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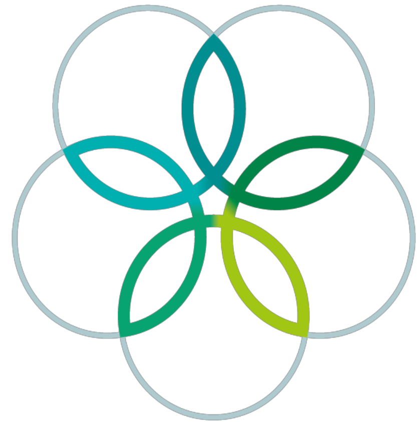

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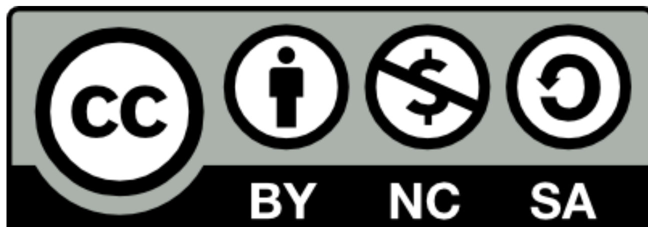

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PRACTICAL EXAM 4 - BIOCHEMISTRY

Max. total points 100
Exam duration 90 minutes
20 questions

INTRODUCTION

PURPOSE

In this exam you will analyse enzyme kinetics with and without an inhibitor of the enzyme.
The exam consists of two main parts, each of which contains three subparts.

Part 1 (57.5 points)
1.1. Introduction to enzyme kinetics (theory) (0 point)
1.2. Enzyme kinetics experiment of an industrial α-galactosidase using a synthetic substrate analogue pNP-Gal (laboratory work) (Questions 1–2: 40 points)
1.3. Data analysis of enzyme kinetics of α-galactosidase (Questions 4–11: 17.5 points)

Part 2 (42.5 points)
2.1. Introduction to enzyme inhibitors (theory) (Questions 11–13: 2 points)
2.2. Inhibition experiment of α-galactosidase (laboratory work) (Question 14: 27 points)
2.3. Data analysis of inhibition kinetics of α-galactosidase (Questions 15–20: 13.5 points)

Before you begin, we advise you to skim the entire exam to get an overview of the content. Since most points are earned on the lab part, **we recommend you to carry out <u>parts 1.2 and 2.2</u>, before starting with calculations and theoretical questions** (Parts 1.3, 2.1 and 2.3).

MATERIALS & EQUIPMENT

First, verify that you have all items listed below in front of you. **Please raise your pink**

card immediately, if anything is missing – and no later than 15 minutes after the start of the exam.

A. 1 p200 pipette. Use pipette p200 for volume interval 20-200 µL, unless otherwise stated
B. 1 p1000 pipette. Use pipette p1000 for volume interval 201-1000 µL, unless otherwise stated
C. 96 tips for p200 in box. A pipette tip should be discarded after each pipetting, unless otherwise stated
D. 96 tips for p1000 in box. A pipette tip should be discarded after each pipetting, unless otherwise stated
E. > 30 microcentrifuge tubes (1.5 ml)
F. 1 rack for microcentrifuge tubes
G. 2 microtitre plates labelled with your country code + A or B
H. 1 microtitre plate template
I. 1 stopwatch
J. 1 pencil
K. 1 marker
L. 1 calculator
M. 1 ruler
N. Pink card for contact with exam personnel
O. 9 ml 2 M (Molar=mole/Liter) Na_2CO_3 (**Stop**)
P. 6.5 ml 15 mM (milli-molar, milli=10^{-3}) pNP-Gal (**Substrate**)
Q. 15 ml Ultra pure water (**Water**)
R. 5 ml 1mM pNP (**Standard**)
S. 2 ml 0.024 mg/ml (**Enzyme**)
T. 5 ml 0.5 M (**Inhibitor**)
U. One touch pen for the tablet

1.1. INTRODUCTION TO ENZYME KINETICS

α-Galactosidases catalyze the hydrolysis of terminal galactosyl residues in α-galactosides. Typically, the activity of these enzymes is assayed using the synthetic substrate analogue para-nitrophenyl-α-galactoside (pNP-Gal), which is hydrolyzed to galactose (Gal) and para-nitrophenyl (pNP) (Figure 1.1). pNP-Gal is colourless, while the pNP product is yellow and its concentration can be measured quantitatively by determining its absorbance A_{405} at 405 nm using a microtitre plate reader.

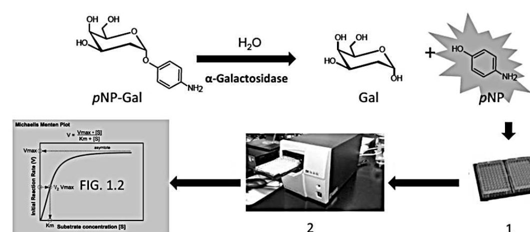


Figure 1.1: Schematic representation of the galactosidase activity assay: pNP is quantified

using a microtitre plate reader (2) that measures the absorbance A at 405 nm. In order to measure enzymatic activity, a standard curve is used to convert absorbance to a product concentration. 1, microtitre plate (Material G).

In Part 1, the dependency of the rate of hydrolysis on the substrate concentration will be investigated. In order to do so, a Michaelis-Menten plot (Figure 1.2), which describes this relationship is available to allow you to estimate the two important parameters V_{max} and K_m (see legend to Fig. 1.2).

The initial reaction rate V_o can be determined from $\Delta[P]/\Delta t$,which is the change in product concentration ([P]) per time (Δt).

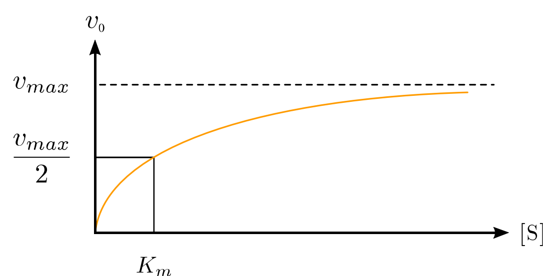


Figure 1.2: Michaelis-Menten plot: Initial reaction rate V_o versus substrate concentration [S]. K_m is the substrate concentration at which the enzyme operates at half its maximum rate, V_{max}, which reflects the saturation of the enzyme active sites with substrate.

Using another plot, the Lineweaver-Burk plot, the parameters V_{max} and K_m can be determined from the Y-axis and the X-axis intercepts, respectively (Figure 1.3). A Lineweaver-Burk plot is generated by plotting the inverse of the initial reaction rate ($1/V_o$) against the inverse of substrate concentration ($1/[S]$).

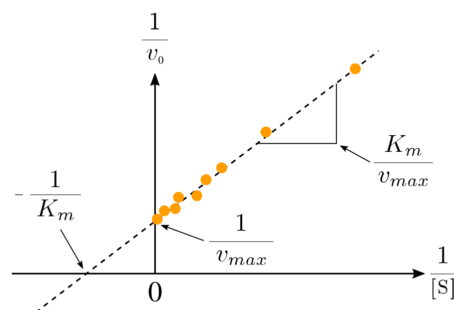


Figure 1.3: Lineweaver-Burk plot: After determining V_o at different substrate concentrations, a Lineweaver-Burk plot is made. A straight line is fitted to the data to determine K_m from the inverse of the intercept of the line with the X-axis, and V_{max} from the inverse of the intercept of the line with the Y-axis. These intercepts are calculated from the equation of the line.

1.2 ENZYME KINETICS EXPERIMENT OF AN INDUSTRIAL ALPHA-GALACTOSIDASE

1.2.1 STANDARD CURVE

Begin by generating the standard curve that will be used to measure the product (pNP) concentration of the enzymatic reactions later on. To generate the standard curve, you will need to dilute the 1 mM pNP standard stock solution **(Standard)** in the stop reagent **(Stop)**.

Q. 1 Standard curve dilution scheme

The 1 mM pNP standard stock solution (Standard) will, when needed, be diluted in the stop reagent (Stop). Calculate the volumes of pNP and stop reagent needed to prepare the final standard concentrations in a total volume of 500 µl. Type your calculated values into the table below (Table 1.1).

Tabel 1.1: Dilution scheme to prepare the standard curve

Tube label	St1	St2	St3	St4	St5
[pNP] standard (mM)	0.2	0.4	0.6	0.8	1
Volume of standard stock solution (Standard) (µl)					
Volume of stop reagent (Stop) (µl)					

Preparation of the standard curve
Protocol

a. With your marker, label five 1.5 ml microcentrifuge tubes according to the first row in Table 1.1: from St1 to St5.

b. Transfer the different volumes of the 1 mM pNP standard solution (**Standard**) to the labelled 1.5 ml tubes according to your calculations in Table 1.1 (**use the same pipette tip**).

c. Transfer the different volumes of the stop solution (**Stop**) to the labelled tubes according to your calculations in Table 1.1. The standard solutions are mixed thoroughly by turning the microcentrifuge tubes upside-down 5 times.

d. Transfer 100 µL ultra pure water (**Water**) into wells A1-A5 and B1-B5 of microtitre plate A (**use the same pipette tip**, see Fig. 1.4 and/or use the microtitre plate template to help you pipette in the correct wells).

e. Transfer 50 µL of each of the final pNP diluted standard solutions (Table 1.1) into the same microtitre plate wells. Each solution is pipetted in duplicates in two different wells (The subscripts I and II designate replicates of the same solution, Fig. 1.4).

f. Add 100 µL stop reagent (**Stop**) using a p1000 pipette to each pNP standard, A1-A5 and B1-B5. Mix thoroughly by pipetting the mixture up and down two times.

Plate A

	1	2	3	4	5	6	7	8	9	10	11	12
A	St_I1	St_I2	St_I3	St_I4	St_I5							
B	$St_{II}1$	$St_{II}2$	$St_{II}3$	$St_{II}4$	$St_{II}5$							
C							Standard curve					
D												
E												
F							Kinetics experiment					
G	S_I1	S_I2	S_I3	S_I4	S_I5							
H	$S_{II}1$	$S_{II}2$	$S_{II}3$	$S_{II}4$	$S_{II}5$							

Figure 1.4: Microtitre plate A: St, standards (see Table 1.1); S, reaction mixtures with different substrate concentrations (see Table 1.2 below).

Proceed now with part 1.2.2, where you will set-up the enzymatic reaction mixtures to your microtiter plates.

Important note: the assistants will not accept any microtitre plates in the last 10 minutes of the exam. If you feel that you will not be able to complete part 1.2.2 in time, hand in your plate now by raising the pink card. You results will be shown in Question 2.

1.2.2 ENZYME KINETICS EXPERIMENT

Protocol

Prepare the pNP-Gal substrate diluted solutions for the kinetics experiment**.**

a. Label five 1.5 mL tubes with a marker with S1 through to S5 (Table 1.2).

b. The 15 mM pNP-Gal substrate stock solution (**Substrate**) is diluted with ultra pure water (**Water**) in the labelled 1.5 ml tubes (see Table 1.2 below). The diluted solutions should be mixed thoroughly by turning the tubes upside-down 5 times.

Table 1.2: Substrate dilution scheme for the kinetics assay.

Tube label	S1	S2	S3	S4	S5
Volume (µl) of 15 mM pNP-Gal **(Substrate)**	40	120	240	400	800
Volume (µl) of ultra pure water **(Water)**	960	880	760	600	200

c. Transfer 50 µL of each diluted substrate solution (Table 1.2) and 50 µL ultra pure water (**Water**) into microtitre plate A, wells G1-G5 and H1-H5. (see Figure 1.4 and/or the microtitre plate template).

d. Set the timer at 5 minutes and start it immediately after you pipetted the enzyme

solution to the first well to start the first enzymatic reaction (S_I1) as described below.

e. Pipette 50 µL of the 0.024 mg/ml α-galactosidase enzyme (Enzyme) into wells G1-G5 and H1-H5 starting with S_I1 and $S_{II}1$, and continue in the same order and tempo throughout to $S_{II}5$ to start the enzymatic reactions in each well (hereafter referred to as the "enzymatic reaction mixture"). Ensure good mixing by quickly but gently pipetting 50 µl of the mixture up and down two times in each well.

f. After 5 minutes incubation time add 100 µL 2 M Na_2CO_3 solution (**Stop**) using a p1000 pipette to stop each of the enzymatic reactions in wells G1-G5 and H1-H5 in the same order and tempo as you started them. Mix well by pipetting the mixture up and down two times.

Q. 2 enzyme kinetics experiment

Hand in your microtitre plate containing your samples from parts 1.2.1 and 1.2.2 by raising your pink card. After measurement, the obtained values will be display in the table below automatically.

Note: no microtiter plates will be accepted in the last 10 minutes of the exam!

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

1.3 DATA ANALYSIS OF ENZYME KINETICS

Your task is now to determine the kinetics parameters of substrate hydrolysis by the α-galactosidase.
First the standard curve linear function for the product (pNP) should be determined using data from table 1.3
Access to a standard curve enables you to calculate product concentrations in the reaction mixtures, which further allows the determination of the initial reaction rate (V_0) of the enzyme for each substrate concentration.
The standard data set resembling microtitre plate A and shown below (Table 1.3) should be used for the calculation. This will avoid error carry-over penalty from part 1.2. However, your own data will be measured and used in the evaluation of your exam.

Table 1.3: Provided absorbance data for calculations (columns 1-5 in microtiter plate format).

	1	2	3	4	5
A	0.882	1.681	2.473	3.251	3.964

B	0.858	1.657	2.449	3.227	3.940
⋮					
G	0.304	0.728	1.049	1.272	1.512
H	0.307	0.716	1.009	1.234	1.466

Q. 3 Mean Absorbance of standards

Calculate the mean absorbance for each duplicate measurement for the standard curve given in Table 1.3. Enter all answers with three digits after the decimal point.

Table 1.4: Mean absorbance for standard curve

Tube label	St1	St2	St3	St4	St5
[pNP] (mM)	0.2	0.4	0.6	0.8	1
Mean A_{405} nm of duplicates					

Q. 4 standard curve linear function

In the figure below (Figure 1.5), the concentration of pNP (mM) is plotted against the absorbance (Mean A_{405} nm calculated in Question 3).

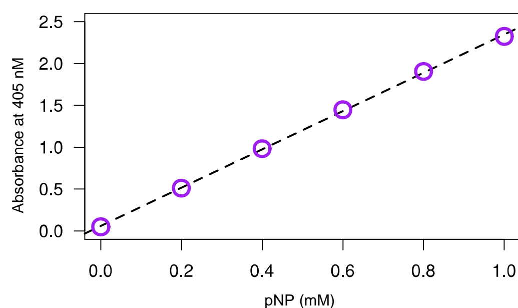


Figure 1.5: Hypothetical pNP product standard curve. The purple circles represent means of measured absorbances and the black dashed line is a linear regression to them.

Determine a and b of the standard curve linear function (see below) mathematically using only the mean absorbances of the two data points St1 and St5. Give a and b with three digits after the decimal point.:

A_{405} (absorbance units at 405 nm) = a·[pNP] (mM) + b, where a is the slope and b is the Y-axis intercept

a (A_{405}/mM)	
b (A_{405})	

The volume of the enzymatic reaction mixture from the experiment in Part 1.2.2 is 150 µl.

Q. 5 Reaction Time

Convert the reaction time given in the protocol into seconds.

Reaction time (seconds)	

Q. 6 Analysis of kinetics data (uninhibited enzyme)

Use the following standard curve equation to calculate the product concentration for each reaction mixture:

A_{405} absorbance = 2.29*[pNP] (mM) + 0.058.

The initial reaction rate V_0 can be determined from Δ[Product]/Δtime, i.e. the change in product concentration per time. Give all numbers with three digits after the decimal point.

Table 1.5: Analysis of uninhibited data

Tube label	S1	S2	S3	S4	S5
Volume of Stock solution (Substrate) (μl) (from table 1.2)	40	120	240	400	800
Volume of ultra pure water (Water) (μl) (from table 1.2)	960	880	760	600	200
Substrate concentration [S] prior to adding into the reaction mixture (mM)					
Substrate concentration [S] in reaction mixture (mM)					
Mean A_{405} absorbance, calculated from Table 1.3					
[$Product_{mean}$] (mM)					
V_0 (μM/second)					
1/[S] (1/mM)					
1/V_0 (second/μM)					

Q. 7 Michaelis-Menten PARAMETERS (graphical estimate)

Shown below (Figure 1.6) is a theoretical Michaelis Menten plot (V_0 versus [S]) resembling the reaction mixtures S1–S5 in Table 1.3.

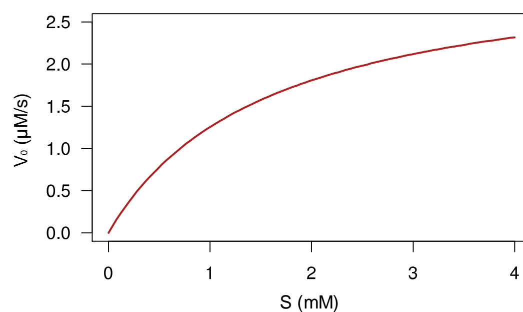


Figure 1.6: Theoretical Michaelis-Menten plot for the kinetics experiment in the absence of inhibitor

Estimate V_{max} and K_m graphically from the Michaelis-Menten plot (Fig. 1.6). Give answers with one digit after the decimal point.

V_{max} (µM/s)	
K_m (mM)	

Q. 8 Lineweaver-Burk linear function

Shown below (Figure 1.7) is the Lineweaver-Burk plot ($1/V_0$ versus 1/[S]) of the S1–S5 data point in Table 1.3.

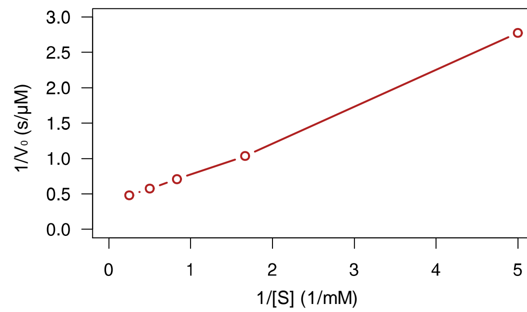


Figure 1.7: Lineweaver-Burk plot of the uninhibted enzyme kinetics data

Determine the linear function of in the Lineweaver-Burk plot (Figure 1.7) in the form shown below mathematically from the two data points for S1 and S5.
Give a and b with three digit after the decimal point.

$1/[V_o] = a \cdot 1/[S] + b$,

a (mM·s/μM)	
b (s/μM)	

Q. 9 determination of Vmax and Km

Using the linear function calculated above (Q. 8), determine K_m and V_{max} mathematically from the intercepts with the axes. Give numbers with three digits after the decimal point (no unit conversions should be done).

V_{max}	
K_m	

Q. 10 Enzyme concentration in reaction mixture

Calculate the enzyme concentration in the reaction mixture in μM from the enzyme stock concentration= 0.024 mg/ml and the enzyme's molar mass (75 000 gram/mole). Give the concentration with three digits after the decimal point.

Enzyme stock (mg/ml)	0.024
[E] (μM) in reaction mixture (micro=10^{-6})	

Q. 11 Turnover rate constant

The catalytic turnover rate constant k_{cat} (reaction rate of 1 enzyme molecule) has the unit 1/second and is calculated as follows:

$$k_{cat} = \frac{v_{max}}{[\mathrm{E}]}$$

Determine k_{cat} . Give number with three digits after the decimal point.

k_{cat} (1/second)	

2.1 INTRODUCTION TO INHIBITORS

Inhibitors are compounds that can specifically bind to enzymes, thereby reducing their activity and resulting in apparent changes in either K_m, V_{max} or both. Change in apparent kinetic parameters can be determined from the Lineweaver–Burk plot of an enzymatic reaction performed in the presence of an inhibitor. Reversible inhibitors can be competitive, non-competitive, or uncompetitive, depending on the mode of binding to their enzyme targets.
The inhibition of enzyme activity and apparent change in kinetic parameters can also be visualised in Michaelis-Menten and Lineweaver-Burk plots (Fig. 2.1).

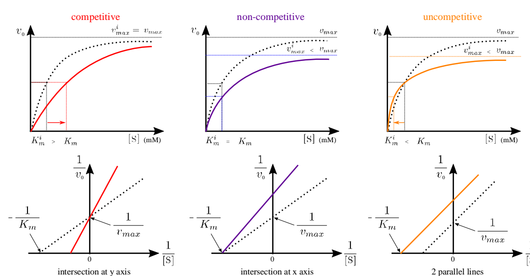


Figure 2.1: Inhibition of enzyme activity in Michaelis-Menten and Lineweaver-Burk plots. Dashed black curves are without an inhibitor and solid curves in the presence of inhibitor. v_0 is

initial reaction rate.

Inhibitors are characterized by their inhibition equilibrium constant K_i, defined as

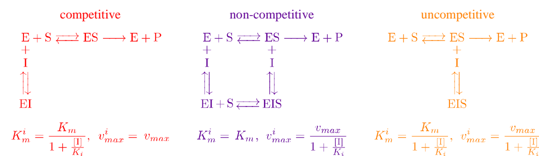
$$K_i = \frac{[I]\,[E]}{[EI]}$$

Where [I], [E] and [EI] are the concentrations of the free inhibitor, free enzyme and enzyme-inhibitor complex, respectively.

For **competitive inhibition**, the apparent K_m in the presence of inhibitor is designated as K_m^i. The chemical equilibrium for substrate (S) and inhibitor (I) binding to the enzyme (E) is shown below. K_m and K_m^i are related according to the equation below:

For **non-competitive inhibition**, the apparent V_{max} in the presence of inhibitor is designated as V_{max}^i. V_{max}^i and V_{max} are related according to the equation below:

For **uncompetitive inhibition**, the apparent K_m and V_{max} in the presence of inhibitor are designated as K_m^i and V_{max}^i, respectively. K_m and V_{max} are related to K_m^i and V_{max}^i according to the equations below:



Equation 2.1: The chemical equilibria for substrate (S) and inhibitor (I) binding to the enzyme (E) is shown in the top part of the figure for different inhibition types. The lower part shows the equations relating the change in apparent kinetic parameters to the inhibitor concentration and to the inhibition equilibrium constant.

Q. 12 factors affecting inhibition

For all inhibition types, the degree of inhibition, i.e. reduction in enzymatic reaction rate, is dependent on: (choose the best of the answers below).

1. INHIBITOR CONCENTRATION [I]
2. SUBSTRATE CONCENTRATION [S]
3. K_I OF THE INHIBITOR
4. CONCENTRATION OF [ES]
5. STATEMENTS 1, 2 AND 3
6. STATEMENTS 1 AND 3

Q. 13 Competitive Inhibition signature

Indicate if the following statement is true or false

	TRUE	FALSE
In competitive inhibition, the increase in substrate concentration [S] reduces or overcomes inhibition.		

2.2. INHIBITION OF ALPHA-GALACTOSIDASE (27 POINTS)

This part is experimentally similar to Part 1b. An inhibition kinetics experiment of α-galactosidase will be conducted in the presence of 50 µL inhibitor, which has a concentration of 0.5 M (mole/Liter).

Protocol

Substrate preparation for inhibition kinetics experiment

a. Prepare the substrate solutions according to Table 2.1, similarly to what you have done in Part 1.2.2. Remember to mix the solutions by turning the tubes upside down 5 times.

Table 2.1: Substrate dilution scheme for kinetic assay.

Tube label	IS1	IS2	IS3	IS4	IS5
Volume (µl) of substrate stock solution (Substrate)	80	160	320	600	840
Volume (µl) of ultra pure water (Water)	920	840	680	400	160

b. Transfer 50 µL inhibitor (**Inhibitor**) into the microtitre plate B wells A1-A5 and B1-B5 using the same pipette tip. (See Figure 2.2 and/or the microtitre plate template).
c. Transfer 50 µL of each final substrate solution (Table 2.1) to the same well positions (A1-A5 and B1-B5).

Plate B

	1	2	3	4	5	6	7	8	9	10	11	12
A	IS_I1	IS_I2	IS_I3	IS_I4	IS_I5							
B	$IS_{II}1$	$IS_{II}2$	$IS_{II}3$	$IS_{II}4$	$IS_{II}5$							
C												
D												
E												
F												
G												
H												

Figure 2.2: Microtitre plate B: IS, samples are reaction mixtures in the presence of inhibitor at different substrate concentration (see Table 2.1 above).

d. Set the timer at 5 minutes and start it immediately after you start the first enzymatic reaction by adding the enzyme solution to the first well (IS_I1) as described below.
e. Pipette 50 µL of the α-galactosidase (**Enzyme**) into the wells A1-A5 and B1-B5, starting with IS_I1 and $IS_{II}1$, and continue in the same order and tempo throughout to $IS_{II}5$ to start the enzymatic reaction in each well.
Ensure good mixing by quickly but gently pipetting 50 µl of the mixture up and down two times in each well immediately after you pippette the enzyme.
f. After 5 minutes incubation, add 100 µL stop reagent (**Stop**), using a p1000 pipette, to stop each of the enzymatic reactions in the wells A1-A5 and B1-B5 in the same order and tempo as you started them.
Mix thoroughly by pipetting the mixture up and down two times immediately after you pipette the stop solution.

Q. 14 ENZYME inhibition KINETICS EXPERIMENT

Hand in your microtitre plate containing your samples from part 2.2 by raising your pink card. After measurement, the obtained values will be displayed in the table below automatically. Please use the standard data given below (Table 2.2) for your calculations

Note: no microtitre plates will be accepted in the last 10 minutes of the exam!

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

2.3. DATA ANALYSIS OF INHIBITION KINETICS OF ALPHA-GALACTOSIDASE

In this section you will utilize the theory of Part 2.1 and the supplied inhibition data from Part 2.2 (see Table 2.2 below) to calculate enzyme kinetic parameters in the presence of inhibitor. The Lineweaver-Burk equation for the inhibited data will be compared to the supplied hypothetical Lineweaver-Burk equation for the uninhibited reaction to deduce the type of inhibiton. When you identify the type of inhibition, you will use these two Lineweaver-Burk equations (supplied hypothetical uninhibited and in the presence of inhibitor) to determine the change in the relevant kinetic parameters and to use the relevant equation to determine the inhibition equilibrium constant (K_i).

Table 2.2: Provided absorbance data for inhibition experiment (the first two rows and columns 1-5 in microtiter plate format).

	1	2	3	4	5
A	0.251	0.375	0.507	0.596	0.634

B	0.252	0.380	0.501	0.598	0.635

Q. 15 Analysis of Inhibition kinetics Data

Calculate and fill in the table below. In order to calculate product concentrations in mM, use the standard equation given in Q6:

Absorbance A_{405} = 2.29 * [pNP] (mM) + 0.058

Tube label	IS1	IS2	IS3	IS4	IS5
Volume (µl) of Stock solution (Subtrate) (from table 2.1)	80	160	320	600	840
Volume of (µl) ultra pure water (Water) (from Table 2.1)	920	840	680	400	160
Substrate concentration [S] prior to adding into the reaction mixture (mM)					
Substrate concentration [S] in reaction mixture (mM)					
Mean A_{405} absorbance from table 2.2					
[$Product_{mean}$] (mM)					
V_0 (µM/second)					
1/[S] (1/mM)					
$1/V_0$ (second/µM)					

A Lineweaver-Burk plot is produced based on the inhibition kinetics data IS1-IS5 in table 2.2. The hypothetical Lineweaver-Burk equation of the uninhibited reaction is: 1/[Vo] = 0.363·1/[S] + 0.908, and this line is plotted in Fig. 2.3. This supplied equation should be used for the calculations below, and **NOT the equation you have determined in part 1.3 (Fig. 1.7).**

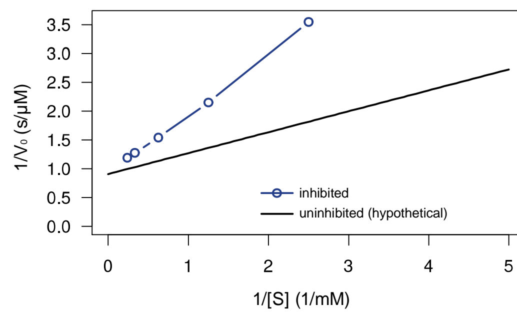


Figure 2.3 Lineweaver-Burk plot of the inhibited data and hypotheical unhibited data.

Q. 16 Lineweaver-Burk linear function (inhibited reaction)

Determine the linear function of the Lineweaver-Burk plot (Fig. 2.3) in the presence of inhibitor in the form shown below mathematically using only the data from IS1 and IS5. Give a and b with three digits after the decimal point.

$1/[Vo] = a \cdot 1/[S] + b$,

a (mM·s/μM)	
b (s/μM)	

Q. 17 apparent Kinetic Parameters with inhibitor

Determine the apparent kinetic parameters in the presence of inhibitor from the Lineweaver-Burk plot of the inhibited reaction. Give the parameters with three digits after the decimal point (no unit conversions should be done in this calculation).

$V_{max}{}^{i}$		
$K_m{}^{i}$		

Q. 18 Type of Inhibition

What type of inhibition does the inhibitor exert on the α-galactosidase? Choose the most likely type of inhibition based on the size of changes in kinetic parameters in the presence of the inhibitor as compared to the hypothetical data for the uninhibited enzyme

1. COMPETITIVE
2. NON-COMPETITIVE
3. UNCOMPETITIVE

Q. 19 Effect of Substrate concentration

Based on the type of inhibition you have chosen above, how would an increase in substrate concentration influence inhibition? Choose one of the statements below.

1. LESS INHIBITION
2. NO CHANGE
3. MORE INHIBITION

Q. 20 Inhibition Constant

Determine the inhibition constant (K_i) if the concentration of the 50 µL inhibitor added to the reaction mixture is 0.5 M. Give numbers with three digits after the decimal point (no unit conversions should be done in this calculation).

Inhibitor concentration in the reaction mixture (mM)	
K_i (mM)	

END