Determining the Initial Velocity, Maximum Velocity, and Michaelis Constant of Pure Acid Phosphatase and Calculating the Amount of Acid Phosphatase Found in Wheat Germ Extract

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Abstract

The experiments performed in this work were designed to determine the maximum velocity and Michaelis constant of acid phosphatase and calculate the amount of acid phosphatase found in wheat germ embryos. Both experiments tested the catalysis of nitrophenol phosphate into nitrophenol by acid phosphatase. The first experiment calculated the amount of acid phosphatase found in 1 gram of wheat germ extract by comparing the initial velocities of a pure acid phosphatase reaction to the wheat germ extract reaction. The second experiment determined the maximum velocity and Michaelis constant of acid phosphatase by creating and analyzing Michaelis-Menton and Lineweaver-Burke plots. The amount of acid phosphatase found in wheat germ extract was determined to be 9.3 µg of acid phosphatase per 1 gram of wheat germ. The maximum velocity of acid phosphatase was determined to be 12.79 nmoles of nitrophenol per minute. The Michaelis constant of acid phosphatase was determined to be $0.314x10^{-8}$ M which indicates that acid phosphatase has a high affinity for nitrophenol phosphate.

Introduction

The definition of an enzyme is "a protein or RNA that catalyzes a biological reaction," which means that enzymes help speed up the chemical reactions of life.[1] Enzymes are produced by cells and different enzymes aid in the catalysis of different biological functions of cells. Some of these functions include the breakdown of unwanted material, the synthesis of new material, and the transfer of chemicals.[2] Enzymes work by binding with a substrate and then aiding in the change from substrate to product. A substrates is any molecule that is acted upon by an enzyme.[1] Enzymes can work by either binding two substrates together or breaking one substrate apart. During both reactions, the enzyme remains unchanged and can therefore be used on more than

one substrate molecule in a reaction. Enzymes are very specific in that they will only bind to the substrate(s) to which they chemically couple, which means that every reaction is catalyzed by its own enzyme.[2] An enzyme's effectiveness is based upon the degree of affinity for the substrate it catalyzes. The affinity of an enzyme is defined by how well it binds to the substrate and can be expressed by finding the Michaelis constant for the enzyme's reaction. The higher the Michaelis constant, the lower the affinity meaning that the enzyme does not bind to the substrate quickly. The lower the Michaelis constant, the higher the affinity meaning that the enzyme binds quickly to the substrate.

The Michaelis constant is the substrate concentration when half of the maximum velocity has been reached.[2] The maximum velocity of an enzymatic reaction is the initial velocity at which the rate of the reaction begins to plateau and no longer increases as the amount of substrate increases. The initial velocity of a reaction with a specific concentration of substrate is found by calculating the change in amount of product divided by the change in time. However, pH and temperature can affect the affinity of the enzyme as enzymes are made of proteins and unsuitable conditions can cause the enzyme to denature.[2]

To test an enzyme, it must first be extracted from a cell. In this case the specific enzyme tested was acid phosphatase which can be found in wheat germ. In the wheat germ embryo, acid phosphatase is used to separate phosphate groups from other molecules because the free phosphate groups promote growth in the embryo.[2] For this laboratory nitrophenol phosphate was used as a substrate because once broken down, the nitrophenol turns yellow in an alkaline solution.[2]

Two experiments were performed during this laboratory. The first experiment focused on determining the amount of acid phosphatase contained within the tested amount of wheat germ

extract. First, the amount of produced nitrophenol for the pure acid phosphatase reaction and the wheat germ extract acid phosphatase reaction were found by comparing the absorbencies of known amounts of nitrophenol. Next, the initial velocities for both reactions were found. The initial velocities were then used to calculate the amount of acid phosphatase in the wheat germ extract by comparing it to the known amount of acid phosphatase in the pure acid phosphatase reaction. The second experiment determined the Michaelis constant (Km) and the maximum velocity (Vmax) of the pure acid phosphatase reaction by comparing the absorbencies of the reaction to the absorbencies of a known concentration of nitrophenol. From there, equations were derived from Michaelis-Menton and Lineweaver-Burke plots in order to calculate the Michaelis constant and maximum velocity of acid phosphatase.

Materials and Methods

A few changes were made to the methods in both Experiment 1 and Experiment 2. Aside from these changes, all methods and materials remained the same as described within the BIOL 333 lab book.[2] In Experiment 1 during step 1 of the "Preparation of the Enzyme Extract", 10 mL of enzyme extraction buffer was used instead of 5 mL. Also in Experiment 1 during the second portion of "Measurement of the Product (Nitrophenol) in the Reaction" it was found that the pure acid phosphatase reactions in multiple samples had not taken place, so further testing commenced and the inclusion of KOH was ensured. In Experiment 2 during the 4th step of the procedure, instead of discarding 2 mL of the substrate from tube 2, only 1 mL was discarded. In step 6 of the procedure of Experiment 2, instead of adding 10 µL of acid phosphatase to each tube, it was added to only the tubes labeled 1-8. Also in Experiment 2 in step 8 of the procedure,

instead of adding 3 mL of water to each tube, 3 mL of water was added to tubes 1-8 and 4 mL of water was added to the standard tubes C1-C6.

Results

The purpose of Experiment 1 was to determine the amount of nitrophenol produced by separate reactions of pure acid phosphatase and wheat germ acid phosphatase by comparing the absorbencies of each reaction at 410 nm to the absorbencies of known amounts of nitrophenol at 410 nm. After determining the amount of nitrophenol produced by both reactions, the initial velocities for both reactions were calculated and the initial velocities helped calculate the amount of acid phosphatase present in the wheat germ extract. Figure 1 shows the standard curve for Experiment 1. By using the trend line generated by Excel, the amounts of nitrophenol produced by the other two reactions could be determined simply by replacing "y" with the absorbance and then solving for "x" as seen in Calculation 1 which shows the calculation for tube 1 of the pure acid phosphatase reaction. All of the amounts of produced nitrophenol were calculated using the same calculation for the first experiment. Figure 2 shows the amounts of nitrophenol produced by each reaction. During the majority of the reaction, the wheat germ extract acid phosphatase was producing more nitrophenol but by the end the pure acid phosphatase had produced more nitrophenol than the wheat germ extract acid phosphatase reaction. The initial velocity of the pure acid phosphatase was 7.195 nmoles of nitrophenol per minute and the initial velocity of the wheat germ extract acid phosphatase was 6.695 nmoles of nitrophenol per minute as shown in Calculation 2. By using the initial velocity of both reactions and the known amount of acid phosphatase present in the pure acid phosphatase reaction, it was calculated using Calculation 3

that the 0.5 g of wheat germ extract contained 4.65 µg of acid phosphatase which means that 1 g of wheat germ contains 9.3 µg of acid phosphatase according to Calculation 4.

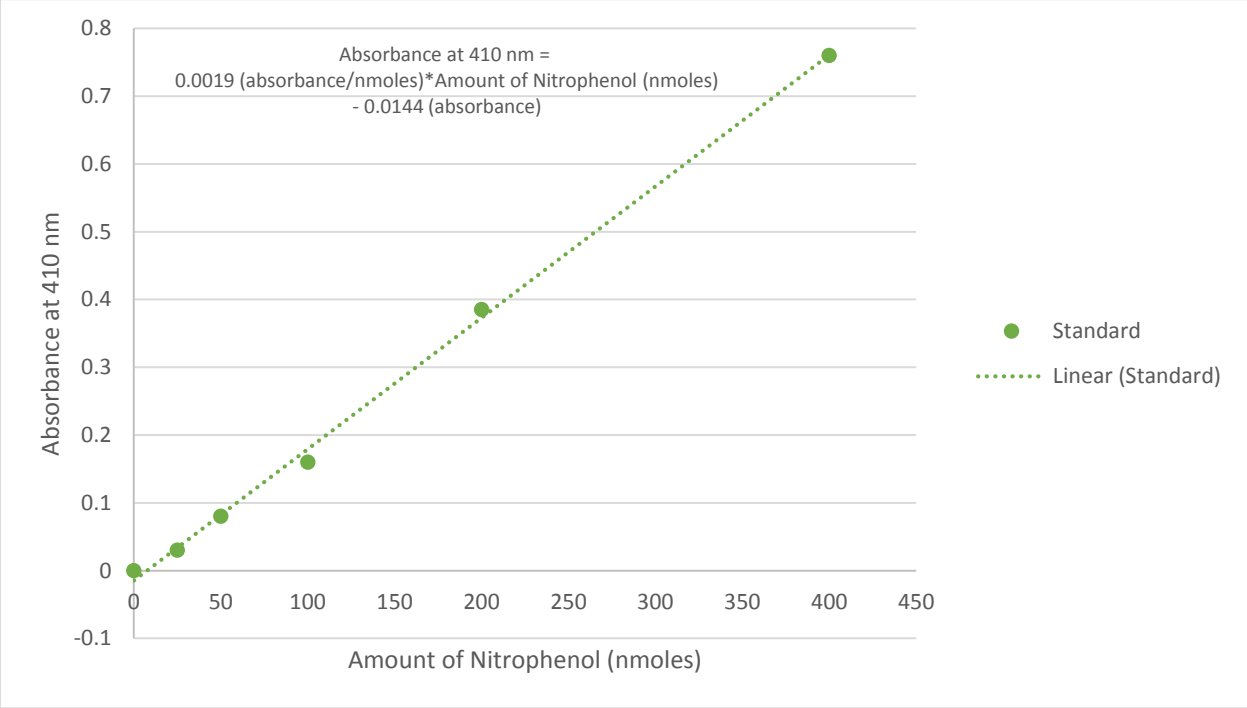

Figure 1: Plot shows the standard curve for Experiment 1 as a function of absorbance at 410 nm vs. the known amount of produced nitrophenol in nanomoles. The trend line and its equation expressed in the figure was used in calculating the amounts of nitrophenol produced by the pure acid phosphatase and the wheat germ extract acid phosphatase reactions for Experiment 1. Figure 1 was derived from the data in Table 1 of the Appendix.

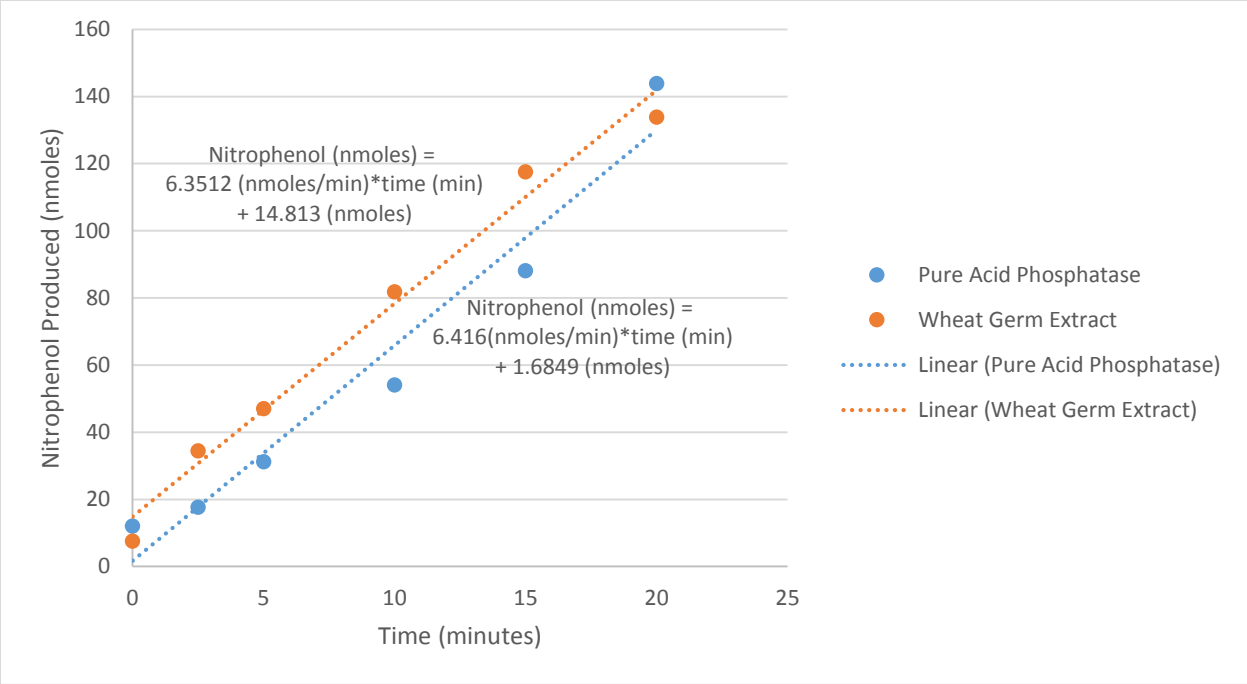

Figure 2: Plot shows the amount of nitrophenol in nanomoles produced by both the pure acid phosphatase and wheat germ extract acid phosphatase reactions in Experiment 1 as a function of time. The amounts of nitrophenol for the corresponding absorbencies at each time were determined using the standard curve trend line equation from Figure 1. Figure 2 was derived from the data in Table 2 of the Appendix.

Calculation 1

$$y = 0.0019 \times x - 0.0144$$

$$Absorbance = 0.0019\left({absorbance}/{nmoles}\right) \times Nitrophenol(nmoles) - 0.0144(absorbance)$$

$$Nitrophenol = \frac{Absorbance + 0.0144}{0.0019}$$

$$Nitrophenol = \frac{0.0084 + 0.0144}{0.0019} = 12\ nmoles$$

Calculation 2

$$V_0 = \frac{nmoles\ at\ time\ 2 - nmoles\ at\ time\ 1}{Time\ 2 - Time\ 1}$$

$$V_0\ of\ Pure\ Acid\ Phosphatase = \frac{(143.89 - 0)}{(20 - 0)} = 7.195\ {}^{nmoles\ nitrophenol}/_{minute}$$

$$V_0\ of\ Wheat\ Germ\ Extract = \frac{(133.89 - 0)}{(20 - 0)} = 6.695\ {}^{nmoles\ nitrophenol}/_{minute}$$

Calculation 3

$$\frac{5 \mu g\ pure\ acid\ phophatase}{7.195\ (\frac{nmoles}{minute})} = \frac{x\ \mu g\ acid\ phosphatase\ in\ wheat\ germ}{6.695\ (\frac{nmoles}{minute})}$$

$$x = \frac{(\frac{5}{7.195})}{6.695} = 4.65\ \mu g\ acid\ phosphatase\ in\ wheat\ germ$$

Calculation 4

$$1\ g\ wheat\ germ \times \frac{4.65\ \mu g\ acid\ phosphatase}{0.5\ g\ wheat\ germ} = 9.3\ \mu g\ acid\ phosphatase$$

Experiment 2 was conducted in order to find the effects of the concentration of the substrate on the initial velocity of the reaction with pure acid phosphatase. The initial velocities of each concentration were then used to create Michaelis-Menton and Lineweaver-Burke plots in order to calculate the Vmax and Km of pure acid phosphatase. Figure 3 shows the standard curve of the correlation between the standard's absorbance at 410 nm and the amount of nitrophenol the sample contained. The trend line for this graph was found by using Excel and was used to

calculate the amount of nitrophenol that was produced in tubes 1-8 of the second experiment. The y-intercept for the trend line was set at the origin to ensure that the 0 absorbance contained 0 nanomoles of nitrophenol. The amount of produced nitrophenol was calculated using the same method as in Experiment 1, but the Experiment 2 standard trend line equation was used instead. Once the amounts of nitrophenol were calculated for each of tubes 1-8, the initial velocities could be found. The initial velocities were found using the same method as in Experiment 1. Figure 4 shows how the initial velocity of the reaction changes as the concentration of the substrate also changes and is also a Michaelis-Menton plot. The reciprocal of the data used to in Figure 4 was used to create Figure 5. The trend line for Figure 5 was then used to determine the maximum velocity and Michaelis constant of the catalyzed reaction. The maximum velocity was found to be 12.79 nmoles of nitrophenol per minute and the Michaelis constant was found to be 314.31 µM by using Calculation 5. The Michaelis constant converts to $0.314x10^{-8}$ M.

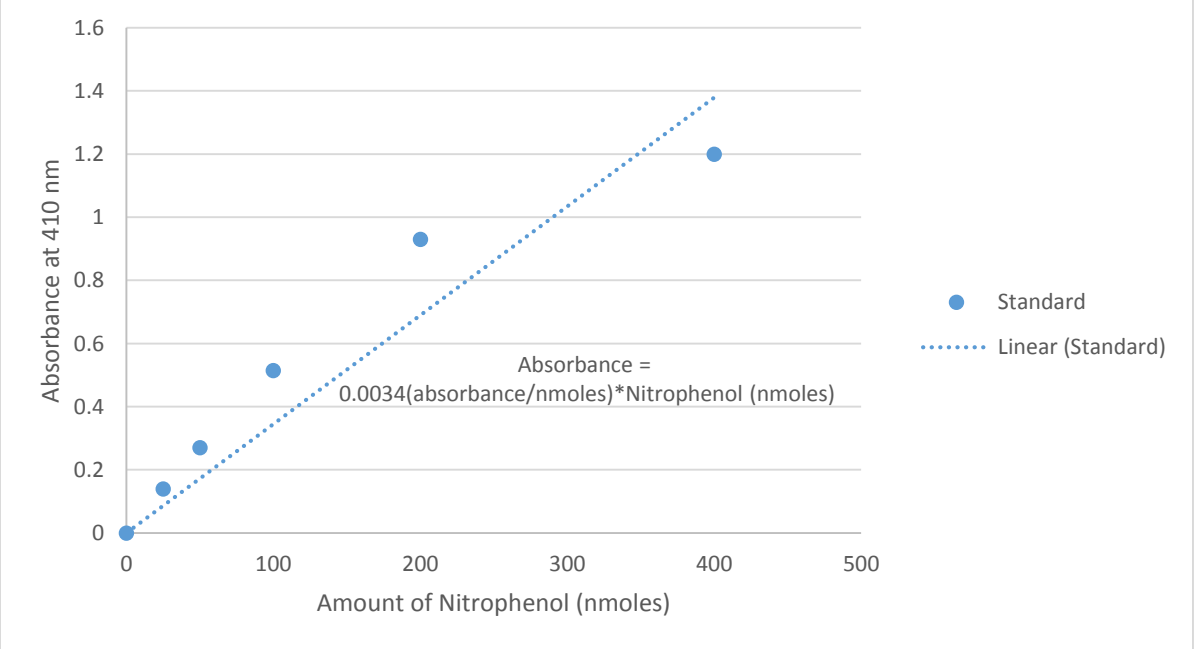

Figure 3: Plot shows the standard curve of Absorbance at 410 nm and the corresponding known amount of nitrophenol in nanomoles. The equation in the figure was used to calculate the

amounts of nitrophenol in tubes 1-8 of Experiment 2. Table 3 in the Appendix was used to create this figure.

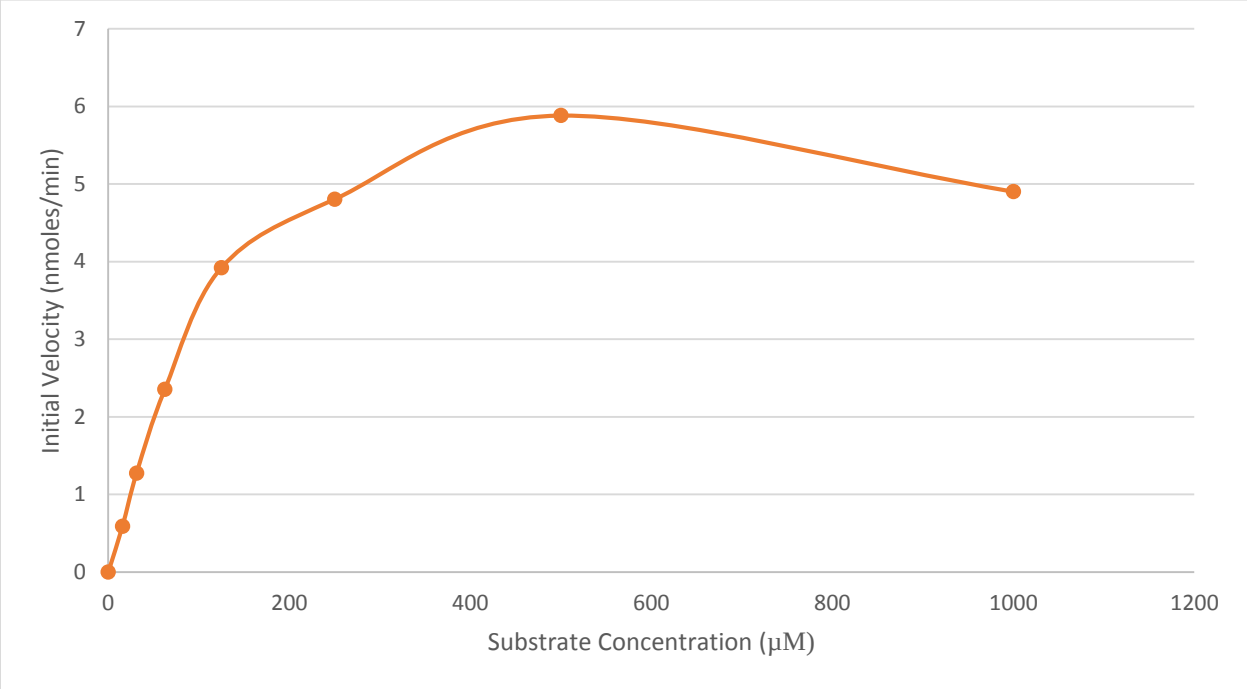

Figure 4: Shows the trend in initial velocity (nmoles/min) as the concentration of the substrate (µM) is increased. This figure was created using the data from Table 4 in the Appendix.

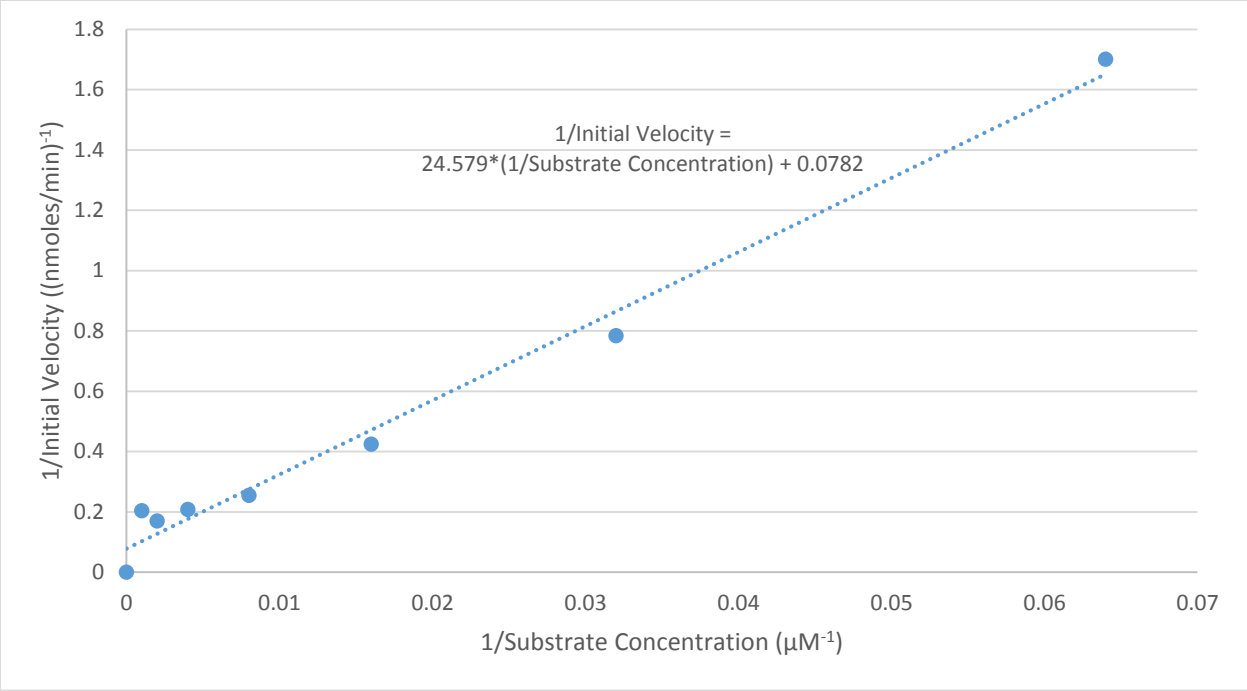

Figure 5: Plot shows the Lineweaver-Burke plot of Figure 4. The trend line for Figure 5 was used in calculating the Michaelis constant and maximum velocity of the reaction. The data from Table 5 in the appendix was used to create Figure 5.

Calculation 5

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}}$$

$$\frac{1}{V_0} = 24.579\left(\frac{1}{[S]}\right) + 0.0782$$

$$V_{max} = \frac{1}{0.0782} = 12.79\ {nmoles\ of\ nitrophenol}/{minute}$$

$$K_m = 24.579 \times 12.79 = 314.31\mu M$$

Discussion

The wheat germ extract was found to contain 9.3 µg of acid phosphatase per gram of wheat germ. Since no published values of acid phosphatase in wheat germ could be found, the data here can be assumed to be representative until further studies are conducted. According to the data collected the maximum velocity of the reaction catalyzed by acid phosphatase is 12.79 nmoles of nitrophenol per minute. According to an experiment done by Tso and Chen[3], the maximum velocity of this reaction should have been closer to approximately 34 nmoles of nitrophenol per minute. The discrepancies between the two may have occurred due to the initial velocity at 1000 µM. Since the experiment was only conducted once, there was no way to find out if this value should have been higher as estimated by the standard or if it indeed portrayed the beginning of a plateau in the velocity. The difference could also be contributed to the fact that the acid phosphatase used in Tso and Chen's experiment was extracted from rice plants and the environments of the reactions were likely different from the ones used in this experiment. The Michaelis constant for acid phosphatase was determined to be 0.314×10^{-8} M, which is similar to Tso and Chen[3], who found the Michaelis constant for acid phosphatase to be 0.33×10^{-8} M. Some reasons for differences between constants could be the human error associated with any scientific experimentation and the natural variation of biological molecules and enzymes. The low Michaelis constant for acid phosphatase indicates that it has a high affinity for its substrate, nitrophenol phosphate. The experiment can be considered successful because it produced defensible values for the variables in question. This work can serve as a model for other enzyme kinetic systems that are useful in nutrition, medicine, and industrial applications.

References

[1] Geoffrey M. Cooper and Robert E. Hausman. **The Cell: A Molecular Approach.** *Sinaur Associates, Inc.* Sixth Edition. 2000; Chapter 3, pp. 73-101.

[2] Darby Harris, Chris Beyer, and Sabine Rundle. **BIOL333: Cell and Molecular Biology Laboratory.** *Western Carolina University*. Part I, pp. 1-14.

[3] S. C. Tso and Y. R. Chen. **Isolation and characterization of a group III isozyme of acid phosphatase from rice plant.** *Department of Botany, National Taiwan University, Taipei, Taiwan, Republic of China.* Botanical Bulletin of Academia Sinica, Vol. 38, 1997. Accessed 16 February, 2014. < http://ejournal.sinica.edu.tw/bbas/content/1997/4/bot384-04.html>

Appendix

Table 1: The Absorbance of Standard Known Amounts of Nitrophenol at 410 nm for Experiment 1		
Test Tube	**Amount of Nitrophenol (nmoles)**	**Absorbance at 410 nm**
Standard 1	0	0.000
Standard 2	25	0.030
Standard 3	50	0.080
Standard 4	100	0.160
Standard 5	200	0.385
Standard 6	400	0.760

Table 2: Nanomoles of Nitrophenol Produced by the Pure Acid Phosphatase and Wheat Germ Extract Acid Phosphatase Reactions Over a Twenty Minute Time Period		
Amount of Nitrophenol Produced for Pure Acid Phosphatase Reaction (nmoles)	**Amount of Nitrophenol Produced for Wheat Germ Extract Acid Phosphatase Reaction (nmoles)**	**Time (minutes)**
12.00	7.58	0
17.68	34.42	2.5
31.16	47.05	5
54.11	81.79	10
88.11	117.58	15
143.89	133.89	20

Table 3: Absorbance of Standard Known Amount of Nitrophenol at 410 nm for Experiment 2		
Test Tube	**Amount of Nitrophenol (nmoles)**	**Absorbance at 410 nm**
Standard 1	0	0.000
Standard 2	25	0.140
Standard 3	50	0.270
Standard 4	100	0.515
Standard 5	200	0.930
Standard 6	400	1.200

Table 4: The Change in Initial Velocity Due to Increasing Substrate Concentration		
Test Tube	**Substrate Concentration (µM)**	**Initial Velocity (nmoles/min)**
1	0	0
2	15.625	0.588
3	31.25	1.275
4	62.5	2.353
5	125	3.922
6	250	4.804
7	500	5.882
8	1000	4.902

Table 5: Reciprocal Data of Substrate Concentration and Initial Velocity for Experiment 2	
1/Substrate Concentration (μM^{-1})	**1/Initial Velocity ($(nmoles/min)^{-1}$)**
Undefined	Undefined
0.064	1.701
0.032	0.784
0.016	0.425
0.008	0.255
0.004	0.208
0.002	0.17
0.001	0.204