a 3:4:1 (v/v/v) mixture of the stock solution–0.2 *M* Tricine + 0.1 *M* Tris–water, degassed in an evacuated vial by ultrasonic shocking for 1 min.

Partial gel immobilization

Procedure A: a one-end-modified capillary is filled with a gel as described above. The gel will be immobilized at the modified part of the capillary during polymerization [10,11]. *Procedure B*: a capillary without any modification is filled with a gel according to the polymerization process. After 4 h, its ends are dipped into MS for 10 min. The bifunctional silane will diffuse into the capillary tips and forms chemical bonds between the gel and the capillary tips. The capillary ends are then dipped for 10–15 min into a solution of 2% T + 3% C/TT15 + 1% TEMED + 1% APS to enhance the immobilization. This method immobilizes the gels at the capillary tips.

By combining procedures A and B, four types of capillaries can be prepared: CapA, with gel immobilized by A; CapAB, with gels immobilized first at one end by A then at the other end by B; CapB, with gels immobilized at one end by B; CapBB, with gels immobilized at both ends by B.

Full-length gel immobilization

For low-concentration gels (below 8% T), the full-length-modified capillaries are filled with the gels by the same polymerization process and the gels are immobilized along the entire capillary wall during polymerization.

The prepared capillaries are generally equilibrated with running buffer at 200 V/cm for 1 h and then stored at room temperature with both ends dipped into the running buffer. The capillary tips are re-immobilized by procedure B every month for long term storage (up to 6 months). The detection window is made just before separation of samples. About 2 mm of the polyimide over-coating are removed manually with a scalper and cleaned with methanol.

2.4. Electrophoresis

Electrophoresis was performed using the Beckman P/ACE system 2100, controlled by an IBM computer of Model SP/2 with the System Gold software (version 7.0). The running buffer was

the same as that used for preparing monomer solutions, degassed just before use and renewed every five runs. The temperature was set at 25 ± 0.1°C. The detection wavelengths were 220 nm for poly(Asp)$_{5400}$ [4] and 254 nm for poly(A). The data rate was 1 Hz and the rise time 1 s. The sample was introduced into the negative end by applying voltage for a few seconds.

3. Results and discussion

3.1. Gel immobilization and stability

To produce stable capillaries, gels are generally immobilized along the entire capillary wall by a co-polymerization method, that is, by polymerization of acrylamide in the capillaries pre-coated with bifunctional silanes [14–20]. Without immobilization, gels migrate from the capillary ends due to electroosmosis and/or electrostriction [11], resulting in irreproducible separation and a short life time (running stability) of the capillaries. However, this immobilization technique dramatically enhances the formation of voids because the gel shrinkage, which is caused by the volumetric losses of the resulting gels compared to the initial solution [5], is inhibited. If the gels are unable to shrink or can only shrink locally because of forming chemical bonds or strongly adsorbing to the capillary wall, the formation of voids becomes a natural way to compensate the volumetric losses which leads to current drop, serious disruption of the applied electric field and irreproducible or no separation. Void-free and stable capillaries are thus the prerequisite in CGE. A simple method to overcome the problem is to confine the immobilization to within a short section [10,11] so that most parts of the gels can shrink more freely. An even better idea is to immobilize the gel after polymerization which requires an immobilization agent able to form bonds with the gels by a different mechanism. At present, when using the

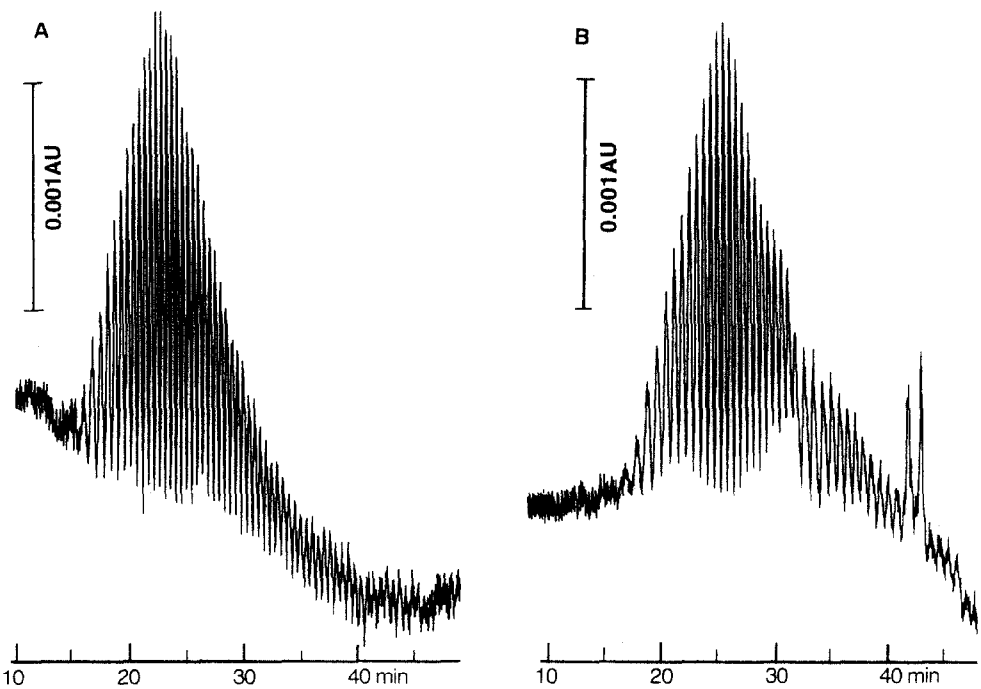

Fig. 2. Electrophoresis of poly(Asp)$_{5400}$ with CapB, obtained from the 20th injection (A) and 27th injection (B). Capillary: 27 cm (effective length 20 cm) × 75 μm I.D.; gel: 20% T + 5% C; buffer: 0.2 *M* Tricine–Tris, pH 8.3; constant current: 16 μA (6.2 kV); injection: 5 kV, 30 s.

reported silane [14–20], we can only immobilize the gels at the capillary tips (procedure B). The question arises: whether the gels which are immobilized over such a short section are stable enough?

To examine the running stability, the resulting capillaries are run continuously at about 200 V/cm until current drop occurs, with poly(Asp)$_{5400}$ as a testing sample (60–90 min per separation). The results (Fig. 2) show that the capillaries with gels immobilized at the tips by procedure B (both of CapB and CapBB) can be used for more than 20 injections. The gels may crack (from large voids) unexpectedly after about 25 injections as shown in Fig. 2B where the 27th separation failed because a current drop occurred at about 25 min after injection. CapB and CapBB are therefore less stable than the isotachophoretically produced capillaries (up to 7 days [4]) but are comparable to the low-concentration gel-filled capillaries without immobilization (10–30 h [6,7]).

The stability of the capillaries can be improved

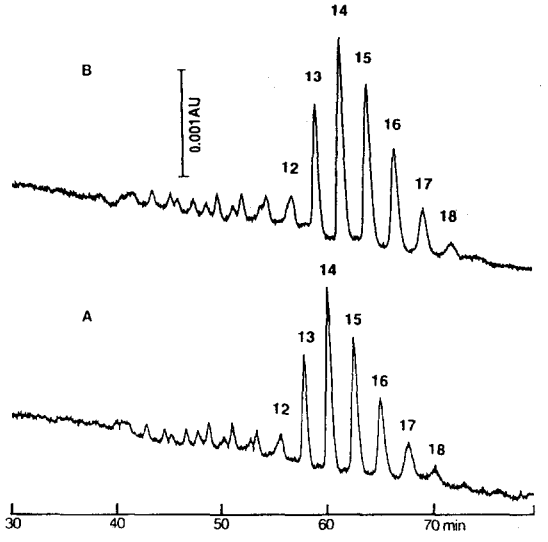

Fig. 4. Electrophoresis of poly(A)$_{12\text{-}18}$ with a newly prepared (A) and a 6-month-stored (B) capillary. The numbers show the estimated size of the peaks; the first part of the peaks (before peak 12) seemed to be impurities and/or a partial hydrolysate of the sample. Capillary: 27 cm (effective length 7 cm) × 25 μm I.D.; gel: 30% T + 5% C; buffer: TT15; constant voltage: 5.4 kV (*ca.* 0.9 μA); injection: 5 kV, 10 s.

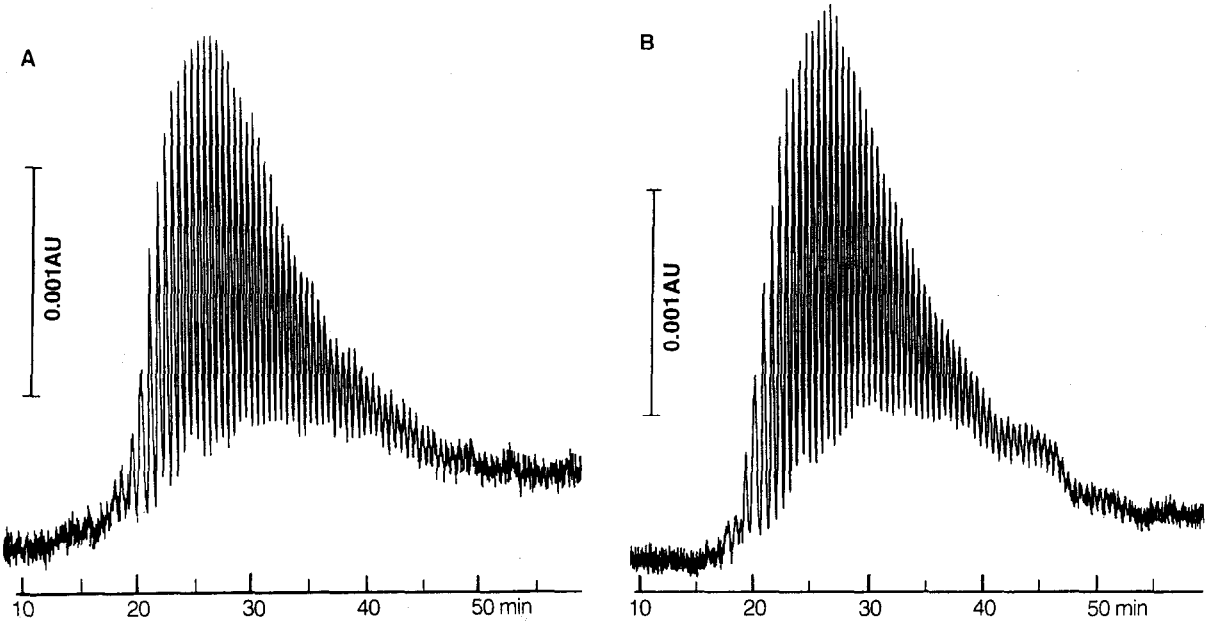

Fig. 3. A comparison of the 10th (A) and 75th (B) separation with CapA. Capillary: 37 cm (effective length 30 cm) × 75 μm I.D.; gel: 15% T + 5% C; buffer: TT15; constant voltage: 7.4 kV (7.9 μA); injection: 5 kV, 15 s.

for up to more than 70 injections by immobilization of the gels over a longer section of at least 0.5 cm with procedure A (CapA and CapAB). Fig. 3 shows that, for the tested capillary, the resolution loss at the 75th injection (Fig. 3B) is less than 10% compared to the 10th injection (Fig. 3A). Interestingly, the stability of CapA is similar to CapAB if positive voltage is applied to the longer immobilized end. When negative voltage is applied to this end, the current may drift in the first several runs but no gel cracking in the tube has been observed. (This orientation phenomenon was also observed for CapB). After running for one week, the gels may crack at the tube end which had been cooled during polymerization. In this case, the capillaries can be repaired by cutting off the damaged end.

The storage time or shelf-life of the prepared capillaries is generally 2 to 3 months, but may be up to more than 6 months, which is comparable with the isotachophoretically produced capillaries (between 1 and 2 months [4]). Fig. 4 shows a comparison between a newly prepared and 6-month-stored capillary of 25 μm I.D. The testing sample is poly(A)$_{12-18}$ instead of poly(Asp)$_{5400}$ which has much more poorer detection sensitivity.

3.2. Polymerization considerations

Reducing the gel immobilization length is only a prerequisite condition to producing void-free capillaries, the elimination of the voids finally depends on how to polymerize the acrylamide inside the capillaries. There are a number of polymerization conditions which can suppress the formation of voids, but the most effective ones seem to be the polymerization pressure and the control of gelatinization direction.

The gelatinization direction can be controlled in several ways [5], of which the axial-programmed-temperature polymerization is the simplest one. By assuming that a radical induced polymerization, once it starts, may quickly spread from one region to a neighbouring region, we simplified the procedure by just keeping the capillary ends at different temperatures (see polymerization process). No strict axial thermal gradient could be developed by this control but about 30% of the voids were eliminated. The data were obtained as following: ten unmodified capillaries of 45 cm × 75 μm I.D. cm with a 20% T + 5% C/TT15 gel were prepared without the application of pressure, of which five were controlled by temperature but the other five not. The voids were counted under a microscope and averaged.

It also seems possible to control the radial gelatinization direction by a radial thermal gradient. We hence cooled the tubing wall by washing it with an ice-cooled monomer solution. About 10% of the voids were eliminated by this procedure. Further study showed that, using a 0.5% HPMC solution instead of the monomer solution, about 12% of the voids were eliminated. This means that this is probably due to a coating effect but not due to the radial thermal gradient. When the capillary wall is coated by a viscous material, the lateral gelatinization can clearly be inhibited for a certain time until the polymerizing solution diffuses into or replaces this coating layer. To keep this coating effective, the injection of the polymerizing solution into the capillary should be very quick and once the solution reaches the outlet, the injection should be stopped immediately.

The thermal and coating controls can eliminate only under 50% of the voids. However, when a slight pressure is applied to the column, the voids can be eliminated nearly completely. Pressurizing polymerization was first developed by Bente and Myertson [8] who used high pressure to pre-compress the polymerizing solution. In our method, the pressure is reduced to *ca.* 5 atm and is applied only to the upper end of the tubes. Our purpose is not to compress the solution but to force the unpolymerized or partially polymerized solution and/or gel to move towards the polymerized end in order to compensate for the volumetric loss. By our simple temperature control, an irregular polymerization easily occurs between the two tube ends where the capillary wall is in contact with air. The irregularly formed gels resist or block the solution flow. Even if there is no irregular gel formed in the tube, the highly viscous solution does not

necessarily flow toward the polymerized end without pressure. Interestingly, a slight pressure associated with isotachophoretic polymerization can also improve the success rate of the preparation.

In addition, capillary position during polymerization influences the elimination of voids. A vertical position yields the best result. In an arched position, voids accumulate in the bend, and in a horizontal position, voids form mostly in the centre of the tube and partially at the upper wall side. The vertical position can also accumulate liquid in the upper capillary end. Under a microscope, the liquid movement in the upper end of a 100 μm I.D. capillary with a 5% T + 5% C gel, newly prepared by setting the upper end at a higher level than the water in the vial to prevent the water from flowing into the tube, was clearly observed by warming and cooling this end during observation. This movement was not observed in the ends of an arched or horizontally positioned capillary. About 2–4 cm of this end should be cut off to eliminate the liquid and empty space resulting from gel shrinkage. This liquid is possibly exuded due to the volumetric losses of the gels. If irregularly accumulating somewhere inside the tube, it will disrupt the applied electric field although it may not cause

Table 1
The success rate in producing CapA with x% T + 5% C gels

	Gel (% T)	Buffer[a]	TEMED[b] (%, v/v)	APS[b] (%, w/v)	Capillary[c]			Success rate (%)
					Dimension	Tatol	Usable	
A	10	TT15	0.05	0.05	45 cm × 50 μm	20	20	100
	15		0.05	0.05		36	35	97.2
	20		0.05	0.05		33	30	90.9
	25		0.05	0.04		7	6	85.7
	30		0.05	0.05		10	6	60.0
	30		0.04	0.035		15	13	86.7
B	20	TT15	0.05	0.05	45 cm × 100 μm	11	10	90.9
					45 cm × 75 μm	20	19	95.0
					45 cm × 50 μm	20	18	90.0
					35 cm × 25 μm	5	4	80.0
C	20	TT15	0.05	0.05	45 cm × 75 μm	20	19	95.0
		BT21	0.05	0.05		8	3	37.5
		BB25	0.05	0.05		17	6	35.3
		BB25	0.05	0.035		15	11	73.3
		BB25	0.04	0.03		20	17	85.0
D	15	TT15	0.03	0.03	45 cm × 50 μm	20	20	100
			0.05	0.05		36	35	97.2
			0.03	0.10		20	15	75.0
			0.05	0.10		20	11	55.0
			0.07	0.10		20	9	45.0
			0.10	0.10		20	5	25.0
			0.10	0.07		20	10	50.0
			0.10	0.05		20	12	60.0
			0.10	0.03		20	16	80.0

[a] TT15 = 0.1 *M* Tricine + 0.05 *M* Tris; BT21 = 0.2 *M* boric acid + 0.1 *M* Tris; BB25 = 0.25 *M* boric acid/borax, pH 8.3.
[b] At the total concentration (TEMED + APS) of < 0.08%, the hanging time of the capillaries was prolonged to 8 h.
[c] A usable capillary for CapA is defined as a void-free capillary with gel immobilized for ≥ 0.5 cm and a total length of ≥ 27 cm which is the shortest manageable length using the Beckman CE system.

current problems. It is thus better to prevent the liquid from accumulating inside the tube.

3.3. Success rate

Void-free capillaries can successfully be prepared with an I.D. as small as 25 μm and gel concentrations of up to more than 30% T. For the gels immobilized after polymerization, the success rate is 95–100%. If the gels are immobilized during polymerization, the success rate typically ranges between 85 and 100% as shown in Table 1, parts A and B. The success rate depends largely on buffer components (Table 1, part C) and on the concentrations of TEMED and APS (Table 1, part D). Generally, organic buffers such as Tricine–Tris can prevent the formation of voids much more effectively than inorganic buffers such as boric acid and phosphate in the same polymerization conditions. When inorganic

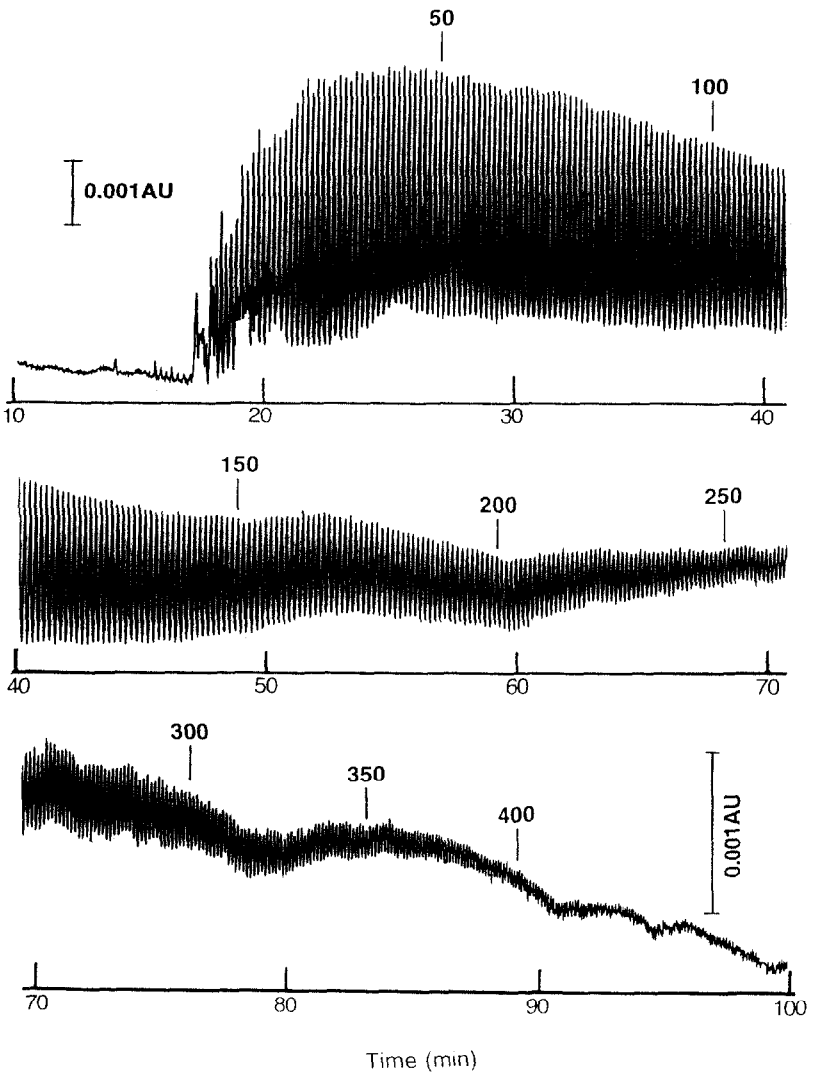

Fig. 5. Electrophoresis of partially hydrolyzed poly(A) with entirely immobilized low-concentration gel-filled capillary. The numbers show the size of the bands measured with poly(A)$_{12\text{-}18}$ as a reference. Capillaries: 37 cm (effective length 30 cm) × 100 μm I.D.; gel: 5% T + 5% C; buffer: 7 *M* urea, 0.1 *M* Tris–0.25 *M* boric acid; voltage: 7.4 kV (8.7 μA); injection: 5 kV, 3 s.

buffers such as boric acid are used, low concentrations of TEMED and APS are preferred to improve the success rate (Table 1, part C). TEMED and APS control the polymerization speed or the starting point of gelatinization. High concentration of TEMED or APS speeds up the preparation but increases the possibility of forming voids. In contrast, low concentration reduces the voids but raises the polymerization time and also the exuding of liquid from gels. At too low concentrations of TEMED and APS, polymerization may not occur. The suggested total concentration of TEMED + APS is 0.06–0.14%. These studies show that, besides the pressure and the control of the gelatinization direction, the buffer components and the concentrations of catalyst and radicals are very important in preparing the highly condensed gel-filled capillaries.

Finally, we would like to mention that the suggested polymerization method can also be used for producing the capillaries with gels of below 8% T + 5% C, immobilized along the entire capillary wall. Fig. 5 shows a separation of poly(A) in a highly denatured gel (5% T + 5% C/7 *M* urea). A 30-cm separating length can yield 400 bands in 90 min without any optimization of the separating conditions. The plate number $[5.54(t_R/w_{1/2})^2$, $w_{1/2}$ = peak width at the half height, t_R = elution time] of 150mer is $1.2 \cdot 10^7$ plates/m. This is comparable to the high performance gel-filled capillaries without immobilization [7].

Acknowledgements

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