β-galactosidase: Competitive inhibition

In the past few years the Biology Team at SSERC have published details of several enzyme assays which could be used to support Higher Biology and Higher Human Biology; such enzyme systems include phosphatase [SSERC, 2015], catalase [SSERC, 2018], and β-glucosidase [SSERC, 2015].

Figure 1a - β-galactosidase catalyses the following reaction.

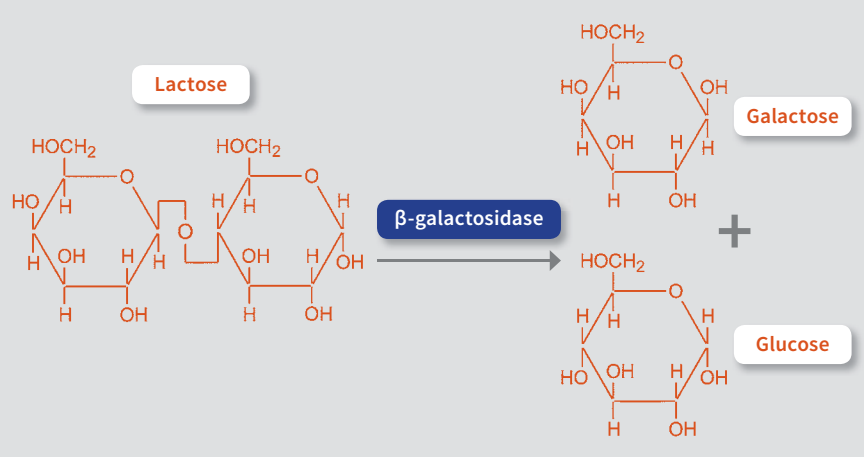

Figure 1b - β-galactosidase catalyses the following reaction.

The most recent SQA Course Specification documents [SQA, 2019] for Higher Biology and Higher Human Biology both contain the suggestion that one of the Learning Activities which students might follow is to 'Carry out *experiments on the effect of inhibitors on reactions. Examples could include the inhibition of β-galactosidase by galactose and its reversal by increasing ONPG concentration'.* Interestingly neither specification suggests Learning Activities which involve non-competitive inhibitors.

In this article we explore an activity which sets out how one might assay β-galactosidase and study the effects of a competitive inhibitor.

The enzyme system

The enzyme β-galactosidase catalyses the reaction as shown in Figure 1a. In more detail this can be represented as seen in Figure 1b.

Galactose acts as an inhibitor of the forward reaction and in this capacity can be described as a feedback inhibitor.

ONPG (o-nitrophenyl β-D-galactopyranoside) also acts as substrate for the enzyme, see Figure 2a. In more detail this can be represented as seen in Figure 2b.

Galactose acts as a competitive inhibitor of the enzyme, competing with ONPG for the active site. At high galactose concentrations the reaction of β-galactosidase with ONPG will be supressed because >>

Figure 2a - β-galactosidase catalyses the following reaction.

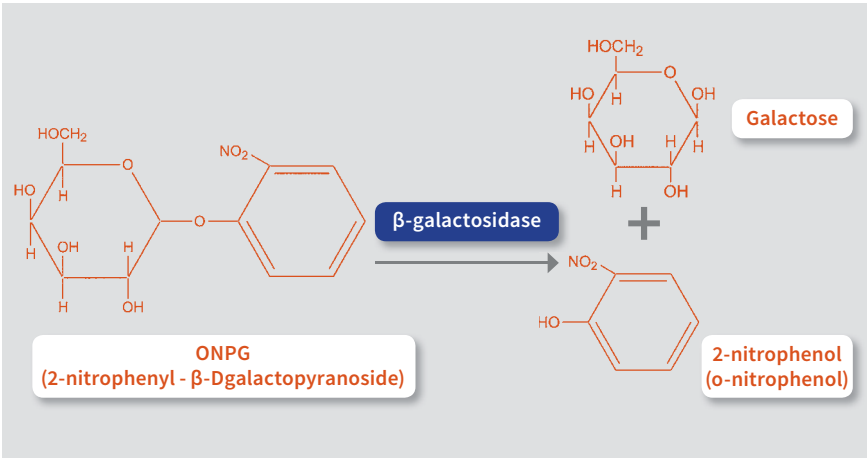

Figure 2b - β-galactosidase catalyses the following reaction.

of competition for the active site. This inhibition can be overcome if the ONPG concentration is sufficiently increased.

Experimental design

In common with many enzyme-based practicals, the inhibition of β-galactosidase offers huge scope for students to become involved in the process of experimental design. On the SSERC website (see https://www.sserc.org.uk/subject-areas/biology/higher-biology/enzymes/) we have provided a detailed protocol for the experiment (this includes a Technical Guide) as well as a PowerPoint which we use when running this practical as part of CPD sessions. The protocol on the website has all the steps laid out in a prescriptive manner. However, we believe that there is scope for students to be shown the basic technique (i.e. the mixing of colourless solutions of β-galactosidase and ONPG leading to the formation of coloured o-nitrophenol) and invite them to produce an experimental plan for how they might show that the reaction is competitively inhibited by galactose. At one level one might just add galactose and show that the rate of colour formation is slowed or stopped; the formulation of a detailed, robust protocol which allows for the generation of quantitative results, accompanied by suitable controls, is by contrast a demanding exercise.

We use *Lactase* (Lactozym); from the National Centre for Biotechnology Education as our source of enzyme.

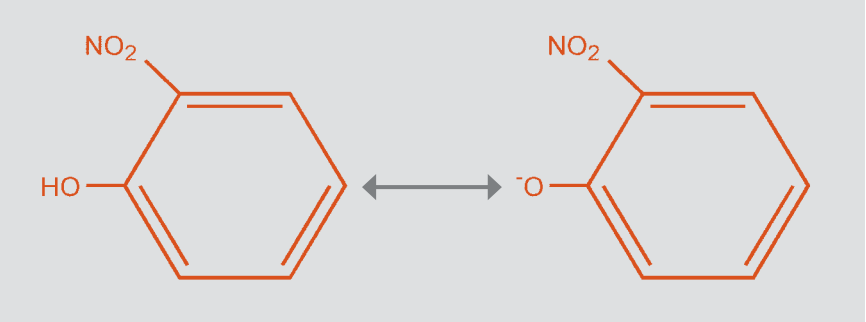

Figure 3 - in solution o-nitrophenol, the product of the reaction, exists in 2 forms viz the protonated form (which is colourless) and the anionic form (which has a yellow colour).

If kept refrigerated the enzyme maintains its declared activity for 6 months or longer. Whilst the optimum pH for activity is reported [National Centre for Biotechnology Education, 2019] to be 6.5 the protocol suggests that experiments be carried out in buffer at pH 8.0. The reason for this difference is that in solution o-nitrophenol, the product of the reaction, exists in 2 forms *viz* the protonated form (which is colourless) and the anionic form (which has a yellow colour), see Figure 3.

The pK_a for the equilibrium shown is 7.2 (http://www.zirchrom.com/organic.htm). In solutions at pH 7.2 equal amounts of the protonated and anionic forms will be present. In order to take advantage of the coloured nature of the anionic form (i.e. making it easier to follow the reaction colorimetrically), the enzyme assay is carried out at pH 8.0 where the majority (some 80+%) of the o-nitrophenol will be in its coloured form.

Tube	20% galactose in buffer (cm^3)	ONPG stock (cm^3)	Buffer (cm^3)
1	2	0.2	0.8
2	2	0.4	0.6
3	2	0.6	0.4
4	2	0.8	0.2
5	2	1.0	0.0

Table 1 - Solutions as prepared.

Steps in the protocol

All solutions, (apart from the 'working enzyme solution' see step 1 below), are prepared in pH 8.0 buffer. A stock solution of ONPG at a concentration of 2.8×10^{-2} mol dm^{-3} is prepared in buffer (pH 8.0).

1) Preparing a diluted enzyme solution of suitable activity

The first stage in the protocol is to produce a 'working enzyme solution'. 10-15 drops of the *Lactase* stock are diluted with distilled water (approximately 20 cm^3).

2) Testing enzyme activity

A solution (3 cm^3) is prepared which contains buffer (2 cm^3, pH 8.0, 0.1 mol dm^{-3}) and ONPG (0.5 cm^3, 2.8 x10-2 mol dm^{-3}). Working enzyme solution (0.5 cm^3) is added and the absorbance of this mixture is measured after 2 minutes. What you are looking for is an absorbance change after 2 minutes of between 0.3 and 0.5. If the activity of the working enzyme solution is too low (absorbance <0.3) then its concentration needs to be increased; if too high (absorbance >0.5) then the working enzyme solution needs to be diluted.

3) Measuring the effect of increasing ONPG concentration (the substrate) on β-galactosidase activity in the presence of a constant concentration of galactose

Solutions as prepared are shown in Table 1. >>

In turn 0.5 cm^3 of the working stock enzyme solution is added to each tube and the absorbance measured after 2 minutes (wavelength of observation is in the range 420-450 nm depending on choice of colorimeter). Typical data are shown in Table 2 and plotted in Figure 4.

From the data in Figure 4 it can be seen that an increase in ONPG concentration leads to an increase in ONP concentration after 2 minutes incubation. This is taken as evidence that galactose on the active site of the enzyme can be replaced by increasing ONPG concentration in behaviour that is classically displayed by competitive inhibitors.

Extension work

- Investigate the rate of o-nitrophenol production by measuring absorbance changes at regular time intervals over a period of some 5-6 minutes (in the absence of an inhibitor).
- Investigate the effect of enzyme concentration on the rate of o-nitrophenol production (in the absence of an inhibitor).
- Investigate the effect of increasing concentration of galactose whilst keeping [ONPG] constant.
- Substitute glucose for galactose (the other product of the reaction) to see if it has an inhibitory effect on enzyme activity.
- Investigate the effect of a non-competitive inhibitor (I_2/KI) on enzyme activity - the protocol on the SSERC website has experimental details for this. <<

Tube	Final concentration of galactose (% w/v)	Final concentration of ONPG (mol dm^{-3})	Absorbance (450 nm)
1	11.4	1.6×10^{-4}	0.05
2	11.4	3.2×10^{-4}	0.11
3	11.4	4.8×10^{-4}	0.15
4	11.4	6.4×10^{-4}	0.23
5	11.4	8.0×10^{-4}	0.27

Table 2 - Typical data.

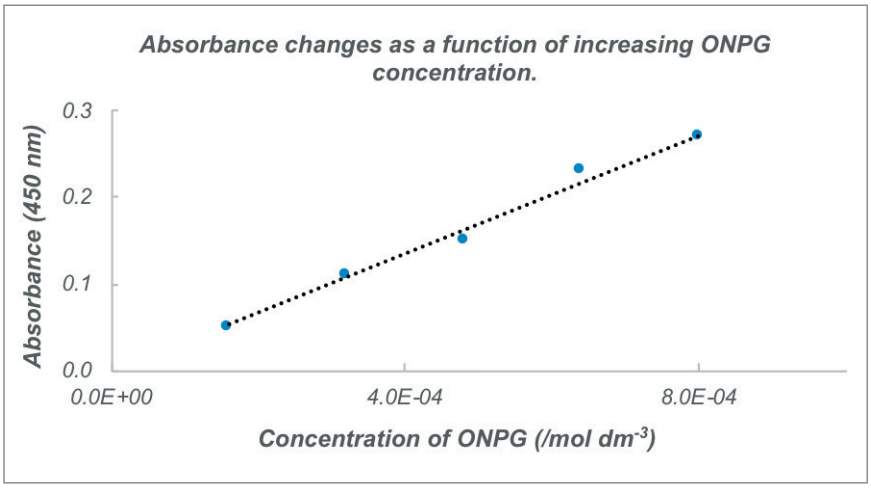

Figure 4 - Absorbance changes observed (450 nm) in solutions of increasing o-nitrophenyl β-D-galactopyranoside (ONPG) concentration in the presence of β-galactosidase and galactose (11.4% w/v). Further experimental details are given in the text.

References

- National Centre for Biotechnology Education (2019), *Lactase* (β-galactosidase). Details are available at http://www.ncbe.reading.ac.uk/MATERIALS/Enzymes/lactozym.html (accessed April 5th 2019).
- SQA (2019), Higher Biology and Higher Human Biology Course Specifications. These can be downloaded at https://www.sqa.org.uk/files_ccc/HigherCourseSpecBiology.pdf and https://www.sqa.org.uk/files_ccc/HigherCourseSpecHumanBiology.pdf respectively (accessed 25th March 2019).
- SSERC (2018), Catalase activity in immobilised yeast - effect of inhibitors, STEM Bulletin, **265**, 2-3.
- SSERC (2015), Fun with phosphatase, SSERC Bulletin, **251**, 6-8.
- SSERC (2015), Kinetic studies with β-glucosidase, SSERC Bulletin, **252**, 12-14.