Assessment of the Enzymatic Activity and Inhibition using HPFA with a Microreactor, Trypsin, Absorbed on Immobilized Artificial Membrane

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Abstract

The purpose of this study was to develop a new method to assess the activity and inhibition of the immobilized enzyme trypsin based on the frontal analysis of enzymatic reaction products. This novel technique was performed by on-line monitoring of the absorption at 253 nm of N-benzoyl-L-arginine (BA) from the hydrolysis of *N*-benzoyl-L-arginine ethyl ester (BAEE). A microreactor was constructed by immobilizing trypsin on an immobilized artificial membrane (IAM)-packed column. Trypsin was non-covalently and dynamically immobilized on-line in the hydrophobic interface of an IAM liquid chromatographic stationary phase. The trypsin-IAM stationary phase was bioactive. Due to the enzymatic reaction, the substrate of BAEE was completely hydrolyzed when the BAEE concentration was below 0.5 mmol/L and partly hydrolyzed when the BAEE concentration ranged from 0.75 to 1.0 mmol/L. By the addition of soybean trypsin inhibitor (STI), phenylmethane sulfonyl fluoride (PMSF), and benzamidine into the substrate solution, the results obtained from the frontal analysis showed that the activity of trypsin on IAM was strongly inhibited not only by STI but by both benzamidine and PMSF.

Introduction

Trypsin is a pancreatic serine protease. Trypsin was widely used in industrial and biomedical applications, such as the food industry, drink clarification, and leather processing. In biotechnology, trypsin is often used to perform enzymatic reactions of proteomics study. Because it is capable of selectively cleaving proteins at arginine and lysine residues that typically provide peptides in a mass range compatible with mass spectra for amino acid sequence determination (1,2). In comparison with use of the soluble form, the use of immobilized trypsin for protein cleavage has many advantages, including higher enzyme to substrate ratio, high digestion efficiency, and the reusability of the enzyme (3,4). Moreover, the rate of enzyme denaturation or inactivation is lower than that of the free enzyme, which often quickly loses its catalytic activity, leading to a lack of reproducibility. Therefore, the use of immobilized trypsin provides good reproducibility, better suitability for trace-level samples, is amenable to high-throughput automation, and provides overall cost-effectiveness.

Suitable matrices and immobilization methods are the key points in enzyme immobilization. It is well-known that the characteristics of the support (i.e., shape, particle size, porosity, chemistry, and mechanical strength) may strongly affect the basic characteristics of the immobilized enzyme (5–7). Therefore, selection of the matrix is a key factor influencing the activity and the applicability of the resulting bioreactor. The IAM liquid chromatography (LC) stationary phase used in this study was derived from the covalent immobilization of 1-myristoyl-2-[(13-carboxyl)tridecanoyl]-sn-3-glycerophosphocho-line on aminopropyl silica, and resembles one-half of a cellular membrane (8). It is non-toxic, amphiphilic, biocompatible, and simulates half of the cell membrane. This silica in the core of the bead could act as a rigid support, and the microporous phosphatidylcholine layer on the bead surface could provide a suitable steric position for enzyme absorption.

The assessment of the immobilized enzymes, including their activity and reaction kinetics, was commonly performed by off-line or column-switching analysis of products (9,10). High-performance frontal analysis (HPFA) (11) has been widely used to investigate and characterize the specific interaction between biomolecules, in which no conversion of substrate has been involved. For an instance, frontal analysis can be used to measure the affinity constant between an immobilized protein and a ligand to measure the number of solute binding sites in the column and to determine the type of binding (single- or multi-site). Several types of proteins have been mainly reported in recent studies, including plasma proteins, receptor proteins, and transporter proteins. (*i*) The binding properties of plasma proteins with drugs (small molecules) (12), such as R- or S-warfarin (13–15, 20) and to D- or L-tryptophan (16–18) to HSA, and salicylate to BSA (19, 21, 22) were measured; (*ii*) The receptor proteins (23-25), such as immobilized β_2-adrenergic receptor with

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R- or S-propranolol were reported, and the affinity rank order of nicotinic acetylcholine receptors with ligands was found to be: (±)-epibatidine > A85380 > (–)-nicotine > carbachol > atropine (23); (*iii*) The binding affinity (K value) of transporter proteins (26–30), such as P-glycoprotein (PGP), with vinblastine and the human red cell glucose transporter with cytochalasin B and D-glucose was studied.

A microreactor by immobilized trypsin on the activated glycidyl methacrylate-modified cellulose membrane packed column was constructed (31), and HPFA was used to characterize the activity and reaction kinetics of the immobilized enzyme in a membrane-packed column through the on-line frontal analysis of enzymatic reaction products (31–33). The trypsin was covalently immobilized on the membrane, and, thus, the technique is difficult to widely apply to the application. The aim of this paper is to develop a new method for the construction of a trypsin-immobilized microreactor based on the capillary IAM column. The activity of the immobilized enzyme was characterized through on-line frontal analysis of the enzymatic reaction for which an artificially synthesized low-molecular-mass substrate BAEE was employed. The inhibitions with the STI, benzamidine, and PMSF for trypsin-IAM were also examined by the frontal chromatography with the microreactor column.

Experimental

Materials and methods

Trypsin and tris [tris(hydroxymethyl)aminomethane] were purchased from Sigma (St. Louis, MO). Immobilized artificial membrane phosphatidylcholine drug discovery (IAM.PC.DD_2) silica beads (12-µm particle size, 300-Å pore size) were obtained from Regis Chemical (Morton Grove, IL). Polyamide-clad fused-silica capillaries (375-µm o.d., 100-µm i.d. and 690-µm o.d., 400-µm i.d.) were obtained from Yongnian Optical Fiber Factory (Hebei, China). BAEE was purchased from Fluka (Buchs, Switzerland). STI, benzamidine, and PMSF were purchased from Amresco (Solon, OH); methanol (Merck, Darmstadt, Germany) and acetonitrile (Fisher, Fair Lawn, NJ) were chromatographic-grade. Ultra-pure water was purified with a Milli-Q Plus system (Millipore, Bedford, MA) and was used to prepare all aqueous solutions. All other reagents were of analytical reagent-grade and obtained from Beijing Chemical Reagents Factory (Beijing, China).

Capillary liquid chromatography was carried out on a Trisep TM 2010GV system (Unimicro Technologies, Pleasanton, CA), a data acquisition module, and a UV detector. Electrophoretic measurements were made on a G1601BA CE system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) and air thermostating of the capillary Milli-Q system.

Preparation of packed capillary column

In constructing the microreactor, the retaining frits must possess high permeability to buffer flow and yet be mechanically strong enough to contain the packing material and to resist the pressures for packing or flushing the column. The most common approach to frit construction is by sintering silica materials (34). In this trial, one end of the 100-µm i.d. capillary tubing was pressed into a few millimeters of sodium silicate and sintered. It was then used to heat the sodium silicate to form a frit at the end of the capillary, by applying a nichrome coil with appropriate voltage on it.

A 5-cm length of 400-µm i.d. quartz capillary was flushed with ultra-purity water for 20 min and dried by nitrogen gas. One end of the tubing was then glued to a 7-cm length of 100-µm i.d. open capillary using epoxy resin glue. Using gravitational force (35), IAM was then packed into the 400-µm i.d. capillary to a length of ~ 3.0 cm, and an end-frit of 100-µm i.d. capillary was glued to the open end of the 400-µm i.d. quartz capillary to seal the packing materials. The polyimide outside the capillary adjacent to the frit was burned, thus, forming a window for detection.

Immobilization of trypsin on IAM

In the capillary LC mode, 10 mmol/L Tris-HCl buffer (pH 7.4) was used as the mobile phase. Before use, all sample solutions were passed through a 0.22-µm filter and the mobile-phase solution was degassed in an ultrasonic bath for 30 min. The IAM column was placed in a capillary LC system with a flow rate of 5 µL/min. During the experiment, the column was rinsed with methanol, ultra-pure water, and the mobile phase solution for 30 min, respectively, and a steady baseline was observed. Unless otherwise stated, the temperature of column was kept at room temperature (25°C). The conditions to dynamically immobilize trypsin onto IAM were optimized. Three hundred microliters of trypsin solution (0.1 mg/mL in the Tri-HCl buffer, pH 7.4) was loaded into the injector, and the valve switched to the inject position. After 45 min, the valve was switched to the load position. At the same time, the eluent was collected to determine protein concentration by Bradford assay. The amount of immobilized trypsin was calculated by the difference between the added protein amount and that found in the eluent (36). By this approach, the amount of trypsin absorbed on IAM is ~ 21.3 µg protein per mg IAM.

Determination of trypsin activity

The activity of the trypsin immobilized on IAM supports was determined by two methods: (*i*) On-line frontal analysis experiments were performed on the capillary trypsin-IAM columns. When the substrate solution was perfused through the microreactor at a fixed flow rate, reaction of the substrate with immobilized trypsin appeared and hydrolysis was observed by frontal analysis method as the elution profiles reached the plateau. With on-line UV detection, BA can be detected at 253 nm due to a greater absorbance of BA over BAEE at the same wavelength. The activities of the trypsin-IAM were measured at a series of concentrations, ranging from 0.1 to 1.0 mmol/L. (ii) The second method was the off-line method, where the reactions of the immobilized trypsin enzymes and substrates were performed similar to the on-line experiments, but the reaction products were measured by capillary electrophoresis at 228 nm.

Enzyme activity and inhibition studies on trypsin-IAM column

The enzymatic activity of the trypsin-IAM column was also studied. The substrate concentrations examined ranged from

0.25 to 1.0 mmol/L at a flow rate of 5.0 µL/min. The effect of the known inhibitors STI, PMSF, and benzamidine on the enzymatic activity of the trypsin-IAM was also investigated. The inhibition of the trypsin-IAM was carried out using injections of a series of substrate and inhibitor mixtures. Substrate solutions with various concentrations of inhibitor were perfused through the trypsin-IAM column, and the elution was determined for each inhibitor concentration from the elution profile. On the trypsin-IAM, the concentrations of STI ranged from 1.0×10^{-8} to 1.0×10^{-7} mol/L. The concentrations investigated for PMSF and for benzamidine were 5, 7.5, and 10 µmol/L, respectively.

Results and Discussion

Activity of immobilized trypsin by on-line frontal analysis

In this study, the 0.3 mL volume injector loop was fully loaded with the sample solution and connected to the mobile phase for 30 min by switching the injector valve, which resulted in a sample injection of desired volume. The injector valve was then switched to the load position, and the loop was detached from the mobile phase.

Bradford assay was employed for the amount of trypsin immobilized on the IAM stationary phase, but the data are the absolute amount of trypsin not the activity of the immobilized trypsin. For the measurement of the enzymatic activity, a frontal analysis was established. A flow rate at 5 µL/min was applied to the capillary trypsin-IAM system at a concentration of 0.5 mmol/L BAEE. Figure 1 shows the profiles of frontal analysis for hydrolysis of BAEE to BA on the trypsin-immobilized column as well as of BAEE on IAM column without immobilization of trypsin, respectively. It can be seen that once the plateau is reached, the column is in a state of dynamic equilibrium of the substrate and product diffusion. The plateau of BAEE on the trypsin-immobilized column was higher than that of the IAM column, but the breakthrough time became shorter by ~ 5 min. This result shows that the interaction between BAEE and IAM was stronger than that of BA and IAM. Because the molar absorbance coefficient of BA was higher than that of BAEE at 253 nm, the curve of BAEE on the trypsin-IAM column and BA on the IAM column was almost overlapped, which indicates that BAEE on the trypsin-IAM column was mostly hydrolysis for the product BA. As shown in Figure 1, trypsin was successfully immobilized on IAM and kept its bioactivity.

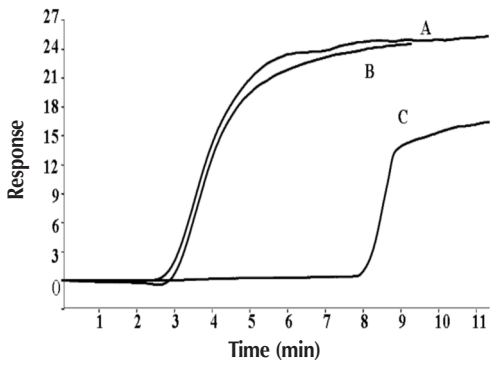

Figure 1. The elution profile of frontal chromatography. BAEE on trypsin-IAM column (A); BA on IAM column (B); BAEE on IAM column (C). The conditions as follows: columns, a capillary packed with IAM or immobilized-trypsin IAM (400 µm i.d. × 3.0 cm), and the mobile phase, 10 mmol/L Tris-HCl buffer (pH 7.4), a flow rate at 5 µL/min, a concentration of BAEE or BA at 0.5 mmol/L.

The frontal chromatographic studies with the trypsin-IAM demonstrated that the immobilized trypsin was active in the flow system. Figure 2 shows that when a series of the substrate BAEE was injected into the trypsin-IAM, the height of plateau of the eluents appeared. As positive and negative controls, BA and BAEE were injected into the IAM system without immobilized trypsin, respectively. When the concentration of BAEE was below 0.5 mmol/L, the BAEE substrate was completely hydrolyzed by the microreactor. However when the concentration of BAEE was higher than 0.75 mmol/L due to saturation of the active sites of immobilized trypsin, the substrate was partly hydrolyzed as shown in Figure 2. As a result, the line A shifted due to the difference in the molar absorbance coefficient between residue BAEE and product BA.

For quantitative determinations, standard curves were drawn based on the frontal analysis assay. When the injection volumes were large enough, it was eluted as a zonal peak, and the peak height was proportional to sample concentration (37,38). Here the consideration of concentrations include two aspects:

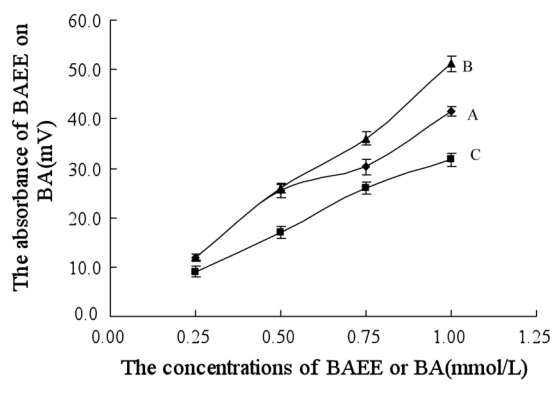

Figure 2. Relationship between the concentrations and absorbance of BAEE (0.5 mmol/L) or BA plateaus: BAEE on trypsin-IAM column (A); BA on IAM column (B); BAEE on IAM column (C).

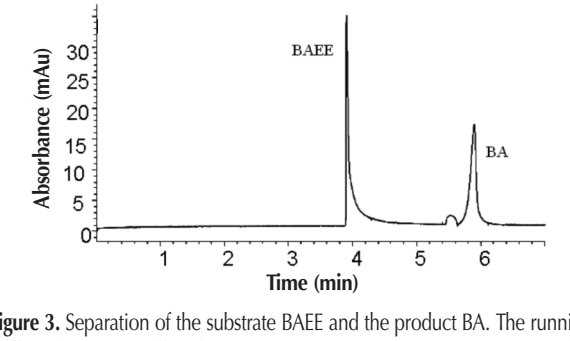

Figure 3. Separation of the substrate BAEE and the product BA. The running buffer: phosphate buffer of 50 mmol/L (pH 8.3), the applied voltage: 15 kV, the capillary: 50 µm i.d × 45 cm (36 cm to the detector), the pressure injection: 50 mbar for 5 s, the detection wavelength: 228 nm.

quantitative analysis and saturation of the active sites of enzyme in a microreactor. The saturated concentration of BAEE to trypsin in the microreactor was found at the concentration between 0.5 and 0.75 mmol/L. Therefore the concentrations of BAEE and BA were set from 0.25 to 1.0 mmol/L, respectively, and injected to IAM column three times. By plotting peak height versus concentrations, the regression equation of these curves (average high of plateau, y, versus concentration, x, mmol/L) and the correlation coefficients were $y = 30.84x + 1.7$ ($r^2 = 0.99$) for BAEE and $y = 51.04x - 0.6$ ($r^2 = 0.99$) for BA.

In order to test the validity of the frontal analysis method, the off-line detection of the product is applied to measure the activity of trypsin immobilized on IAM support by capillary electrophrosis (CE). Figure 3 shows the separation of BAEE and BA. The product BA from hydrolysis of substrate BAEE was measured at different concentrations of BAEE. When the concentration of BAEE through the trypsin-IAM column was 0.5 mmol/L, only assayed the product BA, which demonstrates that BAEE was completely hydrolyzed by trypsin-IAM. The result was consistent with the frontal analysis method (Figure 1).

Activity of BAEE on immobilized trypsin inhibited by protein inhibitor (STI) by on-line frontal analysis

Studies of the inhibition of enzymatic reactions have played a dominant role in the elucidation of the specificity of enzymatic systems. The inhibition studies were carried out using 0.5 mmol/L BAEE and STI in a concentration range of 1.0×10^{-8} to 1.0×10^{-7} mol/L. Figure 4 shows the frontal analysis profiles for hydrolysis of BAEE to BA on the trypsin-IAM column with different concentrations of STI as well as BAEE on the IAM column without immobilization of trypsin, respectively. The hydrolytic activity of the trypsin-IAM was measured by the frontal analysis method as described. When 0.5 mmol/L BAEE was injected into the trypsin-IAM column, the product BA was produced in the mobile phase. The interaction between BA and trypsin-IAM was weaker than that of BAEE and trypsin-IAM, and thus following continuous sample loading, the product BA was separated from BAEE. The zone of BA appeared in the column, then the plateau zone of the BAEE and BA mixture was eluted from the column. The plateau region extended with increasing injection volume, but the peak height remained constant, which is dependent on the substrate and product concentration in the mobile phase.

Because the substrate BAEE and the inhibitor STI compete for the same binding site of trypsin surface, increased concentrations of STI caused a decrease in height of the eluent profile of BA and in the plateau region from the trypsin-IAM column. The results indicate that the activity of trypsin was significantly reduced by the inhibitor STI.

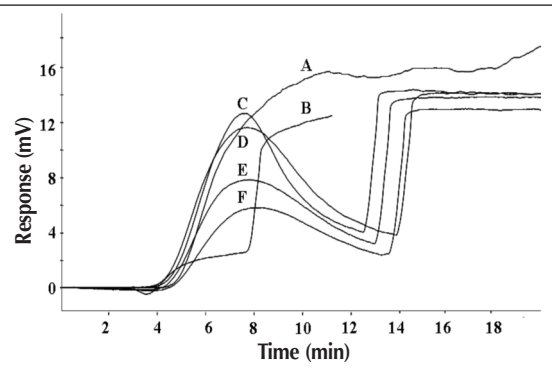

Figure 4. Frontal chromatography elution profile of BAEE through the trypsin-IAM column with different STI concentrations: trypsin-IAM column (A); IAM column (B); trypsin-IAM column with 1.0×10-8 mol/L STI (C); trypsin-IAM column with 2.5×10^{-8} mol/L STI (D); trypsin-IAM column with 7.5×10^{-8} mol/L STI (E); trypsin-IAM column with 1.0×10^{-7} mol/L STI (F).

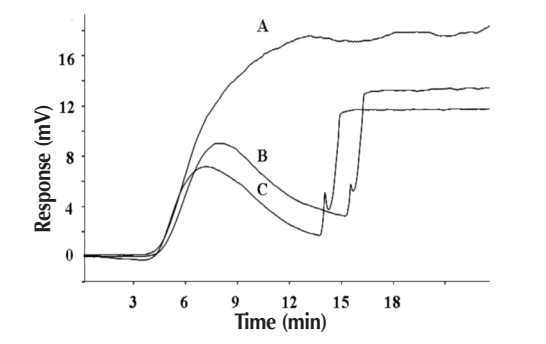

Figure 5. Frontal chromatography elution profile of BAEE passing through the trypsin-IAM column with different benzamidine concentrations: without benzamidine (A); benzamidine at 5.0 µmol/L (B); benzamidine at 7.5 µmol/L (C).

Activity of BAEE on immobilized trypsin inhibited by small molecules (benzamidine and PMSF) by frontal analysis

Inhibitory behaviors of small molecules, including both benzamidine and PMSF for the activity of trypsin immobilized on IAM were investigated, respectively. Figure 5 shows the trypsin-

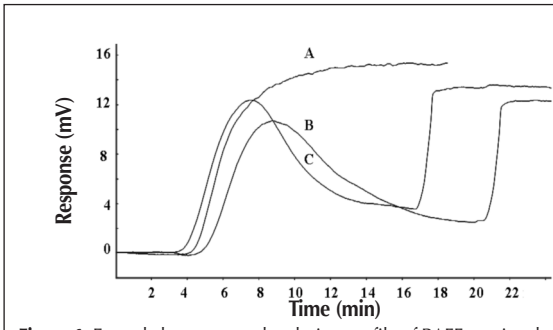

Figure 6. Frontal chromatography elution profile of BAEE passing through the trypsin-IAM microreactor with different PMSF concentrations: without PMSF (A); PMSF at 5.0 µmol/L (B); PMSF at 7.5 µmol/L (C).

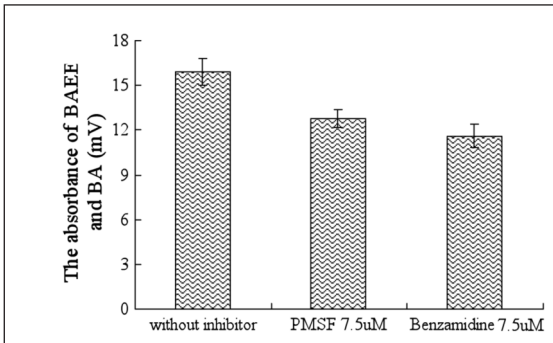

Figure 7. Comparison of the difference in inhibition of the activity of trypsin on the IAM column between PMSF and benzamidine.

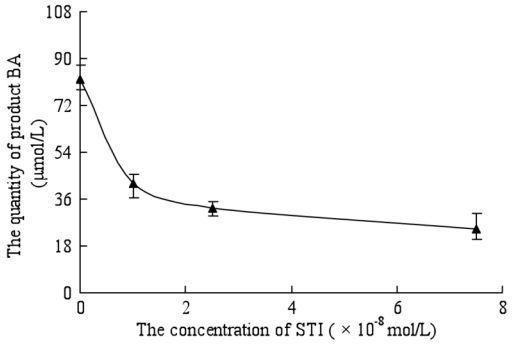

Figure 8. Effect of the concentrations of STI on the production of BA from the hydrolysis of BAEE.

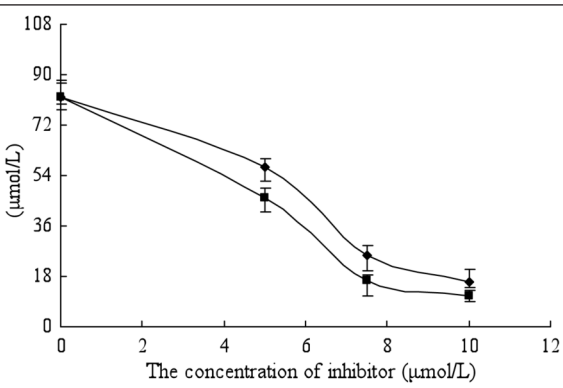

Figure 9. Inhibition curves of benzamidine and PMSF to trypsin activity measured by the off-line CE assay: PMSF (A) and benzamidine (B).

IAM column inhibition curve at the different concentrations of benzamidine in the mobile phase, which the absorbance curves of the eluent were going down as concentrations of benzamidine increased. A similar phenomena was observed with different concentrations of PMSF (Figure 6). Comparing to the case without inhibitors (refer to the curve A in the figures), both benzamidine and PMSF significantly decreased the production of BA from the enzymatic reaction of BAEE catalyzed by trypsin on the IAM column. Figure 7 gives a comparison of the inhibition between benzamidine and PMSF at the concentration of 7.5 µmol/L, representing that benzamidine is more potent to trypsin than PMSF. In this study, we demonstrate that frontal chromatography equipped with the trypsin-IAM microreactor may be a quick and efficient way for the screening of trypsin inhibitors.

Off-line determination of inhibited trypsin-IAM activity using CE

The off-line measurement of inhibition to trypsin-IAM activity by CE is applied to confirm the results obtained from the frontal analysis with the microreactor. The same CE assay as in Figure 3 was employed. The product BA from hydrolysis of substrate BAEE in the trypsin-IAM microreactor was collected and then detected by CE. As shown in Figure 8, with increasing concentrations of STI, the production of BA was decreased. Referring to the frontal analysis in Figure 4, in which the inhibition are presented by the reduction of peak heights of BA produced from hydrolysis with the concentration of STI from 1.0×10^{-8} mol/L to 1.0×10^{-7} mol/L, an identical tendency of inhibition was observed in the off-line detection. The agreement between the two cases implicates that the on-line trypsin-IAM microreactor is able to offer reliable results for the inhibition study. Furthermore, the inhibition curves of both benzamidine and PMSF in Figure 9 obtained by the off-line detection shows that the inhibition of benzamidine to trypsin activity is more potent than that of PMSF, which is also observed in the frontal analysis in Figures 5, 6 and 7.

Conclusion

In summary, a novel method to characterize the trypsin activity has been developed based on the frontal analysis with a microreactor. The microreactor was prepared in a manner in which trypsin was dynamically but not covalently fixed on IAM packed in capillary. Comparing to the zone chromatography, the frontal analysis provide a possibility to do the on-line inhibition study for enzymes. The inhibitory effect of STI, benzamidine, and PMSF to trypsin obtained from the frontal analysis are identical with that reported by previous methods, and also confirmed by the off-line CE analysis in this study. The results implicate that it provides a rapid and reliable method for evaluating enzyme activity and screening inhibitors.

Ackowledgement

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