Published in final edited form as:

Electrophoresis. 2010 January ; 31(2): 346–352. doi:10.1002/elps.200900055.

Improved Peak Capacity for Capillary Electrophoretic Separations of Enzyme Inhibitors with Activity-Based Detection Using Magnetic Bead Microreactors

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Abstract

A technique for separating and detecting enzyme inhibitors was developed using capillary electrophoresis with an enzyme microreactor. The on-column enzyme microreactor was constructed using NdFeB magnet(s) to immobilize alkaline phosphatase-coated superparamagnetic beads (2.8 μm diameter) inside a capillary before the detection window. Enzyme inhibition assays were performed by injecting a plug of inhibitor into a capillary filled with the substrate, AttoPhos. Product generated in the enzyme microreactor was detected by laser-induced fluorescence. Inhibitor zones electrophoresed through the capillary, passed through the enzyme microreactor, and were observed as negative peaks due to decreased product formation. The goal of this study was to improve peak capacities for inhibitor separations relative to previous work, which combined continuous engagement electrophoretically mediated microanalysis (EMMA) and transient engagement EMMA to study enzyme inhibition. The effects of electric field strength, bead injection time and inhibitor concentrations on peak capacity and peak width were investigated. Peak capacities were increased to ≥ 20 under optimal conditions of electric field strength and bead injection time for inhibition assays with arsenate and theophylline. Five reversible inhibitors of alkaline phosphatase (theophylline, vanadate, arsenate, L-tryptophan and tungstate) were separated and detected to demonstrate the ability of this technique to analyze complex inhibitor mixtures.

Keywords

Capillary electrophoresis; Electrophoretically mediated microanalysis; Enzyme inhibition; Magnetic beads

1 Introduction

Because enzyme catalysis plays a central role in biological chemistry, enzymes are one of the most important classes of drug targets, and enzyme inhibitors have been central to the drug discovery and development process [1]. Rapid, inexpensive and information-rich analytical techniques are needed for enzyme inhibitor screening [1]. When potential enzyme inhibitors are part of a synthetic or natural mixture of compounds, it would be desirable to use analysis methods that simultaneously separate compounds in the mixture and detect their inhibition of a target enzyme.

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The authors have declared no financial or commercial conflict of interest.

Capillary electrophoresis (CE) has been successfully applied for studies of enzyme kinetics and enzyme inhibition [2,3]. The sample volume required for CE is extremely small (pL-nL). Capillary electrophoresis is a powerful separation technique and is capable of separating reaction products from substrates and enzymes with high efficiency. Electrophoretically mediated microanalysis (EMMA) is a CE-based technique, which was first described by Bao and Regnier in 1992 [4] and is commonly applied for performing enzyme assays and studying enzyme inhibition [2,3,5]. In EMMA, an enzyme-catalyzed reaction is carried out by mixing reaction components electrophoretically in a capillary column. There are two formats of EMMA, continuous engagement EMMA and transient engagement EMMA. In continuous engagement EMMA, enzyme assays are performed by injecting a zone of enzyme into a capillary that has been filled with a solution containing the substrate for the enzyme [5]. Product is formed as the enzyme migrates through the capillary, and the product is detected as a plateau. In transient engagement EMMA, the enzyme and the substrate are injected into the capillary as distinct zones [5]. Product is formed when the enzyme and substrate zones mix electrophoretically in the capillary, and the product is detected as a separate peak.

Enzyme inhibition studies have been performed using EMMA in both capillaries and microchips [3]. The first report using EMMA to study enzyme inhibition by Saevels et al. investigated the inhibition of adenosine deaminase by erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) in a capillary using transient engagement EMMA [6]. The inhibitor, EHNA, was added to the running buffer, the enzyme solution and the substrate solution. The voltage was turned off when the zones of substrate and enzyme were overlapped to allow more product to form for detection, and it was then reapplied to separate the zones of enzyme, substrate, and product. Hadd et al. studied the inhibition of β -galactosidase by phenylethyl β -D-thiogalactoside, *p*-hydroxymercuribenzoic acid and D-lactose in a microchip device [7]. Substrate, enzyme and inhibitor were electrokinetically pumped from separate reservoirs and were mixed at a four-way intersection to develop the enzyme-catalyzed reaction at the downstream reaction channel. A method combining continuous engagement EMMA and transient engagement EMMA was developed by Whisnant et al. and used to study the inhibition of alkaline phosphatase in a capillary [8,9]. The capillary was first filled with an electrophoresis buffer that included the substrate. The inhibitor and enzyme were then injected electrokinetically into the capillary as separate zones. A product plateau was formed as the enzyme zone migrated through the capillary and interacted continuously with the substrate. The reaction product was fluorescent and detected by laser-induced fluorescence (LIF). A negative inhibition peak was created on the product plateau when the enzyme and inhibitor zones mixed and then separated electrophoretically. Several other groups have reported studies of enzyme inhibition based on variations of EMMA in capillaries and microchips [3].

In 1999, Hadd et al. reported an EMMA-based method in a microchip device, which could separate mixtures of inhibitors and detect them based on their inhibition of the target enzyme [10]. A mixture of four inhibitors of acetylcholinesterase, tetramethylammonium chloride, tetraethylammonium chloride, tacrine and edrophonium, was injected electrokinetically through the first intersection on the microchip as a short zone into a separation channel. Enzyme also flowed continuously through the first intersection into the separation channel. The enzyme-catalyzed reaction was initiated after electrophoretic separation of the inhibitors by using confluent mixing to add substrate at the second intersection to the enzyme and inhibitor stream. A final fluorescent product was generated from the enzyme-catalyzed reaction product by adding a derivatization reagent at the third intersection. The four inhibitors of acetylcholinesterase were detected as negative peaks due to reduced product formation. Despite the promise of this early study, no subsequent reports have appeared in the literature applying this approach or further developing it. In principle,

the EMMA method for studying enzyme inhibition developed by Whisnant et al. [8,9] could be used to separate mixtures of inhibitors and detect them based on their inhibition of the target enzyme; however, poor peak capacity limits this approach in practice. For example, the peak capacity based on studies of the reversible inhibition of alkaline phosphatase by theophylline is calculated to be 3 [9].

The goal of this study was to develop a simple, on-column CE method in a capillary for separating mixtures of inhibitors and detecting these molecules based on their inhibition of a target enzyme. To overcome the limited peak capacity of the EMMA method developed by Whisnant and coworkers [8,9], the enzyme was immobilized in the CE capillary before the detector by immobilizing enzyme-coated paramagnetic beads with a magnetic field [11]. The enzyme inhibitors were separated by CE before reaching the enzyme microreactor. The effects of several experimental variables on the assays, such as bead injection time and separation potential, were investigated. Five reversible inhibitors of alkaline phosphatase were separated, and their individual inhibition peaks were observed using this approach. This method was directly compared to the EMMA method reported by Whisnant et al. [8,9] using the same enzyme, substrate and mixture of inhibitors.

2 Materials and methods

2.1 Reagents

Alkaline phosphatase (EC 3.1.3.1 from calf intestine) and AttoPhos (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate) were obtained from Promega (Madison, WI). Sodium phosphate and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium vanadate was from Acros Organics (Pittsburgh, PA). Other chemicals were supplied by Sigma-Aldrich (St. Louis, MO). All solutions were prepared in ultrapure water ($> 18 \text{ M}\Omega\text{-cm}$) from a Modulab water purification system (United States Filter Corp.; Palm Desert, CA).

2.2 CE-LIF instrumentation

The CE-LIF instrument was constructed in house and is similar to previous instruments [8,9]. A schematic representation of the instrument is presented in Figure S1. The LIF detector is not shown in detail. The 457.9-nm line of an air-cooled argon ion laser (543R-AP-A01, Melles Griot; Carlsbad, CA) was used for excitation. The laser beam was focused onto the capillary by a CaF_2 plano convex lens ($f = 20.0 \text{ mm}$) (Thorlabs; Newton, NJ). The laser power at the capillary was 24.0 mW. The fluorescence was collected at 90° relative to the excitation beam by a $20\times$ microscope objective (0.5 NA; Melles Griot; Carlsbad, CA), and was filtered by a $560 \pm 10 \text{ nm}$ bandpass filter (53900, Oriel; Stratford, CT) and an $800 \mu\text{m}$ diameter pinhole (Oriel). The fluorescence was then detected by a PMT (HC120-01, Hamamatsu; Bridgewater, NJ) at a potential of 1000 V. The PMT output was filtered by a low-pass filter at 50 Hz, and the data were collected at 20 Hz by the data acquisition board (PCI-6229, National Instruments; Austin, TX). A LabVIEW program (Version 7.1, National Instruments) was written and used for data acquisition. The data were analyzed using OriginLab 7.5 (Northampton, MA).

A Spellman CZE1000R high-voltage power supply (Hauppauge, NY) was used to apply the electrophoretic potential. Fused-silica capillaries with a $50 \mu\text{m}$ i.d. and $220 \mu\text{m}$ o.d. from SGE (Austin, TX) were used. For all experiments, the capillaries were 60.0 cm total length and 45.0 cm to the detection window. The detection window was made by removing the polyimide coating using a window maker (MicroSolv Technology; Eatontown, NJ). Each new capillary was rinsed before use with 0.1 M NaOH, water, and then diethanolamine (DEA) buffer (50.00 mM, pH 9.50) using a manual syringe pump for 10 min, respectively.

All solutions used for CE were filtered through a 0.2- μ m membrane filter (Whatman; Hillsboro, OR).

2.3 Enzyme immobilization on magnetic beads

Superparamagnetic polystyrene beads (Dynabeads M-270 Epoxy) with a diameter of 2.8 μ m were purchased from Invitrogen Dynal (Oslo, Norway). The covalent attachment of alkaline phosphatase to the magnetic beads was performed using the protocol provided by the manufacturer. Briefly, a batch of magnetic beads (1.5 mg, 1.0×10^8 beads) was washed twice with 100 μ L of 100.0 mM sodium phosphate buffer at pH 7.40. In each washing step, the beads were separated from the wash buffer by fixing the bead suspension with a NdFeB magnet and removing the supernatant. The washed beads were then resuspended in 30 μ L of the sodium phosphate buffer (3.3×10^9 beads/mL). After mixing the 30- μ L suspension of washed beads and 30 μ L of 14 μ M alkaline phosphatase in the same phosphate buffer, 30 μ L of 3.000 M ammonium sulfate in the pH 7.40 phosphate buffer was added to enhance binding of alkaline phosphatase to the beads. The resultant mixture was incubated with slow tilt rotation on a rocking platform for 24 h at room temperature. After incubation, the beads were washed four times with 150 μ L aliquots of DEA buffer (50.00 mM, pH 9.50) to remove alkaline phosphatase molecules that were not covalently attached to the beads. Finally, the beads were resuspended in 200 μ L DEA buffer (5.0×10^8 beads/mL) and stored in the refrigerator at 4 $^{\circ}$ C until use. The coated bead suspensions were diluted to 1.7×10^8 beads/mL, and the suspension was homogenized with a vortex mixer before injection into the CE capillary. Different injections from the same batch of beads were used for all of the experiments reported in this paper.

2.4 Magnetic bead immobilization in the capillary

A copper holder was constructed to hold the magnets that fixed the magnetic beads inside the capillary and to control the temperature in this region of the capillary. This holder is illustrated in Fig. S1. The holder positioned the magnet(s) near the capillary surface, 27.0 cm from injection end of the capillary. Water from a thermostatted bath was circulated through the copper holder to control the temperature of a 5.0-cm section of the capillary, centered at the magnet. Permanent NdFeB magnets (B442 and D24) used in this work were purchased from K&J Magnetics (Jamison, PA). A B442 magnet (3700 Gauss) was secured in the holder with one pole perpendicular to the capillary bore. The distance from the center of the capillary bore to the face of the magnet closest to the capillary was 350 μ m. In a 2-magnet configuration, a pair of D24 magnets (3895 Gauss) was secured in the holder. The two magnets were arranged at $\pm 20^{\circ}$ relative to the longitudinal axis of the capillary bore with their north poles facing the capillary bore [12]. The distance from the inner edge of each magnet to the center of the capillary bore was 395 μ m. To create an enzyme microreactor with magnetic beads, the beads coated with alkaline phosphatase were electrokinetically injected into the capillary at 300 V/cm and then transported to the immobilization zone by electrophoresis (200 V/cm) in DEA buffer (50.00 mM, pH 9.50). After a series of experiments, the beads could be easily removed by rinsing the capillary with DEA buffer using a manual syringe pump.

An inverted microscope (Nikon ECLIPSE TE 300) was used with a 10 $\times$ objective to image fixed magnetic beads inside the capillary. Images for the 1-magnet configuration were captured by a CCD camera and imaging software (WinView Software Version 32) from Princeton Instruments (Trenton, NJ). A digital camera (Sony DSC-W55/P; Sony Electronics, Inc.) was used with the 2-magnet configuration to record images of bead plugs observed through the eyepiece diopter of the microscope. The bead plug lengths were measured using the inner diameter of the capillary (50 μ m) as a reference.

2.5 Enzyme assays

For heterogeneous enzyme assays in a capillary with fixed magnetic beads, the running buffer was 0.100 mM AttoPhos in 50.00 mM DEA at pH 9.50. AttoPhos is a fluorogenic substrate for alkaline phosphatase. All inhibitor solutions were prepared in the running buffer. Inhibitors were electrokinetically injected at 200 V/cm for 3.0 s. The applied electric field for carrying out the enzyme assays was 200 V/cm unless otherwise noted. The electrode and the capillary inlet were dipped in DEA buffer before and after each injection of inhibitor to prevent cross contamination of the running buffer and inhibitor solution. The capillary was thermostatted at 25.0 °C. For heterogeneous enzyme inhibition assays, peak capacity (n_c) was calculated using Equation 1:

$$n_c = 0.5887 \frac{t}{w_{1/2}} \quad (1)$$

where t is the migration time of an inhibitor, and $w_{1/2}$ is the full width at half maximum of an inhibition peak [13].

For homogeneous CE enzyme inhibition assays, the basic experimental procedures were the same as those described previously [9]. The capillary was filled with the same running buffer used for the heterogeneous enzyme inhibition assays, including substrate. A zone of an alkaline phosphatase inhibitor, theophylline, was first injected for 3.0 s at 12.0 kV into the capillary. A potential of 12.0 kV was then applied for 30.0 s (40.0 s when a mixture of inhibitors was injected). Next, a plug of 51 pM alkaline phosphatase was injected for 3.0 s at 12.0 kV. Finally, a separation potential of 12.0 kV (200 V/cm) was applied. The enzyme concentration used for homogeneous assays was selected so that the activity of the enzyme zone was approximately the same as that for a plug of magnetic beads with immobilized enzyme. Zones of enzyme in solution or beads with immobilized enzyme were injected with AttoPhos in DEA buffer, and the resulting fluorescent product signals were compared. The inhibitor solutions contained the inhibitor as well as 0.100 mM AttoPhos in 50.00 mM DEA at pH 9.50. The electrode and the capillary inlet were dipped in DEA buffer before and after each injection to prevent cross contamination of the running buffer, enzyme solution and inhibitor solution. The thermostating system for the homogeneous assays was constructed as described previously [9]. To thermostat the capillary, Teflon tubing was used to enclose the capillary from the injection end to the detection window, and N₂ (25.0 °C, 8 psi) flowed through the Teflon tubing around the capillary. The temperature of the N₂ was controlled by passing it through a coil of tubing in a temperature-controlled water bath before it passed over the capillary to control the capillary temperature.

3 Results and discussion

3.1 Enzyme microreactors for enzyme inhibition assays

The goal for this work was to expand the ability of CE enzyme inhibition assays to separate and detect mixtures of inhibitors by increasing the peak capacity compared to our previous work [8,9]. The approach presented here is to immobilize the enzyme of interest inside the capillary before the detector. The running buffer contained a fluorogenic substrate for the target enzyme. In this work, the fluorogenic substrate was AttoPhos, and the enzyme studied was alkaline phosphatase. Inhibitor mixtures will separate by CE before reaching the enzyme microreactor, and each inhibitor zone will produce a negative inhibition peak (reduced fluorescent product formation) as it migrates through the enzyme microreactor and inhibits the enzyme. Chetwyn and Susan Lunte used a related approach to separate and detect mixtures of acetylcholinesterase inhibitors based on an enzyme-modified electrode

placed at the end of a CE capillary [14]. In the work presented here, the fluorescent reaction product is detected by LIF downstream from the enzyme microreactor.

The enzyme microreactors were constructed using rare earth magnets to fix enzyme-coated magnetic beads inside the capillary before the detection window [11]. Two different magnet configurations were used in this work, and for both configurations the magnets were placed at the same distance from the injection end of the capillary. In the 1-magnet configuration, a single magnet was placed with its pole facing the capillary bore. In the 2-magnet configuration, two identical magnets were positioned at $\pm 20^\circ$ relative to the long axis of the capillary with their north poles facing the capillary [12]. In both configurations, the magnets were able to hold magnetic beads in place inside capillary at the electrophoretic field strengths used in this study.

The use of magnetic immobilization of enzyme-labeled beads greatly simplified the development and optimization of this approach to CE-based enzyme inhibition assays. Enzyme microreactors could be constructed simply by electrokinetically injecting enzyme-coated magnetic beads into a capillary and then transporting the beads to the immobilizing magnet by electrophoresis in DEA buffer. No modification of the inner capillary surface was required. A single injection of beads was routinely used for multiple injections throughout the day with no apparent loss of enzyme activity. Although no attempt was made to determine the maximum number of consecutive experiments that could be performed from a single bead plug (1 bead injection), up to 19 consecutive experiments were performed for this work using a single bead plug, and more than 10 consecutive experiments were performed routinely using a single bead plug. A new microreactor could be created at any time by injection of a new bead plug from the same batch of enzyme-coated beads.

3.2 Optimization of the enzyme assay

The temperature at the enzyme microreactor and the concentration of substrate in the running buffer were optimized before beginning enzyme inhibition studies. The pH value used for these studies (pH 9.50), was based on optimization for a previous study using EMMA with alkaline phosphatase [8]. This pH value also falls within the pH range listed by the manufacturer for this alkaline phosphatase substrate (pH 9.0–10.3). A 5.0 cm section of the capillary centered at the magnet was temperature controlled by circulating water from a thermostatted bath through the copper holder. The enzyme activity was nearly constant from 25–45 °C, using the 1-magnet configuration for immobilizing alkaline phosphatase-coated magnetic beads (Fig. S2). A temperature of 25 °C was used for subsequent experiments. Although the temperature of the capillary was controlled externally at 25 °C, it is important to note that the temperature in the bore of the capillary will be higher than this due to Joule heating [15]. The electrophoretic current for this experiment was 3.9 μ A.

The substrate concentration was optimized by measuring the fluorescence signal when buffers with AttoPhos concentrations ranging from 0.005 to 0.500 mM were allowed to electrophorese through the capillary at 200 V/cm. The fluorescence signal due to product formation at each AttoPhos concentration was recorded when it became stable. The value of K_m was determined to be 0.026 mM by fitting the experimental data in the resulting plot of fluorescence vs. substrate concentration (Figure S3) nonlinearly to the Michaelis–Menten equation. An AttoPhos concentration of 0.100 mM was used for subsequent experiments.

3.3 Inhibition assays

An electropherogram of an enzyme inhibition assay for sodium arsenate, a reversible competitive inhibitor of alkaline phosphatase, [16] is presented in Fig. 1. The enzyme microreactor was constructed using a single magnet. The capillary first was filled with a

0.100 mM AttoPhos solution to obtain a constant fluorescence signal from the enzyme-catalyzed reaction product (Fig. S4). Then, 0.125 mM sodium arsenate was injected for 3.0 s at 12.0 kV. Finally, a separation potential of 12.0 kV was applied. At 11.2 min, a decrease in product formation was observed due to the inhibitor zone passing through the plug of enzyme-coated magnetic particles. After the inhibitor zone migrated past the plug of beads, the product fluorescence returned to its original level, and the enzyme activity was restored, demonstrating that sodium arsenate is a reversible inhibitor of alkaline phosphatase.

The electropherogram also contains several artifact peaks. The two unresolved peaks at ~2.3 min resulted from product formation during zero-potential incubation, which occurred during injection. The first peak near 2.3 min was generated when the high voltage was turned off just before the inhibitor was injected. The second peak was generated when the high voltage was turned off just after the inhibitor injection and before application of the separation potential. There are also two reproducible artifact peaks (a positive peak and a negative peak) at ~4.9 min, and the cause of these two peaks is unclear. Electropherograms for the same experiments carried out in an enzyme microreactor constructed with two magnets (Fig. S5) are similar in appearance to those obtained using a single magnet.

The inhibition of alkaline phosphatase by theophylline was also studied with both enzyme microreactors. Theophylline is a reversible, noncompetitive inhibitor of alkaline phosphatase [17]. An electropherogram for a 3.0-s injection of 1.00 mM theophylline is shown in Fig. 2. The negative peak at 8.4 min is due to theophylline inhibition. The artifact peaks are almost identical to those observed for arsenate.

3.4 Effects of electric field strength and bead injection time on peak capacity

The effects of the electric field strength and bead injection time on the peak capacity (n_c) for separation of reversible inhibitors were investigated to optimize the ability of the enzyme inhibition assays to resolve mixtures of inhibitors. Inhibition assays of alkaline phosphatase by arsenate were performed at different electric field strengths using 1 and 2-magnet enzyme microreactors. A plot of the peak capacity vs. field strength is presented in Fig. 3. For both types of enzyme microreactors, the peak capacity decreased with increased electric field strength. Taking into consideration both the peak capacity and the total analysis time, an electric field strength of 200 V/cm was chosen for later experiments ($n_c \approx 20$).

The results of inhibition assays (arsenate, alkaline phosphatase) with different bead injection times using both microreactor configurations are presented in Fig. 4. Increasing the bead injection time increased the total number of beads injected and the bead plug length (Fig. S6). The image of a bead plug for an injection time of 15 s for the 2-magnet configuration is shown in Fig. S6. The density and total number of magnetic beads in a plug cannot be readily determined from 2-dimensional images of the 3-dimensional bead plug.

Figure 4 shows a plot of peak capacity as the bead injection time was increased from 3.0 s to 90 s. For both magnet configurations, the peak capacity decreased sharply when the bead injection time was increased from 15 to 30 s. There appears to be a slight increasing trend in peak capacity as the injection time was increased from 30 to 90 s. For inhibition assays by arsenate, the migration time of the inhibitor did not change much as the bead injection time was increased, and the changes in peak capacity vs. bead injection time resulted mainly from variation of the inhibition peak width. The inhibition peak width for inhibition assays using enzyme microreactors with magnetic beads is a complex and interesting phenomenon. The inhibition peak width ultimately reflects the decrease in production of enzyme-catalyzed reaction product over time and does not necessarily represent the physical width of the inhibitor zone. In this study, our goal was to improve the peak capacity for inhibitor separations, so we focused on the peak capacity for optimizing these assays.

The increase in bead injection time also affected the S/N for the inhibition peaks for both enzyme microreactor types (Figure S7a). The fluorescence signal due to enzyme-catalyzed product formation increased as the number of beads immobilized increased due to increased bead injection time. With longer magnetic bead injection time, the inhibition peak height increased; however, the baseline noise also increased. The S/N of the inhibition peak only changed slightly for the bead injection times investigated here. A magnetic bead injection time of 15.0 s was used for later experiments to obtain an enhanced peak capacity ($n_c \approx 20$) and S/N.

Inhibition of alkaline phosphatase by theophylline with different magnetic bead injection times also was studied for both magnet configurations. These results are shown in Fig. S7 (b and c). The peak capacity for theophylline was less impacted by the bead injection time compared to arsenate, ranging only from 17–25 for all bead injection times and both enzyme microreactor configurations. The S/N increased for theophylline by approximately a factor of two for both magnet configurations when the bead injection time was increased from 3.0 to 15 s. At longer bead injection times, the S/N ratio was relatively stable as was observed for arsenate inhibition.

3.5 Inhibition peak shape and inhibitor concentration

To investigate the effect of inhibitor concentration on inhibition peak shape, different concentrations of sodium arsenate were injected for 3.0 s using both 1 and 2-magnet enzyme microreactors. Figure 5 shows electropherograms for inhibition assays at different arsenate concentrations using a 1-magnet enzyme microreactor. The electropherograms are artificially offset vertically to more clearly show the inhibition peaks, but the fluorescence baselines were the same in the original electropherograms. The inhibition peaks became deeper and broader as the inhibitor concentration increased. Plots of the inhibition peak width at half maximum ($w_{1/2}$) vs. the inhibitor concentration (Fig. 6) for both enzyme microreactor types show that the peak widths increased by more than a factor of 20 in both cases. The positive peaks at ~8.9 min are fluorescein, which was used as a control since it is not expected to interact with the immobilized enzyme and magnetic beads. The fluorescein peak widths were constant at ~0.04 min at inhibitor concentrations from 0.0125 mM to 1.25 mM, and decreased to 0.01 min at an inhibitor concentration of 12.5 mM. These relatively sharp fluorescein peaks indicate that physical obstruction of the capillary by the bead plug is not contributing significantly to the band broadening observed for the inhibition peaks. The broadening of the inhibition peaks likely results from interaction between inhibitor and enzyme when the inhibitor migrates through the bead plug. The artifact peaks at ~5.0 min were similar for the assays at lower arsenate concentrations, but became deeper and wider at arsenate concentration of 12.5 mM. Presumably the changes to the fluorescein peak and the artifact peaks for the 12.5 mM arsenate sample were caused by the increased ionic strength of the sample due to arsenate.

The effect of inhibitor concentration on inhibition peak shape was also studied for theophylline using both enzyme microreactors. The electropherograms for these experiments with a 1-magnet microreactor are presented in Fig. S8. Theophylline inhibition was also studied using the EMMA method previously reported by Whisnant et al. [8,9]. The electropherograms for the EMMA studies are presented in Fig. S9. Figure 7 shows plots of the inhibition peak widths at half maximum ($w_{1/2}$) vs. theophylline concentration for all three assays. For all three assays the peak width increases by a factor of 6 as the theophylline concentration increases from 0.500 to 10.0 mM. At all inhibitor concentrations, the inhibition peak width for heterogeneous assays is larger than that for the homogeneous assay (1.5–2×). These results are consistent with the hypothesis that the immobilization of the enzyme on magnetic beads is not contributing significantly to the band broadening observed here. Because the inhibitor is present at such high concentrations relative to the

enzyme in both heterogeneous and homogeneous assays, it is unlikely that the overall inhibitor zone is physically broadened by interaction with the enzyme. The exact nature of the broadening of the inhibition peaks and its dependence on inhibitor concentration remains unclear. It is important to note that separation efficiency and peak capacity values are compromised when the inhibitor concentration is high.

3.6 Separation and detection of enzyme inhibitor mixtures

Figure 8 presents a separation of a mixture of 5 inhibitors of alkaline phosphatase using a 1-magnet enzyme microreactor. The inhibitors were electrophoretically separated in the capillary before reaching the enzyme microreactor, and the inhibitor zones produced negative peaks due to reduced product formation as they passed through the enzyme microreactor. All 5 analytes, tryptophan, theophylline, vanadate, arsenate and tungstate, are reversible inhibitors of alkaline phosphatase [16–19]. The same experiment was performed with a 2-magnet enzyme microreactor (Fig. S10). All 5 inhibitors as well as fluorescein (peak 6) were resolved from each other and from artifact peaks in both experiments. Inhibition assays of the five species also were carried out individually so that the inhibition peaks could be identified according to their migration times (data not shown).

The same separation was attempted using the homogeneous method combining continuous EMMA and transient engagement EMMA [8,9]. An electropherogram for this experiment is presented in Fig. 9. Only two peaks are apparent in the product plateau in the electropherogram from 6.2 to 7.0 min. Based on the migration order of the inhibitors from the experiments in Figs. 8 and S10 as well as experiments using the homogeneous EMMA method in which the samples were spiked with arsenate or tryptophan, these peaks were identified as theophylline (6.5 min) and vanadate (6.8 min). Tryptophan migrated fastest among the five inhibitors, and the enzyme zone probably did not overtake the tryptophan zone before it reached the LIF detector. Arsenate and tungstate have the slowest migration times and were overtaken first by the enzyme zone. This will result in inhibition of the enzyme, but the inhibitors were probably unresolved, and the inhibition was observed at the far edge of the plateau (~6.9 min). This will not produce distinct inhibition peaks, but will make the product plateau appear narrower. These results show that the homogeneous EMMA method has very limited peak capacity due to the constraints of the reaction product plateau width, and in this case it cannot separate and detect all five inhibitors of alkaline phosphatase.

4 Concluding Remarks

A simple CE method has been developed that is capable of separating mixtures of enzyme inhibitors and detecting the inhibitors based on their inhibition of a target enzyme using an on-column enzyme microreactor. This approach resulted in greatly increased peak capacity ($n_c \approx 20$) compared to previous work, which combined continuous engagement EMMA and transient engagement EMMA for enzyme inhibition studies [8,9]. This new method was able to resolve a mixture of 5 inhibitors of alkaline phosphatase, while the same sample only produced 2 distinct inhibition peaks using the previous method. The enzyme microreactor was created by electrophoretically loading magnetic beads coated with the target enzyme into a capillary and fixing the beads in the capillary using inexpensive rare earth magnets. This approach avoids the complications of carrying out reactions inside the capillary to immobilize the enzyme. The immobilized enzyme can be replaced by simply rinsing the capillary at high pressure and injecting a new batch of enzyme-coated magnetic beads. Enzyme microreactors based on one magnet and two magnets were used in this work. There was no significant advantage obtained using the more complicated 2-magnet microreactor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Rattikan Chantiwas for her technical suggestions. Ryan Picou and Rachel Henken are acknowledged for their comments on the manuscript. This work was supported by the National Institutes of Health Grant GM066984. The authors would like to dedicate this manuscript to Angela R. Whisnant (June 25, 1974 – May 28, 2009).

Abbreviations

DEA	diethanolamine
EMMA	electrophoretically mediated microanalysis

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Figure 1.

Electropherogram of an enzyme inhibition assay (inhibition of alkaline phosphatase by arsenate) using a 1-magnet enzyme microreactor. A single B442 magnet (3700 Gauss) was used to immobilize magnetic beads inside the capillary. Magnetic beads (1.7×10^8 beads/mL) coated with alkaline phosphatase in DEA buffer (50.00 mM, pH 9.50) were injected for 30.0 s at 18.0 kV (300 V/cm). Next, a potential of 12.0 kV (200 V/cm) was applied to the capillary filled with DEA buffer to transport the beads to the immobilizing magnet. The running buffer (50.00 mM DEA, pH 9.50) contained 0.100 mM AttoPhos. The inhibitor, 0.125 mM sodium arsenate, was injected for 3.0 s at 12.0 kV. Finally, a separation potential of 12.0 kV was applied.

Figure 2.

Electropherogram of an enzyme inhibition assay (inhibition of alkaline phosphatase by theophylline) using an enzyme microreactor constructed with a single magnet. The inhibitor, 1.00 mM theophylline, was injected for 3.0 s at 12.0 kV, and then the separation potential of 12.0 kV was applied. Magnetic beads (1.7×10^8 beads/mL) were injected for 15.0 s at 18.0 kV. All other experimental conditions are the same as in Fig. 1.

Figure 3.

Effect of electric field strength on peak capacity (n_c) for assays of alkaline phosphatase inhibition by arsenate. Experimental conditions are the same as in Fig. 1, except that the electric field strength was varied as indicated. The electric field strength for each arsenate injection matched the separation field strength for that experiment.

Figure 4. Effect of bead injection time on peak capacity (n_c) for assays of alkaline phosphatase inhibition by arsenate. Experimental conditions are the same as in Fig. 1, except that the bead injection time was varied as indicated.

Figure 5.

Electropherograms of enzyme inhibition assays at different arsenate concentrations using a 1-magnet enzyme microreactor. Magnetic beads (1.7×10^8 beads/mL) were injected for 15.0 s at 18.0 kV. A mixture of 0.5 μ M fluorescein and the inhibitor, arsenate, was injected for 3.0 s at 12.0 kV, and then the separation potential of 12.0 kV was applied. All other experimental conditions are the same as in Fig. 1. The electropherograms were artificially offset along the vertical axis so the inhibition peaks could be viewed without overlap.

Figure 6.

Plots of inhibition peak width at half maximum ($w_{1/2}$) vs. inhibitor concentration for assays of alkaline phosphatase inhibition by arsenate. Experimental conditions are the same as in Fig. 1, except that magnetic beads (1.7×10^8 beads/mL) were injected for 15.0 s at 18.0 kV.

Figure 7.

Plot of inhibition peak width at half maximum ($w_{1/2}$) vs. inhibitor concentration for assays of alkaline phosphatase inhibition by theophylline. Experimental conditions for heterogeneous assays using enzyme microreactors are the same as in Fig. 1, except that magnetic beads (1.7×10^8 beads/mL) were injected for 15.0 s at 18.0 kV. For homogeneous assays based on EMMA, a zone of theophylline (0.500, 1.00, 5.00 and 10.0 mM) was injected for 3.0 s at 12.0 kV into a capillary filled with 0.100 mM AttoPhos in 50.00 mM DEA at pH 9.50. Next, a potential of 12.0 kV was applied for 30.0 s. Then, a plug of 51 pM alkaline phosphatase was injected into the capillary for 3.0 s at 12.0 kV. Finally, a separation potential of 12.0 kV was applied.

Figure 8.

Separation of alkaline phosphatase inhibitors using a 1-magnet enzyme microreactor. Peak identities are: 1. tryptophan; 2. theophylline; 3. sodium vanadate; 4. sodium arsenate; 5. sodium tungstate; 6. fluorescein. Magnetic beads (1.7×10^8 beads/mL) were injected for 15.0 s at 18.0 kV. A mixture of five inhibitors was injected for 3.0 s at 12.0 kV. Other experimental conditions are the same as in Fig. 1.

Figure 9.

Separation of alkaline phosphatase inhibitors using homogeneous EMMA. Peak identities are: 1. theophylline; 2. sodium vanadate. A mixture of five alkaline phosphatase inhibitors was injected for 3.0 s at 12.0 kV into a capillary filled with 0.100 mM AttoPhos (50.00 mM DEA, pH 9.50). Next, a potential of 12.0 kV was applied for 40.0 s. Then, a plug of 51.0 pM alkaline phosphatase was injected into the capillary for 3.0 s at 12.0 kV. Finally, a separation potential of 12.0 kV was applied.