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Original Papers

Automated Method, Based on Micro-Sequential Injection, for the Study of Enzyme Kinetics and Inhibition

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A micro-reactor system with continual spectrophotometric detection has been operated in Sequential Injection lab-on-valve (SI-LOV) mode and applied to enzyme kinetics and inhibition studies, using acetylcholinesterase (AChE) and angiotensin-converting enzyme (ACE) as model systems. With the advantages of automation, real-time kinetic measurement, and thorough mixing, the SI-LOV micro-reactor system allows for the monitoring of initial reaction rates and determination of reactant concentrations in the reaction mixture, both of which are essential for the determination of kinetic constants for enzymes and inhibitors. Enzyme, substrate, and inhibitor are precisely metered by the syringe pump and delivered to a stirred micro-reactor, followed by a reference scan that establishes the baseline for the following reaction rate measurement. Michaelis constants (K_m) for AChE and ACE were determined to be 0.16 mM and 0.30 mM, respectively, which are consistent with literature values. The type of inhibition (competitive, uncompetitive, or mixed), the dissociation constants for the inhibitors, and the inhibitor dose-response curves were also determined.

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Introduction

Enzyme kinetics and inhibition studies provide information about the physiological properties of enzymes and the mechanistic properties of enzyme-catalyzed biochemical processes. Many enzyme inhibitors have important pharmaceutical applications. Acetylcholinesterase (AChE) is an enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh) and reduces its concentration at the cholinergic synapses. ACh deficiency has been associated with Alzheimer's disease, a common form of dementia characterized by memory loss, emotional instability, and progressive loss of mental ability.[1,2] AChE inhibitors, such as tacrine, have been used in the treatment of Alzheimer's disease by preventing the degradation of ACh and prolonging its function.[1,2] Edrophonium, another AChE inhibitor, has been used for the diagnosis of Myasthenia Gravis (MG), an autoimmune disease characterized by episodic muscle weakness. MG results when antibodies interfere with the conduction of nerve impulses by binding to ACh receptors, preventing ACh from binding.[3,4] An injection of edrophonium rapidly leads to marked improvement in most people with MG weakness by blocking the action of AChE and increasing ACh levels, allowing the receptors to function more efficiently.

Angiotensin-converting enzyme (ACE), also called dipeptidyl carboxypeptidase, is a proteolytic enzyme that catalyzes the removal of dipeptides from a variety of compounds, as in the conversion of angiotensin I to angiotensin II. Angiotensin I has little biological effect until it is converted to angiotensin II, a potent vasoconstrictor that causes arteries and veins to constrict and builds up blood pressure.[5,6] ACE is also involved in the inactivation of bradykinin, a potent vasodilator that lowers blood pressure by relaxing blood vessels.[7] ACE inhibitors have been used to treat hypertension and heart failure by restraining the action of ACE, which decreases the formation of angiotensin II and the inactivation of bradykinin.

Based on programmable flow, micro-Sequential Injection carried out in the lab-on-valve (SI-LOV) format[8–10] has been used for a number of biomolecular assays, including enzymatic assays aimed at the determination of the concentration of the substrate[10] or the activity of the enzyme.[9] Since the system used for such serial assays is calibrated using a series of standards, processed in the exact same way as unknown samples, it is not necessary to know the exact concentration of the reactants captured in the flow cell. In addition, these assays are based on the reproducibility of fluid handling by the SI system and, thus, complete mixing of different reactants is not required.

While previous uses of SI-LOV system were aimed at the automation of serial assays, the novel aspect of this work is the use of the system to automate a research investigation that will yield numerical values of kinetic constants, obtained by a systematic variation of the reactant concentrations. In enzyme studies aimed at determining kinetic constants, the concentration of each reactant in the reaction mixture must be precisely known, which makes it necessary to thoroughly mix reactants at the onset of measurement. In this work, reactants are delivered to a stirred, batch-mode micro-reactor and mixed thoroughly. The concentrations of the reactants in the micro-reactor are determined by using dye injection experiments. Additionally, maintaining the advantage of SI, SI-LOV micro-reactor system uses fiber optic spectroscopy to monitor the initial rate of reaction in real time, which is also essential for the determination of kinetic constants for enzymes and inhibitors.

Experimental

Instrumentation

The Sequential Injection system (FIAlab-3000, FIAlab

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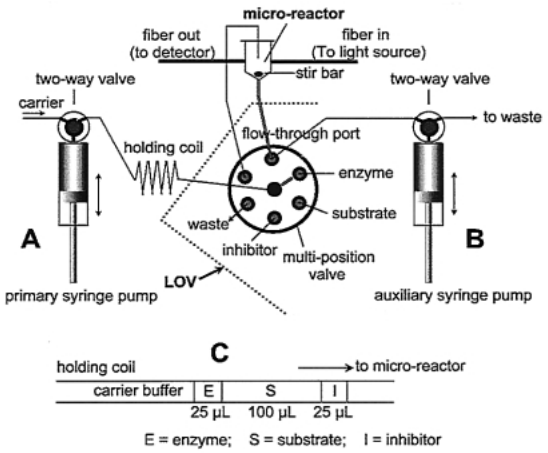

Fig. 1 The Sequential Injection system consists of two syringe pumps (A and B), two two-way valves, a holding coil, and a "lab-on-valve" manifold mounted on a multi-position valve. A stirred micro-reactor is attached to one channel of the flow-through port on the LOV. The fiber-optic cables connect the micro-reactor to the light source and the spectrophotometer. By adjusting the distance between the two fiber-optic probes, the light path of the optical cell is set to 5 mm. The primary syringe pump (A) is used to sequentially aspirate enzyme, reagent, inhibitor, or buffer into the holding coil and subsequently deliver the stacked zones (C) into the micro-reactor by flow reversal. At the end of each measuring cycle the auxiliary syringe pump (B), connected to the other channel of the flow-through port, is used to empty the micro-reactor.

Instruments, Inc., http://www.flowinjection.com) consists of two 2500 µL high-precision, bi-directional syringe pumps, two two-way valves, a holding coil, and a "lab-on-valve" sample-processing unit mounted on a multi-position valve (Fig. 1). A stirred micro-reactor is attached to one channel of the flow-through port on the LOV. The capacity of the micro-reactor is 1000 µL, and a minimum volume of 350 µL is required in order to fully submerge the fiber optic probes. The fiber-optic cables (Ocean Optics, Inc., http://www.oceanoptics.com) connect the micro-reactor to the light source and the spectrophotometer (S2000, Ocean Optics, Inc.). By adjusting the distance between the two fiber-optic probes, the light path of the optical cell is set to 5 mm. The UV light source is a deuterium lamp (Model D 1000, Analytical Instrument Systems, Inc., http://www.aishome.com). FIAlab software (version 5.9.137) is used to control and automate the SI-LOV system.

Materials

The carrier solution was PBS buffer (phosphate buffered saline, pH 7.4) for AChE and HEPES (pH 7.5) for ACE. AChE from electric eel (catalog # C3389), acetylthiocholine (ASCh, # A5751), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, # D218200), tacrine (# A3773), edrophonium (# E3256), ACE from rabbit lung (# A6778), *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG, # F7131), and alanine-proline (Ala-Pro, # A3253) were purchased from Sigma-Aldrich (www.sigma.com). The AChE substrate solution contained equal moles of ASCh and DTNB at various concentrations in PBS buffer. This solution was prepared fresh daily and kept on ice to minimize the spontaneous hydrolysis of ASCh. The ACE substrate solution contained various amounts of FAPGG in HEPES buffer. PBS buffer and HEPES buffer were also used to prepare AChE and ACE inhibitor solutions, respectively. A green fabric dye (097, Procion MX, Jacquard, http://www.jacquardproducts.com) solution was used to determine the dilution factors and concentrations of the reactants by measuring the absorbance at 418 nm. A series of dilutions of the dye were prepared to assure that the absorbance was linearly proportional to the concentration in the range investigated.

Table 1 Measurement protocol

Step	Measurement protocol
1	Aspirate 1500 µL buffer + 25 µL enzyme + 100 µL substrate + 25 µL inhibitor
2	Deliver 370 µL to the stirred micro-reactor by flow reversal
3	Perform reference scan after a delay period
4	Reaction rate measurement
5	Flush the holding coil and empty the reactor

Method

The primary syringe pump (Fig. 1A) was used to aspirate the reactants into the holding coil and subsequently deliver the stacked zones (Fig. 1C) to the micro-reactor by flow reversal. At the end of each measuring cycle, the auxiliary syringe pump (Fig. 1B) was used to empty the micro-reactor *via* the flow-through port. The measurement protocol (Table 1) consisted of five steps: 1) 1500 µL of buffer were aspirated into the holding coil using the primary syringe pump, followed by 25 µL of enzyme, 100 µL of substrate, and 25 µL of inhibitor (for inhibition studies) or buffer (for determination of Michaelis constant). 2) The flow was reversed and the stacked zones were sent to the stirred micro-reactor by pumping a total of 370 µL. 3) Following a delay period (1 s for AChE and 16 s for ACE), a reference scan was performed to establish a baseline. 4) Next, the reaction rate was measured for a suitable period of time (15 s for AChE and 45 s for ACE). 5) At the end of the measurement, the remaining contents in the holding coil were flushed through the waste port and the reactor was emptied by the auxiliary syringe pump. Each experiment was performed in triplicate, and the reactor was rinsed twice with buffer between different runs. The volumes of the reactants, the final volume in the reactor, the activity of the enzymes in the reactor (0.369 U for AChE and 0.0492 U for ACE), and temperature (22˚C for AChE and 25˚C for ACE) were kept the same for all experiments.

The initial slope of the response curve represents the initial rate of the enzymatic reaction. Lineweaver–Burke (double reciprocal) plots of initial rate and substrate concentration were used to determine the Michaelis constants (K_m) for the enzymes, inhibition type, and the dissociation constants for the inhibitors.[11] Inhibitor dose-response curves were constructed by plotting the percent remaining activity of the enzyme against the logarithm of the inhibitor concentration.

Results and Discussion

Monitoring of the enzymatic reactions

The activity of AChE was measured spectrophotometrically using a method developed by Ellman *et al.*[12] The method employs acetylthiocholine (ASCh) as a synthetic substrate of AChE. ASCh is hydrolyzed to produce thiocholine (reaction (1)), which reacts with a chromogen, DTNB, to yield a yellow product (reaction (2)) that can be monitored by measuring the increase in absorbance at 412 nm.

$$\text{ASCh} \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetate} \quad (1)$$

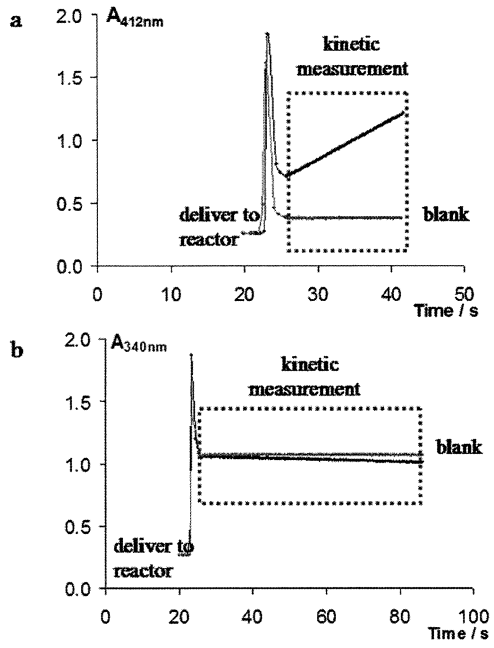

Fig. 2 Complete response curves of AChE (a) and ACE (b) enzymatic reactions in the absence of inhibitor. The reference scans in these initial experiments were performed when the reactor was filled with buffer. The initial peak was due to change in refractive index as the reactants entered the micro-reactor. Once the reactor was filled, the slope of the linear response was due to the enzymatic reactions. Blank values were obtained by performing the experiments in the absence of the enzymes. The reactions of AChE resulted in a rapid increase in absorbance while the reaction of ACE produced a slow decrease in absorbance. Substrate concentrations were 0.420 mM (a) and 0.146 mM (b). All concentrations in this work refer to the concentrations in the micro-reactor. In subsequent experiments, data was only collected for the kinetic portion of the response curve.

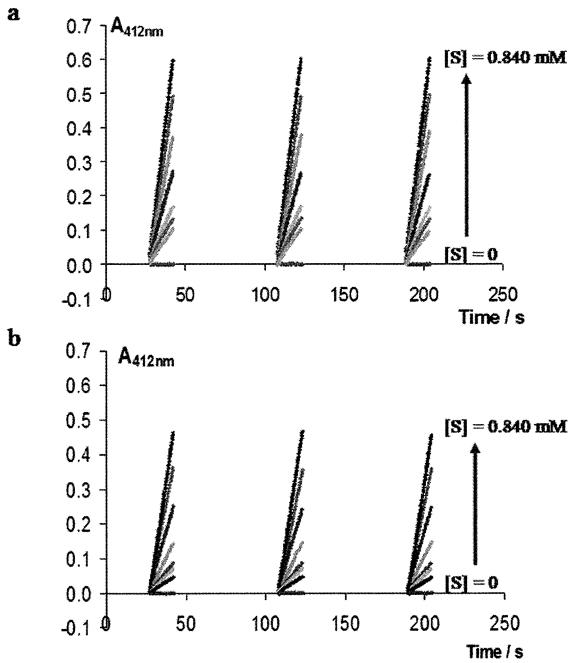

Fig. 3 Superimposed kinetic response curves of AChE reactions at different substrate concentrations in the absence (a) and presence (b) of inhibitor, edrophonium, at 0.115 μM. Triplicate runs. The substrate concentrations were 0, 0.0263, 0.0394, 0.0526, 0.105, 0.210, 0.420, and 0.840 mM.

$$\text{Thiocholine} + \text{DTNB} \longrightarrow \text{2-nitrobenzoate-5-mercaptothiocholine} + \text{5-thio-2-nitrobenzoate (yellow, 412 nm)} \quad (2)$$

For ACE, its substrate *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycylglycine (FAPGG) was used for the continuous spectrophotometric monitoring of the enzyme activity.[13] Hydrolysis of FAPGG (reaction (3)), the rate of which is directly proportional to ACE activity, results in a decrease in absorbance at 340 nm.

$$\text{FAPGG} \xrightarrow{\text{ACE}} \textit{N}\text{-[3-(2-furyl)acryloyl]-L-phenylalanine (FAP)} + \text{glycylglycine (GG)} \quad (3)$$

Stopped-flow techniques,[8] in which stacked reactants in the holding coil are directly transported into the flow cell and stopped there for measurement, have been used for serial enzymatic assays of glycerol,[9] glucose,[9,10] and ethanol.[10] For the present study, however, it is necessary to modify the stopped-flow technique by using a micro-reactor within which the injected zones are thoroughly mixed, since the concentration of reactants in the mixture must be known in order to calculate the kinetic constants. Figure 2 shows the complete response curves that are comprised of two distinct sections. The initial peak was due to change in refractive index as the reactants entered the micro-reactor. Once the reactor was filled with thoroughly mixed reactants, the slope of the linear response was due to the enzymatic reactions.

For both AChE and ACE, blank values were obtained by performing the experiments in the absence of the enzymes and a horizontal line was observed in both cases (Figs. 2a and b). The reference scans in these initial experiments were performed when the micro-reactor was filled with buffer. In the presence of the enzyme, the reaction of AChE was fast and a rapid increase in absorbance was observed. Since the reactants were already partially mixed and beginning to react on the way to the reactor, the starting point of the enzymatic reaction curve was elevated compared to the blank value (Fig. 2a). In subsequent experiments (Fig. 3), the reference scans were performed after the reactants were delivered to the micro-reactor, so that all kinetic curves have the same starting point. In contrast to AChE, the reaction of ACE was notably slower, and the reaction rate curve fell gradually under the level of the blank (Fig. 2b). The advantage of stopped-flow kinetic measurement is emphasized by these experiments, showing that small changes in absorbance, in the presence of high background, can be reliably detected and used for reaction rate measurement. In the case of ACE, the high background absorbance of the substrate would make it very difficult to detect the changes in absorbance over the same time period using endpoint measurements.

Determination of dilution factors of reactants

Since the concentrations of substrates and inhibitors in the micro-reactor must be known for computation of the kinetic constants, and only part of the zones stacked in the holding coil was delivered to the micro-reactor to avoid excessive dilution, dye injection experiments were conducted to determine the dilution factors of the reactants. For example, in order to determine the dilution factor of the substrate, a dye solution was used as a substitute for the substrate solution, while colorless buffer was used as substitutes for the enzyme and inhibitor solutions. Using the same protocol as outlined in Table 1,

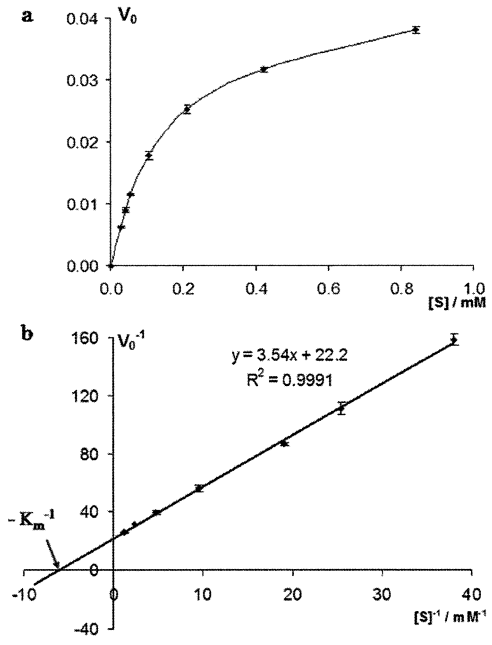

Fig. 4 (a) Michaelis–Menten plot of AChE in the absence of inhibitor. The plot shows the relationship between initial rate and substrate concentration. (b) Corresponding Lineweaver–Burk plot. The K_m for AChE was determined to be 0.16 mM using the x intercept of the Lineweaver–Burk plot. The unit of reaction rate in this work is (absorbance unit)/s.

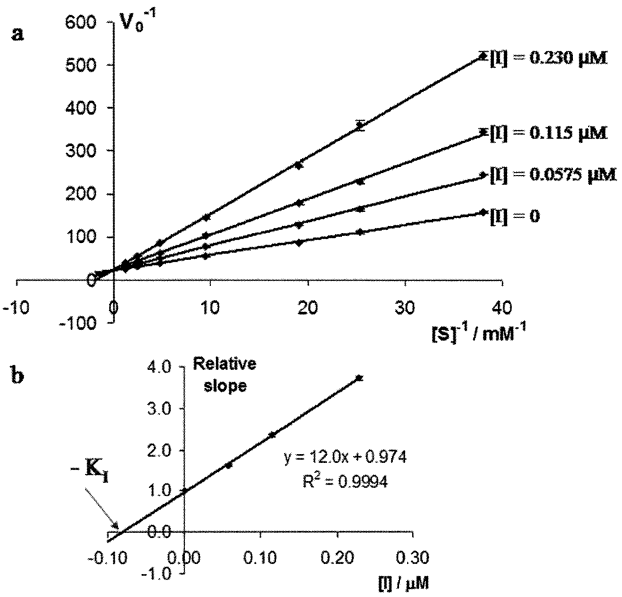

Fig. 5 (a) Superimposed Lineweaver–Burk plots at four different edrophonium concentrations (0, 0.0575, 0.115, and 0.230 μM). The four lines intersect at the same point on the y-axis, which shows that edrophonium is a competitive inhibitor of AChE.[11] (b) Plot of the relative slope of the Lineweaver–Burk plot *versus* edrophonium concentration. The plot gives a straight line (relative slope = 1 + [I]/K_I), with the x-intercept equal to $-K_I$. The K_I for edrophonium was determined to be 0.081 μM.

except that the reference scan was performed when the reactor was filled with buffer, the absorbance of the mixture (A_s) was determined to be 0.284. Next, the same dye solution was directly pipetted into the micro-reactor without any dilution,

Table 2 Different types of enzyme inhibition

Inhibition type	$K_{m,app}$	$V_{max,app}$	Relative slope $(K_{m,app}/V_{max,app})/(K_m/V_{max})$	Relative y-intercept $(1/V_{max,app})/(1/V_{max})$
Competitive	αK_m	V_{max}	α	1
Uncompetitive	K_m/α'	V_{max}/α'	1	α'
Mixed	$\alpha K_m/\alpha'$	V_{max}/α'	α	α'

$K_{m,app}$ = apparent K_m in the presence of inhibitor.
$V_{max,app}$ = apparent V_{max} in the presence of inhibitor.
$\alpha = 1 + [I]/K_I$; $\alpha' = 1 + [I]/K_I'$, where [I] = inhibitor concentration, K_I = competitive dissociation constant for inhibitor, K_I' = uncompetitive dissociation constant for inhibitor.
Relative slope: slope of the Lineweaver–Burk plot in the presence of inhibitor divided by that in the absence of inhibitor.
Relative y-intercept: y-intercept of the Lineweaver–Burk plot in the presence of inhibitor divided by that in the absence of inhibitor.

yielding an absorbance (A_0) of 1.17. The dilution factor for the substrate was then calculated, by using the ratio of A_s to A_0, to be 0.243. In a similar fashion, the dilution factors for the enzyme and the inhibitor were determined to be 0.0492 and 0.0661, respectively.

The dye experiments explained above also proved that the solutions in the micro-reactor were completely mixed before kinetic measurements took place. After the dye solution and buffer were injected to the micro-reactor, the absorbance value remained constant throughout the data collection. If the solutions were still being mixed during the measurement, the absorbance value would have changed until it reached a constant value at which the solutions were fully mixed.

Acetylcholinesterase (AChE)

Determination of Michaelis constants (K_m). The relationship between the substrate concentration ([S]) and the initial rate (V_0) of the enzymatic reaction is expressed by the Michaelis–Menten equation (Eq. (1)), in which V_{max} is the maximum reaction rate, and K_m is the Michaelis constant for the enzyme.

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

The Michaelis–Menten equation can be rearranged into equations more useful in plotting experimental data, such as the Lineweaver–Burk equation (Eq. (2)), in which V_0^{-1} is directly proportional to $[S]^{-1}$.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

In this work, the initial reaction rate was determined from the initial slope of the kinetic response curve (Fig. 3a), with the unit equal to (absorbance unit)/s. The Michaelis–Menten and Lineweaver–Burk plots of AChE in the absence of inhibitor are shown in Figs. 4a and 4b, respectively. Using the x-intercept of the Lineweaver–Burk plot, the K_m value for AChE was determined to be 0.16 mM, which is consistent with literature value 0.173 mM.[14]

Determination of inhibition type and dissociation constants (K_I and K_I') for the inhibitors. Reversible inhibition can be competitive, uncompetitive, or mixed, depending on the interaction of the inhibitor with the enzyme and the enzyme-substrate complex.[11] In each type of inhibition, K_m and V_{max} are

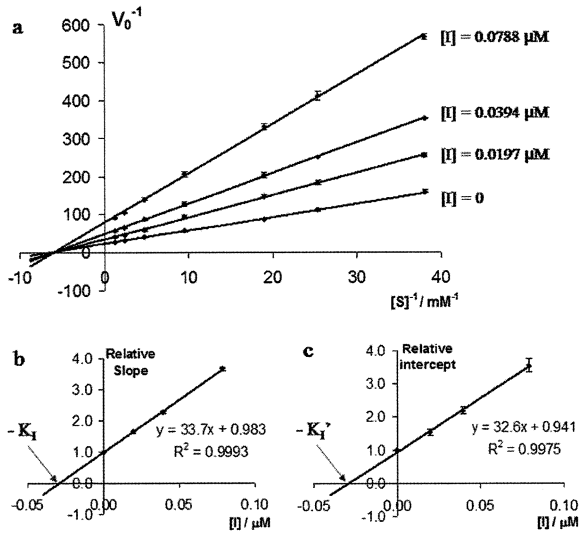

Fig. 6 (a) Superimposed Lineweaver–Burk plots at four different tacrine concentrations (0, 0.0197, 0.0394, and 0.0788 μM). The four lines intersect at the same point on the *x*-axis, which shows that tacrine is a noncompetitive inhibitor ($K_I = K_I'$) of AChE.[11] (b) Plot of the relative slope of the Lineweaver–Burk plot *versus* tacrine concentration. The plot gives a straight line (relative slope = 1 + [I]/K_I). K_I for tacrine was determined to be 0.029 μM using the *x*-intercept. (c) Plot of the relative *y*-intercept of the Lineweaver–Burk plot against tacrine concentration. The plot also gives a straight line (relative *y*-intercept = 1 + [I]/K_I'). K_I' for tacrine was determined to be also 0.029 μM using the *x*-intercept.

affected differently (Table 2). Non-competitive inhibition, in which $\alpha = \alpha'$ and $K_I = K_I'$ (see Table 2 for definitions of α, α', K_I, and K_I'), is a special case of mixed inhibition.

The inhibition of AChE by edrophonium was investigated at four different inhibitor concentrations (0, 0.0575, 0.115, and 0.230 μM). As an example, superimposed response curves in the presence of 0.115 μM edrophonium are shown in Fig. 3b. Superimposed Lineweaver–Burk plots at four different edrophonium concentrations are shown in Fig. 5a. The four lines intersect at the same point on the *y*-axis,[11] showing that edrophonium is a competitive inhibitor of AChE. The replot (Fig. 5b) of the relative slope (see Table 2 for definition) of the Lineweaver–Burk plot *versus* edrophonium concentration gives a straight line (relative slope = 1 + [I]/K_I), with the *x*-intercept equal to $-K_I$. The K_I for edrophonium was determined to be 0.081 μM.

For another AChE inhibitor, tacrine, the superimposed Lineweaver–Burk plots at different inhibitor concentrations (0, 0.0197, 0.0394, and 0.0788 μM) are shown in Fig. 6a. The four lines intersect at the same point on the *x*-axis,[11] showing that tacrine is a noncompetitive inhibitor ($K_I = K_I'$) of AChE. Similar to edrophonium, the K_I for tacrine (0.029 μM) was determined by plotting the relative slope *versus* tacrine concentration (Fig. 6b). To determine K_I', the relative *y*-intercept (see Table 2 for definition) of the Lineweaver–Burk plot was plotted against tacrine concentration (Fig. 6c). This plot also gives a straight line (relative *y*-intercept = 1 + [I]/K_I'). The K_I' for tacrine was determined to be 0.029 μM using the *x*-intercept of the plot.

Dose-response curves. Figure 7 shows the dose-response curves of inhibition of AChE by edrophonium and tacrine. The percent remaining activity of the enzyme is plotted against the logarithm of the inhibitor concentration. The percent remaining activity was calculated as the ratio of the initial rate in the presence of the inhibitor to that in the absence of the inhibitor. The curves have a characteristic sigmoidal shape. Tacrine produced more inhibition than edrophonium at the same concentration and appeared to be a more potent inhibitor of AChE.

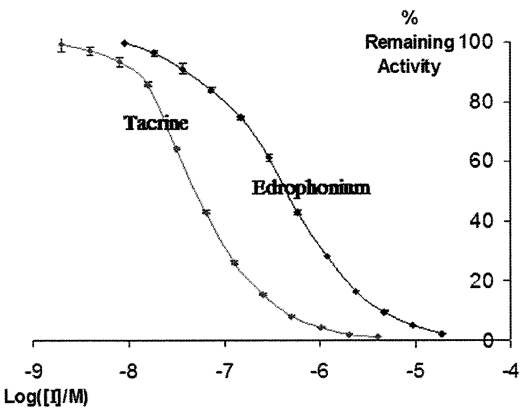

Fig. 7 Dose-response curves of edrophonium and tacrine. The percent remaining activity of the enzyme is plotted against the logarithm of the inhibitor concentration. The percent remaining activity was calculated as the ratio of the initial rate in the presence of the inhibitor to that in the absence of the inhibitor. The substrate concentration was 0.840 mM.

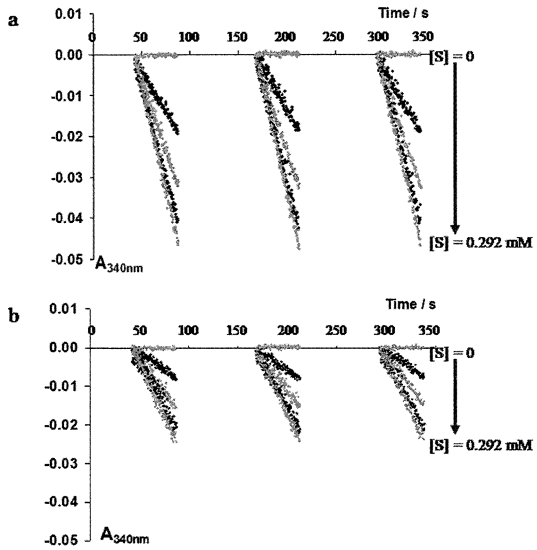

Fig. 8 Superimposed response curves of ACE reactions at different substrate concentrations in the absence (a) and presence (b) of inhibitor, Ala-Pro, at 49.7 μM. Triplicate runs. The substrate concentrations were 0, 0.0729, 0.146, 0.219, and 0.292 mM.

Angiotensin-converting enzyme (ACE)

The SI-LOV micro-reactor system was applied to the kinetics and inhibition studies of a different enzyme system, ACE, using the same protocol as for AChE but with a longer monitoring period appropriate for the slower reaction. Figure 8 shows the superimposed response curves of the enzymatic reactions in the (a) absence and (b) presence of the inhibitor, Ala-Pro, at 49.7 μM. Compared to AChE, the reaction of ACE with its substrate was much slower and the absorbance decreased as a result of

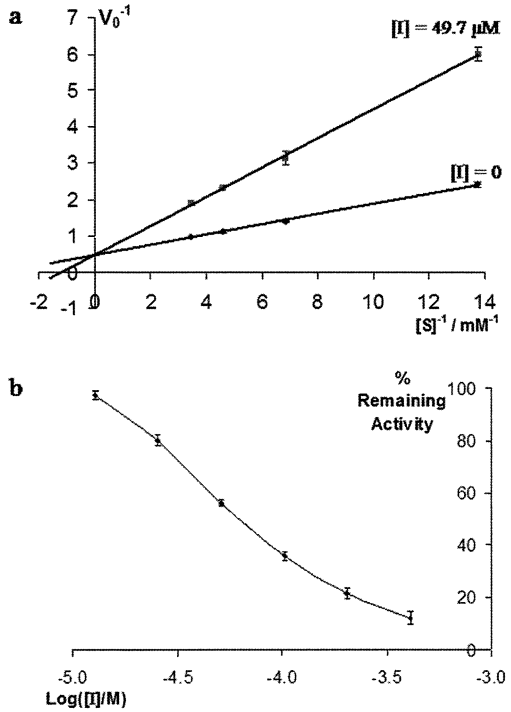

Fig. 9 (a) Superimposed Lineweaver–Burk plots in the absence and presence of Ala-Pro at 49.7 µM. Ala-Pro appeared to be a competitive inhibitor of ACE.[11] The K_m for ACE was determined to be 0.30 mM, and the K_I for Ala-Pro was determined to be 27 µM. (b) Dose-response curve of Ala-Pro. The substrate concentration was 0.292 mM.

the enzymatic reaction. Figure 9 shows the superimposed Lineweaver–Burk plots of ACE reactions in the absence and presence of Ala-Pro (a) and the dose-response curve of Ala-Pro (b). With the two lines of the Lineweaver–Burk plot intersecting on the *y*-axis,[11] Ala-Pro appeared to be a competitive inhibitor of ACE. The K_m for ACE was determined to be 0.30 mM, consistent with literature value 0.30 mM.[13] The K_I for Ala-Pro was determined to be 27 µM.

Conclusion

Since its introduction in 1989, Sequential Injection technology has been used for serial assays for environmental,[15,16] pharmaceutical,[17,18] and process monitoring applications.[9,19] In this work, the SI-LOV technique was combined with a micro-reactor system for the first time, with the aim to carry out enzyme kinetics and inhibition studies under well-controlled conditions. While maintaining the advantages of flow-mode SI-LOV, such as automation and real-time measurement, the stirred, batch-mode micro-reactor ensures thorough mixing, which makes it possible to determine reactant concentrations that are essential for the determination of kinetic constants. While AChE and ACE were used as model systems in this work, the SI-LOV micro-reactor system can also be used to study other enzymes with spectrophotometric detection, to perform automated kinetic assays of reaction systems not involving enzymes, and to carry out automated micro-scale titrations, which is an ongoing project in our research group.

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