Measurement of Enzyme Kinetics Using a Continuous-Flow Microfluidic System

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This paper describes a microanalytical method for determining enzyme kinetics using a continuous-flow microfluidic system. The analysis is carried out by immobilizing the enzyme on microbeads, packing the microbeads into a chip-based microreactor (volume ~1.0 nL), and flowing the substrate over the packed bed. Data were analyzed using the Lilly−Hornby equation and compared to values obtained from conventional measurements based on the Michaelis−Menten equation. The two different enzyme-catalyzed reactions studied were chosen so that the substrate would be nonfluorescent and the product fluorescent. The first reaction involved the horseradish peroxidase-catalyzed reaction between hydrogen peroxide and *N*-acetyl-3,7-dihydroxyphenoxazine (amplex red) to yield fluorescent resorufin, and the second the *β*-galactosidase-catalyzed reaction of nonfluorescent resorufin-*β*-D-galactopyranoside to yield D-galactose and fluorescent resorufin. In both cases. the microfluidics-based method yielded the same result obtained from the standard Michaelis−Menten treatment. The continuous-flow method required ~10 μL of substrate solution and 10^9 enzyme molecules. This approach provides a new means for rapid determination of enzyme kinetics in microfluidic systems, which may be useful for clinical diagnostics, and drug discovery and screening.

Microfluidic chip-based devices have been developed for a number of important assays, including DNA analysis,[1,2] protein analysis,[3,4] and immunoassays.[5−7] During the past decade it was demonstrated that, compared to traditional analytical instruments, the microfluidic platform provides a means for reducing both analysis time and the amount of reagent necessary to perform each analysis.[8−11] However, profitable commercialization of microanalytical systems awaits development of methods that capitalize upon these enhancements without introducing complex problems that outweigh the advantages. Here, we address this need by describing an analytical method for measuring enzyme kinetics using a continuous-flow microfluidic system. In this approach, a microfluidic channel is filled with enzyme-modified microbeads, and then a nonfluorescent substrate is moved over the bead bed by convective flow. It is possible to correlate the enzyme reaction rate to the appearance of the fluorescent product of the enzyme-catalyzed reaction. Accordingly, this method is potentially useful for high-throughput screening of enzymatic activity.

The first report of using a microfluidic system for analyzing the kinetics of an enzymatic reaction was provided by Hadd et al.,[12] who used computer-controlled electrokinetic transport for diluting and mixing reagents. Specifically, precise concentrations of a fluorogenic substrate were mixed with *β*-galactosidase, and the kinetics of the reaction was obtained by monitoring the fluorescence of the hydrolysis product. More recently, Duffy et al.[13] developed a centrifugal microfluidic system capable of carrying out multiple simultaneous homogeneous enzymatic assays using colorimetric detection.

Most previous quantitative reports of enzyme activity measured using microfluidic systems have been carried out in homogeneous solutions.[14−18] However, heterogeneous assays using immobilized enzymes have the potential for providing the following advantages: ease of enzyme placement within arrays, ability to assay membrane proteins (which of course is not possible in homogeneous solution), simplification of enzyme recycling, and continuous-flow analysis. As discussed later, a potential drawback of our approach is that immobilization of enzymes may alter their

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10.1021/ac034155b CCC: $25.00
Published on Web 00/00/0000

intrinsic kinetic rates. Interestingly, only a few reports describe the use of biomolecules immobilized either directly on the walls of microchannels or on a support contained within the channel. For example, a very early report from Karube and co-workers described a sensor for glucose detection based on immobilization of glucose oxidase on the walls of a micromachined capillary.[19] More recently, Harrison et al. showed that a microfluidic device containing trypsin immobilized on beads could be used to digest proteins prior to analysis by mass spectrometry.[20] Mao et al. reported a method for immobilizing enzymes on fluid bilayers supported on the walls of microfluidic channels and then evaluating their kinetics in one shot using laminar flow-controlled dilution.[21] Finally, Peterson et al. reported the preparation of reactive porous monoliths within microchannels, their use as supports for the immobilization of trypsin, and the characterization of the resulting conjugates for the digestion of proteins.[22]

In previous reports, we described microbead-based microfluidic systems for carrying out multiple, sequential enzymatic reactions and DNA hybridization analyses.[23,24] A key finding of these studies is that microbeads offer the convenience of fluidic handling, a high degree of synthetic and characterization flexibility, very high surface-to-volume ratio compared to open-channel microfluidic devices, and the capacity to efficiently mix reagents even under laminar flow conditions.

The intrinsic kinetics of soluble enzymes in homogeneous solution are generally described by the Michaelis−Menten relationship.[25] However, the kinetics for immobilized enzymes may be different from those measured for the same enzyme in solution because of diffusional restrictions or interactions with the support. The kinetics of immobilized biocatalysts are often referred to as the apparent kinetics. Under steady-state conditions, the apparent kinetic parameters in packed-bed enzyme reactors are usually calculated using a formalism known as the Lilly−Hornby model.[26]

In the experiments reported here, horseradish peroxidase (HRP) was immobilized on microbeads via streptavidin−biotin conjugation, and the kinetics of the HRP-catalyzed reaction between hydrogen peroxide and *N*-acetyl-3,7-dihydroxyphenoxazine (amplex red) was monitored under continuous-flow conditions. The results compare favorably to literature values and to values we obtained using conventional analysis methods. To investigate the generality of this approach, the β-galactosidase (β-Gal) catalyzed hydrolysis of resorufin β-D-galactopyronoside (RBG) was also studied.

EXPERIMENTAL SECTION

Chemicals. Positive photoresist (AZP4620) and developer solution (AZ421K) were obtained from the Clariant Co. (Somerville, NJ). Poly(dimethylsiloxane) (PDMS) microfluidic devices were fabricated by curing the prepolymer components of Sylgard 184 (Dow Corning, Midland, MI). Microbeads were Proactive Streptavidin-Coated Microspheres (Bangs Laboratories, Fishers, IN) that were found by optical microscopy to be 15.5 μm in diameter. Biotin-labeled horseradish peroxidase, biotin-labeled β-galactosidase, and sodium resorufin (7-hydroxy-3*H*-phenoxazin-3-one) were purchased from the Sigma Chemical Co. (St. Louis, MO). Amplex red (*N*-acetyl-3,7-dihydroxyphenoxazine) and resorufin β-D-galactopyranoside were obtained from Molecular Probes (Eugene, OR). Hydrogen peroxide (30%) and dimethyl sulfoxide (DMSO) were obtained from EM Science (Gibbstown, NJ). All chemicals were of reagent grade quality or better. Milli-Q (Millipore) water (18 MΩ·cm) was used to prepare aqueous solutions.

Fabrication of Microfluidic Devices. The fabrication of microfluidic devices and the associated weir structures were described in our previous reports.[23,24] Briefly, positive photoresist was spin-coated onto a glass slide and illuminated with UV radiation through a mask for 5 min to create a master. A slit-type photomask having a width of 100 μm was aligned on the glass master to fabricate weirs within the microchannels. After UV exposure for 5 min, the glass master was developed in 60% AZ421K solution for 30 s. PDMS polymer molds were prepared by casting a PDMS prepolymer against a positive photoresist master and curing at 65 °C for 2 h. The PDMS mold was irreversibly sealed to a clean glass substrate after both were treated with an oxygen plasma (60 W, model PDC-32G, Harrick Scientific, Ossining, NY) for 60 s. The depth and width of the microchannel and weir were determined using a Veeco Dektak 3 profilometer (Veeco Instruments, Plainview, NY).

Enzyme Immobilization on Microbeads. HRP and β-Gal were conjugated to biotin via amide coupling of an aminocaproyl spacer. HRP−biotin (240 units/mg) and β-Gal-biotin (350−700 units/mg) contain 2−3 mol of biotin/mol of HRP and 2−4 mol of biotin/mol of β-Gal, respectively. The conjugation of streptavidin-coated microbeads with the biotinylated enzymes was carried out using the following procedure. A 30-μL sample of stock beads (4.8×10^6 bead/mL) were rinsed in 200 μL of phosphate buffered saline (PBS) solution (pH 7.4, 150 mM NaCl, 4 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4) and centrifuged at 4000 rpm for 5 min. The microbead pellet was resuspended in 30 μL of PBS, and then 30 μL of the biotinylated enzymes (1 mg/mL) in PBS (pH 7.4) was added to the microbeads to yield the microbead−enzyme complex. The mixture of microbeads and biotinylated enzymes was incubated for 2 h at room temperature (18−25 °C) with gentle mixing. After conjugation, the mixture was centrifuged to remove unreacted biotinylated enzymes and resuspended in 500 μL PBS. These microbead−enzyme complexes were stored at 4 °C prior to use.

Enzyme Kinetic Assays. To carry out enzyme assays in continuous-flow microfluidic systems, microbead−enzyme complexes were packed into on-chip microreactors using pump-driven pressure. Stock solutions of amplex red and RBG were prepared in DMSO and stored at −20 °C. Immediately before use, the thawed stock solutions were diluted in buffer solution. Various concentrations of H_2O_2 (in 50 mM Tris-HCl plus 10 μM amplex red, at pH 7.4) and RBG (in 100 mM Tris-HCl, 2 mM KCl, and 0.1 mM $MgCl_2$, at pH 7.8) were introduced into the microchannels at various flow rates using a microsyringe pump. Optical and fluorescence images of the enzyme reactions were acquired with

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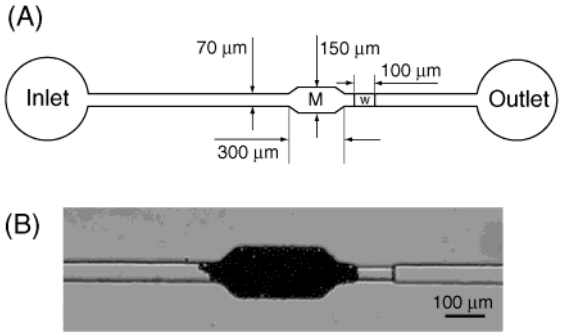

Figure 1. (A) Schematic illustration of the microfluidic device used for analysis of enzyme kinetics. (B) Optical image of a microchamber after packing with microbeads. The regions labeled M and W are, respectively, the microreactor and weir.

a fluorescence microscope (Nikon Eclipse TE 300, Nikon Co., Tokyo, Japan), equipped with band-pass filters (a cube-type XF 102-2 filter set, Omega Optical, Inc., Brattleboro, VT), a 100-W mercury lamp, and a charge-coupled device camera (Photometrics Ltd., Tucson, AZ). Fluorescence images were analyzed using V++ precision digital imaging software (Digital Optics, Auckland, New Zealand).

To compare results of continuous-flow, on-chip enzyme assays with a conventional homogeneous assay method, the HRP-catalyzed reaction between H_2O_2 and amplex red, and the hydrolysis of β-Gal and RBG, were performed by monitoring the fluorescence of resorufin at 563 nm using a conventional spectrofluorometer. The fluorescence signal was calibrated using a standard solution of resorufin.

RESULTS AND DISCUSSION

A schematic illustration of the microfluidic device used in this study is shown in Figure 1A. The device was fabricated from PDMS using standard photolithographic and replica molding methods[27] (fabrication details are provided in the Experimental Section). The key components of the device are the microreactors (labeled M in Figure 1A) and weirs (labeled W in Figure 1A). The width and depth of the weirs ranged from 40 to 60 μm and 7 to 12 μm, respectively. Therefore, microspheres having a diameter of 15.5 μm are retained within microreactors (Figure 1B). Microspheres were coated with streptavidin, a tetrameric protein having four biotin binding sites, so that nucleic acids or proteins modified with biotin could be conjugated to the microsphere surface. Microspheres were introduced into microreactor using pump-driven pressure. The volume of the microreactor was 1.0 nL, and 250−300 microbeads could be packed into each microreactor.

Oxidase enzymes, and particularly HRP, are used in a wide variety of bioassays. Accordingly, we chose to benchmark this continuous-flow study of enzyme kinetics using the HRP-catalyzed reaction between H_2O_2 and amplex red. In the presence of HRP, amplex red reacts with H_2O_2 in a 1:1 stoichiometry to produce highly fluorescent resorufin (Figure 2A).[28]

Before carrying out a continuous-flow study of enzyme kinetics, the inner walls of the microdevice were passivated against

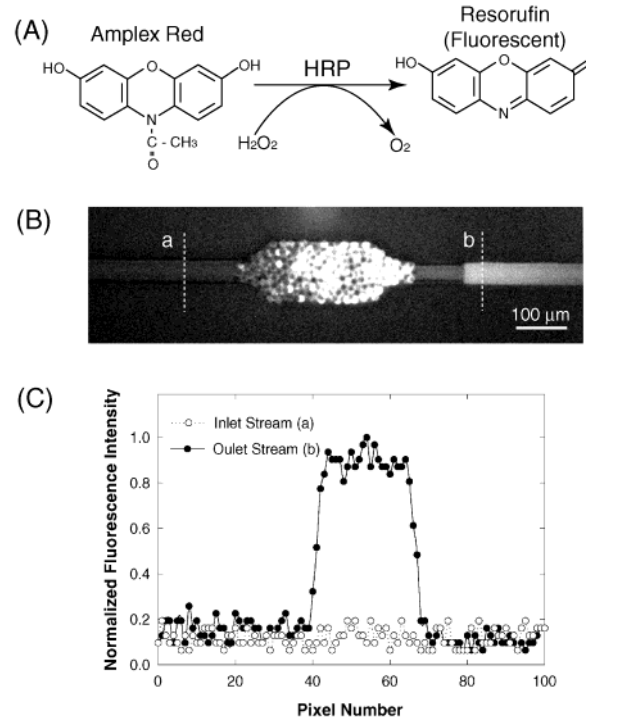

Figure 2. (A) HRP-catalyzed reaction between nonfluorescent amplex red and H_2O_2 to yield fluorescent resorufin. (B) Fluorescence micrograph of the microreactor during continuous-flow operation. The substrate solution contained 5 μM H_2O_2 and 10 μM amplex red in 50 mM Tris-HCl buffer (pH 7.4) and was introduced into the microreactor from left to right. Flow rate, 0.5 μL/min. (C) Normalized fluorescence intensity line scans obtained at the locations indicated by the dashed lines in (B). The excitation and maximum emission wavelengths were 563 and 587 nm, respectively.

nonspecific adsorption of proteins with 1 mg/mL BSA in PBS solution for 3 h at room temperature. Biotin-labeled HRP was then conjugated to the streptavidin-coated microspheres, and these modified beads were packed into the microreactor. Aqueous solutions containing 5 μM H_2O_2 and 10 μM amplex red in 50 mM Tris-HCl buffer (pH 7.4) were introduced into the main channel at a flow rate of 0.5 μL/min from the inlet reservoir. Figure 2B shows a fluorescence image of the resulting HRP-catalyzed reaction in the continuous-flow microreactor. Fluorescence line scans (Figure 2C) taken across the inlet and outlet streams (obtained at positions a and b, respectively, in Figure 2B) indicate that amplex red is a fluorogenic substrate having very low background fluorescence but that its fluorescent product, resorufin, exhibits strong fluorescence.

Enzyme kinetics were evaluated using the Lilly−Hornby model, which was developed for packed-bed reactor systems.[26] This model is embodied in eq 1. Here, f is the fraction of substrate

$$f[A_0] = C/Q + K_{m(app)} \ln(1 - f) \qquad (1)$$

converted to product during the reaction, Q is the flow rate of the substrate, $[A_0]$ is the initial substrate concentration, C is the reaction capacity of the microreactor, and $K_{m(app)}$ is the apparent Michaelis constant. The value of f was determined experimentally by subtracting the fluorescence signal in the inlet stream from the fluorescence signal in the outlet stream of the microreactor.

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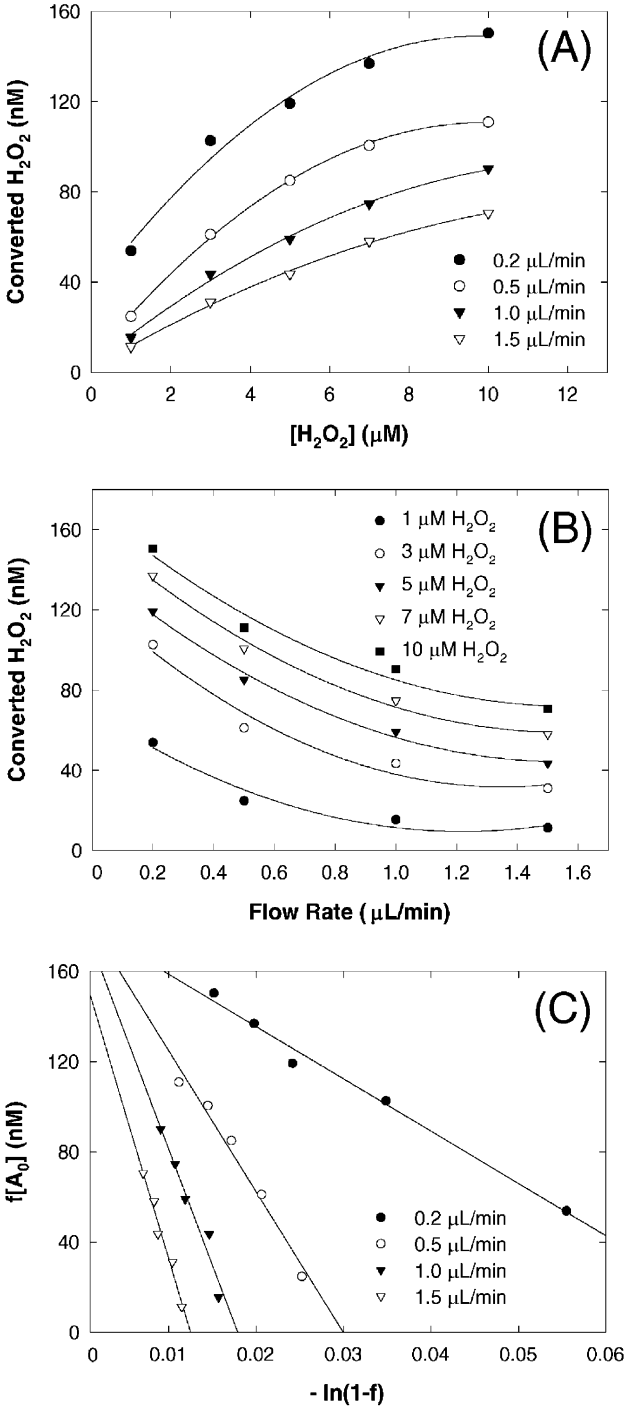

Figure 3. Analysis of data obtained under continuous-flow conditions for the HRP-catalyzed reaction between H_2O_2 and amplex red to yield fluorescent resorufin. The HRP-functionalized microbeads were packed in a microreactor like that shown in Figure 2B. (A) Plots of the concentration of H_2O_2 converted to product vs the initial H_2O_2 concentration as a function of flow rate (Q). The flow rates ranged from 0.20 to 1.5 μL/min. (B) Plots of the concentration of H_2O_2 converted to product vs flow rate as a function of the concentrations of H_2O_2. The concentrations of H_2O_2 ranged from 1.0 to 10.0 μM. (C) Plots of $f[A_0]$ vs $-\ln(1 - f)$. The flow rates ranged from 0.20 to 1.5 μL/min.

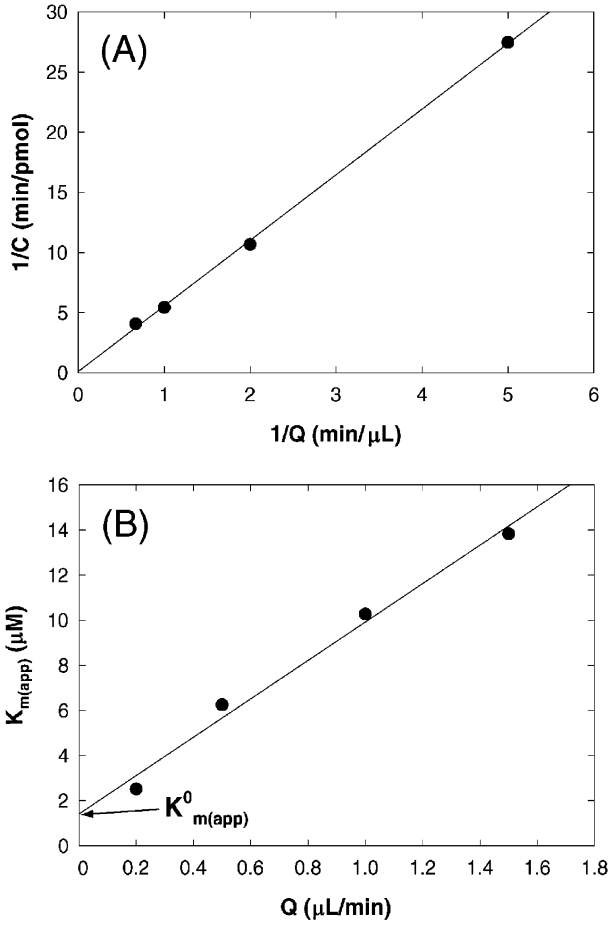

Figure 4. Analysis of data obtained under continuous-flow conditions for the HRP-catalyzed reaction between H_2O_2 and amplex red to yield fluorescent resorufin. (A) Plot of $1/C$ (C is the microreactor capacity) vs $1/Q$ (Q is the flow rate). (B) Plot of $K_{m(app)}$, the apparent Michaelis constant, vs Q.

Table 1. Effect of Flow Rate, Q, on $K_{M(app)}$ and the Reactor Capacity, C, of the Microreactor for the HRP-Catalyzed Reaction

Q (μL/min)	$K_{m(app)}$ (μM)	C (fmol/min)
0.2	2.32	36.4
0.5	6.25	93.5
1.0	10.3	184
1.5	13.0	245

Because the appearance of resorufin and the disappearance of H_2O_2 are related by a 1:1 stoichiometry (Figure 2A), the turnover of H_2O_2 could be evaluated from calibration curves obtained from known concentrations of resorufin and its corresponding fluorescence intensity. In this study, the initial H_2O_2 concentrations ranged from 1 to 10 μM and the amplex red concentration was held constant at 10 μM. These solutions were flowed through the microreactor at rates ranging from 0.2 to 1.5 μL/min.

Panels A and B of Figure 3 show substrate concentration and flow rate effects, respectively, on the HRP-catalyzed hydrolysis of H_2O_2. From these data, linear plots of $f[A_0]$ versus $-\ln(1 - f)$ were obtained (Figure 3C). Values for $K_{m(app)}$ and the reaction capacity of the microreactor (C), as a function of Q, were derived from a weighted least-squares fit to the points shown in Figure 3C. The results are summarized in Table 1. The maximum reaction capacity, C_{max}, of the microreactor can then be obtained from these data.[29] Specifically, a plot of $1/C$ versus $1/Q$ (Figure 4A) yields a

C_{max} value of 9.03 pmol/min. The value of $K_{m(app)}$ should approach the value of the true Michaelis constant of the free enzyme, K_m, at zero flow rate if only mass-transfer effects are responsible for masking the intrinsic enzyme kinetics. This assumption was tested by plotting the $K_{m(app)}$ values obtained against Q (Figure 4B). The intercept on the vertical axis yields a zero-flow $K_{m(app)}$ value ($K^0_{m(app)}$) of 1.51 μM. As discussed next, this value compares favorably with measurements of K_m determined using standard methods.

Enzyme kinetics in homogeneous solution were evaluated using the Michaelis−Menten equation (eq 2). This equation

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

relates the initial rate of the enzyme reaction, V_0, to the concentration of substrate, [S], a maximum rate, V_{max}, corresponding to the velocity of the reaction when the active sites of the enzyme are saturated with substrate, and the Michaelis constant, K_m. The values of K_m for HRP were determined using the following procedure. First, the background fluorescence was measured 100 s after mixing various concentrations of H_2O_2 with 10 μM amplex red. Second, 0.01 unit/mL HRP in 50 mM Tris-HCl (pH 7.4) was added to these solutions, and then fluorescence intensity was measured for 500 s. V_0 was determined for each H_2O_2 concentration (0.1−5.0 μM) from the initial slopes of plots of normalized fluorescence intensity as a function of time (Figure 5A). A plot of the Lineweaver−Burk equation (eq 3) then yields K_m and V_{max}

$$1/V_0 = (K_m/V_{max})/[S] + 1/V_{max} \quad (3)$$

(Figure 5B). The results of this treatment yield V_{max} = 1.90 μM/min and K_m = 1.55 μM. This value of K_m is very close to the value of $K^0_{m(app)}$ (K_m = 1.51 μM) determined using the continuous-flow approach described earlier.

To demonstrate the generality of this approach, the β-Gal-catalyzed hydrolysis of nonfluorescent RBG to D-galactose and fluorescent resorufin was also investigated (Figure 6A). To validate the microfluidic-based method for measuring enzyme kinetics, the value of K_m was first determined in homogeneous solution. Figure 6B shows plots of fluorescence intensity, arising from the appearance of resorufin, as a function of time for RBG concentrations ranging from 50 to 250 μM. The initial slopes from these plots and the Lineweaver−Burk equation (Figure 6C) were used to determine a Michaelis constant, K_m, of 363 μM.

Next, the kinetics for RBG hydrolysis by β-Gal were evaluated using the continuous-flow approach. RBG solutions varying in concentration from 25 to 200 μM (buffer: 100 mM Tris-HCl, 2 mM KCl, and 0.1 mM $MgCl_2$ at pH 7.8) were pumped into the inlet of the microfluidic device at flow rates of 10, 30, 50, 70, and 100 nL/min. As before, the reaction rates were determined by capturing fluorescence images at the inlet and outlet of the microreactor (Figure 7A) and then converting the corresponding fluorescence intensities (determined from line scans, Figure 7B) to RBG concentrations using a calibration curve constructed by flowing resorufin only through the channel. From data obtained

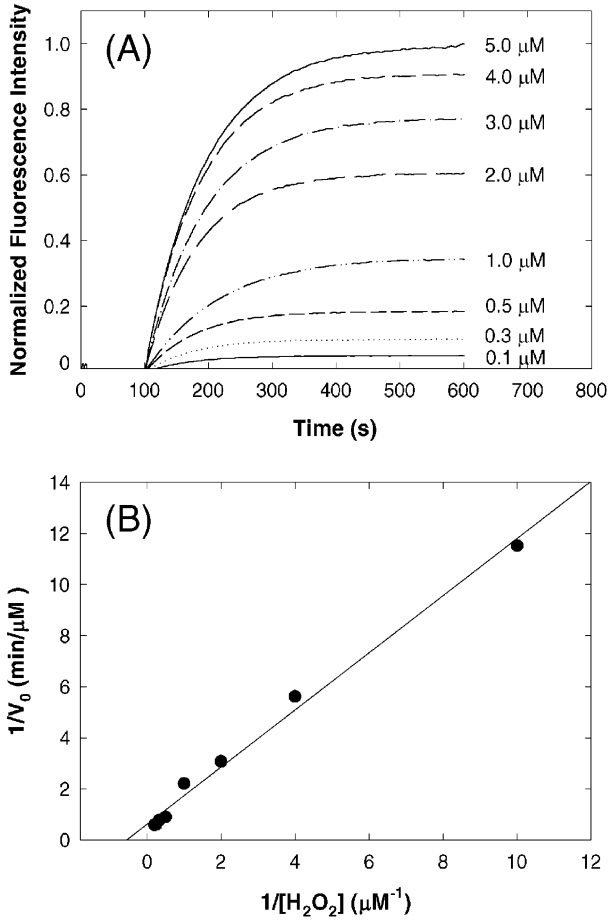

Figure 5. Analysis of data obtained under homogeneous reaction conditions for the HRP-catalyzed reaction between H_2O_2 and amplex red to yield fluorescent resorufin. (A) Plot of normalized fluorescence intensity vs time. H_2O_2 concentrations ranged from 0.1 to 5.0 μM. Conditions: 0.01 unit/mL HRP plus 10 μM amplex red in 2.0 mL of 50 mM Tris-HCl buffer (pH 7.4). (B) Lineweaver−Burk plot of the reciprocal initial reaction rates obtained from (A) vs the reciprocal of the H_2O_2 concentration.

using various RBG concentrations and flow rates, primary plots of $f[A_0]$ against $-\ln(1 - f)$ were used to generate $K_{m(app)}$ values. A secondary plot of $K_{m(app)}$ versus Q yields a $K^0_{m(app)}$ value of 409 μM (Figure 7C). This value compares reasonably well with that determined in homogeneous solution (K_m = 363 μM) and with literature values (K_m = 380 μM and 500 ± 200 μM).[12,30]

SUMMARY AND CONCLUSIONS

We have described a continuous-flow method for measuring enzyme kinetics in microfluidic systems. The approach is conveniently implemented using bead-immobilized enzymes contained within microreactors having a liquid volume (the difference between the total microreactor volume and the beads) of 0.45 nL. It is possible to estimate the total number of enzyme molecules required for the kinetic analysis by assuming each enzyme has a projected area of 80 nm^2, that the total surface area of the 275 beads in each microreactor is 2.1 × 10^5 μm^2, and that the enzymes are close-packed on the bead surface. This treatment suggests a maximum of 3 × 10^9 enzyme molecules are required for the analysis.

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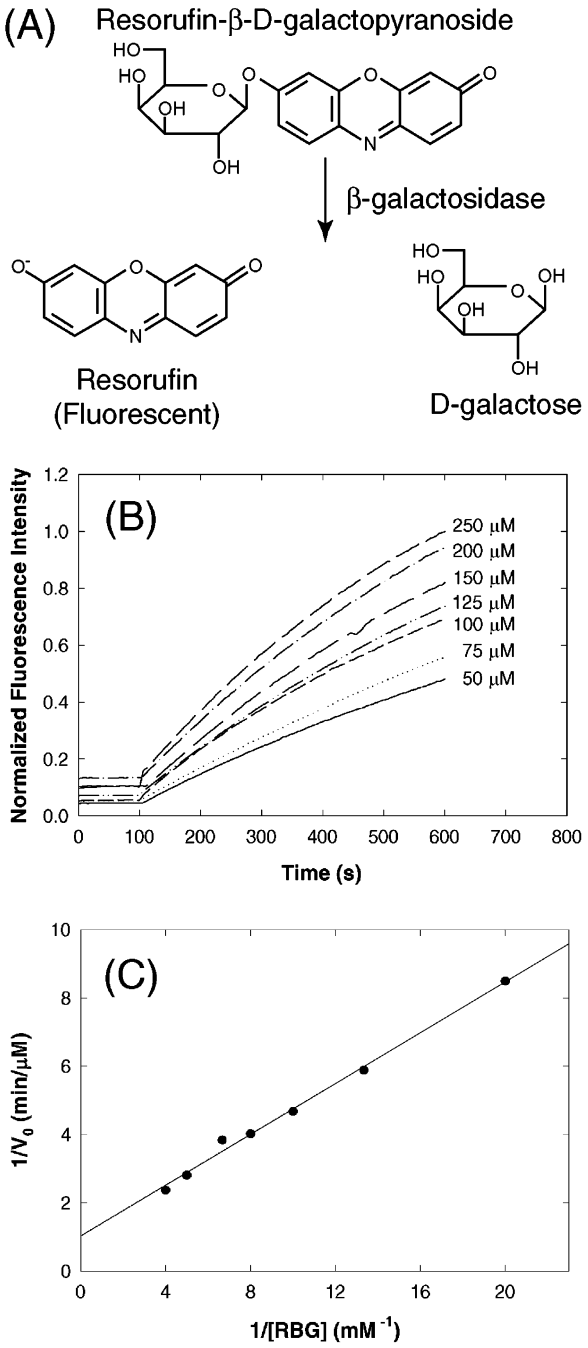

Figure 6. (A) β-Gal-catalyzed reaction of nonfluorescent RBG to yield D-galactose and fluorescent resorufin. (B) Normalized fluorescence intensity vs time for this reaction carried out in homogeneous solution. RBG concentrations ranged from 50 to 250 μM. Conditions: 0.1 unit/mL β-Gal in 2.0 mL of 100 mM Tris-HCl buffer (pH 7.8), 2 mM KCl, and 0.1 mM $MgCl_2$. (C) Lineweaver–Burk plot of the reciprocal initial reaction rate vs the reciprocal of the RBG concentration.

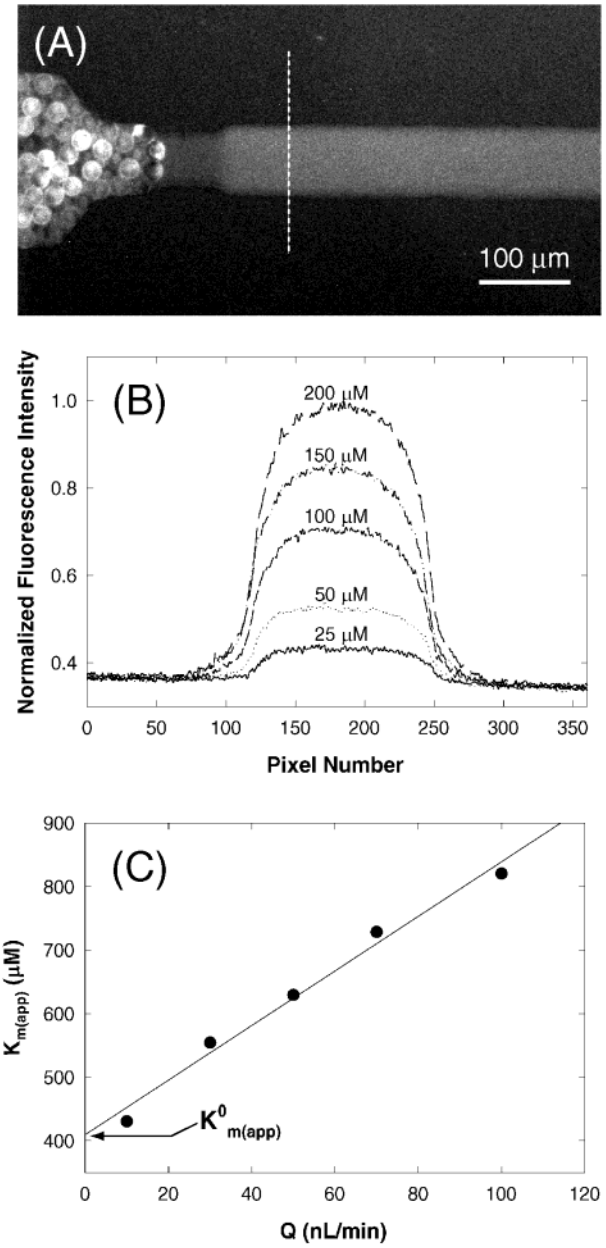

Figure 7. (A) Fluorescence micrograph showing the enzymatic production of fluorescent resorufin in the outlet stream of a microreactor packed with β-Gal-functionalized microbeads. Flow rate, 50 nL/min. (B) Normalized fluorescence intensity line scans, obtained at the location indicated by the dashed line in (A), at RBG concentrations ranging from 25 to 200 μM. (C) Plot of $K_{m(app)}$, the apparent Michaelis constant, vs Q.

The kinetic analysis relies on the Lilly–Hornby model, which has been previously used to evaluate apparent kinetic parameters in packed-bed enzyme reactors under steady-state conditions. The Michaelis constants determined using the new approach reported here and traditional methods based on the Lineweaver–Burk relationship are indistinguishable within the estimated precision of the methods. A significant outcome of this study was the finding that the kinetics of both β-Gal and HRP were the same in solution and after immobilization on the microbeads. Similar findings have previously been reported for enzymes immobilized in fluid lipid bilyaers.[21]

Because of the reduced cost, reagent economy, and increased throughput associated with microfluidic-based assays, it is quite likely that many analytical determinations presently carried out in microtiter plates will be performed using microfluidic systems in the not-too-distant future. This paradigm shift will necessitate development of new standard methods for measuring kinetic and thermodynamic properties of reactions. The results reported here contribute to this important goal, and they are therefore likely to

be of value for clinical diagnostics, and drug discovery and screening.

ACKNOWLEDGMENT

Financial support from the U.S. Army Medical Research & Material Command is gratefully acknowledged. We also acknowledge the support of the Texas Institute for Intelligent Bio-Nano Materials and Structures for Aerospace Vehicles, funded by NASA Cooperative Agreement NCC-1-02038. Some of the instrumentation used to carry out this work was provided by the Center for Integrated Microchemical Systems at Texas A&M University.

Received for review February 17, 2003. Accepted April 8, 2003.

AC034155B