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Enzyme kinetics: Teaching using polyphenoloxidase with a practical science activity

Olcay Sinan*

Balikesir University Necatibey Education Faculty, Department of Primary Science Education, Balikesir, Turkey

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Abstract

Enzymology is one of the fundamental areas of biochemistry and involves the study of the structure, kinetics, and regulation of enzyme activity. Research in this area is often conducted with purified enzymes and extrapolated to in vivo conditions. The specificity constant, Km (Michaelis-Menten constant), Vmax (catalytic power), IC_{50} and Ki (inhibition constant) expresses the efficiency of an enzyme as a catalyst. These parameters are usually determined for enzyme catalysis and inhibition study. In this work we propose a classroom experiment for its determination using polyphenol oxidase (PPO) enzyme from banana. In addition, inhibition experiment was carried out by L-cysteine and p-aminobenzene sulfonamide which is classical PPO inhibitors. The Km and Vmax values of banana PPO towards catechol and 4-methyl catechol were determined by Lineweaver Burk method. Although there were a few variations in the kinetic parameters results among students, the students generally carried out the program successfully by correctly identifying inhibition type. The inquiry-based problem solving approach of this curriculum facilitates an understanding of the basic concepts of biochemistry and its application.

Keywords: Biochemistry education; Experimental design; Enzyme kinetics; Polyphenol oxidase

1. Introduction

Characterization of enzyme kinetics today plays an essential role in important areas such as medical diagnostics, proteomics research, or even enzyme inhibition based biosensors for environmental monitoring. Of all subjects covered in a general biochemistry textbook enzyme kinetics and inhibition are perhaps the one topic that has the most tangible relevance to everyday life [1]. Inhibitors of enzymes are used as pharmaceutical agents in human and veterinary medicine as well as herbicides and pesticides [2]. However, as instructors may know, students typically find this topic difficult. Some reasons for this, as well as one possible solution involving computer simulations, have been addressed by Gonzalez-Cruz et al. [3].

*Correspondence author. Tel.: +90-266-241-2762; fax: +90--266-241-2700.
E-mail address: osinan@balikesir.edu.tr or olcaysinan@yahoo.com (O. Sinan).

Our current biochemistry course for majors has a required laboratory component. Students at our university offer 4-hr elective biochemistry lab for a week. For the above reasons, I conducted an experiment that allowed students to understand enzyme kinetics much better after carrying out an offered lab experiment in two week. Harlen and Wake [4] also indicated that practical work in the classroom increases the effectiveness of teaching science by providing students with hands-on experience with science and allowing them to infer the theory behind the experimentation [4].

Many different enzymes are used in undergraduate laboratory kinetics experiments published in standard laboratory texts. These enzymes include tyrosine hydroxylase [5, 6] alkaline phosphatase [7] and lactate dehydrogenase (LDH) [6]. Polyphenol oxidase is chosen in this study since it catalyzes a reaction that is often familiar to students. Many students have seen the browning of fruits and vegetables and thus have an immediate connection this enzyme. Students also have the opportunity to see this enzyme again during the everyday life. In addition, polyphenol oxidase may be a good model enzyme for teaching practical classes ranging from beginning level to more advanced biochemistry courses, in order to demonstrate important principles of kinetic studies and parameter determination (Vmax, Km, Ki) [8]. In fact, a review of 20 general biochemistry textbooks commonly used in undergraduate and medical school courses revealed that the two methods namely Lineweaver-Burk and Eadie Hofstee showed the determination of the Michaelis constant (Km) and maximal velocity (Vmax) [1, 9].

The aim of this work is to design newly practical experiment for biochemistry laboratory to learning enzyme kinetics for undergraduate students. Polyphenol oxidase enzyme extract was prepared from banana. The students determined Km and Vmax values using catechol and 4-methylcatechol as substrate with Lineweaver-Burk graphs. IC_{50} and Ki values of different inhibitors (sodium azide and dithiothreitol) also determined on PPO. In order to determine $IC_{50,}$ which is inhibitor concentrations causing up to 50% inhibition values, % activity-inhibition graph were drawn.

2. Background on PPO

PPO, often called tyrosinase, is an enzyme that synthesizes in many species of plants and fungi. It is homologous to mammalian tyrosinase, an enzyme involved in melanin formation. PPO is a metalloenzyme which contains two active-site copper ions that are essential for enzymatic activity. It catalyzes a redox reaction in which various phenolic compounds are oxidized (Figure 1), ultimately leading to the production of dark-colored pigment molecules which cause the familiar browning of vegetables, fruits, and mushrooms as they age or when they are bruised. Due to its role in the production of these melanin-like polymers, PPO is considered to be an important part of plant/fungal defense mechanisms against insects and pathogens. Melanin-like product formation by PPO is of great concern to the agricultural industry because postharvest browning of product results in considerable economic loss because of the alteration of color, flavour, and nutritional value [10–15]. For more information, see the reviews by Yoruk and Marshall [10] and Mayer [11].

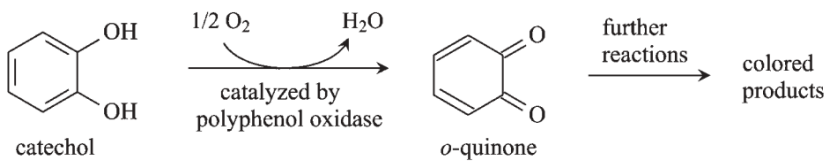

Fig. 1. Catechol's oxidize and subsequent reactions.

Although there are commercially available enzyme assay kits and calculation program for kinetic parameters produced by companies for educational purposes, they are too simple to motivate students or to satisfy their inquisitiveness. Hence, a suitable protocol is suggested for determination of kinetic parameters in universities. This protocol facilitates understanding of students to important of inhibitor as pharmaceutical agents in human and veterinary medicine as well as herbicides and pesticides, another important enzyme biotechnology. This protocol allows students to explore the biotechnology used in a variety of fields and to understand the concepts of enzyme kinetics.

3. **Materials and methods**

3. 1. Materials

All chemicals used in this experiment were the best grade available and used without further purification because of obtaining from Sigma Chem. Co. Enzyme activity were measured with the aid of a Cary -1E-g UV-Visible Spectrophotometer (Varian). Banana fruits used in this study were bought from local market.

3. 2. Extracting enzyme from banana

The extraction procedure was adopted from Wesche-Ebeling and Montgomery [16]. Firstly banana fruits were peeled. Secondly, to prepare the crude extract, 10g of sample tissue was cut quickly into thin slices and homogenized in a warring blender for 2 min using 100 ml of 0.1M phosphate buffer (pH:6.5) containing 5% poly (ethylene glycol) and 10mM ascorbic acid. The 0.1 M concentration was chosen to avoid the influence of enzymatic extract ionic strength on PPO activity, as described by Angleton and Flurkey [17]. After filtration of the homogenate through muslin, the filtrate was centrifuged at 15000g for 20 min, and the supernatant was collected. The supernatant obtained was used as crude extract.

3. 3. Enzyme assay

Enzyme activity was determined by measuring the increase in absorbance with a Cary |1E|gUVVisible Spectrophotometer (Varian) set 420nm when using catechol and 4-methycatechol as substrates [18]. In each measurement, the volume of solution in a quartz cuvette was kept constant as 3mL. About 0.1mL of PPO was used with 0.6mL of 0.1 M catechol and 2.3mL of 0.1 M phosphate buffer (catechol substrate pH6.5) and 0.2mL of 0.1 M 4-methylcatechol and 2.7mL of 0.1 M phosphate buffer (4-methylcatechol substrate pH6.5). The 0.1 M concentration was chosen to avoid the influence of enzymatic extraction ionic strength on PPO activity, described by Angleton and Flurkey [18]. One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001min-1mL-1. PPO activity was assayed at 20 ^{0}C [18].

4. Kinetic studies and determination of IC_{50} and K_i values

In order to obtain Km and Vmax values separately for catechol and 4-methylcatechol at optimum pH and temperature the enzyme activity was measured at five different substrate cuvette concentrations. Km and Vmax values were determined by means of Lineweaver-Burke graphs. IC_{50} and Ki values of different inhibitors (sodium azide, dithithreitol) were

determined on PPO. In order to determine IC_{50} values 10mM catechol was used as substrate. At first the activity of enzyme was measured without inhibitor. This measure was accepting 100% activity for graph and then enzyme activity assay with different inhibitor concentration. In order to determine IC_{50} values were drawn by using % activity-inhibition graph. The inhibitor concentrations causing up to 50% inhibition were determined from the graphs. This way was followed to determine Ki values. In the media with or without inhibitor, the substrate concentrations were 0.6, 0.8, 1.0 and 1.2 mM. For this purpose, the substrate was used between 0.6 and 1.2 ml. Inhibitor solutions were added to the reaction medium as 0.1, 0.2 and 0.3ml resulting in three different fixed concentrations of inhibitor. The Lineweaver-Burke graphs were obtained and Ki values were calculated.

4. 1. Procedures

4. 1. 1. Step 1. Introduction

The first step in this practical experiment was to divide the students into several groups, where each group consisted of 4-5 students. If students have not already studied enzyme, this experiment may provide an opportunity to introduce concepts such as the following: enzymes are proteins; enzymes are biological catalysts; substrate; active site; inhibitors. In our case, this background information was covered in the lecture course that accompanied the lab course and was then reviewed in a brief pre-lab lecture immediately before the experiment was carried out. Specific background information about the enzyme studied in this experiment should also be given. We provided this information to students in a handout that also contained the experimental instructions, data sheets, and post-lab questions.

4. 1. 2. Step 2. Experiments

The protocol for determination of kinetic measurements has three main stages:

- ☑ *Enzyme extracting from banana (Approximately 30 min.).* Each group prepared the banana extract. For this purpose, each group prepared the extraction buffer and then peeled banana was homogenized with the buffer using warring blender for 2 min. Enzyme solution was isolated with muslin filtration and centrifugation (see Materials and Methods above).
- ☑ *Determination of Km and Vmax values.* Each group determined Km and Vmax values using Lineweaver-Burk graphs. In order to determine Km and Vmax values, enzyme assay was applied at five different substrate concentration using different substrate (catechol and 4-methyl catechol) (45min).
- ☑ *Determination of IC_{50} and Ki values.* Each group determined IC_{50} values for two inhibitors (see Materials and Methods above). Viewing inhibition type and Ki values by Lineweaver-Burk graphs. As the same substrate concentration were used. (Approximately 30 min for determination of IC_{50} values and 45min for Ki).

4. 1. 3. Step 3. Presentation

Students were asked to present their data and conclusions about the differentiation of each substrate. The presentation from each group included an introduction describing the concept of enzyme kinetics, methods, results and their conclusion.

5. Results and discussion

The developed protocol was assigned to five different groups of students as described in the previous section. Students in each group carried out enzyme extracting, enzyme assay, enzyme inhibition and obtained results that were used to approximately determine the Km, Vmax, IC_{50}, inhibition type and Ki values.

Table 1. Kinetic values determined from students using catechol and 4-methly-catechol as substrates.

	Catechol			4-methly-catechol		
Groups	Km (mM)	Vmax (U/min)	Vmax/Km (U/min)/mM	Km (mM)	Vmax (U/min)	Vmax/Km (U/min)/mM
A	12.4	3450	278,2	8.5	4170	490,6
B	14.3	3200	223,7	10	3663	366,3
C	22	560	25,4	8.3	4470	538,5
D	15.4	2612	169,6	6.6	4612	698,8
E	20	2157	107,85	11.5	682	59,3

To characterize the properties of polyphenol oxidase, 4methylcatechol and catechol have been widely used as major substances [16, 18, and 19]. In order to investigate enzyme kinetics, Lineweaver–Burk graphs are drawn to calculate the Km and Vmax values. Kinetic values obtained in the absence of inhibitor for Vmax ranged from 560 to 3450 EU/mL.min with an average of 2396 EU/mL.min (n=5) for using catechol as substrate. Vmax ranged from 682 to 4612 EU/mL.min with an average of 3519 EU/mL.min for using 4-methylcatechol as substrate. (Data were only considered from student pairs with an $R^2 \geq 0.99$ on the Lineweaver-Burk plot). Values determined for Km ranged from 12.4 to 22mM for catechol and 6.6 to 11.5mM for 4-methylcatechol as substrate, with an average of 16.8 and 8.98, respectively. Figure 2 and 3 shows the Lineweaver-Burk graphs for the determination of Km and Vmax values by one of the groups. The affinity of banana PPO (lowest Km) was the highest for 4-methyl-catechol. Considering the ratio Vmax/Km, it was concluded that 4-methylcatechol is the most suitable phenolic substrate for banana (Table 1). Student kinetic values are also similar to those in previous studies [20, 21]. Km and Vmax values determined each groups was almost identical. All of them was correctly determined the efficient substrate for banana PPO. Different values found by each group may result from environmental conditions and their own mistakes.

Many substances may alter the activity of an enzyme by combining with it in a way that influences the binding of substrate and/or its turnover number. Substances that reduce an enzyme's activity in this way are known as inhibitors. Many inhibitors are substances that structurally resemble their enzyme's substrate but either does not react or react very slowly compared to the substrate. Such inhibitors are commonly used to probe the chemical and conformational nature of a substrate-binding site as part of an effort to elucidate the enzyme's catalytic mechanism [9]. Students also have the opportunity to see enzyme inhibition study during the discussion of inhibition in lecture. Thus, students determined IC_{50}, Ki and inhibition type for two inhibitor using 4-methylcatechol as substrate. Different types of inhibitors have different effects on enzyme kinetics. The preventing of enzymatic browning by a specific inhibitor may involve a single mechanism or be the result of interplay of two or more mechanisms of inhibitor action. There are various mechanisms through which enzyme inhibitors can act [22].

5. 1. Competitive inhibition

A competitive inhibitor has a chemical similarity to the substrate and competes with the substrate for binding to the active site of the enzyme. The Lineweaver–Burk equation for competitive inhibition is:

$$\frac{1}{Vo}=\left(\frac{\alpha Km}{V\,max}\right)\cdot\frac{1}{[S]}+\frac{1}{V\,max}\ \text{where}\ \ \alpha=\left(1+\frac{[I]}{Ki}\right) \tag{1}$$

In this equation, [S] is substrate concentration (mol L^{-1}); Ki is the dissociation constant of the enzyme–inhibitor complex; Vmax is the maximum velocity at saturating concentration of the substrate (EU mL^{-1} min^{-1}); V_0 is the enzyme activity value (EU mL-1 min-1); and Km is the Michaelis constant (M) [9]. Ki values were obtained by plotting 1/v0 vs. 1/[S]. A plot of 1/v0 vs. 1/[S] for competitive inhibition of banana PPO using 4-methylcatechol as a substrate is given in Figure 2. The Lineweaver–Burk plots for PPO using five concentrations of substrates in the absence and presence of dithiothreitol show that inhibition type was competitive.

5. 2. Non-competitive inhibition

Lineweaver–Burk equation for non-competitive inhibition is given in the following Eq. (2):

$$\frac{1}{Vo}=\left(\frac{\alpha Km}{V\,max}\right)\cdot\frac{1}{[S]}+\frac{\alpha'}{V\,max} \tag{2}$$

In a classic example of pure non-competitive inhibition, the uninhibited reaction and the enzyme in the presence of the inhibitor yield the same Km value. The plot of this equation consists of lines that have the slope (1+([I]/Ki))(Km/Vmax) and intercept (1+([I]/Ki))(1/Vmax) [25]. Banana PPO was non-competitive inhibition using 4-methylcatechol as substrate. Again, Ki values obtained are given in Table 2.

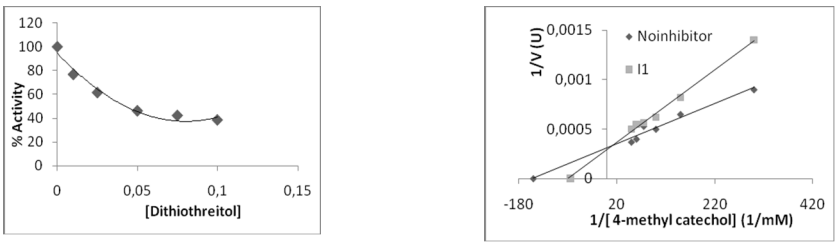

Fig. 2. Effect of dithiothreitol on banana PPO from one group data.

Fig. 3. Effect of sodium azide on banana PPO from one group data.

According the reports obtained from groups, students determine the type of inhibition observed for their inhibitor and support their conclusion with the Lineweaver-Burk plot results and with their numerical values of Vmax, Vmax [I], Km and Km [I]. They discuss whether or not their conclusion is reasonable based on comparison of the structures of inhibitor and substrate. The structure of dithiothreitol is more appropriate to active site of enzyme than glutathione and would be expected to fit into the 4-methylcatechol binding site, exhibiting competitive kinetics. Student data almost always allow the correct determination that dithiothreitol is competitive and sodium azide is non-competitive in this assay. I routinely use duplicate samples and one inhibitor concentration enough to determine for inhibition type with student. I recommend having the teaching assistant check the raw data before the student leaves the lab. If precision is low and the expected trend is not observed, student should repeat the experiment.

Table 2. IC_{50}, Ki values and inhibition type determined student groups

	Sodium azide			Dithiothreitol		
Groups	IC_{50}	Ki (mM)	Inhibition Type	IC_{50}	Ki (mM)	Inhibition Type
A	$2.0x10^{-4}$	3.31	Non-competitive	$1.5x10^{-4}$	2.7	competitive
B	$2.33x10^{-4}$	5.02	Non-competitive	$2.97x10^{-4}$	0.2	competitive
C	$4.0x10^{-4}$	2.49	Non-competitive	$1.35x10^{-4}$	15.7	competitive
D	$3.2x10^{-4}$	3.33	Non-competitive	$3.30x10^{-4}$	3.13	competitive
E	$1.5x10^{-4}$	4.98	Non-competitive	$4.32x10^{-4}$	2	competitive

IC_{50} and Ki values of inhibition studies obtained of all groups (Table 2). The average of IC_{50} for sodium azide was $2.6x10^{-4}$mM and for dithiothreitol $2.7x10^{-4}$mM using 4-methylcatechol as substrate. This is consistent with data from Nathalie et al [20, 24] which shows that sodium azide and dithiothreitol were effective inhibitor for banana PPO under similar conditions. These inhibitors especially were chosen for understanding of the student with two inhibition type. Student's inhibition data was similar to a number of studies for PPO [20, 21, 25, and 26]. Students determined the Ki values. The medium Ki for sodium azide was 3.8mM and for dithiothreitol 4.7mM.

Dithithreitol is a competitive inhibitor for banana PPO. In competitive inhibition, only the apparent Michaelis constant is affected by the inhibition [9]. Lineweaver-Burk plots for banana PPO using different concentrations of 4-methyl catechol as substrate in the absence and presence of inhibitors used were typical of competitive inhibition as illustrated of one group in the Fig. 2, which Vmax values have not changed when Km values have increased. This result suggested that the reaction between inhibitor and substrate catalyzed by banana PPO is a competitive inhibition. This mode of inhibition reveals that inhibitor binds only to active site of the free enzyme, prohibiting the substrates from undergoing oxidation.

In this experiment, banana PPO was non-competitively inhibited by sodium azide. In reversible noncompetitive inhibition, both inhibitor and substrate can bind simultaneously to the enzyme molecule. Clearly two molecules must bind to different sites on the enzyme. The presence of inhibitor does not affect substrate binding but does interfere with the catalytic functioning of the enzyme. A linear regression method was used to determine whether the experimental data fitted with the inhibition equations. As can be seen from figure 2 and 3, the fact that regression coefficient values are in the range 0.98–0.99 shows that the experimental data fit better with related inhibition equations. In the literature, there are also other inhibitors for PPO. Various inhibitors may also be used in the experiment of this study.

5. 3. Possible modifications

If there is enough time, students can compare the Vmax and Km values obtained from the Michaelis-Menten plot with those of Lineweaver-Burk plot. This allows them to see the

limitations of the graphs. At the highest concentration of substrate in this experiment, Vmax is not reached. Thus, the Vmax which students estimate from the v vs. [S] graph will be lower than that of the Lineweaver-Burk plot.

Students could also determine whether the pH and temperature affect the enzyme activity. For this purpose, PPO activity as a function of pH was determined using 4-methyl catechol as substrate. The buffers may be used 0.1 M acetate (pH 4.0-6.0) and 0.1 M phosphate (pH 6.0-9.0) adjusted with 0.1 M NaOH and HNO_3 [27]. For determining the optimum temperature values of the enzyme, PPO activity was measured at different temperatures in the range of 10-60 ^{0}C using 4-methyl catechol as substrate as indicated above. The effect of temperature on the activity of PPO was tested by heating the standard reaction solutions (buffer and substrate) to the appropriate temperatures before introduction of the enzyme. The desired temperatures were provided by using a Tempette Junior TE-85 temperature controller attached to the cell-holder of the spectrophotometer. Once temperature equilibrium was reached, enzyme was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals [28].

6. Conclusions

It is my opinion that these experiments such used PPO enzyme assay to teach students the key concepts and practical skills in enzyme kinetics will benefit our students remarkably in their future professional lives. This experiment can provide an opportunity for students to understand some concepts such as enzymes, biological catalysts, substrate, active site; inhibitors. Students in this course learned the determination of Km and Vmax values. They were asked to identify which substrate is appropriate for banana PPO. Students determined that inhibitor concentration of causing up to 50% inhibition with two inhibitors. They also investigated inhibition type and discuss whether or not their conclusion is reasonable based on comparison of the structures of inhibitor and substrate. These open-ended, inquiry-based and micro teaching exercises also may help our students develop critical thinking, scientific process and problem-solving skills [29-42]. Students in the current study gained the ability to independently search enzyme kinetics to obtain enzyme and its inhibition using spectrophotometric analysis for their projects. This lab course is also easily adaptable for multi-week studies. Also a study of different PPO source could be used and compare in this respect of enzyme kinetics.

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