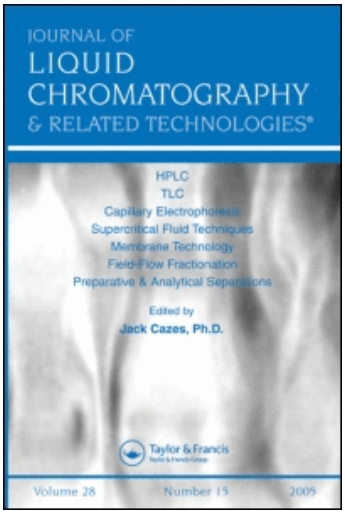

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Selective On-Line Preconcentration of Proteins by Tandem Metal Chelate Capillaries-Capillary Zone Electrophoresis

Jianyi Cai[a]; Ziad El Rassi[a]
[a] Department of Chemistry, Oklahoma State Universit, Stillwater, Oklahoma

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SELECTIVE ON-LINE PRECONCENTRATION OF PROTEINS BY TANDEM METAL CHELATE CAPILLARIES-CAPILLARY ZONE ELECTROPHORESIS*

JIANYI CAI AND ZIAD EL RASSI**
Department of Chemistry
Oklahoma State University
Stillwater, Oklahoma 74078-0447

ABSTRACT

Fused-silica capillaries having surface bound iminodiacetic acid metal chelating functions were developed for the selective on-line preconcentration of dilute protein samples prior to capillary zone electrophoresis. The metal chelate capillaries with immobilized zinc proved effective in the selective accumulation of detectable amounts of proteins from dilute samples. They permitted the detection of 25 times less concentrated samples than by CZE alone with concentration sensitive detectors. Large sample volumes could be introduced without affecting separation efficiency. Besides demonstrating the effectiveness of metal chelate capillaries in the quantitative determination of proteins from dilute samples, the effects of pH, composition of electrolytes and sample feeding time on the analytical signal were examined with carbonic anhydrase, a zinc binding protein.

INTRODUCTION

In capillary zone electrophoresis (CZE), small sample volumes must be introduced into the capillary column so that high separation efficiencies and high

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** To Whom all correspondence should be addressed

resolution can be achieved. This requirement renders CZE unsuitable for the analysis and detection of dilute samples. To overcome this inherent problem of the technique and to allow the analysis of dilute samples with concentration sensitive detectors, several methods have been reported for performing on-line preconcentration prior to CZE separation.

Electrophoretic stacking techniques have been evaluated in CZE of dilute samples. The simplest approach has been the introduction by electromigration of samples dissolved in straight water or in buffer of very low ionic strength (1, 2). This leads to an enhanced field strength at the injection point, thus permitting the introduction of a relatively large amount of ions. Another alternative has been the so-called field amplified sample injection technique in which a small plug of water is introduced into the capillary prior to sample introduction (3, 4). The plug of water creates a large field amplification which allows a 100-fold enhancement in the amount of ions injected without any substantial loss in separation efficiencies. Very recently, another modification of electrophoretic stacking has been introduced for on-line preconcentration of dilute samples (5). This method, which was evaluated with peptides, relies on the introduction of the sample in a pH higher than the isoelectric points of the sample components and that of the running buffer. The components will be first negatively charged and migrate in the opposite direction to the bulk flow. As they will reach the sample solution-electrophoresis buffer interface they become positively charged and concentrate at this interface before they will dissipate and separate. The major drawback in the electrophoretic concentration or stacking methods has been the limited loadability, which means that only a small sample volume can be introduced.

Another approach for allowing the analysis of dilute samples by CZE has been the on-line preconcentration by isotachophoresis (6-12). However, the isotachophoretic concentration mode is difficult to automate and is limited by the

choice of electrophoresis buffers. Also, positive and negative analytes cannot be determined at the same time.

Very recently, we have introduced on-line preconcentration schemes in which tandem octadecyl capillary–capillary zone electrophoresis proved useful for the determination of dilute samples of environmental significance (13). The on-line octadecyl capillaries enhanced the detectability in terms of solute concentration by a factor of 10 to 35 as compared to that obtained by CZE alone. In this report, we wish to extend the utility of on-line preconcentration with capillaries having interactive walls to the analysis of dilute samples of proteins. In this regard, we have developed capillaries with surface bound metal chelating functions, *e.g.*, iminodiacetic acid functions, for the selective preconcentration and subsequent separation of dilute protein samples by tandem metal chelate affinity capillaries–capillary zone electrophoresis. The preconcentration with metal chelate capillaries is based on the affinity between proteins and the immobilized metal chelates on the capillary wall. Since this type of interaction is selective, only proteins with affinity for the chelated metal can be concentrated.

EXPERIMENTAL

Instrument

The capillary electrophoresis instrument used in these studies closely resembles that described earlier (13, 14). It consisted of a 30-kV dc power supply Model EH30P03 of positive polarity from Glassman High Voltage Inc. (Whitehouse Station, NJ, U.S.A.) and a Linear (Reno, NV, U.S.A.) Model 200 UV-Vis variable wavelength detector equipped with a cell for on-column detection. The electropherograms were recorded with a computing integrator Model CR601 from Shimadzu (Columbia, MD, U.S.A.).

Capillaries

Fused-silica capillaries were purchased from Polymicro Technologies Inc. (Phoenix, AZ, U.S.A.). Capillary tubes with 50 μm or 75 μm inner diameter and 375 μm outer diameter were used to prepare the metal chelate capillaries. The separation capillaries consisted of fused-silica tubes of 50 μm I.D. with interlocked polyether coatings (denoted as I-200, and having surface bound polyethylene glycol 200 moieties) prepared in our laboratory as described earlier (15). The hydrophilic coatings were essential to minimize solute-wall interactions during solutes differential migration.

Reagents and Materials

Carbonic anhydrase from bovine erythrocytes was purchased from Sigma (St. Louis, MO, U.S.A.). γ-Glycidoxypropyltrimethoxysilane (Z-6040) was a gift from Dow Corning (Midland, MI, U.S.A.). Iminodiacetic acid was donated by Hampshire (Nashua, NH, U.S.A.). Reagent grade ethylenediaminetetracetic acid disodium salt (EDTA) and other chemicals used in the preparation of the running electrolytes were from Fisher Scientific (Pittsburgh, PA, U.S.A.). Colloidal silica Ludox HS-40 was a gift from Du Pont (Wilmington, DE, U.S.A.). Deionized water was used to prepare the running electrolyte. All solutions were filtered with 0.2 μm Uniperp Syringeless filters from Fisher Scientific to avoid capillary plugging.

Capillary Surface Modification

To increase the surface concentration of metal chelating ligands and in turn increase the linear capacity of the preconcentration capillaries, the inner surface of the tubes was increased by chemical and/or physical treatments before the bonding

of the metal chelating functions to the capillary surface. These surface roughening which were described earlier (13) involved etching, and in some cases subsequent coating with colloidal silica.

The etching of the inner surface of the metal chelate preconcentration capillaries was carried out as follows. Short fused-silica capillaries were filled with a 5% (w/v) solution of ammonium hydrogen bifluoride in methanol and allowed to stand for 1 hour before the solution was removed with a flow of nitrogen gas. The capillaries were then sealed in flame and heated at 300 °C for 5 hrs (16). Thereafter, the capillaries were flushed with 0.1 *M* HCl and water. Finally, the capillaries were stored in HPLC grade methanol. To prepare support coated capillaries, the etched tubes were filled with a 10% (w/v) colloidal silica solution and heated at 250 °C for one hour. This treatment was repeated 3 times. The etched and/or support coated capillary was filled with a solution of γ-glycidoxypropyltrimethoxysilane and heated at 100 °C for 30 min. This treatment was repeated twice. Subsequently, the epoxy activated capillaries were allowed to react with a 10% (w/v) solution of iminodiacetic acid at 65 °C in an oven (17). This treatment was repeated twice. Finally, the capillaries were flushed with water and then stored in HPLC grade methanol.

Other Procedures

Dilute solutions of carbonic anhydrase were prepared by dissolving the protein in the binding electrolyte. All the samples were freshly prepared for each set of experiments. Before each run, the capillaries were flushed successively with debinding electrolyte containing 30 m*M* EDTA, water, 0.2 *M* $ZnCl_2$ solution, water again and then the binding electrolyte. Finally, the capillaries were allowed to equilibrate for 10 to 20 min with the binding electrolyte.

Dilute samples were introduced at the anode end by hydrodynamic flow, *i.e.*, gravity-driven flow. The electrolyte reservoir at the anode end was replaced by a sample reservoir and was raised to a certain height above the cathodic reservoir for a certain period of time. The sample volume introduced, V, was estimated experimentally by the equation:

$$V = \pi r^2 L t_i / t_0 \qquad (1)$$

where t_0, t_i, L and r are the time it takes the sample front to reach the detection point, the injection time, the length of the capillary to the detection point and the inner radius of the capillary, respectively.

RESULTS AND DISCUSSION

Carbonic anhydrase was selected as model protein to illustrate the principles and demonstrate the potentials of on-line preconcentration using tandem metal chelate capillaries-capillary zone electrophoresis. The affinity of this protein to Zn(II)-IDA sorbents is well documented (18).

Operational and Basic Principles of Preconcentration with Metal Chelate Capillaries

Figure 1 depicts the idealized structure of the metal chelate capillaries used in this study. The capillary surface was either etched or etched and then coated with colloidal silica. As can be seen in Fig. 1, a hydrophilic coating was introduced in order to shield the silica surface toward proteins and minimize protein adsorption by non specific interactions. Iminodiacetic acid (IDA) functions were covalently attached to this hydrophilic coating to serve as the metal chelating ligands. In all the studies, Zn(II) was immobilized on the capillary surface and the corresponding metal chelate capillary tubes are denoted by Zn(II)-IDA-Cap. Naked IDA capillaries (i.e., without chelated metal) are coded by IDA-Cap.

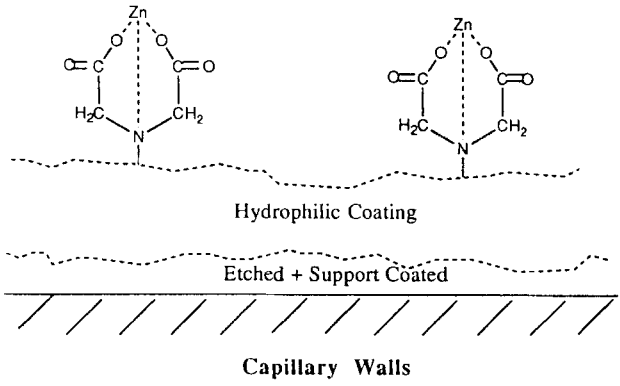

Figure 1. Schematic illustration of the idealized structure of the inner surface of metal chelate preconcentration capillary.

The surface coverage with metal chelating functions increased by coating the etched capillaries with colloidal silica. However, the colloidal support leached out after prolonged use. Therefore, most of the studies presented in this report were carried out with preconcentration capillaries having etched walls, which exhibited constant performance for longer period of time. Similar observations were reported earlier with octadecyl capillaries (13).

The Zn(II)-IDA-Cap was connected in series to a separation capillary having polyether coating (denoted as I-200, see Experimental) via a PTFE tube the inner diameter of which has the same size as the outer diameters of the two capillaries. The preconcentration capillary served as an open-tubular metal chelate affinity chromatography column whereby the analytes accumulated at the wall as the dilute sample was introduced.

Similar to metal chelate affinity chromatography (for a recent review on this type of chromatography, see Ref. 19), proteins are retained by the immobilized *metal through* interaction with electron donor side chain groups situated on the

protein surface. These groups are histidine, cysteine, and to a lesser extent tryptophan (20). Therefore, metal chelate capillaries would allow selective preconcentration of a protein or a group of proteins having affinity to the metal chelate walls.

The on-line preconcentration involves two steps, the accumulation followed by the desorption of the solutes in which binding and debinding electrolytes are used successively. The accumulation step was best carried out by gravity-driven flow (see below for details), whereas the desorption step was achieved by electromigration. Since the accumulated solute on the inner wall should be stripped off the wall and introduced in the separation capillary as a thin plug, the debinding electrolyte should contain a strong competing agent that desorbs the metal and the protein from the binding sites on the surface of the metal chelate capillary. In this regard, ethylenediaminetetraacetic acid (EDTA) is an excellent candidate since it forms stronger complex with metals than the covalently attached IDA functions on the inner surface of the capillary. Thus, the binding electrolytes used in this study were sodium phosphate solutions, whereas the debinding electrolytes were sodium phosphate containing EDTA. In addition, the concentration of the debinding agent should be carefully adjusted to ensure a fast desorption kinetic and minimize band broadening during the process of debinding.

Electroosmotic Flow

In order to examine the influence of the preconcentration capillary on the magnitude and direction of the average electroosmotic flow in the tandem IDA-Cap→I-200, the flow through both capillaries was measured with phenol as the inert tracer under various electrolyte compositions and capillary surface contents. The results are listed in Table 1.

Table 1. Influence of the preconcentration capillary and its surface content on the average electroosmotic flow (EOF) of the coupled capillaries. Calculated intrinsic electroosmotic flow of the preconcentration capillary in its various forms are also presented. Preconcentration capillary, IDA-Cap at various surface content, 20 cm x 50 μm I.D., etched at 300 °C; separation capillary, interlocked polyether 200 (I-200), 30 cm (to the detection point), 60 cm (total length) x 50 μm I.D.; running voltage, 15 kV; binding electrolyte, 10 m*M* sodium phosphate, pH 6.0; debinding electrolyte, 10 m*M* phosphate, 50 m*M* EDTA, pH 3.5; tracer, phenol; injection, electromigration, 3 seconds; detection, 210 nm.

Tandem capillaries	Status of IDA-Cap	Measured EOF (nL/min) of tandem capillaries	Calculated EOF (nL/min) of IDA-Cap[c]
I-200 [a]	---	43.00	---
IDA-Cap→ I-200 [a]	Naked	62.97	122.90
Zn(II)-IDA-Cap→ I-200 [a]	With Zn	35.25	12.00
Zn(II)-IDA-Cap→ I-200 [a]	With Zn & adsorbed protein	30.62	-6.52[d]
Zn(II)-IDA-Cap→ I-200 [b]	With Zn & adsorbed protein	43.61	----

[a] Running electrolyte, binding electrolyte.

[b] Capillaries were first equilibrated with the binding electrolyte; phenol was injected and then debinding electrolyte was applied. These sequences are the same as those used in the on-line preconcentration.

[c] Calculated EOF using eqn 2.

[d] The negative sign is to indicate that the direction of the flow is anodal.

Very recently, we have introduced from our laboratory two-dimensional electrophoretic systems for the control of electroosmosis in CZE, which involved capillaries coupled in series having different magnitude of electroosmotic flow (21). Also, we have introduced a simple relationship that relates the average electroosmotic flow in the coupled capillaries, v_{eo}, to the intrinsic electroosmotic velocities of the connected segments (21). This relationship is as follows

$$v_{eo} = (v_{eo,1}l_1 + v_{eo,2}l_2)/l_t \qquad (2)$$

where $v_{eo,1}$ and $v_{eo,2}$ are the electroosmotic velocities of segment 1 and 2 in the coupled format, respectively, l_1 and l_2 are their corresponding lengths and l_t is the total length. This relationship states that the average electroosmotic flow in the coupled capillaries is a weighted average of the intrinsic electroosmosis of the connected segments.

According to eqn 2, the average electroosmotic flow in the coupled capillaries is largely influenced by the intrinsic flow velocity in each of the connected segments. As can be seen in Table 1, the electroosmotic flow for the coupled IDA-Cap→I-200 capillaries in the absence of chelated metal increased by a factor of 1.46 with respect to the flow in I-200 for the same total length. The flow in the IDA-Cap without chelated metal on the surface was calculated by eqn 2, and found to be about 122.90 nL/min, see Table 1. This is because at pH 6.0 the two carboxylic groups of the naked iminodiacetic acid (*i.e.*, without chelated metal) function are fully ionized. In the presence of the chelated metal on the inner surface of the preconcentration capillary, the average electroosmotic flow for Zn(II)-IDA-Cap→I-200 was greatly reduced. The immobilized Zn(II) decreased the negative zeta potential of the preconcentration capillary and, as a result, the electroosmotic flow was decreased. As calculated by eqn 2, the amount of flow in the presence of chelated zinc was reduced by a factor of 10 with respect to the naked IDA capillary,

see Table 1. In the presence of adsorbed carbonic anhydrase on the surface of the preconcentration capillary, the flow across the tandem capillaries was reduced even further. Through eqn 2, the calculation of the intrinsic flow of Zn(II)-IDA-Cap with adsorbed protein on the surface reveals a small anodal flow (i.e., a reversal in the polarity of the flow has occurred). This can be explained by the slightly net positive charge of the adsorbed protein at this particular pH (pI of the protein = 6.1). In the debinding step the average electroosmotic across the tandem capillaries remained cathodal (i.e., towards the cathode), but was reduced by a factor of ca. 0.7 with respect to the IDA-Cap→I-200, see Table 1.

Besides their fundamental significance, the above results demonstrated that in all cases a moderate cathodal flow can be still generated due to the fact that the preconcentration capillary is shorter than the separation capillary in the tandem format. This is essential for the desorption step and subsequent separation of the accumulated solutes. However, due to the changes in the zeta potential of the preconcentration capillary and consequently in the magnitude of the flow, the accumulation step is best achieved by hydrodynamic means, which would allow a better control of the amount of sample introduced.

Normal Detection Limit

Figure 2 shows a plot of peak height versus sample concentration for carbonic anhydrase obtained under normal injection conditions, i.e., without on-line preconcentration step, whereby a small volume is injected. As can be seen in Fig. 2, the plot was linear in the concentration range studied, *i.e.*, for up to 1 mg/mL. However, the detection limit in terms of concentration was 25 µg/mL(*i.e.*, *ca.* 8.0×10^{-7} *M*). To analyze samples of lower concentrations, a means for preconcentration is therefore necessary.

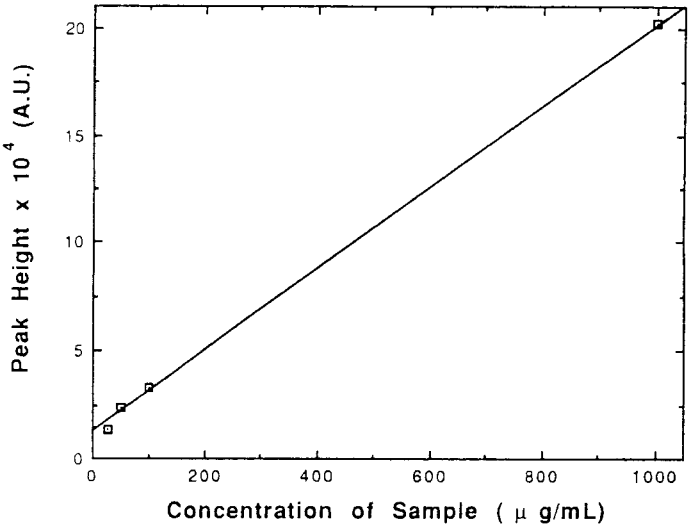

Figure 2. Plot of peak height *versus* sample concentration obtained by CZE alone. Separation capillary, interlocked polyether 200, 50 cm (to the detection point), 80 cm (total length) x 50 μm I.D.; running electrolyte, 100 m*M* sodium phosphate, pH 6.0; sample injection, electromigration, 20 kV, 5 seconds; running voltage, 20 kV; detection, 210 nm. Sample, carbonic anhydrase.

Quantitative Determination of Dilute Samples with Metal-Chelate Preconcentration Capillaries

Bovine carbonic anhydrase was employed as the model solute to examine the usefulness of tandem Zn(II)-IDA-Cap→CZE in the quantitative determination of proteins from dilute samples. The results are depicted in Fig. 3 in terms of peak height versus sample concentration. The peak height increased linearly with sample concentration over the range studied. The detection limit was approximately 1 μg/mL (*i.e., ca* . 3.2 x 10^{-8} *M*), which is 25 folds lower than the detection limit with normal CZE, under otherwise the same detection conditions.

The above results show that on-line metal chelate preconcentration capillaries are suitable for the quantitative determination of proteins from dilute

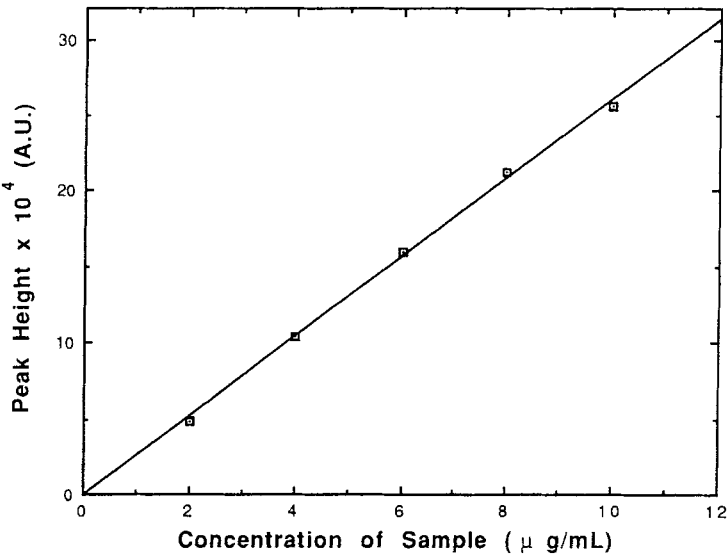

Figure 3. Plot of peak height *versus* sample concentration obtained by on-line preconcentration of dilute bovine carbonic anhydrase samples. Preconcentration capillary, Zn(II)-IDA, etched at 300 °C, 20 cm x 50 μm I.D.; separation capillary, I-200, 30 cm (to the detection point), 60 cm (total length) x 50 μm I.D.; binding electrolyte, 10 m*M* sodium phosphate, pH 6.0; debinding electrolyte, 10 m*M* sodium phosphate, 50 m*M* EDTA, pH 3.5; sample injection, hydrodynamic, Δh = 20 cm, 30 min; volume introduced, ca. 390 nL; running voltage, 15 kV; detection, 210 nm.

samples. The linear relationship between peak height and sample concentration at relatively low concentration is an indication of a linear adsorption isotherm and is in agreement with earlier observations (13).

Figure 4 portrays typical electropherograms for carbonic anhydrase obtained with tandem Zn(II)-IDA-Cap→CZE. Sharp peaks were obtained even though the sample volumes introduced were relatively large, *ca.* 120 nL. It has to be noted that in normal CZE, the maximum sample volume that can be introduced is about 5-10 nL. Above this amount, severe band broadening will result. This illustrates the

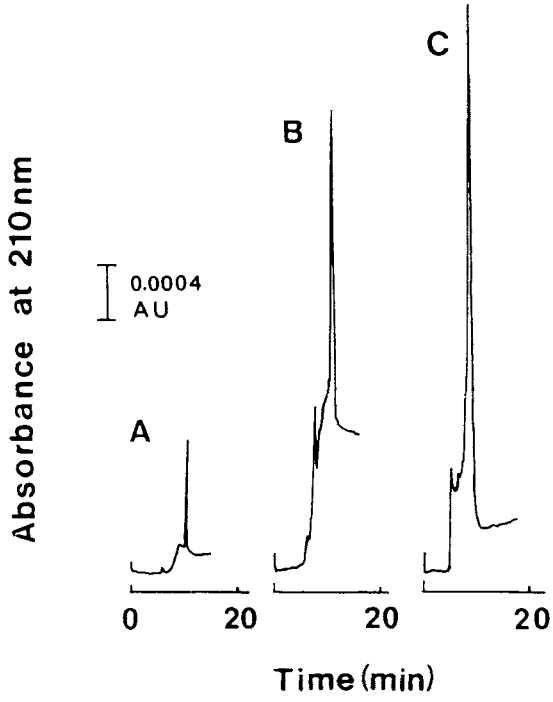

Figure 4. Typical electropherograms illustrating the preconcentration of carbonic anhydrase by tandem Zn(II)-IDA-Cap→CZE. Preconcentration capillary, Zn(II)-IDA, etched at 250 °C and support coated with colloidal silica, 20 cm x 75 μm I.D.; separation capillary, as in Fig. 3; binding electrolyte, 10 m*M* sodium phosphate, pH 6.0; debinding electrolyte, 10 m*M* sodium phosphate, 30 m*M* EDTA, pH 3.8; sample volume introduced, ca. 120 nL; running voltage, 20 kV; detection 210 nm; sample concentration, 10 μg/mL in A, 40 μg/mL in B, 100 μg/mL in C.

effectiveness of on-line preconcentration with metal chelate capillaries as far as the separation efficiencies and detection limit are concerned.

Furthermore, Fig. 4 shows that at concentration above 10 μg/mL the peak height is no longer a linear function of concentration. This corresponds to saturating the adsorption sites on the capillary wall with adsorbed solute molecules and consequently the adsorption isotherm flattens out, i.e., the concentration of the solute in the adsorbed phase has approached a maximum value.

Table 2. Effect of pH of the binding electrolyte. Binding electrolyte, 10 m*M* phosphate; debinding electrolyte, 10 m*M* sodium phosphate, 50 m*M* EDTA, pH 3.5; sample, 10 μg/mL carbonic anhydrase; other experimental conditions are same as in Fig. 3.

pH	Peak height x 10^3 (A.U.)
5.0	0.52
6.0	2.56
7.0	3.54

Effects of Operational Parameters

To determine the optimum conditions for on-line preconcentration, the effects of various operational parameters were investigated. These parameters include the composition of the binding and debinding electrolytes, and the duration of sample introduction.

pH of Binding Electrolyte. The effect of pH of the binding electrolyte was studied and the results are listed in Table 2. As shown, the peak height increased with the pH of the binding electrolyte. High pH favors stronger interactions between the protein and the chelated metal (19), thus allowing the accumulation of a larger amount of the analyte on the inner surface of the metal chelate capillary.

Concentration of Debinding Agent. As mentioned above, the choice and the concentration of the debinding agent is very important in the desorption step. The graph in Fig. 5 shows that the detector signal increased with increasing the concentration of EDTA in the debinding electrolyte. An optimum concentration for the debinding agent is about 50 m*M* whereby the current is still in the range that does not lead to system overheating. At this optimum concentration of EDTA, a good recovery of the sample is achieved and fast desorption is obtained.

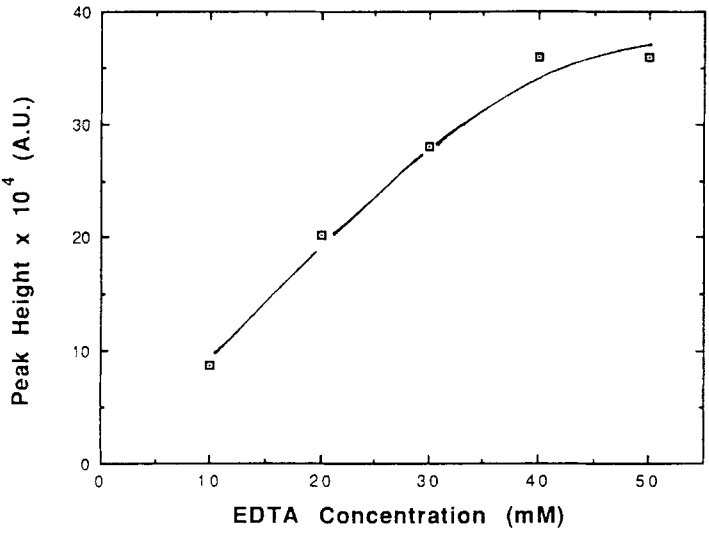

Figure 5. Plot of peak height versus EDTA concentration in the debinding electrolyte. Debinding electrolyte, 10 m*M* sodium phosphate containing different concentration of EDTA, pH 3.5; sample, 10 μg/mL bovine carbonic anhydrase. Other experimental conditions are as in Fig. 3.

Feeding time. The duration of sample introduction determines the volume introduced and consequently influences the amount of solute accumulated on the capillary wall from a given solution. The results of this study are shown in Fig. 6 (dashed line). The sample volume introduced, which was calculated using eqn 1, as a function of time is also shown. In all cases, the amount of solute accumulated on the wall first increased and then leveled off at relatively longer feeding time. The volume of the preconcentration capillary is ca. 390 nL, which under the conditions of the experiment can be filled up with the feeding solution in ca. 30 min. As can be seen in Fig. 6, a feeding time above 30 min did not lead to more adsorption from the dilute samples.

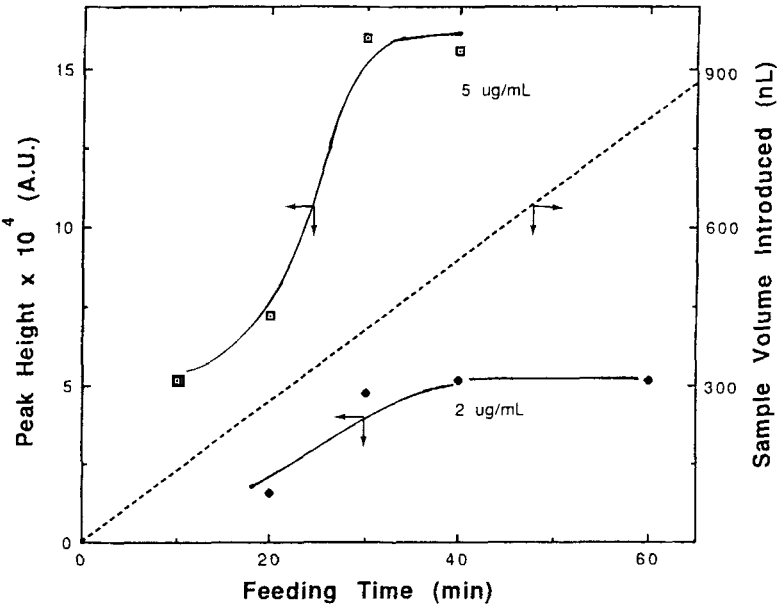

Figure 6. Plots of peak height *versus* the feeding time. Other experimental conditions are as in Fig. 3.

CONCLUSIONS

The on-line preconcentration method described here with capillary having metallic walls offers a means by which dilute samples of proteins can be analyzed by CZE. The metal chelate preconcentration capillary permits continuous sample loading, i.e., large sample volume can be introduced, and consequently low detection limit in terms of concentration can be obtained. Furthermore, with preconcentration capillaries plots of peak height versus sample concentration are linear, thus permitting the quantitative analysis of dilute samples. Most importantly the on-line preconcentration with interactive capillaries requires simple instrumentation that are customarily used in CZE, and can greatly increase detection

sensitivity with respect to sample concentration. These are initial studies, and broader investigation involving other metal chelate and affinity ligands are being conducted in our laboratory.

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