Unit: Cell and Molecular Biology (AH): Molecular interactions in cell events: Catalysis
Title: Investigating the effect of competitive and non-competitive inhibitors on the enzyme β-galactosidase

TEACHER/LECTURER GUIDE

Type and purpose of activity

This experiment can be used to:
- provide evidence for assessment of Outcome 3
  (For advice on marking Outcome 3 report, please contact the SAPS Scotland office.)
- develop knowledge and understanding of the effect of competitive and non-competitive inhibitors on enzyme activity
- develop problem solving skills and in particular Outcome 2 PCs:
  (c) conclusions drawn are valid and explanations given are supported by evidence
  (d) experimental procedures are planned, designed and evaluated appropriately.

Background information

The enzyme β-galactosidase catalyses the following reaction:

$$\text{LACTOSE} \xrightarrow{\beta\text{-galactosidase}} \text{GLUCOSE} + \text{GALACTOSE}$$

The chemical ONPG (o-nitrophenyl β-D-galactopyranoside) is also degraded by the enzyme:

$$\text{ONPG} \xrightarrow{\beta\text{-galactosidase}} \text{ONP} + \text{GALACTOSE}$$

The ONP produced is yellow, allowing the rate of this reaction to be followed colorimetrically.

Galactose acts as a competitive inhibitor, competing with ONPG for the active site of the enzyme. At a sufficiently high concentration, it will inhibit the reaction by preventing ONPG making contact with the active site. The enzyme, however, is still capable of activity. Thus, when the ONPG concentration is increased it will eventually overcome the inhibition.

Iodine solution on the other hand is a non-competitive inhibitor. When it combines with the enzyme the shape of the active site is altered sufficiently to prevent the substrate combining with it. Increasing substrate concentration will therefore not overcome the inhibition.

Classroom management

Students can work individually or in pairs for this experiment.

If there are several groups of pupils requiring to use the colorimeter, a rotation system could perhaps be employed i.e. each group could start the reaction (by adding the enzyme) 20-30 seconds apart. The colorimeter would just require to be zeroed once for each ‘run’. In this way 4-6 groups could carry out the experiment at about the same time.

Estimated time: 50-60 minutes should be sufficient to collect all the data.

The enzyme solution MUST be kept in crushed ice. If allowed to reach room temperature its activity will rapidly decrease.

Supply of materials

In order to satisfy the core skill in problem solving, students will be required to identify and obtain resources required for themselves. Further advice on supply of material is given in the Technical Guide.

Extension work

Substitute galactose with glucose (the other product of the reaction) to see if it has a similar effect on enzyme activity.

Investigate the rates of reaction in the above experiment by regularly measuring absorbance/transmission over 5-6 minutes.

Investigate the nature of the inhibition using the enzyme phosphatase and the inhibitors phosphate and iodine

The rate of reaction (V_0) at low substrate concentrations can be calculated. If $1/V_0$ is plotted against 1/[substrate] then the maximum velocity and the Michaelis constant for the reaction can be calculated. See Hames reference on enzyme kinetics (or any good biochemistry textbook).

References

Adds, Larkcom and Miller (eds.), (1996) *Cell Biology and Genetics*, Nelson Advanced Modular Science, ISBN 0–17–448266–3

Hames, B.D., Hooper, N.M. and Houghton, J.D. (1997) *Instant Notes in Biochemistry*, Bios Scientific.

Russo, S. E. and Moothart, L. (1986) Kinetic study of the enzyme lactase. *Journal of Chemical Education*, **63**(3), 242–243.

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TECHNICAL GUIDE

Materials required

Materials required by each student/group:

6 cuvettes (or test tubes if suitable colorimeter is used)
2 boiling tubes
beaker of crushed ice
6 x 1 cm^3 droppers
10 cm^3 syringe
6 cm^3 ONPG stock solution ($3x10^{-2}$ M in buffer)
40 cm^3 buffer (0.1 M potassium phosphate, pH 8)
15 cm^3 20% galactose in buffer
5 cm^3 I_2/KI solution in buffer
25 cm^3 distilled water
eye protection
gloves

Materials to be shared:

colorimeter (420-440 nm filter)
1 cm^3 dropper
distilled water
β-galactosidase stock solution

Preparation of materials

The buffer: 0.1 M K_2HPO_4 adjusted to pH 8 with 0.5 M HCl. Each student/group will require 80-100 cm^3. About half the volume made up will remain as plain buffer. The rest will be used to make up other solutions.
CARE!: Avoid direct skin and eye contact, wear eye protection and gloves.

ONPG stock solution: $3x10^{-2}$ M in buffer. Each student/group will require 6 cm^3. For every 10 cm^3 required, weigh out 0.09 g and dissolve in 10 cm^3 buffer. Shaking for 5-10 minutes will be required for the powder to be completely dissolved. The ONPG stock solution is best made up fresh (or no more than 2 days in advance and stored in the fridge).
ONPG available from Sigma Aldrich, Fancy Road, Poole, Dorset BH12 4QH. Catalogue no. N1127, 1g for £9.70 1g for £14.80 (2005 price)

Galactose solution: 20% in buffer. Each student/group will require 10-15 cm^3. To make up 50 cm^3, dissolve 10 g galactose in 50 cm^3 buffer. It dissolves readily.

I_2/KI solution: Each student/group will require about 5 cm^3. Dissolve 0.3 g iodine and 1.5 g potassium iodide in 100 cm^3 water to make a stock solution (this will keep for months stored in a dark glass bottle). Take 1 cm^3 of this stock solution and make up to 80 cm^3 with buffer. This diluted I_2/KI solution is the solution to be used by the students in the experiment.
CARE!: Iodine is classified as HARMFUL. Wear gloves when preparing the solution.
N.B. The diluted I_2/KI solution MUST be made up IMMEDIATELY before the experiment is carried out (it will remain effective as an inhibitor for 1 hour).

β-galactosidase is available as 'Lactozym' from NCBE, Science and Technology Centre, Earley Gate, University of Reading, Whiteknights, READING, RG6 6BZ. Tel: 0118 987 3743. Cost £12.50 (2005 price) for 100 cm^3.
CARE! Avoid direct skin and eye contact, wear eye protection and gloves. Enzyme powder can cause allergies. Do not allow any spillages to dry up. Wipe up spillages immediately and rinse cloth thoroughly with water.

For guidance on sources of colorimeters see SSERC Bulletin No. 208, Spring 2003, pages 208-209.

Supply of materials
It is not appropriate to provide all equipment and materials in, for example, a tray system for each student/group. Equipment and materials should be supplied in a way that students have to identify and obtain resources. Normal laboratory apparatus should not be made available in kits but should generally be available in the laboratory. Trays could be provided containing one type of specialist equipment or materials.

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PREPARING FOR THE ACTIVITY

Read through the Student Activity Guide and consider the following questions.

Analysis of activity

- What is the aim of the activity?
- What is being varied in the activity?
- What variables must be kept constant?
- What measurements are you going to make?
- Why should the enzyme activity be measured without either inhibitor both at the beginning and at the end of the experiment?

Getting organised for experimental work

- What safety measures are you required to take?
- In your group decide how the activity will be managed by allocating tasks to each member. For Outcome 3 it is important that you play an active part in setting up the experiment and in collecting results.

Recording of data

- Prepare tables to record your group results.
- You should use a ruler, correct headings and appropriate units.

Evaluation

- Has the activity of the enzyme remained about constant for the duration of the experiment?
- Cross-contamination will seriously affect the results. Have sufficient measures been taken to avoid cross-contamination?
- Why is it more difficult to completely inhibit the enzyme with galactose than with iodine solution?
- Is the range of ONPG concentrations used suitable to show clearly if the inhibitor is competitive or non-competitive?

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STUDENT ACTIVITY GUIDE

Introduction

Inhibitors are substances that reduce the activity of enzymes.

When the inhibitor binds reversibly to the active site of the enzyme it is known as a COMPETITIVE INHIBITOR. Often a competitive inhibitor is a similar shape to the substrate. Its association with the active site of the enzyme reduces the rate of binding between the substrate and the enzyme, thus lowering the rate of reaction. However, this type of inhibition can be overcome by increasing the substrate concentration as this will decrease the chances of enzyme and inhibitor binding.

When a NON-COMPETITIVE INHIBITOR combines with an enzyme, the active site may still be free. When it combines with the enzyme the shape of the active site is altered sufficiently to prevent the substrate combining with it. Increasing substrate concentration will therefore not overcome the inhibition.

In this experiment you will use the enzyme β-galactosidase. Its normal substrate is lactose but you will use a synthetic substrate, ONPG. When the enzyme is active, it breaks down the ONPG to a yellow substance. Thus, the rate of reaction is proportional to the intensity of the yellow colour formed.

$$\text{ONPG} \xrightarrow{\beta\text{-galactosidase}} \underset{\text{(ONP)}}{\text{YELLOW SUBSTANCE}} + \text{GALACTOSE}$$

The reaction will firstly be carried out without an inhibitor, using a low concentration of substrate. An inhibitor will then be used at a concentration that prevents this enzyme/substrate mixture from reacting. While keeping the inhibitor concentration constant, the substrate concentration will be gradually increased. If the inhibition is overcome by this action, the inhibitor is competitive. If the inhibition is unaffected, the inhibitor is non-competitive.

Equipment and materials

Materials required by each student/group:

6 cuvettes (or test tubes if suitable colorimeter is used)
2 boiling tubes
beaker of crushed ice
6 x 1 cm^3 droppers
10 cm^3 syringe
6 cm^3 ONPG stock solution
40 cm^3 buffer pH 8
15 cm^3 20% galactose
5 cm^3 I_2/KI solution
25 cm^3 distilled water
eye protection
gloves

Materials to be shared:

colorimeter (420-440 nm filter)
1 cm^3 dropper
distilled water
β-galactosidase stock solution

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Instructions

CARE! Wear eye protection and gloves throughout this experiment to avoid direct skin and eye contact with some of the chemicals used.

1. Put 20 cm^3 of distilled water in a boiling tube. Surround the tube with crushed ice and add 4 drops of β-galactosidase stock solution.

   This diluted enzyme is the solution you will use throughout the experiment. Do NOT allow it to reach room temperature as this will reduce the enzyme's activity considerably. Ensure the stock β-galactosidase is returned to the refrigerator as soon as possible.
   CARE!: Enzyme powder can cause allergies. Do not allow any spillages to dry up. Wipe up spillages immediately and rinse cloth thoroughly with water.

2. In a boiling tube mix 0.5 cm^3 of the stock ONPG solution with 9.5 cm^3 of 0.1 M buffer (pH 8). Label x20 dilution.

3. Put 2 cm^3 of buffer and 1 cm^3 of this x20 diluted ONPG solution into a cuvette. Mix by inverting the cuvette 2-3 times. Zero the colorimeter with this solution.

4. Add 0.5 cm^3 of the diluted enzyme to the cuvette. Start the stopclock and invert the cuvette 2-3 times.

5. Read the absorbance/transmission two minutes after adding the enzyme. This should be between 0.3 and 0.5 absorbance units (50-32% transmission).

   If the absorbance is above 0.5 units, dilute the enzyme solution with distilled water and repeat steps 2-4 until an appropriate absorbance is obtained after 2 minutes. If the absorbance is below 0.3 units, add 1-2 drops of the stock β-galactosidase to your diluted enzyme.

You are now going to investigate:
(i) the effect of galactose (an inhibitor) on the activity of the enzyme
(ii) the effect of increasing the ONPG concentration (the substrate) in the presence of galactose

6. Mix the solutions, as shown in the following table, in different cuvettes.

cuvette no.	20% galactose (cm^3)	ONPG stock solution (cm^3)	buffer pH 8 (cm^3)	* ONPG x20 dilution (cm^3)
1	2	-	-	1.0
2	2	0.25	0.75	-
3	2	0.5	0.5	-
4	2	0.75	0.25	-
5	2	1.0	0	-

* Note - the volume of ONPG stock solution in the x20 dilution is 0.05 cm^3

7. Treat each cuvette in turn as follows:
   Invert 2-3 times, put in colorimeter and zero the instrument (CARE! If you are sharing the colorimeter with other groups, only the first group should zero it for each 'run').
   Add 0.5 cm^3 of the diluted enzyme solution. Start the stopclock and invert cuvette 2-3 times.
   Take an absorbance/transmission reading 2 minutes after adding the enzyme.
   Rinse out the cuvettes several times with water and dry.

You are now going to investigate:
(i) the effect of iodine solution (another inhibitor) on the activity of the enzyme
(ii) the effect of increasing the ONPG concentration in the presence of the iodine solution
CARE! Iodine is HARMFUL. Wear gloves and eye protection.

8. Again, using the following table as a guide, mix the solutions in different cuvettes.

cuvette no.	I_2/KI solution (cm^3)	ONPG stock solution (cm^3)	Buffer pH 8 (cm^3)	ONPG x20 dilution (cm^3)
1	1.0	-	1.0	1.0
2	1.0	0.5	1.5	-
3	1.0	1.0	1.0	-

9. Treat each cuvette in turn as follows:
Invert 2-3 times, put in colorimeter and zero the instrument (CARE! If you are sharing the colorimeter with other groups, only the first group should zero it for each 'run').
Add 0.5 cm^3 of the diluted enzyme. Start the stopclock and invert cuvette 2-3 times.
Take an absorbance/transmission reading 2 minutes after adding the enzyme.
Rinse out the cuvettes several times with water and dry.

10. To ensure that enzyme activity has remained constant, repeat steps 3-5. These results should be similar to the ones obtained initially.

11. Present your results in a table with suitable headings. Draw a graph of volume of stock ONPG added against absorption/transmission after two minutes (i) in the presence of galactose (ii) in the presence of iodine solution.