Effect of substrate concentration on soil enzyme acid phosphatase and linear transformation of michaelis menten plot

Dr. J Aruna Kumari and Dr. A Padmasri

Abstract

Substrate concentration of enzyme is one of the factor which effect the enzymes kinetics, to study the role of substrate concentration on soil enzyme Acid phosphatase in selected soils. Forty soil samples were collected and assayed for the activity of soil acid phosphatase and four soil samples, two Alfisols and two Vertisols soils with high activity were selected for further study. The activity of acid phosphatase as expressed in terms of µg of 4-nitrophenol released g⁻¹ soil h⁻¹ ranged from 18.93 to 51.2 with an average value of 31.79 activities of the surface soils Soil acid phosphatase increased with increase in substrate concentration upto 30mM and almost reached a plateau at a substrate concentration of more than 30mM at higher concentrations the enzyme activity remained almost constant in all the four soils. With further increase in substrate concentration, minimal change in enzyme activity was observed. Characteristics of enzyme activities like maximum enzyme reaction velocity (Vmax) and Michaelis constant (Km) were determined using Michaelis – Menten equation similar to those determined in homogenous system. The maximum reaction velocity of soil acid phosphatase for soils under study were calculated (µg of 4-nitrophenol g⁻¹ soil h⁻¹) and varied from 40.0 to 56.6 and followed the sequence VS I > AS II > AS I > VS II using Lineweaver – Burk plot. The values compared well with Hanes – Wolf transformation 46.0 to 56.0 and followed the sequence VS I > VSII > AS II > AS I under and Eadie – Hofstee transformation the values varied from 44.0 to 54.4 and followed the order VS I > VS II > AS II >ASI. Michaelis constant (Km) of the soil acid phosphatase calculated using Lineweaver – Burk transformation plot varied from 0.44mM to 0.60mM and followed the order is VS I >VSII > AS II >AS I. Michaelis – Menten equation, Substrate concentration, Acid phosphatase activity and Vertisols

1. Introduction

The enzymes play a very important role in the mineralization of nitrogen, phosphorus and sulphur. Acid phosphatases is the enzyme which play a very important role in the mineralization of phosphorus. Phosphatases have been used to describe a large group of enzymes that catalyzes the hydrolysis of esters and anhydrides of H₃PO₄ (Tabatabai, 1994) [24]. This enzyme plays a major role in the mineralization of soil organic P. There are five groups of phosphatases phosphoric monoester hydrolases, phosphoric diester hydrolases, triphosphoric monoester hydrolases, phosphoamidase polyphosphatases (Tabatabai and Bremner, 1969 [20], Eivazi and Tabatabai, 1977 [7], Brownman and Tabatabai, 1978) [1]. The phosphomonoesterases are classified as acid and alkaline phosphatases, because they show optimum activities in acid and alkaline ranges, respectively. They play a critical role in P cycle and are correlated to P stress and plant growth. When there is a signal indicating P deficiency in soil, acid phosphatase secretion from the plant root is increased to enhance the solubilisation and remobilization of phosphatidic acid and thus influencing the ability of the plant to cope up with P stressed conditions. Michaelis - Menten constants of soil acid phosphatase have been reported by several workers (Tabatabai and Bremner, 1971 [22], Cervelli et al., 1973 [2] and Thornton and McLaren, 1975). The apparent Km values of acid phosphatase in soil range from 1.3 to 4.5 mM The Km values obtained for phosphomonoesterases are affected by shaking the soil substrate mixture using incubation, normally the values are more uniform among soils when shaking than when static incubation technique is employed (Eivazi and Tabatabai, 1977)
By changing the buffer system and pH used by Tabatabai and Bremner, (1969) [20] in assay of phosphatase activity in soils and Cervelli et al., (1973) [25] showed that the substrate 1-nitrophenyl phosphate is absorbed by soils in the presence of 0.5 M NaOAc buffer pH 4.7. They used adsorption parameters derived from the Freundlich Isotherm to determine concentration of the free substrate and to calculate the Km values of acid phosphatase. Irving and Cosgrove, (1976) [9] examined the graphical techniques used in calculations of the Km values of acid phosphatase and concluded that the linear transformation of Eadie – Hofstee is superior. Presumably this graph shows the greatest deviation from the classical Michaelis – Menten equation but recent work shows that the three linear transformations are equally applicable for estimation of the apparent Km values of enzymes in soils (Dick and Tabatabai, 1978 [4] and Tabatabai and Singh, 1979 [21]). Each transformation gives different weightage to errors in the variables (Dowd and Riggs, 1965) [8] and this is reflected in the variation of estimated Km and Vmax values derived for any soil enzyme by using different plots Kinetic parameters (Vmax and Km) are often used to characterize enzymes, they are considered to be constant for a specific enzyme under defined experimental conditions (Marx et al., 2005) [11], but they may vary independently. Maximum reaction velocity (Vmax) of an enzyme catalyzed reaction imply splitting velocity or rate of dispersion of enzyme-substrate complex into enzyme and reaction products, which reflects the conjunction affinity between enzyme and substrate. The higher or lower Vmax value can be used as an indicator to speedy or slow enzymatic process. Vmax and Km of an enzyme express the quantity of an enzyme and substrate affinity, respectively (Marx et al., 2005) [11]; Davidson et al., 2006 [3]. Enzymes catalyzing the same reaction, but derived from different sources of soil have different Km values (Nannipieri et al., 1990) [2].

Theories and mathematical analysis of enzyme reactions are based on the concept that an enzyme acts by forming a complex or compound with substrate presumably the complex of enzyme and substrate is unstable and proceeds through one or more steps or re-arrangement to form the product plus the original enzyme. This theory of enzyme was proposed by Michaelis and Menten and may be expressed by the following equation:

\[ S + E \stackrel{k_1}{\rightleftharpoons} ES \rightarrow E + P ... (1) \]

Where S is the substrate, E is the enzyme, ES is the intermediate enzyme-substrate complex, P is the product of the reaction and K_1, K_2 and K_3 are the respective reaction velocity constants or rate constant of the three processes.

It can be shown that with the soluble substrate in excess, the rate of reaction, that is, the decrease in concentration of the substrate with time or the increase in concentration of the product is given by:

\[ \frac{-dS}{dt} = \frac{dp}{dt} = k_3[ES] = \frac{k_3[E][S]}{K_m + [S]} = \frac{V_{max}[S]}{K_m + [S]} \]

Where S and ES are the concentration of substrate and enzyme-substrate complex respectively, K_m is Michaelis constant.

\[ K_m = \frac{K_2 + K_3}{K_1} \]

\[ V_{max} = K_3E \]

The three linear transformations that commonly used are:

\[ \frac{1}{V} = \frac{1}{v_{max}} + \frac{K_m}{v_{max}} \frac{1}{[S]} \] Lineweaver-Burk transformation

\[ \frac{1}{V} = \frac{K_m}{v_{max}} \frac{1}{[S]} \] Hanes-Wolf transformation

\[ V = V_{max} - K_m \frac{V}{[S]} \] Eadie-Hofstee transformation

Plots of the variables of such relationships normally give straight lines. The value of the slope and intercept are commonly used for determination of the constants from a set of experimental data. Once the Km and Vmax are known for a particular enzymatic reaction under a given set of conditions, the reaction velocity, V can be calculated for any substrate concentration. The Michaelis constant is by far the most fundamental constant in enzyme chemistry. It has the dimensions of concentration (that is, moles per liter) and it is a constant for the enzyme only under rigidly specified conditions. The Km value is useful in estimating the substrate concentration necessary to give a maximum velocity. Kinetic parameters (Vmax and Km) are often used to characterize free enzymes in solution, they are considered to be constant for a specific enzyme under defined experimental conditions (Marx et al., 2005) [11], but they may vary independently. Maximum reaction velocity (Vmax) of an enzyme catalyzed reaction simply splitting velocity or rate of dispersion of enzyme-substrate complex into enzyme and reaction products, which reflects the conjunction affinity between enzyme and substrate. The higher or lower Vmax value can be used as an indicator to a speedy or slow enzymatic process. Vmax and Km of an enzyme express the quantity of an enzyme and substrate affinity, respectively (Marx et al., 2005) [11]. However, Michaelis constant (Km) represents the endurance of an enzyme-substrate complex, which is related with the substrate. The efficiency of the enzymes to decompose substrate at low concentration is directly related to their Km value (Marx et al., 2005) [11]. Higher is the endurance of an enzyme-substrate complex, lower will be the Km value. Enzymes catalyzing the same reaction, but derived from different sources of soil have different Km values (Nannipieri et al., 1990) [2]. Besides, Km is independent of enzyme concentration and kinetically reflects the apparent affinity of enzyme for the substrate. In other words, smaller the Km value, the greater will be the affinity for the substrate (Masciandaro et al., 2000) [12]. However, estimating Km is challenging due to the uncertainty regarding the relative contribution of artificial and naturally occurring substrate under non saturating conditions (Stone et al., 2011) [19]. Moreover, enzymes may operate under non-saturating conditions in soil, which supplements Km an important parameter that merits increased attention (Davidson et al., 2006 [3] and German et al., 2011) [9]. If substrate concentration is similar to Km, the measure of affinity for substrate/enzyme can provide information about the adsorption level or enzyme accessibility. Besides, Km influences enzyme activity at low substrate concentration (Davidson and Janssens, 2006 and Davidson et al., 2006) [3]. Many investigations have dealt with
the kinetic properties of enzymes (Masciandaro et al., 2000, Zhang et al., 2009 and 2010, Juan et al., 2010) [12, 28, 29, 10]. Although, the literature on soil enzyme is on the increase, reports on kinetic constants like Michaelis constant and V_{max} and their correlations with soil properties are limited. Values for both K_{m} and V_{max} vary with the type of soil and also its physical fractions. Then, values are also influenced by assay conditions like choice of substrate and buffer, use of shaken or unshaken soil suspensions.

When the Michaelis–Menten model is applied to ecological systems, V_{max} and K_{m} no longer reflect the biochemical attributes defined in its original context. In such cases, these parameters are more accurately described as apparent V_{max} (AppV_{max}) and apparent K_{m} (AppK_{m}) with AppV_{max}, a relative measure of enzyme abundance; and AppK_{m}, a relative measure of substrate (Wallenstein et al., 2011) [27].

K_{m} values may also fluctuate, depending on whether it is in the free or in an absorbed state (McLaren and Packer, 1970) [13]. While investigating the enzyme splitting of urea in the presence of bentonite, (Durand, 1966) [10], obtained higher K_{m} values for adsorbed than for free enzyme. K_{m} values also varied with pH of assay, being lowest at the pH optimum. In general K_{m} for soil enzymes are greater than that for the corresponding pure enzymes. Paulson and Kurtz, (1970) [15], indicating a much lower apparent affinity of the adsorbed enzyme for the substrate compared to that of the native enzyme. Shaking of soil suspension during assay decreased K_{m} values and increased V_{max} values for soil urease (Tabatabai, 1973) [23].

### Materials and Methods

The procedure of Tabatabai and Bremner (1969) [20] and Eivazi and Tabatabai (1977) [7] were adopted for the assay of acid and alkaline phosphatases respectively. Modified Universal Buffer (MUB) Stock: The stock of MUB was prepared by mixing 12.1 g of Tris (hydroxymethyl) aminomethane (THAM), 11.6 g of maleic acid, 14 g of citric acid and 6.3 g of boric acid in 488 ml of 1N sodium hydroxide and the solution was diluted to 1 litre with distilled water. Modified Universal Buffer (pH 6.5): 200 ml of MUB stock was transferred to 1 litre beaker and kept on a magnetic stirrer and the pH of the solution was adjusted to 6.5 with 0.1N HCl and volume was made up to 1 litre with distilled water. The MUB buffer was wrapped with carbon paper and stored in a refrigerator. P-nitrophenyl phosphate solution (0.025M): This was prepared by dissolving 0.420 g of disodium salt of p-nitrophenyl phosphate in 40ml of MUB pH 6.5 and the solution was diluted to 50 ml with MUB of the same pH. The solution was wrapped with carbon paper and stored in a refrigerator. Calcium chloride (0.5M): This was prepared by dissolving 73.5g of CaCl_{2}·2H_{2}O in distilled water and made up to 1 litre. Sodium hydroxide (0.5M): 20 g of sodium hydroxide was dissolved in 700 ml of distilled water and diluted to 1 litre with water. Standard p-nitrophenol solution: Primary stock solution of 1000 µg ml^{-1} of p-nitrophenol was prepared by dissolving 1 g of p-nitrophenol in distilled water and made up to 1 litre. From this, secondary stock of 100 µg ml^{-1} and 20 µg ml^{-1} solutions were prepared. Working standards of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg ml^{-1} were prepared from 20 µg ml^{-1} stock and the absorbance of these standards were recorded at 420nm in spectrophotometer. This was used for the standard curve. To 1 g of soil sample taken in glass tubes, 4 ml of modified universal buffer pH 6.5 was added followed by addition of 1 ml of 4-nitrophenyl phosphate solution. Different concentrations viz. 2, 4, 6, 8, 10, 20, 40, 60, 80 and 100 mM of substrate solution i.e. 4 – nitrophenyl phosphate were prepared separately and 1 ml solution was added to each glass tube in triplicates. The final concentrations of substrate in the incubation mixtures were 1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 mM. After 2 hours of incubation at 37°C, the glass tubes were removed and 1 ml 0.5 M CaCl_{2} and 4 ml 0.5 M NaOH was added to each tube. The acid phosphatase activity was determined by estimating the 4-nitrophenol released. The glass tubes were swirled and the soil suspension was filtered through Whatman No. 42 filter paper. The absorbance of yellow color of 4-nitrophenol liberated due to hydrolysis of the substrate by phosphomonoesterases was measured at 420 nm. Controls were run simultaneously following the same procedure except adding 1 ml of 4-nitrophenyl phosphate after the addition of 1 ml of 0.5M CaCl_{2} and 4 ml of 0.5M NaOH. Corrections were made for control / blank values.

### Results and Discussions

The effect of substrate concentration on acid phosphatase activity is presented in (Table 1) and is depicted in (Figure 1). Increase in substrate concentration increased soil acid phosphatase activity upto 30mM in all the soils and almost reached a plateau at a substrate concentration of more than 30mM at higher concentrations the enzyme activity remained almost constant. Eivazi and Tabatabai, (1977) [7], Tabatabai and Bremner, (1971) [22], Srinivas and Raman, (2008) [18] and Vandana, (2012) [26] also obtained similar results for phosphomonoesterases. Plots of three linear transformations of Michaelis-Menten’s equation for acid phosphatase are shown in (Figure 2, 3 and 4).

<table>
<thead>
<tr>
<th>Substrate concentration (mM)</th>
<th>Acid phosphatase activity (µg of 4-nitrophenol released g⁻¹ soil h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VS1</td>
</tr>
<tr>
<td>1.0</td>
<td>25.6</td>
</tr>
<tr>
<td>2.0</td>
<td>28.2</td>
</tr>
<tr>
<td>3.0</td>
<td>30.4</td>
</tr>
<tr>
<td>4.0</td>
<td>32.9</td>
</tr>
<tr>
<td>5.0</td>
<td>34.6</td>
</tr>
<tr>
<td>10.0</td>
<td>36.1</td>
</tr>
<tr>
<td>20.0</td>
<td>38.0</td>
</tr>
<tr>
<td>30.0</td>
<td>38.7</td>
</tr>
<tr>
<td>40.0</td>
<td>38.9</td>
</tr>
<tr>
<td>50.0</td>
<td>38.9</td>
</tr>
</tbody>
</table>

Table 1: Effect of substrate concentration on soil acid phosphatase activity
Fig 1: Effect of substrate concentration on soil acid phosphatase activity

Fig 2: Lineweaver - Burk plot of soil acid phosphatase activity

Fig 3: Hanes - Wolf plot of soil acid phosphatase activity
The linearity of these plots is indicated that the values obtained for acid phosphatase activity fitted the three linear transformations of Michaelis-Menten’s equation. The values of V\text{max} and K\text{m} obtained by least square analysis of these plots are presented in (Table 2). From the graph, it is observed that with all the soils, reasonably linear plots were obtained in all the cases. The values of V\text{max} and K\text{m} obtained from the least square analysis of these plots are presented in (2) and (Figures 2, 3 and 4).

The maximum reaction velocity of soil acid phosphatase for soils under study were calculated (µg of 4-nitrophenol g\textsuperscript{-1} soil h\textsuperscript{-1}) and varied from 40.0 to 56.6 and followed the sequence VS I >AS II > AS I > VS II using Lineweaver – Burk plot. The values compared well with Hanes – Wolf transformation 46.0 to 56.0 and followed the sequence VS I > VS II > AS II > AS I under and Eadie – Hofstee transformation the values varied from 44.0 to 54.4 and followed the order VS I > VS II > AS II > AS I.

**Table 2: Maximum enzyme reaction velocity (V\text{max}) and Michaelis Constant (K\text{m}) values of soil acid phosphatase activity**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>VS I</td>
<td>52.6</td>
<td>56.0</td>
<td>54.4</td>
<td>0.60</td>
<td>0.67</td>
<td>0.65</td>
</tr>
<tr>
<td>VS II</td>
<td>40.0</td>
<td>54.0</td>
<td>44.0</td>
<td>0.46</td>
<td>0.86</td>
<td>0.58</td>
</tr>
<tr>
<td>AS I</td>
<td>44.0</td>
<td>46.0</td>
<td>45.0</td>
<td>0.44</td>
<td>0.78</td>
<td>0.51</td>
</tr>
<tr>
<td>AS II</td>
<td>46.0</td>
<td>48.0</td>
<td>47.0</td>
<td>0.52</td>
<td>0.63</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Michaelis constant (K\text{m}) of the soil acid phosphatase calculated using Lineweaver – Burk transformation plot varied from 0.44mM to 0.60mM and followed the order is VS I>AS II > AS I> VS II. The values compared well with those obtained from Hanes – Wolf (0.63 to 0.86) and followed the order is VS II > AS I > VS I > AS II. In Eadie – Hofstee plots the values ranged from (0.51 to 0.65) and the order followed is VS I > AS II > AS I > VS II. Irving and Cosgrove, (1976) [9] concluded that the linear transformation of Eadie – Hofstee values were found to be superior but the work shown by others (Dick and Tabatabai, 1978 [4], Tabatabai and Singh, 1979 [21], Rao, 1989 [16], Srinivas, 1993 [17] and Vandana, 2012 [20]) shows that the three linear transformations are equally applicable for estimation of K\text{m} and V\text{max}, values of enzymes in soil. significant difference was not noticed when K\text{m} and V\text{max} were calculated from the linear transformation of Michaelis plot.

**References**