

## **Measuring Amylase activity in Cereal Grains**

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Cereal grains produce amylase enzymes during germination and early seedling growth. These amylases catalyze the breakdown of starch stored in the endosperm to produce maltose. Maltose can then be further broken down into glucose, which is used for the growth of the emerging embryo. You will use amylase from germinating wheat grains to study the enzyme kinetics of amylase, how amylase activity changes during and after germination and how factors such as pH affect enzyme activity.

For this assay we will not use purified amylase, but a crude extract from wheat grains that will contain many other compounds in addition to amylase. It is essential to keep the grain extract cold at all times to prevent degradation of the amylase by proteases.

In order to assay for the activity of an enzyme one must be able to quantitatively measure either the disappearance of the substrate or the appearance of the reaction product(s). In this experiment we will measure the disappearance of the substrate, starch, as this can be easily done using a spectrophotometer.

### **Preparation of wheat grain extracts**

Place 10 wheat grains (imbibed for 2 days) in a mortar on ice. Measure out 25 ml of cold 10 mM citric acid-sodium citrate buffer (pH 5). Add a few ml of the buffer to the grains and grind them thoroughly with a pestle. Add more buffer as you go, using about 15 ml total.

Transfer the homogenate to a 50 ml centrifuge tube, rinse out the mortar with the last 10 ml of buffer, and add this to the 50 ml tube.

Centrifuge your sample at 15,000 x g for 10 minutes at 4°. This will pellet starch grains, cell walls, mitochondria, and nuclei, leaving soluble proteins such as amylase in the supernatant.

Carefully pour the supernatant into a 50 ml tube and store it on ice at all times.

### **Basic assay for enzyme activity**

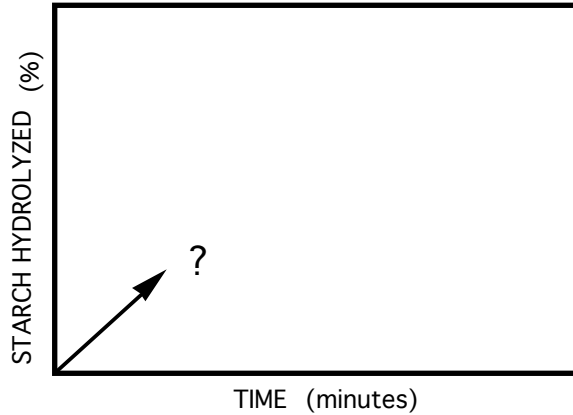
Label 24 test tubes for triplicates of the following time periods: 0, 0.5, 1, 2, 3, 4, 5, and 10 minutes. Add 1 ml of soluble starch (0.5 mg/ml) in pH 5 buffer to each. Label 1 more tube as a blank and add 1 ml of water to it.

Add 3.5 ml of 1 N HCl into the 0 time treatments and to the blank tube before adding the seed extract. For each of the other tubes, note the starting time and add 0.5 ml of the grain extract. Let the reaction run for the designated time period, then stop the reaction by adding 3.5 ml of 1 N HCl and mixing.

Add 0.5 ml of the iodine solution to each of the stopped reactions. The iodine solution develops a blue color when mixed with starch. The amount of light absorbed by the solution (at 580 nm) will be proportional to the amount of starch present. Keep in mind that the amount of starch that has been hydrolyzed (which is what you want to know) will be the amount of starch that has been lost from each tube over the course of the assay.

Set the spectrophotometer at 580 nm and use your blank tube to set the zero point. Measure and *record the absorbance of each sample.*

*Prepare a graph of the % starch lost over time.*



*Calculate the amount of amylase activity (amount of starch hydrolyzed  $s^{-1}$  grain $^{-1}$ ) that was present in your seed extract.*

*For how long did you continue to observe a linear relationship between the amount of starch hydrolyzed and the assay time. Use a reaction time within this linear range for the remaining assays.*

### **Effect of pH on enzyme activity**

Label 18 test tubes making triplicates for each of the following pHs: 3, 4, 5, 6, 7, and 8. Add 1 ml of starch solution at the correct pH to the appropriate test tubes. Add 0.5 ml of your grain extract. Let the reaction proceed for the appropriate time (see above). Stop the reaction by adding 3.5 ml of 1 N HCl to each tube and mixing.

Add 0.5 ml of the iodine solution to each of the stopped reactions. Measure the absorbance of each sample at 580 nm. You will then need to subtract this value from the absorbance of a tube incubated for 0 minutes to determine the amount of starch that was actually hydrolyzed.

*Graph the amount of starch hydrolyzed versus pH. What was the optimal pH for amylase activity? Why do you think that pH might have an effect on the activity of enzymes?*

### **Amylase activity during germination and early seedling growth**

Extracts have been prepared from wheat grains that were imbibed for 0, 1, or 2 days. Assay the amount of amylase activity in each of these extracts (in triplicate). Use the reaction time and the pH that you have determined to be optimal in the previous experiments.

*Graph the amount of amylase activity in imbibing grains versus time. What sort of pattern do you see in the amount of amylase activity during grain germination and early seedling growth? Can you explain why such a pattern might exist?*

**10 mm citric acid/sodium citrate buffer (pH 5)**

citric acid (monohydrate)                      2.1 g  
add about 800 mL water

adjust pH to 5.0 with NaOH

add water a total volume of 1 liter

**50 mm citric acid/sodium citrate buffer**

citric acid (monohydrate)                      10.5 g  
add about 800 mL water

adjust pH to 3, 4, 5, 6, 7, or 8 with NaOH

add water a total volume of 1 liter

**Iodine solution**

KI        5.0 g  
KIO<sub>3</sub>    0.36 g

dissolve in 1 liter of 2 mM NaOH