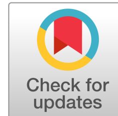

A Laboratory Work to Introduce Biochemistry Undergraduate Students to Basic Enzyme Kinetics -Alkaline Phosphatase as a Model

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Abstract

Enzyme kinetics is an essential topic in undergraduate Biochemistry courses. A laboratory work that covers the principal basic concepts of enzyme kinetics in steady state is presented. The alkaline phosphatase catalyzed reaction of phenylphosphate hydrolysis was studied as a model. The laboratory experience was designed to reinforce the concepts of initial velocity dependence on substrate and enzyme concentration, and to highlight the importance of the accurate determination of initial reaction rate. The laboratory work consists in two parts, in which students first determine the enzyme concentration and the time to be used in the following session to obtain the kinetic parameters (K_M and V_{max}) by non-lineal fitting of the Michaelis–Menten equation to the initial velocity dependence with substrate concentration results. The experimental methodology is robust, the cost per student is low and the equipment and reagents used are of easy access. © 2018 International Union of Biochemistry and Molecular Biology, 00 (00):1–7, 2018.

Keywords: *Enzyme kinetics; alkaline phosphatase; experimental design; hands-on learning; laboratory experiment*

Introduction

Enzyme kinetics is an essential topic in undergraduate biochemistry courses. Steady-state kinetic studies, usually initial rate measurements, are a first approach in the characterization of enzyme function. The variation of initial rate with substrate and/or product concentration provides not only quantitative values of macroscopic reaction constants but also important information about the mechanism involved in the catalysis [1,2]. Through kinetic analysis, a model for an enzyme-catalyzed reaction can be proposed, certain kinetic constants determined and a kinetic equation deduced. Although this approach alone cannot completely elucidate the mechanism of enzyme catalysis, it can provide useful information regarding the steps involved in the catalysis, that is, the order in which substrates add and products leave the enzyme.

With the purpose of introducing undergraduate students to basic enzyme kinetics, a laboratory experiment was designed in order to address the concepts of time course measurements, reaction rate determination, definition and importance of initial reaction velocity in steady-state conditions, initial rate dependence with substrate and enzyme concentration, and nonlinear regression analysis to obtain kinetic parameters. A week before the enzyme kinetics module starts, students attend two lectures, in which general concepts of enzymes, catalytic mechanisms, allosterism, and cooperativity are discussed. The enzyme kinetics module is organized in one seminar followed by two laboratory sessions (4 hr each). During the seminar, the principal concepts of basic enzyme kinetics are introduced, including transition state theory, steady state and initial velocity concepts, deduction of Michaelis–Menten equation, and enzyme inhibition. Even though only Michaelis–Menten kinetics is discussed, it is pointed out that there are non-hyperbolic behaviors on the initial velocity versus substrate concentration dependence.

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The significance of the kinetic parameters obtained and the limitations in terms of describing the enzyme catalytic mechanism are also discussed.

In the laboratory sessions, alkaline phosphatase is used as a model. This enzyme not only has been used for academic and teaching purposes [3–7], but also its activity is determined in the clinical laboratory, as its increase in blood is associated with multiple pathologies, mainly hepatobiliary and bone disorders [8]. Alkaline phosphatase is a promiscuous enzyme with broad substrate specificity that catalyzes the hydrolysis of phosphate esters, with optimum *in vitro* activity at a pH of 8–10 [9,10]. One of the substrates used to determine phosphatase activity is sodium phenylphosphate, which is hydrolyzed to phenol and phosphate. As neither the substrate nor the products absorb in the visible region of the spectra, the reaction can be monitored by measuring the inorganic phosphate release with the colorimetric reagent Fiske-Subbarow [6,11]. Given that the addition of this reagent not only allows phosphate quantification but stops the reaction as well by an abrupt change on the reaction media pH, fixed-time assays are performed to ascertain product generation after a period of time. To point out that enzyme activity can also be determined by continuously monitoring changes in substrate or product concentration when these can be directly detected, a data sheet of a commercial kit for the determination of alkaline phosphatase activity in serum using 4-nitro-phenylphosphate as a substrate is discussed with students.

It is important to remark that we have successfully used this reaction system for several years in different laboratory experiences as part of a course for third-year undergraduate Biochemistry students and it proved to be very robust. The assay is inexpensive and utilizes commercially available reagents and basic equipment. In the present work we describe the experimental approach used for the past 4 years.

Objectives

The general objective of the laboratory work described is to obtain the kinetic parameters K_M, V_{max}, and k_{cat} for the hydrolysis of phenyl-phosphate catalyzed by alkaline phosphatase. For this purpose, the initial reaction rate at different substrate concentrations must be determined. Therefore, it is crucial that the measurements of the rate of phosphate release are performed in initial velocity conditions and therefore the reaction time must be carefully chosen. On this basis, laboratory work was designed in two sessions. In the first, the amount of phosphate release as a function of reaction time at three different enzyme concentrations is measured. In the second, and based on the results of the first session, variation of the initial velocity with substrate concentration is determined and kinetic parameters are obtained by nonlinear regression.

The learning goals of this experience are that students:

- Understand the importance of measuring initial reaction rate in steady-state studies.
- Comprehend the experimental design for an accurate determination of the initial rate, particularly in fixed-time assays.
- Obtain and analyze time courses of product release to estimate the time interval to measure initial rate and its dependence with enzyme concentrations.
- Determine the dependence of initial rate with substrate and enzyme concentrations.
- Employ nonlinear regression to obtain K_M, V_{max}, and k_{cat} values of the Michaelis–Menten equation.
- Gain experience in the setup and execution of time dependent pipetting of different reagents in a set of test tubes.
- Learn how to process, present and analyze experimental data.

Materials

Chemicals

Sodium phenyl phosphate dibasic dihydrate (P7751, Sigma Aldrich, St Louis, Missouri, United States); alkaline phosphatase from bovine intestinal mucosa (P7640, Sigma Aldrich, lyophilized powder); reagent grade K_2HPO_4, Na_2CO_3, $NaHCO_3$, aminonaphtolsulfonic acid, sodium sulfite, sodium bisulfite, ammonium molibdate, and sulfuric acid.

Working Solutions

Substrate solution: Sodium phenyl-phosphate solution is prepared from stock solution in reaction buffer; 2 mM and 20 mM concentrations are used for laboratory sessions 1 and 2, respectively.

Enzyme solution: Alkaline phosphatase is prepared from stock solution in reaction buffer; 2.5, 10, and 20 μg/mL solutions are used.

The solutions are kept in ice until the experimental procedure.

Fiske-Subbarow reagent: To prepare 1 L of the colorimetric reagent, 0.49 g of the powder mix (reducing reagent) is dissolved in approximately 600 mL of distilled water, then 181.4 mL of acid molybdate reagent are incorporated and finally distilled water is added to reach 1 L volume.

Details of the preparation of stock solutions and equipment needed are given in Supporting Information Appendix 2.

Hazards

The major safety issue is the use of reagents with sulfuric acid. Concentrated sulfuric acid is extremely corrosive and can cause serious damage when not properly handled; appropriate personal protective equipment should be worn. The handling and disposal of these solutions requires special attention, institutional regulations must be followed. The

preparation and handling of solutions with concentrated sulfuric acid is performed by instructors before the laboratory work session. Students use the Fiske-Subbarow reagent, which contains diluted sulfuric acid. The other chemicals used are aqueous solutions of salts and proteins in low concentrations with minimal hazards and can be handled with standard laboratory safety procedures. Personnel and students should be informed of the safety issues and instructed in the proper handling of the reagents.

Experimental Procedure

Laboratory Session 1: Initial Velocity Conditions. Product Versus Reaction Time Curves with Different Enzyme Concentrations

During this session, time courses of product release in the presence of three different enzyme concentrations are performed. First, laboratory instructors discuss with the students the objectives of the experiment, the details of the experimental procedure, the importance of controls and data processing. Special emphasis is given to the accurate control of the reaction time, as this is a fixed time assay in which the colorimetric reagent stops the reaction.

It is important to remark that the product versus reaction time assay is performed at the lower substrate concentration to be used for the substrate curve experiment in laboratory session 2. Under these experimental conditions, the time interval in which the reaction rate remains constant (initial velocity condition) will be the shortest, and thus guarantees determination of initial reaction rate for all substrate concentrations to be used during lab session 2. In the example provided in Supporting Information Video 1, it can be observed that the initial-rate phase of the reaction persists longer at higher substrate concentrations [12].

Details of reaction mixtures, controls and standards preparation are provided in Supporting Information Appendix 2. Briefly, the reaction is initiated by substrate addition in different test tubes containing fixed enzyme concentration. Test tubes are incubated for different length of times, then the reaction is stopped by the addition of the Fiske-Subbarow reagent and the product amount is determined spectrophotometrically.

In Table I, there is a summary of the test tubes. To calculate the product generated for every time point assessed, the concentration of phosphate after each incubation time (test tube No. 11–20 in Table I) is calculated by means of the equation of the linear plot obtained for the standard phosphate curve (test tube No. 1–6 in Table I). As the reaction product is the phosphate released by the enzymatically catalyzed hydrolysis of phenyl-phosphate, the presence of phosphate in the reagent solutions (buffer, enzyme, and substrate) should be checked. For this purpose, different controls are included (test tubes No. 7–9 in Table I). Even though the substrate proved to be stable under the experimental conditions of the assay, a control of non-catalyzed hydrolysis of the phenyl-phosphate is performed for pedagogical purposes (test tubes No. 10 in Table I).

Students work in pairs in order to facilitate the addition of reagents at the stipulated times. The whole class is divided in three groups, each one working with a different enzyme concentration. For each laboratory shift, data obtained by students are loaded into spreadsheets where the individual and the pooled results of all the students working with the same enzyme concentration are depicted (spreadsheets in Supporting Information Appendix 3). The averaged results of the product generated for every time point for the three enzyme concentrations are then available *via* the virtual campus (an online educational platform) to students for analysis and writing of a laboratory report. In this report, the individual results are presented, indicating the raw and processed data. In addition, the averaged results of the class are included in the report for analysis and used to determine the time interval of linear product appearance (steady state) and the initial rate for each enzyme concentration.

Laboratory Session 2: Determination of K_M, V_{max}, and k_{cat}. Initial Rate Versus Substrate Concentration Curve.

In this lab session, the variation of initial velocity with substrate concentration is determined. Before the experimental procedure starts, laboratory instructors discuss with the class the results obtained during the previous lab session and decide an enzyme concentration and incubation time to be used. The importance of choosing a reaction time that guarantees initial rate determination for all the assay conditions is explained. The experimental protocol for assessment of initial rate dependence with substrate concentration, controls and data processing are also discussed.

Students work individually in this lab session, as the protocol is simple and easy to perform in terms of reagents addition. Different substrate concentrations are incubated with the enzyme for a fixed time period. Students prepare standards for the calibration curve (test tubes No. 1–6), controls (No. 7–10) and reaction mixtures (No. 11–18) (Table II). It is important to point out that the initial rates at all substrate concentrations are obtained by measuring product generated after a fixed reaction time that must be within the initial conditions time interval obtained in lab session 1. The time of addition of reagents to each tube is not provided; instead, students are encouraged to design the time table for reaction initiation (by enzyme addition) and termination (by Fiske-Subbarow addition) taking into account the chosen reaction time interval. An example of reaction set up for the determination of initial rate versus substrate concentration for a reaction time of 5 min is provided in Supporting Information Appendix 2.

Initial velocity can be calculated as the concentration of product generated per reaction time used. Students load initial velocity values in spreadsheets (Supporting Information

TABLE I *Reaction setup for the determination of the linearity interval of product appearance in time*

Test tube No.	Description	Phosphate (mM)	Enzyme (μg/ml)	Substrate (mM)	Reaction time (min)
1–6	Standard curve	0 to 1	–	–	–
7	Buffer control	–	–	–	–
8	Enzyme control	–	1.25, 5 or 10	–	–
9	Substrate control	–	–	1	–
10	Substrate control (37 °C)	–	–	1	41
11–20	Reaction mix	–	1.25, 5, or 10	1	0–40

See Supporting Information Appendix 2: Supporting Information Table S1 for further details

Appendix 4) and plot the experimental points of initial rate for each substrate concentration. Michaelis–Menten equation is fitted to the experimental data by nonlinear regression using the spreadsheets tool Solver [13–16], which allows obtaining the best fitting values of K_M and V_{max}.

Results and Analysis

Data collected from four cohorts of approximately 300–400 students each was analyzed. Each year, students are distributed in eight shifts and work in two separate laboratories simultaneously. Therefore, the experience was conducted with a maximum of 30 students and at least two instructors per laboratory. The results presented here correspond to those obtained in one laboratory shift and are representative of the whole data analyzed (Supporting Information Appendix 2). Details of the data processing and analyses of controls is provided in Supporting Information Appendix 2.

Laboratory Session 1: Initial Velocity Conditions. Product Versus Reaction Time Curves with Different Enzyme Concentrations.

The goal of this experiment is to choose an enzyme concentration and an incubation time that ensures initial velocity conditions for every substrate concentration to be used in the next session.

Experimental data of product concentration versus reaction time obtained for each of the three enzyme concentrations used are shown in Fig. 1*A*. It can easily be evidenced that as the enzyme concentration rises, an increment in the initial slope is observed. As the reaction time is not long enough, the reaction does not reach equilibrium, but given enough time, it is expected that the three curves would approach the same product concentration. In this graph, it can also be observed that the time interval in which initial velocity can be determined (i.e. linear product appearance) is longer, as enzyme concentration decreases. However, to better estimate the time interval of initial rate

TABLE II *Reaction setup for the determination of kinetic parameters*

Test tube No.	Description	Phosphate (mM)	Enzyme (μg/ml)	Substrate (mM)	Reaction time (min)
1–6	Standard curve	0–1	–	–	–
7	Buffer control	–	–	–	–
8	Enzyme control	–	(a)	–	–
9	Substrate control	–		10	–
10	Substrate control (37 °C)	–	–	10	(b)
11–18	Reaction mix	–	(a)	1–10	(b)

For detail see Supporting Information Appendix 2: Supporting Information Table S3. (a) Alkaline phosphatase in the concentration selected (i.e. 5 μg/ml). (b) Selected reaction time (i.e. 5 min).

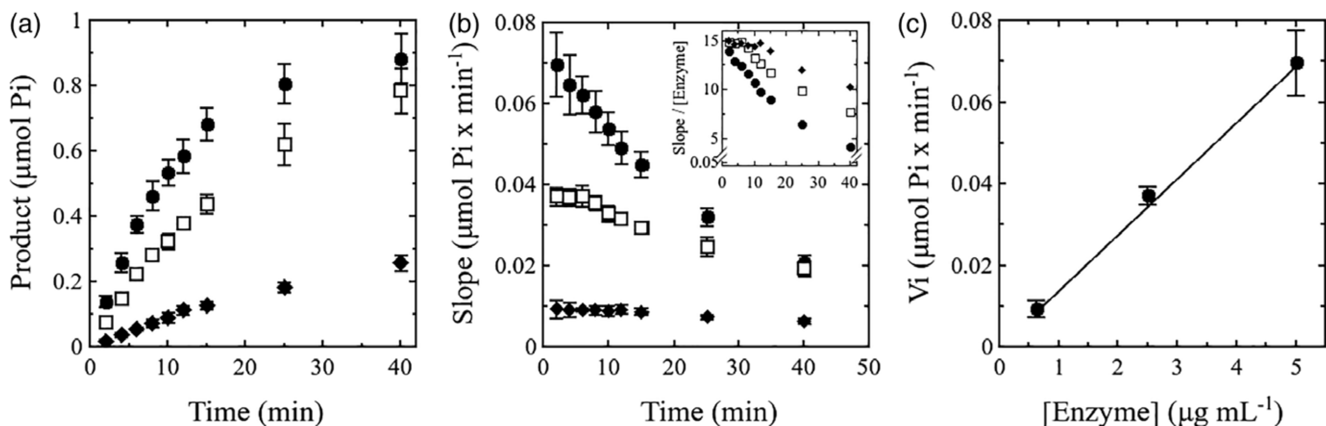

FIG 1 ***Assessment of the reaction linearity for different enzyme concentrations (1.25 ◆, 5 □, or 10 ● μg/mL in panels A and B) at 1 mM phenyl-phosphate.*** *Panel A, Time course of reaction. Panel B, Plot of slope versus reaction time; inset: slope/[enzyme] versus reaction time. Panel C, Plot of initial velocity (Vi) versus enzyme concentration. Every point represents an average of six independent determinations; the error bars show the standard deviation. Pi, inorganic phosphate.*

conditions, the slope for each reaction time point (including all the experimental points from 0 to the reaction time under analysis) was calculated (Fig. 1*B*). Note that this slope is not indicative of the instantaneous velocity, but would represent the "average velocity" for each time interval under analysis. It can be seen that the average velocity remains constant for a time period and then decreases. Thus, in this period, reaction progresses in steady state condition and the average velocity would correspond to the initial rate. This representation clearly shows that the period during which the slope of the product versus reaction time plot remains constant decreases with increasing enzyme concentration. To improve the visualization of this, the slope was normalized by the enzyme concentration (Fig. 1*B*, inset). This analysis reinforces the concept that the reaction rate remains constant for a period of time, corresponding to initial velocity. Finally, the value of the initial rate for each enzyme concentration was obtained (Fig. 1*C*). It is important to point out that, as expected for enzymes obeying Michaelis-like rate behavior, a linear plot is observed, indicating that the specific activity (Vi/[enzyme]) remains constant within the enzyme concentration interval used [12].

From the analyses of these graphs, students decide the enzyme concentration and reaction time to perform the substrate curve in the second laboratory session. The reaction time to be used should be chosen considering that it must be comprised within the time interval where the reaction rate remains constant, it should allow the operator to add reagents to the different tubes with no rush, and the product generated in that time must be enough for its accurate detection. Considering the experimental design to be used, the reaction time should be longer than 4.5 min (Supporting Information Appendix 2).

From the plots shown in Figs. 1*A* and 1*B*, the reaction rate remains constant for less than 2, 6–8, or 12–15 min for enzyme concentrations 10, 5, or 1.25 μg/ml, respectively. If the enzyme in the higher concentration is used, the experimental procedure will be difficult, since the addition of reagents to initiate and stop the reaction would have to be too fast. Therefore, 1.25 and 5 μg/mL concentrations could be used for the experiment to be performed in session 2. Considering that there is no need in prolonging the experiment and the error attained with low absorbance data, the enzyme concentration chosen was 5 μg/mL and a reaction time between 5 and 7 min.

Laboratory Session 2: Determination of K_M, V_{max}, and k_{cat}. Initial Rate Versus Substrate Concentration Curve.

With the purpose of characterizing the enzymatic reaction under study, the effect of substrate concentration on the initial velocity is assessed. A representative curve showing the initial velocity dependence on substrate concentration is shown in Fig. 2. As expected, the curve obtained seems to have a hyperbolic behavior compatible with Michaelis–Menten kinetics mechanism. In this sense, a linear plot is observed after performing a Lineweaver-Burk or double reciprocal transformation (Fig. 2, inset), indicating a hyperbolic behavior of the experimental data. Notice that, although in many textbooks and reports K_M and V_{max} values are determined using Lineweaver-Burk plots, the linear transformation distorts the experimental error and, thus, it is not appropriate for an accurate estimation of the kinetic parameters [5,13,17–19]. Therefore, students obtain the best fitting values of K_M and V_{max} by fitting Michaelis–Menten equation [Eq. (1)] to the initial velocity (Vi) versus substrate concentrations [S] results using nonlinear regression in a spreadsheet template and the Solver tool (Supporting Information Appendix 4).

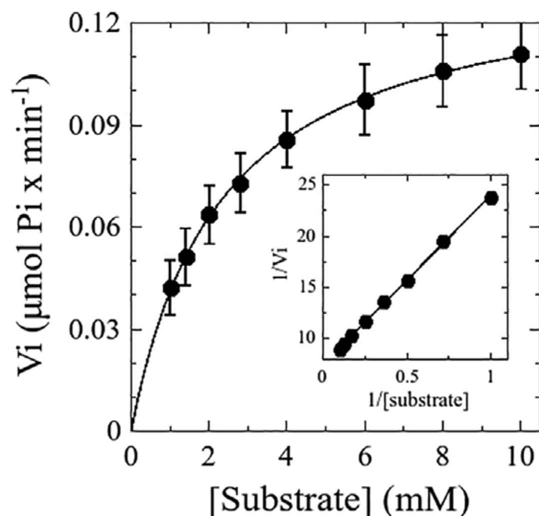

FIG 2 ***Initial velocity dependence on substrate concentration***. *Each point represents an average of 16 determinations; the error bars show the standard deviation. The continuous line is the plot of Michaelis–Menten equation [Eq. (1)] with the best fitting values of K_M and V_{max} (2.3 mM and 0.136 mM min^{-1}, respectively). The inset shows a Lineweaver-Burk or double reciprocal plot. Pi, inorganic phosphate.*

$$V_i = \frac{V_{max}[S]}{K_M + [S]} \tag{1}$$

where V_{max} is the initial rate at $[S] \rightarrow \infty$ and K_M is the substrate concentration where Vi = $V_{max}/2$. The catalytic constant (k_{cat}), expressed in min^{-1} units, is calculated as the ratio between V_{max} and enzyme concentration in molarity units. For this, students have to calculate the enzyme concentration in the reaction mix and transform it from µg/mL to molarity units, assuming that the enzyme preparation is pure and considering a molecular weight for the dimer of 160 kDa. The spreadsheet template is available to students *via* the virtual campus for further analyses of results and elaboration of a laboratory report.

Conclusion

The laboratory work described introduces students to the basic experimental work with enzyme kinetics. It reinforces the concepts of initial velocity dependence on substrate and enzyme concentration, and allows the determination of kinetic parameters as a first approach in enzyme characterization. The experimental procedure was designed to highlight the importance of the accurate determination of initial reaction rate. Students gain practice not only on laboratory skills (i.e. reaction setup and time-dependent pipetting), but also on experimental design, data processing, analyses, and representation. The two-part laboratory work in which students first determine the enzyme concentration and the reaction time to be used in the following session to find the kinetic parameters is especially important to make use of the theoretical concepts in the analysis of the results and the design of further experiments. The graphical analysis of the variation of the slope with time reinforces the concept that the reaction rate remains constant for a certain period (initial rate conditions), and also serves as a useful tool to visualize the duration of this period. Through the determination of the kinetic parameters K_M and V_{max} by fitting the Michaelis–Menten equation to the experimental data, students are introduced to non-linear regression. In this regard, the use of spreadsheets and solver tool constitutes an excellent approach to visualize and understand the process of fitting an equation to experimental data [19]. The assay is robust, as can be seen from the comparison of results obtained by different students, laboratory shifts and cohorts (Supporting Information Appendix 2). Additionally, the assay is economical and does not need sophisticated equipment, so it can easily be implemented in undergraduate classes, even in those with large number of students. Furthermore, the experimental design and the approaches described to analyze the results could easily be applied to the study of other enzyme reactions, especially if fixed-time assays are used.

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