BIOLOGY 163 LABORATORY
Amylase Activity in *Hordeum* and *Mya*

(Reviewed Fall 2011)

An understanding of the nature and control of enzymatic action is essential to understand energetics at the cellular and molecular level. Since most enzymes are proteins, any factor that alters protein structure will influence the biological activity of the enzyme. Proteins are more than long chains of amino acids held together by peptide bonds. They have a complex and highly specific pattern of folding and coiling -- called the tertiary structure -- held in place by disulfide bridges and by weak non-covalent interactions (WNCs) such as hydrogen bonds. Since many of the forces that hold a protein molecule in its specific configuration are much weaker than the covalent bonds between the amino acids, the shape is subject to alteration by temperature, acidity, ionic concentrations, and other factors. Anything that affects the folding pattern of the enzyme molecule is likely to influence the ability of the enzyme to perform its function. Thus a study of enzyme activity may give insight into the more general mechanisms of protein structure and function.

Amylases are enzymes found in a wide variety of organisms, microbial, plant, and animal. Their function is to hydrolyze starch, a major form of stored energy. You will investigate some of the properties of amylases from two sources: the crystalline style from the salt-water clam (*Mya*), and barley (*Hordeum*) kernels. The crystalline style is a rod-shaped structure in the stomach of the clam. Cilia in the stomach continuously rotate the rod. As it rotates, the end of the rod wears away, releasing the amylase contained within it. In barley, cells in the outer layer of the endosperm (the aleurone) synthesize amylase to be released during seed germination. This allows the seed to break down nutrients stored in the endosperm to provide energy for the growing embryo. Although plant and animal amylases are fundamentally similar in function, their structural properties may differ.

Enzymes are studied by measuring the rate of the chemical reaction that the enzyme catalyzes. Once a reliable measure, called an assay, is worked out, various factors that might affect enzyme action can be tested by comparing the reaction rate with a normal control reaction, usually done under optimal conditions. A number of methods are used to measure the rate of a chemical reaction. One may measure the rate at which the substrate is used, or, alternatively, the rate at which the product is formed. Making quantitative determinations of small chemical changes often is difficult, but one convenient way to do so is by measuring a color change. You can measure color changes accurately with a spectrophotometer, an instrument widely used in biochemical analyses. The technique is so valuable that much effort has been spent in adapting it to situations where no obvious color change is involved in the reaction being studied. Often substances undergoing chemical change do have differences in their "color" but only in the ultraviolet range of the spectrum. Also, all molecules have characteristic absorption properties in the infrared end of the spectrum. Thus ultraviolet, visible, and infrared spectrophotometers are valuable tools of the biologist.

In this experiment you will use an interesting trick to make a color change as the amylases digest the starch -- a reaction that normally does not produce any visible color. An artificial substrate for the enzyme is made by reacting the starch molecules with an intensely blue dye. The dye molecule attaches to the sugar units of the starch molecules in such a way that they do not interfere with the enzymatic reaction. In an undigested state starch is insoluble and, since the dye remains bound to the starch granules, none is in the solution. (You will find more about the nature of the starch molecule in Appendix I.) When the amylase enzymes split starch to form the soluble sugars, the dye also goes into the solution with the sugar, coloring the solution blue. After removing the undigested starch particles, you can measure the color quantitatively with a spectrophotometer.

Since the amount of color in the solution is directly proportional to the amount of starch digested, you can make quantitative measurements of the enzyme activity. In other words, the more intense (dark) the blue is, the higher the rate of enzyme activity.

EXPERIMENTAL PROCEDURES

(Work as sides of tables, using *either* clam *or* barley, as directed by your instructor.)

A. ENZYME PREPARATIONS *(Keep materials cold during enzyme preparations!)*

1. CRYSTALLINE STYLE

Carefully remove one valve of the salt-water clam, *Mya*, by prying the shells apart with a scalpel and cutting the large adductor muscles. Locate the stomach, ventral to the umbo, and make a small cut in the "window" on the dorsal surface (your instructor will likely demo this for you). The end of the crystalline style should slip out. Remove the style with forceps and place it in sea water. You will need five styles for the enzyme preparation.

a. Weigh five styles after blotting them on filter paper to remove excess water. Record the weight on the data sheet in your lab notebook.
b. Place the weighed styles in a mortar with a small pinch of sand.
c. Grind the dry styles, making a paste.
d. Add 5 ml seawater, suspend the style paste by mixing thoroughly, and pour into a conical centrifuge tube.
e. Add another 5 ml sea water to rinse the mortar and pestle; add that to your style suspension in the conical centrifuge tube.
f. Chill the suspension on ice for 10 minutes.
g. Centrifuge the suspension at ~ 300 X g for 2 minutes.
h. Carefully decant the **supernatant** (solution above the pellet) into a test tube, and place on ice. THIS IS YOUR CRYSTALLINE STYLE ENZYME PREPARATION.

2. BARLEY ALEURONE ENZYME

a. Obtain a dish of germinated Barley seeds (about 120 seeds) and remove all roots and shoots.
b. Weigh the seeds (minus roots and shoots). Record the weight (about 5-6g) on the data sheet at the end of this handout.
c. Place the barley into a mortar with a small pinch of sand and grind to a paste.
d. Add 10ml distilled water to the paste, and suspend the enzyme thoroughly to give a very thick, milky suspension (like chowder).
e. Scrape the suspension into a small beaker and place it on ice for 15 minutes.
f. Filter the suspension through miracloth into a large (40-50 ml) centrifuge tube.
g. Your instructor will assist you in centrifuging your sample in a refrigerated, high-speed centrifuge at 15,000-18,000 rpm (~ 31,000 X g) for 10 minutes.
h. *Immediately upon removing your sample from the centrifuge*, carefully decant the **supernatant** (solution above the pellet) into a test tube, and place on ice. THIS IS YOUR BARLEY ENZYME PREPARATION.

B. MATERIALS

Before starting this experiment, you must become familiar with the procedure because you need to work very carefully and fairly rapidly. Have the following items ready:

1. Five small beakers, labeled “1” to “5”.
2. Watch, stop-clock, or clock with second hand.
3. Five centrifuge tubes, labeled to correspond to the labeled beakers.
4. Pipettes for the enzyme solution, the substrate, buffers, and inhibitor. (Buffers control the pH or acidity of a solution. See Appendix II for more about pH and buffers.)

Carefully study the sample data sheet provided so that you understand what measurements you are to make in order to calculate enzyme activity. You must record:

1. the conditions in each test beaker
2. the time when the enzyme is added to the substrate
3. the time when the digestion is terminated by centrifuging the mixture
4. the absorbance measured with the spectrophotometer.

C. TEST PROCEDURES

1. PREPARATION FOR ENZYME ASSAY

Prepare 5 beakers as indicated in the table below. Since no digestion takes place without the enzyme, you can pipette all reagents, EXCEPT THE ENZYME, into the beakers at any time.

			Beaker Number		
Reagent	1	2	3	4	5
	Control	pH 5	pH 6	pH 7	pH 8
Starch-Azure Substrate	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Buffer, pH 5		2.5 ml			
Buffer, pH 6			2.5 ml		
Buffer, pH 7	2.5 ml			2.5 ml	
Buffer, pH 8					2.5 ml

2. DIGESTION

Study the procedure before you begin. You will need work quickly but carefully to add the enzyme preparation to each of the beakers in sequence.

1. Add 0.5 ml of DISTILLED H2O to your CONTROL beaker (Number 1). *Record the exact time at which the water is added to each beaker!* Mix by swirling the beaker.

2. Add 0.5 ml of the ENZYME PREPARATION (crystalline style or barley) to each of the remaining four beakers (Numbers 2-5). *Record the exact time at which the enzyme is added to each beaker!* Mix by swirling the beaker.

3. Swirl each beaker briefly every minute thereafter.

4. After approximately 10 minutes (the exact time is not critical), pour the reaction mixture from each beaker into an appropriately labeled centrifuge tube. Centrifuge the tubes at ~ 1100 X g for 4 minutes, *noting the exact time at which you begin the centrifugation.* (The digestion effectively ends at the beginning of centrifugation since the starch granules immediately go to the bottom of the tube where they have little contact with the enzyme.)

5. At the end of the centrifugation, carefully decant the **supernatant** into a spectrophotometer tube. It is NOT necessary to get every drop of fluid--the spectrophotometer tube need not be full and you must not stir up any undigested starch from the bottom of the centrifuge tube!

6. Measure the color intensity, comparing the value for each tube with that of your control (see Part 3 below for method for spectrophotometer readings). *As a qualitative check, also record the relative intensity of color for each tube as perceived by eye.*

3. SPECTROPHOTOMETER READINGS

A spectrophotometer has two different scales on its meter. One is in units of percent transmittance and the other is absorbance. Because absorbance is directly proportional to the concentration of the colored substance in solution, use the absorbance scale.

The spectrophotometer you use has a variable light source that can be set to any wavelength in the visible spectrum. For maximum sensitivity to the blue dye used in this experiment, the light is set to the wavelength corresponding to the complementary color.

1. Set the wavelength to 620 nm.

2. For each wavelength you use, you must set the ends of the spectrophotometer's electronic scale. To do this:

   a. Using the **left** control knob, set the absorbance at ∞ with **nothing** in the sample well. Be sure the well cover is down.

   b. Place a tube containing the control solution in the sample well (in this case tube "1") and adjust the **right** control knob so the scale reads 0 absorbance. (By doing this, your experimental readings are made relative to the absorbance of the control tube. In effect, the control absorbance is subtracted automatically from all experimental readings, simplifying the arithmetic!)

3. After setting both ends of the spectrophotometer scale as directed above, place each experimental tube in the spectrophotometer and record the absorbance value.

D. USING SPECTROPHOTOMETER READINGS TO ASSESS ENZYME ACTIVITY

The enzyme activities you measured are for 0.5 ml of your enzyme preparation. Since the weights of individual styles are not uniform from one clam to another and the amount of enzyme in the barley may be quite different from that in the style, the quantity of enzyme in your 0.5 ml samples may vary considerably from that in someone else's preparation. It is also important to consider the elapsed time during which the starch is exposed to the enzyme, as this may vary from assay to assay.

To have a standard basis for comparing the enzyme activity in different preparations, you need to express the enzyme activity on a per minute, per unit weight basis. Pay careful attention to the calculations as described in the data sheet!

APPENDIX I

STARCH DIGESTION

The official nomenclature for enzymes consists of a suffix, "-ase" preceded by either the name of the substrate molecule (e.g., a "ribonuclease" is an enzyme that acts on ribose nucleic acid) or of the particular chemical reaction catalyzed (e.g., an "oxidase" is an enzyme that oxidizes its substrate). The chemical bond between the glucose molecules in a starch polymer is an amyl bond, and the digestive enzyme that splits this bond is an amylase. (“Polymer” means many, "poly", segments or parts, "mer", thus starch is composed of many sugar molecules.) Plant cells are able to combine many glucose molecules into a large storage molecule consisting of long chains, which are relatively unbranched. The technical name for this starch molecule is amylose. (The suffix "ose" means a sugar.) A more branched form, called amylopectin, occurs in many fruits and animal cells make a still more highly branched polymer called glycogen.

When amylase splits the amyl bond, a molecule of water, in its ionized form, is added to the product. Thus one end of the split chain accepts a hydrogen while the other end receives an OH^-. Splitting a large molecule into fragments by adding a water molecule is called hydrolysis. Amylase then, like all other digestive enzymes, is a hydrolytic enzyme.

As the starch chain is split into progressively shorter fragments, the chemical and physical properties of the polymer change. The small pieces of partially hydrolyzed starch are dextrans. The glue often used on postage stamps is dextran. It has a mildly sweetish taste, and is edible. The dextrans are broken down finally to two and three unit chains of glucose, called maltose and malto-triose. These are quite soluble and in the body are acted upon by a different enzyme for the final digestion to glucose. In the experiment using the dye-labeled substrate, the product is maltose with a dye molecule attached to give color to the solution.

Figure 3 shows a starch molecule. The asterisk indicates the point where the azure dye attaches to the molecule. The labeled glucose units are distributed randomly along the chain. Although shown as a straight chain, the molecule is coiled into a loose helix. Dotted lines indicate points for hydrolytic cleavage by the enzyme.

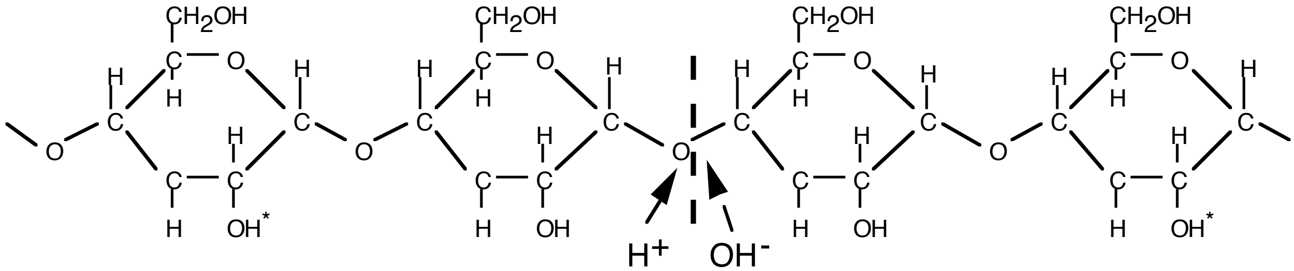

Figure 3. Amylose hydrolysis. Amylase cleaves the insoluble starch (amylose) into smaller soluble sugars. As the sugars dissolve, they take the attached azure dye (represented by an asterisk) with them into the solution, which turns blue as a result.

APPENDIX II

pH AND BUFFERS

“Buffer” means "something that lessens or absorbs the shock of impact." (The American Heritage Dictionary.) In biological and chemical terminology, a buffer is a chemical that shields or protects a reaction system from sudden changes in acidity or alkalinity (pH). If you wish to study a reaction at a particular pH, a buffer is used to establish and maintain the desired pH.

In your study of enzymes, you will use a citrate buffer. This buffer is a mixture of citric acid and disodium phosphate in the appropriate proportions required to give the desired pH. In solution, these two chemicals are able to "absorb" either added acid (hydrogen ions, H^+) or added base (hydroxyl ions, OH^-) by reacting with these ions and effectively neutralizing them.

Similar buffering systems occur in living organisms and serve to protect the internal body fluids of the organism from sudden or drastic changes in pH.

APPENDIX III

CALCULATIONS

Enzyme was not added to the CONTROL sample. Since we know there is no enzymatic activity in the control, we use it to set the absorbance scale of the spectrophotometer to “zero.”

Any enzymatic activity that takes place in the *experimental* samples will result in a color change and a corresponding increase in the measured absorbance of the sample. Therefore, we are able to *quantify* “enzyme activity” by measuring Δ absorbance over a given period of time.

In order to facilitate comparisons among samples, absorbance measurements will be standardized for time and tissue weight. A few simple calculations yields “Δ absorbance/minute/gram of tissue” or “enzyme activity/gram of tissue.”

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DATA SHEET

Beaker Number	Conditions	Qual. Visual Obs.*	Time		**A** Total Elapsed Time (minutes)**	**B** Absorbance for Total Elapsed Time	**B / A = C** Δ Absorbance per Minute	**C / D*** = E** Enzyme Activity / Gram of Tissue
			Initial	Final				
Barley 1	Control							
2	pH 5							
3	pH 6							
4	pH 7							
5	pH 8							
Style 1	Control							
2	pH 5							
3	pH 6							
4	pH 7							
5	pH 8							

* *Qualitatively* rank the depth of color of each sample as observed with the naked eye.

** To facilitate calculations, convert “minutes and seconds” to “minutes and tenths of minutes” using the following formula:

number of seconds / 60 = tenths of minutes (e.g., 9:30 = 9.5 minutes)

*** **D =** Grams Tissue per 0.5 ml Enzyme Preparation as follows:

Barley Kernels

(_____ gm barley / _____ ml original liquid) x 0.5 = _____ gm barley per 0.5 ml enzyme preparation = **D**

Clam Style

(_____ gm style / _____ ml original liquid) x 0.5 = _____ gm style per 0.5 ml enzyme preparation = **D**

ASSIGNMENT

You will present this study in the form of a complete scientific paper. Consult the "Guide to Writing Scientific Papers" and "Working with Statistics" for general ideas and assistance. Use the following guidelines to focus your efforts:

Introduction (two or three well-developed paragraphs)
Provide some relevant background information, but stay focused on the key point of your study. (HINT: Think about the structure-function relationship of enzymes!) Be sure to clearly state the *specific* question you are investigation and what you hypothesize regarding the outcome of your experiment.

Materials and Methods (one paragraph)
It is not necessary to re-write detailed procedures that have been previously published. Summarize the general protocol in a few concise sentences, and then **reference the laboratory handout** for the details. *Be sure to cite the handout as you would any other source!*

Results (figure(s) and one or two short paragraphs)
Using group data provided by your instructor, present the results of the study in a meaningful and informative way. Include text that references the figure(s) and summarizes the key points or trends shown therein. (i.e., How did barley/clam amylase activity vary over the range of pH tested?)

Discussion (two or three well-developed paragraphs)
Address each of the following questions thoroughly but concisely. (HINT: Again, keep the structure-function relationship of enzymes in mind when developing your responses!)

- Does pH affect amylase activity? If so, why might this be?
- Which pH is the best pH for style amylase function? Which is best for barley amylase function? How might you account for differences you observe?
- Would significant changes in pH within a living organism be undesirable? Explain.

Your paper should also include a proper *title*, as well as *acknowledgements* and *literature cited* sections.

Your instructor may provide additional detailed instructions.