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Journal of Chromatography A, 756 (1996) 245–253

JOURNAL OF CHROMATOGRAPHY A

Preparation of linear polyacrylamide gel step gradients for capillary electrophoresis.

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Received 18 March 1996; revised 9 July 1996; accepted 9 July 1996

Abstract

A means for casting step-gradients in linear polyacrylamide gel concentration for capillary electrophoresis is presented. A UV–Vis whole-column detector is used to profile the gel gradients cast in the capillary, while detection of fluorescein-labeled proteins is accomplished with an epi-illumination laser-induced fluorescence whole-column detection system. Fluorescence images of the capillary during the separation indicate a sharpening of the zones as they traverse the interface between gel concentrations. The potential of gel gradients in capillary electrophoresis for the analysis of wide molecular mass range protein or peptide samples is demonstrated. A mixture of proteins that range in molecular mass from $6 \cdot 10^3$ to $97.4 \cdot 10^3$ is separated in less than 15 min with baseline resolution of closely sized proteins that were not be resolved in a single concentration gel. Finally, analysis of several capillary images acquired during solute migration through the gel gradient permits the generation of a Ferguson plot from a single electrophoretic run.

Keywords: Capillary columns; Step gradients; Polyacrylamide gradients; Proteins

1. Introduction

Capillary electrophoresis (CE) has become a powerful analytical tool for separating and quantitating samples of biomedical and biological origin. The capillary format permits rapid separations, sensitive detection and is compatible with different separation media. In particular, utilization of polymer gels as separation media has transferred well from conventional slab-gel electrophoresis to the instrumental capillary systems. Capillary gel electrophoresis (CGE) has proven to be very powerful for high-resolution DNA sequencing (for example see references [1–3] and forensic DNA analysis [4,5]. CGE has also been applied to the size-based separation of proteins and polypeptides [6–11]. Early work with gel-filled capillaries [2] involved cross-linked gels with a fixed pore structure, however fabrication problems and limited lifetime have hindered their widespread acceptance. More recently, linear gels (polymer networks) have been used with considerable success for DNA applications [1,3–5] and for size-based separations of proteins [7,11].

An area that holds some promise for CE applications is the implementation of gel-density gradients as the separation medium. Slab gels cast with gradients in density along the separation axis have been widely used in biomedical research for high-resolution separations of wide molecular mass range samples and particularly for glycoprotein analysis (12). The variation of gel density with distance along the separation bed permits separation of a wider

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0021-9673/96/$15.00
PII S0021-9673(96)00630-9

molecular mass range of solutes than is possible with single-concentration gels, without compromising resolution of closely sized species. Furthermore, if the density gradient is continuous, a migrating zone will eventually reach a point along the gradient when it can no longer penetrate the pore structure. The net result is a focusing effect as the electric field drives the trailing edge of the zone against the gel; leading to more sensitive detection of individual zones and greater peak capacity.

To date, there have been few reports dealing with gel gradients in capillary electrophoresis [13–15]. Sepaniak [14,15] described the preparation of a two-step gradient in methyl cellulose (MC) concentration, used in conjunction with a spatial-scanning laser-induced fluorescence (LIF) detection system to optimize resolution per unit time for separation of DNA fragments [14]. Effective column length was altered by positioning the capillary on a translational stage and re-positioning the detection point between runs [14]. In a subsequent report [15] refinements to the scanning detector and its application to fundamental studies of band dispersion and other electrophoretic phenomena were described. The scanning detection system was used to probe the effects on peak width upon migration across an interface between two different MC concentrations. The results indicated an unexpected broadening of the zone upon crossing the interface. It was suggested that hydrodynamic introduction of the different density polymer solutions into the capillary (and the concomitant parabolic flow profile) resulted in a severe radial viscosity gradient at the interface region [15].

Chen and co-workers [13,16] reported a method to prepare highly condensed (30% T+5% C), cross-linked polyacrylamide gel-filled capillaries and establish gradients in gel concentration along the length of the capillary [13]. The highly condensed gels appear to be critical for high-resolution of oligosaccharides and polyamino acid polymers [13,16,17]. The described procedure for preparing the dense gels included application of slight pressure during polymerization, control of the polymerization direction and careful control of initiators and catalysts [13,16]. With this procedure cross-linked gels with concentrations as high as 30% T+5% C [16] as well as step gradients [13] over a range of gel concentrations (10% T+5% C to 15% T+5% C) were prepared. Using the step gradient a decrease in separation time (fixed point detection) for a polyaspartate homopolymer was demonstrated.

In this paper, a simple method for casting step gradients along the capillary is demonstrated. A micro-chamber device that allows for changing the gel solution reservoirs without removing the capillary tip from solution is described. In this manner, air bubbles are not introduced into the interface region between gel steps. Gels are profiled using a whole-column UV–Vis absorbance detector, operating at 214 nm. The fluorescein isothiocyanate (FITC)-labeled solutes are detected as they migrate through the separation capillary by a whole-column LIF detector (argon ion laser at 488 nm), that can be operated in either a fixed detection point mode or scanning mode to image the entire capillary during the separation [18]. Focusing of solutes as they traverse the interfaces between gel concentrations is observed. The application of a gel gradient to the separation of a wide molecular mass range sample of FITC-labeled proteins is shown. Furthermore, if the gel profile is known, a single electrophoretic analysis provides sufficient data to generate a Ferguson Plot.

2. Experimental

2.1. Materials

Electrophoretic grade acrylamide and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Labs. (Richmond, CA, USA). The bi-functional reagent γ-methacryloxypropyltrimethoxysilane was purchased from Huls America (Bristol, PA, USA). Fluorescein isothiocyanate isomer I was purchased from Aldrich (Milwaukee, WI, USA). The protein samples utilized in these studies, FITC-labeled insulin (Ins, molecular mass: 6000), cytochrome *c* (CytC, 12 400 dal), lysozyme (LYZ, 14 400), horse heart myoglobin (MYO, 17 000), soybean trypsin inhibitor (TPI, 21 500), chymotrypsinogen (CTPG, 25 000), bovine carbonic anhydrase (CA, 29 000), bovine serum albumin (BSA, 66 000) and phosphorylase *b* (97 400) and ammonium persulphate (APS), Tris(hydroxymethyl)aminomethane (Tris), boric acid, sodium dodecyl sulphate (SDS)

were reagent grade from Sigma (St. Louis, MO, USA). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Preparation of FITC labeled proteins

All proteins, except insulin (which was purchased as the FITC conjugate), myoglobin and phosphorylase *b*, were dissolved in 50 m*M* sodium carbonate buffer pH 9.5. Myoglobin was dissolved in 50 m*M* potassium phosphate buffer pH 6.8 while phosphorylase *b* was dissolved in 50 m*M* sodium carbonate buffer pH 9.5 containing 15% glycerol, 1% 2-mercaptoethanol and 1% SDS. The conjugation with FITC (dissolved in acetone) was carried out with a 2.5:1 mol ratio of FITC to protein for all samples except myoglobin (1:1, FITC to protein) and phosphorylase *b* (10:1, FITC to protein). The FITC–protein mixtures were allowed to react in dark at 25°C for 3.5 h, and then dialyzed extensively against 5 m*M* phosphate or carbonate buffer to remove unreacted FITC and low molecular mass reaction by-products. All FITC-labeled proteins were kept in the freezer before use.

2.3. Preparation of polyacrylamide gel-filled capillaries

Single-concentration linear polyacrylamide gels were prepared by dissolving 0.2 g of acrylamide in 5 ml of TB buffer (250 m*M* Tris and 100 m*M* borate, 0.1% SDS, pH 8.2) followed by degassing for 25 min under sonication and vacuum. Upon degassing, polymerization of gel solutions was initiated by adding 20:1 TEMED (10%) and 20:1 APS (10%). The capillaries were filled with the gelling solutions by applying vacuum to the distal end. Gel-filled capillaries were kept at room temperature for at least 10 h to ensure complete polymerization.

2.4. Preparation of step-gradient polyacrylamide gel-filled capillaries

The micro-chamber system utilized for casting gel step gradients is shown in Fig. 1. The caps and bottoms of microcentrifuge vials (0.6 ml, Fisher Scientific, Pittsburgh, PA, USA), were cut off for use as the gel solution chambers. Each chamber was separated by Parafilm to avoid mixing of gel solutions in the chambers. Fused-silica capillaries, 75 μm

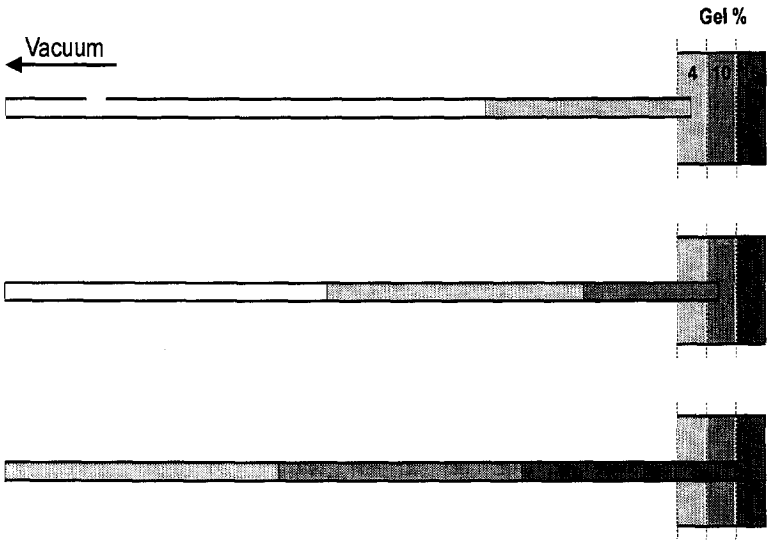

Fig. 1. Illustration of the micro-reservoir system used to cast step gradients in the capillary. Each reservoir chamber was separated by parafilm and filled with gelling solutions containing the different monomer concentrations. To change the composition of the solution drawn into the capillary, the parafilm was pierced and the capillary inserted into the next chamber.

I.D., were pre-coated as described by Hjerten [19] using γ-methacryloxypropyltrimethoxysilane and 4% linear polyacrylamide. All solutions were thoroughly mixed and de-gassed, then chilled in an ice bath prior to initiation of polymerization. Immediately after addition of the polymerization agents, each micro-chamber reservoir was filled with the appropriate gelling solution. Gradients of at least six steps can be easily cast with this device.

The capillary, with polyimide outer coating removed [18] was mounted on a clear, flat plexiglas platform. To cast the gel, one end of capillary was inserted into the first chamber and the first gelling solution was drawn into the capillary by applying light vacuum from an aspirator using a two-arm glass 'T' with PTFE stopcocks in each arm to act as a splitter device. The applied vacuum (controlled by slightly opening the side-arm stopcock) was just enough to draw the gelling solution through the capillary. The flow of gelling solution in the capillary can be monitored easily with the unaided eye or under the microscope for precise control. As each gelling solution filled the capillary to desired length, measured by a mark made on the platform adjacent to the capillary, the capillary was pushed through the Parafilm barrier into the next gelling solution chamber. The next gelling solution could then be pulled into the capillary without introducing an air pocket between the gels. The gelling solutions of various concentration were gradually pulled into the capillary until the front of the first solution reached the tip of the capillary. In this manner it is possible to cast a gel step gradient with precise control over the length of each step. The capillary was kept flat and undisturbed overnight at room temperature. Columns prepared in this fashion have a useful lifetime of approximately 2 weeks.

2.5. Step-gradient gel profile

The step gradient gel was profiled using a scanning UV–Vis detection system [20,21] available in our laboratory. In this system, the column is translated along its axis between a fiber optic and photodiode connected to a Linear Instruments Model UV-200 (SpectraPhysics, Piscataway, NJ, USA) UV–Vis absorption detector to monitor absorbance along the length of the capillary. Schematic diagrams and a description of the UV–Vis scanner are available to anyone requesting this information from the corresponding author.

2.6. Capillary gel electrophoresis

The gel electrophoresis was performed using an laboratory-constructed CE system consisting of a Spellman 0–30 kV power supply (Plainview, NY, USA). LIF detection was accomplished with a spatial-scanning, whole-column detection system developed in our laboratory [18]. This detector can be operated using a fixed detection point or images of the entire capillary can be generated during the migration of solutes through the capillary be translating the column through the probe beam.

The operating buffer for the gel electrophoresis was the same as that used for preparing gel solutions. All gel capillaries were pre-run by applying 5 kV for 25 min to pre-condition the column. The FITC-labeled proteins were diluted in water containing 0.1% SDS followed by heating at 90°C for 10 min. After cooling to room temperature, protein samples were injected into the gel column electrokinetically from the cathode end. All separations of protein samples were performed at 5 kV, and the current through the gel capillaries did not exceed 20 μA.

3. Results and discussion

In conventional slab-gel electrophoresis the density gradients commonly employed are cross-linked polyacrylamide in which the concentration (pore size) of the gel varies continuously along the separation path [12]. The gels usually range from a very loose pore structure (i.e. 5% T+3% C) at the sample injection end of the separation bed to highly condensed, tight gel structures (30% T+3% C). The gradients can be cast with very precise control over the final gradient profile (linear or exponential) using a simple device consisting of two plastic syringes [22].

The situation in CE is somewhat different. The small column volume and stringent restrictions on external mixing volumes, make casting continuous gradients along the length of the capillary more difficult. Preparing gel gradients in which the distal

end of the capillary consists of highly condensed, cross-linked gels is further complicated by the well-known difficulties associated with preparing dense, cross-linked gels [16,17].

The gels reported herein consist of step gradients in linear polyacrylamide concentration. It was our experience that linear acrylamide in concentrations up to 20% T can be routinely cast without significant air bubble formation within the gel. The most significant problem encountered in this work was bubble or void formation at the interface between steps, even when the time to transfer the capillary between the different gel vials was short [13] and only light vacuum applied. Utilization of the micro-chamber system eliminated bubble formation at the interface region of the capillary since the capillary is never removed from the gel solution.

To evaluate the shape of the gel gradients, UV–Vis absorption profiles of the entire column were examined. Capillary images were generated by scanning the length of the column with a modified UV–Vis detector at 214 nm. Subsequent to polymerization of the gel (at least 10 h after the initiation of polymerization), the absorbance and refractive index differences of the different gel concentrations allow for visualization of each interface. A typical whole-column absorbance profile of a three-step gel, formed using the micro-chamber reservoir device, is shown in Fig. 2. The peak 21 cm from the distal end of the capillary is a reference mark made on the outside wall of the capillary with a permanent marker. The shaded area shown below the position axis represents the expected gel profile, based on the length of each solution drawn into the capillary. With the micro-chamber reservoir the transition interface between gels was never more than 2 cm. This is in direct contrast to the profiles of gels formed using full vacuum from the aspirator to rapidly draw gel solutions from individual reservoirs into the capillary. In this cast the transition interface region between gel steps was never less than 3 cm.

The performance of a gel concentration step gradient is illustrated in Fig. 3, a series of five fluorescence images of the capillary acquired as FITC–BSA migrates through a two-step gradient. The gel cast in this capillary was a two-step gradient at the extremes of gel concentration, 4%–20% T, the expected profile is shown in the shaded area above the position axis. As the solute (FITC–BSA) migrates through the first 12 cm of capillary (4% T) it encounters the transition interface between the gels (center image, acquired 10 min after injection), indicated by a sharpening of the band and a decrease of migration velocity. This particular gel was not

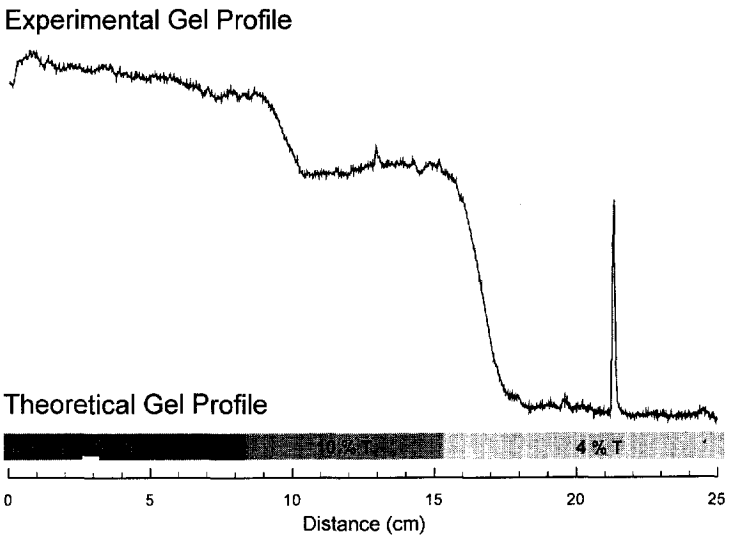

Fig. 2. UV–Vis absorption profile of a three-step denaturing gel gradient cast in a 75 μm I.D. capillary. The column was positioned in the UV–Vis scanner and a mark was made on the capillary with a permanent marker 21 cm from the distal end of the capillary. The expected gel profile, based on observation of the meniscus position during the filling process, is shown in the shaded area above the position axis.

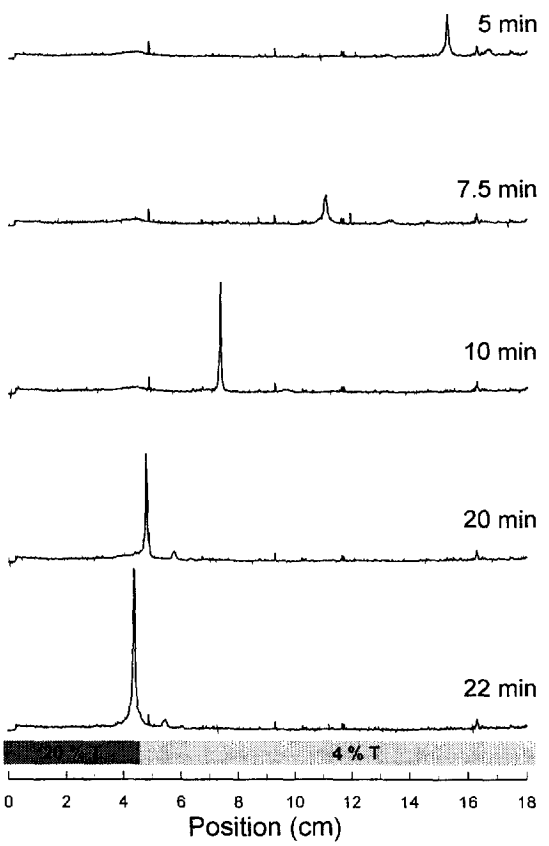

Fig. 3. Five fluorescence images acquired during the migration of FITC–BSA through a capillary in which a 4–20% T two-step gradient was cast. Capillary, 30 cm×75 μm I.D.; applied voltage, 8 kV; injection, 2 kV for 1 s; buffer composition and other operating conditions are given in Section 2. The expected gel profile is shown in the shaded area just above the position axis. The capillary was scanned at 1.5 cm/s with a pixel width of 50 μm to generate each image.

cast with the micro-chamber system and as such a much broader interface region is expected as there is less control over the gel profile. In fact, analysis of several scans acquired during solute migration through this gel indicated the apparent interface between the gels occupies about 4 cm of the capillary, starting 8 cm from the anode. The expected interface should be 4.5 cm from the anode.

Once BSA migrates completely into the more dense gel it has focused into a narrow zone and its velocity has decreased considerably. In fact, migration entirely through the remaining 4 cm of the 20% T gel portion of the capillary requires an additional 30 min. Inspection of the last two images indicates a small peak that begins to appear about 5.5 cm from the anode. This may be due to a dimer or trimer of BSA that is detectable only after focusing in the dense gel. This higher molecular mass species is observed when using short effective column lengths in single-concentration gels, but the signal from this species is rapidly lost during migration through longer columns.

The application of a multi-step gel gradient to the separation of a wide molecular mass range sample is shown in Fig. 4 and Fig. 5. Fig. 4 depicts an electropherogram of nine FITC-labeled proteins (detection is fixed point LIF with an effective column length of 22 cm) separated in a single concentration (4% T) denaturing linear acrylamide gel. The proteins range in molecular mass from 6000 to $97.4 \cdot 10^3$ (peak identities are given in the figure caption). In this gel, two of the observed peaks are actually composed of co-migrating solutes (lysozyme and myoglobin, peak nos. 3, 4 and chymotrypsinogen and carbonic anhydrase, peak no. 6, 7). The individual solutes in these zones can be resolved using higher concentration gels, but at the expense of analysis time for the BSA and phosphorylase *b* (about 50 min for 15% T gel).

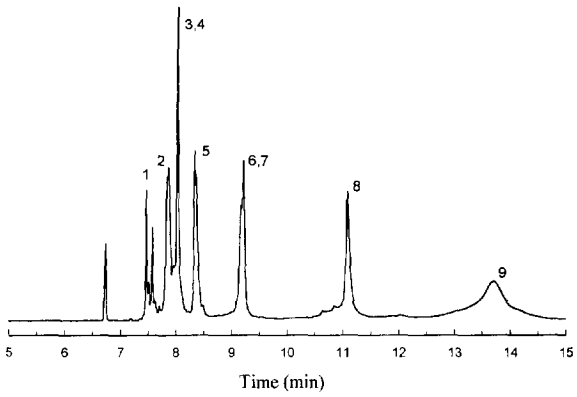

Fig. 4. Separation of FITC-labeled proteins in a denaturing 4% T linear acrylamide, single concentration gel with LIF detection. The detection point is fixed 22 cm from the cathodic end of the capillary. Peak identifications are as follows: 1=insulin; 2=cytochrome *c*; 3=lysozyme; 4=myoglobin; 5=soybean trypsin inhibitor; 6=chymotrypsinogen; 7=carbonic anhydrase; 8=bovine serum albumin and 9=phosphorylase *b*. Capillary, 30 cm×75 μm I.D.; applied voltage, 8 kV; current, 20 μA; injection 2 kV for 1 s.

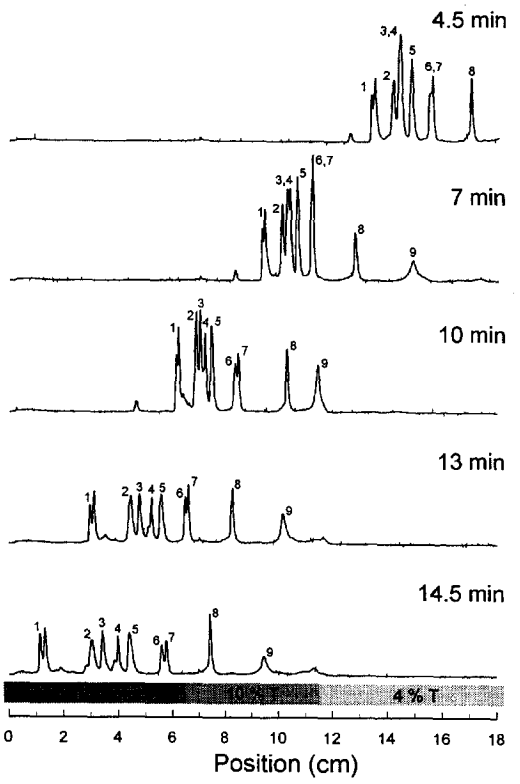

Fig. 5. Five fluorescence images of the separation capillary during the migration of 9 FITC-proteins through a three-step gradient (4%–10%–16% T). The peak identifications are as in Fig. 4. Capillary, 30 cm×75 μm; applied voltage, 8 kV, current 15 μA; injection, 2 kV for 1 s; operating conditions are given in Section 2. The capillary was scanned at 1.5 cm/s with a pixel width, 50 μm; the position axis is distance from the anodic end of the capillary.

Separation of the same nine FITC-labeled proteins in a capillary in which a gel gradient has been cast is illustrated in the series of fluorescence images shown in Fig. 5. A three-step gradient, cast with the microchamber reservoir system, consisting of 4%–10%–16% T was established in the capillary. After sample injection, a series of images were acquired at 1-min intervals. In less than 5 min all the zones are separated to approximately the same extent as seen after migration through the entire single-concentration gel (Fig. 4), phosphorylase *b* has not yet migrated into the detection window. As the solutes migrate further through the gel, focusing is evident at each interface. Within 15 min there is baseline resolution between those zones that could not be adequately resolved in the 4% T single-concentration gel (cytochrome *c*, lysozyme and myoglobin).

Step gradients in gel concentration, combined with whole-column detection, permit the facile construction of Ferguson plots. By acquiring capillary images at sufficient intervals to measure solute position at two points in each gel, the apparent mobility of each solute in each gel can be calculated. If the separation mechanism is size-based, plots of log (mobility) vs. gel composition will yield a straight line (different slope for each sample component) with a common *y*-intercept, equal to the free-solution mobility. All the mobility data for the Ferguson plot shown in Fig. 6 was generated in a single electrophoretic run on the three-step gradient column used for the separation shown in Fig. 6.

In this work no special efforts were made to control the FITC-labeling reaction [23,24] and limit the number or position of the attached FITC labels. The reaction of FITC with myoglobin was carried out at a lower pH than the other reactions because the myoglobin derivative resulting from the higher pH reaction (9.5) featured a very broad electrophoretic zone. Lowering the pH most likely restricted the number of attached labels. Quantitation of FITC-labeled proteins by size-sieving should pose no problems since the molecular mass differences due to the incorporation of more than one FITC label are generally not sufficient to produce multiple peaks. This is supported by the fact that all the solutes investigated for this work migrated as a single zone, with the exception of the smallest component, insulin.

4. Conclusions

We have described a simple method to cast step gradients in linear polyacrylamide gel concentration for capillary electrophoresis. The length of each step is controlled by filling the capillary with the gelling solutions using gentle vacuum and the microscope to monitor the filling process. Such gradients have been used in conjunction with LIF whole-column detection to demonstrate their potential as an alternative to single concentration gels for the separation of wide molecular mass range samples. Whole-column detection permits visualization of the expected

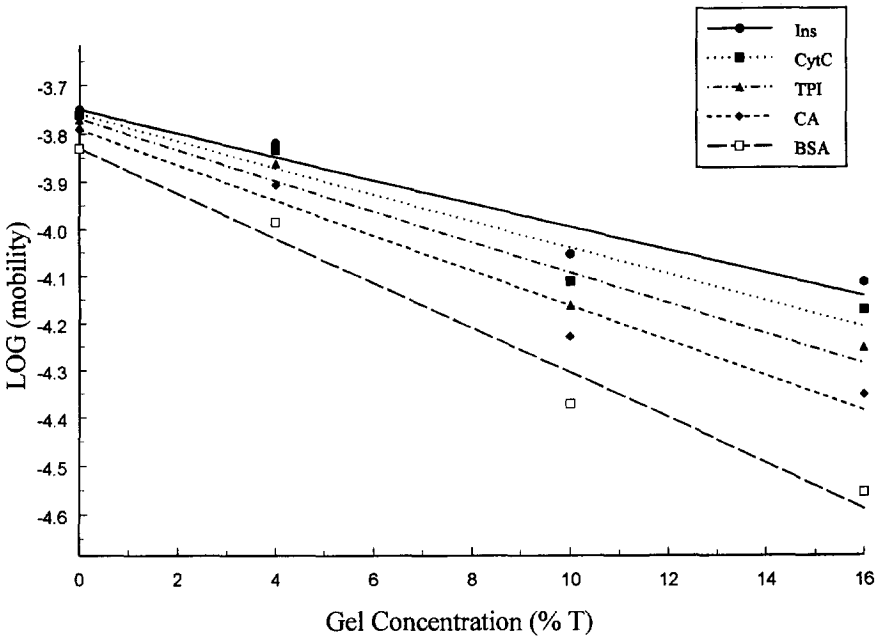

Fig. 6. Ferguson plot for 5 FITC-labeled proteins (insulin, cytochrome *c*, soybean trypsin inhibitor, carbonic anhydrase and bovine serum albumin). The mobility data for this curve was generated in a single electrophoretic run. Operating conditions and column (three-step gradient) are the same as given in Fig. 5.

sharpening of the zones and slowing of their migration velocity at the interface region between steps. When using the linear acrylamide gels it was not possible to completely inhibit migration through the gel. Presumably the dynamic nature of entangled polymer matrices will not afford such focusing. The more rigid pore structure of cross-linked gels may be required to impede migration along the capillary to achieve narrow focusing of the solute zones.

Acknowledgments

We thank Dr. Donald Rose of Glaxo Wellcome, Inc. for the kind donation of the UV–Vis scanner. The work of machinist Jerry Sewel is acknowledged for modifications made to the capillary stage. This work was supported, in part, by the National Science Foundation under Grant No. CHE9624884. We also acknowledge the UAB Department of Chemistry and UAB Graduate School for additional financial support.

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