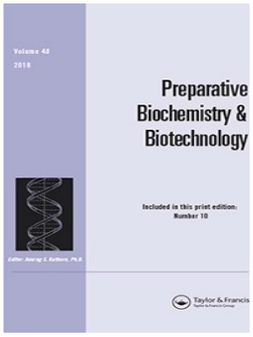

Preparative Biochemistry and Biotechnology

ISSN: 1082-6068 (Print) 1532-2297 (Online) Journal homepage: https://www.tandfonline.com/loi/lpbb20

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To cite this article: Pawel Jankowski, Adam Samborski, Ryszard Ostaszewski & Piotr Garstecki (2019): Evaluation of droplet-based microfluidic platforms as a convenient tool for lipases and esterases assays, Preparative Biochemistry and Biotechnology, DOI: 10.1080/10826068.2019.1605527

To link to this article: https://doi.org/10.1080/10826068.2019.1605527

PREPARATIVE BIOCHEMISTRY AND BIOTECHNOLOGY
https://doi.org/10.1080/10826068.2019.1605527

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Evaluation of droplet-based microfluidic platforms as a convenient tool for lipases and esterases assays

Pawel Jankowski[a]*, Adam Samborski[a]*, Ryszard Ostaszewski[b], and Piotr Garstecki[a]

[a]Institute of Physical Chemistry, PAS, Warsaw, Poland; [b]Institute of Organic Chemistry, PAS, Warsaw, Poland

ABSTRACT

The accurate estimation of kinetic parameters is of fundamental importance for biochemical studies for research and industry. In this paper, we demonstrate the application of a modular microfluidic system for execution of enzyme assays that allow determining the kinetic parameters of the enzymatic reactions such as V_{max} – the maximum rate of reaction and K_M – the Michaelis constant. For experiments, the fluorogenic carbonate as a probe for a rapid determination of the kinetic parameters of hydrolases, such as lipases and esterases, was used. The microfluidic system together with the method described yields the kinetic constants calculated from the concentration of enzymatic product changes via a Michaelis–Menten model using the Lambert function *W(x)*. This modular microfluidic system was validated on three selected enzymes (hydrolases).

KEYWORDS

Enzyme assay; lipases; esterase; microfluidics; kinetic enzymatic reactions; Lambert function

Introduction

Enzymes catalyze a variety of organic and biochemical reactions. Consequently, the determination of the enzyme activity is extremely important in medical diagnostics, in biotechnology, and in research. The most important property of all enzymes is their catalytic power – measured by the kinetic parameters of an enzymatic reaction. Measurement of rapid enzyme kinetics is essential to an understanding of many biological and chemical processes.[1] Special attention has been paid to hydrolytic enzymes due to their ability to accept a wide range of substrates and to their stability in aqueous environments. Interestingly, hydrolases may also be active in organic solvents. This makes hydrolases attractive both in academic research and in industrial applications. Often, the kinetic properties of hydrolases do not meet the requirements of a particular application. Directed evolution techniques are widely used for the generation of enzymes expressing desired kinetic properties in respect to particular substrates.[2] Fast and efficient test methods for the determination of hydrolase hydrolytic activity are very valuable for high-throughput screening in biotechnology.

Several methods were proposed for hydrolase assays. Polymer membrane ion-selective electrodes can be used as a convenient method for hydrolase assay, especially for turbid and nontransparent samples.[3] For transparent samples, other methods are of special attention. These include mass spectrometry,[4] fluorescence, and UV-visible spectroscopy[5] which are definitely the most popular ones. Fluorescence assays are very sensitive in respect to a small amount of enzymes and less susceptible to impurities causing background signals. Fluorescence assays can be used for crude cell lysate and for turbid solutions.[2] For hydrolase activity assays, respective fluorogenic probes should be used. The important factor is associated with enzyme amount. For classical assays, a relatively large amount of enzyme solution is necessary (2–5 mL). Application of microfluidic chip systems together with a fluorescent substrate can diminish the amount of enzyme solution to 0.01 mL only. Unfortunately, until now, the validation of experimental data from different methods is unavailable, what makes the purpose of these studies important for researchers and technician. Esters are commonly used as fluorescent probes because those compounds are nonfluorescent while the product formed causes strongly blue fluorescence emission.[6] Commonly used esters of 4-methylumbelliferone are poor probes due to its fast hydrolysis rate in water.[7] Our systematic studies proved that achiaral[8] and chiral[9] mixed carbonates of 4-methyl-7-hydroxycoumarin are promising substrates for fingerprinting hydrolase activity. Also, enzymatic self-immolative probes sensitive towards hydrolase detection through a cascade reaction were designed.[10] Although this work was very successful, determination of hydrolase key kinetic parameters still requires a huge number of experiments.

The key parameters for the enzyme kinetics are (i) V_{max} – the maximum rate of reaction, (ii) K_M – Michaelis constant (related to the dissociation constant, indicates the affinity of the substrate, low K_M values indicating high affinities). In order to simplify and automate measurements of

CONTACT Pawel Jankowski pjankowski@ichf.edu.pl; Adam Samborski asamborski@ichf.edu.pl Institute of Physical Chemistry, PAS, Warsaw, Poland
*Authors have contributed equally to this work.

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kinetic parameters, we propose a droplet-based microfluidic assay. Droplet microfluidic systems are highly promising tools for a range of analytical and chemical processes due to their flexibility to change the reagents and reaction conditions within the same chip and operating on ultra-small samples. Droplets typically contain reagents and are separated from each other and from the walls of the microfluidic chip by an immiscible oil, thus avoiding major limitations of single-phase (continuous phase) microfluidics,[11] such as dispersion of the time of retention of reagents and diffusion-limited mixing. Each droplet can be used as an independent microreactor. As multiple droplets can be formed easily, the systems open also a facile vista for a high number of repetitions of any reaction, thus yielding statistically sound measurements for determination of the parameters of interest. All these advantages of droplet systems can be very well used also in kinetic analyses of enzyme activity.

Droplet microfluidic systems have already been demonstrated to support enzymatic reactions in droplets for analysis of the kinetics of enzymatic reactions.[11–16] In all these demonstrations, the kinetic parameters were determined via the Limeweaver–Burke scheme, which is based on measuring the initial reaction rate at varying substrate concentrations.[17] This means that it is necessary to make many drops with different substrate concentrations and consequently, the microfluidic systems that generate gradient concentration profiles are needed.

Ismagilov et al. used a microfluidic chip to perform kinetic measurements of ribonuclease A (RNase A) with better than millisecond resolution (single-turnover kinetics).[18] Huck et al. presented the parallel microdroplets technology (PmD) [19] and used it to measure the steady-state kinetics of the enzyme hydrolysis of fluorescein diphosphate by *E. coli* alkaline phosphatase. In turn, Hong et al. demonstrated enzyme (β-galactosidase) reaction on a chip designed for generations of a gradient of reagent concentrations in 11 parallel processors. By changing the reactant concentrations and measuring the reaction rates, they determined the key kinetic parameters of the enzyme.[20] In a different approach, Bui et al.[21] presented a microfluidic system that uses diffusive mixing under laminar flow to generate a linear concentration gradient of the substrate at the cross-section of the channel. In the end, the channel branches off into four downstream channels followed by a simple Y-junction for mixing with enzyme solution and standard T-junction geometry for a droplet formation. A high-speed camera was used for fluorescence imaging of droplets. Next, another approach to control dilution gradients was presented by Gielen et al.[22,23] They used a robotic compartment-on-demand (COD) platform for the automatic formation of droplets of the enzyme (β-glucosidase) with different substrate concentrations.

The methods referenced above used spectroscopic methods to determine the concentration of the product of the enzymatic reaction. Han *et al.* integrated the electrochemical detection method into a droplet-based microfluidic system for measuring enzyme kinetics.[24] They used this system for measuring the Michaelis–Menten kinetics of the decomposition of H_2O_2 by catalase.

Here, we demonstrate an automatic microfluidic system dedicated to a screening of enzymatic reactions with the full control over such parameters as: (i) concentration of substrates, (ii) ratio of reagents, (iii) time of reaction, and (iv) the temperature control. The system allows to generate droplets with any composition of components (enzymes, enzymatic substrates, and other reagents) using the droplet-on-demand (DoD) method. Droplets are moving in an oscillating motion inside the reactor module, where the enzymatic reaction takes place. We measured the fluorescent signal each time the drop passes through the detector and in this way, we can collect data on the progress of the reaction over time. The time in which the data will be collected can be freely changed by changing the number of passes (oscillations) of the droplets in the reaction module. The changes in the concentration of the enzymatic reaction product – measured over time – are used to determine the kinetic constants of the enzymatic reaction. We create a numerical code that will use the experimental data to fit the Michaelis–Menten (M–M) model.

Experimental

Materials and methods

We purchased commercial enzyme substrates from Fluka and Sigma-Aldrich. We used perfluorodecaline purchased from Alfa Aesar. All chemicals were commercial products of the analytical grade. We synthesized the fluorogenic probe according to the procedure described in the literature.[9] We recorded the fluorescence measurements with OceanOptics QEPro-F spectrofluorometer connected to a microfluidic system using optical fibers. We used a UV diode LTPL-U034UVH385 (LITEON) as the illumination source. We made an electronic system: valve controller, drop detectors, power supply in our laboratory.

The design of the microfluidic experimental setup

The experimental setup for the enzymatic reaction is shown in Figure 1. The system comprises three main modules: reagent dosing system(A), formation of droplets and mixing part(B), and reactor and detection system(C). This setup provides full control over parameters such as the concentration of the enzyme and substrate, time, and temperature of the reaction.

The reagent dosing system(A)

We used a droplet-on-demand system (DOD)[25], which allows to dose the desired volumes of the liquids. The DOD system is operated by a digital driver that opens electromagnetic valves (V165, Sirai, Italy) for each liquid in a preset sequence (time and order). To minimize the amount of reagents and avoid contact of reagents with the valves system, reaction samples are placed in syringes (1 mL) and then loaded into the fluoroethylene propylene (FEP) tubing

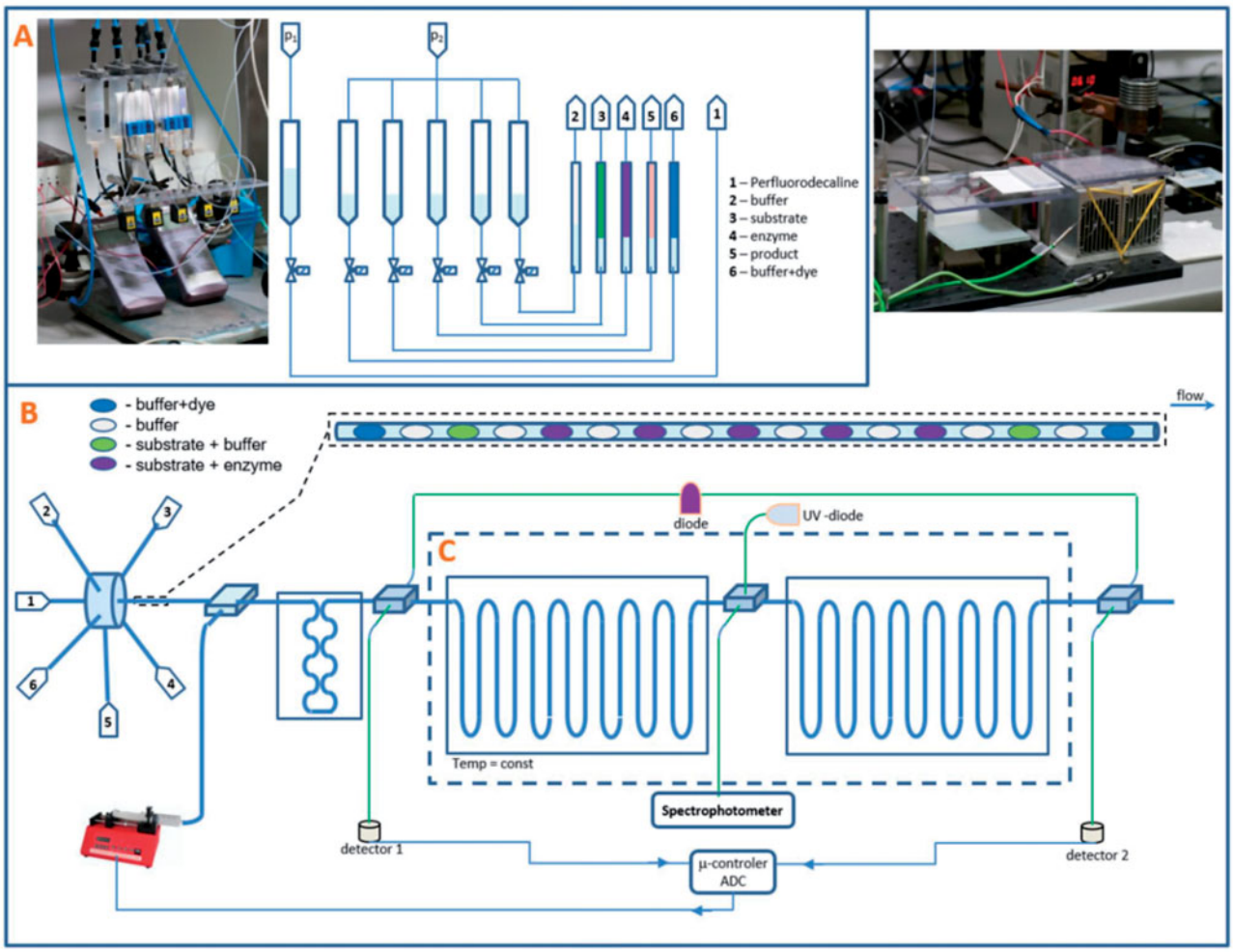

Figure 1. Scheme of experimental setup for determining the kinetic parameters of the enzymatic reaction: (A) reagent dosing system, (B) droplet generation system, and (C) reactor and detection system.

(O.D. 0.9 mm, I.D. 0.4 mm, length 50 cm, BOLA, Grünsfeld, Germany) via aspiration by an immiscible fluid (perfluorodecaline) (Figure 2A) to the second part of the system. The rate of the flow of the perfluorinated oil is controlled by the compressed air using a compressor and a pressure regulator (p_1 and p_2 in Figure 1).

Formation of droplets and mixing part(B)

Droplets are generated in the Teflon cube equipped with six inlets and one outlet. The first inlet (1) is connected with a container containing fluorinated oil (continuous phase). The other five inlets are connected to syringes filled with buffer (2), buffer solution of enzymatic substrate (3), buffer solution of enzyme (4), solution of product (5) and dye (6). It should be emphasized that by using valves (droplets on demand system – DoD) we can independently generate different initial concentrations of enzyme and substrate (by combining buffer, substrate, and enzyme solutions in various proportions into one drop), which enables us to easily find the proper range of these parameters for each reaction.

To determine the concentration profile of the product of enzymatic reaction as a function of time, an appropriate set of droplets is produced. A sequence of n-droplets is equivalent to the parallel conducting of n-experiments, which affects the accuracy of the calculations. After the formation, every droplet passes through the 19.5 cm long meandering channel made of tubing (FEP, O.D. 1.6 mm, I.D. 0.8 mm) for rapid mixing.[18,26,27]

Before the mixer, the syringe filled with perfluorodecaline (placed on the syringe pump) was connected to the system. The syringe pump is responsible for the oscillating flow of the drops inside the reactor.

Reactor and detection system(C)

The reaction part consists of a 120 cm FEP tubing (O.D. 1.6 mm, I.D. 0.8 mm) placed in a holder made of polycarbonate plate (tube was placed in specially milled grooves in the PC plate). The plate with tubes was placed on the two Peltier elements to ensure temperature control. In our experiment, we kept all the time the constant temperature (T = 22 °C) but the described system allows to carry out the experiment in the range of 10–70 °C. In the central part of the reactor, a UV diode was placed directly above the FEP tube and an optic fiber was placed in the special channel milled in the polycarbonate plate. The other part of the optical fiber was connected to the spectrophotometer. On both sides of the reactor, there are two detectors consisting of a photodetector and LED diode. Detectors were used to

detect droplets containing dye (absorbance measurement) and control the syringe pump responsible for oscillating motion.

Enzyme assay

In a typical experiment, we generated a group of droplets (5 µL each, which corresponds to a drop of about 1 cm in length) consisting of: buffer + dye (methylene blue solution in buffer – a marker defining the beginning of the sequence), buffer (flushing the system to avoid cross-contamination), substrate + buffer (to determine non-enzymatic hydrolysis of substrate), five sequences of buffer and substrate + enzyme (enzymatic reaction; each droplet contains 0.25 µg of enzyme and the substrate concentration was set to 9.5 μM), buffer, substrate + buffer, buffer, buffer + dye (a marker defining the ending of the sequence)(Figure 1B).

The reagents in droplets are mixed and then droplets are injected into the reactor part. During the experiment, the group of droplets is moving back and forth along the system between the two detectors 1 and 2. The direction of the flow of droplets in the reactor is controlled by a microcontroller and a syringe pump. The detectors recognize the location of colored droplets and change the pump's operating mode from infusing to withdraw and vice versa (Figure 1C). In the center of the system, we positioned the spectrophotometer detector which measures the fluorescent intensity from the content of the drops as the droplets pass through the detector. The flow parameters (pumping rate = 0.7 mL × min^{-1})) were selected so that the intervals between the measurements for each drop were about 2 minutes.

After the measurement is completed, we generated a sequence of drops of the product diluted with the buffer in various ratios. Thanks to this, we determined the calibration curve that will be used to determine the dependence of fluorescence intensity on product concentration (see Supplemental Information part).

Results and discussion

We choose fluorogenic carbonate carrying fluorescence label ((R)-1-Phenylethyl-(2-oxo-4-methyl-2H-chromen-7-yl) carbonate, Figure 2A) as the versatile probe for examination of hydrolytic activities of enzymes.[8,9] We excite the product of the enzymatic reaction, 4-methylumbelliferone with λ_{ex} = 385 nm wavelength light. The product emits at maximum intensity at λ_{em} = 445 nm (Figure 2B).

Initially, we intended to determine the minimum concentration of the product which we are able to measure in our detector system. Preliminary experiments carried out with various dilutions of the product (drops containing 4-methylumbelliferone) proved that we are able to measure the concentration of the product of an enzymatic reaction with a sensitivity of 10^{-6} M. In the experiment, it is important that there is no cross-contamination between the droplets. We performed an experiment in which we created droplets of 4-methylumbelliferone solution and then two droplets of pure buffer (Figure 3).

We observed that only the first drop of the buffer, directly after drop with fluorescent dye showed fluorescence. This allowed us to conclude that a single spacer droplet of buffer separating droplets with reagents can absorb any material transferred between the droplets, thus preventing any cross-contamination between the reactions. Figure 4 shows the typical spectral data for a set of droplets during the experiment carried out to determine the kinetic parameters of the enzymatic reaction.

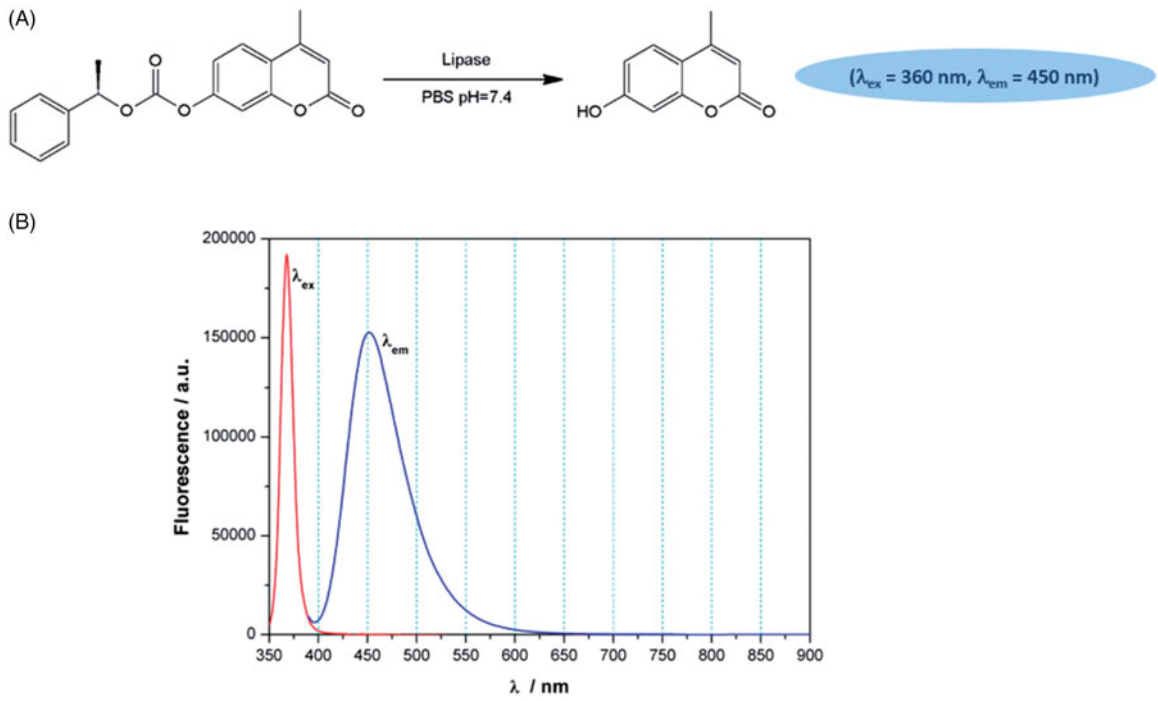

Figure 2. (A) Scheme of enzymatic reaction of carbonate, (B) the fluorescence spectrum of 4-methylumbelliferone (R = CH_3).

Methods of estimating enzyme kinetic parameters

We calculate the kinetic constants of the enzymatic reaction V_{max} and K_M from the signal of fluorescence intensity that we directly measure in our experiment. The fluorescence intensity is proportional to the product concentration of an enzymatic reaction $P(t)$, where $P(t) = S_0 - S(t)$; $S(t)$ is the substrate concentration and S_0 is the initial substrate concentration. Obviously, $P(t)$ and $S(t)$ are the functions of time. To find the relation between the fluorescence intensity and the product concentration, we prepared a calibration curve in the way that we described previously. For $S(t)$, we use the known solution of the differential Michaelis–Menten (M–M) equation:[28–32]

$$\frac{dS(t)}{dt} = -\frac{V_{max}S(t)}{K_M + S(t)} \tag{1}$$

This solution is given in terms of the Lambert function $W(x)$[33], which satisfies the transcendental equation $W(x)e^{W(x)} = x$

$$S(t) = K_M\ W\left\{\frac{S_0}{K_M}exp\left(\frac{S_0 - V_{max}t}{K_M}\right)\right\} \tag{2}$$

In our experiment, we generated packets of five droplets with substract + enzyme (enzymatic reaction; comprised the mixture of substrate and enzyme at equal concentrations; Figure 4: violet color), and two droplets with substrate + buffer (non-enzymatic hydrolysis reaction; Figure 4: green color). For every droplet, we make measurements of the intensity of fluorescence over 10 times during each passage through the detector and for these values, we calculate the average value of intensity. To determine the real concentration profile of enzymatic reaction, all values of the intensity for droplets (substract + enzyme) are reduced by the

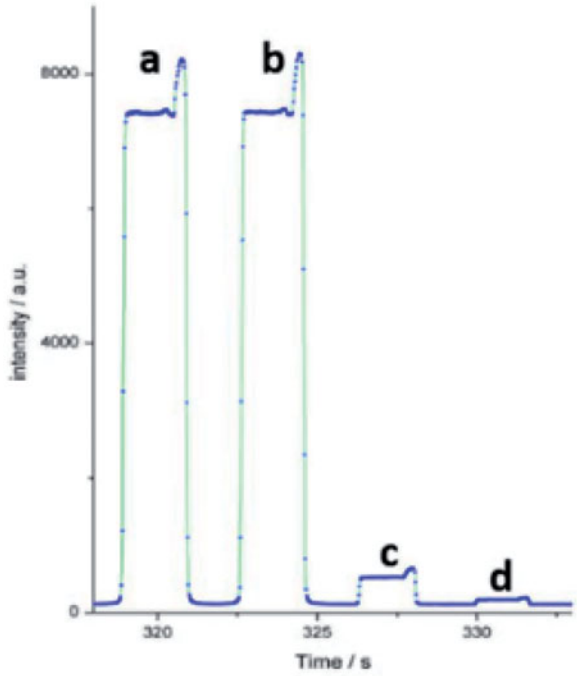

Figure 3. Testing of cross contamination between the droplets. a,b – droplets of the solution of the product of the enzymatic reaction, c – the first drop with buffer, d – the second drop with buffer.

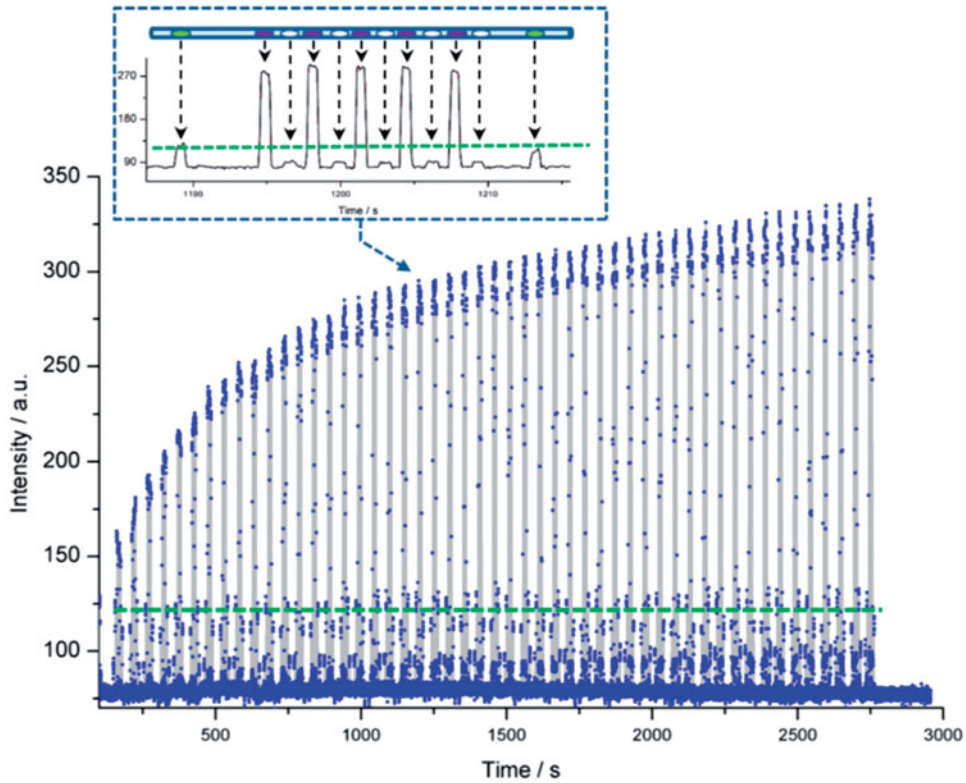

Figure 4. Measurement of the fluorescence (blue dots) of droplets flowing through the detector during the enzymatic reaction. The inset shows a single sequence of drops: a substrate (green), enzyme + substrate (purple) and the buffer (white). The green line indicates the level of fluorescence for the hydrolysis reaction. The temperature during measurements was set at 22 °C.

intensity for non-enzymatic hydrolysis reaction. Five droplets are equivalent to five microreactors, in which five identical reactions take place simultaneously. We calculate for them the average values of the concentration and the standard deviations as a function of time and create a numerical code to fit our results to the M–M model. This code comprises two parts: in the first part, we calculate the average concentration of the product of enzymatic reaction as a function of time and in the second part, we determine fitting curve, using the M–M model to the concentration profile that was obtained from our experiment. For estimation of the parameters of the enzyme kinetic reactions: V_{max} and K_M, we use the nonlinear least-squares routines (Levenberg–Marquardt).[34,35] In this interactive routine, it is necessary to have the initial estimation of V_{max} and K_M, that is obtained through linearization (in three different ways) of the M–M equation:[36]

$$\frac{t}{ln\left(\frac{S_0}{S}\right)} = \frac{K_M}{V_{max}} + \frac{(S_0 - S)}{V_{max} ln\left(\frac{S_0}{S}\right)} \tag{3}$$

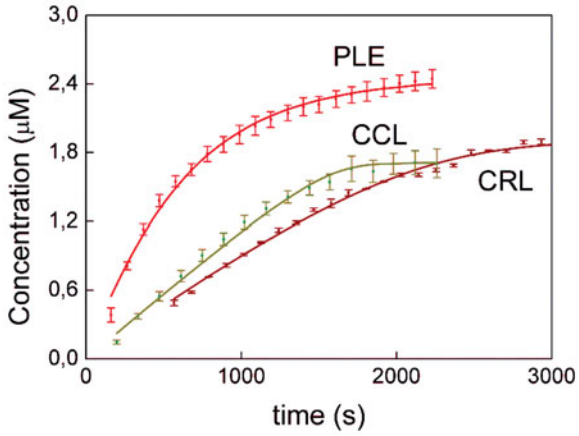

Figure 5. The experimental results in droplets and fitting curve for the product concentration of enzymatic reaction for enzymes: *PLE*, *CCL*, and *CRL*.

Table 1. The kinetic parameters obtained from the reaction for enzymes: *PLE*, *CCL*, and *CRL*.

Enzyme	V_{max}[μM/s]	K_M[μM]	k_{cat}[1/s]	E_0[μM]
PLE	$1.50 \times 10^{-2} \pm 7.09 \times 10^{-3}$	7.32 ± 4.16	1.84×10^{-1}	8.14×10^{-2}
CCL	$1.21 \times 10^{-3} \pm 7.94 \times 10^{-5}$	$0.1 \pm 6.8 \times 10^{-2}$	2.94×10^{-2}	4.12×10^{-2}
CRL	$1.16 \times 10^{-3} \pm 7.83 \times 10^{-5}$	$0.4 \pm 9.9 \times 10^{-2}$	2.37×10^{-2}	4.9×10^{-2}

$$\frac{t}{S_0 - S} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \frac{ln\left(\frac{S_0}{S}\right)}{S_0 - S} \tag{4}$$

$$\frac{S_0 - S}{t} = V_{max} - \frac{K_M}{t} ln\left(\frac{S_0}{S}\right) \tag{5}$$

Independent of the choice of the Equations (3–5) for determining the starting data, we received the same final results.

Figures 5 and 6 show the results of enzymatic reaction for enzymes: *PLE, CCL,* and *CRL*. In Figure 5, we present the product concentration as a function of the interval used for the reaction in the experiment. The points represent experimental data with standard errors: red color is *PLE* enzyme, green color is *CCL* enzyme, and brown color is *CRL* enzyme, while the lines are obtained from the fitting procedure. The data shown in Figure 5 show that our model corroborates with the experimental result. This suggests that the use of M–M equation to fit the experimental data provides a reasonable estimate of the kinetic parameters: V_{max}, K_M, and k_{cat}, where $k_{cat} = V_{max}/E_0$ in Table 1. The values of E_0 are the initial concentration of the enzymes and were calculated based on the available literature data.

Figure 6 shows the reaction rate as a function of the substrate concentration. We obtained these results using our kinetic parameters in M–M relation.

Conclusions

We have shown a simple, modular automatic microfluidic system for enzyme assays that allows determining the kinetic parameters of the enzymatic reactions, including V_{max} – the maximum rate of reaction and K_M – the Michaelis constant. The system allows to generate droplets with any composition of components (enzymes, enzymatic substrates, and other reagents) using the droplet-on-demand (DoD) method, with the full control over such parameters: concentration of substrates, ratio of reagents, time of reaction, and the temperature control. In our experiment, we kept the same temperature, but in the future, we plan the experiments for a different temperature. To calculate the kinetic constants from the experiment, we used the known solution of Michaelis–Menten model in terms of the Lambert function $W(x)$ and created a numerical code to fit this model

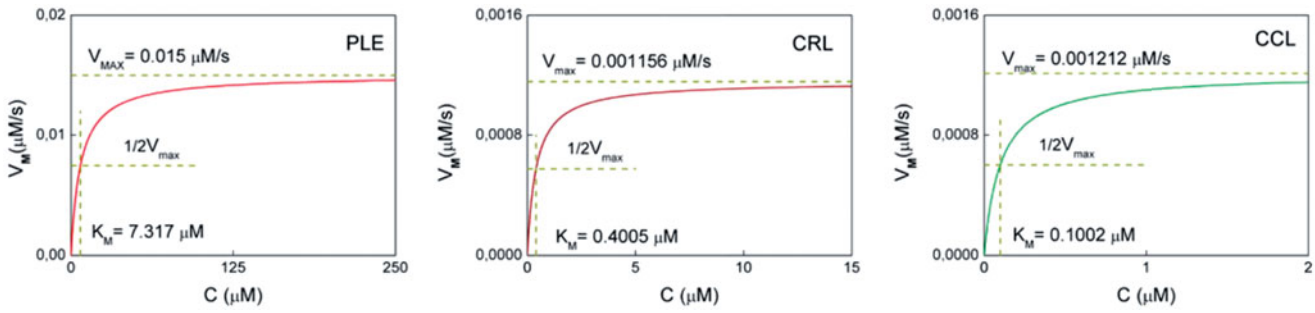

Figure 6. Michaelis–Menten curves for an enzyme reaction – the relation between the substrate concentration and reaction rate.

using the nonlinear least-square fitting routines. For this procedure, we need the functional form of the substrate concentration $S = h(t; V_{max}, K_M)$ and its derivatives. Therefore we use the Schnell–Mendoza equation[28–32] because the integrated form of M–M equation[29] gives us the implicit function $t = f(S(t); V_{max}, K_M)$.

The experimental data and the fitting curve suggest that the M–M model corroborates with the experimental result and gives a reasonable estimate of the kinetic parameters. In this simple modular microfluidic system, we are validated on three hydrolases: *PLE, CHL,* and *CRL*. Using our experimental data, we calculated kinetic parameters that are in full accordance with literature data.

Generally, in experiments with enzymes, the kinetic parameters were determined via the Lineweaver–Burke scheme[13,17,20,21] which is based on measuring the initial rate at varying substrate concentrations. Consequently, the microfluidic systems that generate gradient concentration profiles are needed. Therefore, in our method, we measure changes in the concentration of the product of the enzymatic reaction over time and we use these data to determine the kinetic constants of the enzymatic reaction using a numerical code to fit the experimental data to Michaelis–Menten (M–M) model, all in one experiment only.

It is worth mentioning that optimization experiments can be performed within minutes and the collection of data is fully automatized. Due to the simplicity of the designed system, it can be used in almost all laboratories and for experiments, very small amounts of enzymes are required. For experiments, a very small amount of enzyme solutions are required (typically 0.01 mL) what makes the method discussed easily applicable, even for small enzyme samples. Further experiments on the development of enzymatic assays for other types of enzymes in different temperatures are in progress in our laboratory.

Funding

We gratefully acknowledge the financial support of the National Science Center project Poland OPUS No. 2016/23/B/ST5/03307 for financial support.

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