Experiment Objective:

In this experiment, students will learn general principles about several plant enzymes which have important uses in biotechnology.

Storage:
Store entire experiment in the refrigerator.

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.
**Experiment Components**

This experiment is designed for 10 laboratory groups.

A. *Hordeum vulgare* seeds  
B. *Hordeum vulgare* seeds (malted)  
C. *Hordeum vulgare* seeds (roasted)  
D. Concentrated iodine solution/stain  
E. Concentrated reaction buffer (potassium acetate buffer, pH 6.9)  
F. Starch  
G. Amylase enzyme powder

- Petri plates (100 mm)  
- Starch indicator paper  
- 1 ml pipets  
- Graph paper template

**Storage:**  
Store entire experiment in the refrigerator.

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Requirements

- 1 N HCl
- Distilled Water
- Spectrophotometer
- Microscope or Magnifying Lens
- Test tubes (approximately 350 tubes, 13 x 100 mm)
- Test tube racks
- 2 Waterbaths (37°C and 60°C)
- Hot plate
- Ice and ice bucket
- Scalpel or single edge razor blades
- Cutting Board
- Forceps
- Paper Towels
- Tape
- Gloves
- Micropipetor (5-50μl) and tips
- Beakers, flasks and graduated cylinders
- Pipet pumps (for 1 ml pipets)
BACKGROUND INFORMATION

Biochemical Analysis of Plant Enzymes

Biotechnology combines the principles and technology involved in a variety of scientific disciplines including biochemistry, chemistry, cell biology, and genetics. DNA contains the blueprint for all cell proteins which are responsible for information retrieval and processing. Enzymes do most of the catalytic work of biotechnology.

Many plant enzymes have interesting and important biotechnology applications. One which may be familiar is bromelain, the active ingredient in meat tenderizer. This enzyme is a protease, a protein that degrades other proteins. Bromelain is obtained from pineapple and makes your mouth feel irritated when you have eaten too much of the fruit.

The plant-derived enzyme being studied in this experiment is the enzyme amylase. Amylase is a starch degrading enzyme and like bromelain has a biotechnology application. Amylase is one of the enzymes responsible for the conversion of the starch stored in barley seeds to sugar during seed germination. At the industrial level, this is known as malting. Malted grains are allowed to germinate under controlled conditions, then are treated with heat to stop seedling development and dry the malt. In some instances, the grain may be roasted to give the malt a different flavor. This of course is very important to the brewing industry.

In a barley seed, as germination begins, the embryo produces a hormonal signal that induces production of amylase in the aleurone layer of the seed. The embryo and aleurone are identified in Figure 1, depicting a barley seed. With production of amylase by aleurone cells, starch molecules stored in the seed are broken down into simple sugar molecules. This sugar serves as a source of carbon for the developing, pre-photosynthesis embryo.

This experiment consists of two activities. The first part deals with tissue prints which will be performed to investigate amylase activity in seeds that have been treated at different temperatures. The second set of experiments will explore various factors that can influence amylase activity in vitro. Quantitative results can be measured using a spectrophotometer.
EXPERIMENT OBJECTIVE:
In this experiment, students will learn general principles about several plant enzymes which have important uses in biotechnology.

LABORATORY SAFETY
Gloves and goggles should be worn routinely as good laboratory practice. Use extreme caution when working with razor blades.

Student Experimental Procedures

PART I

Quantitative Amylase Enzyme Analysis

1. Label the starch indicator paper as follows:

<table>
<thead>
<tr>
<th></th>
<th>Barley</th>
<th>Malted Barley</th>
<th>Roasted Barley</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Place the starch indicator paper on the lab bench.

2. Take 3 seeds from the treatment "barley". Cut 2 of the seeds longitudinally and make a cross section of the other seed. Gently blot the seeds on a paper towel.

3. Firmly print each seed onto the starch indicator paper in the barley section. Place the cut surface down and press firmly onto the paper surface with a gloved finger or eraser end of a pencil.

4. Remove the printed seed carefully with forceps or the edge of the razor blade. Try to avoid smearing the tissue print. Save the seeds for a later step.

5. Proceed in the same manner to print the seeds from the other two treatments ("malted barley" and "roasted barley"). Make sure you apply the same pressure to the seeds from all treatments. Save the seeds for a later step. The roasted seed is somewhat fragile and may become squashed on the paper. Do not be concerned if this occurs.
Student Experimental Procedures, continued

6. After all seeds have been printed, allow the starch indicator paper to dry thoroughly. During this time, proteins transferred to the paper from the seed will bind to the paper so they are not washed away. Examine the paper with a magnifying lens, dissecting scope, or light microscope at low magnification. Notice the physical imprint on the paper left by the seeds. You should be able to see the structure of the seed in amazing detail.

7. Allow the paper to continue drying for 15-20 minutes.

8. Stain the starch indicator paper by placing it into the dish of iodine stain. Use the forceps to slowly and carefully cover the paper with the stain. Remove the paper from the stain when it has developed an even purple color (soak several seconds).

9. Drain excess stain from the paper and transfer it to a clean, dry paper towel. Gently blot the indicator paper with another paper towel.

10. Use the iodine solution in the dish to stain the seeds from the three treatments. Dip the seeds into the iodine for a few seconds and remove them to a paper towel. Keep the treatments separated.

11. When the tissue prints have dried, examine them to determine the location of the amylase in each seed. The presence of amylase is indicated by the disappearance of starch and the lack of purple color on the starch indicator paper.

12. Compare the patterns of staining between the seeds and the corresponding tissue print on the starch indicator paper.

PART II

Quantitative Amylase Enzyme Analysis

A. Preparation of Standard Curve

1. Dilute enzyme concentrate stock [4 units/ml] as follows:

   4 U/ml = enzyme concentrate stock [4 units/ml]
   3 U/ml = 3 ml of 4 units/ml + 1 ml distilled water
   2 U/ml = 2 ml of 3 units/ml + 1 ml distilled water
   1 U/ml = 1 ml of 2 units/ml + 1 ml distilled water
   0.5 U/ml = 1 ml of 1 unit/ml + 1 ml distilled water
   0.25 U/ml = 1 ml of 0.5 unit/ml + 1 ml distilled water

   Use a new/clean pipet for each dilution, or rinse pipet between dilutions. Save the solutions for Part D.
2. Label 7 tubes as follows: control, 4 U/ml, 3 U/ml, 2 U/ml, 1 U/ml, 0.5 U/ml, and 0.25 U/ml.

3. Mix 3 ml 1% starch solution with 27 ml diluted buffer (E). Add 2 ml of this mixture to each of the 7 tubes. Save the remaining solution for Part D.

4. Label 1 tube blank and add 2 ml of diluted buffer (E).

5. Starting with the smallest dilution (0.25 U/ml), add 50 μl of 0.25 U/ml enzyme solution to the tube labelled 0.25 U/ml. Mix and proceed with the remaining dilutions; do not add enzyme to the blank or the control.

6. Place tubes in a rack or beaker and incubate at 37°C for 5 minutes.

7. After the incubation period is complete, remove all tubes from the waterbath and add 1 ml 1 N HCl to each tube and mix.

8. Add 0.2 ml iodine solution to all tubes and mix.

9. Use the blank tube to blank the instrument and read and record the absorbance of each tube on the spectrophotometer at 620 nm.

10. Graph the data to show the change in absorbance (y-axis) as a function of enzyme concentration (x-axis).

B. Determination of Specific Activity in Malted Barley

1. Weigh one malted barley seed and record the weight.

2. Crush the seed and place in a 100 ml beaker. Add 50 ml diluted buffer (E) and mix well.

3. Allow the debris to settle completely or transfer several milliliters to a centrifuge tube and spin briefly in a tabletop centrifuge.

4. Remove 1.8 ml of the extract (avoid any seed debris) and transfer to a test tube.

5. Add 0.2 ml of 1% starch, mix and incubate at 37°C for 5 minutes.

6. Stop the reaction by adding 1 ml 1 N HCl and mix.

7. Add 0.2 ml iodine solution and mix.

8. Read and record the absorbance on the spectrophotometer at 620 nm.

Useful Hint!

When adding enzyme from most dilute to most concentrated, it is not necessary to change pipet tips.
9. Determine the unit activity/mg seed. From the standard curve from Part A, locate the absorbance reading (from the seed) on the y-axis and find the corresponding enzyme concentration on the x-axis. Calculate the approximate enzyme activity/mg seed.
   - seed weight = 30 mg
   - dissolved in 50 ml diluted buffer, extract = 0.6 mg seed material/ml
   - $A_{620}$ 1 ml extract + starch = 0.2
   - from standard curve, corresponds to 1 U/ml
   - each mg of seed has approximately 1.7 units of enzyme activity

C. Enzyme Activity Related to Time

1. Crush 1 malted barley seed and place in a 15 ml tube and add 15 ml diluted buffer (E) and mix well.

2. Allow the debris to settle or spin briefly in a tabletop centrifuge.

3. Label 7 tubes as follows: 0, 1, 2, 3, 5, 7, 10.

4. Transfer 1 ml of the fresh extract to each of the 7 tubes.

5. Label 1 tube “blank” and add 1 ml diluted buffer (E).

6. Quickly add 0.1 ml 1% starch to all tubes except the blank and start a timer for 10 minutes, immediately add 1 ml 1 N HCl to the tube labelled 0 and mix. After 1 minute has passed, add 1 ml 1 N HCl to the tube labelled 1 and mix. Continue adding 1 N HCl at the appropriate times until 10 minutes has expired. Add 1 ml N HCl to the blank.

7. After all reactions have been stopped with 1 N HCl, add 0.2 ml iodine solution to each tube and mix.

8. Use the blank tube to blank the instrument, read, and record the absorbance on the spectrophotometer at 620 nm.

9. Graph the data to show the change in absorbance (y-axis) as a function of time (x-axis).

D. Enzyme Activity Related to Temperature

1. Label 8 tubes as follows: C 4°, 4°, C 37°, 37°, C 60°, 60°, C 100°, 100°. (C represents the control tube for each temperature.)

2. Add 1 ml of the starch/buffer solution from Part A, Step 3 to each of the 8 tubes.
EXPERIMENTAL PROCEDURES

Student Experimental Procedures, continued

3. Add 50 μl of 1 unit/ml (from Part A, Step 1) to the tubes labelled 4°, 37°, 60°, and 100°. Do not add the enzyme to the tubes labelled with C (control).

4. Mix the tubes and transfer to the appropriate temperature station and incubate for 5 minutes.

5. After 5 minutes, add 50 μl of 1 unit/ml enzyme to the tubes labelled control and immediately add 1 ml 1 N HCl to all tubes and mix.

6. Add 0.2 ml iodine solution to all tubes and mix.

7. Use a blank (1 ml buffer, 1 ml 1 N HCl, 0.2 ml iodine) to blank the spectrophotometer. Read and record the absorbance at 620 nm.

8. Graph the data to show the change in absorbance (y-axis) as a function of temperature (x-axis).

Study Questions

PART I

Qualitative Amylase Enzyme Analysis

1. Compare the patterns of stain between the different types of seeds. Which type exhibited the most amylase enzyme activity?

2. What does the amylase do to the starch in the indicator paper?

PART II

Quantitative Amylase Enzyme Analysis

1. Is greater amylase activity correlated with more or less purple/brownish color in the test tube?

2. At which temperature is the enzyme most active?

3. What is the purpose of a no enzyme control in the experiment?
Pre-Lab Preparations

PART I

Qualitative Amylase Enzyme Analysis

15 minutes before the lab:

1. Label 3 beakers "barley", "malted barley", and "roasted barley".

2. Add approximately 50 of each of the seeds labeled A, B, and C, to the appropriate beakers. Soak in distilled water for 15 minutes.

3. Mix 20 ml concentrated iodine solution/stain with 180 ml distilled water.

For Part I, each student group requires:

- 3 seeds from each treatment (A, B, and C)
- Single edge razor blade
- 1 pair of forceps
- 1 sheet of starch indicator paper
- Paper towels
- 20 ml diluted iodine stain in petri plate (D)
- Gloves

PART II

Quantitative Amylase Enzyme Analysis

On the day of the lab:

1. Dilute the concentrated reaction buffer (E) by adding 100 ml of concentrated buffer to 900 ml of distilled water.

2. Prepare the 1% starch solution by adding 1 g starch (F) for a final volume of 100 ml with distilled water. Stir and gently heat the solution to dissolve the starch. Refrigerate until ready to use.

3. Prepare 1 N HCl by adding 25 ml concentrated HCl (12 N) to 275 ml distilled water. Stir and label "Caution! 1 N HCl".

4. Set up temperature stations: 0-4°C = bucket of crushed ice or refrigerator; 37°C and 60°C = waterbaths; and 100°C = boiling waterbath. Use a test tube rack or place beakers in waterbaths to hold tubes.
5. Mix 50 ml concentrated iodine solution/stain with 50 ml distilled water.

Within 30 minutes of lab:

6. Dilute amylase enzyme powder (G) by adding enzyme to 100 ml distilled water. The concentration is 4 units/ml.

For Part II, each student group requires:

- Test tubes (35 - 13 X 100 mm)
- 100 ml diluted reaction buffer (E)
- 10 ml 1% starch solution (F)
- 10 ml amylase enzyme concentrate [4 units/ml] (G)
- 30 ml 1 N HCl
- 8 ml diluted iodine solution (D)
- 50 and 100 ml beakers
- 1 test tube rack
- 1 50 ml or 100 ml graduated cylinder
- 10 1 ml pipets and pump
- 1 micropipetor (5-50 μl) and tips
- 3-4 dry malted barley seeds (B)
Expected Results

PART I

Qualitative Amylase Enzyme Analysis

The presence of amylase enzyme is indicated by a clearing zone on the starch indicator paper within or around the area of the seed print. The roasted seed should give the most amylase activity, followed by the malted seed. You should see some amylase activity in the untreated barley seed, mostly near the aleurone section of the seed.

PART II

Quantitative Amylase Enzyme Analysis

Results may vary widely from the examples listed below.

A. Preparation of Standard Curve
Expected Results, continued

C. Enzyme Activity Related to Time

D. Enzyme Activity Related to Temperature
Please refer to the kit insert for the Answers to Study Questions