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An Additional Method for Analyzing the Reversible Inhibition of an Enzyme Using Acid Phosphatase as a Model

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Abstract: Using wheat germ acid phosphatase and sodium orthovanadate as a competitive inhibitor, a novel method for analyzing reversible inhibition was carried out. Our alternative approach involves plotting the initial velocity at which product is formed as a function of the ratio of substrate concentration to inhibitor concentration at a constant enzyme concentration and constant assay conditions. The concept of initial concentrations driving equilibrium leads to the chosen axes. Three apparent constants can be derived from this plot: K_{max}, K_{min}, and $K_{inflect}$. K_{max} and K_{min} represent the substrate to inhibitor concentration ratio for complete inhibition and minimal inhibition, respectively. $K_{inflect}$ represents the substrate to inhibitor concentration ratio at which the enzyme-substrate complex is equal to the inhibitory complex. These constants can be interpolated from the graph or calculated using the first and second derivative of the plot. We conclude that a steeper slope and a shift of the line to the right (increased x-axis values) would indicate a better inhibitor. Since initial velocity is not a linear function of the substrate/inhibitor ratio, this means that inhibition changes more quickly with the change in the [S]/ [I] ratio. When preincubating the enzyme with substrate before the addition of inhibitor, preincubating the enzyme with inhibitor before the addition of substrate or with concurrent addition of both substrate and inhibitor, modest changes in the slopes and y-intercepts were obtained. This plot appears useful for known competitive and non-competitive inhibitors and may have general applicability.

Keywords: Acid phosphatase, enzyme kinetics, inhibition mathematical model.

1. INTRODUCTION

Quantitative assessment of the inhibition of enzymatic reactions has implications in a wide variety of important areas such as the biochemical, pharmaceutical, medical, veterinarian, and agricultural fields. These fields collaborate in order to investigate new solutions to common problems. Therefore, it is important for the biochemical field to have graphical methods for determining the degree of enzyme inhibition thus allowing information to be easily interpreted among several fields. These graphic methods generally involve Michaelis and Menten assumptions [1] under first order product formation conditions (v_o). If the v_o as a function of changing substrate concentration plots as a hyperbolic curve, linear transformations (such as Lineweaver-Burk [2], Eadie-Hofstee [3], Hanes-Wolfe [4], among others) have been used to assess the type and extent of inhibition by reversible inhibitors. The IC50, which compares inhibitors by reporting inhibitor concentrations that result in 50% inhibition at a given enzyme and substrate concentration, is also widely used. The Dixon plot [5] is used to empirically determine a K_i value. Cornish-Bowden [6] reported the use of S/v_o plotted against [I] as a more useful graph than the Dixon plot. These methods have been compared by Dowd and Riggs [7] and Atkins and Nimmo [8]. In general these graphs involve use of v_o (in some form) on the y-axis with substrate (in some form) on the x-axis using several different inhibitor concentrations or using inhibitor concentrations (in some form) on the x-axis. The use of the three important participants, (enzyme, substrate and inhibitor) is not directly compared as independent variables. The types of reported values include the IC50 value, the K_i value, and the calculation of the kcat/Km ratio with and without the presence of a suspected inhibitor. In general, however, these approaches focus on the inhibitor concentration effects and not the substrate and enzyme concentrations and how their changes may affect the kinetic parameters measured. Thus these graphical analyses ignore one or more key variables for inhibition. None of these well-established plots directly compare substrate or enzyme concentrations with inhibitor concentrations. Conceptually for a competitive inhibitor, we have substrate and inhibitor competing for the same enzyme active site leading to apparent inhibition dependent on the ratio of substrate to inhibitor. For uncompetitive inhibition or negative allosteric modulation, it is assumed that this inhibition is dependent on the ratio of enzyme to inhibitor. Therefore, while these methodologies have been widely used, the development of new models is important to increase our understanding of the complexities of enzyme inhibition. Thus we present an experimental approach in which we vary inhibitor concentrations and measure initial velocities; these data are then presented as a function of the molar ratio of enzyme/inhibitor or substrate/inhibitor to assess line trends which are then related to their effectiveness. To do this, we used a standard enzyme, wheat germ acid phosphatase (EC# 3.1.3.2) with the artificial substrate, *para*-nitrophenylphosphate, incubated with and without sodium orthovanadate previously reported to be a competitive inhibitor of the enzyme as a model system [9]. We also assessed the order of additions of substrate and inhibitor on initial velocity.

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2. MATERIALS AND METHODS

2.1. Wheat Germ Acid Phosphatase Kinetic Studies

Wheat germ acid phosphatase, type 1, was purchased from Sigma and used at a final concentration of 1.2 µM in all assays. A time dependent assay at 37°C was performed in a total reaction volume of 1.0 mL containing 0.5 M sodium acetate buffer (pH=4.5). The substrate used was *para*-nitrophenylphosphate (*p*Npp), and the reaction was stopped with the addition of 100 µL of 10 M sodium hydroxide. Product formation was measured by spectroscopy at 405 nm (ε=18,000$M^{-1}cm^{-1}$)[10]. Kinetic studies were performed by varying substrate concentration (4 µM-1.8 mM) under apparent first order reaction conditions (30 minutes, *p*Npp; 22.8 µM) and steady state assumptions [11]. All reactions were performed in triplicate and data reported as mean ± standard deviation (SD of 5% or less not shown).

2.2. Sodium Orthovanadate Inhibition Studies

Using the assay conditions employed in the kinetic studies, enzyme assays were performed with substrate concentrations 45 µM, 90 µM, 228 µM, and 456 µM (Half K_m, K_m, V_{max} Condition 1, and 2* V_{max} Condition 1, respectively) with varying inhibitor concentration. The inhibitor used for these studies was sodium orthovanadate (Na_3VO_4) from Acros Organics. Inhibitor concentrations were chosen on a log scale ratio of [substrate]/ [inhibitor] ([S]/[I]log range: -3 to 4). Substrate and inhibitor were added to the enzymatic reaction concurrently in some experiments.

2.3. Preincubating Effects on Inhibition

To assess the effects of preincubating either substrate or inhibitor with enzyme, product formation was assessed using the previous assay conditions by preincubating enzyme with inhibitor for 5 minutes at 37°C before substrate addition, preincubating substrate with inhibitor for 5 minutes at 37°C before addition to the enzyme reaction, or preincubating substrate with enzyme for 5 minutes before inhibitor addition. The final product formation for preincubation with substrate was determined by subtracting the product formation of a control reaction (5 minutes) under the same conditions and concentrations.

3. RESULTS

3.1. Wheat Germ Acid Phosphatase Kinetic Studies

From the time dependent assay, apparent first order product formation occurs between 15 minutes and 45 minutes (data not shown). For subsequent studies under apparent initial velocity conditions, incubation time was 30 minutes. Kinetic studies, performed by varying substrate concentration under initial velocity conditions, are shown in Fig. (**1**). The insert shows the Lineweaver-Burk transformation of these data with a linear slope. From Fig. (**1**), V_{max} and K_m were extrapolated as 50 µM/30 min and 90 µM, respectively.

3.2. Sodium Orthovanadate (Na_3VO_4) Inhibition Studies

Data shown in Figs. (**2-5**) are from assays for which substrate and inhibitor were added simultaneously. A Dixon Plot

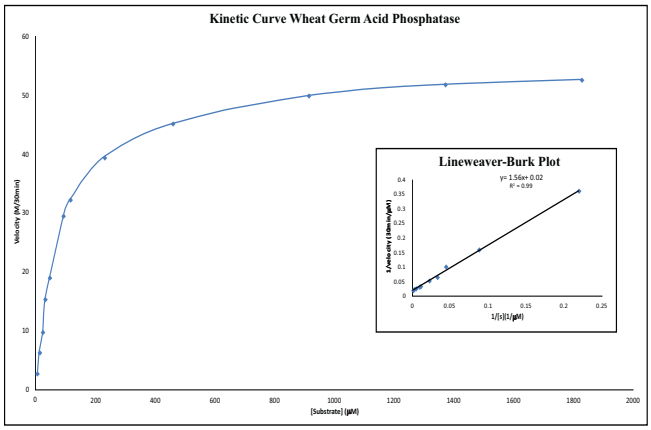

Fig. (1). Initial velocity of product formation as a function of time with varying substrate concentration.

for inhibition data is shown in Fig. (**2**) (concurrent addition of substrate and inhibitor) as a semi log plot. The plot is non-linear over the tested inhibitor concentration range in both the log scale (shown) and traditional non-log scale (not shown). Plotting the data on either the log or non-log scale did not result in a linear relationship of $1/v_o$ as a function of inhibitor concentration. In general a useful Dixon Plot uses substrate concentrations such as multiples of K_m that result in an apparent first order product formation [5]. Using these same data, a plot of initial velocity versus the log ratio of substrate to inhibitor concentration is shown in Fig. (**3**). A plot of percent of control velocity versus the log ratio of substrate to inhibitor concentration is shown in Fig. (**4**) to correct for the differences in maximum velocity (concurrent substrate and inhibitor addition). Linear inhibition ranges (from K_{max} to K_{min}) are approached with increasing substrate concentrations towards V_{max} from R^2= 0.92 to 0.99 (Fig. **5**). Non-V_{max} substrate concentrations were fit to third order polynomial functions solely in order to probe for an inflection point for these data (equations not shown). The third polynomial best fit functions yield the same equation and R^2 value (0.98 for K_m and Half K_m substrate concentrations) for the velocity and percent of control plots and can be used interchangeably. The x values at the inflection point of the K_m and Half K_m substrate concentrations were calculated by the second derivative of the best fit function in Fig. (**4**) (0.80 for K_m and 0.68 for Half K_m). The percent of control velocity was calculated at the inflection point by using the determined x value in the best fit equations of Fig. (**4**) (equations not shown). The percent inhibition at the inflection point was 55% and 53% for K_m and Half K_m, respectfully. Inflection points cannot be determined due to the linearity in this portion of the Fig. (**5**) plot. The substrate/inhibitor concentration ratio yielding 50% of control for the non-first order conditions are 1.07 for 2*V_{max} condition 2 (R^2=0.99) and 0.95 for V_{max} condition 1 (R^2=0.97).

Key for Figs. 3-5:

Red square= 2* V_{max} Condition 1

Blue diamond= V_{max} Condition 1

Green triangle= K_m

Purple X= Half K_m

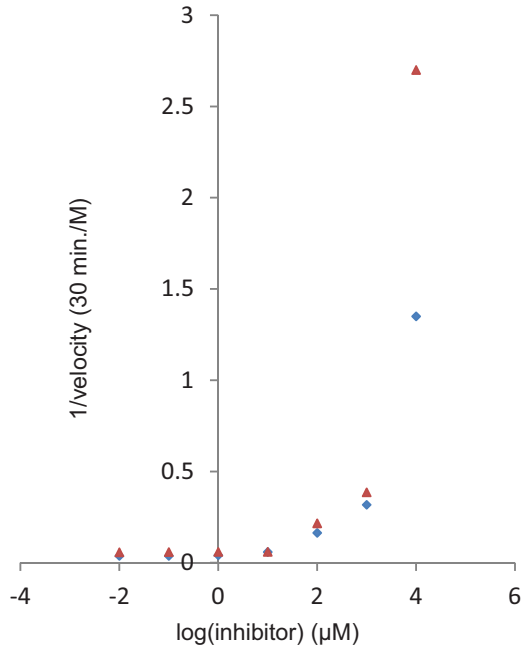

Fig. (2). Non-Linear Dixon Plot of Na_3VO_4 inhibition at two different substrate concentrations.

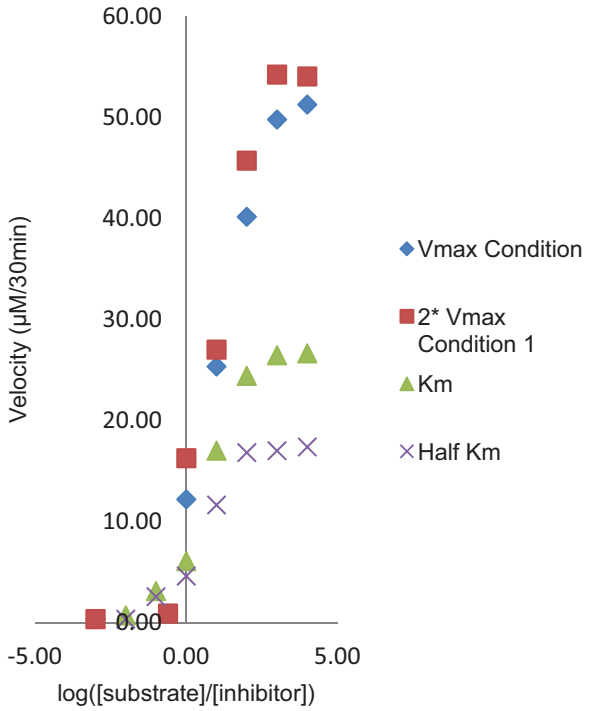

Fig. (3). Velocity of product formation as a function of the log ratio of substrate to inhibitor concentrations. *(The color version of the figure is available in the electronic copy of the article).*

3.3. Preincubating Effects on Inhibition

The results from preincubating enzyme with substrate or inhibitor at Half K_m and V_{max} substrate conditions for five minutes are shown in Figs. (**6** and **7**). Inflection points of the Half K_m substrate conditions were assessed as described and determined to be 0.67, 0.67, or 0.44 for preincubation with inhibitor, concurrent addition, or preincubation with substrate. These inflection points yielded 51%, 54%, and 52% (with R^2= 0.96, 0.98, and 0.99), respectively. For example, with an inflection of 0.67, for every one inhibitor molecule there are approximately 5 substrate molecules at 50% inhibition. The percent of control values significantly varied from each experiment as the x-value approaches the inflection point. At apparent V_{max} substrate concentrations, the slope of the inhibition range changed from 26.5 to 29.7 to 31.4 (Concurrent addition, substrate preincubation, or inhibitor preincubation, respectively) with R^2 values essentially remaining constant.

When plotting percent of control as a function of the log ratio of substrate to inhibitor, we define the slope of the resulting line as the sensitivity of inhibition to a change in this

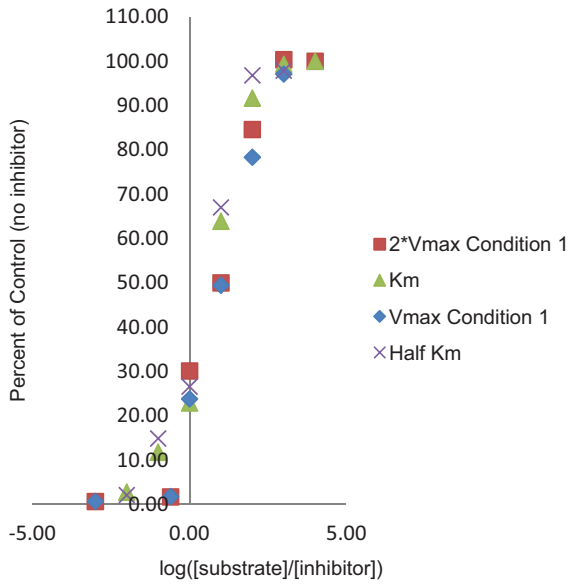

Fig. (4). Percent of control velocity as a function of the log ratio of substrate to inhibitor concentrations. *(The color version of the figure is available in the electronic copy of the article).*

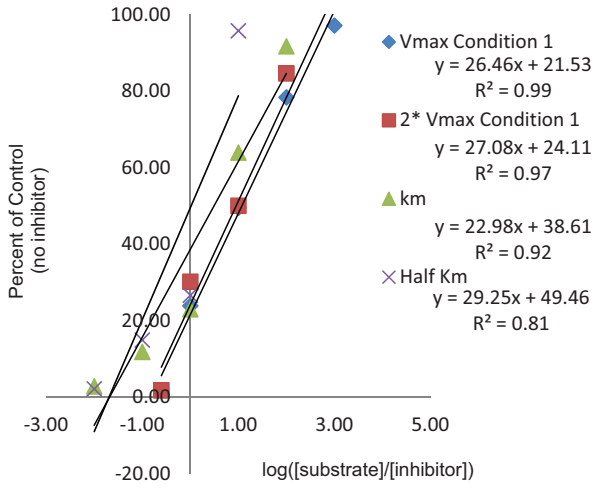

Fig. (5). Linear Analysis of Inhibition Range from K_{max} to K_{min} (Equations listed from top to bottom: highest substrate concentration to lowest). *(The color version of the figure is available in the electronic copy of the article).*

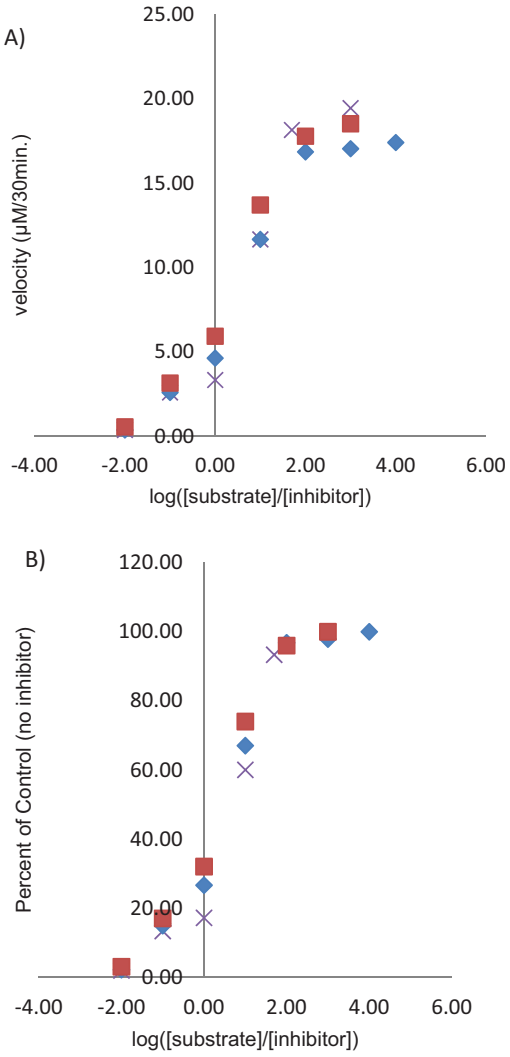

Fig. (6). (**A**). Velocity of product formation as a function of the log ratio of substrate to inhibitor concentrations with preincubating enzyme with substrate (square) or inhibitor (X) or concurrent addition (diamond) at Half K_m substrate conditions. (**B**) Percent of control as a function of the log ratio of substrate to inhibitor concentrations with preincubating enzyme with substrate (square) or inhibitor (X) or concurrent addition (diamond) at Half K_m substrate conditions.

ratio (Fig. **7**). When preincubating the enzyme with substrate before addition of inhibitor, preincubating the enzyme with inhibitor before the addition of substrate or with concurrent addition of both substrate and inhibitor, modest changes in the slopes and y-intercepts were obtained as shown in Fig. **7**. For example, a slope of 31.40 was obtained for enzyme preincubation with inhibitor, which is the most sensitive slope of the three lines shown. Physically this means that inhibition changes more quickly with the change in the ratio. We conclude that a steeper slope and a shift of the line to the right (increased x axis) would indicate a better inhibitor. A steeper slope means a larger percent of control for every 10 fold increase (one log) in substrate relative to inhibitor, and a larger x axis value at a relative inhibition percentage means less inhibitor is required for a given percent of control (degree of inhibition).

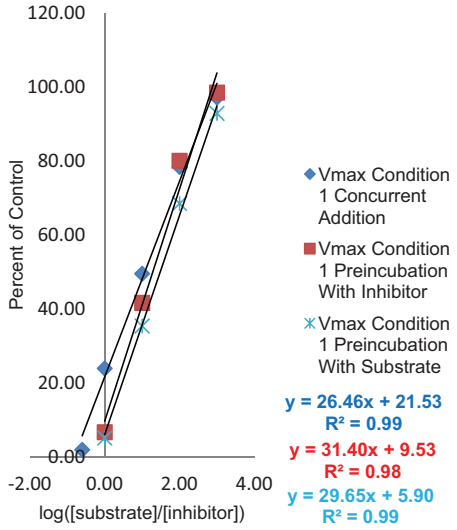

Fig. (7). Linear analysis of preincubating enzyme at V_{max} substrate conditions.

3.4. Comparison of [Substrate]/[Inhibitor] and [Enzyme]/[Inhibitor] Plots

Percent of control velocity plotted as a function of the log ratio of enzyme to inhibitor concentration (Fig. **8B**) shows an apparent increased shift in [E]/[I] x axis values (approximately two logs) with lower substrate concentration while percent of control velocity as a function of the log ratio of substrate to inhibitor concentration does not show a significant shift (Fig. **8A**).

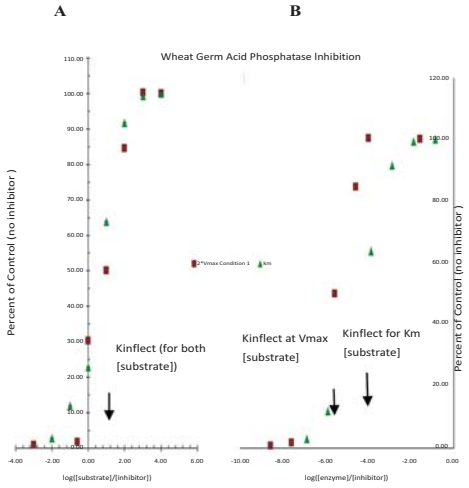

Fig. (8). Comparison between substrate/inhibitor (**A**) and enzyme/inhibitor (**B**) data.

3.5. Developing the 50% of Control Concept

A kinetic equation was derived from a rate law for a simple competitive inhibitor as shown in Scheme A using Michaelis and Menten and steady state assumptions [1, 9]. This equation shows a relationship of [S]/[I] to [ES]/[EI]. Therefore the ratio of [S]/ [I] when [ES] = [EI] (at 50% of control) is equal to $\frac{k_3 k_{!1}}{k_{!3} k_1}$.

Rate Laws: $[E] + [S] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_2} [E] + [P]$

and

$[E] + [I] \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} [EI]$

Using Steady State Assumptions:

Equation 1: $\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] = 0$

Equation 2: $\frac{d[EI]}{dt} = k_3[E][I] - k_{-3}[EI] = 0$

Solve for [E]

From Equation 1: $[E] = \frac{k_{-1}[ES]}{k_1[S]}$

From Equation 2: $[E] = \frac{k_{-3}[EI]}{k_3[I]}$

Combine Equations 1 and 2: $\frac{k_{-1}[ES]}{k_1[S]} = \frac{k_{-3}[EI]}{k_3[I]}$

When [ES] = [EI] $\frac{[S]}{[I]} = \frac{k_3 k_{-1}}{k_{-3} k_1}$

Scheme A. Kinetic Equation for a Competitive Inhibitor:

3.6. Applying the 50% of Control Concept

Using the linear fit for V_{max} Condition 1 (Fig. **5**) to solve for 50% inhibition yielded an x value of 0.95 on the log scale. On a non-log scale this value is calculated as 8.9 ([substrate]/[inhibitor]) using the following:

[ES]=[EI] at 50%

50.00=27.08x+24.11

X=0.95 on log scale

[S]/[I]=10^0.95

[S]/[I]=8.9

Therefore $k_3\ k_{-1}/(k_{-3}k_1) = 8.9$ for these enzyme, substrate, and inhibitor conditions. Thus the overall steps leading away from [ES] formation dominate relative to the [ES] forming steps at 50% inhibition. Therefore a larger ratio should indicate a much better inhibitor when using this analysis. Here we eliminate the IC50 problems of comparisons from lab to lab that likely use different proportions of enzymes and substrates in the experimental design. We propose that the x value that gives 50% of inhibition be reported for a more facile comparison of inhibitors. However, the slope of the plots will also be of comparative value.

4. DISCUSSION/ CONCLUSION

4.1. Enzyme/Inhibitor vs. Substrate/Inhibitor

A. Wheat Germ Acid Phosphatase Data

Orthovanadate is a well-known inhibitor of phosphatases, including wheat germ acid phosphatase [9]. The inhibition of this wheat germ acid phosphatase enzyme by orthovanadate, as seen in Fig. (**8A**), occurs at approximately the same substrate to inhibitor ratio for two different substrate concentrations. Other substrate concentrations (2* V_{max} condition and Half K_m) also illustrate the same correlation for this plot. This is the expected result for a competitive inhibitor since inhibition is consistently dependent on the ratio of substrate to inhibitor. Fig. (**8B**) shows that there is not a similar correlation between inhibition and the ratio of enzyme to inhibitor concentration with different substrate concentrations. Therefore, inhibition is dependent on the ratio of substrate to inhibitor concentration and not dependent upon the enzyme to inhibitor concentration ratio. Other mechanisms of inhibition, such as uncompetitive or noncompetitive, are hypothesized to show a correlation in the enzyme to inhibitor plot. The enzyme to inhibitor plot theoretically may be used for allosteric modulation as well.

4.1.1. Non-constant Acceleration

Evaluating the plots of velocity or percent of control as a function of the log or non-log ratio of substrate to inhibitor concentration yields an apparent "S-shaped" curve from K_{max} to K_{min} for substrate concentrations lower than V_{max} saturated conditions as shown in Fig. (**9**). Fig. (**9**) illustrates the graphical representation of this concept where K_{min}, $K_{inflect}$, and K_{max} are interpolated constants and that the dominant intermediate changes along the curve. Enzyme concentration and substrate concentration are kept constant for these plots to control for non-inhibitory changes in product formation (due to changes in enzyme saturation or diffusion rates). At K_{min}, enzyme-substrate (ES) complex is the dominant state so that there is little apparent effect of the inhibitor on product formation, hence minimum inhibition. At K_{max}, the inhibited enzyme complex (such as EI, or ESI) is the dominant state resulting in the largest decrease in product formation. The $K_{inflect}$ theoretically represents the ratio value at which enzyme-substrate complex and inhibited enzyme complex are equal. Experimentally this can be confirmed by 50% inhibition occurring at the inflection point ($K_{inflect}$). Therefore, there is a non-constant acceleration that occurs throughout this inhibition range. This concept is important for comparing inhibition data represented by the Dixon plot, IC50 value, or k_{cat}/K_m ratio. Differential accelerations for those three methods may bias interpretation of results. The range of the x values from K_{max} to K_{min} for different inhibitors can be easily compared. If two or more inhibitors' ranges are determined, the inhibitor whose range values are largest is considered a 'better' inhibitor because it will take less inhibitor per starting material to inhibit the reaction. This theoretical approach was tested experimentally for limitations and the potential to compare inhibitors in this research.

4.1.2. Inflection Point Significance

Our data suggest that approximately 50% of inhibition occurs at the inflection point of enzymatic reactions that are not saturated. This conclusion is drawn from the error in best fit used for our mathematical determinations. Since 50% inhibition roughly occurs at this inflection point, the ES complex is approximately equal to the inhibited enzyme complex at that point. Therefore, non-saturated systems can be used to evaluate where the ES complex is approximately equal to the inhibited enzyme complex. However, this relationship can be more accurately described by using satu-

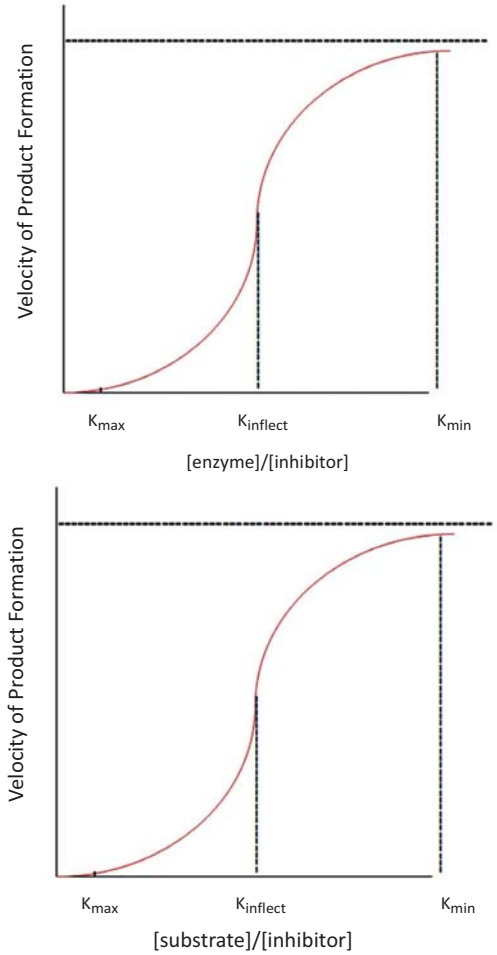

Fig. (9). Theoretical plot of velocity of product formation as a function of the ratio of starting materials (E/I or S/I).

rated conditions and solving for the x value at 50% of control.

4.1.3. Comparing Competitive Inhibitors Using Our Model

In order to compare inhibitors with this model, the range of x values from K_{max} to K_{min} should be evaluated. A molecule that inhibits at a range that occurs at a larger x value is considered a 'better' inhibitor because less inhibitor molecules per substrate molecules are needed for inhibition. A steeper slope means a larger percent of control for every 10 fold increase (one log) in substrate relative to inhibitor, and a larger x-axis value means less inhibitor is required for a given percent of control (degree of inhibition). This method may provide valuable information to other fields that want to study enzyme inhibition.

4.1.4. Effects of Preincubation

Preincubating enzyme with either substrate or inhibitor under not-saturated conditions shows an apparent change in percent of control as a function of the substrate to inhibitor ratio as the inflection point is approached. Ratios farther away from the inflection point converge to essentially the same values. This information is important when deciding how to design experiments for inhibitors. The preincubation can bias inhibition data at ratios closer to the inflection point. However, there was not a large shift from K_{max} to K_{min} for our substrate, enzyme, or inhibitor combinations. Under saturated conditions there is also a slight effect noticed in the slope of these plots as seen in Fig. (**7**).

B. Actin Inhibition of Plasmin

To evaluate other possible types of inhibition, published data from Lind and Smith [12] were evaluated. These authors had reported that actin is a noncompetitive plasmin inhibitor. Their data from this study, re-plotted as percent of control as a function of the log ratio of enzyme to inhibitor or as a function of the log ratio of substrate to inhibitor, are shown in Fig. (**10A** and **B**), respectively. These plots suggest that inhibition is dependent on the ratio of enzyme to inhibitor concentrations and not the ratio of substrate to inhibitor concentration under their experimental conditions. This supports our hypothesis that noncompetitive inhibition depends on the enzyme to inhibitor concentration ratio.

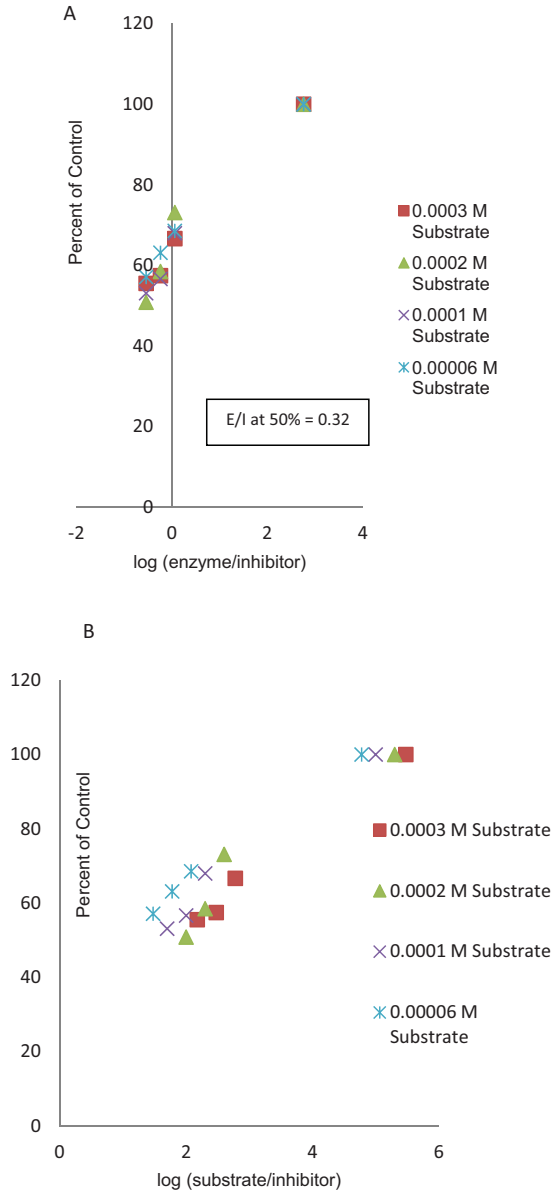

Fig. (10). Actin is a noncompetitive plasmin inhibitor with data from Lind and Smith [12] plotted as a log function of E/I (**A**) or S/I (**B**) ratios.

CONCLUSION

Wheat germ acid phosphatase and plasmin inhibition were evaluated to assess an additional method for analyzing reversible enzyme inhibition. Previous models such as the IC50 and Dixon Plot lack proper incorporation of key variables involved in different known types of inhibition such as competitive and noncompetitive and can lead to controversy when reporting inhibition data. This additional method not only directly compares the fundamental factors in each type of inhibition, but also shows biological relevance in the form of the necessary concentration of inhibitor relative to its competing complex to inhibit enzymatic product formation. This biological relevance has the potential to translate into the pharmaceutical field. In the case of a generic competitive inhibitor, if the ratio of substrate to inhibitor concentrations is calculated alongside an approximation of endogenous substrate concentration, then the effective delivered dose of inhibitor yielding the desired inhibition percent can be estimated. The same concept can be applied to noncompetitive inhibition by using the endogenous enzyme concentration. The approach can also be used to estimate the enzyme concentration of a system at 50% of control inhibition assuming a 1:1 inhibitor: enzyme complex.

In addition to the main focus of this work, the data suggest that the sequential order of addition of materials in enzyme reactions can bias inhibitor results. This bias is more prevalent under non-saturated conditions, closer to the $K_{inflection}$ defined here. Depending on the application of the inhibition, adding the starting materials in different sequences can lead to differential results and should not be overlooked. Therefore, it is important to report how the sequential additions in enzyme assays are performed.

This model should be tested using additional enzyme, substrate, and inhibitor combinations to see if the patterns found in this work can be universally applied to all types of reversible enzyme inhibitors. Although rigorously tested here, this model is nascent and the authors encourage other scientists interested in improving enzymatic inhibition studies to examine inhibitor targets with these concepts in mind. Experiments with additional enzyme, substrate, and inhibitor combinations are needed to evaluate whether or not the conclusions from these experiments can be generally applied to other enzyme classes as well as other types of inhibition. The idea of modeling enzymatic product formation as a function of the ratio of starting materials can also be applied to allosteric effects and catalytic efficiencies in future experiments.

AUTHORS CONTRIBUTION

JMB and BMD carried out the experiments as well as most of the experimental designs and data interpretations, manuscript preparation and reviewing; CCM contributed to the data interpretation and manuscript preparation and reviewing; MAJ contributed to experimental design, manuscript preparation and reviewing.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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