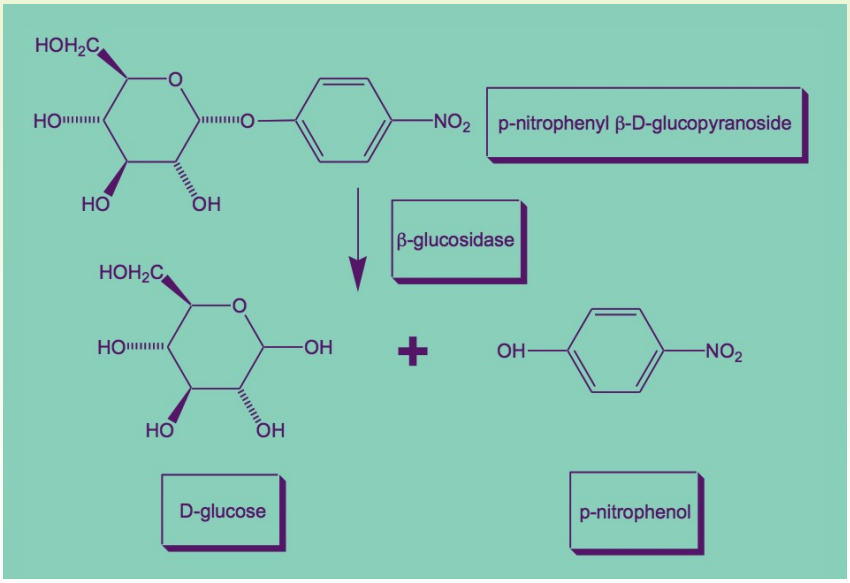
Kinetic studies with

As mentioned in a recent article in the SSERC Bulletin [1] the Biology Team in SSERC is always on the look-out for 'good' enzyme systems to support the new Higher and Advanced Higher qualifications in Biology and Human Biology [2-3].

Robust enzyme assays should ideally meet a number of criteria, for example:

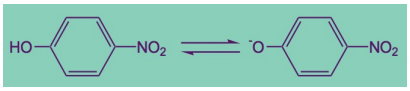
- it should be possible to monitor the change in either product or substrate concentration (preferably product)
- the effects of pH and temperature on enzyme rate should be minimised
- there should be a convenient method for measuring initial reaction rates
- the initial reaction rate should be proportional to enzyme concentration.

Many enzymes meet the above criteria and in this article we explore the merits of β-glucosidase which is found in both prokaryotic and eukaryotic systems and has been shown to play important roles in a variety of biochemical processes. In particular, β-glucosidase is capable of hydrolysing the β-glucosidic linkages of disaccharides, oligosaccharides or conjugated glucosides and it is this property that is utilised in the assay system described here. Typically, both substrates and products of reactions involving β-glucosidase are colourless and so they do not lend themselves to assays involving colorimetric determination.
To overcome this problem the substrate of choice for reactions in the school/college laboratory is likely to be 4-nitrophenyl β-D-glucopyranoside (also called p-nitrophenyl β-D-glucopyranoside or p-nitrophenyl β-D-glucose).



The enzyme reacts with the substrate to produce glucose and p-nitrophenol as shown above.

In solution p-nitrophenol exists in 2 forms *viz* the protonated form (which is colourless) and the anionic form (which has a yellow-green colour); the pK_a for the following equilibrium has been reported to be 7.4:



Consequently in solutions at pH 7.4 equal amounts of the protonated and anionic forms will be present. In order to take advantage of the coloured nature of the anionic form, the enzyme assay described in the following sections is carried out at pH 7.4.

Materials and methods

The enzyme, β-glucosidase, and substrate, p-nitrophenyl β-D-glucopyranoside, can both be obtained from Sigma Aldrich (www.sigmaaldrich.com/).

All solutions should be prepared using a phosphate buffer (0.2 mol dm^{-3}, pH 7.4). Stock solutions of the enzyme (β-glucosidase, 1 mg in 10 cm^3 phosphate buffer) and substrate (p-nitrophenyl β-D-glucose, 2.5 x 10^{-2} mol dm^{-3}, prepared in phosphate buffer) will need to be provided.

The change in colour should be measured at, or close to, 405 nm. Such a choice of wavelength will be problematic for many colorimeters used in schools but it should noted [4]

β-glucosidase

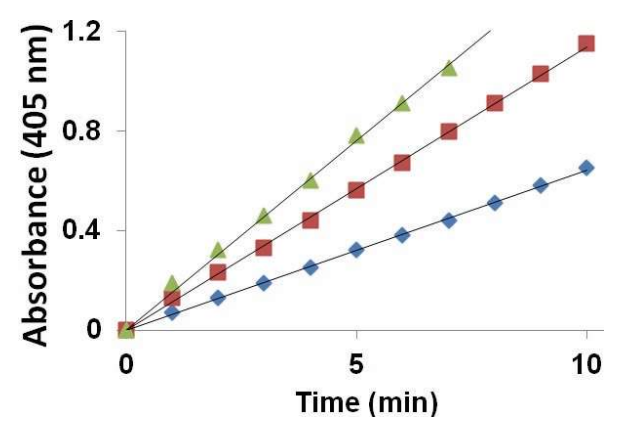


Figure 1 *- Effect of increasing enzyme concentration on the rate of breakdown of p nitrophenyl β-D-glucose by β-glucosidase: ▼ 0.25 cm³ of enzyme stock, ■ 0.5 cm³ of enzyme stock, ▲ 0.75 cm³ of enzyme stock. Further details are given in the text.*

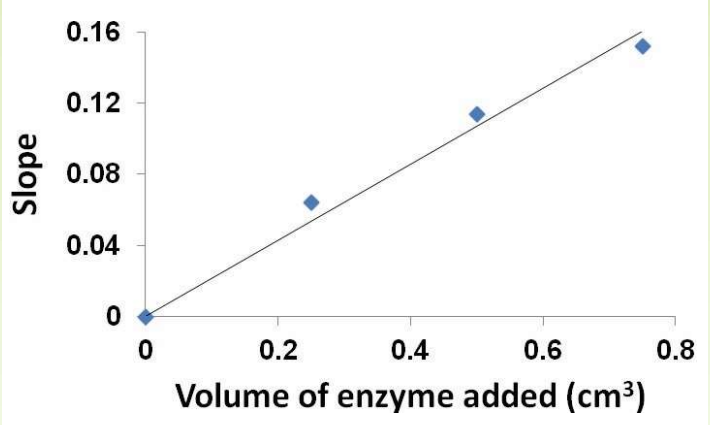


Figure 2 *- Slopes of the plots shown in Figure 1 as a function of added volume of β-glucosidase.*

that during 2014 SSERC was able to provide a spectrophotometer for each local authority and this would be an ideal piece of equipment to use for such experiments.

The experimental protocol

Please note - when dispensing solutions it is important that cross-contamination is avoided!

1) In a **clean** cuvette mix substrate (0.25 cm³) and buffer (2.0 cm³).
2) Place into the colorimeter and set the absorbance at 405 nm to read 0.00. This will be your t = 0 reading.
3) Now add enzyme (β-glucosidase) 0.25 cm³.
4) Mix quickly and measure and record the absorbance at 405 nm every minute for 10 minutes or until the absorbance is > 1.2.
5) To measure the effect of enzyme concentration on the rate of reaction repeat steps 1-4 but use 1.75 cm³ of buffer and 0.5 cm³ of enzyme (don't forget substrate (0.25 cm³)!).
6) A further increase in enzyme concentration can be achieved by using 1.5 cm³ of buffer and 0.75 cm³ of enzyme.

Results and discussion

Typical data sets for the 3 different enzyme concentrations are shown in Table 1 and plotted in Figure 1.

As can be seen there is an increase in production of the product, p-nitrophenol, as the volume of enzyme present is increased from 0.25 cm³ (▼) to 0.5 cm³ (■) with a further increase as the enzyme volume is increased to 0.75 cm³ (▲).

The plots in Figure 1 represent a measure of enzyme activity. Under the conditions of the experiments the linear relationship between absorbance and time means that it is possible to calculate the slopes of the lines in Figure 1 and present these as a function of enzyme volume and this is shown in Figure 2.

	Absorbance at 405 nm		
Time (min)	Volume enzyme 0.25 cm³	Volume enzyme 0. 5 cm³	Volume enzyme 0.75 cm³
0.0	0.00	0.00	0.00
1.0	0.07	0.13	0.19
2.0	0.13	0.23	0.32
3.0	0.19	0.33	0.46
4.0	0.25	0.44	0.60
5.0	0.32	0.56	0.78
6.0	0.38	0.67	0.91
7.0	0.44	0.80	1.05
8.0	0.51	0.91	
9.0	0.58	1.03	
10.0	0.65	1.15	

Table 1 *- Effect of increasing β-glucosidase concentration on the absorbance at 405 nm in a cuvette of 1 cm pathlength. All solutions were prepared on 0.2 mol dm⁻³ phosphate buffer at pH 7.4. Total volume in the cuvette was 2.5 cm³ and the initial substrate concentration was 2.5 x 10⁻³ mol dm⁻³. See text for more details.*

Solution N°	Volume stock p-nitrophenol (cm^3)	Volume buffer (cm^3)	Concentration of p-nitrophenol ($mol\ dm^{-3}$)	Absorbance (405 nm)
1	0.0	10.0	0	0.00
2	1.0	9.0	2×10^{-5}	0.21
3	2.0	8.0	4×10^{-5}	0.40
4	3.0	70	6.0×10^{-5}	0.63
5	4.0	6.0	8.0×10^{-5}	0.77
6	5.0	5.0	1.0×10^{-4}	0.98

As described the β-glucosidase enzyme system is convenient for showing the relationship between rate of reaction and the concentration of enzyme. Such a robust system lends itself to a range of investigative work. We acknowledge that both the substrate and enzyme are not 'cheap items' but it should be remembered that quite small amounts are needed for the assay.

The Biology Team within SSERC has been asked on a number of occasions if we can provide data sets which could be used to support students following Higher Biology and Higher Human Biology programmes. Providing students with the data in table 1 and inviting them to generate and explain the plots in Figures 1 and 2 may be a useful exercise.

Extension activity

There is no need for students to calculate the concentration of p-nitrophenol present in the cuvette although you may wish them to do so for the sake of completeness. In order to make such calculations it will be necessary for students to produce or interpret a standard curve of the absorbance of p-nitrophenol solution as a function of concentration. This can be conveniently achieved as follows:

1) Prepare a stock solution of p-nitrophenol in 0.2 mol dm^{-3} phosphate buffer (pH 7.4) at a concentration of 2×10^{-4} mol dm^{-3}.
2) Make dilutions of the stock p-nitrophenol solution as per the table above. **Note** all dilutions should be made using 0.2 mol dm^{-3} phosphate buffer (pH 7.4).
3) Measure the absorbance of these solutions at 405 nm (or as close to 405 nm as your colorimeter will allow).
4) Use solution 1 to zero the colorimeter at 405 nm.

Plots of absorbance against concentration of p-nitrophenol (see Figure 3) would allow conversion of the data in Figure 1 into plots of product concentration as a function of time and the generation of slopes with 'more meaning'.

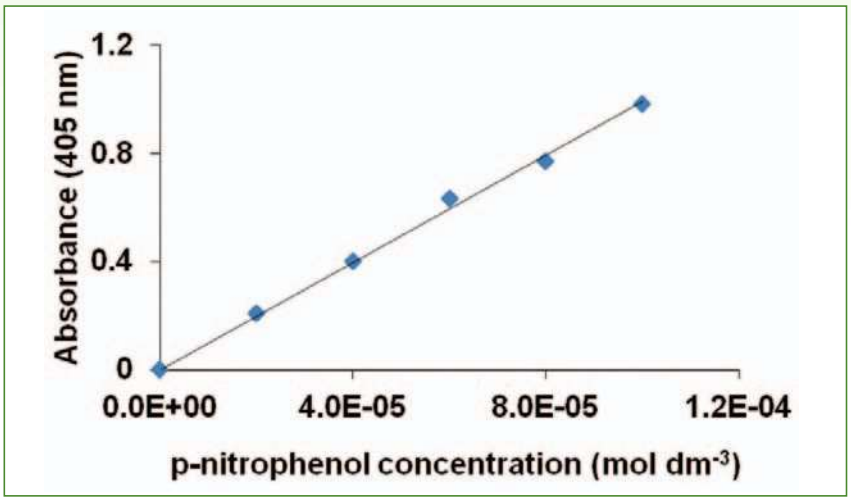


***Figure 3** - Absorbance (405 nm) of p-nitrophenol as a function of concentration. All solutions were prepared in 0.2 mol dm^{-3} phosphate buffer at pH 7.4. Cuvette pathlength was 1.0 cm.*

Health and safety

β-glucosidase, like many enzymes, is a sensitiser. When making up solutions, avoid raising dust, wear gloves and perhaps a mask. p-nitrophenol is harmful if swallowed, inhaled or in contact with skin. To make up solutions, wear eye protection and possibly gloves. The solutions are of low hazard.

References

[1] Fun with phosphatase (2015) SSERC Bulletin, **251**, 6-8.
[2] SQA (2014) Higher Biology Course Support Notes - can be downloaded at www.sqa.org.uk/files_ccc/CfECourseUnitSupportNotes_Higher_Sciences_Biology.pdf (accessed July 8th 2015).
[3] SQA (2014) Higher Human Biology Course Support Notes - can be downloaded at www.sqa.org.uk/files_ccc/CfECourseUnitSupportNotes_Higher_Sciences_HumanBiology.pdf (accessed July 8th 2015).
[4] A present for everyone (2014). SSERC Bulletin, **247**, 2.