Chapter 16

Using Bromelain in Pineapple Juice to Investigate Enzyme Function

William V. Glider[1] and Mark S. Hargrove[2]

[1]School of Biological Sciences
University of Nebraska-Lincoln
Lincoln, NE 68588-0118
wglider1@unl.edu

[2]Biochemistry, Biophysics, & Molecular Biology
4114 Molecular Biology
Iowa State University
Ames, IA 50011-3260
msh@iastate.edu

Bill Glider received a B.S. in Science Education from Cornell University (1970), a M.S. in Botany from the University of Maine-Orono (1975), and a Ph.D. in Biological Sciences from the University of Nebraska-Lincoln (1983). Bill is a Senior Lecturer in the School of Biological Sciences at the University of Nebraska-Lincoln (UNL) where he lectures in the General Biology Program, serves as the Biology Teaching Lab Coordinator, and is Coordinator of the TA Training Program for the School of Biological Sciences and the College of Arts and Sciences. Bill was the recipient of a Distinguished Teaching Award from the College of Arts and Sciences (2002). His research interests are biology education, with emphasis on the use of web-based technology as a pedagogical tool.

Mark Hargrove is an Assistant Professor in the Department of Biochemistry, Biophysics, and Molecular Biology at Iowa State University. He received a B.S. in Chemistry and Biological Sciences from the University of Nebraska-Lincoln (1992) and a Ph.D. from Rice University (1993). His research interests are the structure, function, folding, and expression of heme proteins. He teaches courses in biochemistry and biophysics.

Reprinted From: Glider, W. V. and M. S. Hargrove. 2002. Using bromelian in pineapple juice to investigate enzyme function. Pages 275-295, in Tested studies for laboratory teaching, Volume 23 (M. A. O'Donnell, Editor). Proceedings of the 23rd Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 392 pages.

Contents

Introduction

This investigation introduces students to the structure and function of the proteolytic enzyme, bromelain, which is present in large quantities in the fruit, leaves, and stems of pineapple. In this lab exercise, students study the rate at which bromelain catalyzes the hydrolysis of gelatin (substrate) at different temperatures and pH. Strips of developed black and white photographic film are placed in freshly squeezed pineapple juice under different experimental conditions. The time it takes for the film to clear is used as an indicator of the rate at which bromelain catalyzes the hydrolysis of the substrate (gelatin), which binds the black silver grains to the plastic backing of the film. This lab exercise has been used in traditional non-majors and mixed majors/non-majors General Biology labs. It can easily be adapted to an investigative approach.

Materials

The lab rooms are equipped with six moveable lab benches that have four lab stations per table for a total of 24 students per lab. Aluminum dissecting pans (18 x 28 x 3 cm) without wax are used to organize student equipment and solution containers on the lab benches.

Materials On Each Student Lab Bench

- 4 plastic 16-ounce beverage cups
- 4 styrofoam coolers marked with fill lines to denote the amount of water required to cover the solutions in the test tubes
- 12 disposable culture tubes (13 x 100 mm)
- 4 plastic test tube racks (Nalgene Unwire Test Tube Racks for 13mm tubes, 72 places (Fisher Cat. No. 14-809-22**)**
- 1, box Kimwipes7
- 1 metal pan containing one box of pH indicator strips containing only enough strips needed for each lab section, plus a few extra (approximately 15)
- 4 alcohol thermometers
- 2 grease pencils
- 2 pairs forceps

Materials on Lab Bench at Front of Room

Large Metal Dissecting Tray Containing:

- 1 electric juicer
- 1 800-mL beaker for collecting juice
- 1 600-mL beaker for collecting filtered juice
- 1 250-mL graduated cylinder
- 1 sponge for clean-up
- 1 large, sharp knife for cutting pineapple

11 Small Metal Pans:

- 1 labeled "Fresh Juice" containing:
  - 1 250-mL beaker labeled "Fresh Pineapple Juice" to be used in the gelation experiment
  - 3 14.4-cm Pasteur pipets with bulbs labeled "Fresh Juice" and marked with a black ring indicating a volume of 1 ml
- 2 labeled "Pineapple Juice"
- 1 labeled "pH 3.5 Buffer"
- 1 labeled "1.0 N HCl"
- 1 labeled "1.0 N NaOH"
- 1 labeled "pH 5 Buffer"
- 1 labeled "pH 7 Buffer"
- 2 labeled "Distilled Water"
- 1 labeled "Wax Paper" containing wax paper cut into squares (5 x 5 cm)

Miscellanous Items

- rubber bands
- cheesecloth, 1-layer thick, cut into squares (20 x 20 cm)
- plastic bucket lined with garbage bag for disposal of pineapple pulp
- 3 boxes of latex gloves in sizes S,M,L
- 24 stopwatches
- 1 cooler filled with ice
- 2 plastic jars filled with film strips
- 1 film clearing demonstration card

Materials Next to Sinks

- 3 test tube brushes
- Dilute liquid soap
- Plastic containers for collecting silver solution for disposal as a hazardous waste

Temperature Experiment

Lab Bench at Front of Room

- 2 Repipet Jr. dispensers each labeled with the appropriate solution and placed in a metal pan
- 1 pH 3.5 Buffer with syringe set to 5 mL
- 1 Pineapple Juice with syringe set to 5 mL

Side Lab Bench

- Water bath set at 70^{o} C containing:
  - 2 plastic test tube racks
  - Distilled water to cover the test tubes 0.75 of their length
  - Plastic water bath balls (VWR Cat. No. 26396-466) to cover the entire water surface to decrease evaporation.
  - 1 250-mL beaker labeled "Hot Juice" containing three 14.4-cm Pasteur pipets with bulbs labeled "Hot Juice" and marked with a black line indicating a volume of 1 mL.

Gelation Experiment

- 1 slide warmer

- 1 250-mL beaker labeled “Gelatin”containing 3 Pasteur pipets labeled “Gelatin” each marked with a line indicating a volume of 1 mL.
- 1 250-mL beaker labeled “Water” containing 3 Pasteur pipets labeled “Water” each marked with a line indicating a volume of 1 mL.
- 1 250-mL beaker labeled “Canned Juice” containing about 150 mL of juice (put in a small styrofoam cooler with an ice pack) and 3 Pasteur pipets labeled “Canned Juice” each marked with a line indicating a volume of 1 mL.

pH Experiment

- 9 Repipet Jr. dispensers each labeled with the appropriate solution and placed in a metal pan.
- 1 1.0 N HCl - syringe set at 2mL
- 1 1.0 N NaOH - syringe set at 2mL
- 2 Distilled Water - syringe set at 5mL
- 2 Pineapple Juice - syringe set at 5mL
- 1 pH 3.5 Buffer - syringe set at 2mL
- 1 pH 5 Buffer - syringe set at 2 mL
- 1 pH 7 Buffer - syringe set at 2 mL

Demonstration Materials and Teaching Aides

- Demonstration of a Multiple Fruit
  - Cut a pineapple in half lengthwise and put it in plastic storage container with a large ice pack.

- Photographs
  - pineapple plant with fruit
  - people working in pineapple factories and plantations

- Overhead Transparencies
  - Enzyme activation energy graph
  - Enzyme-substrate diagrams
  - Hemoglobin protein structure
  - Amino acid structures
  - Protein structure
  - pH scale including pH of common solutions
  - Levels of protein organization (primary, secondary, etc.)
  - Bromelain assay diagram

- Models
  - Wire protein structure models: helix (coil)
  - Space filling protein and amino acid model

- Box of JELL-O®. Highlight the statement on the box that cautions against adding fresh pineapple to the gelatin dessert.

- Bottle of unseasoned Adolf's ® Meat Tenderizer. The meat tenderizer contains the enzyme papain, a proteolytic enzyme similar to bromelain, which is found in papaya. Ask students how treating meat with papain results in tenderizing the meat.

Web Sites: Commercial Uses of Bromelain and Background Information

http://www.thorne.com/altmedrev/fulltext/bromelain1-4.html
http://www.worldwideshoppingmall.co.uk/pets-corner/pet-health-bromelain-papain.asp
http://www.lef.org/prod_hp/php128.html
http://www.ars.usda.gov/is/AR/archive/nov99/bossy1199.htm

Notes for the Instructor

Pineapple Juice Extraction

- It is recommended that the preparation and extraction of the pineapple juice be done in front of the students in order to emphasize that the enzyme is extracted from the fresh pineapple fruit.
- Be sure to provide a copy of the operating instructions for the electric juicer to the lab instructors.

Safety Equipment

- Students are required to wear gloves when handling the solutions used in the pH experiment.
- Students are required to wear safety glasses throughout the lab exercise.

Disposal of Silver Solutions

- Since the silver released from the film during the experiment is a heavy metal, it is considered a hazardous waste and must be disposed of according to EPA guidelines. All solutions containing silver as well as the used film strips are emptied into plastic milk containers labeled "Used Chemicals" placed at each sink in the lab rooms. The University Environmental Health and Safety Unit picks up the silver waste for proper disposal.

Pre-lab Instructions and Reminders to Students

Describe the basis of the bromelain enzyme assay.

- Photographic film is composed of a piece of plastic that has been dipped in a solution of gelatin and silver crystals to form a thin coating. The gelatin coating the film is probably denatured but not hydrolyzed.
- When the gelatin (a protein) holding the silver crystals to the film is hydrolyzed with the aid of bromelain, the primary structure of the gelatin is destroyed and the silver grains slough off the film, since there is nothing to bind them to the plastic.
- Students have difficulty in understanding how an enzyme, which is a protein, can catalyze the breakdown of another protein, in this case gelatin.

♦ Emphasize the fact that it is *not* the acid in the juice that is catalyzing the breakdown of the gelatin. In the pH experiments, the students actually demonstrate this with their control tubes.
♦ Emphasize the function of control tubes in these experiments. Point out that the gelatin on the film could be being digested by the water, sugar, or the acid in the pineapple juice.

Demonstrate:

- what vigorous agitation means. Tell students to agitate the film at the time intervals stated. Continuous agitation will lead to erroneous results.
- what the film looks like when it has cleared. The film in the 70 ^{o}C and certain of the pH solutions will turn brown. This is not clearing! There is a laminated demonstration card at each TA desk that shows the difference between cleared and uncleared film. Show this card to the students.
- how to operate the repipet dispensers. When dispensing the fluid, slowly press the plunger *all the way down* until it stops. Then release the plunger slowly so that the syringe chamber is fully filled.

Remind Students

- that the test tubes must be cleaned with soap and water before use and between experiments. Rinse the tubes thoroughly to remove soap.
- that the film strips must be handled by the taped end only so as not to get oil from their hands on the film surface.
- that they will only need the number of test tubes present at each station (total of 12). Do not let them raid the prep room for extra tubes.
- to carry the styrofoam coolers with both hands on the bottom.
- that if the film in the control tubes does not clear at the same time the film in the juice clears, record "no reaction" in Table 1.

Part I: Effect of Temperature on Enzyme Activity

Remind Students to

- maintain the water baths at the correct temperature throughout the experiment. This is especially important for the 20°C experiment. The film in the 20 °C experimental tube will clear within 40-60 minutes if the temperature is maintained at 20°C. Plastic cups are used for adding and removing water from the water baths.

Procedure at 70 °C:

- Make sure the water bath temperature is at least 70°C. I have had problems with the film clearing at temperatures as high as 68 °C.
- It is extremely important that the pineapple juice is placed in the 70°C water bath and allowed to equilibrate for at least 5 minutes before the film is placed in the tube. It takes at least 5 minutes at 70°C for the bromelain to denature.
- Make sure the water level in the water bath is slightly higher than the level of the pineapple juice in the tube. This ensures that the entire volume of juice reaches a temperature of 70°C before the film is placed in the tube.
- Do not let students remove hot water from the water baths.
- The lab instructor should place a tube of fresh juice in the 70°C water bath in order to demonstrate protein coagulation and precipitation. Often times, the students don't observe coagulation in their own tubes because they are constantly agitating the contents.
- Tape down the temperature control on the water bath so that it cannot be tampered with and changed.

Part II: Effect of Bromelain on Gelation

Remind Students to:

- use the fresh juice in the beaker, **not** that in the Re-pipet dispenser. Otherwise, they will dispense 5 mL rather than the 1 mL dispensed by the Pasteur pipet.
- add the fresh, hot, canned juice or water to the test tubes before adding the gelatin and to *immediately mix* the gelatin-juice mixture by placing a piece of wax paper over the mouth of the tube and inverting.
- use a plastic cup to hold the ice.
- *not* allow the tubes to remain in the ice *over* 5 minutes. The difference in consistency of the gelatin becomes less obvious the longer the tubes remain in the ice.

Part III: Effect of pH on Enzyme Activity

Procedure

After the students have completed the temperature and gelation experiments:

- Move the "Fresh Juice" and "pH 3.5 Buffer" Re-pipet dispensers used in the temperature experiments to the metal pans labeled "Fresh Juice" and "pH 3.5 Buffer" for the pH experiment.
- Bring the Re-pipet dispensers containing the solutions for the pH experiments into the lab room and place them in the appropriately labeled metal pan.

Remind Students:

- that one student in each pair should time the reaction while the other one does the agitation and makes the observations for clearing.
- to test the pH of the contents of the experimental *and* control tubes.
- to use only the number of pH strips called for (*i.e.*, 6 strips per student pair #1 and 4 strips per student pair #2)
- to read the results on the pH strips while they are still moist. They should not drop the pH strips into the solution and try to read the results through the test tube. Forceps should be provided for retrieval of pH strips from the tubes.
- that the pH of each of the solutions in the experimental tubes should be different.
- if the color of the pH strip is between two pH units, record it as the nearest 0.5 pH unit.

Approximate pH of Solutions in Experimental Tubes

Tube	Solution in Experimental Tubes	Approximate pH
1	5 mL juice + 2 mL 1 N HCL	1
3	5 mL juice + 2 mL pH 3.5 buffer	3.5
5	5 mL juice + 2 mL pH 5 buffer	5
7	5 mL juice + 2 mL pH 7 buffer	7
9	5 mL juice + 2 mL 1 N NaOH	13

Suggested Time Budget

Quiz = 20 minutes maximum

Pre-lab = 20 minutes maximum

Part I (Effect of Temp.) = 1 hour

Part II (Gelation) = 15 minutes

Part III (Effect of pH) = 45 minutes

Post Lab = 10 minutes

Student Outline

Introduction

A catalyst is any substance that affects the rate of a chemical reaction without itself being used up. **Enzymes** are organic catalysts that speed up the rates of biochemical reactions in living organisms. All but two known enzymes are proteins. In this exercise, you will study the enzyme **bromelain,** which is present in large quantities in the fruit, leaves, and stems of several varieties of pineapple.

Biology of Pineapples

The pineapple (*Ananas comosus*) is placed in the Order Bromeliales and the Family Bromeliaceae. Pineapples are short stemmed, herbaceous plants having basal rosettes of stiff, spiny leaves. The flowers are clustered together on a short stalk that arises from the rosette of leaves. The cluster of flowers resembles a pine cone, thus the name pineapple. Leaves also grow from the top of the flower cluster. As the ovaries of the individual flowers on the stalk ripen, they swell up to form the pineapple fruit. The pineapple fruit is considered a **multiple fruit** because it consists of the enlarged ovaries of many flowers more or less grown together into one mass. Since cultivated pineapples are seedless, the plant is propagated asexually from various plant parts including small shoots (slips), which arise from the stem at the base of the fruit. The pineapple is native to northern South America and was first brought to Europe at the beginning of the 16th century. Today, the plant is cultivated in Hawaii, Brazil, the West Indies, Malaysia, Thailand, South Africa and China. At one time, Hawaii produced nearly 35% of the world's crop. However, Thailand is currently the world's largest producer of fresh and canned pineapple. Machines cut the fruits into cylinders, drill out the woody center, and divide the fruit into slices. The leftover fruit pulp is turned into juice or canned for use in jam and ice cream. Some varieties of pineapple are cultivated for the strong white silky fibers that can be extracted from the leaves. In the Philippines, this fiber is woven to produce "pina," a light-weight sheer fabric, which is often embroidered and used for table linen or clothing.

Bromelain

Bromelain is a proteolytic enzyme or protease. Such enzymes catalyze the breakdown of proteins into their amino acid building blocks through a hydrolysis reaction. **Hydrolysis** (L. *hydro* = water; Gk. *lysis* = loosening or breakdown) is the decomposition of large molecules into smaller units by combining them with water. In the case of protein digestion, the peptide bonds are broken with the insertion of the components of water, -H and -OH, at the cleaved (broken) ends of the chain.

In this lab exercise you will study the rate at which bromelain catalyzes the breakdown of the protein gelatin (the substrate) at different temperatures and pH. Gelatin (the main component of JELL-O®) is a purified structural protein derived from animal tissues high in collagen, like tendons and cartilage.

Bromelain Assay (Analysis):

To determine the rate at which gelatin is degraded by the bromelain you could measure the rate at which the gelatin (substrate) is broken down or the rate that the products of this digestion (amino acids) are produced. In this exercise, you will measure the rate at which the substrate is degraded by the enzyme. To do this you will use strips of exposed and developed black and white photographic film. Photographic film is composed of a plastic sheet coated with a thin coating (emulsion) of gelatin impregnated with silver grains. The gelatin simply serves to bind the silver grains to the plastic. If the gelatin is destroyed, the black silver grains will simply slough off, leaving the relatively clear plastic backing of the film intact.

In these experiments, strips of developed film will be placed in pineapple juice under different experimental conditions. The time it takes for the film to clear will be an indication of the rate at which the enzyme bromelain catalyzes the breakdown (digestion) of the substrate (gelatin).

Part I: Effect of Temperature on Rate of Enzyme Activity

Procedure

A. Based on your knowledge of enzymes, write down your hypothesis as to the effect of temperature on the rate of bromelain activity. Be precise. For example, will the rate of bromelain activity increase, decrease, or remain the same as the temperature increases? Will there be a lower and upper temperature limit to this rate change?

B. In this experiment your table will work as a group to collect the data as follows:

1. One person at the table will determine the rate of enzyme activity at 70°C and 20°C. This part of the experiment will require four clean test tubes. Before using the test tubes, make sure they have been washed with soap and hot water and rinsed thoroughly.
2. The remaining three people at the table will determine the rate of enzyme activity at either 50, 40, or 30°C. Each temperature experiment will require two clean test tubes. Before using the test tubes, make sure they have been washed with soap and hot water and rinsed thoroughly.

C. Instructions for Using the Re-pipet Dispensers:

1. In this lab you will be using automatic pipeting dispensers, which allow you to accurately dispense pre-measured amounts of fluid. When dispensing a fluid into a test tube, *slowly* press the plunger *all the way down* until it stops. Then release the plunger *slowly* so that the syringe chamber is fully filled.

D. Procedure for Testing Enzyme Activity at 70 °C

1. From the dispenser labeled "Juice," fill one test tube with 5 mL of freshly squeezed pineapple juice. Using a grease pencil label the tube with your initials and "70°C juice."
2. From the dispenser labeled "pH 3.5 Buffer," add 5 mL of pH 3.5 buffer to a second test tube. Label the tube with your initials and "70°C Buffer."
3. Place the test tube containing pineapple juice and the one containing the buffer in the 70°C electric water bath. Make sure both tubes are properly labeled.
4. Allow the solutions in the two test tubes to equilibrate at 70°C for 5 minutes.
5. Obtain two strips of film. *Handle the strips of film by the taped end only. Do not get oil from your fingers on the film surface*. Place a strip of film in each of the two test tubes. Record the time you put the strips of film in the test tubes.
6. Every 10 minutes *vigorously* agitate the film for 5 seconds and check for clearing. Record the time it takes for the film to clear in Table 1 under the column labeled "Reaction Time." *If the film in the experimental and/or control tubes shows no signs of clearing after 60 minutes, record "No reaction."*

   Note: You should start the 20 °C experiment at your desk between the times you agitate the film in the 70 °C water bath.
7. After 60 minutes in the 70°C water bath, hold the tube containing the heated pineapple juice up to the light and compare it to fresh pineapple juice. What is visually different between the fresh pineapple juice and the juice incubated at 70°C?

Do not throw out the pineapple juice in the 70 °C tube. Allow the tube to remain in the water bath. You will need this juice for Part II of the lab.

E. Procedure for Testing Enzyme Activity at 20 °C

1. From the dispenser labeled "Juice," fill a *clean* test tube with 5 mL of pineapple juice and label it

"20°C juice." Fill a second clean test tube with 5 mL of pH 3.5 buffer and label it "20°C buffer."

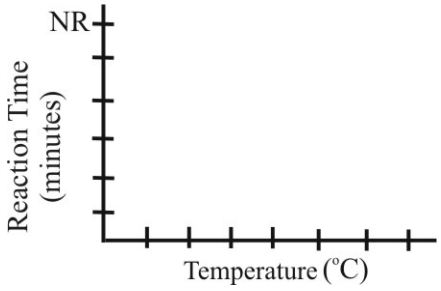
2. Construct a 20°C water bath by filling a styrofoam cooler with 20°C water to the fill line marked on the inside of the water bath.
3. Place the test tube containing pineapple juice and the one containing buffer in a test tube rack and place the rack in the 20°C water bath.
4. Allow the solutions in the two test tubes to equilibrate at 20°C for 5 minutes.
5. Obtain two strips of film from the TA desk. *Handle the film by the taped end only! Do not get oil from your fingers on the film surface***.** Set your stopwatch to zero. Place a strip of film in each of the test tubes and start your stopwatch.
6. *Every 5 minutes vigorously agitate the film for 5 seconds and check for clearing.* You must maintain the 20°C temperature throughout the experiment. Record the time in Table 1 when the film clears. If the film in the experimental and/or control tubes shows no signs of clearing after 60 minutes, record **"No Reaction."**

F. Procedure for Testing Enzyme Activity at 50, 40 and 30 °C

1. From the dispenser labeled "Juice,"fill one **clean** test tube with 5 mL of freshly squeezed pineapple juice. Using a grease pencil label the tube with your initials and "50°C juice."
2. From the dispenser labeled "pH 3.5 Buffer," fill a second **clean** test tube with 5 mL of pH 3.5 buffer. Label the tube with your initials and "50°C Buffer."
3. Construct a 50°C water bath by filling a styrofoam cooler with 50 °C tap water to the fill line marked on the inside of the cooler.
4. Place the test tube with pineapple juice and the test tube with buffer in a test tube rack and place the test tube rack in the 50°C water bath.
5. Allow the solutions in the two test tubes to equilibrate at 50°C for 5 minutes.
6. Obtain 2 strips of film. *Handle the strips of film by the taped end only. Do not get oil from your fingers on the film surface.* Set your stopwatch to zero. Place a strip of film in each of the two test tubes and start your stopwatch.
7. *Every minute vigorously agitate the film for 5 seconds and check for clearing. You must maintain the 50 °C temperature throughout the experiment*. Stop your stopwatch when the film is clear. Record the time required for the film to clear under the column labeled "Reaction Time" in Table 1. *If the film in the experimental and/or control tubes shows no signs of clearing after 60 minutes, record "No reaction."*
8. After the film has cleared at 50°C, empty the contents of the tubes (liquid and film strips) into the **waste containers** located next to the sink. *Do not empty the contents of the tubes down the sink drain!* Wash the two test tubes with soap and hot water, making sure that all of the soap has been rinsed off the tubes. Then repeat steps 1-5 above for 40 and 30°C (in that order).
9. Repeat steps 1 - 8 above for 40 and 30°C.

Questions:

1. Construct a line graph showing the effect of temperature on the rate of bromelain activity (see sample graph to the right). On the y-axis, plot the time it took for the film to clear (reaction time) in the experimental *and* control tubes and on the x-axis plot the temperature.
   The abbreviation NR = No Reaction**.**



2. a. Based on the data from **your** graph, what effect does temperature have on the rate of bromelain activity?
   b. In order for an enzyme to catalyze a reaction the enzyme and substrate must come in contact with each other and bind together momentarily. Based on this fact, what is the mechanism by which temperature affects the rate of an enzyme reaction. (Hint: How do changes in temperature affect the rate at which the enzyme and substrate come in contact?)
   c. Based on the data from your graph, what is the optimum temperature for bromelain activity? Explain

why you chose your answer.

3. a. For each 10˚ C increase in temperature, calculate the *change in rate* of the enzyme reaction. For example, if it took 5 minutes for the film to clear at 20˚C and 20 minutes for the film to clear at 30˚C, the change in rate is 4 fold (20 minutes/5 minutes = 4 fold decrease in rate).
   b. Does the *change in rate* of the bromelain catalyzed reaction increase, decrease, or remain the same as the temperature approaches 70˚C. Explain why based on your knowledge of enzyme structure and function.
4. a. What effect did the 70˚C temperature have on the rate of bromelain activity? Based on your knowledge of how enzymes catalyze a reaction and on enzyme structure, explain this observed effect.
   b. Why is it incorrect to say the bromelain enzyme was **"**killed**"** by exposure to the 70˚C temperature?
5. a. In the experiments testing the effect of temperature on bromelain activity, why was a test tube containing pH 3.5 buffer incubated along with the pineapple juice at each temperature tested?
   b. Why was pH 3.5 buffer used rather than distilled water?
6. Assuming the silver grains from the film have no effect on the function of bromelain, explain why or why not the *same* enzyme solutions could be used over again to repeat the temperature experiments (with the possible exception of that at 70˚C)?
7. Other than to protect their skin from being cut by the prickly leaves and being irritated by the acidity of the juice, why do pineapple pickers wear protective gloves when harvesting pineapples?
8. If you were to place a strip of photographic film in a test tube with a *lipase* solution, would the film clear? Explain why or why not.

Part II: Effect of Bromelain on Gelation (Solidification) of Gelatin

Introduction:

Gelatin is a soluble mixture of polypeptides (protein) that is produced by boiling collagen extracted from the hide, bones, hoofs, tendons, and other connective tissue of horses and cows. Collagen is the most abundant of all proteins in higher vertebrates, making up one-third or more of the total body protein.

If powdered gelatin is mixed with water and warmed, it will dissolve. Then if allowed to cool, the polypeptide chains cross-link with each other and water becomes trapped within this network of inter-connected polypeptides. This process results in the formation of a semi-solid "gel." If the peptide bonds between the amino acids of the gelatin polypeptides are broken, gelatin will not solidify when cooled.

Procedure: (Work in a Group of Four)

A. Based on your knowledge of enzymes and the process of gelation, write a hypothesis as to the effect of bromelain enzyme on the gelation of gelatin.

B. Obtain four clean test tubes and carry out the following steps:

1. Label one of the tubes "Fresh Juice." Using the Pasteur pipet labeled "Fresh Juice" located next to the water bath, fill the pipet to the 1-mL line with fresh pineapple juice and dispense it into the tube.
2. Label a second tube "Hot Juice." Using the Pasteur pipet labeled "Hot Juice" located next to the water bath, fill the pipet to the 1-mL line with your pineapple juice which was heated to 70^{o}C in Part I and dispense it into the tube.
3. Label the third tube "Canned Juice." Using the Pasteur pipet labeled "Canned Juice" located at the "Gelatin Experiment" station, fill the pipet to the 1-mL line with canned pineapple juice and dispense it into the tube.
4. Label the fourth tube "Control." Using the pasteur Pasteur pipet labeled "Water" located at the "Gelatin Experiment" station, fill the pipet to the 1-mL line with water and dispense it into the tube.

C. Using the Pasteur pipet provided, fill it to the 1-mL line with warm JELL-O® and dispense it into the tube labeled "Fresh Juice." *Immediately mix the contents of the tube by covering the mouth of the tube with a piece of wax paper and inverting the tube.*

D. Using the Pasteur pipet provided, fill it to the the 1-mL line with warm JELL-O® and dispense the JELL-O® into the tube labeled "Hot Juice." *Immediately mix the contents of the tube by covering the mouth of the tube with a piece of wax paper and inverting the tube.*

E. Using the Pasteur pipet provided, fill it to the the 1-mL line with warm JELL-O® and dispense the JELL-O® into the tube labeled "Canned Juice." *Immediately mix the contents of the tube by covering the mouth of the tube with a piece of wax paper and inverting the tube.*

F. Using the Pasteur pipet provided, fill it to the 1-mL line with warm JELL-O® and dispense the JELL-O® into the tube labeled "Control." *Immediately mix the contents of the tube by covering the mouth of the tube with a piece of wax paper and inverting the tube.*

G. Place all four test tubes in a plastic cup filled with ice for 5 minutes.

H. After 5 minutes in the ice, remove the tubes, tilt them slightly, and note the consistency of the gelatin in each tube. Return the unused ice to the cooler. Record your observations in Table 2.

Questions:

1. In which tube or tubes did the gelatin fail to solidify? Explain why the gelatin in each tube did or did not solidify.
2. If you want to add pineapple to JELL-O® why must you use canned pineapple rather than fresh pineapple?
3. Why was water added to tube #4?

Part III: Effect of pH on Rate of Enzyme Activity

Procedure

A. Based on your knowledge of enzymes, write a hypothesis as to the effect of pH on the rate of bromelain activity. For example, will the rate of bromelain activity increase, decrease or remain the same as the pH increases, decreases? What is the expected optimal pH for bromelain activity?

B. In this experiment, each pair of students at a table will collect part of the data. At the conclusion of the experiment, you will combine your table's data.

C. Procedure: Student Pair 1

1. Wash and rinse six test tubes. Using the appropriately labeled dispensers, add the following substances to each of six test tubes. *Immediately after adding the solutions to each tube, mix the contents by covering the mouth of the tube with a piece of wax paper and inverting the tube.* Use a new piece of wax paper for mixing each tube.

Note: Read across from left to right.

Tube #	1N HCl (mL)	pH 3.5 buffer (mL)	pH 5 buffer (mL)	water (mL)	pineapple juice (mL)
1	2	0	0	0	5
2	2	0	0	5	0
3	0	2	0	0	5
4	0	2	0	5	0
5	0	0	2	0	5
6	0	0	2	5	0

2. Test the pH of the solutions in *all* six tubes by the following method:
   - To test the pH of each solution, dip a pH strip into the solution in the test tube and withdraw it. *Do not drop the pH strip into the test tube*. While the strip is still moist, match the four colored squares on the pH strip with the four colored squares on the side panel of the pH strip box. *Use a new pH strip for each solution*. Record the pH of each of the six solutions in Table 3.
   - The pH of the solutions in experimental tubes #1, 3, and 5 should be different. If they are not different, consult your TA before starting the experiment.

3. Construct a 45^{o}C water bath by filling a styrofoam cooler to the fill line with 45^{o}C tap water. You must maintain the 45^{o}C temperature throughout the experiment.
4. Place the six tubes in a test tube rack and place the rack in the 45^{o}C water bath. Allow the contents of the tubes to equilibrate for 5 minutes.
5. After the 5-minute equilibration period, place a strip of film in **each** of the six tubes as close to the same time as possible and start your stop watch. *Handle the film by the taped end only! Do not get oil from your fingers on the film surface.*
6. *Every minute vigorously agitate the film in each tube for 5 seconds and check for clearing of the film.* One person should agitate the film and observe for clearing while the other person monitors the stopwatch and records the time of clearing. Record the time required for the film to clear in the column labeled "Reaction Time" in Table 3.
   - If the film in the control tubes (#2,4,6) does not clear at the same time as the film in the experimental tubes (#1,3,5), then record "No Reaction" in the column labeled "Reaction Time" in Table 3.

D. Procedure: Student Pair 2

1. Wash and rinse four test tubes. Using the appropriately labeled dispenser, add the following substances to each of the four tubes. *Immediately after adding the solutions to a tube, cover the mouth of the tube with a piece of wax paper and invert the tube to mix the contents. Use a **new** piece of wax paper for mixing each tube.*

Note: Read across from left to right.

Tube #	pH 7 buffer (mL)	1N NaOH (mL)	water (mL)	pineapple juice (mL)
7	2	0	0	5
8	2	0	5	0
9	0	2	0	5
10	0	2	5	0

2. Test the pH of the solutions in all four tubes by the following method:
   - To test the pH of each solution, dip a pH strip into the solution in the test tube and withdraw it. *Do not drop the pH strip into the test tube.* While the strip is still moist, match the four colored squares on the pH strip with the four colored squares on the side panel of the pH strip box. *Use a new ph strip for each solution*. Record the pH of each of the six solutions in Table 3.

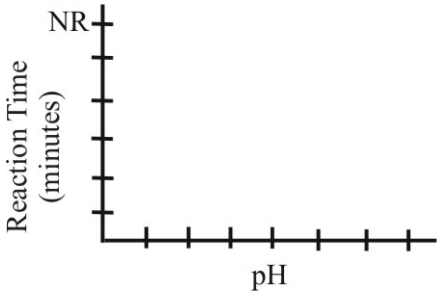
   Note: The pH of the solutions in experimental tubes #7 and #9 should be different. If they are not different, consult your TA before starting the experiment.

3. Construct a 45^{o}C water bath by filling a styrofoam cooler to the fill line with 45^{o}C tap water. *You must maintain the 45^{o}C temperature throughout the experiment.*
4. Place the four tubes in a test tube rack and place the rack in the 45^{o}C water bath. Allow the contents of the tubes to equilibrate for 5 minutes.
5. After the 5-minute equilibration period, place a strip of film in each of the four tubes as close to the same time as possible and start your stopwatch. *Handle the film by the taped end only! Do not get oil from your fingers on the film surface.*
6. *Every minute vigorously agitate the film in each tube for 5 seconds and check for clearing of the film.* One person should agitate the film and observe for clearing while the other person monitors the stopwatch and records the time of clearing. Record the time it takes for the film to clear in the column labeled "Reaction Time" in Table 3.
   - If the film in the control tubes (#8 and #10) does **not** clear at the same time as the film clears in the experimental tubes (#7 and #9), then record "No Reaction" in the column labeled "Reaction Time" in Table 3.

7. At the end of the pH experiment, empty the contents of the tubes (liquid and film strips) into the *waste containers* located next to the sink. *Do not empty the contents of the tubes down the sink drain!* Wash the two test tubes with soap and hot water, making sure that all of the soap has been rinsed off the tubes.

Questions:

1. Using the combined data from both student groups, construct a line graph showing the effect of pH on rate of bromelain activity (see sample graph below). On the y-axis, plot the time it took for the film to clear (reaction time) in minutes in the experimental and control. On the x-axis, plot the pH of the pineapple juice in the experimental and control tubes. The abbreviation NR = No Reaction.



2. a. Based on the data from your graph, what effect does pH have on the rate of bromelain activity?
   b. Based on your knowledge of how enzymes catalyze a reaction and on enzyme structure, EXPLAIN the observed effect of pH on rate of bromelain activity.

3. What was the pH of the fresh pineapple juice? Is it acidic or basic?

4. Based on your data, what is the optimum pH for maximum bromelain activity? Explain why you chose your answer.

5. What was the purpose of including the control tubes (#2,4,6,8,10,12) in this experiment?

6. Why doesn't the enzyme bromelain digest the proteins in your stomach when you eat fresh pineapple? (Hint: What is the pH of the gastric juice in your stomach?)

7. Why were the pH experiments carried out at 45^{o}C?

Acknowledgments

I would like to thank Robert Anderson, General Biology Lab Manager, for assembling the materials used in the presentation of the workshop and for his reading of the final version of this manuscript. In addition, I would like to acknowledge the nearly 10,000 students who have completed this lab exercise over the past 12 years. Numerous revisions of the lab exercise have been based on their comments and the analysis of data collected by them.

Appendix A: Data Sheets

Table 1: The Effect of Temperature on the Rate of Bromelain Activity

Temp. (^{o}C)	Substance	Reaction Time (minutes)
20	juice	
	buffer	
30	juice	
	buffer	
40	juice	
	buffer	
50	juice	
	buffer	
70	juice	
	buffer	

Note: If the film in the control tubes (buffer solution) does **not** clear at the same time the film in the juice clears, record "No Reaction" in the column labeled "reaction time."

Table 2: Effect of Bromelain Enzyme on Gelation

Treatment	Result: record (+) = gelation or (-) = no gelation
Fresh Juice + Gelatin	
Heated Juice + Gelatin	
Canned Juice + Gelatin	
Water + Gelatin	

Table 3: The Effect of pH on the Rate of Bromelain Activity

Tube #	pH	Reaction Time (minutes)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

Note: If the film in the control tubes (#2,4,6,8,10) does **not** clear at the same time as the film in the experimental tubes (#1,3,5,7,9), then record “No Reaction” in the column labeled “Reaction Time”.

Appendix B: Quantities of Materials and Preparation Guide

The lab rooms are equipped with 6 moveable lab benches having 4 lab stations/table for a total of 24 students per lab.

Pineapples

1-2 pineapples are used per section + extra for demonstration
Purchase pineapples as needed and keep in refrigerators. Do not store large quantities of pineapples over a long period of time.

Pineapple Juice Extraction

Fruit Preparation

- Cut the pineapple into thin slices
- Cut off the "rind" of the pineapple
- Cut the peeled slices into wedges which will fit into the extractor

Juice Preparation

- Turn on the electric juice extractor and place the pineapple wedges into the **food opening** and place a 800 mL beaker to collect the juice.
- Place one layer of cheese cloth over the mouth of a 600 mL beaker and secure it with a rubber band. Pour the unfiltered juice through the cheese cloth being careful to keep as much foam out of the juice extract as possible.
- *Dilute the filtered juice 50% with distilled water. All experiments use diluted juice.*
    - Measure 250 mL of undiluted juice in a 500 mL graduated cylinder. Pour the 250 mL of pure juice into a re-Re-pipet container labeled "Pineapple Juice".
    - Add 250 mL of *distilled water* to the juice in the Re-pipet container. *Swirl the diluted juice to make sure that it is completely mixed.*
- Swirl the pineapple juice periodically during the lab period in order to maintain a uniform concentration of enzyme.
- Do not leave the beaker containing the *undiluted* juice where students might mistakenly use it for their experiments.
- About 400 mL of 50% juice (150 mL for Part I, 12 mL for Part II and 180 mL for Part III) are required for 24 students. One pineapple generally will yield this amount of juice.

Preparing, Developing, and Cutting Film into Strips

Sheet Film

- Kodak, Kodalith Ortho Film, Type 3, 6556, 8" x 10" (Cat. # 110 4280)
    Vendor
    National Graphic Supply
    226 North Allen St.
    Albany, New York 12206
    518-438-8411
    800-223-7130

Film Developing: (wear latex gloves when handling and processing the film)

- Make 3.8 liters of Kodak Dektol Developer as per package instructions.
- Remove several sheets of film from the package and expose them to light by placing them purple side up under room lights.
- Place two sheets of exposed film into a photographic tray containing the developer. Agitate it for approximately 2 minutes. Change the developer after every 50 sheets of film developed.
- Rinse two sheets at a time in a tray filled with tap water. Agitate the film for 2-3 minutes.
- Remove the film from the first wash and place it into a second tray of tap water. Agitate the film for 2-3 minutes.
- Remove the film from the second wash and place it into a third tray of tap water. Agitate the film for 2-3 minutes.
- Continue to sequentially rinse the sheets of film two at a time until all sheets have been rinsed.
- Wash the film in an automatic film washer for approximately 30 minutes.
- Rinse the washed film with distilled water.
- Hang each piece of film from a clothespin attached to a clothesline and dry for 12 hours. The sheets of film will initially roll up but will eventually unroll as they dry.

Cutting Developed Film into Strips:

- After the sheets have dried, cut them into 10 x 12.5 cm (4 x 5 inches) rectangles. Wear latex gloves when handling the film and film strips to prevent grease from your hands from getting on the film surface.
- Using a template, place colored tape on the top edge and the 12.5 cm line. Using a paper cutter, cut the film so that there are four pieces that have colored tape on them measuring 10 x 12.5 cm. Using a pasta cutter with the fettuccini head, cut the rectangles into small strips. Each strip is 0.7 cm x 12.5 cm . There are approximately 15 strips per 10 x 12.5 cm rectangle.

Developed Photographic Film

- 120 strips minimum per lab section of 24 students
  20 strips / table X 6 tables = 120 strips
  There are approximately 60 strips per sheet (20 x 25 cm) of developed film
  Two full sheets of film per lab plus extras for mishaps

Disposable Culture Tubes:

- 13 x 100 mm disposable glass culture tubes (VWR cat. # = 60825-571)
- 12 tubes/table x 6 tables = 72 test tubes/section

pH Indicator Strips:

- EM Sciences, pH Indicator Strips - Universal- 0 - 14 (VWR Cat. No. EM -9590-3) (100 strips/pack x 6 packs/case = 600 strips/case)
  - 12 strips/table x 6 tables/section = 72 strips/section

Re-pipetJ Jr. Dispensers

- 10 mL syringes and 976 mL containers (Fisher Cat. No. 13-687-59B)

Temperature Experiment

- 1 pineapple juice with syringe set at 5 mL
- 1 pH 3.5 Buffer with syringe set at 5 mL

pH Experiment

- 2 pineapple juice with syringe set at 5 mL
- 2 distilled water with syringe set at 5 mL
- 1 1.0 N HCl with syringe set at 2 mL
- 1 1.0 N NaOH with syringe set at 2 mL
- 1 pH 7 Buffer with syringe set at 2 mL
- 1 pH 5 Buffer with syringe set at 2mL
- 1 pH 3.5 Buffer with syringe set at 2 mL

When setting the amount to be dispensed by the syringe, the *red line* on the stop collar pointer must be in the downward position. If the red line is in the upward position, the amount dispensed will be incorrect.

Store the Re-pipet syringes at the 10 mL setting to prevent the springs from wearing out. Store the containers with the covers off.

Preparation of Gelatin for Gelation Experiment

Make new gelatin daily.

- In a 250 mL beaker, dissolve 1 packet (7 g) of Knox 7 gelatin in 110 mL hot water.
- Add several drops of red food coloring to desired color intensity.
- Place gelatin solution on a slide warmer in each lab room.

Preparation of Stock Solutions and Buffers for pH Experiment

Solution Quantities

1.0 N HCl and 1.0 N NaOH

4 mL /table x 6 tables = 24 mL of each solution

pH 7 Tris Buffer and pH 5 Acetate Buffer
4 mL /table x 6 tables = 24 mL of each solution

pH 3.5 Formate Buffer
25 mL /table x 6 tables = 150 mL of each solution

Solution Preparation

1.0 N NaOH
Dissolve 40 g NaOH in 500 mL distilled water. Add enough distilled water to make 1 liter.

1.0 N HCl
Add 81.3 mL of concentrated (37% v/v) HCl to 918.7 liters of distilled water. Mix thoroughly. Add enough distilled water to make 1 liter.

pH 7.0 Tris Buffer (3M Tris)
Dissolve 363.3 g Tris Base in about 400 mL distilled water, stirring constantly.
Add 150 mL concentrated HCl (this will cause solution to warm).
Cool the solution to room temperature.
Titrate with concentrated HCl and bring to 1 liter with distilled water.
Cool solution again and recheck the pH.
Titrate with concentrated HCl if necessary to bring to pH 7.

pH 5.0 Acetate Buffer (1M Sodium Acetate)
Dissolve 136.08 g of sodium acetate in 900 mL of distilled water. The pH will be approximately 8.
Add 15 mL concentrated HCl
Titrate with concentrated HCl using a Pasteur pipet until the solution reaches pH 5
Bring the volume to 1 liter with distilled water.

pH 3.5 Formate Buffer
Dissolve 1.36 g of Sodium Formate into 1 liter distilled water. The pH will be approximately 5.9.
Slowly add approximately 1 mL HCl to bring to pH 3.5. Add 3 drops of HCl at a time.

Appendix C: Sources of Purified Bromelain

A crude bromelain extract can be prepared by the following method:

- Add enough ammonium sulfate to the pineapple juice to obtain 0.6 saturation. Stir in the ammonium sulfate slowly.

- Allow the precipitate to form and then centrifuge. Decant the supernatant and vacuum filter the enzyme precipitate until nearly dry. Air-dry the impure enzyme extract. The enzyme extract is not very water-soluble.

Reagent Grade Bromelain from Pineapple Stem: Sigma (Cat. No., B- 4882)