INVESTIGATIONS WITH PHOSPHATASE ENZYMES USING THE SAPS MICROSCIENCE COMBOPlate®

Student Guide

INTRODUCTION

Welcome to the exciting world of modern biochemistry! Your enzyme kit lets you explore the properties and activity of enzymes, using phosphatase – a readily available plant enzyme. The kit also introduces you to small-scale science. These ‘semi-micro’ techniques are widely used in modern university teaching and research laboratories, where test tubes are now rare pieces of equipment. The kit provides you with the information, equipment and reagents that you need to be able to carry out your own investigations on this important plant enzyme.

From your studies in biology you know of the importance of enzymes in living systems. All known enzymes are produced in living cells – indeed, without enzymes, living cells could not function. Enzymes are of many different types – they promote both the synthesis and breakdown of biological molecules as well as a range of other molecular processes. Most enzymes operate within a narrow range of environmental conditions – such as pH and temperature. They are sensitive to a variety of substances that modify or inhibit their action, including the products of the reactions they catalyse.

This kit is based on the use of phosphatases, a group of important enzymes and which are readily available from fresh plant material.

PHOSPHATASE ENZYMES

Phosphatase enzymes occur in a wide range of plant and animal tissues. They catalyse the hydrolysis of phosphate bonds in organic phosphates, between the phosphate group and the rest of the molecule. This releases phosphate ions from a variety of phosphate substrates into the metabolic pool.

Phosphate is an essential component of a number of biologically important molecules, including DNA and RNA, phospholipids of cell membranes, ATP and many metabolites. Phosphatases are key enzymes in liberating and recycling the phosphate that is necessary for many fundamental biological processes.

A range of research interests focus on phosphatases. They have, for example, been implicated in such diverse roles as possible allergens in part of the protein coat of pollen grains and as markers of abnormal prostate gland function.

There are two main groups of phosphatases, classified as acid or alkaline, depending on their pH optima. This protocol uses acid phosphatase from plant sources.

MICROSCIENCE TECHNIQUES

When using the kit, you carry out all the reactions in the small wells of the Comboplate®. You will soon realise that it is necessary to measure very small quantities of the reagents. In modern biochemistry laboratories, volumes much less than 1 ml (1 cm³) are frequently used. This is normally done with a micropipettor, a device that accurately measures very small quantities. When using this kit, you need to measure quantities of 20 µl and 100 µl and it is important that you are able to do this accurately.

Appendix 1 describes techniques for micropipetting very small quantities and you should refer to this appendix to make sure you are familiar with how to do this. Then practise doing some micropipetting before you start working with your Comboplate®, to make sure you can do it successfully and with an appropriate degree of accuracy.

*Note: 1 ml = 1 cm³
Assessing phosphatase activity

This protocol uses **phenolphthalein phosphate (PPP)** as an *artificial* substrate. You may already be familiar with phenolphthalein, which is used as an indicator. Phenolphthalein goes from colourless to magenta as the solution becomes more alkaline (pH 8.3 to 10.0). Its phosphate salt (PPP) is colourless in alkaline solution, but when the phosphate is removed, the magenta colour of alkaline phenolphthalein is observed.

In this protocol, PPP is incubated with a phosphatase enzyme to remove the phosphate and release phenolphthalein. Sodium carbonate is added at the end of the incubation to stop the reaction. The sodium carbonate increases the pH to 9.5 and any free phenolphthalein then gives a magenta colour.

\[
\text{PPP} \rightarrow \text{phenolphthalein} \rightarrow \text{phosphate}
\]

The PPP and enzyme are incubated for a given time under appropriate experimental conditions. The intensity of colour produced (after addition of sodium carbonate solution) is proportional to the concentration of phenolphthalein, and so gives a measure of the level of enzyme activity.

**Equipment and materials required**

- Comboplate®, microfuge tubes, glass rod, micropipettor
- buffer solution, PPP substrate
- germinating mung bean seedlings
- microcentrifuge (per class)

This protocol uses germinating mung beans as the source material for the phosphatase enzyme, though you can also try other plant material.

**Before you start, make sure you can use your micropipettor accurately – see Appendix 1.**

### Practical procedures

**Extraction of phosphatase**

First you prepare a simple aqueous extract of the phosphatase enzyme from suitable plant material.

- Place 2 mung beans seedlings in a microfuge tube.
- Add 0.5 ml of water. Macerate the seed with a small glass rod until you have an even, lump free suspension.
- Take a second microfuge tube and add water to the same level as in the first tube. This is to balance the tubes in the centrifuge.
- Place the tubes in opposite holes in the microcentrifuge. Spin (5 min) at the maximum speed. *(If a microcentrifuge is not available let the tubes stand for about 10 min to obtain a reasonably clear solution.)*
- Use the micropipettor to draw off the clear supernatant and transfer it to the large well E1 in the Comboplate®. You will need to do this several times and be careful not to disturb the pellet. This liquid contains the enzyme and will be used later.

**Estimation of phosphatase activity**

Your standard reaction mixture is as follows.

\[
100 \mu l \text{ buffer solution} \\
20 \mu l \text{ PPP substrate} \\
20 \mu l \text{ enzyme solution}
\]

- Use the small wells (row A) in the Comboplate® for your enzyme reactions. Use a different tip for each solution. Add each solution of the reaction mixture to the well, but always add the enzyme last. Mix well, using the handle of the blue microspatula. *(Make sure you use a clean one for each assay.)*
- Incubate the mixture (at room temperature) for 30 minutes, then add 100 µl 10% sodium carbonate.
- Estimate the intensity of colour using the magenta colour comparator card. Do this by matching the colour in the wells with the graded colour shades on the card. Record the colour value and use this to give a semi-quantitative estimate of enzyme activity.

You can then use these values to plot a graph to compare activity of the phosphatase enzyme with different levels of the factor under investigation, such as pH or temperature.
SUGGESTIONS FOR INVESTIGATIONS WITH PHOSPHATASE ENZYMES

When you are familiar with how to use your Comboplate®, and can carry out the basic practical procedures for estimating phosphatase activity, you can then try some investigations yourself with an extract of phosphatase enzyme. Some suggestions are given here and further information is given in the Technical Guide.

These suggestions are intended to be ideas around which you can build your own investigations. It is your responsibility to design the detail of the protocol, based on the guidance given in the previous section. You should plan carefully, decide what controls might be needed and remember to carry out any necessary risk assessment.

Investigating the effect of pH on phosphatase activity

Use a range of buffers from pH 2.2 to pH 9.2. These can be stored in the big wells. You may wish to float the Comboplate® in a shallow water bath as a means of controlling temperature. Incubate your reaction mixtures for a suitable time then estimate the intensity of the magenta colour using the colour comparator card. Plot your results on a graph.

Select your equipment from the Microscience Comboplate® kit supplied. You will need the following:

• A Comboplate® in which the reactions will be carried out
• 2 x 1.5 ml microfuge tubes – one for making the enzyme extract and the second for balancing in the centrifuge
• A glass rod to crush the germinating seeds
• Micropipettor tips as follows: 1 for each pH value used, 1 for dispensing the enzyme solution, 1 for dispensing PPP, 1 for dispensing sodium carbonate solution. If the full range of buffers (8) is used, you will need 11 tips.
• The colour comparator charts

Questions for discussion

• Why does pH affect enzyme activity?
• Why is it necessary to use buffer solutions?

Investigating the effect of temperature on phosphatase activity

Use pH 5.0 buffer throughout this investigation. You could set up water baths in the range 0°C to 80°C and then place your microfuge tubes containing the reaction mixture in ‘floaters’ in the water baths (see Technical Guide). Incubate your reaction mixtures for a suitable time, transfer your reaction mixture to the well of the Comboplate® which already contains an appropriate volume of Na₂CO₃ solution. Estimate the intensity of the magenta colour using the colour comparator card. Plot your results on a graph.

Select your equipment from the Microscience Comboplate® kit supplied. You will need the following:

• A Comboplate® in which the reactions will be carried out
• 2 x 1.5 ml microfuge tubes – one for making the enzyme extract and the second for balancing in the centrifuge
• A glass rod to crush the germinating seeds
• A small microfuge tube for each temperature to be used
• Micropipettor tips as follows: 1 for the pH 5.0 buffer, 1 for each temperature used, 1 for the enzyme solution, 1 for PPP solution and 1 for sodium carbonate solution.
• The colour comparator charts.

Questions for discussion

• Why are enzyme reactions sensitive to temperature? (Think about the kinetic theory and enzyme structure.)
• What do your results tell you about the temperature optimum for mung bean phosphatase? Can you relate your results to the climate in which mung beans grow?
• Critically discuss your method as a way of investigating the effect of temperature on enzyme activity.
APPENDIX 1

Using a micropipettor

The micropipettor tips are graduated in volumes of 10, 20, 50, 100 and 200 \( \mu \)l. Each successive mark represents the volumes shown in the diagram below.

![Micropipette Tip Diagram]

♦ Fit a micropipettor tip to a 1 ml plastic syringe. Make sure that the nozzle of the syringe has an adapter fitted so that it can take the tip.
♦ Important – before you draw any liquid into the micropipettor, pull the plunger up the barrel for a short distance (about 1 cm). This provides a small reservoir of air in the pipette so that you can then expel fully the whole sample.
♦ Then carry out the following exercise.
  • Draw up 100 \( \mu \)l and pipette it into the small well D1.
  • Remove 50 \( \mu \)l from well D1 and place it in well D2.
  • Remove 10 \( \mu \)l from D1 and transfer it into D3. Repeat this twice more.
  • Draw up the contents of D2. Is this volume 50 \( \mu \)l? Discard this liquid.
  • Remove 20 \( \mu \)l from D1 and transfer it to D3.
  • Draw up the contents of D3. Is this volume 50 \( \mu \)l?

When confident about using the micropipettor, you can start doing investigations with phosphatase.

APPENDIX 2

Your Comboplate® map

To help you work systematically with the Comboplate®, you may wish to use and annotate your copy of the ‘map’ of the Comboplate® (see page 2) and then keep to it in all your work with the Comboplate®. Use extra copies of the map so that you can make sure you know what is in each well!

Small wells
Use the small wells (rows A, B, C and D) to carry out the enzyme reactions and any appropriate controls.

- A1 to A12: Use for enzyme reactions
- B1 to B12: Use for enzyme reactions if doing duplicates
- C1 to C12: These rows can be used for controls
- D1 to D12: These rows can also be used for controls – e.g. enzyme / no substrate; substrate / no enzyme

Large wells
Use the large wells (rows E and F) for storage of the reagents you need to use.

- E1: Store enzyme solution
- E2: Store PPP substrate
- E3: Store sodium carbonate solution
- E4 to E6: Store buffer solution
- F1 to F6: Store buffer solution (If using pH 5 buffer only, this could go in F1)

ACKNOWLEDGEMENTS

SAPS wishes to thank the following:

♦ Dr Barry Meatyard (University of Warwick) who adapted the phosphatase enzyme protocol for use in schools and colleges and carried out the development work for the SAPS Microscience Comboplate® kit.

♦ The teachers, lecturers and technicians who trialled the SAPS Microscience Comboplate® kit and provided helpful feedback.