



## Exploring enzyme renaturation in an introductory biology lab course

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### Abstract

The relationship between the structure and function of proteins is a foundational concept in biology. Enzyme denaturation exercises are a common way to illustrate this concept in introductory-level cell and molecular biology labs. Previously, our students manipulated reaction pH or temperature and quantified the effect of changing conditions on the rate of enzyme activity by measuring production of a colorimetric product in a spectrophotometer. Students watched enzyme rates approach zero as denaturing conditions were imposed. However, first-year students often struggle to link the observed loss in enzyme activity with protein structure changes in their reports. We therefore questioned whether this concept might be reinforced by asking students to take a step further and renature the enzyme by returning to optimal conditions after denaturation. In our updated lab procedure, adapted from Pitkin (1992), students measure turnip peroxidase activity in reactions with increasing pH, finally arriving at pH 9, where activity is minimal. Students then add different volumes of acid to the reactions and observe whether they have (1) decreased pH and (2) recovered enzyme activity. This approach reinforces the connection between reaction conditions and enzyme activity while providing additional data that can be used to discuss the relationship between enzyme structure and function. We present our procedure as a resource for other educators to implement in their own laboratory courses.

**Keywords:** Enzyme activity, denaturation, renaturation, structure and function, spectrophotometry, first-year biology

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### INTRODUCTION

The relationship between structure and function has been identified as one of the core overarching concepts in biology (AAAS 2011, National Research Council 2012). Understanding how changes in biological structure influence function at the molecular level is foundational in order for students to succeed in more advanced biochemistry and molecular biology courses (Tansey et al. 2013). Enzymes are a great model for this concept, as the relationship between protein folding and function is generally established in introductory-level cell and molecular biology classes and can be explored in the lab using simple denaturation experiments. Students manipulate enzyme reaction conditions and quantify the effect that each change has on the rate of product formation – either directly, by measuring the concentration of the product itself, or indirectly, by introducing a secondary reaction with a visible byproduct. Students then use trends in the resulting data to determine what

conditions are optimal for enzyme function, giving them the opportunity to form conceptual links on their own via data analysis and interpretation.

However, first-year students may struggle to link the concrete observations they make in lab with the sub-microscopic processes behind those observations. Students can infer from their data that reaction conditions have a significant influence on enzyme activity, but understanding how activity is conferred by the three-dimensional structure of the enzyme may not be as intuitive, especially for new biologists who are still learning to think about biological processes on the molecular level (Meyer and Land 2006, Ross et al. 2010). Such students can complete lab exercises effectively but are at a loss when asked to expound on the meaning of their results in post-lab assignments.

In order to address this concern, we have adapted Pitkin's (1992) lab procedure for a 100-level cell and molecular biology lab to explore both denaturation and renaturation using peroxidase, an enzyme that is easy to prepare and can be obtained from a variety of sources (The College Board 2012, Flinn Scientific, Inc. 2018). Students use a series of different buffers to shift the pH of an enzyme-catalyzed reaction beyond the enzyme's optimal range. After observing the decrease in activity with increasing pH, students add small volumes of acidic sodium citrate buffer to decrease the pH of the reaction solution until enzyme activity is restored. Our intent was that observing both activity loss and recovery would reinforce the connection between reaction conditions and activity while providing additional data that could be used to discuss the relationship between enzyme form and function. Students decide what volumes of sodium citrate to add themselves and use pH strips to determine to what degree each volume added influenced the final pH of the solution. Allowing students to tinker with the volume of sodium citrate added introduces inquiry-based learning that encourages students to engage with concepts behind the material while they are still in the lab rather than just following the protocol.

In addition to investigating the structure-function relationship of proteins, this laboratory procedure is designed to build competency with spectrophotometry and linear regression. A colorimetric indicator is used to quantify the rate of enzyme catalysis, and students graph absorbance over time for each reaction condition. If the absorbance readings are collected immediately after the reaction starts, a linear fit is possible, and the slope of the line of best fit for the data approximates initial enzyme activity. Changes in linear data are easier for first-year students to interpret than in a more complex model of enzyme kinetics. Students can compare slopes between different reactions to identify optimal conditions. Students can also consider whether peroxidase activity was fully recovered after denaturation by comparing the rate of the re-folded peroxidase to that of the initial rate measurement at optimal pH.

The lab exercise is to be completed over the course of a single 3-hour lab session. Students work in pairs and generally complete the activity in one to three hours, depending on their efficiency and level of preparation. The associated post-lab assignment is a full lab report in the style of a scientific journal, but the post-lab can easily be abbreviated to focus exclusively on data analysis. Further, the procedure can be expanded to include replicates or to cover a wider range of conditions in order to provide additional data for analysis.

## STUDENT OUTLINE

### Objectives

- Understand the function of enzymes and the folding of proteins
- Distinguish enzymes, substrates, and products using the peroxidase-catalyzed reaction as a model
- Investigate how changing the conditions of a reaction influences enzyme activity
- Learn to design experimental procedures

### Introduction

Enzymes are protein molecules present in all living organisms that speed up, or catalyze, biochemical reactions. Many spontaneous chemical reactions occur so slowly that, if left to progress on their own, it can take years for any appreciable change to occur. Crucial biological processes like photosynthesis or cellular respiration consist of a complex, interlocking series of chemical reactions that must occur rapidly and smoothly in order to be of use. Increasing the temperature can increase the rate of chemical reactions, but is indiscriminate and can damage proteins and other important biological compounds. Enzymes significantly speed up reactions without requiring an increase in temperature, allowing living organisms to carry out key cellular functions at a rate that can support life.

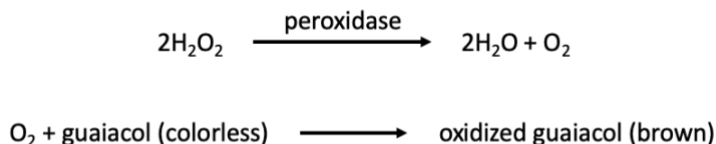
Enzymes can catalyze reactions by bringing reactant molecules together, stretching reactant molecules to encourage the breaking of chemical bonds, providing a favorable microenvironment for a given reaction, or even participating directly in the reaction as a brief intermediary reactant. Enzymes are highly specific and each enzyme will only bind to a particular set of reactants known as the enzyme's substrates. This is due to the enzyme's shape, as the amino acids making up the active site – the region of the enzyme where the substrate binds – interact in specific ways with a particular substrate, allowing the enzyme to recognize its substrate among a group of similar compounds and preventing the enzyme from binding to compounds other than its substrate.

There are hundreds of different kinds of enzymes in the average cell, each accelerating a different group of reactions. However, enzymes are not consumed during the reactions they catalyze, so only a small amount of each type of enzyme may be present, as each enzyme can be reused over and over again to speed up its particular target reaction.

The rate of enzyme activity can be influenced by a variety of factors. Extreme changes in temperature or pH can cause enzymes (and other proteins) to denature, or unfold, losing their three-dimensional structure and, with it, the ability to bind to substrates and catalyze reactions. Depending on the degree of denaturation, this process can be reversed: if an enzyme's environment is returned to favorable conditions, it may be able to refold after denaturation and regain its activity.

In this exercise you will use the enzyme peroxidase. Peroxidases are common in plant and animal cells; we will be sourcing our peroxidase from turnips. Cells produce small amounts of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) during cellular respiration. If allowed to accumulate,  $\text{H}_2\text{O}_2$  can do serious damage to the cell. Peroxidase breaks down  $\text{H}_2\text{O}_2$  into the harmless compounds water and oxygen.

Because both the reactants and products in this reaction are colorless, we will be using a substance called guaiacol as a colorimetric reagent to help us visually follow the reaction. When guaiacol reacts with oxygen, it transformed into a brown oxidized form.



**Figure 1.** Chemical reactions for peroxidase-mediated breakdown of hydrogen peroxide (top) and oxidation of guaiacol (bottom)

The accumulation of oxidized guaiacol in the reaction can be measured with a spectrophotometer due to the color change

of the solution over time. Spectrophotometers contain a light source and a prism, allowing the measurement of absorbance of a particular wavelength of light. Oxidized guaiacol absorbs light at 470nm. The light passes through the sample (in this case the guaiacol-containing solution), and – depending on the amount of oxidized guaiacol – some light will be absorbed. As the concentration of oxidized guaiacol increases, the absorbance will increase accordingly.

Consider how the peroxidase-mediated breakdown of  $H_2O_2$  couples to the color-changing guaiacol reaction. Draw a diagram of what is happening in the tube as these reactions progress. Next, draw what happens when the reaction is placed at high temperature or pH.

## Methods and Data Collection

### Part One: Effect of pH on Peroxidase Activity (adapted from Pitkin 1992)

Standard pipets and micropipettors will be needed to deliver solutions for this exercise. Use a new pipet for each solution, labelling each with sharpie so that they can be reused. Do not cross-contaminate your solutions by using the same pipet or tip in two different solutions.

1. Obtain three test tubes and label them 1, 2, and 3. Pipet the volumes of each solution given in Table 1 into each tube (use a 10-ml pipet for buffer and micropipettors for turnip extract, guaiacol, and  $H_2O_2$ ).

**Table 1.** Solutions for baseline protocol for testing peroxidase enzyme activity

Tube	Guaiacol (ml)	Turnip extract (ml)	0.1% $H_2O_2$ (ml)	pH 5 Buffer (ml)
1	0.1	1.0		8.9
2	0.1		0.2	4.7
3		1.0		4.0

2. Swirl the contents of each tube.
3. Set the spectrophotometer to a wavelength of 470nm. Transfer 1000 $\mu$ L of the solution in Tube 1 to a cuvette and use it to zero the spectrophotometer. This is your reagent blank. Consider the contents of the reagent blank – why do we use a mixture of reactants to zero the spectrophotometer instead of just using water?
4. Prepare your workspace. You need the blanked spectrophotometer, Tubes 2 and 3, a clean cuvette, a P-1000 micropipettor set to 1ml, and a stopwatch. One lab partner should be ready to mix and pipet, while the other should be ready to record numbers and control the timer.
5. As quickly as you can, mix the contents of Tubes 2 and 3 by pouring them together and then back and forth between the two tubes twice. Set the reagent blank aside so that you can use it for subsequent experiments.
6. Pipet 1000 $\mu$ L of the newly-combined contents into the clean cuvette. Immediately place the cuvette into the spectrophotometer, take the first reading, and start the stopwatch. Record the initial reading as Time 0. Leaving the cuvette in the spectrophotometer, record the absorbance every 30 seconds for 2 minutes in a table in your notebook (five recordings total). Do not disturb or invert the cuvette between readings. After collecting all five readings, remove the cuvette and verify the final pH of the solution using a pH strip.
7. Repeat steps 1-6 two more times, substituting in a buffer of a different pH each time (pH 7 buffer for the second experiment, pH 9 buffer for the third). Don't forget to blank the spectrophotometer with your reagent blank before you start each new experiment.
8. Generate a scatterplot of absorbance (y-axis) versus time (x-axis) for the three buffers used. Add a linear trend line to the plot for each buffer. Show the equation of the line and the  $R^2$  value on the figure. What can you conclude about the effect of pH on peroxidase activity? What statistic did you use to draw this conclusion? Was there a condition where activity was

very low? Why do you think this happened? Be sure to label the x and y axes on your figure and write a figure legend describing your results.

### **Part Two: Can Peroxidase Activity Be Recovered After Denaturation?**

Peroxidase activity is very low at pH 9 because the enzyme is at least partially denatured at this pH. Can we recover peroxidase activity by adjusting the solution containing denatured enzyme back to a more favorable pH?

1. Repeat the procedure described in Part One using pH 9 buffer. While one lab partner is taking the five readings, the other should prepare a micropipette with 100 $\mu$ l sodium citrate buffer pH 5 and a small square of parafilm.
2. As soon as your lab partner records the final (2 min) reading, remove the cuvette from the spectrophotometer, add the sodium citrate to the cuvette, place the parafilm over the top of the cuvette, and cover the top with your thumb. Invert once, then *quickly* place the cuvette back in the spectrophotometer and zero it. As soon as the spec zeros, you will get a reading. Record this absorbance as Time 0 and start your stopwatch. Record absorbance every 30 seconds for 2 minutes. At this point you should have 10 readings – five before adding sodium citrate, and five after adding sodium citrate.
3. Remove the cuvette and check the final pH of the solution using a pH strip. How much did 100 $\mu$ l of sodium citrate change the pH of the solution? Did changing the pH change the enzymatic rate of peroxidase? To find out, plot the data for before and after addition of sodium citrate as two separate trendlines and compare.
4. Design and perform two more repetitions of this experiment, adding differing amounts of sodium citrate to the cuvette each time. As above, take five readings at pH 9 before adding sodium citrate, then take five more readings immediately after adding sodium citrate. Don't forget to check the final pH after each iteration of the experiment. How do enzymatic rates change as pH changes? Are the rates of peroxidase activity after renaturation comparable to the rates before denaturation (i.e. the rates measured in Part One of the experiment)?
5. Write up your experimental procedure and all parameters (reagents, volume, equipment, time limits, etc.) in your notebook. You will provide your data analysis in a scientific report, given in the format of a scientific journal article.

### **Post-Lab Assignment: Enzymes Report**

Using the guidelines provided by your instructor, generate a full scientific report on your lab exercise in the form of a journal article, complete with a title, introduction, materials and methods, results section, discussion, and appropriately-formatted references. Your introduction should review the knowledge required to understand the study and should cite four to six primary source references at minimum. Your materials and methods should consist of a concise description of the methods used to conduct your study and should be clear enough that someone could repeat your approach. Your results should summarize your data and point your reader to your figures. Your discussion should interpret your data and present conclusions, implications of your findings, and potential future directions as if you were to continue this research.

As part of your report, you should generate two figures:

Figure 1. A linear regression analysis of time vs. absorbance that compares enzyme activity between the pH 5, pH 7, and pH 9 trials. Include the equation for each line in the figure and provide a key that differentiates the three lines.

Figure 2. A linear regression analysis of time vs. absorbance containing 3 plots – one for each of your sodium citrate trials (including ONLY the readings after you added sodium citrate). Include the equation for each line in the figure and provide a key differentiating the three lines. The pH of the final solution for each trial should be part of either your key or figure legend.

## MATERIALS

Per pair, students will need the following:

- 18 test tubes (16x125)
- 7 cuvettes
- 3-4 strips pH paper
- 4-5 squares of parafilm
- 4 standard pipets (10mL)
- 2 P-1000 micropipettors with tips
- 15 mL turnip extract
- 5 mL diluted guaiacol
- 5 mL 0.1% H<sub>2</sub>O<sub>2</sub>
- 25 mL buffer pH 5
- 25 mL buffer pH 7
- 50 mL buffer pH 9
- 2 mL diluted pH 5 sodium citrate buffer

## NOTES FOR THE INSTRUCTOR

Turnip extract is prepared using purple-topped turnips, which we purchase from the local supermarket. Cut off the rough outer layer of the turnip and weigh out 18g of the inner peeled portion. Blend in a commercial blender with 300mL deionized water for 1 minute, then filter through Miracloth (or a comparable filter cloth or paper) to remove pulp before portioning to students. The extract itself should be made fresh shortly before each lab section, but the turnips retain integrity for at least a week in the refrigerator. Guaiacol dilution is prepared by mixing 0.88mL 99+% guaiacol (CAS 90-05-1) with 400mL deionized water; guaiacol dilutions should be stored in brown or foil-covered bottles and distributed to students in foil-covered test tubes, as the compound is light-sensitive. The 0.1% H<sub>2</sub>O<sub>2</sub> dilution is prepared by creating a 1:3 H<sub>2</sub>O<sub>2</sub> : deionized water dilution using 3% H<sub>2</sub>O<sub>2</sub> from the supermarket or pharmacy. The sodium citrate buffer is a 1:8 1M pH 5 sodium citrate : deionized water dilution (final concentration 125 millimoles). Other dilutions will also work, but will require some testing to determine how drastically they will change the pH of the reaction solution.

Because the entire exercise iterates on the same basic procedure, it is important that students understand how to execute the first six steps of Part One (the initial pH 5 trial) correctly before moving on. We found it helpful to walk the students through the first trial, demonstrating and explaining each step and having the students follow along as a class. It is crucial to emphasize to the students that they should be ready to take their first absorbance reading as soon as they combine the contents of Tubes 2 and 3, as color change progresses rapidly. Students who waited too long to start taking readings did not see any appreciable change in absorbance and incorrectly assumed that enzyme activity was low at the pH in question rather than realizing they had allowed the reaction to plateau.

Using a sodium citrate concentration of 125 mM, students will see the best results if they choose volumes within the 100-300µL range in at least 50µL increments to add to their denatured enzyme solutions in Part Two. Trends in the data will be easier to grasp if students progress from smaller volumes to larger volumes rather than choosing at random. It is not possible for the final pH of the solution to drop below the pH of the citrate buffer being used to decrease the pH, so students should not attempt to add greater volumes once they have identified what volume of sodium citrate brings the pH down to 5.

Turnip peroxidase is stable over a wide pH range, with sources disagreeing on optimal pH but generally observing optimal activity between pH 4-6 (Duarte-Vázquez et al. 2001, Singh and Singh 2007, Motamed et al. 2009). In our classroom trials, roughly half of the student pairs saw optimal activity at pH 5, while the other half saw optimal activity at pH 7. However, all students observed a noticeable decrease in activity at pH 9 relative to the previous pH trials and a noticeable increase in activity upon adding sodium citrate buffer to low-activity solutions. The exact pH at which optimal activity is observed is not as important as seeing the patterns of activity loss and recovery, which all students who correctly graph their data should observe. For further inquiry, students might be encouraged to compare rate of enzyme activity at a particular pH before and after denaturation and renaturation. For example, if the final pH of one of the sodium citrate trials in Part Two is 7, students might compare the slope of the line of best fit for that data to the slope of the line for their pH 7 trial in Part One.

Trends of denaturation and renaturation should be clear within student pairs, but the actual absorbance values recorded and the slopes of the lines of best fit generated for each trial can vary widely between student pairs, even at the same pH. Consequently, the data from student pairs should not be compiled to form a larger data set for analysis unless they are observed to be highly consistent.

At this stage of their education, students may not have been exposed to linear regression analysis. Therefore, it is critical to spend time explaining it prior to the start of lab. Requiring students to generate their graphs for Part One of the lab before they can move on to Part Two is a highly effective way to clear up misconceptions in class. Common mistakes that students make are 1) using line graphs instead of scatterplots and 2) failing to recognize that the slope of the line is the critical statistic. Many students draw their conclusions based on the highest absorbance measurement recorded, failing to recognize that a reaction that both started and ended at a high value can have a lower rate than a reaction that started at a low value but achieved a much higher value as it progressed. Students also struggle to understand the value of the  $R^2$  statistic at this stage. Thus, spending class time creating and interpreting graphs can be a valuable learning experience for students.

Our discussion of protein folding in this lab has been simplified in order to make it palatable to first-year students, but instructors may want to mention that denaturation and renaturation are not always complete – for example, enzymes may recover secondary structure but not tertiary structure, and not all of the enzymes originally present may recover their functional shape. Many enzymes require the presence of other proteins or compounds to refold completely, peroxidases included (Pappa and Cass 1993, Ermolenko et al. 2002). It may be worthwhile to point out to students that the turnip extract used as a peroxidase source in the experiment contains more enzymes than just peroxidase, some of which may participate in the renaturation process.

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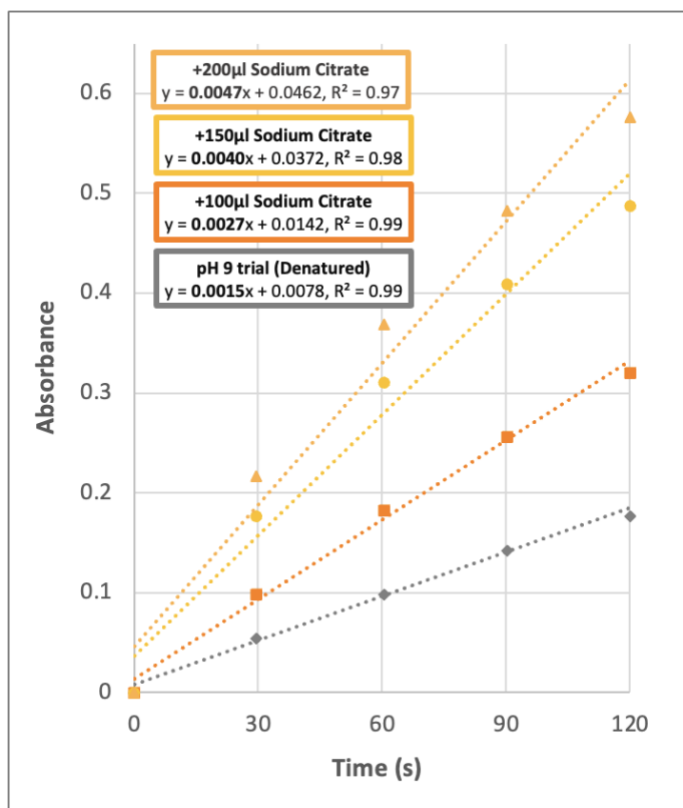
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APPENDIX A

Example of Figure 2 from Part Two of procedure, including data from pH 9 trial for comparison.



**Figure 2.** Initial rate of peroxidase activity increases as solution is adjusted from pH 9 to increasingly acidic pH by adding increasing volumes of 125 mM sodium citrate pH 5. Final pH of the solution after addition of 100µl, 150µl and 200µl of sodium citrate was 8, 7, and 6, respectively.

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