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On-line characterization of the activity and reaction kinetics of immobilized enzyme by high-performance frontal analysis

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Abstract

A microreactor by immobilized trypsin on the activated glycidyl methacrylate-modified cellulose membrane packed column was constructed. Immobilized trypsin mirrored the properties of the free enzyme and showed high stability. A novel method to characterize the activity and reaction kinetics of the immobilized enzyme has been developed based on the frontal analysis of enzymatic reaction products, which was performed by the on-line monitoring of the absorption at 410 nm of *p*-nitroaniline from the hydrolysis of *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). The hydrolytic activity of the immobilized enzyme was 55.6% of free trypsin. The apparent Michaelis–Menten kinetics constant (K_m) and V_{max} values measured by the frontal analysis method were, respectively, 0.12 m*M* and 0.079 m*M* min^{-1} mg $enzyme^{-1}$. The former is very close to that observed by the static and off-line detection methods, but the latter is about 15% higher than that of the static method. Inhibition of the immobilized trypsin by addition of benzamidine into substrate solution has been studied by the frontal analysis method. The apparent Michaelis–Menten constant of BAPNA (K_m), the inhibition constant of benzamidine (K_i) and V_{max} were determined. It was indicated that the interaction of BAPNA and benzamidine with trypsin is competitive, the K_m value was affected but the V_{max} was unaffected by the benzamidine concentration.

Keywords: Immobilized trypsin reactor; Glycidyl methacrylate-modified membrane; Reaction kinetics; Frontal analysis; Enzymes

1. Introduction

Many biocatalysts in their native environment are attached to the cell membrane or entrapped within the cells. This observation led to the concept that pure isolated enzymes may actually perform better when they are immobilized on a solid support. At present, the use of immobilized enzymes is well established in the manufacture of commodity products such as 6-aminopenicilic acid, high-fructose corn syrup and L-amino acids [1]. Immobilized enzymes are also used in medical diagnostics and therapy [2], biosensors [3], chromatographic packings and detectors [4], electronics [5], on-line solid-phase reactors [6], organic synthesis [7] and in many other applications. The carrier that is used for immobilization affects the basic characteristics of the biocatalyst through its properties such as shape, porosity, chemistry, stability and mechanical strength, while porous beads are most frequently

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used as the support. In columns packed with enzyme-modified porous beads, the substrate or the analyte molecules have to diffuse into the pores of the support in order to interact with the active sites of the immobilized enzyme. Because most of the flow in packed columns occurs around the particles through the interparticular voids, the mass transfer of substrate from solution to the immobilized enzyme and back is controlled by the diffusion rate, that, in turn, depends on the particle and pore size, flow-rate and diffusion coefficient of the substrate. Recent reports show that membranes [8,9], perfusion beads [10], fibers [6,11] and continuous tubular filters [12] may also be used for enzyme immobilization. Both theoretical calculations and experimental results clearly document that membrane systems can be operated in a ''dead-end'' or filtration mode at much higher flow-rates than packed beds because all the substrate flows through the support, and the mass transfer is much faster as a result of this convective flow [1,13–15]. This is particularly true for reactions and separations in which macromolecular substrates or high-molecular-mass analytes are involved. The characterization of the immobilized enzymes including their activity and reaction kinetics was commonly performed by the off-line or column-switching analysis of products [16,17]. High-performance frontal analysis [18] has been widely used to investigate and characterize the specific interaction between biomolecules, in which no conversion of substrate has been involved. This report presents a new method 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 product. The apparent Michaelis–Menten kinetic constant (K_m) of *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), the inhibition constant of benzamidine (K_i) and the V_{max} on the immobilized trypsin reactor have been determined simultaneously.

2. Experimental

2.1. Materials and reagents

L-1-(Tosylamido)-2-phenylethyl chloromethy ketone (TPCK)-treated trypsin (EC 3.4.21.4, from bovine pancreas) and benzamidine were purchased from Sigma (St. Louis, MO, USA), HPLC-grade trifluoroacetic acid (TFA) was purchased from Merck (Schuchardt, Germany), BAPNA was from Shanghai Dongfeng Biochemical Technology Company (Shanghai Institute of Biochemistry, Shanghai, China). All the other chemicals were of analytical-reagent grade. The deionized water used in all procedures was purified in the laboratory with a Milli-Q water system (Millipore, Milford, MA, USA).

2.2. Chromatographic instruments

The chromatographic experiments were performed with a modular high-performance liquid chromatography (HPLC) system which consisted of two Shimadzu LC-9A solvent module pumps and a SPD-10AV UV detector (Shimadzu, Kyoto, Japan) controlled by a WDL-95 workstation (National Chromatographic R&A Center, Dalian, China). The column temperature was controlled with a WMZK-02 isotemp controller.

2.3. Preparation of membrane media

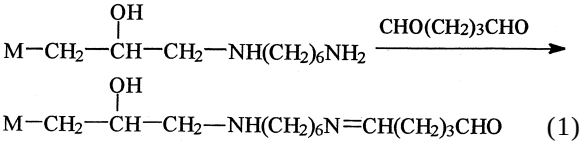
As previously described [8,9], the solid matrix is a composite of cellulose grafted with acrylic polymers formed by polymerizing a glycidyl methacrylate in the presence of dispersed cellulose fiber, the bicomponent fiber thus formed consisted of a cellulosic core as the mechanical support and the acrylic sheath as a chemical functional group carrier. The composite fiber can be further derivatized to yield the required functional groups. The media with amino groups were prepared by reacting the glycidyl groups grafted on the fiber surface with hexyl diamine monomers at 80°C for an additional hour.

2.4. Enzyme immobilized on membrane column

The composite fiber carrying specific functional groups was fabricated in paper form by a conventional paper-forming machine. Piece-by-piece of the membrane was cut off, the columns, which were compatible with the HPLC instrument were packed with the pieces of cut membrane up to the capacity of the column. It has been observed that the mem-

brane-packed column with dimensions of 40×4 mm I.D. could withstand a flow-rate of aqueous buffer of at least 3 ml min^{-1} [19]. Trypsin was covalently immobilized on such membrane material as follows:

(1) Aldehyde groups on the amino membrane were generated from the amino matrices by recirculating 0.05 *M* Tris–HCl buffer (pH 8.4). This was followed by the recirculating of a 1.0% solution of glutaric dialdehyde using the coupling buffer, the reaction was allowed to proceed for 3 h at room temperature. The excess glutaric dialdehyde was removed by washing the column with Tris–HCl buffer. This chemical reaction can be described by following equation:



(1)

(2) The immobilization of trypsin with the above aldehyde groups-generated membranes was performed by recirculating the solutions containing 2.5 mg ml^{-1} trypsin, 0.01 *M* $CaCl_2$ and 0.1 m*M* *N*-benzoyl-L-arginine ethyl ester (BAEE) in a 0.05 *M* Tris buffer solution (pH 8.4), and in the presence of 0.025 *M* $NaCNBH_3$ for 20 h at room temperature. Before the enzyme solution was recycled through the membrane column, the UV absorbance of this solution was measured at 214 and 280 nm. The amount of trypsin immobilized on the membrane is 3.6 mg g^{-1} (≈0.15 μmol g^{-1}) dried membrane.

(3) At the end of reaction, the uncoupled ligand was removed by rinsing the column with buffer solution until the ligand is no longer detectable in the eluent. The UV absorbance of the remaining enzyme solution and that of the washings were measured. An initial estimate of the amount of trypsin coupled to the membrane was determined by comparing the absorbance of the original reaction solution with the final collection solution.

(4) The excess active groups were deactivated by recirculating 1.0% glycine ethylester hydrochloride in 0.05 *M* Tris buffer, containing the reduction agent for 4 h. The microreactor was washed extensively and then equilibrated in the loading buffer for the evaluation and enzymatic reaction.

2.5. Determination of trypsin activity and kinetics of enzymatic reaction

The hydrolytic activity of free trypsin was determined by measuring the initial rate of hydrolysis of a chromogenic trypsin substrate BAPNA at 25°C with a 752C UV–Vis spectrophotometer (Third Shanghai Analytical Instrument Factory, China), which liberates the yellow colored *p*-nitroaniline (*p*-NA) upon hydrolysis. Assays were carried out at pH 8.2 for 10 min in a total reaction volume of 3.0 ml 0.05 *M* Tris–HCl buffer solution in the presence of 0.01 *M* $CaCl_2$ and monitored by following the increase in absorbance at 410 nm.

The activity of the trypsin immobilized on the glycidyl methacrylate (GMA)-modified cellulose membrane was determined by two methods: (1) according to the procedures to measure the activity of the immobilized enzyme in static method [17,20], 20 pieces of trypsin-immobilized membrane of 4 mm I.D. (total mass of membrane 0.0502 g, and amount of the immobilized trypsin 0.181 mg) were placed into 5 ml substrate solution, the suspension of the immobilized enzyme and reagent was vortex-mixed and allowed to stand for 10 min. The suspension was centrifuged and the mixture was rapidly filtered through a filter. The absorbance of the supernatant was measured at 410 nm. The measured activity of immobilized trypsin on the membrane was found to be dependent on the rate of stirring of the reaction mixture. Therefore all assays were carried out at a higher stirring rate which may produce the maximal activity under the conditions of assays. (2) On-line frontal analysis experiments were performed on the membrane columns with dimensions of 20.0×4.0 mm I.D., 20.0×2.0 mm I.D. and 2.0×4.0 mm I.D., respectively, which were compatible with the HPLC instrument. The mobile phase consisted of the same substrate solution as in the static method. The reaction time of substrate on the immobilized enzyme column is adjusted through changing the flow-rate of mobile phase from 1.6 to 0.1 ml min^{-1} (corresponding to the residue time of substrate solution in column from 0.15 to 2.4 min). When substrate solution at a certain concentration was perfused through the microreactor at a fixed flow-rate, reaction of substrate with immobilized trypsin appeared, and hydrolysis extents of substrate have

been observed as the elution profiles reach the plateau by frontal analysis method. Detection of *p*-nitroaniline can be carried out at 410 nm with on-line UV detection due to no UV absorbance of BAPNA at this detection wavelength. Prior to and after the experiments, the relationship between the UV absorbance and the concentration of standard *p*-NA was determined to calibrate the hydrolyzed *p*-NA from BAPNA (0.1 to 0.5 m*M*), and the obtained results showed good linearity with R^2 higher than 0.999. (3) In off-line methods, the reactions of immobilized enzyme and substrate are performed similar to on-line frontal analysis experiments. But the reaction products are detected off-line by another UV–Vis spectraphotometric detection at 410 nm.

For inhibition experiments, substrate solutions with various concentration of inhibitor were perfused through the column and the elution volume was determined for each inhibitor concentration from the breakthrough elution profile. After each experiment, the inhibitor was washed out from the column with Tris–HCl buffer. All the experiments were performed by keeping column temperature at 25°C.

3. Results and discussion

3.1. Direct covalent immobilization of trypsin onto the fiber membrane surface

Trypsin molecules were directly immobilized onto the fiber surface by covalent binding. The immobilized trypsin molecules retained excellent enzymatic activity. The results of this study indicated that the catalytic activity of the membrane-immobilized trypsin exhibited 55.6% of an equivalent molar amount of free trypsin. The stability of the membrane-immobilized trypsin was also investigated. It was stored in 0.01 *M* Tris–HCl buffer (pH 8.2, containing 0.01 *M* $CaCl_2$ and 0.02% NaN_3) at 4°C for 90 days and retained 90% of its original activity [21]. The membrane packed column that preceded the enzyme coupling step sought to create reactive groups on the relatively inert cellulose and to achieve an immobilization of the enzyme some distance away from the hydrophilic membrane surface. The conjugation of trypsin is done by glutaraldehyde, where one aldehyde group reacts with an amine group from the hexyl diamine on the fiber and the other aldehyde group reacts with the amine-containing biomolecules. For comparison, we synthesized the membrane matrix without an arm of hexane diamine and glutaraldehyde. The aldehyde groups on the acrylic membrane were generated directly from the glycidyl groups by acid hydrolysis with HCl at pH 0.6 for 6 h to form vicinal hydroxyl groups, followed by periodate oxidation with $NaIO_4$ for 30 min. Then it was used for the immobilization of trypsin. The results showed the relation activity of the immobilized trypsin is 43.6% of the same amount of free trypsin. It was lower than that of the immobilized trypsin on membrane matrix with the glutaraldehyde arm. The role of the linker, glutaraldehyde, in these procedures was considered to be straightforward on the proximal aldehyde groups in each glutaraldehyde molecule. The glutaraldehyde molecule would link in the classical Schiff's base fashion with the amine groups of the spacer molecules and leaving the distal aldehyde group available for covalent enzyme coupling. It also acted as a spacer arm that allows for greater accessibility and/or freedom to the attached biomolecules. This freedom is essential to the activity of the trypsin molecule reacting with its substrates.

3.2. Activity and reaction kinetics of the immobilized trypsin studied by on-line frontal analysis

For measurement of the enzymatic activity by frontal analysis, the substrate solution at a constant concentration is applied to the enzyme reactor continuously at different flow-rates. The resultant elution profile at each flow-rate is composed of an elution front and a plateau. Fig. 1 shows the profiles of frontal analysis for *p*-NA and hydrolysis of BAPNA to *p*-NA on the trypsin-immobilized column as well as BAPNA on the membrane packed column without immobilization of trypsin, respectively. It can be seen that once the plateau is reached the effects of the substrate and product diffusion and the flow-rate on the profile can be neglected, and the column is in a state of dynamic equilibrium. Then the concentration product of *p*-NA at each plateau can be calculated by the UV absorbance at 410 nm because

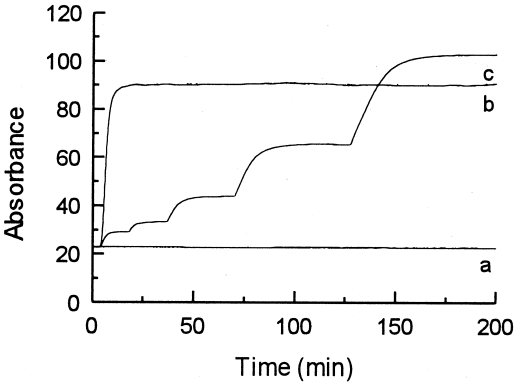


Fig. 1. Elution profiles in frontal affinity chromatography. Curve c is the BAPNA elution pattern of the immobilized trypsin membrane column. Curve a is the BAPNA (0.3 m*M*) elution pattern of the non-immobilized trypsin membrane column. Curve b is the *p*-NA elution pattern by hydrolytic reaction of BAPNA on the immobilized trypsin membrane column. The flow-rates are: 0–18.00 min, 1.6 ml min^{-1}; 18.01–36.00 min, 0.8 ml min^{-1}; 36.01–72.00 min, 0.4 ml min^{-1}; 72.01–126.00 min, 0.2 ml min^{-1}; 126.01–210.00 min; 0.1 ml min^{-1}.

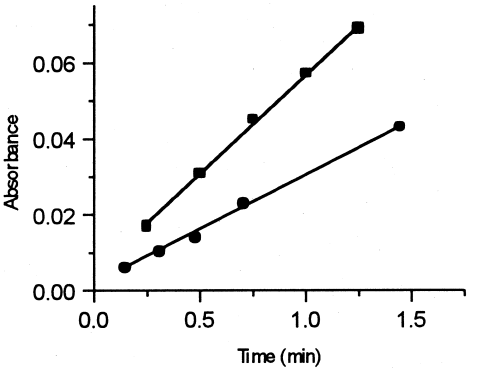


Fig. 2. The hydrolytic activity of free (■) and immobilized trypsin (●) determined from the release of *p*-nitroaniline from DL-BAPNA (0.3 m*M*).

non-hydrolysed BAPNA has no UV absorbance at this detection wavelength. The rate of enzymatic reaction is presented by the amount of *p*-NA formed during the reaction time. The reaction time is defined by the time the substrate resides in the immobilized trypsin membrane column, which is dependent upon the flow-rate in the packed column system. In order to follow the Michaelis–Menten kinetics of the trypsin hydrolysis of BAPNA, the flow-rate is adjusted to keep the experimental conditions within the linear range of the enzyme kinetics. Fig. 2 shows the UV absorbance of BAPNA products by the free and immobilized enzyme at different reaction time, from which it can be calculated that the relative activity of the immobilized trypsin (versus the equivalent free enzyme) was 55.6%. Furthermore, the experimentally obtained data were processed in terms of Michaelis–Menten kinetics by the Hanes plots of [S]/v versus [S] [21] as shown in Fig. 3, where [S] is the substrate concentration and v the velocity of enzymatic reaction. It should be noted that the values of [S] and v can be regarded as the initial values because the total concentration of substrate at the beginning of the reaction is much greater than the amount of the immobilized enzyme. The apparent K_m of immobilized trypsin observed is 0.12 m*M*, which is about 25% of that observed for the free trypsin (0.49 m*M*). The decrease in the K_m values of immobilized enzymes has been widely recognized [20], and has been ascribed to the presence of areas of increased substrate concentration around the polymer due to electrostatic attraction and hydrophobic adsorption of the substrate on the solid materials.

In order to test the validity of the frontal analysis method, both the static method described by Hornby et al. [20] and the off-line detection of products is applied to measure the K_m of immobilized trypsin on membrane material, and the obtained results are listed in Table 1. It can be seen that the K_m values measured by all three methods are very close to each other. However, the V_{max} values measured by the

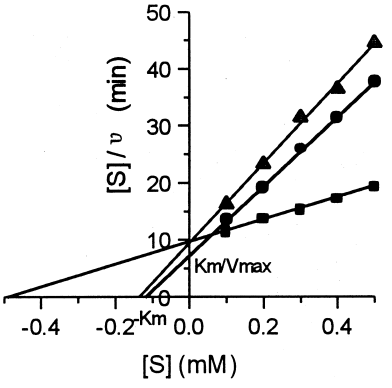


Fig. 3. Hanes plots for free trypsin (■) and immobilized trypsin by the frontal analysis (●) and static methods (△). The enzyme reaction involves BAPNA substrate.

Table 1
Kinetic constants of the immobilized trypsin by various methods

Method	K_m (m*M*)	V_{max} (m*M* min^{-1} mg $enzyme^{-1}$)
Frontal analysis	0.12	0.079
Off-line detection	0.12	0.078
Static method	0.13	0.068

Table 2
Kinetic constants of the immobilized trypsin at different flow-rates

Flow-rate (ml min^{-1})	K_m (m*M*)	V_{max} (m*M* min^{-1} mg $enzyme^{-1}$)
1.6	0.13	0.089
0.8	0.13	0.086
0.4	0.13	0.087
0.2	0.12	0.084
0.1	0.12	0.080

frontal analysis and off-line detection of products are about 15% higher than that by the static method, which is probably resulted from the not completely dynamic mixing during feeding and reaction of substrate in column as that in the static method.

As reported by many authors [1,13–15], the substrate flows through the support and the mass transfer in the membrane column as a result of convective flow is much faster than the conventional porous packing materials caused by the molecular diffusion controlled mass transfer process, which is an important foundation of this developed method. The kinetic reaction constants for enzymatic conversion of BAPNA on the trypsin immobilized membrane column at different flow-rates are measured, and the obtained results are shown in Fig. 4 and the calculated enzymatic reaction constants are listed in Table 2. It can be seen that the effect of flow-rates within 0.1 to 1.6 ml min^{-1} on the apparent K_m is very small, but the V_{max} values slightly decrease with decrease in the flow-rate. Which also support the idea that the mass transfer of substrate in the immobilized enzyme reactor with membrane as solid matrices is mainly controlled by the convective flow, but not by the diffusion rate as in the traditionally porous beads packed column.

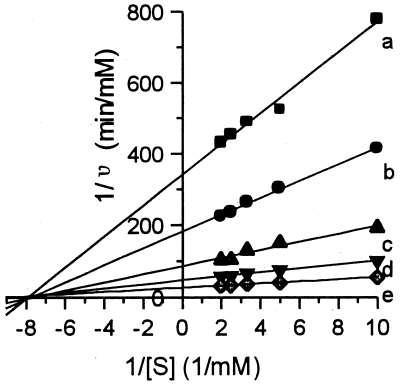


Fig. 4. Lineweaver–Burk plots of immobilized trypsin at different mobile phase flow-rates: (a) 1.6 ml min^{-1}; (b) 0.8 ml min^{-1}; (c) 0.4 ml min^{-1}; (d) 0.2 ml min^{-1}; (e) 0.1 ml min^{-1}.

3.3. Reaction kinetics of BAPNA on the immobilized trypsin inhibited by benzamidine studied by the on-line frontal analysis

Studies of the inhibition of enzymatic reactions have played a dominant role in the elucidation of the specificity and of the kinetics of enzymatic systems. Perhaps the most widely used approach for the determination of inhibitor constants is obtained by treating the experimental data according to the Lineweaver–Burk double reciprocal plot or Dixon method [22,23]. From the slopes and intercepts of these plots drawn for experiments run in the presence and absence of inhibitor, the values of V_{max}, K_m and K_i can be determined. The Dixon method is a simple graphical method that requires only the determination of via two or three substrate concentrations using a series of inhibitor concentrations at each substrate level. The inhibition studies were carried out using 0.1–0.5 m*M* BAPNA and benzamidine in a concentration range of 0.005 to 0.02 m*M*. The hydrolytic activity of the trypsin was measured by the frontal analysis method developed. The kinetics of inhibition depend on the postulated nature of the interaction of the inhibitory compounds with the other components of the enzymatic systems. From the viewpoint of enzyme specificity, the most important type of inhibition is the competitive type where, presumably, substrate and inhibitor compete for the same combing site of the enzyme surface, then such a reaction process can be represented as below:

$$E + S \rightarrow ES \rightarrow E + P$$

$$E + I \rightarrow EI$$

where E, S, ES, P, I and EI denote, respectively, enzyme, free substrate, the enzyme–substrate complex, the traction products, free inhibitor and the enzyme–inhibitor complex. The degree of inhibition depends on both inhibitor and substrate concentrations, and the kinetic constants of reaction can be obtained according to following equation:

$$\frac{1}{v} = \frac{1}{V_{max}} + \left[1 + \frac{[I]}{K_i}\right] \cdot \frac{K_m}{V_{max}} \cdot \frac{1}{[S]}$$

where v is the initial velocity in the presence of the inhibitor, K_i is the dissociation constant of the enzyme–inhibitor complex and [I] is the inhibitor concentration, both in moles per liter.

The data experimentally measured by the frontal analysis method were treated according the Lineweaver–Burk and Dixon plots, respectively. A plot of $(1/v)$ versus [I] from each set of reactions give a series of lines that intersect at the same ordinate intercept (V_{max} remaining constant), whereas the slope was increased by the quantity $(1+[I]/K_i)$. At the intersection, the value of [I] equal to $-K_i$. The obtained results are presented in Figs. 5 and 6. Different straight lines under different benzamidine concentrations were obtained, which are characteristic of competitive inhibition. The K_i of immobilized trypsin calculated from these experiments was 6.0 ± 0.2 μM. The K_i value for benzamidine inhibition of free trypsin was 24.0 ± 0.5 μM. The K_i values for benzamidine and the K_m values for BAPNA determined from the Dixon plots are presented in Table 3. It can be seen that the inhibition constant of benzamidine on the immobilized trypsin is about 25% of that observed for the free enzyme, which is in good agreement with the Michaelis–Menten constant of BAPNA. Those results indicated that the immobilization of enzyme would lead to decrease in the reaction constant of substrate and the inhibition constants of inhibitor simultaneously at the same degree if they are structure related. The Michaelis–Menten constants of both the free and immobilized trypsin for BAPNA in the presence of 0.005 mM benzamidine increase by about 25% from that absence of benzamidine, however, there is almost no effect on the Michaelis–Menten constants with further increase in the benzamidine concentration. As shown in Table 3, the V_{max} values were not affected by the inhibitor concentration of benzamidine, which is the typical characteristic of competitive inhibition.

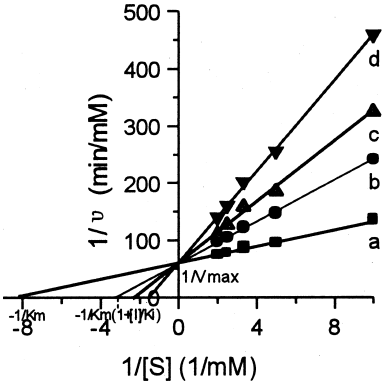


Fig. 5. Lineweaver–Burk plots for benzamidine inhibition of the immobilized trypsin by the frontal analysis method: (a) $C_{benzamidine}=0$ mM; (b) $C_{benzamidine}=0.005$ mM; (c) $C_{benzamidine}=0.01$ mM; (d) $C_{benzamidine}=0.02$ mM.

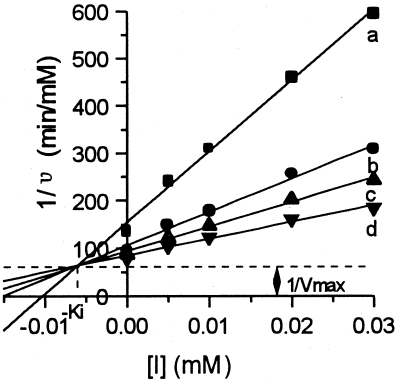


Fig. 6. Graphical determination of benzamidine inhibition constant by the method of Dixon with BAPNA as substrate: (a) $C_{substrate}=0.1$ mM; (b) $C_{substrate}=0.2$ mM; (c) $C_{substrate}=0.3$ mM; (d) $C_{substrate}=0.4$ mM.

4. Conclusion

A novel method to characterize the activity and reaction kinetics of the immobilized enzyme has been developed based on the frontal analysis of enzymatic reaction product, in which the membrane material with mass transfer controlled by the convec-

Table 3
Kinetic constants of the immobilized trypsin evaluated by substrate BAPNA at different inhibitor concentrations of benzamidine

Enzyme status	Benzamidine concentration (mM)	K_m (mM)	V_{max} (mM min^{-1} mg enzyme^{-1})	K_i (μM)
Immobilized trypsin	0	0.12	0.079	6.0±0.2
	0.005	0.17	0.080	
	0.01	0.16	0.079	
	0.02	0.16	0.078	
Free trypsin	0	0.49	0.247	24.0±0.5
	0.005	0.64	0.250	
	0.01	0.68	0.251	
	0.02	0.69	0.253	

tive flow was used as the medium to immobilize the enzyme of trypsin. The apparent Michaelis–Menten kinetic constant (K_m) of BAPNA, the inhibition constant of benzamidine (K_i) and the V_{max} on the immobilized trypsin reactor have been determined simultaneously. Validity of the frontal analysis method has been confirmed by the results obtained from the static method and the off-line detection of products. It was observed that both the Michaelis–Menten constant of BAPNA and the inhibition constant of benzamidine on the immobilized trypsin are about 25% of that observed for the free enzyme, and the effect of flow-rates within 0.1 to 1.6 ml min^{-1} on the apparent K_m and V_{max} values observed on the immobilized trypsin column was negligible.

Acknowledgements

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