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Rapid isoelectric focusing of proteins in hydrolytically stable capillaries

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ABSTRACT

Three new types of capillary coatings for capillary isoelectric focusing that avoid siloxane chemistry, resulting in hydrolytically stable coatings, are described and tested: phenyl–silica, acrylamide-reacted vinyl–silica, and pure PTFE. Capillaries of these three types were compared using standard proteins and a biological mixture of proteins similar to what might be encountered in actual use. Of these, the acrylamide-coated capillary produced the highest-quality results. In contrast to capillaries prepared using siloxane reactions, the capillaries described herein exhibited greatly enhanced stability at high pH.

INTRODUCTION

Capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF) are high-resolution analytical techniques that can be used for separation of proteins [1,2], peptides [3,4], DNA [5], and other biological materials. Because of its high speed compared with slab gel electrophoresis, CZE is ideally suited for rapid analysis of proteins in their native state. One promising use is as a rapid assay at intermediate stages during purification for proteins which do not exhibit a specific measurable enzymatic activity. One limitation of CZE is that, although very minute quantities of solute molecules can be detected, in terms of concentration sensitivity, the current generation of UV detectors produces results slightly inferior to those achieved by high-performance liquid chromatography (HPLC). Additionally, injection of large sample volumes without stacking, or with dilute buffer stacking, reduces the plate count [6]. Thus, for free solution CZE it is necessary to concentrate dilute samples to very small volumes (≈ 1 μl). For proteins, this can cause sample precipitation or aggregation which cause loss of sample, clogging of the capillary, and spurious spikes in the electropherogram caused by particulates.

In principle, cIEF is not subject to this limitation, because the focusing process automatically concentrates the sample during the analysis. Thus, the entire capillary can be filled with sample at a low concentration. In addition, cIEF can provide an indication of a useful physical parameter, the protein's isoelectric point.

To date, however, only relatively few studies have used cIEF to analyze proteins [7–11]. In cIEF, a coated capillary is generally used in order to reduce or eliminate electroosmotic flow (EOF). Alternatively, polymer additives can be added to reduce EOF and wall adsorption by shielding interactions with the capillary wall or increasing solvent viscosity [12,13]. EOF acts to physically pump solvent toward the cathode, and thus interferes with the establishment of a stable pH gradient. Unfortunately, most of the coatings currently in use for CZE of proteins either do not sufficiently reduce EOF, or

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utilize silane derivatives to covalently attach an uncharged organic compound to the silanol groups of the silica capillary. These siloxane linkages, which involve a Si–O–Si–C bond, are slowly hydrolyzed by the high-pH solution used in cIEF. After 5–10 uses, the coating is hydrolyzed away to such an extent that IEF is no longer possible. Therefore, a new type of coating process to eliminate this effect is desired.

This paper describes three types of capillary coatings that avoid siloxane chemistry, resulting in hydrolytically stable coatings: phenyl–silica, acrylamide-reacted vinyl–silica, and pure poly(tetrafluoroethylene), PTFE. Capillaries of these three types were compared using standard proteins and a biological mixture of proteins similar to what might be encountered in actual use. Of these, the acrylamide-coated capillary produced the highest-quality results.

MATERIALS AND METHODS

Coating of capillaries

Acrylamide–vinyl–silica capillaries. An 0.7-m length of 200 μm, 100 μm, or 75 μm I.D. silica capillary tubing was rinsed for 10 min with 1 *M* NaOH, then rinsed with water and dried for at least 2 h in an oven at 150°C with a flow of nitrogen under 5 p.s.i. through the capillary. During all oven treatments, the capillary was kept as straight as possible to prevent the formation of slight permanent bends in the capillary which caused breakage when the Beckman P/ACE autosampler was used. The PTFE septum in a bottle of $SiCl_4$ (Aldrich) was punctured with a 25-gauge needle and one end of the capillary was inserted through the hole. A second 25-gauge needle was inserted through the septum and nitrogen pressure (5 p.s.i.) was applied. Efflux of $SiCl_4$ vapor was verified by pointing the outlet end of the capillary onto a piece of pH paper. The end of the capillary inside the bottle was lowered under the surface and the capillary was filled with $SiCl_4$. The nitrogen pressure was released, the capillary was rapidly removed and its ends connected together by a small piece of silicone tubing (Manostat, 0.015 in. I.D. x 1/32 in. wall). The ends of the capillary were sealed with a small torch and the capillary was incubated overnight at 65°C in an oven. The next day one end was broken and rapidly inserted through a hole in the septum of a bottle of 1 *M* vinyl–MgBr in tetrahydrofuran. The bottle was pressurized with nitrogen as before, and the $SiCl_4$ was blown out. The capillary was flushed with nitrogen for at least 5 min, until the pH paper test indicated that all traces of $SiCl_4$ were removed. The end of the capillary was then submerged in the vinyl–MgBr solution and the solution was forced through the capillary for *ca.* 5 min. The ends of the capillary were sealed as before and the capillary was incubated for 1–2 h at 65°C. The capillary was then flushed with anhydrous THF and treated with $SiCl_4$ and vinyl-MgBr a second time using the same conditions and incubation times. The capillary was then rinsed with tetrahydrofuran, ethanol, water, 0.01 *M* H_3PO_4 and 0.01 *M* NaOH to remove any residual salts or organic compounds, and installed in a cartridge before coating with acrylamide. The final capillary length was 50–55 cm.

The washed capillary was then filled with a solution of 6% acrylamide, 0.4% N,N,N′,N′-tetramethylethylenediamine (TEMED), and 0.04% ammonium persulfate. When the acrylamide solution started to become viscous, the acrylamide was removed by applying pressure to the capillary and the coated capillary was rinsed with water for 10 min and incubated for 1 h at room temperature before use.

For comparison, some acrylamide-coated capillaries were also prepared by the method of Hjertén involving γ-methacryloxypropyltrimethoxysilane [8,14], except that excess silane reagent was removed by washing with ethanol before coating with acrylamide.

PTFE capillaries. A 0.5-m piece of PTFE tubing (0.304 mm I.D. × 0.76 mm O.D.) was flushed with hexane, chloroform, dimethylformamide, then ethanol for 20 min each in a bath of refluxing chloroform to remove any impurities. The tubing was straightened and inserted into a Beckman cartridge. In some cases, the PTFE was surface-dehydrofluorinated [15] by filling the tubing with 4 *M* NaOH containing 10% tetrabutylammonium phosphate solution (Alltech Low UV IPC-A) and incubating at room temperature overnight.

Phenyl-coated silica capillaries. A capillary was chlorinated with $SiCl_4$ as described above, and the chlorinated capillary was filled with phenyllithium (using the same procedure as used to fill the capillary with vinyl-MgBr) and reacted at 65°C for 1 h.

The $SiCl_4$ /phenyllithium treatment was repeated. The phenyllithium was removed by pressurizing the capillary with nitrogen and the capillary was rinsed sequentially with toluene, chloroform, ethanol, and water. Organolithium compounds react with Si–Cl bonds more vigorously than MgBr-containing Grignard reagents [16–18].

Capillary electrophoresis procedure. The coated capillary was installed in a cartridge and filled with a solution containing the protein sample, 2–5% Pharmalytes (pH 3–10), and in some samples, 1% Tween-20. The sample was focused at 10 or 30 kV in a Beckman P/ACE System 2050 with the anode in the inlet solution. The anolyte was 0.01 *M* H_3PO_4 and catholyte was 0.01 *M* NaOH. Absorbance was measured at 280 nm. For PTFE capillaries and 200 μm capillaries, the voltage was reduced to 4 and 5 kV, respectively.

RESULTS AND DISCUSSION

For analysis of protein mixtures from biological samples, isoelectric focusing is an ideal analytical method. Because the entire capillary can be filled with sample (as much as 0.01 ml in a large capillary), sample preconcentration, which results in aggregation and precipitation, is not necessary. In IEF, the proteins are concentrated automatically and can be easily filtered or centrifuged in these larger volumes. Another advantage is that capillary length and diameter are less of a factor in determining resolution, so shorter capillaries with attendant shorter elution times, or larger capillaries with larger sample capacities can be used. Finally, because lyophilization can be avoided, high salt concentrations are less of a problem. High salt concentrations in the sample can modify the appearance of the electropherogram [19,20].

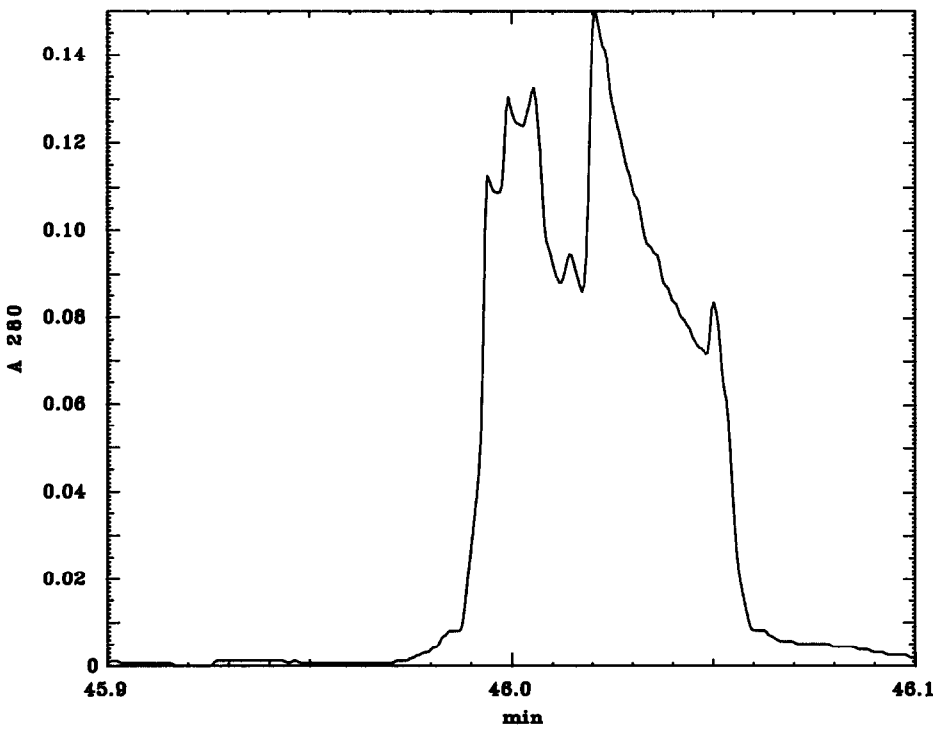
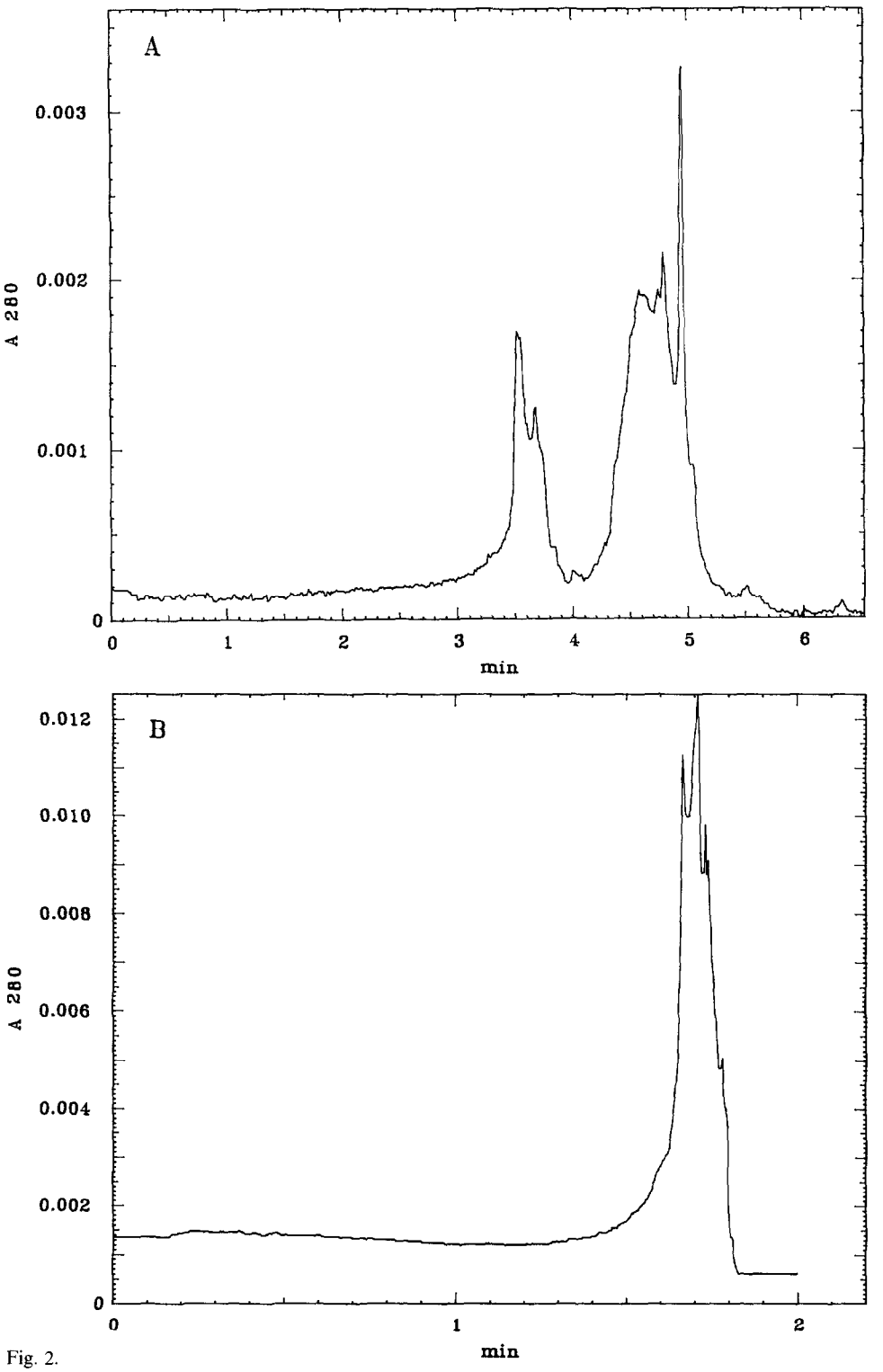


Fig. 1. Focusing of a mixture of bovine hemoglobin and bovine serum albumin in 2% ampholytes (Pharmalytes 3–10) in a 50 cm × 305 μm I.D. PTFE capillary focused for 45.0 min at 4 kV. The anolyte and catholyte contained 0.01 *M* H_3PO_4 in ethanol and 0.01 *M* NaOH in ethanol, respectively. The current decreased from 31 to 1.4 μA during focusing. After 45 min of focusing, the focused proteins were eluted using 0.5 p.s.i. pressure. Only a small degree of focusing is evident.


A
0.003
0.002
0.001
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A 280
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2
3
4
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B
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A 280
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Fig. 2.

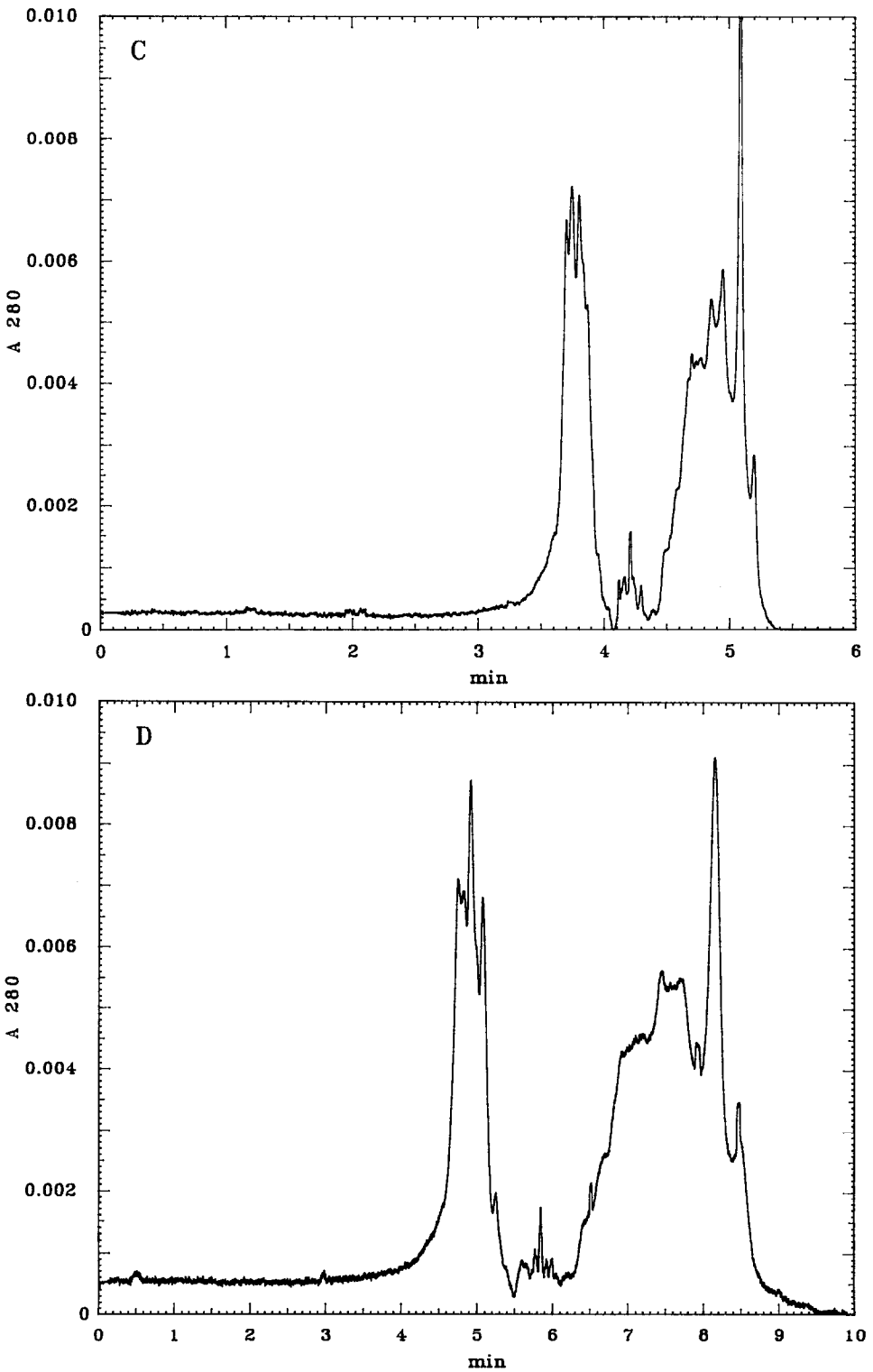

Fig. 2. (A) Focusing of a mixture of bovine hemoglobin and bovine serum albumin in 2% ampholytes (Pharmalytes 3-10) in a freshly prepared 50 cm × 100 μm I.D. capillary coated with acrylamide using the silanol method [8]. Focusing was carried out at 10 kV. During focusing, the current decreased from 8.2 to 4 μA in 6 min. The large peak at 4.95 min is albumin, and the small peak following albumin is an albumin contaminant. (B) Focusing of the protein mixture in the same capillary after 20 sample injections. (C) Focusing of the hemoglobin–serum albumin mixture in 2% ampholytes in a freshly prepared 50 cm × 100 μm I.D. capillary coated with acrylamide using the $SiCl_4$ method. Focusing was carried out at 10 kV. The current decreased from 8.0 to 3.7 μA in 6 min. (D) Focusing of the protein mixture in the same capillary after 30 sample injections.

Polymeric capillaries

Since electroosmotic flow is regarded as the predominant factor interfering with IEF, a variety of hydrophobic capillaries were tried, including PTFE, polyethylene (PE-10), and phenyl-coated silica. Both polyethylene and PTFE required low voltages (< 5 kV) in order to prevent thermally induced erratic increases in current. At higher voltages, heating also caused bubble formation, although this could be prevented by prior degassing of the sample. For PTFE capillaries, resolution and speed of focusing were improved by substuting 0.01 *M* H_3PO_4 and 0.02 *M* NaOH in 98% ethanol for aqueous electrode solutions. PTFE capillaries were sufficiently UV-transparent at 280 nm for direct use without modification. Both PTFE and surface-modified PTFE capillaries had unmeasurably low EOF, and a rapid decline in current occurred indicating that focusing of ampholytes was occurring. Because of the absence of EOF, the focused peaks did not elute spontaneously, but were eluted by pressure or replacement of anolyte with 0.02 *M* NaOH. Despite the lower EOF compared to acrylamide-coated capillaries of similar I.D., the focused proteins were incompletely resolved both with and without Tween-20 in the buffer (Fig. 1). No focusing was observed with phenyl-coated capillaries. These results with hydrophobic coatings suggest that the hydrophilicity of the surface coating is an important factor in IEF. However, these hydrophobic capillaries may be useful in separating other more hydrophilic substances, such as lipids.

Acrylamide-coated capillary

Acrylamide coated capillaries can be conveniently produced by silanization with γ-methacryloxypropyltrimethoxysilane followed by polymerization of acrylamide on the methacrylate moiety [8]. With acrylamide-coated capillaries, in contrast to polymeric capillaries, focusing occurred rapidly. In initial tests, the focusing was allowed to proceed for 3 min, after which the anolyte was replaced with 0.01 *M* NaOH to mobilize the focused proteins [21]. However, the mobilization procedure was found to be unnecessary, since these capillaries had sufficient EOF that the focused proteins eluted spontaneously even in the absence of mobilizing agent (Fig. 2A).

To test the efficiency of the coating procedure, the electroosmotic flow was measured in uncoated and acrylamide-coated capillaries. In uncoated capillaries filled with 20 m*M* sodium phosphate pH 7.0, the EOF was $4.63 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. In the acrylamide-coated capillaries, EOF at pH 7 was reduced below measurable limits ($< 6.94 \cdot 10^{-6}$ cm^2 V^{-1} s^{-1}). However, the acrylamide-coated capillaries coated with the siloxane method exhibited measurable EOF after a few hours of exposure to high pH solution, presumably due to base hydrolysis of the Si–O–Si–C bonds. After a 4-h separation at pH 8.5, the EOF had risen to 1/3 that of an uncoated capillary.

This effect limited the usefulness for IEF of acrylamide-coated capillaries coated with the siloxane method. Fig. 2B shows a cIEF electropherogram of such a capillary after 20 IEF sample injections. Some separation is still evident, but all the peaks eluted by 2.0 min. Eventually, after more samples are analyzed, all peak resolution was lost. Thus, an improved coating technique was sought. An alternative reaction to link organic modifying groups to silica using the hydrolytically stable Si–C bond instead of a Si–O–Si–C bond has been suggested for creating stable stationary phases for liquid chromatography [22,23]. This method involves chlorinating the silica with $SOCl_2$. The chlorinated silicon then reacts avidly with Grignard reagents [17,18,23]. Previous workers showed that capillaries produced by reacting the chlorinated capillary with a Grignard reagent on which acrylamide could be polymerized exhibited reduced EOF [24]. However, these capillaries proved unsuitable for IEF (data not shown). The limiting step in creating this coating is chlorination of the surface silanols, while the Grignard reagent is believed to react instantaneously with the chlorinated silicon [17,23]. Therefore, a more agressive chlorination procedure was tried, using $SiCl_4$ [25]. At least two treatments with $SiCl_4$ were required to achieve measured EOF levels as low as those achievable with the silanol method. EOF can also be evaluated by comparing the current at equilibrium, *i.e.*, after the ampholytes have focused. Using this criterion, the $SiCl_4$ capillaries were also comparable to freshly coated capillaries prepared with the siloxane method (Fig. 2, legend).

Fig. 2C illustrates an electropherogram obtained with a $SiCl_4$ capillary. Acrylamide-coated capillaries coated using the $SiCl_4$ method produced results identical with acrylamide-coated capillaries pro-

duced using siloxane chemistry, but were considerably more stable. Fig. 2D shows an electropherogram of a mixture of standard proteins in the same capillary as in Fig. 2C after injection of 30 samples, some of which contained detergents, high salt, and high pH. Even after 30 runs, no deterioration of the $SiCl_4$ capillary was apparent. In fact, the proteins are retained longer, suggesting that additional residual EOF-inducing substances had been removed.

Fig. 3 shows an IEF pattern from *Hermissenda* tentacle supernatant in an 0.3-m acrylamide-coated capillary. Most of the proteins focused and eluted before the current had reached a plateau value. The entire electropherogram was produced within 3.0 min after sample was applied. Very sharp focusing was observed, with a peak width of *ca.* 0.5 s for the largest peak, while the later, more acidic proteins, were somewhat broader. At least 60 protein components are visible altogether.

Addition of non-ionic detergent (Tween-20) improved resolution for the *Hermissenda* samples, probably by reducing the interaction with uncoated silanols or by increasing the effective size of the proteins. However, for other samples, addition of detergent had little effect (data not shown).

The residence time of the proteins in the capillary could be increased by adding NaCl to the catholyte. However, this did not improve resolution or affect the relative elution order of the peaks (Fig. 4). Increasing the NaCl in the catholyte to 0.04 *M* prolonged the elution period to 10 min. Altering the H_3PO_4 and NaOH concentrations also had little effect on the separation (not shown). Because the proteins tended to elute spontaneously from the capillary, the retention times of the proteins were not a simple function of isoelectric point.

Fig. 5 shows an electropherogram of rabbit cerebral cortex supernatant. Because this sample contained a higher salt concentration than the sample

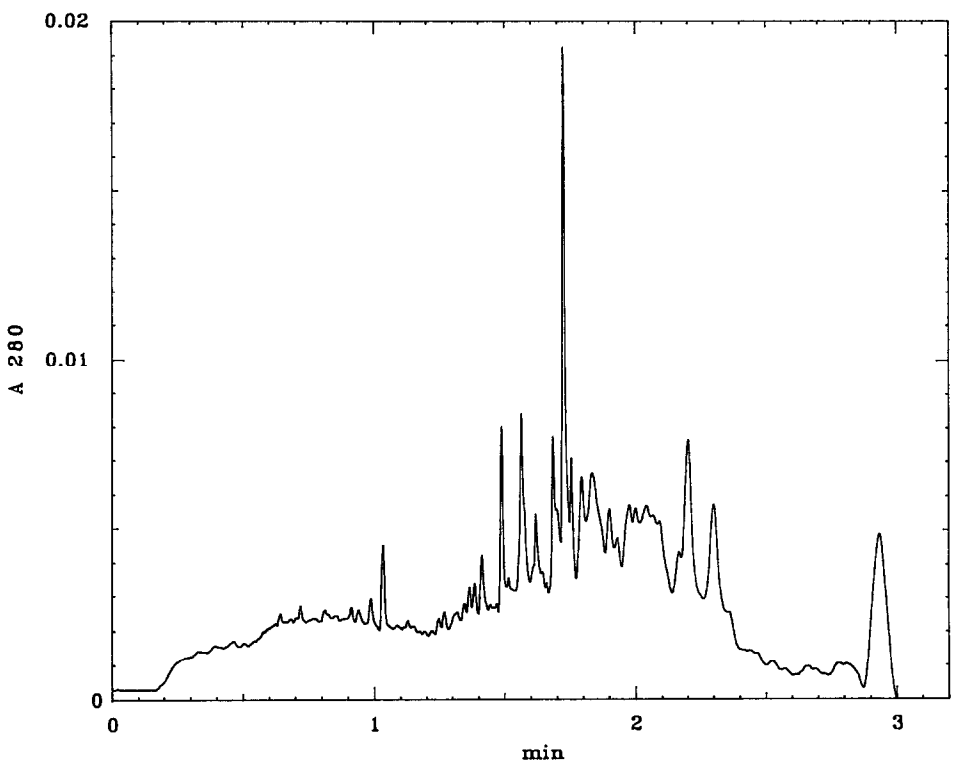

Fig. 3. Focusing of *Hermissenda* tentacle proteins in an acrylamide-coated capillary at 10 kV. The capillary was filled with *ca.* 50 μg/ml total cytoplasmic proteins containing 10% Pharmalytes 3-10 and 1% Tween-20 before focusing. The current decreased from 92 to 32 μA in 4.0 min.

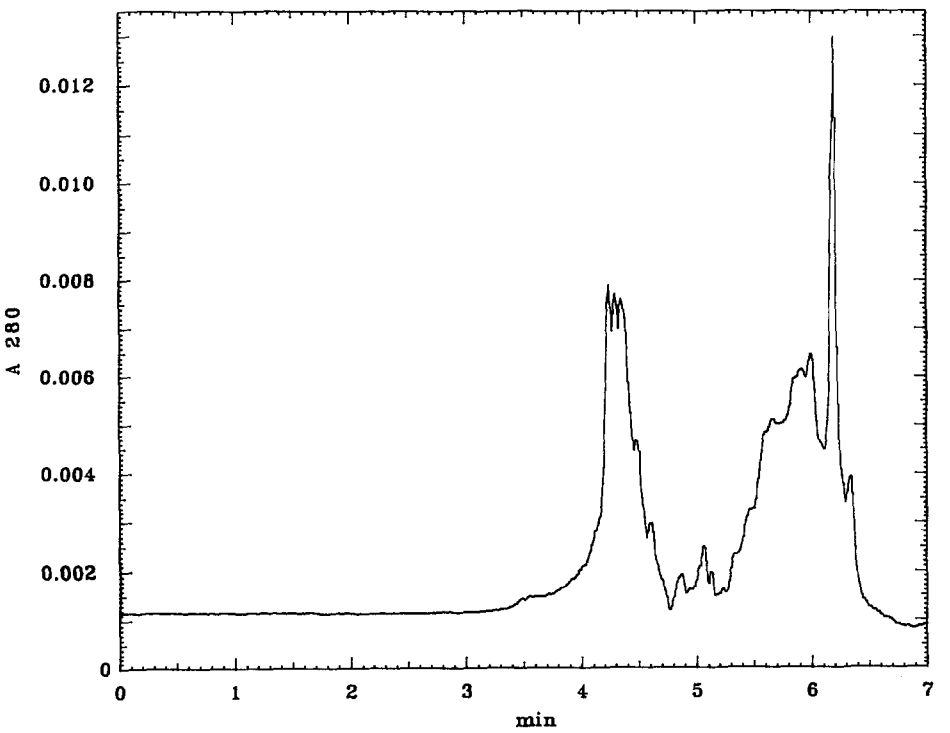

Fig. 4. Focusing of a mixture of bovine hemoglobin and bovine serum albumin in an acrylamide-coated capillary. Conditions were the same as in Fig. 2C except that 0.01 *M* NaCl was added to the catholyte solution. The elution pattern is similar to the pattern in Fig.2C except that the proteins are retained for a longer period.

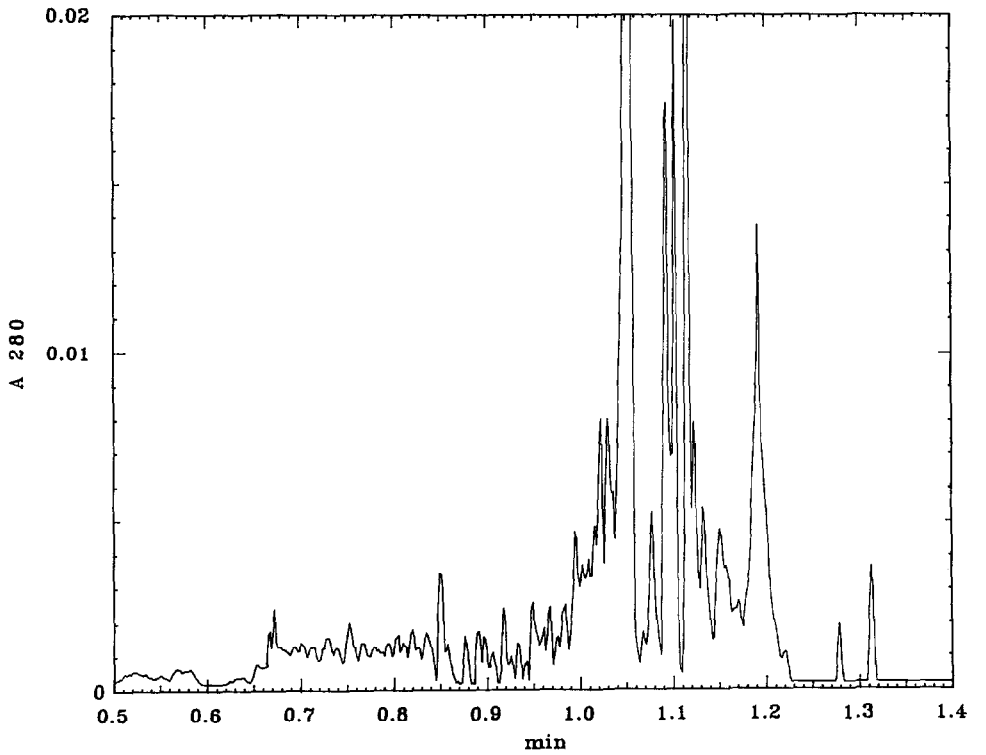

Fig. 5. Focusing of rabbit brain proteins in an acrylamide-coated capillary produced with the $SiCl_4$ method. A sample of rabbit cerebral cortex was homogenized in 10 volumes of water and centrifuged (10 000 *g* × 1 min). A 20-μl aliquot of the supernatant fraction was diluted to 100 μl, and 2 μl of ampholines were added before focusing at 10 kV. Most proteins eluted between 0.6 and 1.4 min.

in Fig. 3, the entire sample was eluted in under 1.5 min. Peaks were also extremely sharp, with minimum peak widths on the order of 0.15–0.25 s. Efficiency calculation was difficult, however, because at the maximum obtainable 10 Hz data acquisition rate, many of the peaks were only 1–2 data points wide. The decrease in absorbance to below baseline values at 1.25 min is caused by displacement of the ampholytes with buffer, and can be eliminated by addition of ampholytes to the anolyte and catholyte solutions.

The rapid elution of focused peaks in Fig. 5 indicates that focusing had occurred within the first $\approx$0.8 min. Since initially the capillary was uniformly filled with sample, this means that the earliest peaks represented proteins that must have travelled at up to 0.5 m/min. It is apparent that some EOF still remains in acrylamide-coated capillaries coated with both methods. This may be caused by a few remaining silanols at the high pH end of the capillary. EOF due to silanol ionization is known to be strongly pH dependent. To test whether focusing was complete, an IEF sample was tested with the proteins only filling the first 1/10 of the capillary nearest the anode or the cathode, with the remainder of the capillary filled with ampholytes alone. In both cases, resolution was greatly reduced.

Although the coated capillaries described here exhibited greater stability than other coatings at high pH, one would expect that after prolonged exposure, the Si–C bonds eventually would begin to hydrolyze. Polyacrylamide could also be hydrolyzed to acrylic acid by high pH, particularly at higher temperatures. Thus, the capillaries should be rinsed with water after use.

The above results demonstrate that cIEF carried out in the hydrolytically stable capillaries results in a rapid, highly reproducible, and high-resolution separation of complex protein mixtures.

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