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Full Length Research Paper

Acid phosphatase from snail heamolymph: a cheap and convenient source of enzyme for kinetic parameters determination

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The study of enzymes occupies a very important position in biological, medical and industrial research. Also, the satisfactory performance of an enzyme assay provides a good introduction to biochemical techniques. This experiment presents a good introductory project to students for the estimation of the kinetic parameters of an enzyme. The spectrophotometric/colorimetric assay of acid phosphatase is very simple with a readily available substrate, p-nitrophenyl phosphate, which can easily be purchased, and other common laboratory reagents; sodium hydroxide, sodium acetate and ethylenediaminetetraacetic acid (EDTA). The snail is cheap and the heamolymph collected required no hemolysis and/or centrifugation. The assay method does not require an additional reagent for colour development. Earlier acid phosphatase had been found to be present in a relatively high activity in the heamolymph of the giant African snail, *Archachatina marginata*, and is highly specific for p-nitrophenyl phosphate.

Key words: Acid phosphatase, snail, *Archachatina marginata,* heamolymph, p-nitrophenyl phosphate.

INTRODUCTION

Acid phosphatase (orthophosphoric monoester phosphorrylase, E. C. 3.1.3.2) are enzymes that catalyze the hydrolysis of orthophosphate esters at acidic pH values. Acid phosphatases occur ubiquitously among plants and animals (Hollander, 1971). They can be divided into groups based on their substrate type: non-specific phosphatases catalyze the hydrolysis of almost any phosphate ester whereas specific ones such as protein phosphatases prefer phosphoproteins or phosphopeptides as substrates. Alkaline and acid phosphatases are non-specific, and are only differentiated based on their optimal pH for catalysis and are thought to recycle phosphate in metabolic reactions. Little is known about their functional significance in plants. They can be further classified as prostatic, lysosomal, erythrocytic, and macrophagic acid phosphatases.

The purification and characterization of acid phosphatase(s) from the heamolymph of the giant African snail have been reported (Ebong and Glew, 1989; Afolayan and Agboola, 1996). While we reported the presence of only a single form using a combination of gel filtration and ion-exchange chromatography (Afolayan and Agboola, 1996), Ebong and Glew (1989) had demonstrated the presence of six isozymes when crude heamolymph was fractionated on an ion-exchange column using stepwise salt gradient elution.

The standard assay for acid phosphatase is an example of a "fixed time assay". The reaction mixture is incubated at 37°C or at room temperature for a fixed time (as opposed to a continous monitoring procedure) and is then stopped by the addition of a solution of sodium hydroxide (to raise the pH to 11-12, which inactivates the enzyme and at the same time converts all the phenoxide to the coloured quinoid form) and the amount of p-nitrophenol formed according to Fig.1 is determined. The product, p-nitrophenol, is yellow in alkaline condition and its concentration can be determined directly by measuring its absorbance at 400 nm without using standard nitrophenol curve. The extinction coefficient of p-nitrophenol is 18.8 ml $\mu mole^{-1}cm^{-1}$ at 400 nm (Barman, 1969).

The significance of the determination of K_m is well documented (Lineweaver and Burk, 1934; Segel, 1975). It should be noted however, that K_m values are not absolute constant but depend on the substrate, temperature and the source of enzyme which is a constant for a given

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Table1. The reaction mixture for varying concentrations of p-nitrophenyl phosphate substrate concentration (mM).

Reagent	0.1mM	0.2mM	0.4mM	0.6mM	0.8mM	1.0mM
10mM NaAc, pH 5	2.0ml	2.0ml	2.0ml	2.0ml	2.0ml	2.0ml
10mM p-NPP	0.03ml	0.06ml	0.12ml	0.18ml	0.24ml	0.30ml
Distilled water	0.97ml	0.94ml	0.88ml	0.82ml	0.76ml	.70ml
Enzyme	0.05ml	0.05ml	0.05ml	0.05ml	0.05ml	0.05ml
Incubate for 5 min at 37°c or room temperature						
1.25 NaOH	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
OD_{400}						

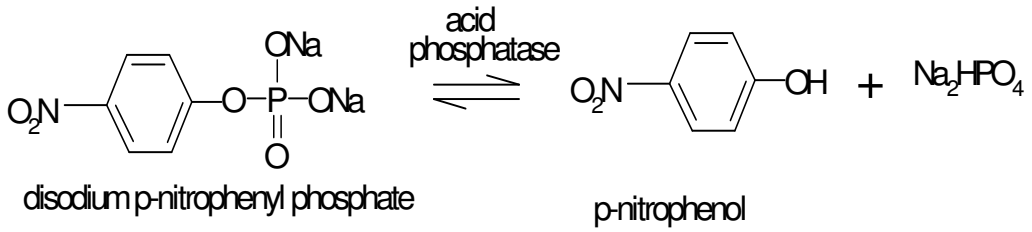

Figure 1. The action of acid phosphatase on p-nitrophenyl phosphate at acid pHs.

enzyme. On the other hand, V_{max} is not a constant; it depends on k_p or k_3, which is constant, and the concentration of the enzyme in the assay ($V_{max} = k_3 [E_t]$) (Segel, 1975).

We present in this paper a simple and inexpensive way of determining kinetic parameters in a teaching laboratory. The availability of snail, the simplicity of the assay method and the readily available substrate make the experiment affordable in any laboratory in the world.

Experimental procedure

The snails were purchased from the local market. The heamolymph was collected by gently breaking the shell at the apex and allow-ing the fluid to flow into a beaker. It was then filtered through a loose-plug of glass wool. p-nitrophenyl phosphate (p-NPP, ditris salt, anhydrous, mol. wt. 461.4) was purchased from Sigma Chemical Co. and all other reagents were of analytical grade. The substrate was dissolved in 10 mM acetate buffer, pH 5.0 containing 1 mM EDTA (the substrate solution is unstable; prepare *in situ*). The substrate concentration was varied between 0.1 and 1.0 mM (Table 1). The reaction was initiated by the addition of 10-50 µl of the diluted enzyme solution (50-100 folds dilution in the acetate buffer). The reaction mixture was incubated at 37°C for 5 min and the reaction stopped by the addition of 0.5 ml of 1.25 M NaOH. The absorbance was read at 400 nm against a blank, for each reaction mixture, without the enzyme.

The velocity of reaction was estimated according to the following expression:

$$\frac{OD_{400} \times V \times D}{\varepsilon_{nitrophenol} \times v \times l \times t} \quad \mu mole/min/ml = units/ml = U/ml$$

where OD_{400} (absorbance at 400 nm), V (total reaction volume), D (dilution factor of enzyme solution), $\varepsilon_{nitrophenol}$ (extinction coefficient of p-nitrophenol = 18.8 $ml\mu mole^{-1}cm^{-1}$), v (volume of enzyme assayed), l (cuvette path length in cm) and t (time of incubation). A unit of enzyme activity was defined as the amount enzyme that would liberate 1.0 µmole of p-nitrophenol per minute under the assay conditions. The Michaelis-Menten, Lineweaver-Burk and Eadie-Hosftee plots were then drawn using SigmaPlot Scientific Graph System. Shown in Table 1 are the reaction mixtures for the varying concentrations of p-NPP.

RESULTS AND DISCUSSION

A typical Michaelis-Menten plot with the corresponding Lineweaver-Burk plot (from the report of a student) of the rates of reaction observed at various concentrations of p-nitrophenyl phosphate is presented in Figure 2. The results of another student using the Eadie-Hosftee plot are presented in Figure 3. The K_m of the enzyme for p-NPP as substrate was 0.056 mM with a V_{max} of 0.146 U/ml from the double reciprocal plot. The latter also gave a K_m of 0.057 mM and a V_{max} of 0.148 U/ml.

Acid phosphatases are found in the cells and secretions of virtually all organisms from *Escherichia coli* to *Homo sapiens* (Hollander, 1971). The presence of a single type of acid phosphatase (orthophosphoric acid monoester phosphohydrolase, EC 3.1.3.2) in the heamolymph of the giant African snail, *Archachatina marginata*, had been shown (Afolayan and Agboola, 1996). This, however, contrasted the work of Ebong and Glew (1989) which has indicated earlier the presence of six iso-

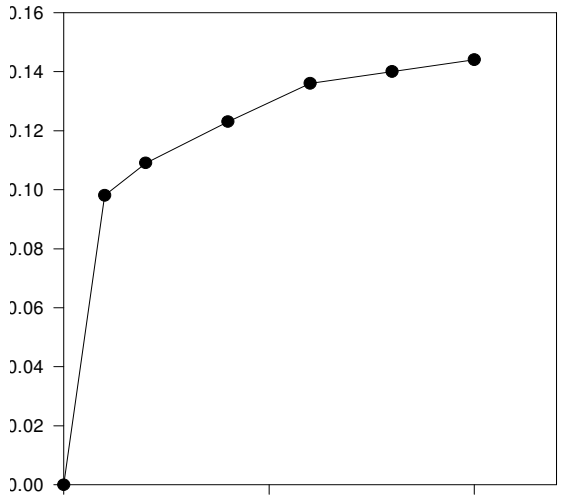

Figure 2a. Michaelis-Menten plot.

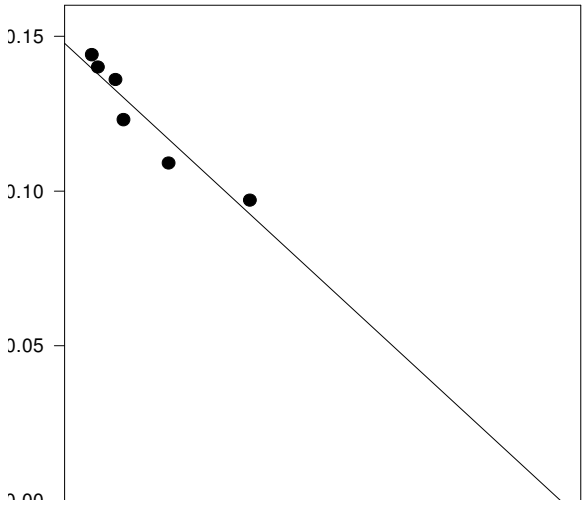

Figure 3. Eadie-Hosftee plot.

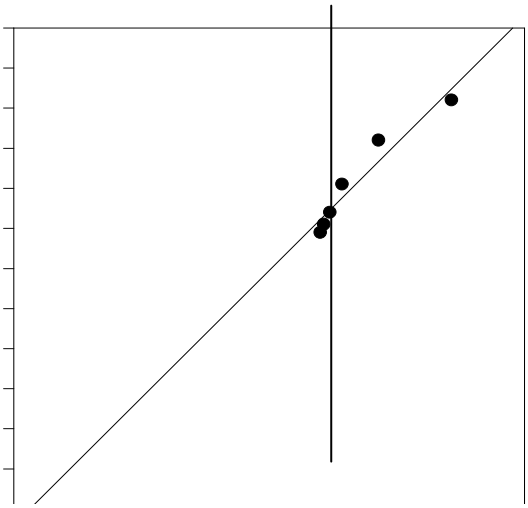

Figure 2b. Lineweaver-Burk plot. The Michealis-Menten plot (Figure 2a) and the Liveweaver-Burk (Double reciprocal) (Figure 2b) plot of the effect of varying the concentration of p-nitropheny phosphate, the substrate **(S**) at a constant amount of enzyme, acid phosphatase, present in the heamolymph of the giant African snail, *Archachatina marginata.* **v** is the initial velocity measured as µmole p-nitophenol released per minute per milliliter of the enzyme solution (µmole/min/ml). The result was that of a student in one of our practical classes. A similar result by another student was plotted according to the Eadie-Hosftee manipulation of the Michealis-Menten equation (Figure 3).

isozymic forms in snail heamolymph. They had used an ion-exchange chromatography step in which each of the stepwise gradient elution yielded an enzyme form while we employed a gel filtration step resulting in only one enzyme activity peak. Further purification and gel electrophoresis did not separate the preparation into any other form (s). Our purified protein catalyzed the formation of nitrophenol and phosphate from p-nitrophenyl phosphate (p-NPP) with limited phosphohydrolytic activity on the following phosphoesters in the order: p-NPP > adenosinediphosphate > adenosinemonophosphate > glucose-1-phosphate > adenosinetriphosphate > glucose-6-phosphate (Afolayan and Agboola, 1996). This led us to the development of a practical class for the demonstration of the determination of kinetic parameters for a single-substrate enzyme. The experiment was intended to introduce the students to some basic techniques of biochemical assays. The experiment can be performed within 1-2 h using crude heamolymph collected from only one snail (20 – 40 ml). With only one form of the enzyme in this fluid, it is appropriate to determine the K_m and V_{max} using the crude preparation (Segel, 1975). In the literature, such experiments were designed to measure phosphorhydrolytic action of either alkaline phosphatase (EC 3.1.3.1) or acid phosphate in human samples especially the serum and prostate(for medical diagnostics) which required a long incubation period of 30-60 min at 37°C (Plummer,1987). We have modified their procedures for the snail heamolymph acid phosphatase assay which requires incubation for just 1-5 min at 37°C or at room temperature. Even where blood was used, the preparation of serum required centrifugation and/or hemolysis. Here, the heamolymph collected requires no centrifugation but a filteration through a loose-plug of glass wool. This was just further diluted to give measurable reaction rates.

The substrates of phosphatases are basically monoesters of orthophosphoric acid. The alcohol esterified to the orthophosphoric acid may be a simple aliphatic alco-

hol, polyhydric alcohol such as glycerol or glucose or any one of a variety of aromatic hydroxyl compounds. The hydrolysis therefore yields an alcohol and a phosphate ion which makes the reaction easy to monitor either by measuring the rate of liberation of inorganic phosphate ion or the other product through colorimetric or fluorometric methods. Substrates that have been employed include disodium monophenyl phosphate (Gutman and Gutman, 1938) (the reaction can be followed by either measuring the phenol formed by colorimetric methods or the inorganic phosphate released), β-glycerophosphate (Bodansky, 1972), p-nitrophenyl phosphate (Hudson et al., 1947), phenolphthalein diphosphate (Huggins and Talalay, 1945), β-naphthyl phosphate (Seligman, 1951) and α-naphthyl phosphate (Babson et al., 1959) and 4-methylumbelliferyl phosphate (Ebong and Glew, 1989) (which can be monitored fluorometrically or the inorganic phosphate released). Moreover, acid phosphatase can be assayed using immunological (Choe et al., 1980) and histochemical methods (Gomori, 1941). It should be noted that every substrate utilized in measuring alkaline phosphatase has also been used in estimating acid phosphatase activity. In fact, methods of acid phosphatase activity measurement are adaptations of those earlier developed for its alkaline counterpart but at acidic pH values.

Today, the p-nitrophenyl phosphate method is the most commonly used colorimetric assay followed by the 4-methylumbelliferyl phosphate assay method where a fluorimeter is available. The chromogenic substituted phenyl-phosphate is colourless, but the reaction product is coloured at alkaline pH and the enzyme reaction can be followed by observing the rate of formation of yellow color of p-nitrophenoxide ion at 400 nm (see Figure 1) and the amount formed estimated using the extinction coefficient. Alternatively, inorganic phosphate released can be estimated by the Fiske and SubbaRow (1925) method. Moreover, instead of a direct spectrophotometric measurement, a standard curve of the p-nitrophenol can be constructed from where the amount of p-nitrophenol formed can be extrapolated. Ebong and Glew (1989) had determine fluorescence using a Turner fluorimeter and values were converted to nanomoles of product using a standard curve prepared from a chloroform solution of the fluorogenic 4-methylumberlliferone. Moreover, it has been shown that the assays employing α or β naphythyl phosphate may not be suitable for acid phosphatase as the p-nitrophenyl phosphate method because of the less affinity of the enzyme for these substrates (Babson et al., 1959). In conclusion, the fact that phosphatases are usually found in secretions and sera and they hydrolyse a large variety of organic phosphate esters made their study convenient. However, studying acid phosphatases has been difficult due to their multiform occurrence (wide isozymic forms) in organisms, small quantity, relative non-specificity and instability in dilute solution (Park and van Etten, 1986). The method described here is simple, straight forward and convenient because of the high specificity and activity of the snail enzyme with p-nitrophenyl phosphate at acid pHs, and the occurrence of only one isozymic form in the snail heamolymph. Moreover, the snails are cheap to purchase or can be picked freely from farmland. Furthermore, the substrate and other reagents required are readily available. In the overall, the experiment is cheap and affordable and it can be performed in any biochemistry laboratory, no matter how rudimentary the set-up may be, and in any economy in the world.

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