Unit: Cell and Molecular Biology (AH): Molecular interactions in cell events: Catalysis
Title: The effect of end product, phosphate, on the enzyme phosphatase

TEACHER/LECTURER GUIDE

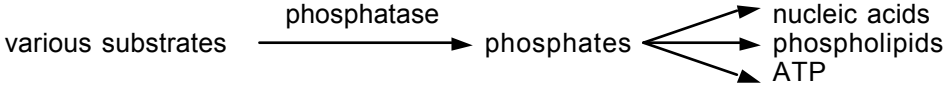
Type and purpose of activity

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 one possible effect of a product on the activity of the enzyme that produced it
- 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

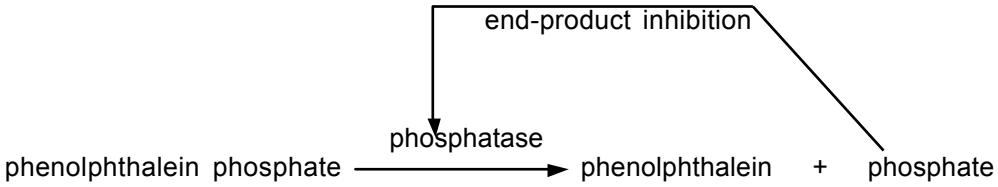
Phosphatases are a group of enzymes which release phosphate groups for cell metabolism. The resulting phosphates can then be used for a variety of purposes e.g. incorporation into the nucleotides of DNA and RNA, to make phospholipids - a component of cell membranes or to make energy rich ATP.



Phosphatases are thus key enzymes in cell metabolism.

There are two main groups of phosphatases, acid or alkaline depending on their optimum pH. This experiment involves an acid phosphatase extracted from germinating mung beans (beansprouts). The enzyme is also found in potatoes, tomato leaves, wheatgerm and in the seeds of many legumes.

A simple aqueous extract, derived from beansprouts is used as the enzyme solution. An artificial substrate, phenolphthalein phosphate is used. It is colourless in acid pH. Increasing concentrations of a phosphate salt are added to a series of test tubes containing the enzyme and the substrate. The phosphate salt, being an end-product, also inhibits the enzyme. This experiment will demonstrate that the higher the concentration of phosphate present the greater the inhibition of the enzyme.



Any free phenolphthalein released can be estimated by the addition of alkali. The alkali does two things:

(i) it stops the reaction by denaturing the phosphatase
(ii) free phenolphthalein turns pink in an alkaline pH.

The intensity of the pink colour produced can be measured quantitatively using a colorimeter.

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Classroom management

Students can work individually or in pairs for this experiment.

The Student Activity Guide asks students to calculate the mass of sodium phosphate to add to achieve certain molarities. Although not required for purposes of assessment it was thought to be a useful and not too difficult task for students to undertake. However, if the class teacher so desires, the students or technicians can be given the masses, by-passing this step in the experiment.

beaker number	molarity of sodium phosphate (NaH_2PO_4) added to buffer	mass of sodium phosphate (NaH_2PO_4) added to buffer /100 cm^3
1	0	0
2	0.05 M	0.69 g
3	0.10 M	1.38 g
4	0.20 M	2.76 g
5	0.30 M	4.14 g

Estimated times: about one hour including a 20 minute incubation period.

There is no point at which it is suitable to leave the experiment overnight. However, any colour formed on addition of the sodium carbonate solution is stable. It is therefore possible to store the coloured tubes in a refrigerator overnight and read the % transmission/absorbance the following day.

If the school does not have a suitable centrifuge this step can be missed out. Instead, filter the beansprout extract through a double layer of muslin. If the tubes are pale pink, colorimeter readings may be unreliable. Students could refer to this discrepancy in the evaluation part of their report.

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

Compare phosphatase activity (i) in plant material as it matures
(ii) in different parts of the plant material
(iii) in different plants.
Investigate the properties of the enzyme by varying temperature, pH and substrate concentration.
Investigate the rate of reaction by varying the times at which the reaction is stopped.
Investigate the inhibiting effect of other phosphates or of a general enzyme inhibitor such as a lead salt.

References

Meatyard, B.(1999) Phosphatase enzymes from plants., *Journal of Biological Education,* **33**(2), 109–112.

Larkcom, J. (1991) *Oriental Vegetables* 60-61. John Murray ISBN 0-7195-4781-4

This practical was based on work initially carried out by Dr Barry Meatyard, SAPS, Warwick Institute of Education, Warwick University, Coventry CV4 7AL.

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TECHNICAL GUIDE

Materials required

Materials required by each student/group:

mortar and pestle
filter funnel
piece of muslin (approx. 12 cm x 12 cm)
centrifuge tube
test tube
5 boiling tubes with rack
marker pen
2 x 5 cm^3 or 10 cm^3 syringe
2 x 1 cm^3 syringes or pipettes
stirring rod
at least 5 cm^3 1% phenolphthalein phosphate solution
beaker containing at least 25 cm^3 10% sodium carbonate solution
gloves and eye protection

Materials to be shared:

water bath at 30°C
at least 500 cm^3 buffer solution pH 5
5 beakers each with an accurate 100 cm^3 graduation mark
spatula
balance
weighing boats/filter paper
Sodium phosphate (NaH_2PO_4)
5 droppers
500 g packet of beansprouts
bench centrifuge
colorimeter with 550 nm filter and cuvettes or test tubes as appropriate.

Preparation of materials

CARE: Wear gloves when preparing the PPP solution and the buffer.

Phenolphthalein phosphate (PPP) can be obtained from Sigma - catalogue no. P 9875 - 2005 price - £9.20 for 1 g. Care should be taken when weighing as the dust may be hazardous. The 1% solution should be made up just before use or if necessary the day before and stored overnight in the refrigerator. PPP slowly degrades to free PP in solution.

N.B. TWO different sodium phosphates are used in this experiment. The dibasic form (Na_2HPO_4) is used as a component of the background buffer which is added to all tubes. The monobasic form (NaH_2PO_4) is added in varying amounts to the background buffer. The monobasic form is used to inhibit the enzyme.

Sodium phosphate (NaH_2PO_4)(the enzyme inhibitor) can be obtained from Sigma - catalogue no. S 9638 - 2005 prices - £12.50 for 250 g. Although other phosphates e.g.Na_3PO_4, Na_2HPO_4, K_2HPO_4 will also inhibit phosphatase they will tend to change the pH considerably when added to the buffer. Such changes in pH must be rectified by adding a few drops of 5 M hydrochloric acid while checking the solution with a pH meter. Using NaH_2PO_4 as the inhibitor avoids readjusting the pH as even at the highest concentration used (0.3 M) the pH will vary as little as 0.1 - 0.2.

To make up 100 cm^3 of buffer add 51.5 cm^3 0.2 M Na_2HPO_4 (the dibasic sodium phosphate) to 48.5 cm^3 0.1 M citric acid. The pH of this mixture should be close to 5.0. Adjust to exactly 5.0 by adding the appropriate solution drop by drop while checking with a pH meter.

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Teachers may wish to get technicians rather than students to add the monobasic sodium phosphate (NaH_2PO_4) to the buffer. If this is the case follow the instructions below:

1. Label 5 beakers 1 - 5.

2. The molarities of sodium phosphate (NaH_2PO_4) to be present in each beaker is shown in the table. Also, shown is the mass of sodium phosphate required in 100 cm^3 of buffer to achieve this molarity.

beaker number	molarity of sodium phosphate (NaH_2PO_4) added to buffer	mass of sodium phosphate (NaH_2PO_4) added to buffer /100 cm^3
1	0	0
2	0.05 M	0.69 g
3	0.10 M	1.38 g
4	0.20 M	2.76 g
5	0.30 M	4.14 g

Weigh out the appropriate mass of sodium phosphate for each molarity. Add it to the beaker along with about 90 cm^3 buffer solution.

3. Stir until it is completely dissolved and then with a dropper carefully add more buffer until the solution reaches the 100 cm^3 mark.

It will usually be more convenient to buy a 500 g packet of beansprouts from a supermarket. The Student Activity Guide has been written assuming that such beansprouts are being used as the source of phosphatase.

Alternatively, soak mung bean seeds in a shallow dish and pour off excess water. Rinse two times each day with fresh water and drain. CARE: Too much water will cause them to rot, too little and they will dry out. Leave in a dark cupboard at about 25°C and harvest after 3 days. When making the enzyme solution from this source 1 cm^3 of water should be added for every seedling used. Most of the water should be added after the seedlings have been crushed using a mortar and pestle.

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

1. What is the aim of the activity?
2. What is being varied in the activity?
3. What variables must be kept constant?
4. What measurements are you going to make?
5. What controls are being used and why?

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.
- You may be asked to calculate the mass of sodium phosphate required to obtain a solution with a certain molarity. If you do not know how to work this out consult the Supplementary Student Information sheet.

Recording of data

Prepare tables to record your group results.

You should use a ruler, correct headings and appropriate units.

Evaluation

The pH of the test tubes you will set up will vary slightly (0.1 - 0.2) due to the different concentrations of sodium phosphate. How could you demonstrate how important this variation in pH is? What could you do to make the pH of all the test tubes exactly the same?

What experimental conditions should be kept constant and why?

How much variation is there between the results of different groups? If this is considerable would it be better to calculate a class average for each test tube before plotting the graph?

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STUDENT ACTIVITY GUIDE

Introduction

Phosphatase enzymes release phosphates from a variety of substrates. These phosphates are required for synthesis of, for example, ATP, phospholipids and nucleotides. They are found in both plant and animal tissues and can be classified as acid or alkaline depending on their optimum pH. This experiment uses an acid phosphatase (optimum pH 5) from germinating mung beans (beansprouts). The same type of enzyme can be found in many other plants including potatoes, legume seeds and tomato leaves.

In this experiment the enzyme is obtained by grinding the beansprouts and collecting the liquid extracted. An artificial substrate phenolphthalein phosphate (PPP) is used. The enzyme and substrate are allowed to react in a buffer solution, the substrate being degraded to phosphate and free phenolphthalein.

$$\text{phenolphthalein phosphate} \xrightarrow{\text{phosphatase}} \text{phenolphthalein} + \text{phosphate}$$

This reaction will be carried out with different molarities of sodium phosphate added to the buffer. As phosphate is a product of phosphatase activity, it may possibly act as an inhibitor of the enzyme. End product inhibition is a type of negative feedback commonly used to control the rate of a metabolic pathway in living things.

After a period of incubation any free phenolphthalein formed can be detected by adding alkali (sodium carbonate) as at this pH phenolphthalein is pink.

$$\underset{\text{phenolphthalein colourless}}{\text{Acid pH}} \xrightarrow{\text{sodium carbonate}} \underset{\text{phenolphthalein pink}}{\text{Alkaline pH}}$$

Thus, the more active the enzyme the more intense the pink colour. The intensity of colour can be measured quantitatively using a colorimeter. The sodium carbonate also denatures the phosphatase and stops the reaction.

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Equipment and materials

Materials required by each student/group:

mortar and pestle
filter funnel
piece of muslin (approx. 12 cm x 12 cm)
centrifuge tube
test tube
5 boiling tubes with rack
marker pen
2 x 5 cm^3 syringes
2 x 1 cm^3 syringes/pipettes
stirring rod
5 cm^3 phenolphthalein phosphate
beaker containing at least 25 cm^3 10% sodium carbonate solution

Materials to be shared:

water bath at 30°C
500 cm^3 pH 5 buffer
5 beakers each with an accurate 100 cm^3 graduation mark
spatula
balance
weighing boats/filter paper
Sodium phosphate (NaH_2PO_4)
5 droppers
500 g packet of beansprouts
bench centrifuge
colorimeter with cuvettes or test tubes as appropriate
550 nm filter for colorimeter
stop clock

CARE: Wear gloves and eye protection whilst carrying out this experiment. The buffer solution, the sodium phosphate and the phenolphthalein phosphate are all possible irritants. If any of these substances come in contact with eyes, wash immediately with plenty of water.

Instructions

N.B. Results from different groups will be averaged. It is therefore important for all groups to carry out the instructions in a similar manner so that other variables are not introduced.

1. Label 5 beakers 1 - 5 and distribute amongst the class.

2. The molarities of sodium phosphate to be present in each beaker is shown in the table. You will have to calculate the mass of sodium phosphate required in 100 cm^3 of buffer to achieve these molarities. The formula weight in grams of the sodium phosphate is 138.0. Help with this calculation is available in the Supplementary Student Information sheet: *Calculating the mass of a chemical required to obtain a solution with a certain molarity*.

beaker number	molarity of sodium phosphate added to buffer
1	0
2	0.05 M
3	0.10 M
4	0.20 M
5	0.30 M

Make out a table showing the mass of sodium phosphate required for 100 cm^3 of each solution.

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Weigh out the appropriate mass of sodium phosphate for each beaker. Add it to the beaker along with about 90 cm^3 buffer solution.

3. Stir until it is completely dissolved and then with a dropper carefully add more buffer until the solution reaches the 100 cm^3 mark.

4. Put about 20 g of beansprouts in a mortar and grind to a paste using the pestle.

5. Filter the liquid through muslin into a clean centrifuge tube.

6. Centrifuge at high speed for about five minutes.

7. Pour the liquid (the supernatant) into a clean test tube being careful not to disturb the pellet. This liquid will be used as the enzyme solution.

8. Collect 5 boiling tubes in a rack and label them 1 - 5. Using a syringe add 5 cm^3 from beaker 1 (containing plain buffer) to tube 1; then using the same syringe add 5 cm^3 from beaker 2 to tube 2 and continue this same procedure step wise to beaker 5.

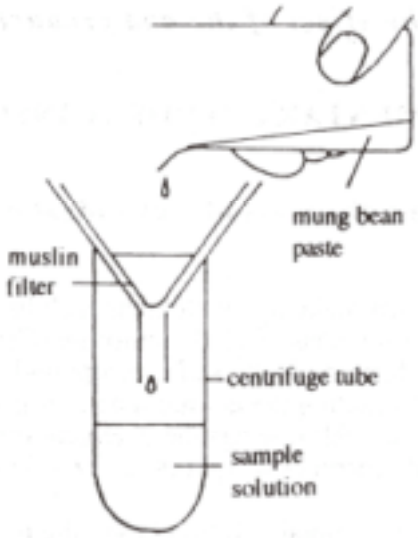
9. Add 1 cm^3 of the substrate, phenolphthalein phosphate to each tube.

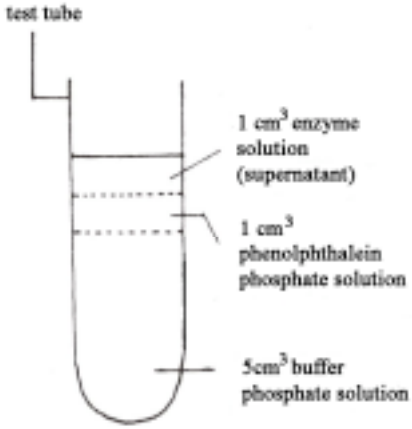
10. Add 1 cm^3 of enzyme solution to each tube and mix well. To avoid serious cross contamination with the stirring rod start at tube 1 and work stepwise to tube 5.

11. Incubate all tubes at 30°C for 20 minutes.

    CARE! Do not incubate for longer. The phosphate is probably a competitive inhibitor. This means that given sufficient time the enzyme will break down all the substrate in all the tubes.

12. Add 5 cm^3 of 10% sodium carbonate solution to each tube and mix as before.
    (Tubes can now be stored in a refrigerator until next day if required)





13. Using water as a blank, measure the intensity of the pink colour using a colorimeter with a 550 nm filter.

14. Present your results in a table with suitable headings. Draw a graph of molarity of sodium phosphate added against transmission or absorbance. Label axes appropriately (a line of best fit is probably most appropriate as a straight line graph is unlikely).

15. Collect results from other groups in the class. They should be very similar! Calculate the average value of transmission or absorption for each tube.

16. Redraw the graph using the average values. For each molarity also plot the highest and lowest values obtained by the class. Draw a vertical line from the highest to the lowest value for each point. This will indicate the range for each point plotted (resuts which differ markedly from the norm should be discussed and on the results of this discussion either be included or ignored).

SUPPLEMENTARY STUDENT INFORMATION

Calculating the mass of a chemical required to obtain a solution with a certain molarity

The atoms that make up an element each have a certain mass e.g. sodium (Na) atoms on average have an atomic mass of 22.99, chlorine (Cl) atoms a mass of 35.44. When different atoms combine to form molecules of a compound, the formula weight of that compound can be calculated by adding the atomic masses together.

e.g. sodium chloride has the chemical formula NaCl
so its formula weight is 22.99 + 35.44 = 58.43

To obtain a one molar solution (1 M), the formula weight in grams is dissolved and made up to one litre with water.

e.g. The formula weight of sodium chloride (common salt) is 58.43
To obtain a 1 M solution of sodium chloride, 58.43 g of it would be dissolved in water and the solution made up to 1 litre.

If you wanted to make just 100 cm^3 of a 1 M solution, 5.84 g of sodium chloride would be in the solution.

If you wanted to make 100 cm^3 of a 0.1 M solution, only 0.58 g of sodium chloride would be required.

How much sodium chloride would you weigh out to make:
(i) 100 cm^3 of a 0.2 M solution?
(ii) 100 cm^3 of a 0.05 M solution?
(iii) 200 cm^3 of a 0.1 M solution? (answers at the bottom of the page)

The chemical you are about to work with is sodium phosphate. Its formula is $NaH_2PO_4.H_20$ and its formula weight is 138.0

How much sodium phosphate would you weigh out to make:
(i) 100 cm^3 of a 1 M solution?
(ii) 100 cm^3 of a 0.1 M solution?
(iii) 100 cm^3 of a 0.05 M solution?
(iv) 100 cm^3 of a 0.2 M solution?
(v) 100 cm^3 of a 0.3 M solution?

Answers to sodium chloride problems (i) 1.16g (ii) 0.29g (iii) 1.16g