**Experiment 3: Activity Determination**

**Introduction:** Specific activity is a method for measuring enzymatic activity and the enzyme purity in a mixture. In order to determine the specific activity of an enzyme, the units of enzyme activity per mg of protein present, the amount of the enzyme's activity and protein content in an unknown mixture is needed. A “Mystery Mix” containing glucose, bovine serum albumin (BSA), and the enzyme acid phosphatase was used to determine the specific activity of the enzyme in the unknown mixture. First, the Bradford Method was used to determine the amount of protein present in the mixture using a colored dye called Bradford Reagent. The absorbance value of the dye was compared to a bovine serum albumin standard curve and the amount of protein contained in the sample was measured. The second procedure required a protein assay to determine the activity of acid phosphatase. The artificial substrate p-nitrophenylphosphate (PNPP) can be used to measure acid phosphatase activity in the unknown mystery mix. Acid phosphatase produces p-nitrophenol (PNP) from PNPP and the resulting product produces a yellow tint that can be detected using a spectrophotometer. The amount of yellow color produced and read on the spectrophotometer is representative of the amount of activity and product produced. The amount of enzyme that catalyzes the hydrolysis of 1.0 micromole PNPP per minute at 37°C measures to the amount of one unit of acid phosphatase activity.
Results: The table below includes data pertaining to the standard curve solutions created and the mystery mix solutions obtained along with their absorbance readings. Attached are the printouts from the Genesys 5 Spectrophotometer date including the plotted standard curve relative to the amount of BSA and the amount of Mystery Mix protein that reacted with the Bradford color reagent.

Procedure 1- Protein Content

Figure 1.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Bovine Serum Albumin (µg)</th>
<th>25 µl/ml BSA (ml)</th>
<th>0.15 M NaCl (ml)</th>
<th>Bradford Reagent (ml)</th>
<th>A595nm</th>
<th>Tube</th>
<th>20 Fold Mystery Mix (ml)</th>
<th>0.15 M NaCl (ml)</th>
<th>A595nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.04</td>
<td>0.96</td>
<td>1.0</td>
<td>0.053</td>
<td>7</td>
<td>0.1</td>
<td>0.9</td>
<td>0.083</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.2</td>
<td>0.8</td>
<td>1.0</td>
<td>0.190</td>
<td>8</td>
<td>0.2</td>
<td>0.8</td>
<td>0.142</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>0.4</td>
<td>0.6</td>
<td>1.0</td>
<td>0.306</td>
<td>9</td>
<td>0.4</td>
<td>0.6</td>
<td>0.240</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>0.6</td>
<td>0.4</td>
<td>1.0</td>
<td>0.402</td>
<td>10</td>
<td>0.6</td>
<td>0.4</td>
<td>0.315</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>0.8</td>
<td>0.2</td>
<td>1.0</td>
<td>0.495</td>
<td>11</td>
<td>1.0</td>
<td>0.0</td>
<td>0.419</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>0.556</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The amount of protein present in the five unknown tubes was calculated from µg to mg/tube.

Tube 7 (0.1 ml Mystery Mix): 1.7386/1000 = 1.7386 x 10^{-3} mg/tube
Tube 8 (0.2 ml Mystery Mix): 3.7257/1000 = 3.7257 x 10^{-3} mg/tube
Tube 9 (0.4 ml Mystery Mix): 7.3270/1000 = 7.3270 x 10^{-3} mg/tube
Tube 10 (0.6 ml Mystery Mix): 10.420/1000 = 10.420 x 10^{-2} mg/tube
Tube 11 (1.0 ml Mystery Mix): 15.456/1000 = 15.456 x 10^{-2} mg/tube
Total amount of protein in the mystery mix number 38 was calculated from the values given from the Genesys 5 and the average was determined.

\[
\text{Mg protein in tube} \times \text{DF} = \text{mg total protein}
\]

\[
\begin{align*}
\text{Sample Volume (ml) in tube} \times \text{Total volume of “stock” protein solution in stock solution} &= \text{mg total protein} \\
\text{Tube 7:} &\quad 1.174 \times 10^{-3} \text{mg/tube} \times 20 \times 6 \text{ ml} = 1.408 \text{ mg} \\
&\quad \text{0.1 ml} \\
\text{Tube 8:} &\quad 3.726 \times 10^{-3} \text{mg/tube} \times 20 \times 6 \text{ ml} = 2.236 \text{ mg} \\
&\quad \text{0.2 ml} \\
\text{Tube 9:} &\quad 7.327 \times 10^{-3} \text{mg/tube} \times 20 \times 6 \text{ ml} = 2.198 \text{ mg} \\
&\quad \text{0.4 ml} \\
\text{Tube 10:} &\quad 1.042 \times 10^{-2} \text{mg/tube} \times 20 \times 6 \text{ ml} = 2.084 \text{ mg} \\
&\quad \text{0.6 ml} \\
\text{Tube 10:} &\quad 1.546 \times 10^{-2} \text{mg/tube} \times 20 \times 6 \text{ ml} = 1.855 \text{ mg} \\
&\quad \text{1.0 ml} \\
\text{Average:} &\quad 1.956 \text{ mg total protein}
\end{align*}
\]

**Procedure 2- Enzyme Activity**

The acid phosphatase activity assay was then performed using amounts of the mystery mix solution ranging from 0.1 ml to 0.8 ml. Other solutions of 0.5 ml 1.0M sodium acetate buffer, 0.5ml 0.05M p-nitrophenyl phosphate, and varying amounts of water to make the total volume of 5.0 ml were added to the tube. A tube containing all the solutions except the enzyme was also created to serve as the blank. The solutions with the varying Mystery Mix amounts were duplicated into samples A and B and incubated in 37°C water bath and KOH was added to stop the assay reaction. After ending the reaction, the solutions and their absorbances were recorded in the table below.

**Figure 2.**

<table>
<thead>
<tr>
<th>Assay Tube</th>
<th>1.0M NaOAc (ml)</th>
<th>0.05M PNPP (ml)</th>
<th>dH₂O (ml)</th>
<th>Mystery Mix (ml)</th>
<th>0.5M KOH (ml)</th>
<th>A405nm A</th>
<th>A405nm B</th>
<th>A405nm Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>3.9</td>
<td>0.1</td>
<td>2.5</td>
<td>0.176</td>
<td>0.168</td>
<td>0.172</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>3.8</td>
<td>0.2</td>
<td>2.5</td>
<td>0.276</td>
<td>0.343</td>
<td>0.309</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>0.5</td>
<td>3.6</td>
<td>0.4</td>
<td>2.5</td>
<td>0.642</td>
<td>0.662</td>
<td>0.652</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0.5</td>
<td>3.2</td>
<td>0.8</td>
<td>2.5</td>
<td>1.120</td>
<td>1.126</td>
<td>1.123</td>
</tr>
<tr>
<td>blank</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
The calculations below were used to convert to \( \mu \) moles/min. A simplified equation was determined to obtain the same values.

**Example Equations** (including sample absorbance value of 1.123)

\[
A = \varepsilon cl \quad c = \varepsilon/c = \frac{1.123}{18800} = 5.973 \times 10^{-5} = \text{molarity}
\]

\[
\begin{align*}
\text{Molarity} & = 5.973 \times 10^{-5} = 1.1946 \times 10^{-5} \frac{\text{moles/litter}}{\text{min}} \\
\# \text{of minutes for assay reaction} & = 5 \text{ min} \\
\text{moles/litter} \times \text{number of liters} & = (1.1946 \times 10^{-5}) \times (0.0075 \text{ liters}) = 8.960 \times 10^{-8} \text{ Units} \\
\text{min} & \quad \text{in reaction assay}
\end{align*}
\]

\[
\begin{align*}
\text{moles/min} \times 1 \times 10^6 \frac{\mu \text{ moles/1 mole}}{} & = (8.960 \times 10^{-8}) \times (1 \times 10^6) = 8.960 \times 10^{-2} \text{ Units}
\end{align*}
\]

**Simplified equations** (including all average absorbance values)

\[
\begin{align*}
(1.123)(0.0798) & = 8.96 \times 10^{-2} \frac{\mu \text{ moles/min}}{} = \text{Units} \\
(0.652)(0.0798) & = 5.20 \times 10^{-2} \frac{\mu \text{ moles/min}}{} = \text{Units} \\
(0.3095)(0.0798) & = 2.47 \times 10^{-2} \frac{\mu \text{ moles/min}}{} = \text{Units} \\
(0.172)(0.0798) & = 1.37 \times 10^{-2} \frac{\mu \text{ moles/min}}{} = \text{Units}
\end{align*}
\]
The level of activity attributed to the amount of protein containing solution used to initiate the reaction was calculated.

\[
\frac{\text{Units}}{\# \text{ml of protein solution added to assay}} = \frac{8.96 \times 10^{-2}}{0.8 \text{ ml}} = 0.112 \text{ Units/ml}
\]

\[
\frac{\text{Units}}{\# \text{ml of protein solution added to assay}} = \frac{5.20 \times 10^{-2}}{0.4 \text{ ml}} = 0.13 \text{ Units/ml}
\]

\[
\frac{\text{Units}}{\# \text{ml of protein solution added to assay}} = \frac{2.47 \times 10^{-2}}{0.2 \text{ ml}} = 0.12 \text{ Units/ml}
\]

\[
\frac{\text{Units}}{\# \text{ml of protein solution added to assay}} = \frac{1.37 \times 10^{-2}}{0.1 \text{ ml}} = 0.137 \text{ Units/ml}
\]

The Total Units of Enzyme Activity was calculated by multiplying the Units/ml obtained by the dilution factor of 1.

\[
\text{Units} \times \text{DF} \times \text{ml of stock protein solution} = \text{Units/ml}
\]

\[
0.112 \text{ units/ml} \times 1 \times 6\text{ml} = 0.67 \text{ Units of Enzyme Activity}
\]

\[
0.13 \text{ units/ml} \times 1 \times 6\text{ml} = 0.78 \text{ Units of Enzyme Activity}
\]

\[
0.12 \text{ units/ml} \times 1 \times 6\text{ml} = 0.72 \text{ Units of Enzyme Activity}
\]

\[
0.137 \text{ units/ml} \times 1 \times 6\text{ml} = 0.82 \text{ Units of Enzyme Activity}
\]
Finally, the specific activity amount was calculated using the Total units of enzyme activity calculated in procedure 2 divided by the average total protein calculated in procedure 1.

<table>
<thead>
<tr>
<th>Total Units of Enzyme Activity</th>
<th>Total Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.672</td>
<td>1.956</td>
</tr>
<tr>
<td>= 0.344 Units/mg</td>
<td></td>
</tr>
<tr>
<td>0.78</td>
<td>1.956</td>
</tr>
<tr>
<td>= 0.400 Units/mg</td>
<td></td>
</tr>
<tr>
<td>0.72</td>
<td>1.956</td>
</tr>
<tr>
<td>= 0.368 Units/mg</td>
<td></td>
</tr>
<tr>
<td>0.82</td>
<td>1.956</td>
</tr>
<tr>
<td>= 0.419 Units/mg</td>
<td></td>
</tr>
</tbody>
</table>

Average = 0.383 Units/mg

**Discussion:** Specific Activity was used to determine the units of enzyme activity per mg of protein present in the mystery mix solution that contained unknown amounts of glucose, bovine serum albumin, and acid phosphatase. Specific activity is used to represent the amount of substrate a given enzyme converts per mg of protein in the solution through a given amount of time. Because the solution contains unknown amounts of its contents, the protein content and enzyme activity were necessary before the specific activity of the enzyme could be determined. Enzymologists determine specific activity in order to determine the purity of the enzyme sample mixture. As the enzyme becomes more pure in a mixture, the specific activity value increases. Purified proteins are valuable for production of growth factors, hormones, DNA polymerases, and antibodies that are useful in the medical technology and research fields where they can create life-saving products and make a profit. The specific activity of acid phosphatase was calculated at 0.383 Units/mg protein. The expected range of the specific activity is known to be 0.05-2.0 units/mg of protein. The calculated value therefore falls within the expected range of the specific activity for the enzyme acid phosphatase.