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The Enzyme Kinetics of Phospholipase A

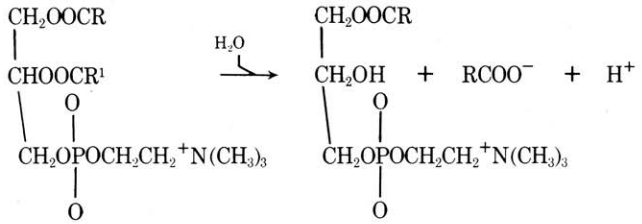
A student experiment

One of the most important topics in the study of elementary biochemistry is enzymes. Unfortunately, since good demonstrative experiments are not readily available, many students never fully appreciate the subject of enzyme kinetics. The experiment presented in this article is one which can easily be conducted by a student using a minimum amount of equipment. The main body of the experiment can be conducted in a single two- or three-hour laboratory session while additional experiments are suggested which can be carried out in a follow-up laboratory period.

While the choice of phospholipase A from snake venom for study in this experiment may at first seem somewhat strange, there are a number of reasons for its choice. During the course of other investigations in this laboratory[1] it has been found that the phospholipase A from *Vipera r. russelli* is unusually stable at room temperature and can thus be utilized without excess concern about denaturation. It has a temperature optimum of 45°C, and exhibits a broad *p*H optimum near 9.0, thus making it an ideal enzyme for this type of experiment. In addition a number of other snake venoms can be used as sources for the same enzyme,[2,3] and thus chemical taxonomic experiments can be used for follow-up studies. It can be also noted that this experiment can be conducted at a cost of less than $1 per student.

Purpose

The purpose of this experiment is to characterize the enzyme phosphoplipase A (EC 3.1.1.4) contained in the venom of the snake *Vipera r. russelli.* The enzyme, reconstituted from crude lyophilized venom, will be used to catalyze the reaction



The enzyme catalyzed reaction will be monitored by following the decrease in *p*H caused by the liberation of the fatty acid with respect to time. A Lineweaver-Burk plot will be used to determine K_M and V_{max}.

Reagents and Equipment

To carry out this experiment the following reagents and equipment will be required

Vipera r. russelli, lyophilized venom (Miami Serpentarium, Miami, FL)
Lecithin, soybean (Sigma Chemical Co., St. Louis, MO)
NaOH, 0.005 *N*
HCl, 0.005 *N*
$CaCl_2$, 0.010 *N*
Venom dilution solution, (NaCl, 1.287 g/100 ml, $CaCl_2$, 0.22 g/100 ml, EDTA, 0.037 g/100 ml)
*p*H meter (with recorder if available)
tissue grinder, 25 ml
syringe, 50 μl
pipets, serological, 2, 5, 10 ml
pipet, volumetric 25 ml
magnetic stirrer with small stirring bar and beaker
stop watch

Procedure

During the course of this experiment at least six kinetics reactions will be run in two parts. The first part of the experiment will be used to show reaction linearity within the *p*H range of 8.50–9.50 used in this experiment. The second part of the experiment will be used to determine the K_M and V_{max} of the enzyme.

Reaction Linearity

Emulsify 250 mg of lecithin in 25 ml of 10 *mM* $CaCl_2$. Weigh out 1–2 mg of the venom. The venom will be reconstituted with venom solution to a concentration of 0.5 mg/ml when reactions are to be run. (*Do not* reconstitute the venom until ready for use as proteolytic enzymes in the crude venom sometimes cause slight inactivation.)

Three reactions will be run in immediate succession. The reactions will have 10 mg lecithin in 10 ml 10 *mM* $CaCl_2$ and will have 5, 10, and 15 μg venom added to catalyze the lecithin hydrolysis. The following procedure should be followed

1) Measure 1 ml of 10 mg/ml lecithin emulsion into beaker and add 9 ml 10 *mM* $CaCl_2$ to bring to 10 ml.
2) Place beaker on magnetic stirrer, insert *p*H electrodes, and adjust *p*H to 9.50.
3) Add 10 μl of 0.5 mg/ml venom solution (5 μg venom) to lecithin with stirring and start timer.
4) Record *p*H every 10 s for 3 min or until p*H* reaches 8.50.
5) Repeat steps 1 through 4 with 10 and 15 μg venom.

These three reactions should show increased enzyme activity with increased amounts of enzyme. If the reaction rate does not appear linear or if activity does not increase with larger amounts of enzyme, it is suggested that this part of the experiment should be repeated.

Determination of K_M and V_{max}

Three (and perhaps six if time permits) reactions will be run. Using the lecithin emulsion and the venom solutions made in the previous part of this experiment, reactions will be run using 5, 10, and 15 mg lecithin in 10 ml of 10 *mM* $CaCl_2$ and each catalyzed with 5 μg venom. The following procedure should be followed

1) Measure 0.5 ml of lecithin emulsion into the beaker and add 9.50 ml of 10 *mM* $CaCl_2$.
2) Place beaker on magnetic stirrer, insert *p*H electrodes, and adjust *p*H to 9.50.
3) Add 10 μl of 0.5 mg/ml venom solution (5 μg venom) to lecithin with stirring and start timer.
4) Record *p*H every 10 s for 3 min or until *p*H reaches 8.50.
5) Repeat steps 1 through 4 with 10 and 15 mg lecithin. (Run duplicate reactions if time permits.)

Treatment of Data

Data from part I of the procedure should be plotted on a graph of *p*H versus time. From the graph, calculate the relative activities for each reaction in Δ*p*H/min. On the graph, the three lines should show constant slope for most of the reaction period, with activities increasing as a function of enzyme concentration. A sample graph is shown in Figure 1.

[1] Toom, P. M., and Devine, J. E., manuscript in preparation.
[2] Marinetti, G. V., *Biochem. Biophys. Acta,* **98,** 554 (1965).
[3] Nutter, L. I., and Privett, O. S., *Lipids,* **1,** 258 (1966).

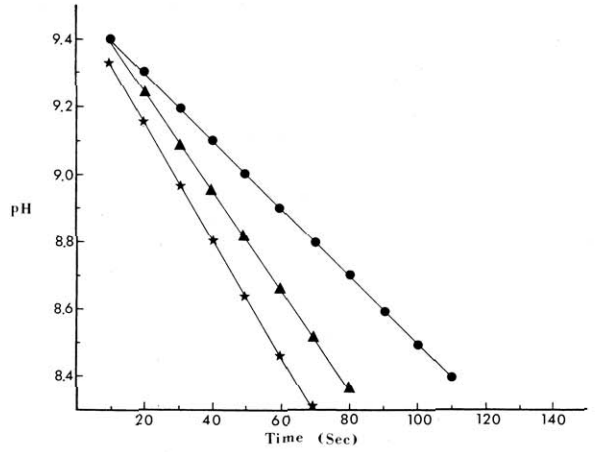


Figure 1 Activity profile of *Vipera r. russelli* phospholipase A. Each reaction mixture contained 10 mg lecithin in 10 ml of 10 *mM* $CaCl_2$. (●—●—● 5 μg enzyme; ▲—▲—▲ 10 μg enzyme; ★—★ 15 μg enzyme).

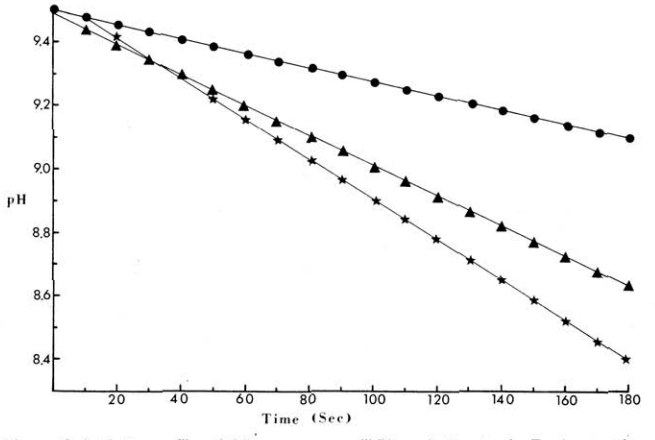


Figure 2 Activity profile of *Vipera r. russelli* Phospholipase A. Each reaction mixture contains 5 μg enzyme. (●—●—● 5 mg lecithin; ▲—▲—▲, 10 mg lecithin, ★—★ 15 mg lecithin).

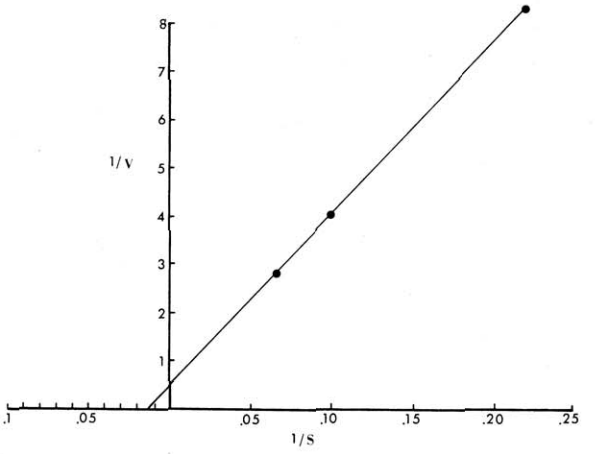


Figure 3 Lineweaver-Burk plot of phospholipase A. Velocity expressed in Δ*p*H/min. Substracts expressed in mg/10 ml.

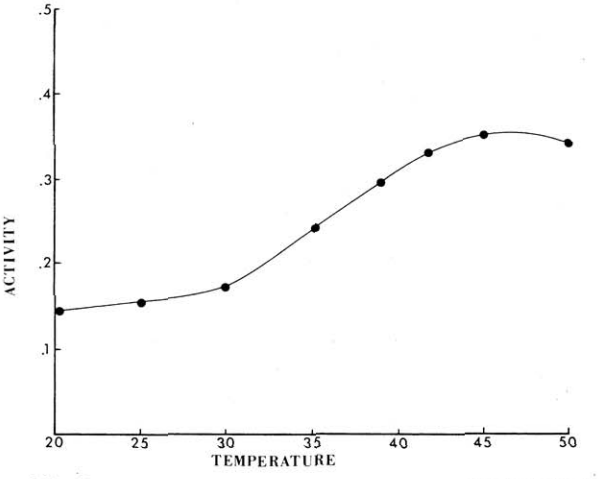


Figure 4 Activity profile of phospholipase A as a function of temperature. Activity expressed as mequiv/min, mg enzyme. Reaction *p*H = 9.0.

Data from part II is first plotted on a graph of *p*H versus time so that the activities may be determined as done with the previous reaction series. A sample graph is shown in Figure 2. The data obtained from this graph will be used to determine K_M and V_{max} for the enzyme by means of a Lineweaver-Burk plot. The inverse of the reaction velocities are plotted versus the inverse of the lecithin concentration in mg. If duplicate or triplicate reactions are run, an averaging or least-squares treatment can be done to make the data more meaningful. Considering the Lineweaver-Burk equation

$$\frac{1}{v} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

it can be seen that the slope of the graph will be K_M/V_{max}, the y intercept will be $1/V_{max}$ and the x intercept will be $-1/K_M$. A sample graph is shown in Figure 4.

K_M should be determined in units of mole/l (the molecular weight of lecithin is 790). V_{max} should be determined in units of mequiv/min utilizing the relationship

$$-\Delta pH = \log [H^+]$$

Treatment of data from Figure 3 shows $K_M = 0.009\ M$ and $V_{max} = 0.6$ mequiv/min.

Additional Experiments

1) Certain metal ions have been shown to inhibit phospholipase A. Run a series of enzyme reactions using 10 mg lecithin in 10 ml 10 *mM* $CaCl_2$ to which varying amounts of either Fe^{3+}, Mn^{2+}, Zn^{2+}, or Al^{3+} have been added. Ion concentrations in the range of 1–10 *mM* should be tested. Dioxan, iodoacetate, and formaldehyde can also be tested.[2]

2) Phospholipase A is relatively heat stable but will denature at higher temperature. Run a series of reactions using 10 mg lecithin and 5 or 10 μg venom while changing the temperature of the reaction mixture. This can be done by using a hot water bath or similar device. Run the first reaction at about 25°C and run subsequent reactions increasing the temperature by about 5° each time. A graph of activity versus temperature should show a temperature optimum of about 45°C as seen in Figure 4.

This experiment was designed to give the elementary biochemistry student an introduction to enzyme kinetics. The straightforward procedure of the experiment directs the student sufficiently so that meaningful data can generally be obtained without bogging him down in complicated procedural detail. It is advantageous to note that once data is obtained, the experiment is not over as the data is not the complete goal of the experiment. Rather the interpretation of the data to obtain the K_M and the V_{max} values is the ultimate goal of the experiment, an exceedingly important point to bring home to the student.

This experiment can be run with any one of a number of snake venoms. To use a different venom it is only necessary to determine the *p*H optimum of the venom and the broadness of the activity versus *p*H graph to determine if linearity can be obtained over a broad enough range to allow for the experiment to be run.

For *Vipera r. russelli*, a K_M value of approximately 40 mg or 0.050 mmole (lecithin molecular weight = 790) should be obtained with a V_{max} value of 0.617 mequiv/min. The temperature optimum of this venom is 45°C. These values compare quite favorably with values obtained using a *p*H stat to determine enzyme activity.