BCH2333 LAB 3: Enzymes

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Purpose

For this lab, acid phosphatase is used to help analyze enzyme kinetics. PNPP (p-nitrophenyl phosphate) is used in the lab to react and produce PNP (p-nitrophenol). Three experiments will be conducted with the use of a control and an inhibitor and a total of fourteen test tubes in experiments two and three and seeing the difference between the two. Two graphs will be plotted in order to view any trends. Also, absorbance values at 405 nm will be recorded and will use the zero test tube to be subtracted from each value. The effects of the inhibitor in comparison to the control will allow to identify the inhibitor type and view if it is non-competitive, noncompetitive or competitive. This data will be calculated and recorded in numerous tables.

R1

Table 1: Time dependence of product formation for acid phosphatase in 0.5mg/mL assay. Six test tubes containing 2.0 mL of 0.5M KOH were prepared along with a seventh tube (R) containing 1.0 M of sodium acetate buffer (pH 5.7) and 3.9 mL of water. 0.1 mg/mL of acid phosphatase solution was added to tube R and incubated at 37 degrees celsius for 3 minutes. 0.5 mL of 0.020 M PNPP was added to test tube R

and then transferred the 0.5 mL of the solution to test tube 0 indicating the zero time sample. This was repeated for all tubes. The absorbance at 405 nm was measured for each test tube and subtracted the zero from each reading. Both the PNPP to PNP % conversion was calculated as well as the PNP concentration using Beer-Lambert law.

Time (min)	A_{405}	A_{405} corrected	PNP (mM)	PNP (μmol)	Percentage of PNPP converted
0	0.010	0.000	0.000	0.000	0.000
2	0.169	0.159	0.00856	0.0214	2.14
4	0.363	0.353	0.00188	0.0047	0.47
6	0.635	0.625	0.00332	0.0083	0.83
10	1.076	1.066	0.00567	0.0142	1.42
15	1.271	1.261	0.00671	0.0168	1.68

Sample calculations for t=2minutes.

a) Sample calculation → corrected absorbance value

$A_{t=0} = 0.010$

$A_{t=2} = 0.169$

$A_{\text{corrected (t=2)}} = A_{t=2} - A_{t=0}$

$A_{\text{corrected (at t=2)}} = 0.169 - 0.010 = 0.159$

Therefore, 0.159 is the corrected absorbance value at time = 2 mins.

b) Sample calculation → PNP (mM) concentration

$A_{\text{corrected (t=2)}} = 0.159$
$\varepsilon_{405} = 1.88 \times 10^4\ M^{-1}cm^{-1}$
Path length of cuvette = 1 cm
c = [PNP] =?
$c = A_{\text{corrected (t=2)}} / ((\varepsilon_{405})(l\ cm))$
$c = 0.159/((1.88 \times 10^4\ M^{-1}cm^{-1})(1\ cm))$

c = 8.46x10^{-6} M = 0.00846 mM

In conclusion, the concentration of PNP at t=2 mins is 0.00846 mM.

c) Sample calculation → Amount of PNP (μmol)

c = 8.46x 10^{-6} M
V = 2.5 mL
nPNP =? μmol
nPNP = (c)(V)
nPNP =(0.00846 mM) (2.5 mL)
nPNP = 0.021 μmol

In conclusion, 0.021 μmol is the amount of PNP at t=2 mins.

d) Sample calculation → Percent conversion of PNPP

$[PNPP]_a$ = 20mM
V_a = 0.5 mL
V_f = 5 mL
$[PNPP]_i = \frac{[PNPP]_a V_a}{V_f}$
$\eta PPNP_i$ = ?
$\eta PPNP_i$ = (20 mM)(0.5 mL) / 5 mL = 1.0 μmol
Therefore, the initial amount of PNPP is 1.0 μmol.

% PNPP converted = (n_{PNP}/n_{PNPP}) (100 %)
% PNPP conversion = (0.021 μmol/1 μmol) (100 %)
% PNPP conversion = 2.1 %

Therefore, 2.1% is the percentage of PNPP converted at t=2mins.
e) Sample calculation → Percentage of acid phosphatase present in assay

[acid phosphatase] $_{initial}$ = 0.4 mg/mL = C_s

$V_{acid\ phosphatase}$ = 0.7 mL

$V_{total\ assay}$ = 5.0 mL

[acid phosphatase] = ?

[acid phosphatase] = ([acid phosphatase]$_{initial}$ * $V_{acid\ phosphatase}$)/(V_{total})

[acid phosphatase] = (0.4 mg/mL)(0.7 mL)/ 5.0 mL

[acid phosphatase] = 0.056 mg/mL

In conclusion, 0.056 mg/mL is the concentration of acid phosphatase in the assay.

R2

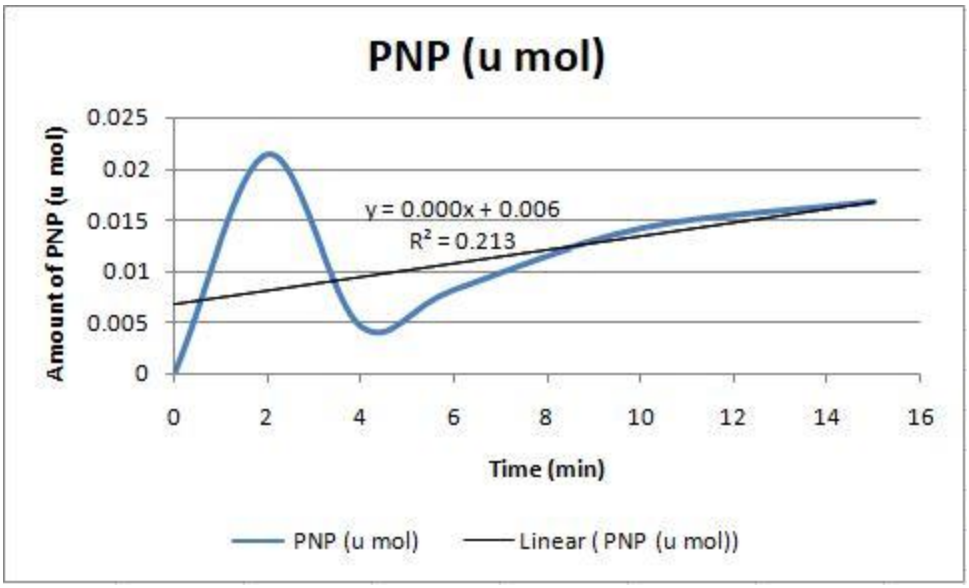

Figure 1: The amount of PNP formed into PNPP over 15 minutes of time in a fixed assay. The preparation of six test tubes was completed with 2.0 mL of 0.5 M of KOH. Each test tube was labelled one of the following, 2,4,6,10, and 15. They remained at room temperature. Test tube R was the seventh test tube made with 3.9mL of water and 1.0M of sodium acetate buffer pH=5.7. Acid phosphatase was added to the mix in this seventh test tube. This reaction began with 0.5 mL of 0.20 M of PNPP being added to it. The amount of product for every tube was calculated and shown in the figure in μmol. The equation is y=0.000x + 0.006 and the correlation value being R^2= 0.213.

Analysis of results:

In figure 1, it shows the amount of product in μmol in six test tubes plotted against the time in minutes for 2,4,6,10 and 15 mins. The correlation value is R^2= 0.213, and the linear regression line gave the equation y = 0.000x + 0.006. The trend line shows an increase in product with an increase in time. It seems to have been an error in the beginning calculations or experimental data as the amount of product has a high jump and goes back down to continue to increase at a steady pace. This can be an error in calculation or an error in the experiment when mixing the solutions together. The remaining of the increase shows that the rate of product that is being formed through time is indeed increasing.

R3

Table 2: Substrate dependence from experiment 2 and experiment 3. For the incubation tubes, water, acetate and PNPP were added and then vortexed. The tubes were incubated for 2 minutes and then enzyme was added. Then they were vortexed and incubated for 10 minutes. KOH was added then vortex and finally was able to read the absorbance at 405 nm. The stock solution, 20 mM, three 0.5 mL solutions were made of 5.0, 0.5, and 2.0 mM PNPP in water. Experiment 2 is the control experiment, and experiment three is including the inhibitor. Seven tubes were used in each. The spectrophotometer read all the absorbance at 405 nm. All test tubes from both experiments were corrected by subtracting the time zero absorbance value from the test tube absorbance values.

Test Tube	[PNPP]* (M)	Control (experiment 2)	Inhibitor (experiment 3)

		A_{405}	[PNP] (M)	PNP (mol)	v (mol/min)	A_{405}	[PNP] (M)	PNP (mol)	v (mol/min)
1	0.00005	0.301	$1.60x10^{-5}$	0.040	0.004	0.102	$5.43x10^{-6}$	0.0136	0.00136
2	0.0001	0.361	$1.92x10^{-5}$	0.048	0.0048	0.161	$8.56x10^{-6}$	0.0214	0.00214
3	0.0002	0.623	$3.31x10^{-5}$	0.083	0.0083	0.241	$1.28x10^{-5}$	0.032	0.0032
4	0.0005	0.779	$4.14x10^{-5}$	0.104	0.0104	0.298	$1.59x10^{-5}$	0.040	0.0040
5	0.00075	0.998	$5.31x10^{-5}$	0.133	0.0133	0.294	$1.56x10^{-5}$	0.039	0.0039
6	0.001	1.006	$5.35x10^{-5}$	0.134	0.0134	0.392	$2.10x10^{-5}$	0.0525	0.00525
7	0.002	1.246	$6.63x10^{-5}$	0.166	0.0166	0.401	$2.13x10^{-5}$	0.0533	0.00533

Sample Calculations: test tube 1

a) Sample calculation → Concentration of PNPP substrate (M)

$[PNPP]_{initial}$= 0.5 mM $V_{initial}$= 50 µL V_{total}= 500 µL

[PNPP] = ?
[PNPP] = $([PNPP]_{initial})(V_{initial})$ / (V_{total})
= (0.5 mM) (50 µL) / (500 µL)
= 0.05 mM = 0.00005 M

In conclusion, 0.00005 M is the concentration of PNPP in test tube 1.

b) Sample calculation → Corrected absorbance value

$A_{\text{test tube 1 incubation}} = 0.318$

$A_{\text{test tube zero tube}} = 0.017$

$A_{\text{corrected}} = A_{\text{test tube 1 incubation}} - A_{\text{test tube time zero tube}}$

$A_{\text{corrected}} = 0.318 - 0.017 = 0.301$

Therefore in test tube 1 the corrected absorbance is 0.301.

c) Sample calculation → Concentration of PNP substrate (M)

$A_{\text{corrected}} = 0.301$
$\varepsilon_{405} = 1.88 \times 10^4\ M^{-1}cm^{-1}$
Path length of cuvette = 1 cm
[PNP] = c = ?
$c = A_{\text{corrected}} / (\varepsilon_{405})(l\ cm)$
$c = 0.301/(1.88 \times 10^4\ M^{-1}cm^{-1})(1\ cm) = 1.6x10^{-5}\ M$

In conclusion, $1.6x10^{-5}$ M is the concentration of PNP in test tube 1.

d) Sample calculation → Amount of PNP (μmol)

$c = [PNP] = 1.6x10^{-5}\ M$
$V = 0.0025\ L$
$\mu mol = \eta_{PNP} = ?$
$\eta_{PNP} = [PNP](V)$
$= (1.6x10^{-5}\ M)(0.0025\ L)$
$= 4.0x10^{-8}\ (1.0x10^{6}) = 0.04\ \mu mol$

Therefore, there is 0.04 μmol of PNP in test tube 1.

e) Sample calculation → Reaction rate (μmol/min)

nPNP = 0.04 μmol
incubation time = 10 min
v = ?

$$v = \frac{\text{nPNP}}{\text{incubation time}}$$

$$v = \frac{0.04\ \mu\text{mol}}{10\ \text{min}} = 0.004\ \mu\text{mol/min}$$

Therefore, the reaction rate velocity in test tube 1 is 0.004 μmol/min.

R4

Table 3: Data used for the Lineweaver-Burk graph. Control and inhibitor values are calculated correspondingly for seven test tubes each.

Test Tube	[S]* (M)	Control			Inhibitor		
		v (μmol/min)	1/v (min/μ)$^{-1}$	1/[S] (M)$^{-1}$	v (μmol/min)	1/v (min/μ)$^{-1}$	1/[S] (M)$^{-1}$
1	0.00005	0.004	250.00	20,000	0.00136	735.29	20,000

2	0.0001	0.0048	208.33	10,000	0.00214	467.29	10,000
3	0.0002	0.0083	120.48	5000	0.0032	312.50	5000
4	0.0005	0.0104	96.15	2000	0.0040	250.00	2000
5	0.00075	0.0133	75.19	1333.33	0.0039	256.41	1333.33
6	0.001	0.0134	74.63	1000	0.00525	190.48	1000
7	0.002	0.0166	60.24	500	0.00533	186.62	500

Sample calculation for control tube 2

a) Sample calculation → [S]
[PNPP] = 0.0001 M [S] = [PNPP] = 0.0001 M
Therefore 0.0001 M is the concentration in tube 2 in the control.

b) Sample calculation → v (µmol/min)
Time = 10 mins µmol = 0.048
V= µmol / time = 0.048 µmol/10min = 0.0048
Therefore, the initial reaction rate is 0.0048 µmol/min in tube 2 in the control.

c) Sample calculation → 1/[S] (M)$^{-1}$
[S] = 0.0001M
1/[S] = 1/0.0001 M = 10000 M^{-1}
Therefore, 10000 M^{-1} is the value for **1/[S] (M)$^{-1}$** in tube 2 in the control.
R5

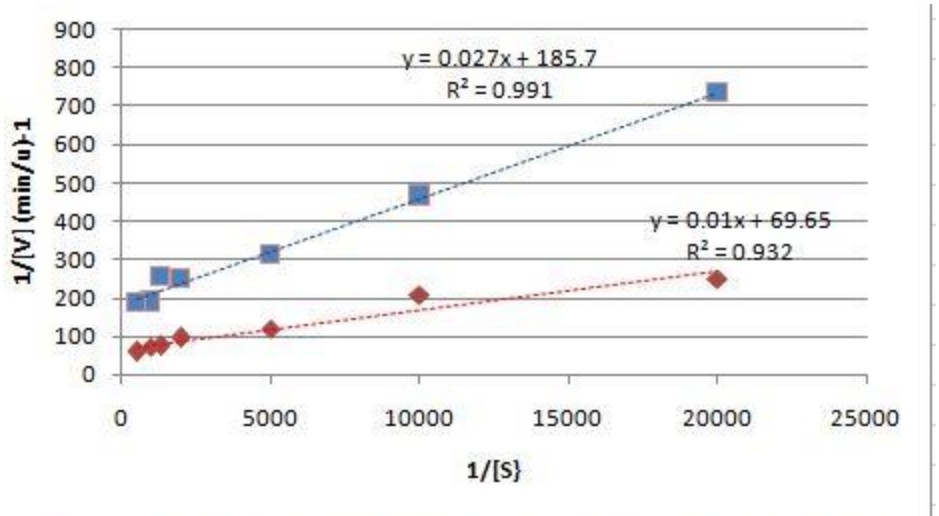

Figure 2: Lineweaver-Burk plot for control and inhibitor reactions with 1/[S] and 1/[V] (min/μ)$^{-1}$. Both linear regression trend lines are shown with their corresponding equations. For the control reaction, an equation of y=0.01x + 69.95 is given with a correlation value of R^2= 0.932. For the inhibitor reaction, the equation is y=0.027x + 185.7, with a correlation value of R^2= 0.991. These experiments each used seven test tubes and the inhibitor ones had the inhibitor included before the addition of the enzymes. A time zero test tube was used which was subtracted from the absorbance values received at absorbance 405 nm.

R6

Table 4: Kinetic parameters from experiments 2 and 3. Experiment 2 was completed without an inhibitor whereas experiment 3 included an inhibitor. The inhibitor was included before the addition of acid-phosphatase enzyme. The Lineweaver-Burk plot was made. The Vmax and Km were calculated for both control and inhibitor reactions. Vmax represents the initial speed of the reaction and Km represents the ½ of the maximum speed of the enzyme. These values are calculated using the linear regression trend lines from Figure 2. The equations used were y= 0.01x + 69.95 and y=0.027x +

185.7. The slopes calculated to have a 170% difference and the y-intercepts had a 165.8% difference. This is not above the 25% difference.

Method	Control		Inhibitor	
	V_{max} (µmol/min or U)	K_M (M)	V_{max} (µmol/min or U)	K_M (M)
Lineweave-r Burk	0.01436	0.000143 M	0.005385	0.000145 M

Sample Calculations

a) Sample Calculation → V_{max} (µmol/min) for control

y= 0.01x +69.95 ; x=0

V_{max} (µmol/min) = 1/69.65
V_{max} = 0.01436 µmol/min

In conclusion, the max initial velocity is 0.01436 umol/min for control reaction.

b) Sample calculation → K_M (M) for control

y= 0.01x +69.95 ; y=0

-1/K_M= -69.95/0.01

K_M= -1/(-69.95/0.01) = 0.000143 M

In conclusion, 0.000143 M is the $K_M$of the control.

c) Sample calculation → V_{max} (µmol/min) for inhibitor

y= 0.027x +185.7 ; x=0

V_{max} (µmol/min) = 1/185.7
V_{max} = 0.005385 µmol/min

In conclusion, the max initial velocity is 0.005385 umol/min for inhibitor reaction.

d) Sample calculation → K_M (M) for inhibitor

y= 0.027x + 185.7 ; y=0

-1/K_M= -185.7/0.027

K_M= -1/(-185.7/0.027) = 0.000145 M

In conclusion, 0.000145 M is the $K_M$of the inhibitor reaction.

e) Sample Calculation → Identification of inhibitor using percent difference of 25%

$$\% = \frac{\text{(Inhibitor slope) – (Control slope)}}{\text{Control slope}} \times 100\%$$

$$\% = \frac{(0.027 – 0.01)}{0.01} \times (100\%)$$

% = 170%

$$\% = \left(\frac{\text{Inhibitor of y – intercept) – (Control of y – intercept}}{\text{Control of y – intercept}}\right) \times 100\%$$

$$\% = \frac{(185.7 – 69.95}{69.95} \times 100\%$$

% = 165.48%

This shows that there is not an increase between the two that is significant since the difference between the two percentages is less than 25%.

Analysis of results:

There was an increase in both the control and in the inhibitor reactions. The inhibitor reaction had a higher reaction rate in comparison to the control reaction. The slope had a higher increase, 170%, whereas the y-intercept had less of an increase in percentage with 165.48% between the two. There is a 4.52% difference which is not above the 25%, showing that not a high change was made.

R7

Table 5: Type of inhibitor. Experiment 2 was done without an inhibitor whereas experiment 3 was completed with an inhibitor. The Vmax shows the maximum initial speed for the reaction. Km represents ½ of the max speed for the enzyme. In figure 2, the slopes , y intercepts, linear regression lines and correlation values were determined. For experiment 2, the linear regression is y= 0.01x + 69.95 and for experiment 3 the linear regression line of trend is y=0.027x + 185.7. Also, the correlation value for the experiment 2 is R^2= 0.932 and for experiment 3 it is R^2= 0.991. The unknown inhibitor # is 20.

Inhibitor Number	[I] (M)	V_{max} (U)	K_M (M)	K_I (M)	$K_{I'}$ (M)	Type of Inhibitor
-	-	0.01436	0.000143 M	-	-	None
20	0.75	0.005385	0.000145 M	-	0.0012 M.	Competitive

a) Sample calculation → Concentration of inhibitor [I] (M)

C_1 = 7.5 mM V_1 = 50 µL
$V_2 = V_{total}$ = 500 µL C_2 = ?
$C_1V_1 = C_2V_2$

$C_2 = (C_1)(V_1)/V_2$
C_2 = (7.5 mM) (50 µL)/500 µL
C_2 = 1 mM = 0.75 M

Therefore, the concentration of the inhibitor is 0.75 M.

b) Sample calculation → K_I' calculation

K_M control = =0.000143 M
K_M inhibitor = 0.000145 M
α_1' = ? = K_M control/ K_M inhibitor
α_1' = 0.000143 M / 0.000145 M
α_1' = 0.986

V_{max} control = 0.01436
V_{max} inhibitor = 0.005385

α_2' = ? = V_{max} control/ V_{max} inhibitor
α_2' = 0.01436/0.005385 = 2.67

α' = (α_1' + α_2')/2
α' = (0.986 + 2.67)/2 = 1.83

α' = 1 + [I] / K_I'
K_I'= [I] / α' – 1
K_I'= 0.001/(1.83 – 1)
K_I'= 0.0012 M

Therefore, the K_I' of the inhibitor is 0.0012 M.

R8

V_{max} = 0.01436 U
V_{enzyme} = 50 µL = 0.05 mL
C_{enzyme} = 0.1 mg/mL
m_{enzyme} = ?

m_{enzyme} = (C_{enzyme})(V_{enzyme})
m_{enzyme} = (0.1 mg/mL) (0.05 mL)
m_{enzyme} = 0.005 mg

specific activity = V_{max}/ m_{enzyme}

specific activity = 0.01436 U/0.005 mg = 2.87 U/mg

In conclusion, 2.87 U/mg is the specific activity of the phosphatase sample.