Kinetic Activity of Alkaline Phosphatase

BACKGROUND AND GOAL

Alkaline phosphatase (AP) cleaves the colorless substrate 4-nitrophenylphosphate (PNPP) forming the yellow product p-nitrophenol (PNP). Like many proteins, the activity of AP is seriously affected by pH, and as its name suggests, appears to work best at alkaline pH values. In this study, students will measure the reaction kinetics of AP at three different pH values: pH 5 (acetate buffer), pH 7 (phosphate buffer), and pH 10 (CAPS buffer). The goal of the experiment is to prove that alkaline phosphatase works best at higher pH values. Evaluation of the activity will be based on determination of $V_{\text{max}}$ and $K_M$ for each pH condition. Since $V_{\text{max}}$ and $K_M$ can vary independently of each other, the best enzyme activity will be the one in which $V_{\text{max}}$ is fastest and $K_M$ is lowest. The ratio of $V_{\text{max}}/K_M$ allows for an easy comparison of the calculated kinetics parameters.

Students will work in teams of 2.

MATERIALS AND REAGENTS

Buffers
- 50 – 100 mM Acetate buffer at pH 5
- 50 – 100 mM Phosphate buffer at pH 7
- 50 – 100 mM CAPS-NaOH buffer at pH 10

Substrate
- 1 mM p-nitrophenylphosphate (PNPP) dissolved in dH2O

Enzyme
- 1 mg/mL Alkaline phosphatase in pH 5 buffer
- 1 mg/mL Alkaline phosphatase in pH 7 buffer
- 1 mg/mL Alkaline phosphatase in pH 10 buffer

Disposable PMMA Cuvettes

Shimadzu UV-1601 spectrophotometer
EXPERIMENTAL PROCEDURES

1. In an earlier laboratory session, students should have prepared 30 mL of each buffer described. Allow the buffers to reach room temperature.

2. Turn on the spectrophotometer and allow it to warm up. Meanwhile, label your cuvettes according to Table 1. Also prepare a table such as that shown in Table 1 in your lab notebook.
   a. Determine the volume of 1 mM PNPP required to make each of the samples listed.
   b. Determine the compensating volume of buffer required to yield a 1-mL reaction sample.
   c. Repeat this table set up in your lab notebook for each pH tested.

3. Combine the appropriate buffer and substrate into each cuvette.

4. Select the KINETICS mode on the UV-1601 instrument. Set the following parameters:
   a. Wavelength: 410 nm
   b. Reaction Time: 60 sec
   c. Rate Time: 60 sec
   d. Lag Time: 0 sec

5. Autozero the instrument using a “Reference” and “Blank” for a given buffer.

6. Place the cuvette into the UV-1601 sample holder. Pipet 100 μL of the appropriate AP solution into the cuvette. Quickly mix with repeated pipetting up and down. Immediately start reading the absorbance change by pressing START.

7. Record the ΔAU/min value for each sample. Plot the reaction velocity as a function of [PNPP] for each condition. Deduce the maximal velocity, $V_{max}$, and $K_m$ from the curvilinear fit. Software such as GraphPad Prism can be used to fit the hyperbola to avoid linearizing the data and thereby unintentionally weighting the date at low substrate concentrations.

Table 1. Example set up of 1-mL reactions for Alkaline Phosphatase kinetics at varying pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Samples</th>
<th>Vol Buffer, mL</th>
<th>Vol 1mM pNPP, mL</th>
<th>Vol of 1 mg/mL AP, mL</th>
<th>ΔAU/min at 410 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Reference</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>300 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>700 μM PNPP</td>
<td>0.9</td>
<td>NONE</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Calculate what the values should be for the areas shaded yellow. Generate an analogous table for each pH tested.
LABORATORY REPORT GUIDELINES

Introduction – 15 points
  ▪ General background on alkaline phosphatase (8 points)
  ▪ Goal (7 points)

Experimental Procedures – 15 points
  ▪ Give one succinct paragraph about the buffer preparation and kinetics experimentation.
  ▪ Include sources of AP and substrate, and instrumentation used

Results – 30 points
  ▪ Text describing results – 15 points
  ▪ Figure of reaction kinetics (velocity vs. [Substrate]) at three pH values – 5 points
  ▪ Description of how the $V_{max}$ and $K_m$ terms were calculated along with their ratio – 10 points

Discussion – 30 points
  ▪ Summary Paragraph
    ▪ Which pH gave the best enzyme activity and what was the basis of your conclusion?
    ▪ How do your results compare to the hypothesis?
    ▪ How does your data compare to the literature?
  ▪ Additional Information
    ▪ Give an additional paragraph on the impact of pH on enzyme activity in general.

References – 10 points
  ▪ Give at least 3 sound references
  ▪ Use ACS guidelines for formatting as shown in the Instructions to Authors for the journal Biochemistry