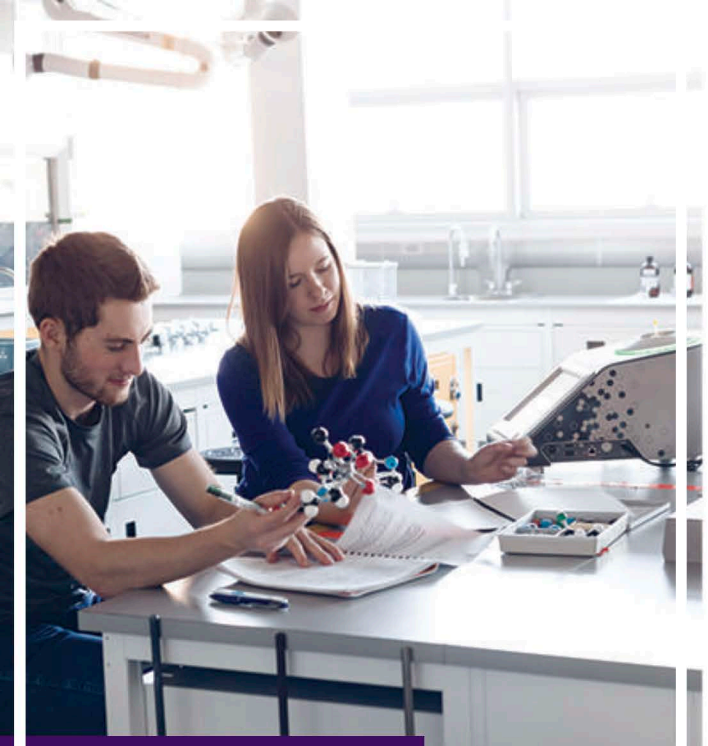

BIOCHEMISTRY

UNDERGRADUATE EXPERIMENT

Determination of Reaction Kinetics for Hydrolysis of *N*-acetyl-DL-methionine

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INTRODUCTION

Enzymes are proteins that catalyze the organic reactions vital to sustain living organisms. The enzymatic reaction begins when the substrate (**S**) reversibly binds to the active site of the enzyme (**E**) to form an enzyme-substrate complex (**E-S**) with rate constants of k_1 and k_{-1}. This is followed by the second step where the enzyme releases the product (**P**) with a rate constant of k_2.[1] The general reaction scheme of an enzyme catalyzed reaction is shown below.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}S \xrightarrow{k_2} E + P$$

To further understand the behavior of enzymes, a kinetic description of their activity is essential. One of the best-known models of enzyme kinetics is the Michaelis-Menten model.[2] The model is defined by an equation that relates the reaction rate, v (i.e. the rate of the formation of [**P**]), to the concentration of the substrate, [**S**]. The Michaelis-Menten equation is given below:

$$v = \frac{d[P]}{dt} = \frac{V_{max}[S]}{K_M + [S]}$$

From the Michaelis-Menten model, two important parameters can be determined, V_{max} and K_M. V_{max} represents the maximum rate of product formation at a saturating substrate concentration and is a measure of the efficiency of the enzyme as a catalyst. The Michaelis constant, K_M, represents the concentration of substrate at which the reaction rate is half of V_{max} and is often used to quantify the affinity of the active site for the substrate (the smaller the K_M value the higher the affinity). Typically, V_{max} and K_M are obtained by determining the initial reaction rate of an enzyme at varying substrate concentrations.[3] The reaction rate is then plotted against concentration to generate a Michaelis-Menten plot. By reciprocating both axes on the Michaelis-Menten plot, the Lineweaver-Burk plot can be obtained from which the V_{max} and K_M can be extracted from the line of best fit.

In this experiment, adapted from a J. Chem. Ed. article published by Olsen and Giles,[4] the enzymatic hydrolysis of *N*-acetyl-L-methionine by porcine acylase (*N*-acyl-L-aminoacid amidohydrolase) is studied. This reaction can be readily monitored via ^{1}H NMR spectroscopy with the NMReady-60. The data obtained from a single reaction can then be used to construct both a Michaelis-Menten and Lineweaver-Burk plot for a fast and semi-quantitative enzyme kinetics analysis.

PROCEDURE

Preparing Stock Solutions

N-acetyl-DL-methionine (0.382 g) was suspended in 2 mL of D_2O along with 0.112 g of KH_2PO_4. Sodium hydroxide (2 M solution in D_2O) was added carefully to bring the pH to 7 using pH paper. The resulting solution is then diluted to 5 mL in a volumetric flask using D_2O. The final solution contained 400 mM of *N*-acetyl-DL-methionine. A stock solution of enzyme is prepared by dissolving 10 mg of porcine acylase and 1.5 mg of $CoCl_2 \cdot 6H_2O$ in 10 mL of D_2O.

Monitoring the Reaction with the NMReady-60

The solution of *N*-acetyl-DL-methionine (500 µL) is transferred to an NMR tube and a ^{1}H NMR spectrum was obtained (spectral width = 20 ppm, spectral centre = 5 ppm, number of scans = 16, delay = 0.5 sec, number of points = 4096). The reaction is initiated by adding 100 µL of the enzyme solution to the NMR tube followed by vigorous mixing. A ^{1}H NMR spectrum is recorded every 4 minutes for 2 hours using the kinetics module on the NMReady-60 (wait type = linear, number of clusters = 40, wait units = seconds, wait time (tau) = 160). To monitor the progress of the reaction, the integrals of the α-methine protons were measured for the reactant (*N*-acetyl-DL-methionine, 4.25 ppm) and product (L-methionine, 3.85 ppm).

Results

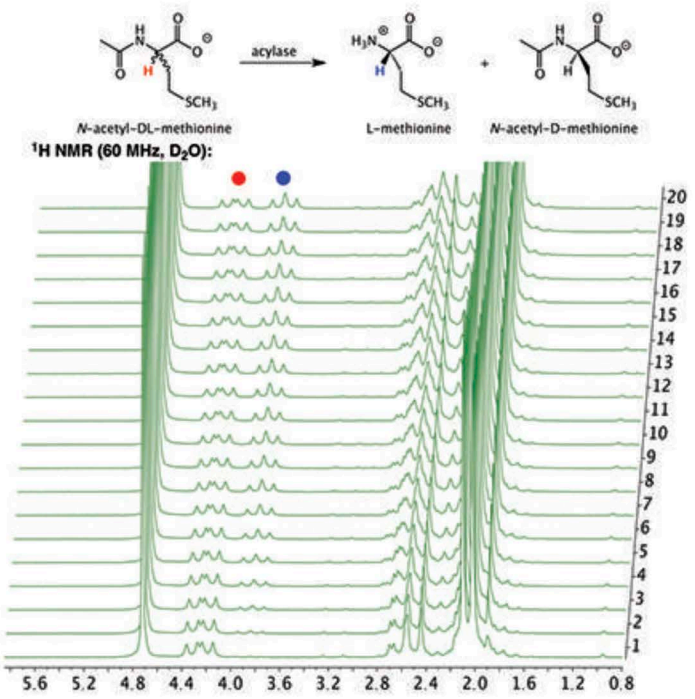

Figure 1. Stacked plot of ^{1}H NMR spectra of the hydrolysis of *N*-acetyl-DL-methionine by porcine acylase to produce L-methionine.

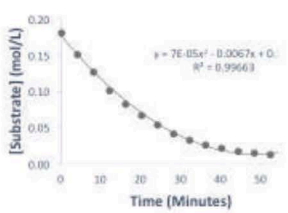

Figure 2. Plot of substrate concentration over time of the reaction.

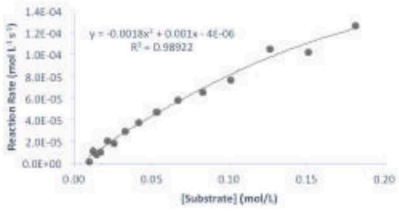

Figure 3. Michaelis-Menten plot of the reaction. The data was fitted to:
$V = (V_{max}\,[S]) / (KM + [S])$.

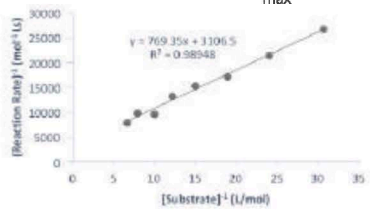

Figure 4. Lineweaver-Burk plot of the reaction. The data was fitted to the equation $1/V = (K_M/V_{max}\,[S]) + 1/V_{max}$ from which the values of K_M (0.24 mol L^{-1}) and V_{max} (0.3152 mmol L^{-1} s^{-1}) were extracted.

Discussion

As seen in Figure 1, the ^{1}H NMR spectrum of the hydrolysis reaction shows the depletion of the substrate, *N*-acetyl-DL-methionine (4.25 ppm), and the simultaneous appearance of the product, L-methionine (3.85 ppm). It is seen that the signal at 4.25 ppm never completely disappears because the D-enantiomer of the racemic mixture remains in the solution and does not get hydrolyzed by the porcine acylase. Figure 2 displays the plot of substrate concentration over time. The reaction is complete within an hour as the substrate concentration reaches a plateau. In Figure 3, the Michaelis-Menten plot illustrates the change of reaction rate as a function of substrate concentration. While the Michaelis-Menten experiment is typically carried out by measuring the reaction rate at several initial substrate concentrations, the experiment is condensed into one reaction in this case. By acquiring multiple ^{1}H NMR spectra as the reaction proceeds, the substrate concentration can be determined from each spectrum and the reaction rate can be approximated by calculating the change in substrate concentration over a known time interval. Therefore, at higher substrate concentration it is seen that the reaction rate begins to reach a plateau which represents the V_{max} at this substrate concentration. From the Michaelis-Menten plot, the Lineweaver-Burk plot (Figure 4) is constructed by reciprocating both axes. Subsequently, it was found that the K_M = 0.24 mol L^{-1} and V_{max} = 0.3152 mmol L^{-1} s^{-1}.

Conclusions

In this experiment the enzymatic hydrolysis of N-acetyl-L-methionine was studied. Due to the difference in chemical shifts of the α-methine protons in the substrate and product, ^{1}H NMR spectroscopy could be used to monitor the progress of the reaction using the NMReady-60 instrument. Furthermore, quantitative data was obtained from the spectra that was used to construct a Michaelis-Menten and Lineweaver-Burk plot which were then used to determine the V_{max} and K_M values of the enzymatic reaction.

References

[1]Le, H.; Algaze, S.; Tan, E. Michaelis-Menten Kinetics https://chem.libretexts.org/Textbook_Maps/Biological_Chemistry/Catalysts/Enzymatic_Kinetics/Michaelis-Menten_Kinetics *(accessed Dec 4, 2018).*

[2]Blanco, A.; Blanco, G. Medical biochemistry; Academic Press: London, United Kingdom, **2017**; pp. 153-175.

[3]Berg, J.; Tymoczko, J.; Stryer, L. Biochemistry; 5th ed.; W.H. Freeman and Co.: New York, **2002**.

[4]Olsen, R., Olsen, J. and Giles, G. "An Enzyme Kinetics Experiment for the Undergraduate Organic Chemistry Laboratory." *J. Chem, Educ.*, **2010**, *87*(9), pp.956-957.

Data Accessibility

The data can be processed directly on the NMReady-60 and printed and/or exported directly to a USB or networked file where it can be worked up using third party NMR processing software.