Inhibition of Human Placental Alkaline Phosphatase by L-Phenylalanine

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ABSTRACT

Human placental alkaline phosphatase (AP) is an enzyme that catalyzes the removal of phosphate and it can be inhibited by L-Phenylalanine (Hoylaerts et al., 1992). The purpose of the project is to investigate the kinetic parameters of human placental AP in the presence and absence of L-Phenylalanine. NPP was used as a substrate in order to get a colored product which allows the measurement of its concentration using spectrophotometry at 450 nm. We hypothesize that L-phenylalanine will be an efficient uncompetitive inhibitor of human placental AP, and that will be determined by a decrease in V_{max} and K_m upon inhibition, and calculated from the Lineweaver-Burke Plot. The results show that the Km value decreased in the presence of 50mM L-Phenylalanine as expected for uncompetitive inhibition. Km insignificantly decreased from 0.269 ± 0.051 mM to 0.224 ± 0.141. However, the Vmax showed a significant increase in the presence of the inhibitor from 0.176 ± 0.180 (µmol/min) to 0.656 ± 0.109 (µmol/min). These observations do not allow us to confirm that L-Phenylalanine is an uncompetitive inhibitor at the concentration of 50mM.

INTRODUCTION

There are four tissue-specific isozymes, or isoforms, of alkaline phosphatase that arise from alternative splicing transcripts. The family of alkaline phosphatase (AP) isozymes includes AP that originates from the liver, bone, intestine, and placenta (Fishman, 1987). The tissue-specific isozyme that will be of interest in this research project is the human placental alkaline phosphatase.

As the role of human placental AP is being discovered, it has become important to find ways to inhibit its activity as its activity has been correlated with the progression of ovary, testis, lung and colorectal tract tumors (Fishman et al., 1976). Inhibition of an enzyme is studied by specific kinetic parameters, including K_m and V_{max}. K_m is the substrate concentration at which the enzyme is catalyzing the reaction at half its maximum velocity (V_{max}). There are three types of enzyme inhibition: competitive, non-competitive, and uncompetitive. Previous research has shown that L-phenylalanine uncompetitively inhibits human placental AP as it binds to the phosphoseryl intermediate of the enzyme at a peripheral binding site located 28 A˚ away from the catalytic site (Llinas et al., 2005). Uncompetitive inhibition occurs when the inhibitor binds to the enzyme-substrate complex, and reduces the K_m and V_{max} (Palmer & Bonner, 2007). We expect V_{max} to decrease because the inhibitor will bind to the enzyme-substrate complex and stop the reaction from continuing. In addition, we expect K_m to decrease because the inhibitor stabilizes the enzyme-substrate complex and makes it difficult for the substrate to dissociate or become converted to product (Dougall & Unitt, 2015).

METHODS

Enzyme Saturation Curve

The purpose of this curve is to find the enzyme concentration that does not limit the rate of the reaction. Reactions were prepared with constant concentration of pNPP substrate (11.2 mM) and varying concentration of human placental alkaline phosphatase from 0.5 to 5 units. In addition Diethanolamine buffer containing 0.50 mM Magnesium Chloride was added bringing up the final volume to 300 uL. Then the absorbance @450 nm of the yellow product was measured over at one second intervals for three minutes using the spectrophotometer. The initial velocity (Vo) was calculated by subtracting the absorbance values then dividing by the time interval. A graph of rate of reaction in absorbance/minute was plotted against enzyme units.

Reactions without Inhibitor

Michaelis-Menton Plot

Reactions were prepared with the enzyme units chosen from the previous step (2 units) while varying the concentration of the pNPP substrate from 0.1 to 0.05 mM and adding buffer to bring the volume up to 300 uL. The absorbance at 450 nm was measured over time as previously. A graph of velocity in umol/min was plotted against substrate concentration in mM.

Lineweaver-Burk Plot

The purpose of this plot to find the Km value which is the concentration of substrate at which the velocity of the reaction is half the maximum velocity. The results from the reactions done in the previous step were used to make generate this plot. A graph of 1/velocity in min/umol was plotted against 1/ substrate concentration in mM.

Reactions with Inhibitor

A Lineweaver-Burk plot was created using a series of reactions where alkaline phosphatase was kept constant at 2 units and the inhibitor concentration was kept constant at 50 mM while varying the substrate concentration from 0.05 mM to 1 mM. The absorbance at 450 nm was measured over time as previously. Then a graph of 1/velocity in min/umol was plotted against 1/substrate concentration in mM^{-1} which is the Lineweaver-Burk plot.

RESULTS

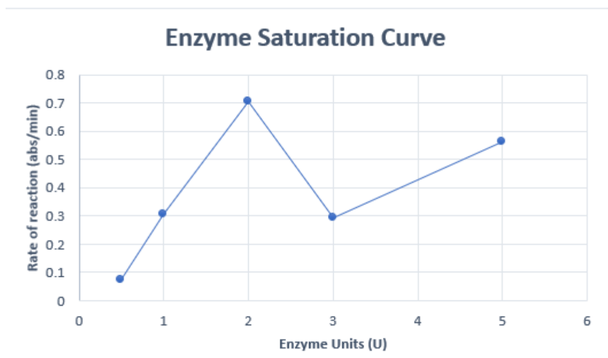

Figure 1 Enzyme Saturation Curve for Human Placental Alkaline Phosphatase. To find the enzyme concentration that is not rate limiting, the substrate PNPP concentration was held constant at 11.2mM with varying the enzyme units (0.5U – 5U) to determine reaction velocity at each enzyme unit. The reaction was performed in 1.0 M Diethanolamine Buffer with 0.50 mM Magnesium Chloride, pH 9.8 at 37°C. The final reaction volume was 300µl. Absorbance at 450nm was measured at 1 second intervals for 3 minutes using UV-Vis Spectrophotometer.

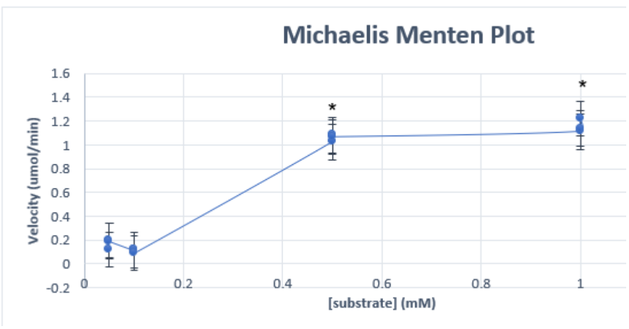

Figure 2 Michaelis Menten Plot. Human placental alkaline phosphatase was held constant at 2U while the PNPP concentration was varied (0.05mM, 0.1mM, 0.5mM, and 1mM), and the rate of the reaction was measured using microplate reader in triplicates. Km is the substrate concentration at ½ Vmax. t-test was performed to determine significance if $p<0.05$. The reaction was performed in 1.0 M Diethanolamine Buffer with 0.50 mM Magnesium Chloride, pH 9.8 at 37°C. *significant

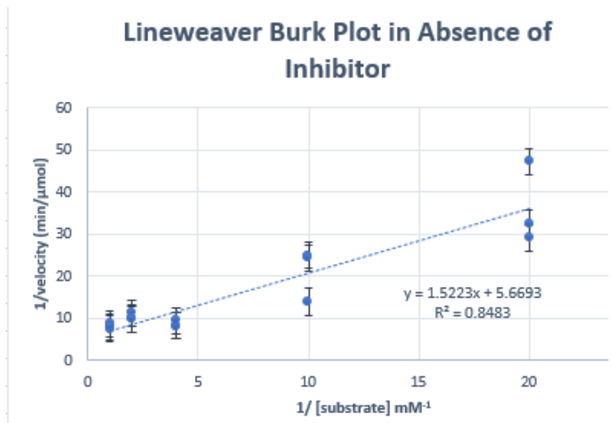

Figure 3 Lineweaver Burk Plot in Absence of Inhibitor. Human placental alkaline phosphatase was held constant at 2U while the PNPP concentration was varied (0.05mM, 0.1mM, 0.5mM, and 1mM), and the rate of the reaction was measured using microplate reader in triplicates. This is to identify Km and Vmax as the y-intercept is 1/Vmax while the x-intercept is -1/Km. t-test was performed to determine significance if $p<0.05$. The reaction was performed in 1.0 M Diethanolamine Buffer with 0.50 mM Magnesium Chloride, pH 9.8 at 37°C. *significant

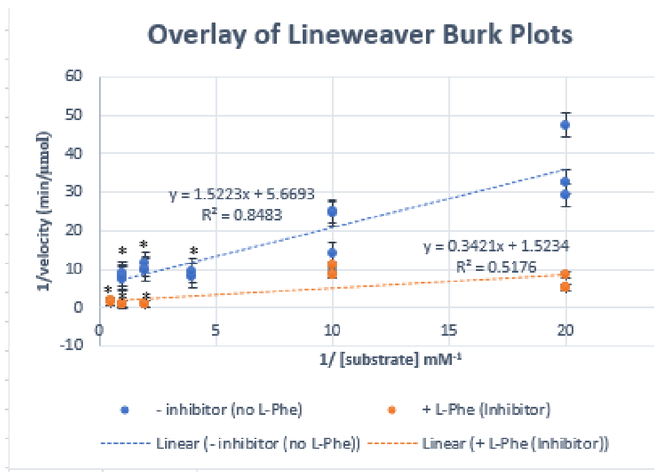

Figure 4 Overlay of Lineweaver Burk Plot in absence and presence of 50mM L-Phenylalanine inhibitor. Human placental alkaline phosphatase was held constant at 2U and the PNPP concentration was varied (0.05mM, 0.1mM, 0.5mM, and 1mM) in the presence of 50mM L-Phenylalanine. t-test was performed to determine significance if $p<0.05$. The reaction was performed in 1.0 M Diethanolamine Buffer with 0.50 mM Magnesium Chloride, pH 9.8 at 37°C. The final reaction volume was 200 µl. *significant

Table 1 Comparison of Vmax and Km Values in the presence and absence of L-Phenylalanine

	Vmax (µmol/min)	Km (mM)
Absence of L-Phenylalanine	0.176 ± 0.180	0.269 ± 0.051
Presence of 50mM L-Phenylalanine	0.656 * ± 0.109	0.224 ± 0.141

ANALYSIS

- As observed in Figure 1, the maximum rate of reaction was 0.704 abs/min for 2U of human placental alkaline phosphatase. The curve was expected to plateau, but that was not observed especially because enzyme units between 2 and 3 were not tested. For further reactions, the enzyme units chosen were 2U.
- Since L-Phenylalanine is known as an uncompetitive inhibitor (Llinas et al., 2005) , it is expected to reduce the Vmax. However, as observed on Table 1, the Vmax increased from 0.176 µmol/min to 0.656 µmol/min in the presence of 50mM of L-Phe inhibitor. Through, t-test analysis this was found to be a significant increase as the p-value is 0.005.
- As expected by an uncompetitive inhibitor, the Km was reduced from 0.269mM to 0.224mM in the presence of 50mM of L-Phe inhibitor, but this was an insignificant decrease as the p-value is 0.642. Since the p-value is greater than 0.05, there is weak evidence to reject the null hypothesis. Thus, future research should include more data replicates.
- The significant increase of Vmax in the presence of the inhibitor is unexpected, and most likely due to technical errors in making the replicates. Thus, the experiment should be repeated with more replicates.

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