Enzyme Exercises Designed for Both Laboratory and Internet Environments

Betty L. Black, Marianne Niedzlek-Feaver, M. Jason Wingate
Department of Biology
Campus box 7617
North Carolina State University
Raleigh, NC 27965-7617
betty_black@ncsu.edu, mnfeaver@unity.ncsu.edu, mjwingat@ncsu.edu

Introduction

We have developed a flexible laboratory exercise that illustrates the principles of enzyme activity and can be used in introductory biology courses for biology majors or non-majors. Additionally, the exercise can be utilized in distance education to bring elements of the laboratory experience on-line. The exercise employs intestinal maltase to illustrate maltose hydrolysis via a colorimetric assay. The goals are to teach students about the properties of enzymes, including the accumulation of product over time, as well as the effects of pH, temperature, and non-competitive inhibition on enzyme activity. Additionally, students will learn skills in equipment use, data analysis and interpretation of graphs.

We have chosen the maltase assay to illustrate enzyme activity for several reasons. First, maltase is an intestinal enzyme found in most mammals, including humans, and so is intrinsically interesting to students. Maltase resides in the microvillus membrane of enterocytes, the cells that line the intestinal lumen, and functions to split maltose into two glucose molecules. Thus it performs the final stage of starch digestion, which is required for intestinal absorption of glucose. Maltase is easily obtained from the intestine of mice, rats, or chicks and is stable during storage for months at -20°C. No specialized equipment or ultra-cold freezers are required, just a source of intestine. Finally, the assay is simple and the intestinal homogenate has high maltase activity, requiring relatively small amounts of tissue.

Instructor's Notes

Preparation of the enzyme solution and assay reagents

While is it possible for students to prepare an enzyme solution from pieces of frozen intestine, we perform this step prior to the laboratory meeting to conserve time. The small intestine is removed from a freshly killed mouse and placed in a petri dish on ice. Since the duodenum is attached to the pancreas and the distal ileum has relatively low maltase activity, we usually use only the jejunal and proximal ileal regions of the intestine. However, the entire small intestine can be used as long as the pancreas is removed. Next, mesentery is removed, the intestine is cut into several pieces, and each piece is flushed with ice-cold water. This is easier if the mouse has been fasted for 12 hours. The flushed intestine is now ready for use: it can be homogenized immediately or cut into small pieces and frozen for later use. Be sure to submerge the intestinal pieces in water before freezing to prevent desiccation.

To prepare the enzyme solution, we simply homogenize the intestine in ice-cold water to give a concentration of 30-100 mg/mL. If a balance is not available, the intestine can be measured instead of weighed; 1 cm of flushed mouse intestine weighs approximately 70 mg. We use a tube and motor driven

teflon pestle for homogenization which gives a uniform solution, free of tissue particles. Other methods that work are a hand-held glass tube and pestle, a Waring blender, or vigorous sonication. Note that if small pieces of tissue remain, they can be removed with forceps. Since all of the maltase activity is in the mucosa (easily homogenized), removing unhomogenized pieces of muscle has no adverse effects. If using rat intestine, scrape the mucosa from the thick, underlying tissue prior to homogenization. The stock intestinal homogenate can be used immediately or stored at -20°C for several weeks before use.

The maltase assay is based on that of Dahlqvist (1968), which has been a standard in intestinal research for 40 years. It is a 2-part assay: first the intestinal homogenate is incubated with maltose, then the released glucose is measured by a colorimetric reaction with tris-glucose oxidase reagent (TGO). Interestingly, the glucometers used by diabetics utilize a similar method of measuring glucose; the reagents are embedded in the glucometer strips. Directions for making up reagents follow.

Enzyme solution:

Dilute the stock intestinal homogenate to 2-3 mg/mL (spectrophotometer method) or 100 mg/ml (glucometer method) with ice-cold deinonized water.

Substrate solution:

Prepare 50 mM maleate buffer by dissolving maleic acid in deionized water and adjusting pH to 6.0.(For 100 ml of buffer, add 0.58 g maleic acid to 90 ml water plus 7 ml of 1N NaOH, adjust pH to 6.0, then bring total volume to 100 ml.) Dissolve maltose in the above buffer to give the desired substrate concentration. We use 50 mM maltose for most spectrophotometric enzyme assays and 300 mM maltose when using a glucometer. Store this solution in the refrigerator or freeze in aliquots for repeated, long-term use.

Components of the TGO solution:

1. 0.5 M Tris-HCl: Dissolve Trizma base in deionized water and adjust pH to 7.0. (For 2 liters, add 121 g Trizma base to 1 liter of water plus ~80 ml of concentrated HCl, adjust pH to 7.0, then add water to bring total volume to 2 liters.) Store in refrigerator--is good for at least a year.
2. detergent: Mix 1 part Triton X-100 with 4 parts 95% ethanol. Store in refrigerator indefinitely.
3. 0.1 % peroxidase solution: Dissolve peroxidase in deionized water. Store in freezer indefinitely.

To make 500 mL of TGO solution, mix together:

5.0 mL detergent

2.5 mL peroxidase solution

2.5 mL o-dianisidine dihydrochloride (Comes in 50 mg bottles to which you add 5.0 mL of deionized water; remaining solution can be frozen for future use.)

~0.5 g glucose oxidase The amount is not critical, but 0.4-0.5 g is usually sufficient.

Bring to a total volume of 500 mL with Tris-HCl

Store TGO in a brown bottle in the refrigerator. It should remain usable for 1 month.

Glucose standards:

Prepare a stock glucose solution of 0.2 mg/mL. Store in freezer indefinitely. Dilute as necessary to provide working solutions of 25-200 µg/mL. We use three standards (50, 100, and 200 µg/mL).

Maltase assay protocols

The maltase assay has two steps. In the first step, equal amounts of intestinal homogenate and substrate solution are added to test tubes kept cold on ice. Tubes are then transferred to a water bath (or other type of incubator) and incubated at 37°C. We use 100 µL of each solution in small, disposable tubes, but amounts can be scaled up or down. The enzyme reaction is stopped at the desired time interval

by returning tubes to the ice bath. Since the enzyme reaction is very slow at 4°C (temperature of ice), the reaction can be precisely timed. The second step of the assay is measurement of the glucose released by maltase action. This can be done by using a spectrophotometer and glucose standard curve or by directly measuring glucose with a glucometer. We use the spectrophotometric method in introductory biology for majors, since these students need to learn the use of scientific equipment. The glucometer method is a shorter assay and more appropriate for non-biology majors.

For the spectrophotometric method, 2 mL of TGO solution is added to each tube using a pump dispenser calibrated for 2 mL. This not only speeds up the work, but also results in adequate mixing of solutions without vortexing the tubes. The Tris component of TGO is a potent inhibitor of maltase, so once TGO has been added the tubes no longer need to be kept on ice. The tubes are then incubated for an additional 20 min at 37°C to develop an orange color. This is actually another enzyme reaction (via glucose oxidase) in which all of the glucose reacts within 15-20 min. To avoid student confusion, we just call it a colorimetric reaction. During the same laboratory period, a glucose standard curve is prepared so that students can quantify released glucose and thus calculate enzyme activity. For the standard curve, 200 µL of each glucose standard is placed in a tube followed by 2 mL of TGO. A tube containing 200 µL of water plus TGO is included to serve as a blank and the tubes are incubated for 20 min at 37°C. Absorption of all solutions is recorded at 420 nm, using the blank to zero the spectrophotometer. We use a Genesys, but any type of spectrophotometer should work. It is also possible to scale down the assay by using 10 uL of homogenate and substrate solution (or 20 µL of standard) plus 200 µL of TGO in microwell plates. The plates (covered to prevent evaporation) can be incubated in an air incubator and read on an Eliza plate reader. This method gives equally good results, although pipetting 10 µL quantities accurately is more of a challenge for students.

A typical standard curve is shown in Figure 1. Students are required to construct a graph, then use it to determine the amount of glucose in each tube of the maltase assay. We prefer that they initially use graph paper and determine glucose content of unknown samples directly from the graph. A quicker method is to calculate a "calibration factor" and multiply each absorption value by that number. The calibration factor is the inverse of the slope of the standard curve (for the example in Figure 1, it is 38).

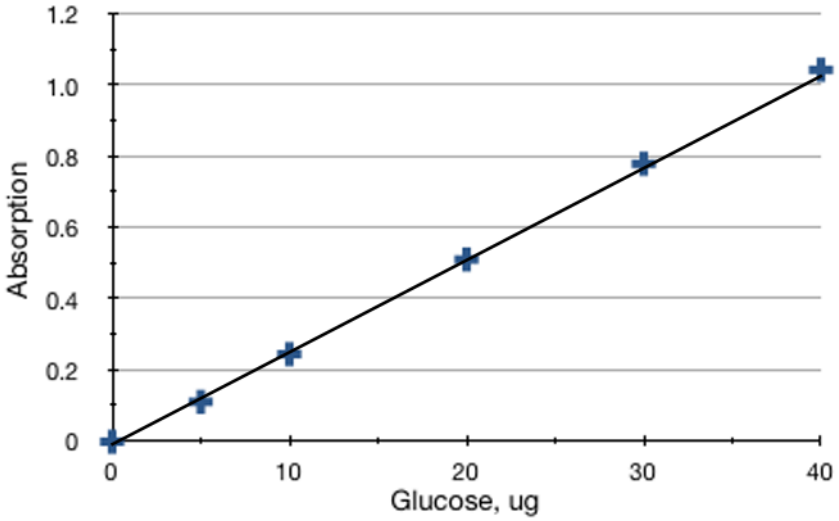

Figure 1. Glucose standard curve

Enzyme activity can then be calculated as µg glucose released/min, with minutes being the time of the first incubation. If the same amount of enzyme will be used for all assays, this is sufficient. If enzyme amount is varied (or if a more rigorous expression of enzyme activity is desired) the above values can be divided by tissue weight or length and expressed as µg glucose/min/mg tissue or /mm tissue.

For the glucometer method, TGO and glucose standards are not needed, since the glucometer strips contain reagents to measure glucose content directly. In this version of the maltase assay, the incubation is stopped by placing the tubes on ice and adding 300 uL of 0.5M Tris-HCl. Tubes should be kept at room temperature after addition of Tris-HCl. A drop from each tube (~30 uL) is placed on a glucometer strip and inserted into the glucometer to obtain a reading in units of mg glucose/dL. Each pack of strips comes with a test strip used to set a zero point and a standard value. Assay conditions using the glucometer differ somewhat, since higher substrate and enzyme concentrations and longer incubation periods are needed to achieve glucose amounts that the glucometer can reliably measure. We recommend a maltose concentration of 300 mM, a working homogenate of 100 mg/mL, and an incubation time of 30-40 min.

Properties of maltase

The maltase assay can be utilized to show the accumulation of product with time--a key feature of enzyme activity. In Figure 2, a time course of maltase activity is shown, using 3 mg/mL working homogenate. If first order kinetics are desired (a straight line for a given incubation length), the homogenate concentration can be lowered to 1 mg/mL, which should give a linear time curve for 15 min.

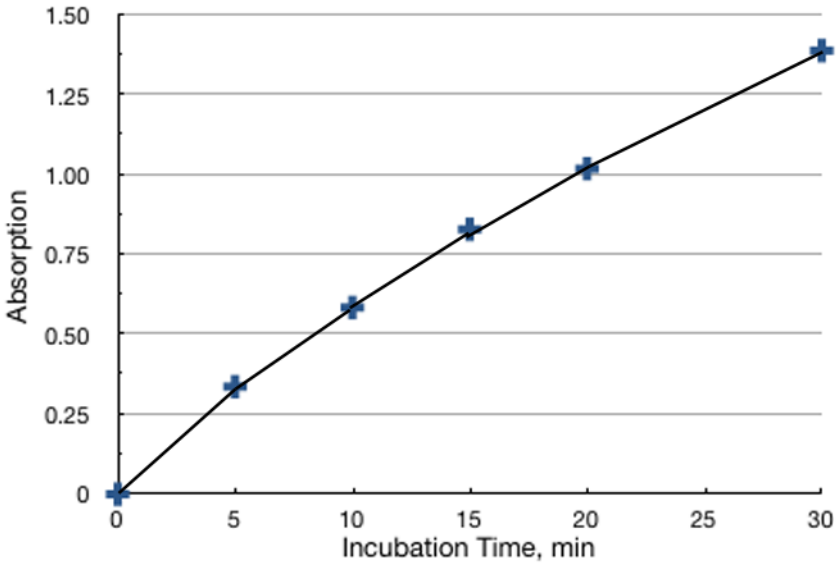

Figure 2. Time course of maltase activity

In this and subsequent graphs of spectrophotometric data, values on the Y axis are the absorption values (directly proportional to enzyme activity). In our laboratory exercise, we give students the

absorption data for the time curve and ask them to graph it. They use their standard curve to calculate µg glucose and plot these values vs. minutes of incubation. This saves time for later assays while introducing students to calculations of enzyme activity and relationship between enzyme activity and incubation time. Students are asked if incubation time changes enzyme activity. If the time curve is a straight line, the answer is no. If it is not a straight line, discussion reveals that this is due to substrate decreasing with time so that enzymes are not always combined with the substrate at longer time points.

The maltase assay is quite versatile and can be used to demonstrate the effects of substrate concentration, pH, non-competitive inhibition, and temperature. Graphs of representative assays are shown below (Figures 3-6). Unless otherwise specified, assay conditions for the spectrophotometric method were: substrate concentration = 50 mM, working homogenate concentration = 2-3 mg/mL, pH = 6, incubation time = 15-20 minutes at 37°C. To save time when students perform the substrate concentration assay, they use only 5, 20, 50, and 150 mM maltose which is sufficient to show saturation at high substrate concentration. For pH assays, they use 4.5, 6.0, 7.5, and 9.0 which illustrates a pH optimum. To inhibit maltase activity, students add $CuSO_4$ at concentrations of 0 (control), 0.2%, 0.5%, and 2.0%. The latter is achieved by using 50 µL of substrate + 50 µL of $CuSO_4$ in substrate solution.

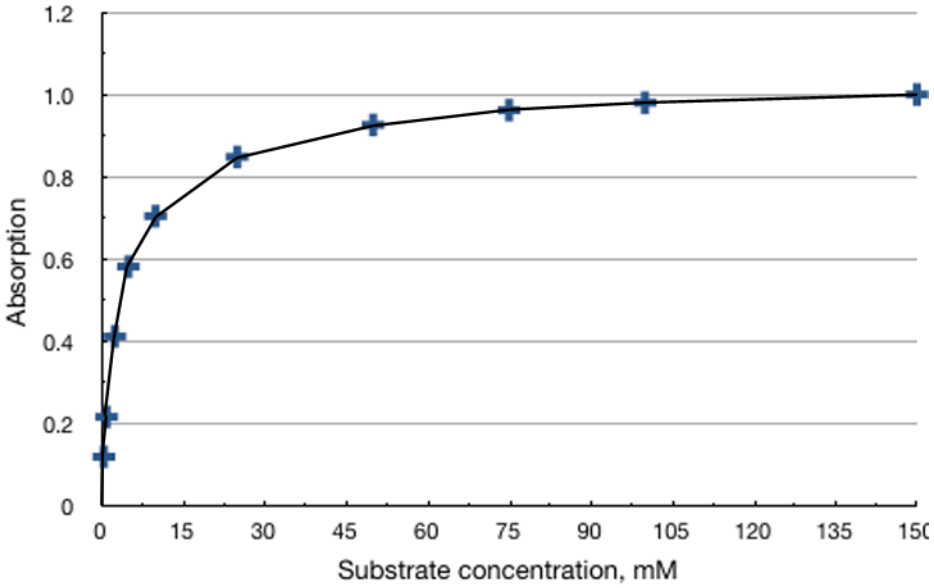

Figure 3. Effect of substrate concentration on maltase activity

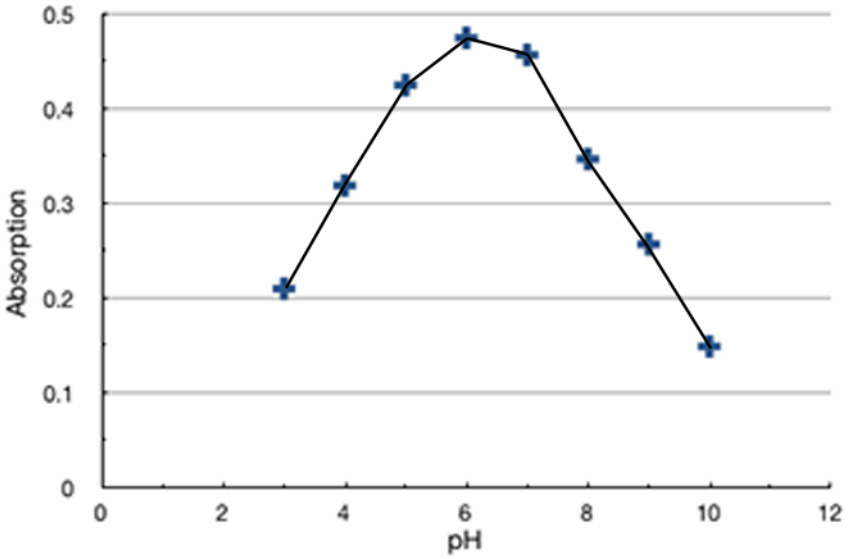

Figure 4. Effect of pH on maltase activity

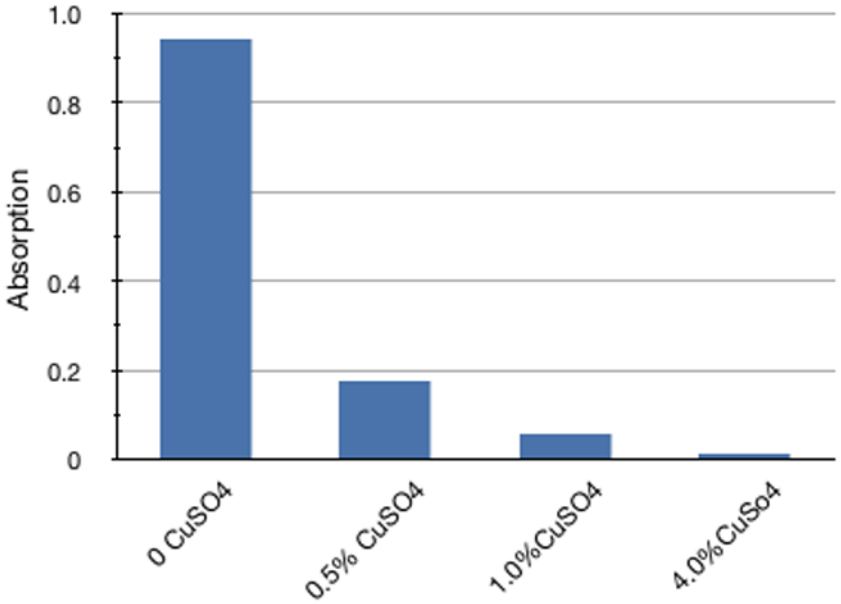

Figure 5. Effect of an enzyme inhibitor on maltase activity

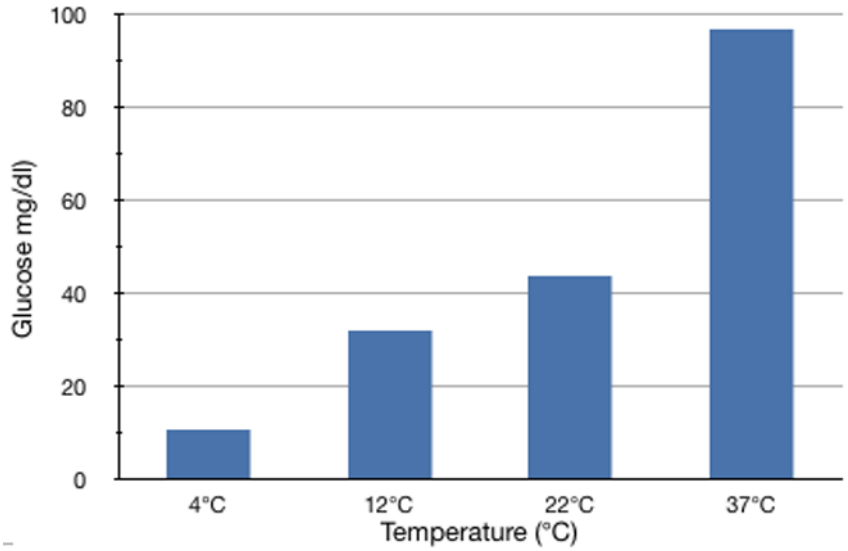

Figure 6. Effect on temperature on maltase activity (glucometer method)
Substrate = 300 mM, homogenate = 100 mg/mL, incubation time = 40 min.
Incubations at 12°C and 22°C were done in a refrigerator and at room temperature.

The above assays are the most that students can perform in a 3-hour lab period that includes data analysis and discussion. Other aspects of enzyme activity that could also be studied are the effect of homogenate concentration on maltase activity and use of different substrates. Examples of such assays are shown below (Figures 7 and 8). Note that the intestinal homogenate also contains the enzymes sucrase and lactase, so activity is found using these substrates. Sucrase activity is dependent on diet and would be much higher if the mice used were fed sucrose rather than a diet high in starch content. Lactase is high in suckling mice, but drops to low levels after weaning (as it does in most mammals) and cannot be altered by diet. We thought that starch as a substrate might be of interest to show that incubation of starch with amylase releases maltose. In the assay shown below, a small amount of activity was found using a saturated solution of corn starch as the substrate, probably because the starch contained some maltose. When 20 mL of starch solution was incubated with 1 mL of saliva for 15 min at 37°C before use, maltase activity was higher. We used a crude approach for the starch/amylase assay; the effect of amylase could probably be optimized to give more dramatic results.

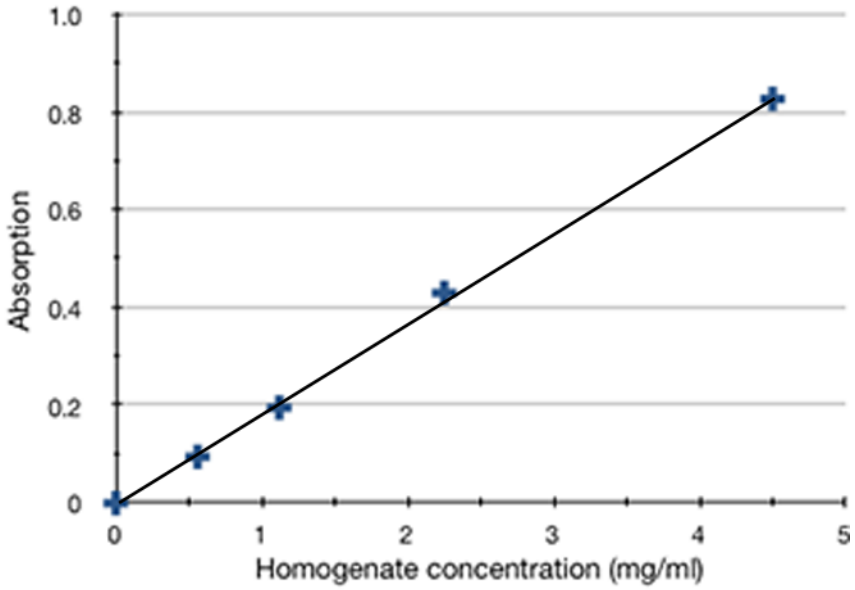

Figure 7. Effect of enzyme concentration on maltase activity

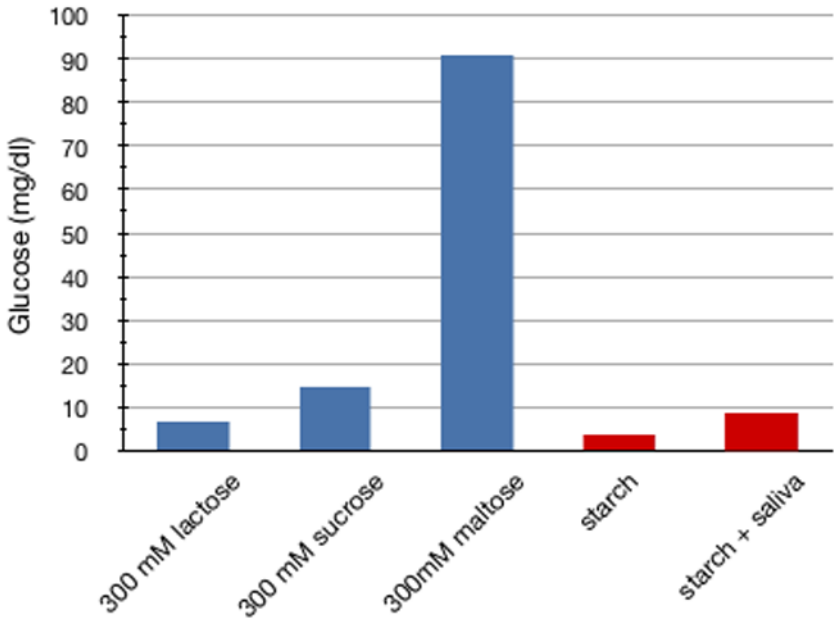

Figure 8. Effect of different substrates on maltase activity (glucometer method) Assay conditions as described in Figure 6, with all incubations at 37°C.

Adaptation of the enzyme exercises for distance education

We also teach a distance education section of introductory biology with on-line laboratories in which the maltase assay is used to illustrate the properties of enzymes. The on-line exercise begins with an introduction to qualitative vs. quantitative assays, then briefly reviews some of the material that students have already learned about enzyme-catalyzed reactions. Next, information about maltase and the assay protocol is given, using videos to illustrate all procedures. A simulated spectrophotometer

provides a way for students to generate and record absorptions for each assay. Students are required to graph the data using an on-line plotter and to analyze the results. As part of their homework assignment for the laboratory, they must submit their graphs and answer questions showing that they understand the properties of enzymes, can explain how a spectrophotometer works, and can interpret the data which they collected. Homework is submitted on-line to a learning management system (WebAssign) in which some answers are automatically graded, while others are hand graded by a teaching assistant.

While the on-line laboratory does not provide hands-on experience with the equipment, it does fulfill the other goals of the enzyme laboratory, including an understanding of how pipets and spectrophotometers function. Because the spectrophotometer and graphing simulations are interactive, students gain realistic experience in the collection and analysis of data. Since they can "perform" the assays in a shorter amount of time than in the classroom laboratory, students have more time to spend on analysis and homework preparation.

In addition to use in distance education, the videos and simulations can be used to prepare on-campus students for an upcoming laboratory, to extend the laboratory experience by assigning additional (on-line) assays as homework, or to provide review material. The interactive spectrophotometer is customizable for any assay in which categories and values are known. The x values (categories such as minutes or temperature) and y values (absorption or transmission) are accessed by viewing the code on the web page with a web editor. All values can be then be changed by typing in new numbers. All simulations are available for download at the North Carolina Learning Object Repository. They may be freely used for any academic purpose. Just go to the following URL and use the guest log in: http://www.explorethelor.org/ .Then use "simulation" as a search term. This should bring up a list of many simulations including the above. For a more specific search, enter "biomovies" and on that page enter the search term "simulation" (you will find a list of simulations by Black and Niedzlek-Feaver). To use the simulations, you may either link to one and use it on the NCLOR site or download the simulation. We recommend the latter, since you can then copy the simulation to computers, burn it on a CD, or upload it to your own web site. Instructions on programming the spectrophotometer for different assays are included with the download, so feel free to adapt this simulation for your own use.

We also provide all videos associated with the enzyme laboratory for download and educational use. These videos can be found by searching the NCLOR for "biomovies", then "enzyme". Additionally, the on-line laboratory exercise, including videos and simulations, can be viewed at http://www.ncsu.edu/project/bio183de/Lab/enzymes_lab/enzymes1.html. If you use any of our resources, we would appreciate feedback and any suggestions for improvement.

Literature Cited

Dahlqvist, A. 1968. Assay of intestinal disaccharidases. Analytical Biochemistry, 22: 99-107.

Appendix

The source and list price of the reagents are as follows. All are provided by Sigma - Aldich.

Chemical	Item Number	List Price	Amount
D-(+)-Maltose	M5885	$25.70	100 g
Maleic acid	M153	$38.50	100 g
Trizma base	T1503	$39	250 g
Peroxidase from horse radish	P8125	$28.18	5 KU
Glucose oxidase from Aspergillus niger	G6125	$122.50	50 KU
Triton X-100	X100	$29.60	100 mL
o-Dianisidine dihydrochloride	F5803	$34	50 mg
D-(+)-Glucose	G5767	$20.50	25 g

The glucometers that we use are old and the strips are expensive. It is now possible to obtain somewhat cheaper glucometers and strips. Two examples are shown below.

Glucometer model	Provider	Monitor cost	Strip cost
Accu-Chek Diabetes Monitoring kit	www.drugstore.com	$20.99	$30.99/pack of 50
Rite Aid TRUEtrack Blood Glucose Monitor	www.drugstore.com	$29.99	$59.99/pack of 100

About the Authors

Betty L Black received her Ph.D. from Washington University (St. Louis). She teaches a course in Developmental Anatomy plus distance education courses in Introductory Biology, Histology, and Animal Diversity. She has received two University awards for “Innovative Excellence in Teaching and Learning with Technology” and has 12 publications on teaching technology topics.

Marianne Niedzlek-Feaver received her Ph.D. from the University of Michigan. As an evolutionary ecologist, she is interested in identifying factors that shape the mating systems of grasshoppers and katydids. She currently teaches Evolution, Invertebrate Zoology and Introductory Biology courses. She has received various grants to improve the laboratory experience, and is a member of the NCSU Academy of Outstanding Teachers.

M. Jason Wingate recently received a M.S. degree in Zoology from NCSU and plans to enter the Ph.D. program. His fields of interest are Cell Biology, Developmental Biology, and Anatomy, and he looks forward to a career of teaching at the College level.