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Enzyme Inhibitor Screening by Capillary Electrophoresis with an on-Column Immobilized Enzyme Microreactor Created by an Ionic Binding Technique

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A novel strategy for screening the enzyme inhibitors from the complex mixtures by capillary electrophoresis with an on-column immobilized enzyme microreactor created by an ionic binding technique is reported. The enzyme microreactor was prepared in two steps: First, the capillary wall was dynamically coated with a polycationic electrolyte hexadimethrine bromide (HDB) by simply flushing the column using the HDB solution. Subsequently, a plug of the enzyme solution was injected and incubated for 5 min to permit the enzyme molecules to immobilize on the positively charged coating via ionic binding. To demonstrate this strategy, angiotensin-converting enzyme (ACE) was employed as a model for the enzyme immobilization, inhibition study, and inhibitor screening. It has been proved that such a prepared immobilized ACE microreactor displays a high enough activity and stability. Furthermore, the immobilized enzyme microreactor could be easily renewed. The inhibition study or inhibitor screening was accomplished through the following procedure: (i) the substrate solution was injected and incubated within the microreactor for a short time span; (ii) subsequently, the voltage was applied to separate the product of the enzyme reaction from the unreacted substrate based on their different mobilities, the peak area of the product representing the enzyme activity; (iii) a certain amount of enzyme inhibitor or candidate compound was spiked into the substrate solution to assay the reduction of the immobilized enzyme activity. Thus, the inhibitors can be easily identified if the reduced peak area of the product is observed in electropherograms. Because the injection volume of the capillary was only 9.8 nL and the enzyme could be reusable, the assay cost could be dramatically reduced. The screening of a small compound library containing natural extracts and commercially available inhibitors was performed. The present approach has proved to be simple, rapid, and robust.

Enzymes are an important class of targets for developing new drugs against various diseases, such as cancer, AIDS, diabetes, and hypertension.[1] In the early stage of the drug discovery associated with the enzyme inhibitors, the major task is to identify the inhibitors for a given enzyme target from a library of compounds.[2] Therefore, the development of rapid, low-cost, and effective techniques for enzyme inhibitor screening has received much attention.[2] Thus far, various methods have been developed for inhibitor screening. The fluorescence-based techniques,[3] such as fluorescence polarization and time-resolved fluorescence, have become the commercially available techniques for enzyme inhibitor screening; however, assays incorporating the fluorometric methods sometimes suffer from interference caused by the autofluorescence or the fluorescence quenching of the probe.[4] Alternatively, various immobilized enzyme reactors have been developed for inhibitor screening. When compared with the free-enzyme-based screening methods, the advantages of using the immobilized enzyme for inhibitor screening are (i) the enzyme can be reused so that the screening cost can be minimized; (iii) the enzyme stability can be promoted; (iii) a mixture of compounds can be screened. Several chromatography-based immobilized enzyme reactors have been developed by Wainer and co-workers for enzyme study and inhibitor screening.[5] In their methods, the enzyme was immobilized on the silica parking materials, which was subsequently packed into the liquid chromatography (LC) column as the enzyme reactor for enzyme study and inhibition screening. Moreover, the enzyme can also be immobilized on the monolithic matrix (EDA CIM disk) as the microimmobilized-enzyme reactor for rapid inhibitor screening.[6] Because mass spectrometry (MS) can provide structure information and permit a label-free enzyme assay, the coupling of an immobilized enzyme reactor with MS has been established as a powerful tool for enzyme inhibitor screening. Frontal affinity chromatography (FAC) coupled with electrospray ionization (ESI)

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MS[7] or matrix-assisted laser desorption ionization (MALDI) MS/MS[8] represents the high-throughput approach for inhibitor screening of a mixture of compounds using immobilized enzyme stationary phases. It not only gives the binding constant between a target enzyme and tested compounds but also provides the inhibition kinetic parameters.[7] Brennan and co-workers prepared enzyme-doped monolithic capillary−enzyme-reactor columns by sol−gel chemistry and directly coupled to tandem MS for screening of enzyme inhibitors.[9] Surface plasmon resonance (SPR) combined with MS has been developed by Borch and Roepstorff for enzyme inhibitor screening.[10] In this approach, the enzyme was immobilized on the sensor chip, and the interactions between the enzyme and the compounds in the mixture was detected by SPR. Concurrently, the activity of the immobilized enzyme was monitored by the coupled MS. Finally, the inhibitor was recovered from the immobilized enzyme and characterized by MS. In addition, Leary and co-workers developed a method using MS combined with immobilized enzyme for high-throughput screening of inhibitors from complex mixtures.[11] Palm and Novotny evaluated PNGase F activity by using an enzyme reactor coupled with off-line MALDI-MS.[12] On-line coupling of LC to a continuous-flow enzyme reactor assayed by MS for screening of enzyme inhibitors in complex samples has been reported by de Boer et al.[13]

Capillary electrophoresis (CE) is not only a separation tool with high separation performance but also a versatile platform for enzyme study and drug discovery. Among the various CE techniques, electrophoretically mediated microanalysis (EMMA) technique is a useful miniature tool for the study of enzymes and for inhibitor screening.[14,15] The screening throughput can be dramatically improved by using multiplex capillary electrophoresis with UV absorption detection.[16] Alternatively, immobilized enzyme microreactors fabricated on capillaries and microfluidic chips represent another promising miniature approach for enzyme study, peptide mapping in proteomics, and diagnostics. Thus far, several methods have been reported for the preparation of on-column enzyme microreactors for capillary electrophoresis (CE), capillary liquid chromatography, and microfluidic chips. Lee and co-workers developed a micro trypsin reactor, which was constructed by fixing a polyvinylidene fluoride membrane with the absorbed trypsin inside the capillary fitting for protein digestion.[17] Sakai-Kato et al. fabricated a trypsin-encapsulated enzyme reactor in the head of the capillary via sol−gel chemistry.[18] Yang and co-workers prepared an encapsulated trypsin enzyme reactor with titania- and alumina-based sol−gel chemistry on the channel of microfluidics.[19] They also reported a method for preparing a trypsin reactor on poly(dimethylsiloxane)-based microfluidics using an ionic binding manner.[20] Peterson et al. developed methods for immobilizing trypsin on the porous polymer monoliths.[21] Amankwa and Kuhr directly immobilized the trypsin onto the inner surface of the capillary via biotin−avidin−biotin technology.[22] Brennan and co-workers developed sol−gel-derived immobilized enzyme microarrays for nanovolumn inhibition assays.[23] Mao et al. immobilized the streptavidin-conjugated enzyme to the surface of the microchannels or capillary tubes coated with biotinylated phospholipid bilayers.[24] Zhan et al. reported a method for fabricating poly(ethylene glycol) hydrogen micropatches containing enzymes within a channel.[25]

In the present work, we describe a new strategy for the preparation of the on-column immobilized enzyme microreactor for rapid screening of enzyme inhibitors via CE. The preparation of the enzyme microreactor is based on the ionic binding immobilization approach, which can be easily and automatically accomplished with the instrument. To demonstrate the feasibility of our strategy, angiotensin-converting enzyme (ACE) was employed as an experimental model. The primary function of ACE is to regulate arterial pressure by converting angiotensin I to vasoconstrictor angiotensin II. Therefore, ACE is a major target for developing drugs used to treat hypertension and heart failure, improve survival following a heart attack, and slow the progression of kidney disease in people with diabetes.[26] The performance and inhibition kinetics of the immobilized ACE were studied in our experiments, and the repeatability of the preparation of the enzyme microreactor was also evaluated. Finally, a small library of compounds containing 3 commercial ACE inhibitors and 31 natural extracts were tested.

EXPERIMENTAL SECTION

Materials and Reagents. Angiotensin-converting enzyme (ACE) from rabbit lung, hippuryl-His-Leu (HHL), and hexa-

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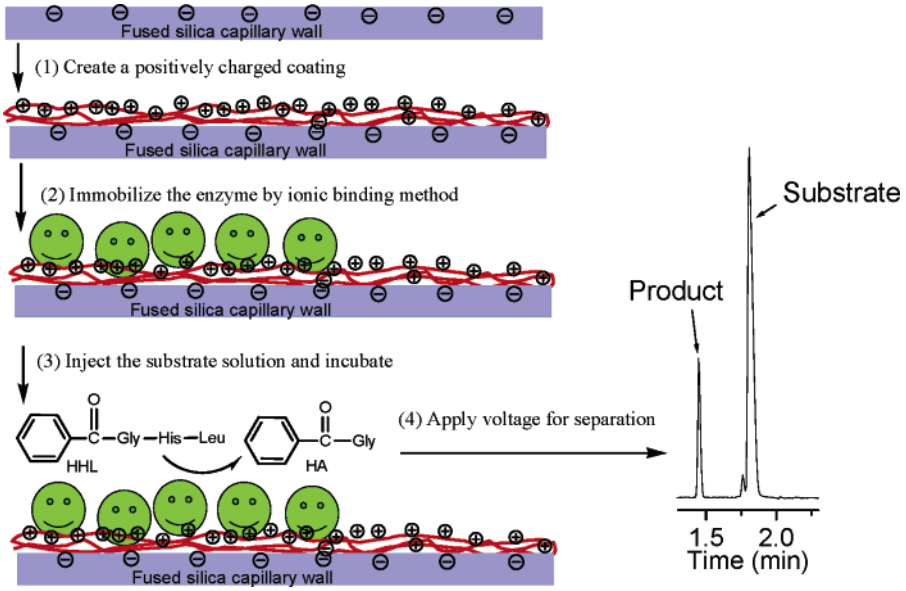

Figure 1. Schematic representation of the on-column immobilized enzyme microreactor prepared by the ionic binding method.

dimethrine bromide (HDB) were purchased from Sigma-Aldrich (Steinheim, Germany). Cilazaprilat was donated by Roche (Basel, Switzerland). ACE solution was prepared with 10 mM borate−phosphate buffer (pH 8.0). Solutions of HHL, captopril, cilazaprilat, and other samples were prepared with 10 mM borate−phosphate buffer (pH 8.0) containing 50 mM NaCl, except if indicated otherwise. All solutions were prepared with deionized water and filtered through a 0.45-μm nylon filter.

Instrumentation. All separations were performed on an Agilent CE system (Waldbronn, Germany) equipped with a DAD detector. A fused-silica capillary with a dimensions of 50-μm i.d. (370-μm o.d.) × 34.5 cm (26 cm to the detection window) were purchased from Yongnian Optical Fiber Co. (Hebei, China). The capillary and carousel were thermostated at 37 °C. All analytes were detected at a UV wavelength of 230 nm.

Preparation of on-Column Immobilized Enzyme Microreactor. A two-step protocol for the preparation of the on-column immobilized enzyme microreactor is shown in Figure 1. First, a capillary was pretreated by 0.1 M NaOH solution for 30 min, followed by rinsing with deionized water, HDB solution (0.1%, w/v), and deionzed water for 5 min, respectively, to create a positively charged coating on the capillary wall. Subsequently, the enzyme solution was charged into the column by pressure at 50 mbar for 10 s, giving a 1.5-cm-long plug of the enzyme solution. The enzyme solution was left in place for 5 min allow the enzyme to immobilize on the capillary wall via ionic binding. The unimmobilized enzyme was thereafter washed out off the column by flushing with the running buffer for 3 min from the outlet end of the capillary. Then the prepared immobilized enzyme microreactor was ready for inhibitor screening.

Immobilized Enzyme Activity Assay and Inhibition Study. The capillary was filled with the running buffer (pH 8.0), consisting of 20 mM sodium borate and 0.01% HDB. Substrate solutions, in the presence or absence of inhibitor, were injected into the enzyme reactor by pressure at 20 mbar for 4 s. Between two injections, the inlet end of the capillary and electrodes were cleaned by dipping them into a vial with water to prevent the enzyme and substrate solutions from contaminating each other. After incubation for a short time, −15 kV was applied to separate the product from the unreacted substrate. Because the positively charged coating reversed the EOF, the voltage polarity also was reversed. The enzyme activity was then assayed by measuring the peak area of the product.

Regeneration of the Enzyme Microreactor. Once the function of the immobilized enzyme reactor becomes poor, it can be renewed conveniently. The capillary column is flushed with 1 M NaCl, 0.1 M HCl, and 0.1 M NaOH, consecutively, to elute the immobilized enzyme (desorption) and then repeats the preparation protocol for enzyme immobilization.

RESULTS AND DISCUSSION

Preparation of the Enzyme Microreactor. As shown in Figure 1, the immobilized enzyme microreactor can be simply prepared using the two-step protocol. The advantages of the present method for preparing the on-column immobilized enzyme microreactor are (i) the protocol is very simple and can be automatically carried out by the instrument; (ii) immobilization is reversible; therefore, the enzyme microreactor can be renewed very easily; (iii) the condition for enzyme immobilization is very mild so that the enzyme activity can be maximally preserved due to the small change in the enzyme structure and activity domain;[27] and (iv) a very small amount of the immobilized enzyme allows detection of low levels of inhibitors; this is useful when working with nature extracts.[15] Furthermore, the major advantage of the immobilized enzyme reactors is the potential to identify inhibitors from the compound mixture.[5] It was proved that the studied enzyme cannot be adsorbed on the bare inner surface of the fused-silica capillary, which inherently possesses a negative charge due to the ionization of the silanol groups. Therefore, it is necessary to modify the capillary wall by a water-soluble polyelectrolyte possessing strong cation groups as the ion exchanger. The polycationic electrolyte HDB has been used to dynamically modify the capillary wall to create a positively charged coating.[28,29] Such a modification approach can be accomplished simply by flushing

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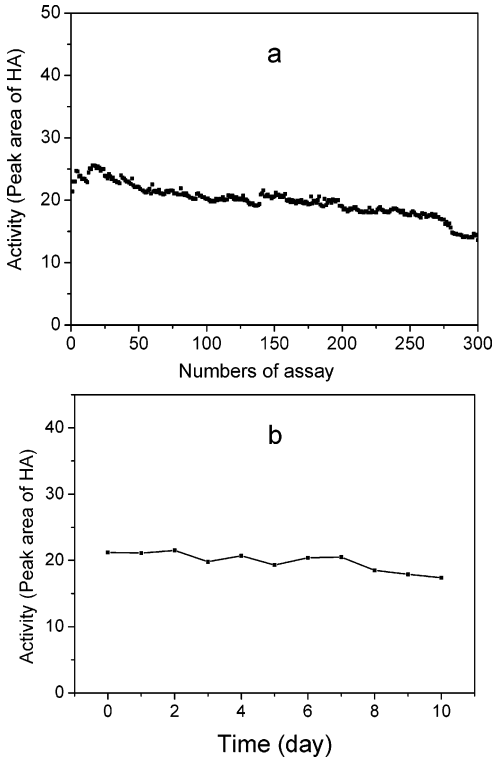

Figure 2. Stability and lifetime of the on-column immobilized enzyme microreactor. (a) Repeatability of enzyme activity for 300 consecutive injections and (b) lifetime of the immobilized enzyme reactor. Conditions: fused-silica capillary, 50-μm i.d. × 34.5 cm (26 cm to detection window); running buffer, 20 mM borate−phosphate buffer (pH 8.0) containing 0.01% HDB; column temperature, 37 °C; sample injection, 20 mbar for 4 s; detection wavelength, 230 nm; incubation time, 1 min.

the capillary with a solution of HDB. The resulting HDB coating is stable enough to withstand the flush with the running buffer. Furthermore, the positively charged coating can produce a constant reversed electroosmotic flow (EOF), which can accelerate the migration velocity of the negatively charged analytes; therefore, very fast separation can be achieved.[28,29] Most importantly, the HDB molecules possess highly dense quaternary ammonium groups, which can act as the strong anionic exchanger for enzyme immobilization. The stability of the immobilized enzyme was investigated by assaying the enzyme activity over 24 h. As shown in Figure 2a, the activity of the immobilized enzyme in terms of the peak area of the produced hippuric acid (HA) (the reaction is shown in Figure 1) decreased slowly with an increase in the assay times, and finally, the immobilized enzyme reactor lost its function by 40% after 300 assays. This is likely due to the immobilized enzyme molecules that are gradually leached from the capillary wall. The lifetime of the immobilized enzyme reactor was also tested, and the result is shown in Figure 2b. In this experiment, the activity of the immobilized enzyme was assayed three times

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Table 1. Repeatability of Enzyme Activity of the Immobilized EnzymeMicroreactor[a]

parameters	RSD %
Intraday Repeatability ($n = 9$)	
corrected peak area of HA	2.4
migration time of HA	0.2
Interday Repeatability ($n = 9$)	
corrected peak area of HA	2.9
migration time of HA	0.9
Batch to Batch ($n = 7$)	
corrected peak area of HA	5.2
migration time of HA	1.4

[a] Conditions: fused-silica capillary, 50-μm i.d. × 34.5 cm (26 cm to detection window); running buffer, 20 mM borate−phosphate buffer (pH 8.0) containing 0.01% HDB; column temperature, 37 °C; sample injection, 20 mbar for 4 s; detection wavelength, 230 nm; incubation time, 1 min.

a day over 10 days. During the testing time, the immobilized enzyme reactor was kept in the running buffer at room temperature. As shown in Figure 2b, a drop of the enzyme activity appeared after the seventh day; however, the free enzyme in solution could keep its activity only for less than 3 days at room temperature.

The repeatability of the immobilized enzyme reactor in terms of intraday, interday, and batch to batch was evaluated. The relative standard deviation (RSD%) for the peak area of the product HA and its migration time are listed in Table 1. These data imply that the present method for the preparation of the immobilized enzyme microreactor was reliable.

It should be noted that although the surface area of the open tubular microreactor is much lower than that of the monolithic enzyme microreactors, the performance of the immobilized enzyme is sufficiently high enough to rapidly catalyze the reaction. This should be due to the high surface-to-volume ratio of the capillary. Furthermore, the open tubular format of the immobilized enzyme microreactor facilitates not only the preparation but also the inhibitor screening, especially for the natural extract samples having a complex sample matrix, because there is no danger of column contamination by the sample matrix.

Because the enzyme immobilization is reversible, it is very easy to renew the enzyme microreactor once its enzyme function becomes poor. It can be renewed just by flushing the capillary with 1 M NaCl to remove the residual enzyme and repeating the preparation protocol. In Table 1, the relative standard deviation for batch-to-batch repeatability in terms of the peak area of HA is 5.4%, implying an acceptable reproducibility.

Performance of the Immobilized ACE. As reported in the literature,[30] ACE displays optimal performance with the borate−phosphate buffer at pH 8.0 at a temperature of 37 °C. These conditions were, therefore, transferred to our experiment. Because ACE is a chloride-ion-dependent enzyme,[30] the NaCl optimal concentration in the substrate solution must be established. As can be seen from Figure 3, the enzyme activity increased with an increase in the NaCl concentration until 40 mM; thereafter, a plateau appeared. Therefore, 50 mM NaCl in the substrate solution

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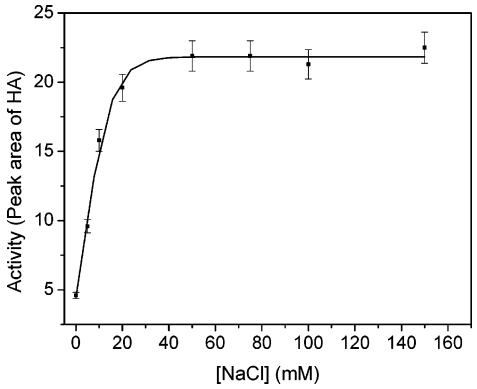

Figure 3. Dependence of the immobilized ACE activity on NaCl concentration in the substrate solution. Conditions: fused-silica capillary, 50-μm i.d. × 34.5 cm (26 cm to detection window); running buffer, 20 mM borate−phosphate buffer (pH 8.0) containing 0.01% HDB; column temperature, 37 °C; sample injection, 20 mbar for 4 s; detection wavelength, 230 nm; incubation time, 1 min.

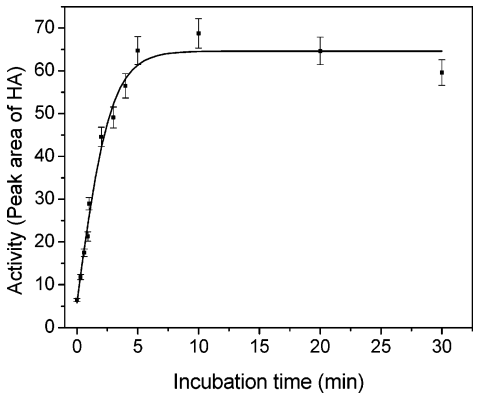

Figure 4. Dependence of the yield of the product HA on the incubation time. Conditions: fused-silica capillary, 50-μm i.d. × 34.5 cm (26 cm to detection window); running buffer, 20 mM borate−phosphate buffer (pH 8.0) containing 0.01% HDB; column temperature, 37 °C; sample injection, 20 mbar for 4 s; detection wavelength, 230 nm.

was fixed for the following experiments. Furthermore, the effect of the incubation time on the yield of product HA was investigated (shown in Figure 4). The maximum yield could be achieved when the incubation time was over 5 min. The drop in the peak area over 8 min reaction time could be due to experimental error resulting from the concentration diffusion of the produce zone. To increase the screening throughput, a 1-min incubation time was selected for the following experiments. In addition, the effect of the buffer concentration on the enzyme activity was investigated, as well. No significant effect on the enzyme activity was observed. In our experiment, we selected 20 mM sodium borate buffer for all experiments because of the sufficient buffer capacity and relatively low Joule heat. The Lineweaver−Burk plots for the immobilized ACE are shown in Figure 5. Each point was measured in triplicate, and the average value was used to fit the plots with the correlation coefficients higher than 0.973. From the plots, the determined K_m value was 0.91 mM, which is smaller than the literature reported values, 2.6[30] and 1.16 mM.[15a]

Inhibition Kinetic and Inhibitor Screening. Two commercially available ACE inhibitors, captopril and cilazaprilat, were employed to evaluate the ACE microreactor. The Lineweaver−Burk plots obtained at different inhibitor concentrations for both

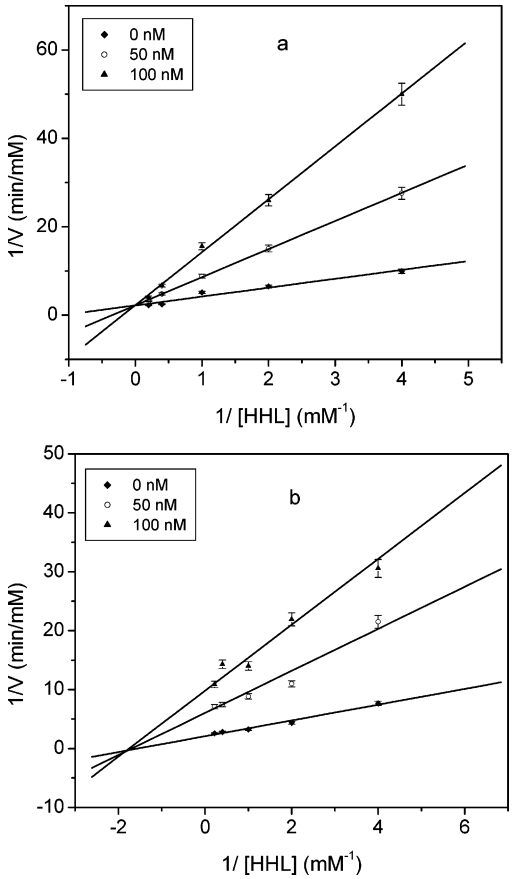

Figure 5. Lineweaver−Burk plots for captopril (a) and cilazaprilat (b). Conditions: fused-silica capillary, 50-μm i.d. × 34.5 cm (26 cm to detection window); running buffer, 20 mM borate−phosphate buffer (pH 8.0) containing 0.01% HDB; column temperature, 37 °C; sample injection, 20 mbar for 4 s; detection wavelength, 230 nm; incubation time, 1 min.

Table 2. Determined IC_{50} and K_i Values of ACE Inhibitors[a]

	IC_{50}, nM		K_i, nM	
inhibitors	determined	reference [15a,31]	determined	reference [15a,31]
captopril	50.1	~2.27 to 580	30.8	~0.33 to 72
cilazaprilat	6.3	1.9	27.4	*b*

[a] Conditions as in Table 1. [b] No reference value is available.

inhibitors are also shown in Figure 5. The resulting plots showed that captopril displayed a competitive inhibition behavior, whereas that of cilazaprilat was noncompetitive. The inhibitor plots for both inhibitors are shown in Figure 6. The determined K_i and IC_{50} values for captopril and cilazaprilat are listed in Table 2. The obtained data in our experiment are comparable with the literature values.[15a,31]

A small compound library listed in Table 3 was employed to evaluate the present immobilized enzyme microreactor. The

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Table 3. Compound Library Used for Inhibitor Screening[a]

sample	% inhibition	sample	% inhibition
captopril[b]	80	*Radix angelicae* sinensis	0
cilazaprilat[b]	98	*Radix* and *caulis acanthopanacis* senticosi	0
dithiothreitol[b]	21	*Radix* and *rhizoma rhei*	0
Chinese honeylocust spine	0	*Radix astragali*	0
Cortex phellodendri	0	*Radix ginseng* rubra	0
Flos carthami	0	*Radix angelicae* sinensis	0
Flos lonicerae	0	*Radix* and *Caulis acanthopanacis* senticosi	0
Fructus aurantii	0	*Radix* and *rhizoma rhei*	0
Fructus aurantii immaturus	0	*Radix astragali*	0
Fructus crataegi	0	*Radix ginseng* rubra	0
Fructus forsythiae	0	*Radix notoginseng*	0
Fructus gardeniae	0	*Radix angelicae* sinensis	0
Fructus schisandrae chinensis	0	*Rhizoma coptidis*	0
Herba hedyotis diffusae	0	*Rhizoma curcumae*	0
Herba scutellariae babratae	0	*Rhizoma fagopyri* dibotryis	0
Herba lycopi	0	*Semen coicis*	0
Lignum dalbergiae odoriferae	0	*Semen ziziphi* spinosae	0

[a] Conditions as in Table 1. [b] The concentration was 500 nM; the concentration of the natural extract was 0.5 mg/mL.

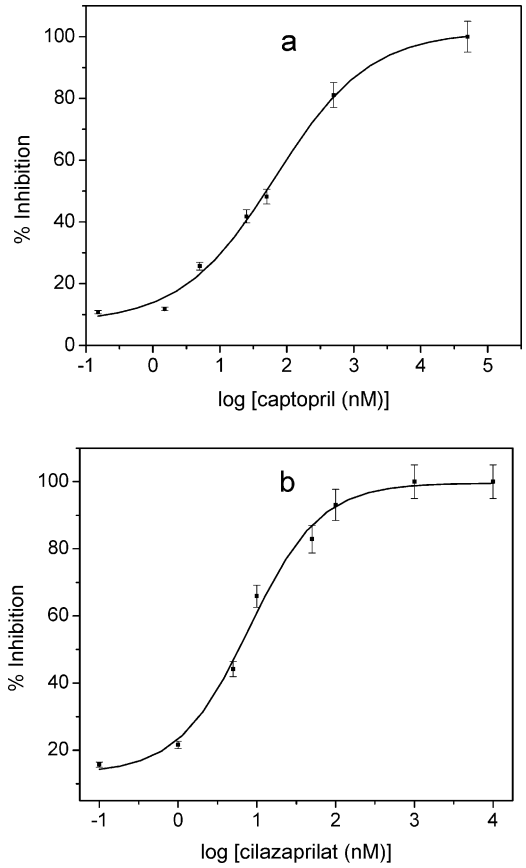

Figure 6. Inhibition plots for captopril (a) and cilazaprilat (b). Conditions: fused-silica capillary, 50-μm i.d. $\times$ 34.5 cm (26 cm to detection window); running buffer, 20 mM borate−phosphate buffer (pH 8.0) containing 0.01% HDB; column temperature, 37 °C; sample injection, 20 mbar for 4 s; detection wavelength, 230 nm; incubation time, 1 min.

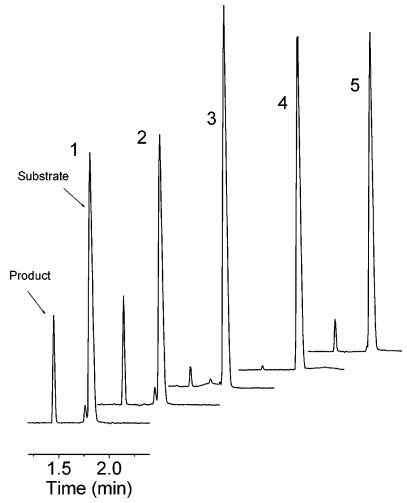

Figure 7. Typical electropherograms for screening the ACE inhibitors. Conditions: fused-silica capillary, 50-μm i.d. $\times$ 34.5 cm (26 cm to detection window); running buffer, 20 mM borate−phosphate buffer (pH 8.0) containing 0.01% HDB; column temperature, 37 °C; sample injection, 20 mbar for 4 s; detection wavelength, 230 nm; incubation time, 1 min. Samples: 1 = blank; 2 = extract of *Rhizoma coptidis* (0.5 mg/mL); 3 = extract of *Rhizoma coptidis* (0.5 mg/mL) spiked with 38.9 μg of cilazaprilat; 4 = cilazaprilat (500 nM); 5 = captopril (500 nM).

library contained three known ACE inhibitors and 31 natural extracts. Unfortunately, we did not find any inhibitor from these natural extracts. For screening of the present library of compounds, the total assay time for each sample was ~4 min. Accordingly, the throughput of the present method is not high; however, the potential to screen a mixture in a complex sample matrix, such as natural extracts, would be the great feature of the present method, as compared with other screening methods. Furthermore, the selectivity due to the combination of CE separation and UV detection can avoid the effect of the sample matrix and inhibitor on the assay. This may be superior to the microplate-based assay, which sometimes suffers from interference by the sample matrix. To demonstrate the potential of the present method for screening a complex mixture, a natural extract sample (0.5 mg/mL) spiked with 38.9 μg of cilazaprilat was assayed, and it yielded a 93.1% inhibition, as compared with the control assay. Thus, screening of a mixture of compounds can dramatically enhance the throughput. Five electropherograms for inhibitor screening are shown in Figure 7. The first electropherogram represents a control assay in which only the substrate solution was injected. the second and third represent assays for

a natural extract sample in the absence and presence of 38.9 μg of cilazaprilat, respectively. It clearly shows that the presence of inhibitor in the complex mixture of compounds can be identified. The fourth and fifth represent the assay for cilazaprilat and captopril, respectively.

CONCLUSIONS

In conclusion, we have developed a novel CE-based immobilized enzyme microreactor for screening of enzyme inhibitors. The method is rapid, simple, and robust. In particular, it greatly minimizes the assay volume and, hence, lowers the consumption of the reagent. Moreover, the immobilized enzyme can be recycled, and its stability was enhanced; therefore, the screening cost can be dramatically reduced. Although the throughput of the present method is low (the assay rate is ~4 min for one sample), the potential for screening mixed compounds can obviously enhance the screening throughput by several times. The method can be easily transferred to a capillary array electrophoresis having 96 capillaries; thus, the sample throughput can be dramatically increased. Our approach greatly facilitates the preparation of the on-column immobilized enzyme reactor and promotes its application not only in screening of enzyme inhibitors, but also in an enzyme activity assay. The study of other enzyme immobilizations with ionic binding on the capillary wall is under going.

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