Bioengineering Laboratory I

Enzyme Assays

Part I: Activity Measurement

2016-2017 Fall Semester

1. Theoretical background

Enzymes are proteins that catalyze biological reactions by lowering the activation energy (Figure 1).

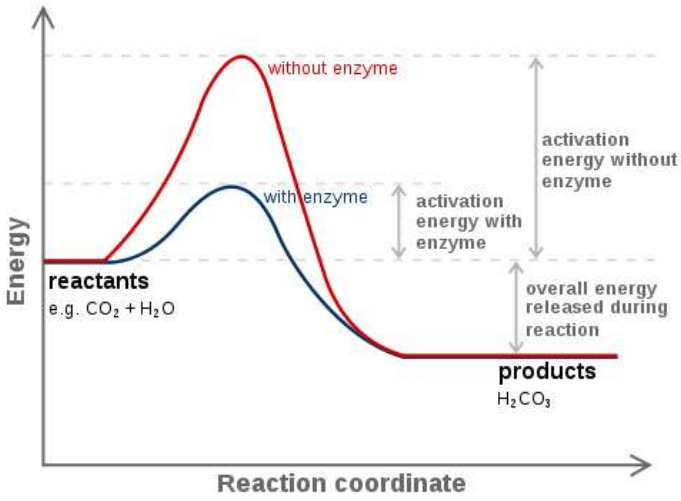


Figure 1. Catalytic behavior of enzyme

Each enzyme molecule contains an active site, to which its specific substrate is bound to form an enzyme-substrate complex as shown in Figure 2.

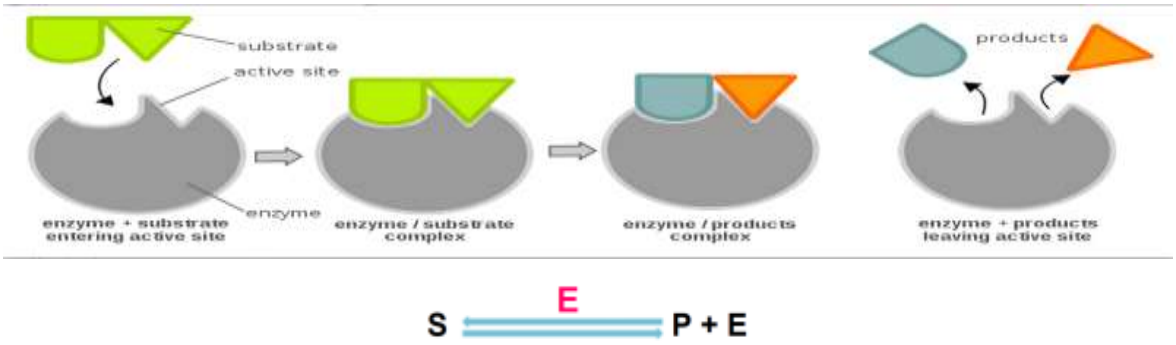


Figure 2: Key and lock model in a catalytic reaction

Enzyme assay are laboratory methods for measuring enzymatic activity. All enzyme assays measure either the consumption of substrate or production of product overtime. Enzyme assays can be split into two groups

1- Continuous or real time assays (spectrophotometric, fluorometric, calorimetric, chemiluminescent, light scattering, microscale thermophoresis) where the assay gives a continuous reading of activity.

2- Discontinuous (stop time) assays (radiometric, chromatographic) where samples are taken, the reaction stopped and then the concentration of substrates/products determined.

Second one is the easiest way to do many assays at one time, **BUT** there are two things that need to be considered before doing this form of the assay. First, is the assay linear? In other words, in the time that I am running the assay, is the product being produced (or substrate converted) at a linear rate? If the conditions of the assay tube are such that the reactants (substrate) are depleted or the products are inhibiting the enzyme, then you **CAN NOT** use this assay. Second, is the compound you are measuring stable enough to wait to read and are the conditions used to stop the enzyme, i.e. acid or base, too harsh to maintain the structure of the readout? We will be doing both stop time and real time/continuous assays.

Among the activity measurements methods, spectrophotometic ones is useful. In spectrophotometric assays, you follow the course of the reaction by measuring a change in absorbance which provides a light source with selectable wavelength in the range 190-1100nm (ultraviolet and visible light) as depicted in Figure 3.

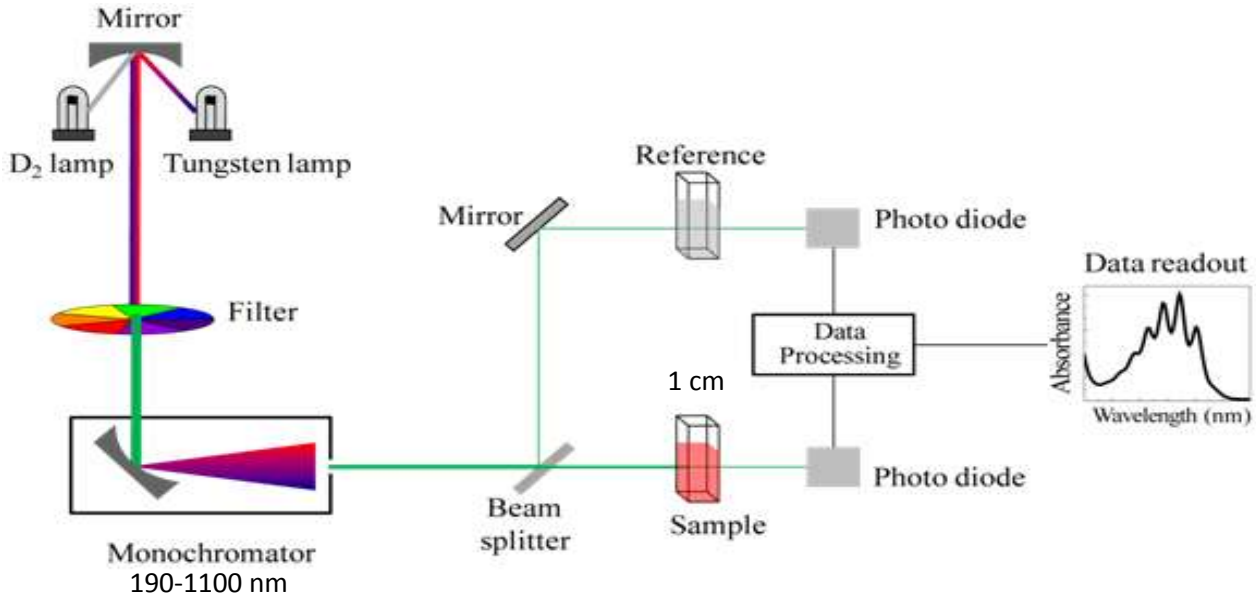


Figure 3. Working principle of UV Spectrophotometer

Sample is contained in a cuvette of exactly 1.00 cm thickness. The light passing through the sample is then measured and recorded by a detector. If this light is in the visible region you can actually see a change in the color of the assay, these are called colorimetric assays. Most

specs can only read between 0.01 and 3.0 abs units. At either end of this range there will be too much noise.

There are two main ways to measure an enzyme's reaction, **coupled or direct**. If the substrate or product has a characteristic absorbance or spectral "fingerprint," the changes in concentration can be directly measured. Meaning the change in absorbance (also known as optical density – OD) vs. time. From this graph (done on the spectrophotometer), you will select a region that is reasonably linear and determine the ΔOD/min and then convert it to Units of enzyme activity per ml.

The *rate*, *R*, of a chemical reaction is the speed or velocity, *v*, at which the reaction proceeds. The reaction rate is expressed as the change in concentration of a reactant or product during a given time interval. For a reaction is written as

$$\mathbf{A} + \mathbf{B} \rightarrow \mathbf{P} \tag{1}$$

where **A** and **B** are the reactants and **P** is the product, the rate can be expressed in terms of either the appearance of the product **P** or the disappearance of either of the reactants **A** or **B**. Thus, for any finite increment of time Δ*t*, one can write

$$R = \Delta[\mathbf{P}]/\Delta t = -\Delta[\mathbf{A}]/\Delta t = -\Delta[\mathbf{B}]/\Delta t \tag{2}$$

Note that there is a minus sign in front of the change in reactant concentrations. This indicates that their concentrations are decreasing. If the interval of time considered becomes smaller and smaller, approaching zero, the calculus can be applied and Equation (4) can be written as

$$R = \mathrm{d}[\mathbf{P}]/\mathrm{d}t = -\mathrm{d}[\mathbf{A}]/\mathrm{d}t = \mathrm{d}[\mathbf{B}]/\mathrm{d}t \tag{3}$$

where the **d function** indicates a derivative.

For kinetic enzyme assays, enzyme “concentrations” in **IU**s is more fundamental, useful, and sensible. An **IU** is typically defined as *the amount of an enzyme that will produce 1 μmol of product per minute or that will consume 1 μmol of reactant per minute*. The **specific activity** of an enzyme is defined as the units divided by the mg of protein present, IU/mg protein. Enzyme activity, of course, is highly dependent on reaction conditions such as temperature, pH, and other solution components.

In our experiment tyrosinase enzyme is used. Tyrosinases are a group of oxidoreductases that catalyze the initial oxidation reaction in melanin formation. They are found in bacteria, fungi, plants, insects, and mammals. Tyrosinases have two distinct activities: (1) the reduction of monophenols (e.g. tyrosine), which is referred to as cresolase activity, and (2) the oxidation of diphenols (e.g. catechol), which is referred to as catecholase activity as depicted in Figure 4. Different tyrosinases will hydroxylate different substrates to varying degrees. For example, the diphenol DHICA is a substrate of human tyrosinase but not of mouse tyrosinase. Diphenols are better substrates of mushroom tyrosinases than monophenols.

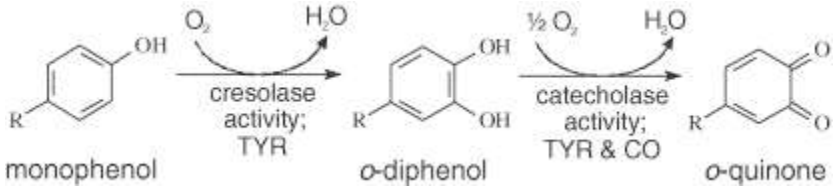


Figure 4. Catalytic reaction of monophenol to o-quinone by tyrosinase enzyme

As described in the general background, tyrosinase catalyzes the oxidoreduction of catechol to o-benzoquinone. Since o-benzoquinone absorbs maximally at 390-410 nm, its production can be tracked in time spectrophotometrically. Unfortunately (at least from the viewpoint of a student trying to analyze the reaction kinetics), o-benzoquinone spontaneously undergoes a series of oxidations and polymerizations that lead to the formation of large, insoluble, dark brown granules called melanins. The complete reaction pathway leading to melanin formation is shown Figure 5, where the initial forward reaction is catalyzed by tyrosinase:

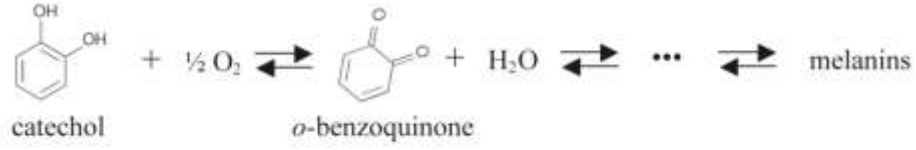
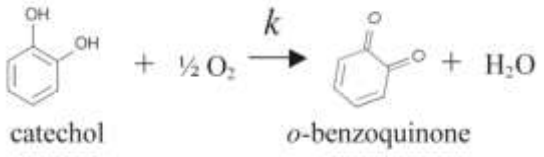


Figure 5. Catalytic reaction of catechol (diphenol) to melanin by tyrosinase enzyme

To precisely predict the rate of formation of o-benzoquinone over time would require knowledge of all the rates that make up this pathway. To simplify this kinetic model, we will look at o-benzoquinone production immediately following the addition of tyrosinase to catechol. Initially, because there is no o-benzoquinone present, we can ignore the reverse reaction from o-

benzoquinone back to catechol. We can also ignore all the reactions downstream of the catalyzed reaction since they cannot occur until o-benzoquinone has been formed. This greatly simplifies out model to the following:



(4)

Over time, however, the concentrations of o-benzoquinone and the other products will increase until we can no longer ignore the numerous other rates in the pathway. Given enough time, the entire system will reach equilibrium, and the rate of o-benzoquinone formation will go to zero. If the concentration of tyrosinase is much greater than the concentration of catechol and the O_2 concentration remains constant, the model in equation (4) predicts that the rate of *o* o-benzoquinone formation will be proportional to the concentration of catechol:

$$\frac{d[P]}{dt} = k[C] \tag{5}$$

In the equation above, P and C denote o-benzoquinone (the product) and catechol, respectively. By using a sufficient amount of catechol and measuring the rate of o-benzoquinone formation rapidly, you can further simplify equation (5) by assuming that the catechol concentration remains constant. Equation (5) then predicts that the concentration of o-benzoquinone will increase linearly with time:

$$[P] = k[C]_o t \tag{6}$$

Here, $[C]_0$ is the initial catechol concentration and k is the rate constant for the catalyzed reaction. Equation (6) only holds for short periods of time. Eventually, both the formation of product and the depletion of catechol will make our assumptions invalid. Your goal is to identify experimental conditions for which equation (6) appropriately describes the formation of o-benzoquinone from catechol in the presence of tyrosinase. This includes determining how long following inititation of the reaction equation (6) remains valid. To find the optimal data collection condition, you will keep $[C]_0$ constant and vary the tyrosinase concentration ([E]). Although [E] is not explicitly in equation (6), varying the concentration of tyrosinase changes the rate constant k. As you decrease [E], k decreases and o-benzoquinone formation is slowed. This is

advantageous because it takes longer for the concentration of o-benzoquinone to reach a level where we can no longer ignore its presence. On the other hand, if the rate of product formation is too slow, the o-benzoquinone that is formed will have time to undergo downstream reactions, invalidating our assumption that we can ignore these reactions. This sets up a Goldiocks dilemma in which you must find the optimal enzyme concentration between these two extremes.

Figure 6 shows plots of product formation over time for high and low enzyme concentrations. Dashed lines are added to make clear the range of times where the plots are linear. Notice that the time range is longer for the lower [E].

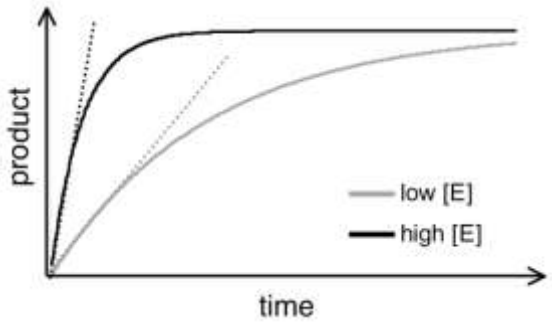


Figure 6. Formation of product with time in an enzymatic reaction

There is one further complication that you must take into account. As melanins polymerize to form larger granules, they become insoluble and slowly sediment during the reaction. This poses a two-fold problem. First, these large granules will scatter light, thus artificially increasing your absorbance signal. Second, as the melanin granules sediment, the concentrations of the various downstream products will change, further complicating the reaction pathway. The formation of sedimenting granules occurs more rapidly at higher enzyme concentrations due to the more rapid formation of o-benzoquinone and the subsequent downstream products.

2. Objective

The aim of this experiment is to provide students experience with a spectrophotometric analysis of enzyme activity. Another scope is to acquaint them with basic enzyme-substrate kinetics and the significance of in-situ enzyme analysis. They find opportunity for the preparation of PBS, enzyme and substrate solutions to conduct the enzyme assay. The students also learn to measure the enzyme activity in µmol/min unit by using Beer Lambert relationship.

3. Materials

Chemicals: Tyrosinase from mushroom (Sigma-Aldrich T3824 -25KU), sodium dihydrogen phosphate monohydrate "$NaH_2PO_4*H_2O$" (98%, Merck), di-Sodium hydrogen phosphate dihydrate "$Na_2HPO_4*2H_2O$" (99%, Merck), pyrocathechol (Sigma-Aldrich C9510). All solution are prepared with ultra pure water (18.2 MΩ) .

Materials: Micropipettes, UV quartz cuvettes (3 ml), stirrer bar, erlenmayer, beaker, spoon, graduated cylinder.

Instruments: Thermo scientific evaluation 201 UV-Visible Spectrophotometer, Thermo Scientific Peltier System, pH meter (WTW), analytical balance (Sartorius), magnetic stirrer.

4. Experimental procedure

Preparation of solutions

a- Prepare 50 mM 50 ml $NaH_2PO_4*H_2O$ and $Na_2HPO_4*2H_2O$ solution by using ultrapure water

b- Measure the pH of these two buffer solution by pH meter.

c- Adjust pH of the phosphate buffer solution (PBS) as 6.5 by mixing these two buffers.

d- Prepare a stock of 2 mM catechol in 30 ml PBS.

e- Prepare 500 µl, 250 U enzyme solution from stock enzyme solution (25 KU/ml PBS).

f- Use catechol and enzyme solutions within 1 or 2 hours. Because these solutions are rather unstable at room temperature.

Determination of maximum absorbace for subsrate (catechol) and product (o-quinone)

a- Turn on UV instrument and open wavelength scanning mode.

b- Turn on peltier system and set temperature to 25 ºC and stirrer speed at level 7 (approximately 700 RPM).

c- Fill both UV quartz cuvette with 3 ml PBS.

d- Put the cuvettes in UV instrument.

e- Scan between 190-1100 nm for base line correction.

f- Cleanout one of your cuvette and fill with 3 ml PBS again.

g- Put the cuvettes in UV instrument.

h- Scan between 190-1100 nm.

i- Cleanout one of your cuvette and fill with 3 ml 2 mM catechol solution to observe the maximum absorption peak of the substrate "catechol".

j- Repeat step g and h.

k- Cleanout one of your cuvette and fill with 3 ml 2 mM catechol solution.

l- Add 100 µl (includes 50 U) tyrosinase enzyme to initiate the enzymatic reaction.

g- Wait 3 min to observe the maximum absorption peak of the product "o-quinone" and also to monitor the occurrence of non-enzymatic reactions.

m- Repeat step g and h.

Activity measurement

a- Open rate mode.

b- Set wavelength at maximum absorbance for the product, temperature to 25 ºC, stirrer speed at 7 level (approximately 700 RPM) and time 70 sec.

c- Fill both UV quartz cuvette with 3 ml PBS.

d- Put the cuvettes in UV instrument for base line correction.

e- Cleanout one of your cuvette and fill with 3 ml 2 mM catechol solution.

f- Start measurement while pipetting 100 µl (includes 50 U) tyrosinase solution which provides in-situ analysis. Formation o-benzoquinone at early time points can be observed, when it is linear with time.

g- According to the max peak of o-quinone, enzymatic activity is determined by monitoring its formation during 70 sec.

h- Cleanout one of your cuvette and fill with 3 ml 2 mM catechol solution.

i- Repeat step from d to h at least two times more.

5. Data evaluation

Plot absorbance versus wavelength (from 190 to 1100 nm) graph for PBS, catechol and the one including catechol and o-quinone to detect the maximum absorbance peak for substrate (catechol) and product (o-quinone). All data can be shown in one graph. If it is necessary you may plot the graph from 240 to 600 nm.

In this experiment tyrosinase activity is measured by evaluating the velocity (rate) at which substrate (catechol) is converted to product (o-quinone). A change in absorbace with time provides an accurate estimation of the initial velocity of the reaction. For this aim, plot absorbance of o-quinone formation versus time graph. It is significant to collect the optimal data. The increase in absorbance should be linear with time. Be sure that the graph is a linear portion at

least 30 seconds and the linear region of your time trace does not exceed 0.8 absorbance value so that you can trust your measurement of the initial product formation rate.

The reaction slope: dA/dt (change of absorbance ΔA over the time interval Δt: 30 sec.) gives the initial steady-state reaction condition

Use the Beer–Lambert relationship; [28].

$$A = \varepsilon p l$$

where "A" is absorbance, "p" is product concentration, "ε" is extinction coefficient (for o-quinone formation from catechol at maximum absorbance was taken as ε=1417 $M^{-1}cm^{-1}$) and "l=1 cm" is path- length. The slope of the kinetic curves is converted to the tyrosinase activity (expressed as μmol/min).

$$\frac{dP}{dt} = \frac{dA}{dt}\frac{1}{\varepsilon l}$$