Wheat Germ Acid Phosphatase Catalyzed Hydrolysis of Para-Nitrophenyl Phosphate: Enzyme Kinetics and Inhibition by Ammonium Molybdate

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ABSTRACT: Wheat germ acid phosphatase hydrolysis of para-nitrophenylphosphate was analyzed and the kinetics of both an inhibited and an uninhibited reaction were determined. Standard curves were constructed using the product, para-nitrophenol. An extinction coefficient of 6.02 μmol·cm⁻¹ was used in the pH determination. An extinction coefficient of 5.78 μmol·cm⁻¹ was used in kinetics determination. The optimum pH of the reaction was approximately 5.0, though the reaction was run using a citrate buffer with a pH of 4.52. The trials for the enzyme kinetics were analyzed in both a Michaelis-Menten plot and a Lineweaver-Burk plot. The Michaelis-Menten plot was used to calculate the following: $K_m$ was 5.00x10⁻⁴ M, $k_{cat}$ was 38.4 sec⁻¹, and the $V_{max}$ was 0.768±.0039 μmol/min. The $k_{cat}/K_m$ was 76800 sec⁻¹ M⁻¹. The Lineweaver-Burk plot gave a $K_m$ of 4.71x10⁻⁴ M, a $V_{max}$ of 0.0724 μmol/min, and a $k_{cat}$ of 36.3 sec⁻¹. In the presence of an inhibitor, the $K_i$ was 1.41 x10⁻⁴ M and the $K_i'$ was 1.46 x10⁻² M. The $k_{cat}/K_m$ ratio was 76900 sec⁻¹ M⁻¹. From the values of both the Michaelis and Menten plot and the Lineweaver-Burk plot, the type of inhibition of wheat germ acid phosphatase by molybdate was mixed non-competitive inhibition.

EXPERIMENTAL PROCEDURES

The procedure published by Pugh, et al. (1) was followed. Deviations from standard procedure are summarized as follows:

Citrate Buffer

The citrate buffer used in the experiment was 0.1 M citrate at pH 4.52. It was made by ten-fold dilution of a 1.0 M citrate buffer of the same pH with deionized water.

Incubation Temperature

The water bath had temperature fluctuations over the course of the incubation. The range of temperature was anywhere from as low as 34°C to as high as 38°C. These fluctuations may have altered enzyme activity.

Enzyme Inhibition

The inhibition of the wheat germ acid phosphatase was achieved using a 10.0 mM solution of ammonium molybdate.

Inhibited Kinetic Constant Determination

The full set of the inhibited reaction quench tubes were not analyzed. In incubation sample 1 and incubation sample 2, the 0 and 10 minute quench tubes were not measured due to procedural error.

RESULTS AND DISCUSSION

In order to properly analyze the hydrolytic activity of the wheat germ acid phosphatase on the substrate para-nitrophenylphosphate (p-NPP), the optimum pH of the catalysis was established. The determination was made by comparing the
rate of product formation to a standard curve constructed with the known product of the reaction, para-nitrophenol (p-NP).

**Standard Curves.** The standard curves were constructed using the product of the catalysis, p-NP, in a 0.02M solution of NaOH. When the p-NP was placed into a strong basic solution it was converted to a para-nitrophenolate anion, which absorbs strongly at 405 nm (1). The same process was present in the quenching of the p-NPP hydrolysis reaction. Since the product that was formed in the catalysis reaction was p-NP, a comparison between the amount of product formed and the standard curve was possible.

As shown in Figure 1, the standard curve used in the pH trials gave an extinction coefficient of 6.02 μmol⁻¹cm⁻¹. The data used to construct the graph had a linear relation and fit the trend with an R² value of 0.9989, indicating a very strong correlation. With the data points representing a large range of values, from an absorbance of 0.0 up to 0.890, the standard curve had a large range of values that it could encompass. The absorbance values of the pH trials ranged from a minimum of 0.08 to a maximum of 0.3831. These values fell within the standard curve, allowing the use of Beer’s law to accurately determine the amount of product in the trials.

Figure 1 also describes a second standard curve used in the enzyme kinetics trials. The extinction coefficient of 5.78 μmol⁻¹cm⁻¹ was given by the slope of the line. The kinetics trials presented findings with a range of absorbances from: 0.49 to 0.86 in the uninhibited trial, and 0.039 to 0.36 in the inhibited trial. These values fell within the range of the standard curve, making it possible to accurately calculate the μmoles of product in the trials. With the standard curve having an R² value of 0.9993, the values calculated from this trend would reflect the actual values with 99.9 percent certainty.

The two standard curves were created with the exact same procedure, and are very similar. The likeness of the two, and the consistency of the significant R² values, indicated that both would return consistent results. Despite the difference in extinction coefficients of 0.24, the reliability of the curves was still significant enough to make supported comparisons.

**Determination and Comparison of Optimal pH.** The enzymatic activity of the wheat germ acid phosphatase, as with any enzyme, was affected by pH. A series of reactions
were run in different pH environments to determine at which pH the wheat germ acid phosphatase catalyzed the hydrolysis of p-NPP the most efficiently. With the general range of the optimum enzyme activity being clustered around a pH of about 5, the most attention was paid to the initial velocities of reaction from pH 4.5 to pH 5.5.

![Figure 2: Comparison of Reaction Velocities of Wheat Germ Acid Phosphatase Catalyzed Hydrolysis of p-NPP at Different pH Values.](image)
The trend of $V_0$ across different pH values indicated the point at which the enzyme activity was the greatest. The trend shown above indicated the highest enzymatic activity at a pH of 5, where the $V_0$ was 0.0318 μmoles/minute.

The pH at which the enzyme was most active at catalyzing the hydrolysis of p-NPP was 5.0, shown in Figure 2. This pH differed from that of the Joyce and Grisolia study, which determined the optimum catalysis pH of a crude fraction containing the p-NPP to be 5.8 (2). The two values differed by a factor of nearly 10. The difference of the two values could be due to the nature of the Joyce and Grisolia study which did not test only p-NPP as a substrate, but instead tested a range of substrates to determine the specificity of the enzymatic activity with regard to substrate. Since the specificity of the enzyme in their paper determined the phospho-diesterase activity of the enzyme to be poor, using the p-NPP as a substrate would not give the actual optimum pH of the enzyme (2). The lesser degree of catalysis of the p-NPP would, therefore, result in a different optimum pH as the activity of the enzyme in hydrolyzing p-NPP is not representative of the optimum pH of the enzyme catalyzing hydrolysis of 3-P-glycerate.

The activity of the wheat germ acid phosphatase must be different at different pH values, a more acidic environment favoring phospho-diesterase activity and a more basic environment favoring hydrolytic activity. The specific substrates that the enzyme catalyzes are more favorably catalyzed at a pH specific to that substrate.

Once the optimum pH for the p-NPP catalysis activity was determined, the enzyme kinetics were studied. Two trials of the reaction were completed, one was an uninhibited reaction over a range of substrate concentrations, while the second was inhibited with 10.0 mM molybdate over that same range of substrate concentrations. In this manner, the extent of the enzyme inhibition was studied, and a mechanism of the inhibition was identified.
The Michaelis and Menten plot is shown in Figure 3. Both the inhibited and uninhibited curves reflect a hyperbolic relation of the initial velocity of the reaction with the concentration of the substrate. The curves were created using the Michaelis and Menten equation which takes the form of 
\[ v = \frac{V_{\text{max}}[S]}{K_m+[S]} \].

Though some of the values at the lower concentrations, namely those at [0.00125] and [0.0025], had lower initial reaction velocities than those anticipated by the hyperbolic trend, the uninhibited reaction date returned a fairly significant \( R^2 \) value of 0.9801. From the \( R^2 \) value it was shown that the data held a fairly strong correlation to the derived Michaelis-Menten equation which was \( y_u = 0.0768x / (0.0005+x) \).

Figure 3 shows that the kinetics of the uninhibited reaction behave as a first order reaction initially, but as the substrate concentration reaches a certain point, the enzyme can only catalyze reactant at a zero order rate as the enzyme had become saturated.

The inhibited reaction was also depicted in Figure 3. The data was treated in the same manner as the uninhibited reaction, using the Michaelis and Menten equation to describe the relation of the data points. The equation \( y_i = 0.0581x / (0.0082+x) \) was thusly derived. In this case, though, the reaction did not follow the same trend as in the uninhibited trial; the first order reaction behavior does not shift to zero order. The substrate range prevented the overall trend from being known, though, as the reaction would have eventually transitioned into a zero order reaction.

From Figure 3, the mechanism of inhibition was not readily apparent. The only conclusion that could be made directly from the Michaelis and Menten plot was that there had indeed been some sort of inhibition taking place. Though the plot was not necessarily informative outright, its values were used to calculate the most accurate kinetics values.

\[ y_u = 0.0768x/(0.0005+x) \quad R^2 = 0.9801 \]

\[ y_i = 0.0581x/(0.0082+x) \quad R^2 = 0.9834 \]
Lineweaver-Burk Plot. Even though the values of the Lineweaver-Burk plot were not as representative of the trend as those of the Michaelis and Menten plot, a much more diagnostic depiction of the type of inhibition was represented in the Lineweaver-Burk plot.

Figure 4 presents the Lineweaver-Burk plot of the inhibited and uninhibited hydrolysis of p-NPP by the wheat germ acid phosphatase. The graph was created using the reciprocals of the substrate concentration and the initial reaction velocity, indicated by the axis titles on the figure.

The uninhibited reaction was described by the line $y_u = 0.0065x + 13.789$. The data used to plot this equation fit the line with an $R^2$ value of about 0.91, which showed that the data was not a perfect fit but was still a positive correlation. Even though the $R^2$ value was not extremely persuasive that the equation fit the trend well enough for a strong depiction of the curve, the graph still showed a fairly consistent linear relation of the points.

The inhibited reaction was much more of an optimum fit with the data fitting the equation $y_i = 0.3758x - 21.343$ with an $R^2$ value of 0.93. The point at 800 M\(^{-1}\) appears to be an outlier from the trend established in the first three samples. The skew of the data towards an increased slope could be due to the aforementioned outlier. The missing point at the lowest concentration of substrate, or the highest $1/[S]$ value, may have drastically affected the line of the uninhibited reaction as well. These two factors may have altered the representative power of the plot by altering the intersection of the points such that they were not as strong indicators of the type of inhibition. In each instance of inhibition on Lineweaver-Burk plots, the $y$-intercepts had stayed positive, whereas in this plot, the inhibited reaction had a $y$-intercept of -21.343. With the loss of the suspect point at 800 M\(^{-1}\) the mechanism of inhibition agrees most with mixed non-competitive inhibition. The three points less than 500 M\(^{-1}\) anticipate an intersection taking place in the second quadrant instead of the first quadrant as is observed in the depicted line of best fit for the inhibited reaction.

The graphical representations of the inhibition mechanism are only qualitative measures of the inhibition. From both the Michaelis-Menten values and the Lineweaver-Burk values, the quantitative measures of the kinetics and inhibition of the reaction describe the findings in a more empirically sound manner.
Comparison of Kinetic Values. Table 1 shows values from Joyce and Grisolia (2), and kinetics values calculated from the Michaelis-Menten plot and Lineweaver-Burk plot. According to the Michaelis-Menten plot the $K_m$ was $5.00 \times 10^{-4} \pm 1.00 \times 10^{-4}$ M, and according to the Lineweaver-Burk plot the $K_m$ was $4.71 \times 10^{-4}$ M. These values were used to determine the dissociation constant of the formation of the enzyme-substrate complex (1). Therefore, it demonstrated how well an enzyme binds to a substrate. A comparison of the $K_m$ values for how wheat germ acid phosphatase and p-NPP and other substrates were used to determine which substrate was best at forming an enzyme-substrate complex.

The $K_m$s taken from Joyce and Grisolia (2) were $2.7 \times 10^{-4}$ M for both 3-P-Glycerate and 2,3-diP-glycerate. The calculated $K_m$s for p-NPP were approximately the same as the $K_m$s for 3-P-Glycerate and 2,3-diP-glycerate.

According to Lineweaver-Burk plot the $K_m$ was $4.71 \times 10^{-4}$ M and according to the Michaelis-Menten plot the $K_m$ was $5.00 \times 10^{-4}$ M. These values further support that wheat germ acid phosphatase is a non-specific enzyme. This also indicates that wheat germ acid phosphatase was better at

### Table 1: A Comparison of Different Kinetics Values Calculated from the Michaelis-Menten Plot and the Lineweaver-Burk Plot, and Kinetic Values from Joyce and Grisolia (2).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Michaelis-Menten</th>
<th>Lineweaver-Burk</th>
<th>Joyce and Grisolia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (M)</td>
<td>$V_{max}$ (μmoles/min)</td>
<td>$V_{max}$ (μmoles/min)</td>
</tr>
<tr>
<td>p-NPP</td>
<td>$5.00 \times 10^{-4} \pm 1.00 \times 10^{-4}$</td>
<td>0.0768±0.004</td>
<td>0.0725</td>
</tr>
<tr>
<td>p-NPP</td>
<td>$4.71 \times 10^{-4}$</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>$2.7 \times 10^{-4}$</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>2,3-diP-glycerate</td>
<td>$2.7 \times 10^{-4}$</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>$K_i$ (M)</td>
<td>n/a</td>
<td>$1.41 \times 10^{-4}$</td>
<td>n/a</td>
</tr>
<tr>
<td>$K_i'$ (M)</td>
<td>n/a</td>
<td>n/a²</td>
<td>n/a</td>
</tr>
</tbody>
</table>

1. Actual value was not given in paper but author claimed this $K_m$ was approximately the same value as the $K_m$ for 3-P-glycerate.
2. This value could not be calculated because the y intercepted on the Lineweaver-Burk plot for the uninhibited wheat germ acid phosphatase was negative.
metabolizing p-NPP than both 3-P-Glycerate and 2,3-diP-glycerate.

The Michaelis-Menten plot had a $V_{\text{max}}$ of 0.768±0.004μmoles/min, and the Lineweaver-Burk plot had a $V_{\text{max}}$ of 0.0724 μmoles/min. $V_{\text{max}}$ indicated the rate that product formed when an enzyme was saturated with substrate (1).

The $k_{\text{cat}}$ for the Michaelis-Menten plot was 38.4 sec$^{-1}$ and the $k_{\text{cat}}$ for the Lineweaver-Burk plot was 36.3 sec$^{-1}$. These calculated $k_{\text{cat}}$ were the turnover rate of the enzyme (3). In other words, it told how fast the enzyme-substrate complex dissociated into enzyme and product.

The $k_{\text{cat}}/K_{\text{m}}$ for the Michaelis-Menten Plot and the Lineweaver-Burk plot were 76800 sec$^{-1}$ M$^{-1}$ and 76900 sec$^{-1}$ M$^{-1}$ respectively. The $k_{\text{cat}}/K_{\text{m}}$ ratio was the understand the cumulative effect of the binding affinity and catalytic rate of wheat germ acid phosphatase and p-NPP (3).

When an inhibitor was present the $K_{\text{i}}$ 1.41 x10$^{-4}$ M . The $K_{\text{i}}'$ could not be calculated because the Lineweaver–Burk plot of inhibited wheat germ acid phosphatase had negative y-intercept. The $K_{\text{i}}$ calculated for the Lineweaver-Burk Plot. The $K_{\text{i}}$ and $K_{\text{i}}'$ were the dissociation constant of the inhibitor and the enzyme (1). $K_{\text{i}}$ is the dissociation constant between the enzyme-inhibitor complex, and $K_{\text{i}}'$ is the dissociation constant for the enzyme-substrate-inhibitor complex. A comparison of these values could have been used to determine what type of inhibition was present. However, since $K_{\text{i}}'$ was not able to be calculated, this was not possible.

Additionally, the kinetics values obtained from Lineweaver-Burk plot and Michaelis-Menten plot were approximately the same. This indicates a consistency between the trends observed in the both of these plot. This also demonstrates that the Lineweaver-Burk plot slope is fairly accurate. An inaccurate slope is a common problem with Lineweaver-Burk plots (1). Thus, the $K_{\text{i}}$ calculated should be accurate. Plus, the inhibited $K_{\text{m}}$, $V_{\text{max}}$, and $k_{\text{cat}}$ obtained from the Lineweaver-Burk plot and Michaelis-Menten plot were used to determine what type of inhibition of wheat germ acid phosphatase was caused by ammonium molybdate.

*Determining the type of inhibition.* $V_{\text{max}}$, $k_{\text{cat}}$, and $K_{\text{m}}$ experienced inhibition. According to Michaelis-Menten plot the inhibited $K_{\text{m}}$ was 0.0176 M, $V_{\text{max}}$ was 0.0469 μmoles/min, and $k_{\text{cat}}$ was 23.4 sec$^{-1}$. According to Lineweaver-Burk plot the inhibited $K_{\text{m}}$ was 0.0082 M, $V_{\text{max}}$ was 0.0581 μmoles/min, and 29.1 $k_{\text{cat}}$ was 29.05 sec$^{-1}$. This indicates mixed inhibition because all of these values decreased. This was concluded because both $K_{\text{m}}$ and $V_{\text{max}}$ were affected by the inhibitor (1).

*Conclusion.* In summary, the results indicates that the Michaelis-Menten plot was better at obtaining accurate kinetic values, but the Lineweaver-Burk gave a better visual representation of the type of inhibition present. The optimum pH for wheat germ acid phosphatase was approximately 5.0. Finally, molybdate causes wheat germ acid phosphatase to undergo mixed non-competitive inhibition.

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REFERENCES

