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Sodium dodecyl sulfate–capillary gel electrophoresis of proteins using non-cross-linked polyacrylamide

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ABSTRACT

Proteins with relative molecular masses of 14 000 to 205 000 were separated by sodium dodecyl sulfate–capillary gel electrophoresis (SDS-CGE) using non-cross-linked linear polyacrylamide gels on both coated and uncoated fused-silica capillaries. It was determined that viscosity of the acrylamide solution was a major factor affecting column stability with linear acrylamide gels. When the viscosity of the acrylamide solution reaches 100 cP, electro-osmotically driven displacement of the gels is insignificant. Uncoated capillaries provided better resolution, stability, and reproducibility than surface coated capillaries when the concentration of linear polyacrylamide was greater than 4%. At lower gel concentrations, non-cross-linked polyacrylamide is easily displaced from the columns. A calibration plot of log molecular mass *vs.* mobility with non-linear polyacrylamide was linear, which indicated that resolution was equivalent to that obtained with cross-linked acrylamide. Separations with model proteins indicated that baseline resolution between protein species that vary 10% in molecular mass can be achieved.

INTRODUCTION

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in the slab gel format has been used for over 25 years to separate proteins by molecular mass [1]. Using this method, proteins are separated on cross-linked polyacrylamide gels with low applied electric fields (10-30 V/cm) and typically detected by staining. Although SDS-PAGE is one of the most commonly used methods for determining molecular masses of proteins, it has some limitations. First, the process is slow. Gel preparation, separation and staining can require hours. Second, quantification is difficult. Stains are non-linear and present problems with amino acids, such as proline. In an effort to overcome some of these problems, Cohen *et al.* [2] examined the separation of proteins in capillaries by SDS-PAGE. SDS–capillary gel electrophoresis (CGE) was much more rapid and of higher resolution than SDS-PAGE in the slab gel format. Separations by SDS-CGE are typically achieved in less than 20 min with on-column absorbance detection [3]. It is even possible to automate SDS-CGE. Furthermore, the CGE column may be used in multiple separations.

Both cross-linked and non-cross-linked polyacrylamide have now been used in SDS-CGE [4–8]. Traditional cross-linked gels create a sieving medium based on a rigid matrix of pores. The pore size is controlled by varying the concentration of acrylamide at a fixed concentration of crosslinking agent. Cross-linked gels were used because they provided a matrix of small pore, were reproducible, and were of sufficient mechanical strength that they could be removed from the system and stained [9]. Unfortunately, cross-linked gels are not very satisfactory in capillary columns. Voids form in the capillary as a result of gel shrinkage during polymerization. The void problem has been addressed both by polymerization under pressure [10] and by adsorbing a layer of linear polymer onto the capillary surface [11]. These techniques reduce, but do not eliminate this problem.

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Attempts to circumvent the problems of cross-linked polyacrylamide in SDS-CGE have led to the examination of non-cross-linked acrylamide. It was apparently anticipated in previous studies that electro-osmosis would be a problem in the use non-linear polyacrylamide. If this were true, electro-osmosis could be effectively eliminated by applying a viscous coating at the capillary wall. This technique was first reported by Hjertén [12] for controlling electro-osmosis in capillary isoelectric focusing. Columns were prepared by coating the capillary with the bifunctional reagent 3-methacryloxypropyltrimethoxysilane (MAPS). Subsequent to silylating capillary walls with MAPS, the capillary was filled with acrylamide and polymerization initiated. Acrylamide polymer was grafted to the capillary wall through MAPS groups. Hjertén suggested the electro-osmosis flow in this capillary will be inversely proportional to the viscosity of the polymer layer attached to the surface.

Previous reports on SDS-CGE have been with surface-modified fused-silica capillaries to reduce electro-osmosis [12,13]. The surface modification most widely used has been to apply a layer of polyacrylamide. Although separations with these capillaries were outstanding, separations on uncoated capillaries were not provided as a control. Thus, it is not possible to assess the contribution of surface coatings in SDS-CGE.

This paper focuses on the importance of surface modification in SDS-CGE with linear polyacrylamide gel filled capillaries. It will be shown that (i) electro-osmosis in CGE is a smaller problem than previously anticipated and (ii) linear polyacrylamide can be used in uncoated capillaries without appreciable gel displacement when the total concentration of acrylamide in the column exceeds 4%. Polypeptides ranging in relative molecular mass (M_r) from 2000 to 200 000 could be accommodated using a set of 4, 6 and 8% linear polyacrylamide gel-filled capillaries.

EXPERIMENTAL

Chemicals

All proteins were purchased from Sigma (St. Louis, MO, USA). Acrylamide, ammonium persulfate (APS), SDS, N,N,N′,N′-tetramethylethylenediamine (TEMED), and Tris base were purchased from Bio-Rad (Richmond, CA, USA). MAPS was purchased from Polyscience (Warrington, PA, USA). All of the buffer solutions were prepared by using double distilled water that was passed through 0.45-μm nylon filters. All of the samples were stored at 0°C between analysis.

Instrumentation

The CGE system was based on an in-house design available in our laboratory. All high-voltage components of the system were contained in a Lucite cabinet fitted with a safety interlock that would interrupt the power supply when the cabinet door was opened. A Glassman PS/EL30P01.5 (Glassman High Voltage, Whitehouse Station, NJ, USA) power supply was used to apply the electric field across the capillary. The power supply was connected to 4-ml buffer reservoirs. On-column detection was performed with an Isco CV^4 (Lincoln, NE, USA) variable-wavelength UV-absorbance detector.

Preparation of acrylamide gel-filled capillaries

Bonded acrylamide capillaries. Fused-silica capillaries of 375 μm O.D. × 75 μm I.D. and 45 cm length with a detector window at 25 cm were obtained from Polymicro Technologies (Phoenix, AZ, USA) and used for all separations. The capillary was first treated with 0.01 *M* NaOH for 30 min followed by 15 min of washing with deionized water. The columns were coupled to a nitrogen gas supply and dried in a gas chromatography oven at 100°C for 1 h. MAPS–methanol (9:1) solution was forced through the capillaries at 50°C for 1.5 h. After the reaction was complete, the solution was purged with nitrogen and the column sequentially washed with methanol for 15 min and with water for another 15 min.

Gel-filled capillaries were prepared by a modified procedure described by Yin *et al.* [14]. A 5-ml volume of 4% acrylamide solution was degassed by aspiration for 30 min. Then 100 μl APS (10%), 20 μl TEMED (10%) and 50 μl SDS (10%) were added. After stirring, the solution was pushed into the capillary under pressure. An hour later the column was ready to use.

Uncoated capillaries. To prepare a gel-filled column with uncoated capillaries, the initial wash procedure was followed. After washing the column, the polymerizing gel solution prepared as above, was

introduced into the capillary.

Protein sample preparation. SDS-Protein samples were prepared in 0.1 *M* Tris–0.25 *M* borate buffer (pH 8.1). The mass ratio of SDS to protein is 2.5:1, which ensures complete absorption of SDS to proteins. SDS–protein samples were electrophoretically injected into the gel column by setting the negative end of the column into the sample vial and applying an electrical field for 10 s at constant voltage.

Viscosity measurements. A dropping ball viscosimeter (Gilmont 2302) was used to measure the viscosity of the polyacrylamide solutions. Different acrylamide solutions were prepared by the procedure described above and the density of each solutions was measured. The viscosimeter was filled with gel solution and the time of a ball falling freely through the gel solution was measured over a constant distance. Two different types of balls, glass and steel, were used at different concentration ranges. Viscosity (η) was calculated from the equation:

$$\eta = K(d_{ball} - d_{soln})t$$

in which, K is a constant, d_{ball} and d_{soln} are the densities of the ball and the solutions and t is the time in minutes.

RESULTS AND DISCUSSION

Comparison of coated and uncoated capillaries

A comparison study was carried out using capillaries with a polymeric surface layer as previously described [14] and uncoated capillaries in which linear polyacrylamide was formed in the capillary. Capillaries with the polymeric surface layer will be referred to henceforth as coated capillaries whereas native fused-silica capillaries will be designated as uncoated capillaries. Polymer concentrations of 3, 4 and 6% were used in both types of capillaries.

A comparison of coated and uncoated capillaries in the separation of protein molecular mass standards is shown in Fig. 1. These standards ranged in from M_r from 14 400 to 78 000 in increments of approximately 10 000. Identification of the protein was accomplished by enrichment with individual standard proteins. The 4% and 6% columns are seen to be comparable in resolution and peak shape, although migration times were longer on the uncoated columns. Resolution is even slightly higher with high-molecular-mass species on the uncoated capillary. Organic surface coating appears to make no contribution to resolution with 4% and 6% linear polyacrylamide filled capillaries.

In contrast, the 3% columns are very different. Resolution is inferior and the migration time shorter with the uncoated capillary. This is attributed to electro-osmotically driven migration of gel in the capillary. After approximately three sample runs, *i.e.* 1 h of operation, much of the acrylamide had eluted from the capillary. Polymer concentration is seen to be an important variable. Even small differences in concentration can have a large impact. This is attributed to a reduction in electro-osmotically driven transport at high polymer concentrations.

Viscosity of linear acrylamide gels

A concentration-dependent reduction of electro-osmosis could be due to one of several phenomena. One could be that electro-omotically driven flow is controlled by increases in viscosity at high polymer concentration. A second could be that ζ potential and electro-osmosis are decreased by adsorption of polyacrylamide onto the capillary wall at high concentrations of polymer.

It can be seen in Fig. 2 that the viscosity of linear polyacrylamide increases exponentially with concentration. Viscosity changes from 40 cP for a 3% gel solution to 103 cP for a 4% solution. There is good correlation between viscosity and the observed difference in longevity of 3 and 4% uncoated capillaries. This does not eliminate the possibility that adsorption also plays some role at higher polymer concentration. Adhesion of polymer to the capillary wall favors both stability and heat transfer from the gel to the capillary wall [15].

Column stability

Properties of a gel, such as viscosity, maybe influenced by the buffer and preparation procedure. To insure consistent polymerization the procedure for gel synthesis was strictly followed. Monomer solutions were always degassed for the same periods of time, and reaction time and temperature were identical for all cases. These procedures insure the reproducibility of capillaries as will be discussed below.

Column stability was studied with coated and uncoated capillaries filled with 4% linear polyacryl-

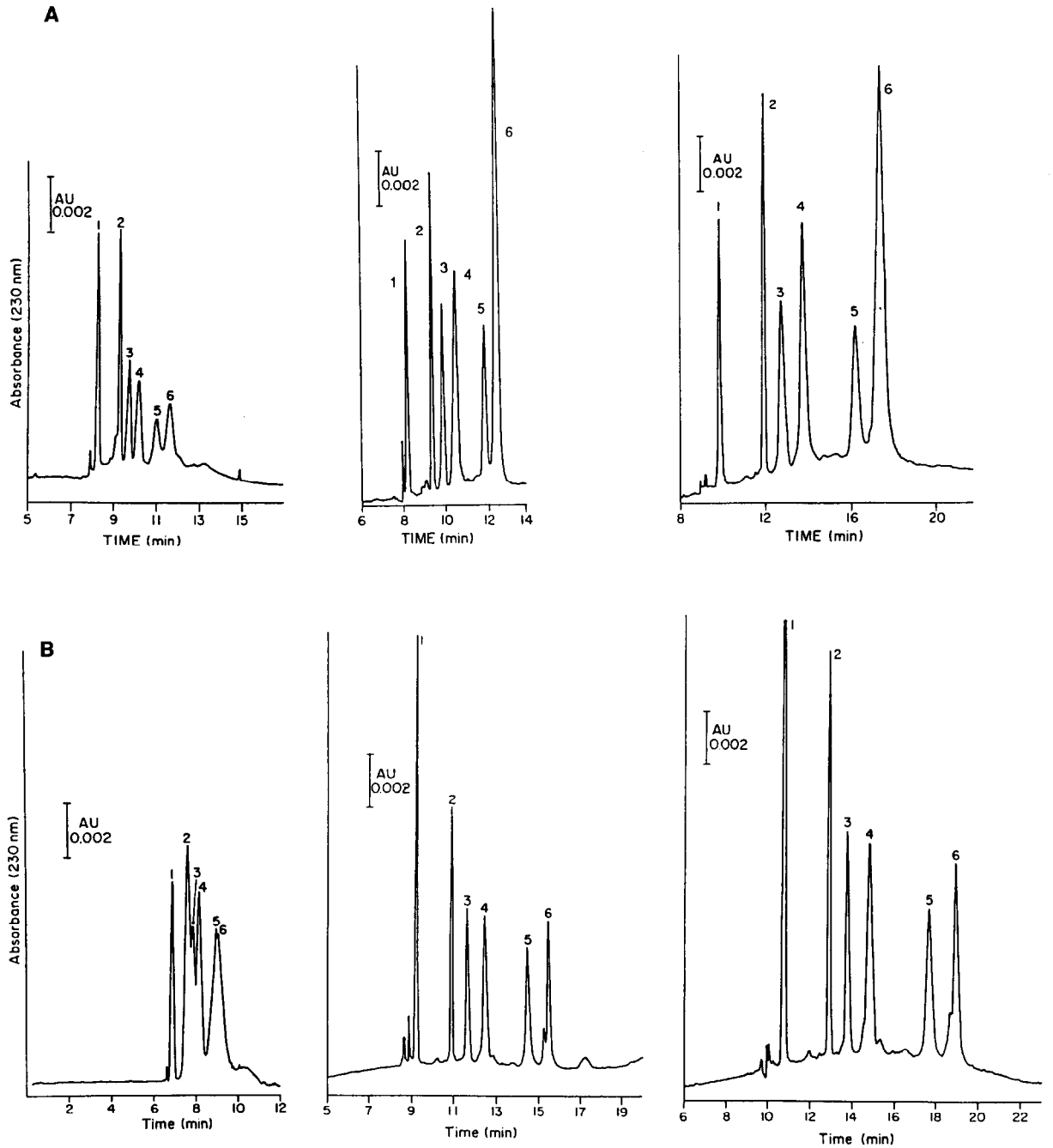


Fig. 1. Capillary gel electrophoretic separation of protein molecular mass standards on coated (A) and uncoated capillaries (B) with 3% (left), 4% (middle) and 6% (right) acrylamide concentration. Conditions: capillary length 45 cm; separation length 25 cm; 75 μm I.D.; separation potential, 12 kV, 267 V/cm, 0.1% SDS. Peaks: 1 = α-lactalbumin (bovine milk) (M_r 14 400); 2 = carbonic anhydrase (M_r 29 000); 3 = glyceraldehyde-3-phosphatedehydrogenase (M_r 36 000); 4 = albumin (chicken egg) (M_r 45 000); 5 = albumin (bovine) (M_r 66 000); 6 = conalbumin (M_r 78 000).

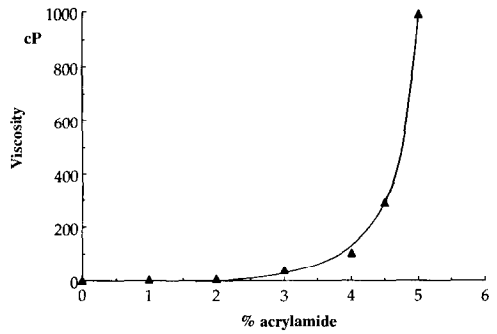


Fig. 2. Plot of the viscosity (η) of the gel *vs.* acrylamide concentration.

amide. Coated capillaries had run-to-run and column-to-column reproducibility of 4% relative standard deviation (R.S.D.) (n = 14) and 2% R.S.D. (n = 21), respectively. These columns could be used for up to 6 h without significant baseline drift. Uncoated capillaries gave a run-to-run reproducibility of 2% R.S.D. (n = 21) and could be used no more than 7 h. This generally allowed 20 samples to be run before baseline fluctuation began. Air bubble formation during use was not observed in either column type.

Baseline fluctuation was noted at 230 nm, but not at 280 nm. This phenomenon is explained in the following way. Free radical polymer polymerization of acrylamide in aqueous 0.1% SDS produces

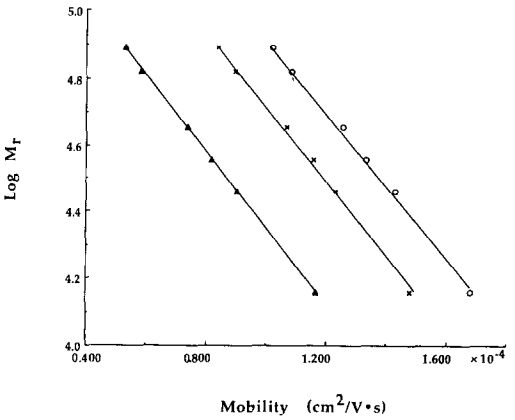


Fig. 4. Plot of log molecular mass of proteins as a function of mobility in gel columns with 4% (○), 6% (×) and 8% (▲) acrylamide in uncoated columns. Separation conditions as in Fig. 1.

polymers of broad molecular mass distribution. At this concentration of SDS there will also be a weak association of the alkyl portion of SDS with the hydrocarbon backbone of polyacrylamide. Under high voltage, these SDS–polyacrylamide complexes will be induced to migrate toward the anode. Low-molecular-mass complexes will migrate more rapidly than those of high-molecular mass. After several hours of operation, an axially asymmetric molecular mass distribution of polyacrylamide should be-

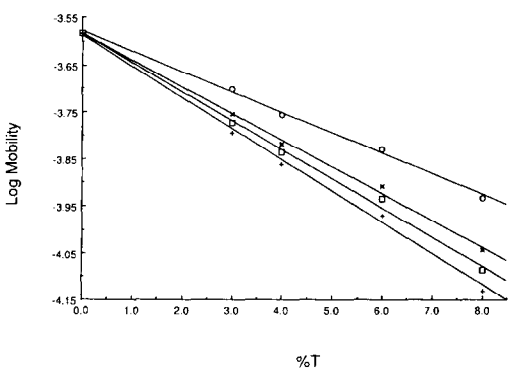


Fig. 3. Ferguson plot for linear acrylamide on uncoated capillary. ○ = α-Lactalumin (bovine milk) (M_r 14 400); × = carbonic anhydrase (M_r 29 000); □ = glyceraldehyde-3-phosphate-dehydrogenase (M_r 36 000); + = albumin (chicken egg) (M_r 45 000). Separation conditions as in Fig. 1.

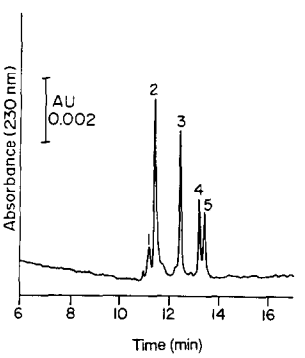


Fig. 5. Separation of myoglobin fragments on 8% acrylamide capillary. Peaks: 1 = myoglobin fragment III (M_r 2510); 2 = myoglobin fragment II (M_r 6210); 3 = myoglobin fragment I (M_r 8160); 4 = myoglobin fragment I + II (M_r 14 400); 5 = myoglobin fragment polypeptide backbone (M_r 16 950). Separation conditions as in Fig. 1.

gin to occur. When the wavelength is set at 230 nm, amide absorbance is sufficiently high that these complexes are observed as they migrate past the detector. Column longevity is better at 280 nm [16].

Separations on uncoated capillaries

The separation of SDS denatured proteins requires a size sieving medium. A Ferguson plot [17], *i.e.* log mobility of proteins *versus* percent acrylamide (%T), exhibits a linear relationship for each protein. Fig. 3 illustrates a Ferguson plot for four proteins on uncoated capillaries containing between 3 and 8% acrylamide. The linear fits of the data were obtained by linear regression. As expected, the slopes increased with M_r and all proteins had identical mobilities at 0% acrylamide. The relationship between electrophoretic mobility and molecular mass with linear polyacrylamide gels is seen in Fig. 4. It is interesting that in these typical plots of log molecular mass *vs.* mobility that there is no statistically significant difference in the slopes. This is similar to cross-linked polyacrylamide gels in the slab gel format. However, higher gel concentrations are better for the separation of low-molecular-mass peptides. This is evident in the separation of myoglobin fragments of M_r 2500 to 17 000 in an 8% linear polyacrylamide capillary (Fig. 5). These peptides coeluted on a 4% linear polyacrylamide gel column.Fig. 6 shows the resolution of polypeptides ranging in M_r from 6500 to 78 000 with a 6% gel while Fig. 7 shows the separation of polypeptides ranging in M_r from 29 000 to 205 000 with a 4% gel.

SDS-CGE was applied to several protein separations problems. Immunoglobulin G (IgG) purified from bovine serum by ion-exchange and reversed-phase chromatography was examined to determine purity. Following partial reduction the sample was run on a coated column with 2% polyacrylamide.

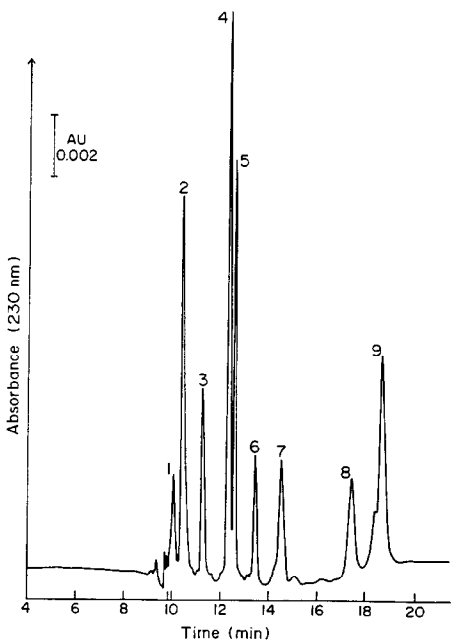


Fig. 6. Separation of protein mixture on uncoated capillary with 6% acrylamide. Peaks: 1 = aprotinin (bovine lung) (M_r 6500); 2 = cytochrome *c* (horse heart) (M_r 12 400); 3 = trypsin inhibitor (soybean) (M_r 20 100); 4 = trysinogen, phenylmethylsulfonyl fluoride (PMSF) treated (M_r 24 000); 5 = carbonic anhydrase (M_r 29 000); 6 = glyceraldehyde-3-phosphatedehydrogenase (M_r 36 000); 7 = albumin (chicken egg) (M_r 45 000); 8 = albumin (bovine (M_r 66 000); 9 = conalbumin (M_r 78 000). Separation conditions are given in Fig. 1.

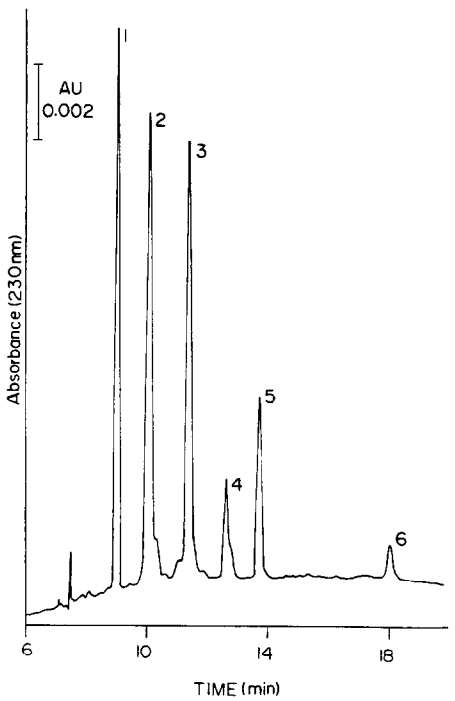


Fig. 7. Separation of protein mixture with wider molecular mass range on 4% acrylamide capillary. Peaks: 1 = carbonic anhydrase (M_r 29 000); 2 = albumin (chicken egg) (M_r 45 000); 3 = albumin (bovine) (M_r 66 000); 4 = phosphorylase B (rabbit muscle (M_r 97 400); 5 = β-galactosidase (*Escherichia coli*) (M_r 116 000); 6 = myosin (rabbit muscle) (M_r 205 000). Separation conditions as in Fig. 1.

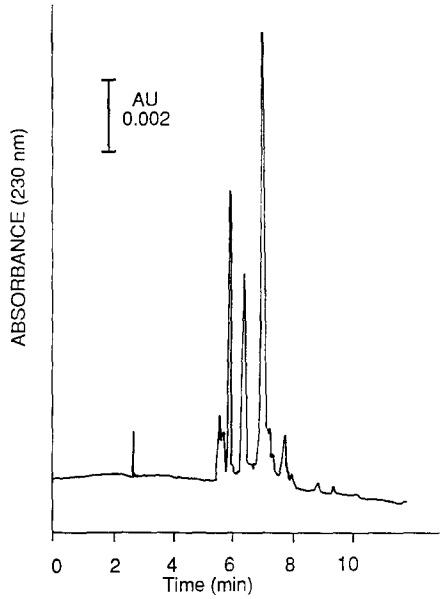
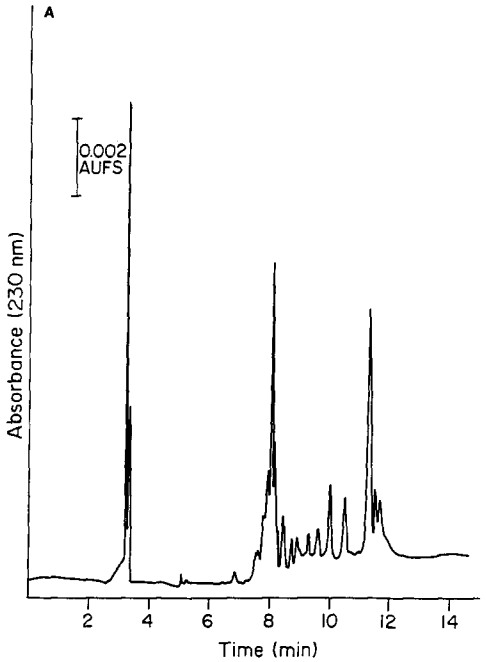


Fig. 8. Analysis of bovine IgG by capillary gel electrophoresis on coated capillary of 2% acrylamide Separation conditions as in Fig. 1.



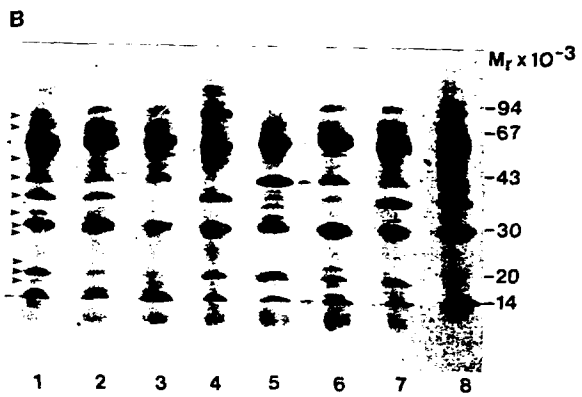


Fig. 9. (A) Separation of human salivary proteins by capillary gel electrophoresis on uncoated surface of 4% acrylamide. Separation conditions as in Fig. 1. (B) SDS-PAGE of human parotid saliva on a 12.5% slab gel. From Beeley [18].

The three major peaks in the electropherogram (Fig. 8) represent the heavy chain if IgG ($M_r \approx$ 53 000), the light chain ($M_r \approx$ 22 000) and the combination of heavy and light chain ($M_r \approx$ 75 000). Impurities with M_r greater than 75 000 and less than 22 000 are also clearly seen, indicating that the IgG sample still is not pure.

Human salivary proteins ranging in M_r from 14 000 to 94 000 have been separated by SDS-slab gel electrophoresis and used in clinical programs designed to monitor oral health [18]. The fact that the electrophoretic separation (Fig. 9B) and staining took over 7 h is a limitation in a clinical assay. The very high proline content of many salivary proteins is yet another complication. Proline-rich proteins are difficult to detect by conventional staining procedures. The separation and detection of human salivary proteins on a 4% linear polyacrylamide SDS-CGE system in 15 min is illustrated in Fig. 9A.

CONCLUSIONS

The importance of surface modification and electro-osmosis in SDS-CGE has been over-estimated in previous studies. When viscosity of a linear polyacrylamide solution reaches 100 cP, electro-osmosis plays no significant role in either determining separation efficiency or pumping gels from the capillary. This allows uncoated fused-silica capillaries to be used with linear polyacrylamide gels for the preparation of capillaries that may be used 20 times

without deterioration of either resolution of detection sensitivity.

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