Application Note

UV-0002

Measurement of Enzyme Activity - ALP Activity (ALP: Alkaline phosphatase)

Measurement and analysis of enzyme activity is often used in the field of life science such as medicines and foods to investigate the enzyme activity and affinity of the enzyme-substrate interaction.
In this paper, the JASCO V-600 series spectrophotometer is used to measure and analyze the enzyme activity of a typical enzyme-substrate reaction to introduce details of the available kinetics calculations.

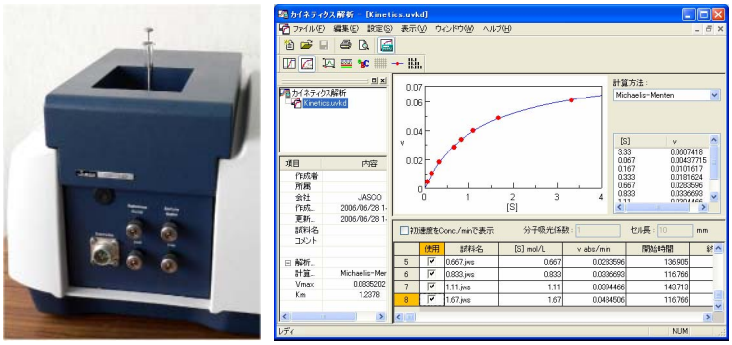

Figure 1 (Left) V-600 series + STR-707 + CSP-748
(Right) Kinetics Analysis Program

Overview

An enzyme (E) binds with a substrate (S) and forms an enzyme/substrate complex. The intermediate complex then further reacts and releases the product (P).

$$\text{Enzyme (E) + Substrate (S)} \rightleftarrows \text{Intermediate Complex (ES)} \rightarrow \text{Enzyme (E) + Product (P)}$$

The time measured for the production of the final product (P) is related to the velocity V:

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad (1)$$

This equation represents the Michaelis-Menten expression for enzyme kinetics. With increasing substrate concentrations [S], the enzyme reactivity is asymptotically approaching its maximum speed [V_{max}]. The [K_m] value is defined by the substrate concentration at its half-maximum speed [V_{max} /2]. This [K_m] value is referred to as the Michaelis-Menten constant.

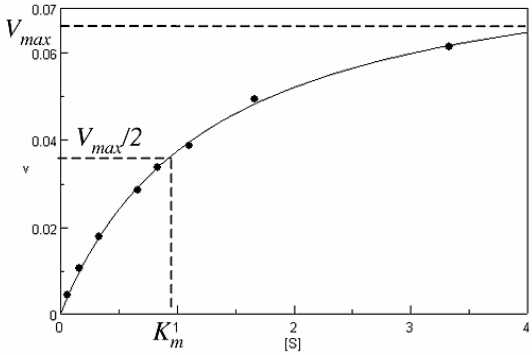

Figure 2 Michaelis-Menten Plot

Based on the equation (1), a saturation curve illustrates the relation between the substrate concentration [S] and velocity [V]. This is commonly called a Michaelis-Menten plot. The [V_{max}] and [K_m] values can then be determined by the plot of the substrate concentration and velocity values derived from a series of experimental determinations of the enzyme activity (Figure 2). Larger values of [V_{max}] denote a higher maximum activity. The [K_m] value represents the affinity of the enzyme for the substrate. Smaller values of [K_m] indicate that the enzyme and substrate are tightly bound and form the ES complex more quickly. On the other hand, larger values of the [K_m] constant indicate that the components are loosely bound and form the ES complex more slowly.

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To conduct an experimental determination of the Michaelis-Menten constant, a kinetics reaction was measured using the following procedures with a JASCO UV-Vis spectrophotometer to calculate the [V_{max}] and [K_m] values.

1. Add substrate solution to an enzyme solution of a specified concentration. Measure the change in the absorbance of the product or substrate during the course of the reaction using the Time Course Measurement Program.
2. Calculate the gradient at the reaction start as the velocity [V].
3. Change the concentration of the substrate solution and follow procedures 1 and 2. Then plot the substrate concentration [S] versus the observed velocity [V].

Determination of the [V_{max}] and [K_m] values by visual observation of the Michaelis-Menten plot (Figure 2) can result in errors for the kinetics values. Therefore, linear curves which can be calculated from variations of equation (1) are used to determine the values. The Lineweaver-Burk, Hofstee, and Eadie plots are expressed as linear plots of the same data derived from the enzyme kinetics reactions. The [V_{max}] and [K_m] values can then be determined by the slope and the intercepts of the vertical and horizontal axes of these plots.

Lineweaver-Burk Plot: 1/[S] vs. 1/v

Taking the reciprocal of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (2)$$

Based on the equation (2), a plot of the experimental data illustrates the relationship between 1/[S] and 1/v (Figure 3). The slope (a) and intercept (b) of the linear curve is then calculated by the least-squares method. Equations (3) and (4) give the [V_{max}] and [K_m] values. This is the most useful expression for enzyme kinetics that result in a smaller [S] value.

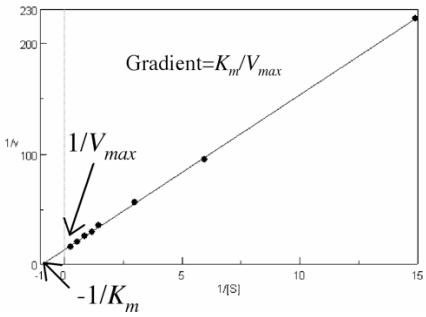

Figure 3 Lineweaver-Burk Plot

$$V_{max} = \frac{1}{b} \qquad (3)$$

$$K_m = a \times V_{max} \qquad (4)$$

Hofstee Plot: [S] vs. [S]/v

$$\frac{[S]}{v} = \frac{1}{V_{max}} \times [S] + \frac{K_m}{V_{max}} \qquad (5)$$

Based on equation (5), a plot of the experimental data demonstrates the relationship between [S] and [S]/v (Figure 4). The slope (a) and the intercept (b) of the linear curve is then calculated by the least-square method. Equations (6) and (7) give the [V_{max}] and [K_m] values based on the slope and intercept from the Hofstee data plot.

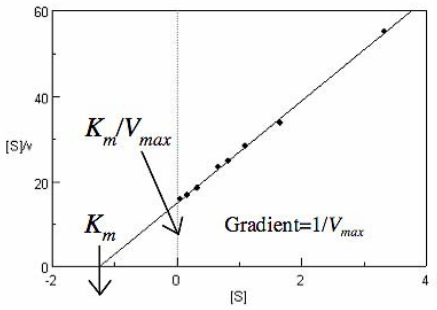

Figure 4 Hofstee Plot

This representation is useful for a kinetics reaction with a larger [S] value.

$$V_{max} = \frac{1}{a} \qquad (6)$$

$$K_m = b \times V_{max} \qquad (7)$$

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Eadie Plot: v/[S] vs. [V]

$$v = K_m \times \frac{v}{[S]} + V_{max} \qquad (8)$$

Based on equation (8), a plot of the experimental data illustrates the relationship between $v/[S]$ and $[V]$ (Figure 5). The slope (a) and intercept (b) of the linear curve is then calculated by the least-square method. Equations (9) and (10) give the $[V_{max}]$ and $[K_m]$ values. This representation is useful for analysis of kinetics reactions with a wide range of $[S]$ values.

$$V_{max} = b \qquad (9)$$

$$K_m = -a \qquad (10)$$

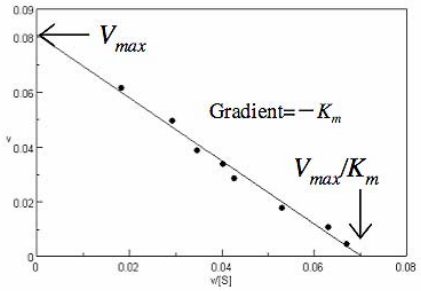

Figure 5 Eadie Plot

Overview of the measurements

When the enzyme (ALP: Alkaline phosphatase) reacts with p-nitrophenylphosphate, inorganic phosphate and p-nitrophenol compounds are produced. The p-nitrophenol has a stable yellow color in an alkaline solution. At the absorption maximum, the substrate concentration $[S]$ and velocity v can be measured using the Time Course Measurement Program to determine the ALP activity.

System Configuration

V-650 Spectrophotometer
STR-707 Water Thermostatted Cell Holder with Stirrer

CSP-478 Sample holder lid with syringe port

Measurement/Analysis Program

Measurement: Time Course Measurement Program

Analysis: Optional Kinetics Analysis Program

Sample

Enzyme solution
- 0.1 M - carbonate buffer solution*1) (at approximately pH 10)
- 10 mM - magnesium chloride solution
- Enzyme solution: physiological saline solution*2) of akaline phosphatase (ALP) 0.0004 µg/mL

*1) Amalgam solution of 27.5 mg of Na_2CO_3 and 22.5 mg of $NaHCO_3$ diluted to 100 mL
*2) Concentration: NaCl 0.9 g/L

Substrate solution
- p-nitrophenylphosphate solution: 0.067, 0.167, 0.333, 0.337, 0.833, 1.11, 1.67, 3.33 mmol/L
  (Final concentrations when mixed with the enzyme solution: 0.0067, 0.0167, 0.0333, 0.0667, 0.0833, 0.111, 0.167, 0.333 mmol/L)

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Detection of the peak wavelength by spectrum measurement

A 0.667 mM substrate solution was added to the enzyme solution. The spectrum was measured after allowing the reactants to react for 2000 seconds. The absorption peak of the p-nitrophenol was observed at the wavelength of 402 nm (Figure 6). The ALP activity in subsequent experiments was determined by measuring the spectrum at 402 nm.

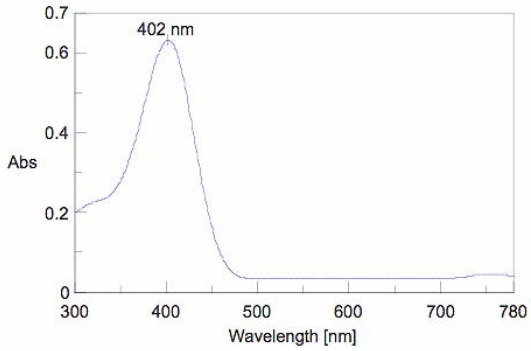

Figure 6 p-nitrophenol spectrum measured after 2000 seconds

Measurement Procedures

1. A blank spectrum was measured with 1.5 mL of a 0.1 M carbonate buffer solution, 0.3 mL of 10 mM magnesium chloride solution, and 0.9 mL of the enzyme solution in a 10 x 10 mm cell.
2. The sample in 1 was measured using the Time Course Measurement software. Shortly after the measurement start, 0.067 mL of the substrate solution was injected into the sample cell, continuing the Time Course Measurement until reaction completion.
3. Procedure 1 and 2 were repeated with the different concentrations of substrate solutions.

Measurement Parameters

Measurement range:	0 - 2000 sec
Data interval:	1 sec
UV/Vis band width:	2.0 nm
Response:	Medium
Measurement wavelength:	402 nm
Temperature:	37° C

Results

Figure 7 illustrates the result of the Time Course Measurement for each substrate concentration. For the time course data of each substrate concentration [*S*], the gradient after the start of reaction (abs/min) was calculated as the velocity *v* shown in Table 1.

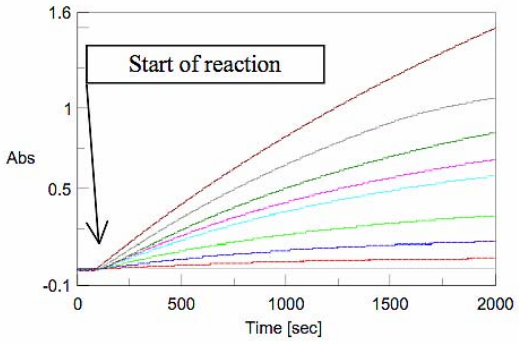

Figure 7 Time Course Data for each substrate concentration

Table 1 Substrate Concentration [*S*] and Velocity *v*

	[*S*] mmol/L	*v* abs/min
	0.0067	0.004
	0.0167	0.010
	0.0333	0.018
	0.0667	0.028
	0.0833	0.034
	0.111	0.039
	0.167	0.048
	0.333	0.061

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The results were plotted using the Michaelis-Menten, Lineweaver-Burk, Hofstee, and Eadie algorithms, and the [V_{max}] and [K_m] values were calculated based on the reaction kinetics. For the Michaelis-Menten values, [V_{max}] and [K_m] values were initially calculated by the Eadie plot and the calculated values were taken as the initial values. The measured v and calculated v from formula (1) were processed with fitting by the least-square method to calculate the final [V_{max}] and [K_m] values.

Table 2 Results of Kinetics Calculations

Calculation Method	Michaelis-Menten	Lineweaver-Burk	Hofstee	Eadie
V_{max}	0.0835	0.0815	0.0828	0.0818
K_m	0.1238	0.1179	0.1212	0.1187
Formula	-	$1/v = 1.447_1/[S] + 12.27$	$[S]/v = 12.0721_[S] -1.4632$	$v = - 0.01187_v/[S]+ 0.08185$

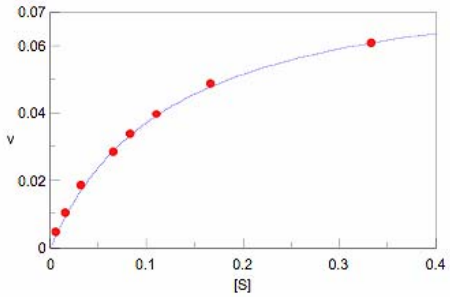

Figure 8-a Michaelis-Menten plot

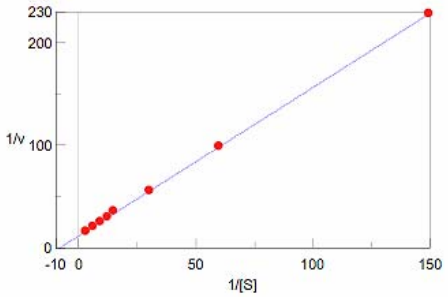

Figure 8-b Lineweaver-Burk plot

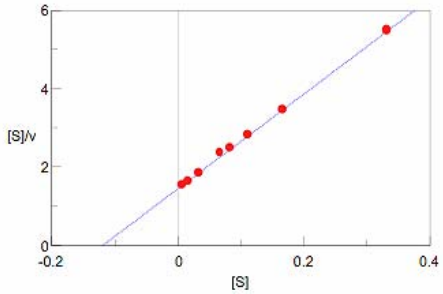

Figure 8-c Hofstee Plot

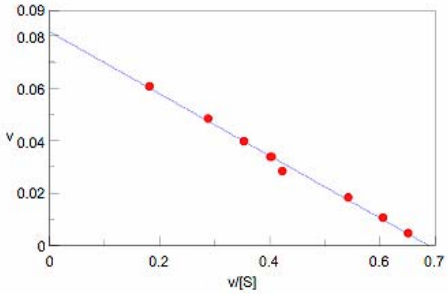

Figure 8-d Eadie Plot

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