Analysis of enzyme activity

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Abstract

Enzyme activity can be easily measured by monitoring product formation or substrate utilisation. In this experiment the progress of the hydrolysis of p-nitrophenylphosphate by alkaline phosphatase, an enzyme reaction, will be measured.
For the first part of the experiment, the enzyme was incubated with the substrate for times between 5 and 30 min, allowing to observe the progress of the reaction. The presence of the enzyme made the assay tubes present a highest absorbance than the control tubes, therefore they had the highest rate of change of concentration, and more product was formed in less time. In summary, it can be concluded that the hydrolysis of pNPP in the presence of enzyme is faster than the hydrolysis without the enzyme.
For the second part of the experiment, the data was analysed to find the kinetic constants V_{max} and Km. The concentration of substrate available to the enzyme was varied and the initial rate of the reaction recorded. The value of Km was low; therefore, the enzyme-substrate interaction is tight. Because the binding is tight, V_{max} will occur at low substrate concentrations.

Introduction

Enzymes are biological catalyst that speed up chemical reactions essential for life. The enzyme catalyses the reaction when the enzyme substrate complex is formed, and the substrates bind to the enzyme. E + S ⟷ ES. At the end of the reaction, the enzyme substrate complex is converted to enzyme and product. ES → E + P. The enzyme doesn't change during the reaction and is used many times.

Measurements of enzyme activities in human body fluids are essential in clinical chemistry for diagnosis of diseases. The determination of enzyme concentrations is of importance in providing a reference point for the measurement of enzyme activity. (N. Kiba, 2005). Usually, the more amount of enzyme the faster the reaction is. The enzyme activity can be easily measured by monitoring product formation or substrate utilisation and the reaction rate is measured by a spectrophotometer.

The maximum rate of the enzyme-catalysed reaction is known as $V_{max.}$ It is theorized that when this maximum velocity had been reached, all of the available enzyme has been converted to ES, the enzyme substrate complex. (Andrew Worthington, Charles C. Worthington and Von Worthington, 2019). This means that $V_{max.}$ will only occur when all the enzyme is found as enzyme substrate complex. The substrate concentration at which this is achieved depends on how tightly the enzyme and substrate bind. We determine the substrate concentration, which will give a rate of half V_{max}, known as the K_m or Michaelis constant. A measurement of a Km for an enzyme gives the biochemist important information about how an enzyme interacts with a particular substrate.

In the first part, the aim of the experiment was to monitory the progress of an enzyme catalysed reaction using a stopped assay (hydrolysis of p-nitrophenylphosphate by alkaline phosphatase). The effect of enzyme concentration on the rate of an enzyme-catalysed reaction is also measured. For some enzyme assays, it is possible to measure the reactant or product directly based on its absorbance properties (Fersht, 1999). The relationship between absorbance and concentration is governed by the Beer-Lambert Law. The enzyme will be incubated with the substrate for times between 5 and 30 min and the progress of the reaction will be plotted. This offers the opportunity to understand the effect of incubation time on the rate of an enzyme catalysed reaction.

In the second part of the experiment, the effect of substrate concentration of the rate of reaction was analysed. The aim was to find the kinetic constants Vmax and Km from the data obtained. Different substrate concentrations can be used to measure the initial rate of the reaction. This way, the interaction between substrate and enzyme is assessed. The Michaelis Menten equation show in a more accurately way the relationship between Vmax, Km and the rate of reaction with a certain substrate concentration (vi).

Method

Part 1

12 test tubes were collected and numbered from 1 to 12. 0.3 mL of triethanolamine, 0.3 mL p-nitrophenylphosphate and 0.4 mL water were added to each one from 1 to 6, making them the control tubes. As the assay tubes, 0.3 mL of triethanolamine and 0.3 mL of p-nitrophenylphosphate were added from 7 to 12. All of the tubes were placed in a water bath at 37°C and were incubated for 5 minutes. When the time was finished, tubes 7 to 12 were removed and 0.4 mL of alkaline phosphatase was quickly added to each one. All of them were then mixed and returned to the water bath.

A single assay tube (7-12) and a single control tube (1-6) were removed at 5-minute intervals and a 4 mL aliquot of 25 mmol L^{-1} NaOH was added immediately. They all were well mixed. A plastic cuvette was filled ¾ full of the contents of one tube using a Pasteur pipette. A spectrophotometer was set to 405 nm and the absorbance was adjusted to zero on a cuvette containing water. The absorbance at 405 nm of each solution was measured and the results were recorded in tabular form in the notebook with the headings ‘Tube number’ and ‘Absorbance at 405 nm’.

Part 2

8 tubes were numbered. The following solutions were added into each tube

1. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.3 Water (mL)
2. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.3 30 mmol L^{-1}p-NPP (mL)
3. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.1 Water (mL) + 0.2 30 mmol L^{-1}p-NPP (mL)
4. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.2 Water (mL) + 0.1 30 mmol L^{-1}p-NPP (mL)
5. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.3 5 mmol L^{-1}pNPP (mL)

6. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.1 Water (mL) + 0.2 5 mmol L^{-1}pNPP (mL)
7. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.2 Water (mL) + 0.1 5 mmol L^{-1}pNPP (mL)
8. 0.3 buffer 0.1 mol L^{-1} Triethanolamine,pH 9.5 (mL) + 0.25 Water (mL) + 0.05 5 mmol L^{-1}pNPP (mL)

When the contents of each tube were thoroughly mixed, they were placed in a water bath at 37°C for 5 minutes. 0.4 ml of enzyme was added to each tube and were mixed and returned to the water bath. In every one of them the total volume was 1.0 mL at this moment.

After 20 minutes, all the tubes were removed from the water bath and 4.0 ml of 25 mmol L^{-1} NaOH was quickly added. All of them had a total volume of 5.0 mL at this moment. The spectrophotometer was set at 405 nm and it was zeroed using the contents of tube 1. The absorbance of each one was measured, and the results were recorded. The initial substrate concentration was calculated in each test tube using C1.V1 = C2.V2 in mmol L^{-1} and it was recorded in a table.

Results and calculations

The protocol given was followed.

Part 1

1. The difference in absorption between the assay and control tube was obtained by subtracting the absorbance at 405 – Assay tube from the absorbance at 405 – Control tube (0.210-0.019= 0.191)

Incubation time (min)	***Absorbance at 405 – Assay tube***	***Absorbance at 405 – Control tube***	***Assay absorbance – control absorbance***
5	*0.210*	*0.019*	*0.191*
10	*0.321*	*0.021*	*0.300*
15	*0.464*	*0.027*	*0.437*
20	*0.588*	*0.045*	*0.543*
25	*0.703*	*0.043*	*0.660*
30	*0.823*	*0.048*	*0.775*

Table 1. A table showing the results from the tubes in absorbance /time

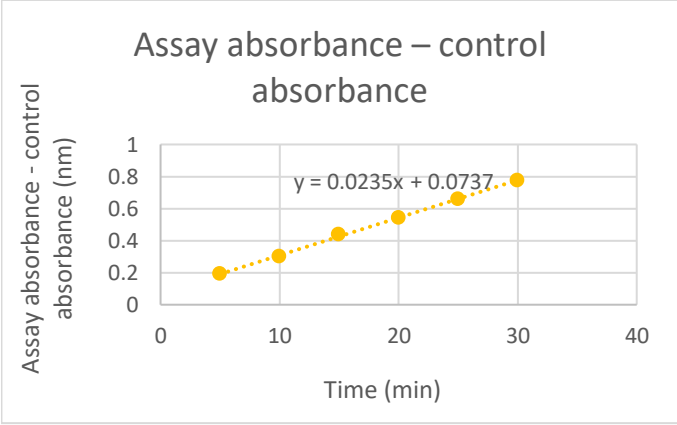

Figure 2. A graph showing the relationship between Assay absorbance – control absorbance and time (min)

2. The initial rate of the enzyme catalysed reaction is 0.0235 ΔA /min^{-1}. It is obtained by finding the gradient of the graph below of the enzyme catalysed reaction against time

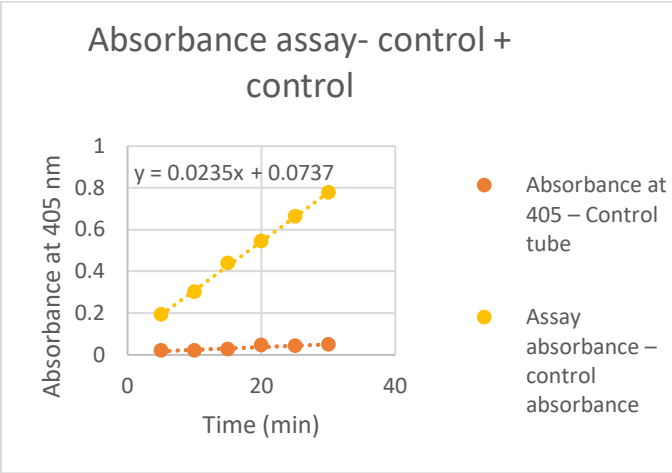

Figure 3. A graph showing the enzyme catalysed reaction against time.

3. The rate of change in absorbance was converted to the rate of change of concentration using the Lambert-Beer law:
   If $\Delta A = \varepsilon \times l \times \Delta c$ then,
   $\Delta c = \Delta A / (\varepsilon \times l) = 0.02/18.3\times10^{3} = 1.28\times10^{-6}$ mol.L^{-1}.1.min-1

4. The amount of p-nitrophenol (moles) produced per minute was calculated to find the rate of pNPP (substrate) hydrolysis as mol min^{-1}.
   1.28×10^{-6} mol.L^{-1}.1.min-1*0.005L= 6.42×10^{-9} mol min-1

5. The rate of pNPP hydrolysis of enzyme was calculated
   6.42×10^{-9} mol min-1/ 0.4 = 1.61×10^{-8}mol min^{-1} mL^{-1}

Part 2

1. We got C2 using C1.V1 = C2.V2. So C2= (C1.V1)/V2= $(30*3\times10^{-4})/1\times10^{-3}$=9
   The initial rate v_i (ΔA min^{-1}) was calculated dividing the absorbance by the time, 20 minutes in this case. (0.327/20min= 0.016 ΔA min^{-1}).

Tube No.	Substrate concentration [S] (mmol-1 L)	Absorbance	ΔA min^{-1}
1	0	0	0
8	0.25	0.327	0.016
7	0.5	0.415	0.021
6	1	0.496	0.025
5	1.5	0.510	0.026
4	3	0.536	0.027
3	6	0.585	0.029
2	9	0.599	0.030

Table 2. A table showing the absorbance obtained from the results, the substrate concentration and the initial rate.

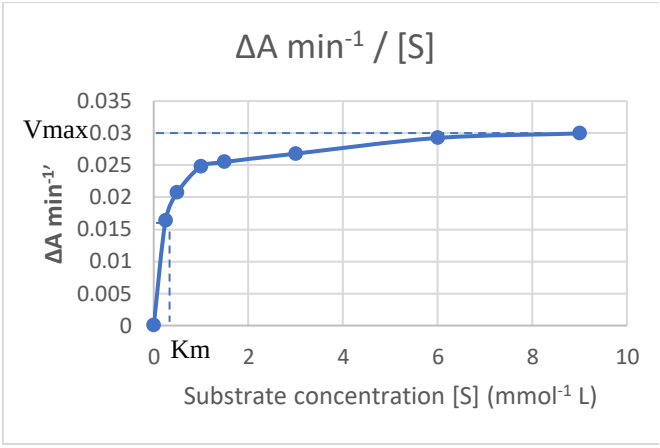

Figure 4. A graph showing the initial rate v_i (Δ A min^{-1}) against substrate concentration (s)

2. The values Vmax and km were estimated from Figure 4
   Vmax = 0.029 mol min^{-1}
   Km= 0.250 mmol^{-1} L

3. The values 1/ ΔA min^{-1} were calculated by dividing 1 by ΔA min^{-1} (1/0.016=62.5 ΔA min^{-1})
   The values 1/[S] (mmol^{-1} L) were calculated by dividing 1 by [S] (mmol^{-1} L) (1/0.25=4 mmol^{-1} L)

Tube No.	ΔA min^{-1}	1/ ΔA min^{-1}	[S] (mmol^{-1} L)	1/[S] (mmol^{-1} L)
1	0	0.000	0	0
8	0.016	62.500	0.250	4.000
7	0.021	47.619	0.500	2.000

6	0.025	40.000	1.000	1.000
5	0.026	38.462	1.500	0.667
4	0.027	37.037	3.000	0.333
3	0.029	34.483	6.000	0.167
2	0.030	33.333	9.000	0.111

Table 3. A table showing the initial rate, $1/v_i$, the substrate concentration (s) and 1/(s).

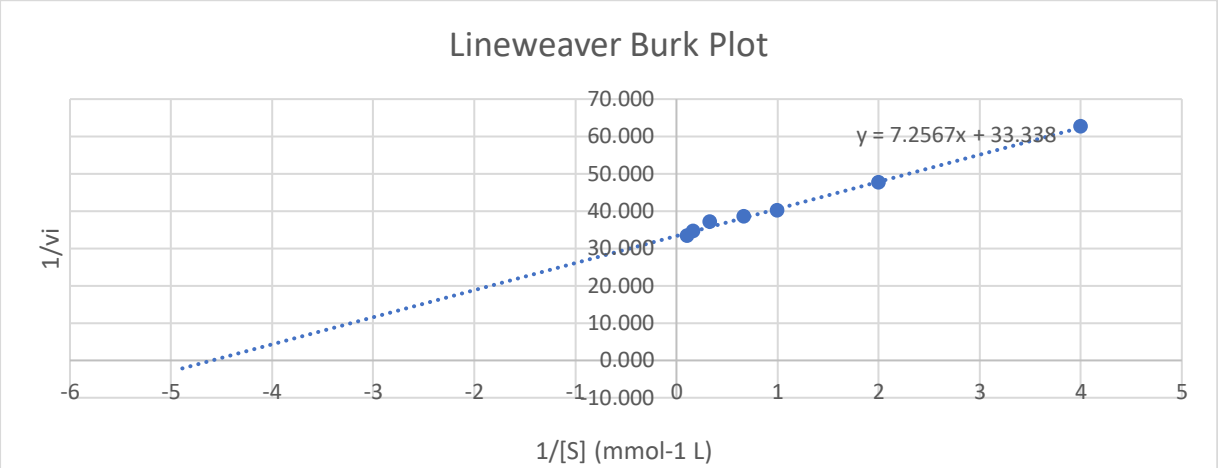

Figure 5. The Lineweaver Burk plot showing the relationship 1/ v_i against 1/(s)

The values of Vmax and Km were calculated by using a rearranged version of the Michaelis Menten equation:

$\frac{1}{vi} = \frac{km}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$; y = mx+c ; y = 7.2567x + 33.338

C = $\frac{1}{V_{max}} = 33.338$ $V_{max} \approx 0.030$ mol min^{-1}

$m = \frac{km}{V_{max}} = 7.2567$ $km = 7.2567 \times 0.029996 = 0.218$ $mmol^{-1}$ L

Discussion

Part 1

As it was mentioned in the introduction, enzymes speed up chemical reactions. In table 1, the assay tubes present highest absorbance than the control tubes. Using the Lambert-Beer law, we can state the relationship between the concentration and the absorbance of the solution. Looking at Figure 3, the initial rate of the enzyme catalysed reaction is greater than the initial rate of the reaction in the control tubes. Consequently, more product is formed at the same time when the enzyme is present. This means that there is a significant hydrolysis of pNPP in the presence of enzyme since the product is formed at a faster speed, supporting the hypothesis and the theory of the experiment.

Some problems occurred while the experiment was being conducted and the final results in tubes 7-12 couldn't be used. The instructor suggested some other data as a refence

and those were used in this report. The problem was probably the pipette used to add the 0.4 mL of alkaline phosphatase. A P1000 was selected instead of a P200. The last one would be more accurately to measure the small aliquot and errors could have been avoided.

Part 2

In the second part of the experiment, we have used a rearranged version of the Michaelis Menten equation and figure 4 to estimate the values of Vmax and Km. Almost the same results were obtained from the graphs. However, the lineweaver burk plot is slightly more accurately since the values are not being estimated directly from the graph, but from a rearranged version of the Michaelis Menten equation. The value of Km is low; therefore, the enzyme-substrate interaction is tight. As a result, the enzyme will act at a more or less constant rate, regardless of variations in the concentration of substrate. Like the theory explains, if the binding is tight, V_{max} will occur at low substrate concentrations. This is shown in figure 5.

This part of the experiment could not be carried out in the laboratory because of time limitations. Even though some practical experience was lost, the data to make the calculations and the results was provided and the kinetic data could be analysed to find the kinetic constants V_{max} and Km, meeting the aim of the experiment.

Conclusions

In summary, an enzyme does increase the rate of the reaction. The aims of the experiment were met, and the results agreed with the theories previously mentioned. The hydrolysis of pNPP in the presence of enzyme was faster than the hydrolysis without the enzyme. And the kinetics constants were obtained and showed that the enzyme-substrate interaction is tight, and V_{max} will occur at low substrate concentrations.

References

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