The effects of pH on Type VII-NA Bovine Intestinal Mucosal Alkaline Phosphatase Activity

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A standard assay for measuring alkaline phosphatase activity is detecting the production of nitrophenol from the artificial substrate p-nitrophenol phosphate. Nitrophenol absorbs light at 420 nm, which provides a convenient detection method. However, the absorbance of nitrophenol varies with pH. This variation complicates the use of the colorimetric assay to study the pH-dependence of alkaline phosphatase activity. We have determined the standard curve relating the absorbance of nitrophenol to buffer pH. These levels were used to normalize data collected from studies of alkaline phosphatase catalysis of different concentrations of p-nitrophenol phosphate across various reaction pH levels. The studies indicate that alkaline phosphatase activity increases over three-fold from pH 7 to pH 9 at saturating concentrations of p-nitrophenol phosphate. Both the $V_{\text{max}}$ and $K_m$ of the enzyme increased as the reaction pH was increased. This indicated a possible conformational relaxation of the alkaline phosphatase catalytic site to allow an elevated rate of catalysis. It is also possible that the ionization state of one or more residues in the catalytic site of alkaline phosphatase has been altered by the pH shift. This too could account for the pH-dependence of the enzyme’s $V_{\text{max}}$ and $K_m$.

Alkaline phosphatases form a large family of dimeric enzymes common to all organisms (1). They catalyze the hydrolysis of phosphomonoesters with the release of inorganic phosphate, which is a vital component of living organisms (5). The mechanism of alkaline phosphatase involves the activation of the catalytic serine by a zinc atom, the formation of a covalent phosphoseryl intermediate, the hydrolysis of the phosphoseryl by a water molecule activated by a second zinc atom, and the release of the phosphate product to a phosphate acceptor (3).

Varying pH levels may have a direct effect on the alkaline phosphatase due to the presence of ionizable residues in the catalytic site of the enzyme. Results from a previous study (3) indicated that alkaline phosphatase has an optimal enzyme activity at approximately pH 10. The previous study was based on a colorimetric assay dependent on the formation of the yellow product nitrophenol from the artificial substrate p-nitrophenol phosphate (pNPP). It was noted that the absorbance of nitrophenol increases from very low levels at pH 6, to maximal levels at pH 9-10. Thus, it is possible that the observed increase in enzyme activity at alkaline pH could merely be due to increased absorbance of nitrophenol at this pH.

Therefore, an experiment was conducted in order to negate the possible interference created by the pH dependent nature of the nitrophenol. In doing so, it was possible to determine whether alkaline phosphatase activity is pH dependent and at which pH optimal enzyme activity is observed, if different from the previous studies (3, 6).

MATERIALS AND METHODS

The materials and methods for this experiment are outlined in the Microbiology 421 laboratory manual (6), experiment A3 pages 1-3. Procedures from the manual were followed, with a number of modifications. Tris buffer was used at pH 7.0, 7.5, 8.0, 8.5 and 9.0. A 50 µM nitrophenol solution was prepared. Data from measuring the 50 µM nitrophenol absorbance under various pH levels were used to construct a standard curve, which allowed for the normalization of the data obtained during the enzyme assays. The nitrophenol assays consisted of 16.67 µM nitrophenol solution, 0.5 ml distilled water, and 0.25 M Tris buffer at pH 7.0, 7.5, 8.0, 8.5 or 9.0, for a final volume of 3.0 ml. The absorbance of each nitrophenol assay was then measured at 420nm using a zeroed Spectronic 20 (a mixture of 1.5 ml distilled water and 0.25M Tris buffer at the corresponding pH level was used as the blank). Data was recorded using the Vernier VIU data acquisition program (Portland, Oregon). The alkaline phosphatase assays consisted of 0.05, 0.1, 0.3, 0.5 or 1.0 ml of pNPP (2.0 mg/ml), 0.24 M Tris buffer at pH 7.0, 7.5, 8.0, 8.5 or 9.0, and distilled water added to a final volume of 3.1 ml. Enzyme activities were calculated based on the absorbance readings of the enzyme product at 420nm. Steps 6 and 7 of the procedure in the lab manual were omitted.

RESULTS

The absorbance of light at 420 nm by nitrophenol (a product of pNPP catalysis) is pH dependent (Table 1). As our standard curve showed, the absorbance at this wavelength increased from a pH of 7.0 up to 8.5, and then decreased
slightly at pH 9.0. This decrease is likely within experimental error, and probably does not represent a reproducible effect. This error caused a slight decrease in the conversion value of nitrophenol absorbance at pH 9.0. However, the decrease is very small and did not cause a significant effect on the calculated enzyme activity, $V_{\text{max}}$ or $K_m$ of alkaline phosphatase at pH 9.0.

Table 1 – Absorbance of 50 µM p-nitrophenol at 420nm under various pH levels.

<table>
<thead>
<tr>
<th>pH level</th>
<th>Absorbance at 420nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.112</td>
</tr>
<tr>
<td>7.5</td>
<td>0.154</td>
</tr>
<tr>
<td>8.0</td>
<td>0.161</td>
</tr>
<tr>
<td>8.5</td>
<td>0.173</td>
</tr>
<tr>
<td>9.0</td>
<td>0.168</td>
</tr>
</tbody>
</table>

Table 2 – $V_{\text{max}}$ and $K_m$ of Alkaline Phosphatase at various pH levels.

<table>
<thead>
<tr>
<th>pH level</th>
<th>$V_{\text{max}}$ (unit/ml)</th>
<th>$K_m$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>1.31</td>
<td>9.3</td>
</tr>
<tr>
<td>7.5</td>
<td>2.25</td>
<td>34.3</td>
</tr>
<tr>
<td>8.0</td>
<td>2.55</td>
<td>40.8</td>
</tr>
<tr>
<td>8.5</td>
<td>2.94</td>
<td>58.9</td>
</tr>
<tr>
<td>9.0</td>
<td>4.52</td>
<td>93.1</td>
</tr>
</tbody>
</table>

Figure 1 - The effect of pH on the enzymatic activity of alkaline phosphatase under different concentrations of substrate pNPP.

Even after correcting for the increased absorbance of nitrophenol at higher pH, the activity of alkaline phosphatase for the substrate pNPP was shown to be pH dependent (Fig. 1). As the alkalinity of the reaction mixture was increased from pH 7.0 to 9.0, a substantial increase in enzyme activity was observed. The increase was especially marked for the shifts from pH 7.0 to 7.5 and from 8.5 to 9.0, while the intermediate increases were more modest. The changes in enzyme activity correlate with the pH dependence of the $V_{\text{max}}$ and $K_m$ of alkaline phosphatase: both values were seen to increase as the reaction pH was increased (Table 2, Figure 2).
Figure 2 - Lineweaver-Burk plot for the effect of pH on the enzymatic activity of alkaline phosphatase under different concentrations of substrate pNPP.

At a reaction pH of 7.0, alkaline phosphatase appeared to be saturated by pNPP at low (0.05 mg/ml) concentrations of substrate (Fig. 1). This concentration increased to 0.5 mg/ml for reactions between pH 7.5 and 8.5. However, at a reaction pH of 9.0, alkaline phosphatase was not saturated, even up to a substrate concentration of 1 mg/ml.

DISCUSSION

Our results demonstrate that pH plays an important role in the enzymatic activity of alkaline phosphatase. If the $V_{\text{max}}$ values were all equal and the $K_m$ values differed from one another, then it would be the substrate that had been affected by the pH levels and not the enzyme. If it was reversed, and the $K_m$ values were all equal while the $V_{\text{max}}$ values were different, this would indicate that the pH had had an effect on the enzyme. If both $V_{\text{max}}$ and $K_m$ varied at different pH levels, then the pH may have affected both the enzyme and the substrate or the enzyme-substrate complex (A. Comeau - personal communication). Because the values of both $V_{\text{max}}$ and $K_m$ of the reaction increased at increased pH (Table 2, Figure 2), it was thought that varying pH affects both the enzyme and substrate structure.

The alteration in enzyme structure could take several forms. It is possible that an increase in pH results in a conformational change in alkaline phosphatase that allows an increase in substrate accessibility to the catalytic site of the enzyme. This "relaxing" of the catalytic site could lead to a decreased affinity (increased $K_m$) of the enzyme for the substrate while permitting a more rapid movement (increased $V_{\text{max}}$) of substrate into and out of the site (W. Ramey - personal communication). In addition to inducing an enzymatic conformation change, increased pH can result in an alteration of the ionization state of residues within the catalytic site. Ionization states can have a great effect on the ability of these residues to catalyze reactions (5). For alkaline phosphatase, it is likely that basic pH plays an important indirect role in active site conformation by creating an optimal ionic environment for the binding of Zn$^{2+}$ and Mg$^{2+}$, which act as allosteric cofactors (4). These changes within the alkaline phosphatase active site may occur at the following amino acids: His 331, His 412, Asp 327, the residues co-ordinated to the first Zn$^{2+}$; His 370, Asp359, the residues co-ordinated to the second Zn$^{2+}$; and Asp 51, the residue co-ordinated to both the Mg$^{2+}$ and the second Zn$^{2+}$ (4).

The low $V_{\text{max}}$ and $K_m$ of alkaline phosphatase at pH 7 (1.254 units/ml) may therefore be due to the protonation of one or more residues within its active site. This protonation may result in a direct effect on substrate catalysis, or it may interfere with important cofactor associations. Conversely, it is possible that a basic pH may lead to the
deprotonation of the phosphate group of pNPP, allowing for a more rapid binding to the catalytic site of alkaline phosphatase. Whatever the causes, a basic reaction pH results in a more efficient conversion of pNPP to p-nitrophenol (Fig. 1).

While each of the enzyme assays at the lower pH ranges (7.0 – 8.5) reached their respective \( V_{\text{max}} \), the enzyme assay at pH 9.0 did not, as if substrate was limiting at this concentration. This was expected because the optimal pH for Type VII – NA Bovine Intestinal Mucosal alkaline phosphatase is 9.8 (www.worthington-biochem.com/manual/P/BAP.html), and the faster reaction should process more substrate. This is in accordance with experiment A3 (6) in which the optimal pH was found to be approximately 10.

It can therefore be concluded that alkaline phosphatase activity is pH dependent. Further experimentation is necessary to identify the exact mechanism by which pH affects the catalytic activity of the alkaline phosphatase active site and the enzyme as a whole.

ACKNOWLEDGEMENTS

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REFERENCES

2. Bingle W.H. 2002. Biology 201: Part I: Section 201 & 204. Department of Microbiology & Immunology, University of British Columbia
Appendix:

Calculations:

1) The following formula was used to adjust the initial absorbance readings based on the data of nitrophenol absorbance over various pH levels (Table A1):

\[
\text{(initial abs) } \times \frac{\text{abs of nitrophenol at pH 8.5}}{\text{abs of nitrophenol at pH of reading}}
\]

Note:

pH 8.5 was chosen by convention as it had the highest absorbance reading. Using any other pH levels would give the same results.

Sample calculation:

Initial absorbance reading at pH 7 and 0.10ml pNPP is 0.09:

The adjusted absorbance reading = 0.09 * 0.173 / 0.112 = 0.139

2) The following formula was used to convert the adjusted absorbance readings into enzyme activity (Reference: Micb421 Lab Manual Exp A3 – 2):

\[
\text{Enzyme activity} = \left(\frac{\Delta A}{\Delta t}\right) \times \frac{1,000}{20,000} \times \frac{Nv}{Ev}
\]

Note:

Enzyme activity is expressed in units where 1 unit equals 1 µmol of nitrophenol product formed per minute

\[\Delta A = \text{change in abs at the 420nm wavelength}\]

\[\Delta t = \text{time (minute) for the observed change in absorbance}\]

\[Nv = \text{total assay volume (ml) at the time of the absorbance reading}\]

\[Ev = \text{volume of enzyme (ml) in assay}\]
Sample Calculation:

At the 10-second interval of pH 7 and 0.1ml pNPP:

Enzyme activity = \[\frac{(0.13902 - 0.12512)}{(1 / 60)} \times (1,000 / 20,000) \times (3.1 / 0.1) = 1.293 \text{ unit}\]

3) The average enzyme activity of each pH levels was obtained by averaging the enzyme activities between the 4 and 14-second time interval. This interval was used as all 25 assays show linear relationship between absorbance and time during this interval.

Sample Calculation:

Average enzyme activity at pH 7 and 0.1ml pNPP =

\[\frac{(1.006 + 1.293 + 1.293 + 1.149 +1.293 +1.149 + 1.006 + 1.149 + 1.006)}{10} = 1.164 \text{ unit}\]

4) The following formula was used to calculate the inverse substrate concentration:

\[1 / [S] = 1 / (2\text{mg/ml} \times \text{substrate vol.} / \text{total vol.})\]

Note:

[S] = concentration of substrate pNPP

The substrate added had an initial concentration of 2mg/ml

Sample Calculation:

When 0.1ml substrate was added:

\[1 / [\text{pNPP}] = 1 / (2\text{mg/ml} \times 0.1\text{ml} / 3.1\text{ml}) = 15.5 \text{ ml/mg}\]

5) The maximal activity of alkaline phosphate (Vmax) at each pH level is represented as the y-intercept of the corresponding line in the Lineweaver-Burk plot. The y-intercepts were calculated using the equation each line.

Sample Calculation:

Vmax at pH 7:
Equation of line at pH 7: \( y = 0.0061x + 0.7973 \)

\[ V_{\text{max}} = \text{y-intercept} = (0.0061)(0) + 0.7973 = 0.7973 \]

6) The negative inverse minimum substrate concentration \((-1/K_m)\) allowing alkaline phosphate to reach its \(V_{\text{max}}\) is represented as the x-intercept of the corresponding line in the Lineweaver-Burk plot. The x-intercepts were calculated using the equation of each line. The \(K_m\) at each pH level were then calculated from its corresponding \(-1/K_m\).

**Sample Calculation:**

\(K_m\) at pH 7:

Equation of line at pH 7: \( y = 0.0061x + 0.7973 \)

\[ -1/K_m = \text{x-intercept} = -0.7973 / 0.0061 = -130.705 \]

\[ K_m = -1 / 130.705 = 0.00765 \text{ mg/ml} \]