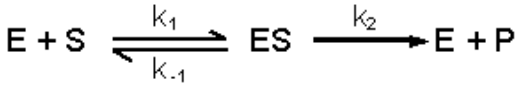
Laboratory 11:
Enzyme kinetics

Objective

- To determine the effect of substrate concentration on enzyme activity (beta galactosidase).
- To determine K_m and V_{max} in the presence or absence of an enzyme inhibitor.

Introduction

Enzymes are essential for cellular function and thus life. **Enzyme kinetics** is the study of chemical reactions catalyzed by enzymes, with a focus on their reaction rates. Enzymes are proteins that enhance the rate (velocity) of a chemical reaction with one or more substrate binding sites. The rate at which an enzyme works is influenced by several factors, including substrate concentration, temperature and pH.
The kinetic behaviour for many enzymes can be explained by the following equation:



Where, E= enzyme; S= substrate; P= product; ES= enzyme-substrate complex; k_1 is the rate constant for enzyme-substrate (ES) formation and k_{-1} is the dissociation rate of the ES complex. K_2 is the rate constant for ES breakdown to yield product.
The rate of catalysis varies with substrate concentration. With a fixed concentration of enzyme, the reaction rate of catalysis rises linearly as substrate concentration increases and then begins to level off and approaches a maximum at higher substrate concentrations. The Michaelis-Menten equation describes quantitatively the relationship between the reaction velocity and the substrate concentration:

$$V_0 = V_{max} [S] / K_m + [S]$$

Where, V_0 = initial velocity (initial linear portion of the enzyme reaction; corresponds to the region of the curve in which the velocity does not change over time)
V_{max} = maximal reaction rate
[S] = Substrate concentration
K_m = Michaelis-Menten constant
At low substrate concentration active sites of enzymes are not saturated by substrate and the reaction rate varies with substrate concentration. As the amount of substrate increases, more sites will be covered. At saturation no more sites are available, the enzyme is working at full capacity and now the rate is independent of substrate concentration (maximal reaction rate).
Michaelis-Menten constant (K_m) describes the affinity of an enzyme to a substrate. K_m is equivalent to the substrate concentration at which the reaction velocity is half of the maximal velocity. This means that a high affinity for the substrate leads to a small K_m and vice-versa.
A reaction curve can be obtained by mixing an enzyme and its substrate together and measuring the product that is generated over time. First the **initial velocity** of the enzyme is determined at different substrate concentrations. This allows us to plot the **saturation curve** (the relationship between substrate concentration and the reaction rate of an enzyme) for the determination of K_m and V_{max}.
Enzyme inhibitors are an important class of pharmacological agents. In many cases

these molecules are competitive, reversible inhibitors of substrate binding. **Competitive inhibitors** bind to the active site of the enzyme, thus preventing the substrate from binding. In the presence of a competitive inhibitor, higher substrate concentrations are required to achieve the same velocities that were reached in its absence and thus K_m is larger. In order to identify competitive inhibition, it is essential to run the enzymatic assay under initial velocity conditions (enzyme catalyzed reaction should exhibit linear kinetics).
Non-competitive inhibitors bind to a different site on the enzyme reducing its catalytic rate. K_m remains unchanged because the active site of those molecules that have not been inhibited is unchanged.
We will determine kinetic parameters of β-galactosidase, an enzyme that catalyzes the hydrolysis of of β-galactosides such as lactose or the lactose analogue orhto-nitrophenil β-D-galactopyranoside (ONPG). Hydrolysis of ONPG to the ONP anion produces a bright yellow colour with a peak absorbance at 420 nm that can be quantified by using a spectrophotometer. We will use Isopropyl β-D-1-thiogalactopyranoside (IPTG) to inhibit β-galactosidase activity.

Determine the effect of substrate concentration on enzyme activity

Each group will do 4 time points, 0.5, 1, 2 and 4 minutes, and determine the effect of substrate concentration (ONPG) on enzyme activity in the presence or absence of inhibitor IPTG. Each group has one 96 well plate (88 samples total) as indicated in Table1.

Materials

- E.coli β-galactosidase (Sigma); 250 units/ml in 1X cleavage buffer
- 1X cleavage buffer (60 mM Na_2HPO_4-$7H_20$, 40 mM NaH_2PO_4-H_20, 10 mM KCl, 1 mM $MgSO_4$-$7H_20$, pH 7.0, supplemented with 38 mM β-mercaptoethanol)
- Substrate: ONPG (Sigma); 4 mg/ml in water
- Inhibitor: IPTG (Sigma); 0.1 M in water
- Stop buffer 1M Sodium Carbonate
- Spectrophotometer (Tecan plate reader measuring A430 nm)
- 96 well (flat bottom; Greiner) see below 8 rows (A to H) x 11 columns (88 wells)

Time(min)			1	2	3	4	5	6	7	8	9	10	11	12
0.5		**A**											NC	-
1		**B**											NC	-
2		**C**											NC	-
4		**D**											NC	-
0.5	IPTG*	**E**											NC	-
1	IPTG*	**F**											NC	-
2	IPTG*	**G**											NC	-
4	IPTG*	**H**											NC	-

Procedure
In a typical enzyme assay, all but one of the components of the reaction mixture are added to the reaction vessel, and the reaction is started at time zero by adding the missing component (either the enzyme or the substrate). We will add the enzyme last. To avoid pipetting errors please organise your workspace and tasks carefully.

A. Prepare Master mix 1: ONPG +/- IPTG

- 1. Label 1.5 ml tubes from 1-11 and 1*-11*.
- 2. In tubes 1-11 mix the corresponding volumes (in µl) of ONPG and H_2O.

  Each tube contains 250 µl (Master mix for 10 reactions)

µl	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
ONPG	25	50	75	100	125	150	175	200	225	250	250
H2O	225	200	175	150	125	100	75	50	25	-	-

- 3. **Transfer** 125 µl Master mix from tubes 1-11 to tubes 1*-11*. Now each tube contains 125 µl.
- 4. Add **10 µl H_2O** to tubes 1-11. Vortex to mix and collect with quick spin.
- 5. Add **10 µl IPTG** to tubes 1*-11*. Vortex to mix and collect with quick spin.
- 6. Pipet **27 µl** Master mix for each concentration of ONPG +/- IPTG into the corresponding wells (4 reactions); column 12 is empty
  - tubes 1-11 into rows A-D.
  - tubes 1*-11* into rows E-H.

B. Negative control without β-Gal

- Pipet **52 µl of cleavage buffer** into column №11, row A-H (total 8 wells).
- Add **125 µl 1 M Sodium Carbonate (stop buffer)**.

C. Prepare Master mix 2: Cleavage buffer + β-Gal

- 1. Mix 4.4 ml cleavage buffer and 176 µl β-Gal in a 15 ml tube. Vortex briefly.
- 2. Transfer 572 µl of the mix 2 in tubes labelled A-H (8 tubes corresponding to rows= timepoints; you need master mix for 10 reactions per row, to have sufficient add the amount corresponding to 11 reactions). This step helps to focus better on single timepoints when you start the reaction and to reduce the risk of contamination between wells.
- 3. Final amount per well: 52 µl= 50 µl Cleavage buffer + 2 µl β-Gal.

D. Start and stop the enzyme reaction

- 1. Start with the 4 min reactions, H1-H10 (NOT H11!): transfer **52 µl master mix** (tube H) into each well and stop the reaction after 4 min by adding **125 µl 1 M Sodium Carbonate**.
- 2. Repeat the step for the remaining time points proceed row by row (G, F, E, D, C, B, A).
- 3. Give the plate to the teachers for absorbance measurement (Spectrophotometer Tecan plate reader at A430 nm). Results will be posted on Moodle.

Summary reagents 96 well plate (see pipetting scheme above)

1. Effect of substrate concentration on b-galactosidase activity (row A-D)

Time points: 30 seconds (A1-A11), 1 minute (B1-B11), 2 minutes (C1-C11), 4 minutes (D1-D11)

Tube Amounts in µl	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
ONPG	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	25
1X buffer	50	50	50	50	50	50	50	50	50	50	52
H_2O	24.5	22	19.5	17	14.5	12	9.5	7	4.5	2	2
β-Gal	2	2	2	2	2	2	2	2	2	2	-
Final volume	79	79	79	79	79	79	79	79	79	79	79

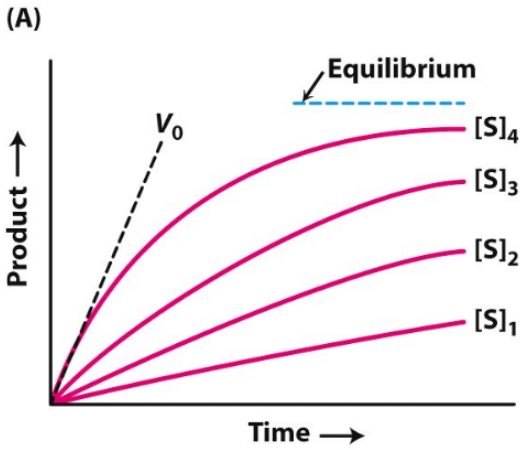
2. Inhibition of beta-galactosidase activity by IPTG (row E to H)

Time points: 30 seconds (E1-E11), 1 minute (F1-F11), 2 minutes (G1-G11), 4 minutes (H1-H11)

Amounts in µl **Tube**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
ONPG	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	25
IPTG	2	2	2	2	2	2	2	2	2	2	2
1X buffer	50	50	50	50	50	50	50	50	50	50	52
H_2O	22.5	20	17.5	15	12.5	10	7.5	5	2.5	-	-
β-Gal	2	2	2	2	2	2	2	2	2	2	-
Final volume	79	79	79	79	79	79	79	79	79	79	79

Data Analysis

1. Determine **Initial Velocity (V_0)**. The amount of product formed at different substrate concentrations is plotted as a function of time. Add a linear trendline in Excel that covers early timepoints. The **initial velocity** (V_0) for each substrate concentration of ONPG with and without IPTG is determined from the **slope** of the trendline at the beginning of the reaction (calculate the amount of product made per unit time right at the beginning of the reaction, remember to subtract the background absorbance)



Abs = time * V_0 **V_0 = Δ Abs/ Δ time**

V_0 is expressed in µM/min.

To convert absorbance to concentration [µM] use the formula (based on the Beer Lambert law)

P = µM of ONPG hydrolyzed = 10^6 * A_{420} / (e * l)
10^6 = for unit µM (instead of M)
e = molar extinction coefficient of ONP (e = 4500 M^{-1} cm^{-1})
l = pathlength in cm (l = 1 cm)

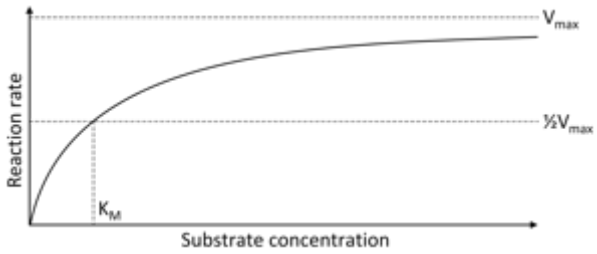
Thus, to get V_0 in µM/min you need to convert the slopes (V_0 [min^{-1}]) use the formula:**V_0 [µM/min] = V_0[min^{-1}] * 10^6 / (e * l)**

Volume of ONPG [µL]	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25
$V_{0\_without\text{-}IPTG}$ [µM/min]										
$V_{0\_with\text{-}IPTG}$ [µM/min]										

2. **You have found** V_0 **for all concentrations measured. Next,** create the Michaelis-Menten plot with V_0 versus substrate concentration [S] and make a logarithmic trendline. To convert the final **substrate concentration** (µM ONPG) use the formula:

   [S] µM = $13.3*10^3$ * V_{ONPG} / 79 µL

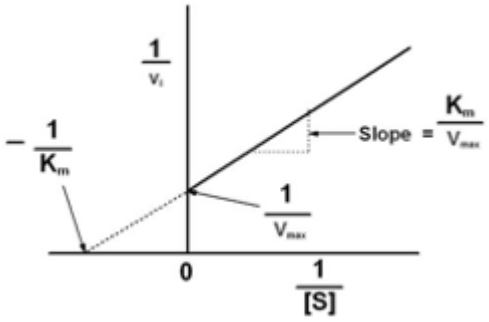
   - Molar concentration 4 mg/ml ONPG : 13.3 mM
   - Total reaction volume : 79 µL
   - V_{ONPG} : Volume of ONPG



Volume of ONPG [µL]	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25
[S] in µM										

3. **Next,** create the **Lineweaver–Burk** plots: Double reciprocal plot 1/v versus 1/ [S] and make a linear trendline and extend it to the left until both lines cut the y-axis and x-axis. Use the plot to determine K_m and V_{max} in the presence and absence of inhibitor.

   Vmax = 1/y intercept
   Km = Vmax*slope of regression line



Questions

1. What kind of inhibition is occurring?

2. Does the V_{max} and K_m change in the presence of the inhibitor?

3. Is your result statistically significant? Justify your answer.

Content for SLIMS

Table 1 - raw values

Table 2 - background corrected values and identifications of outliers + saturation points

Conversion from absorbance to µM and choosing only points with initial linear slope for Vo

Table 3 - values used abs = f(t) - without IPTG
Table 4 - values used abs = f(t) - with IPTG

Table 5 - Vo slopes for increasing concentration of ONPG (µM/min)

Table 6 - Conversion of S from µL used to µM