Study of the Properties of $\beta$-Galactosidase

Theory

The enzyme $\beta$-galactosidase allows lactose metabolism in *Escherichia coli*. As shown in Figure 7-1, this enzyme catalyzes the hydrolysis of lactose into its monosaccharide units, galactose and glucose. A side product of the $\beta$-galactosidase reaction, allolactose, is known to control the expression of the enzyme at the level of transcription.

The gene for $\beta$-galactosidase (*lacZ*) resides within a set of genes and regulatory DNA sequences collectively referred to as the *lac operon* (Fig. 7-2).

An operon is a set of genes whose expression is coordinately regulated. Often, these genes encode proteins or enzymes related in a particular function or to a particular biological pathway. The *lac* operon consists of a promoter sequence, an operator (regulatory) sequence, and three genes. As mentioned above, *lacZ* encodes $\beta$-galactosidase. *LacY* encodes galactoside permease, a transmembrane transport protein that allows lactose to enter the cell. *LacA* encodes thiogalactoside transacetylase, an enzyme whose function is not fully understood. The promoter sequence within the operon is the site where...
RNA polymerase binds the DNA and initiates transcription of \( \text{lacZ} \), \( \text{lacY} \), and \( \text{lacA} \). As we shall see, the operator sequence adjacent to the promoter is an important regulatory sequence that will participate in the control of the expression of the \( \text{lac} \) operon.

Transcription of the \( \text{lac} \) operon can be induced (turned on) in the presence of lactose and repressed in the absence of lactose when the components required for its metabolism are not needed. This regulatory system ensures that the cell will not waste
valuable energy producing proteins that it does not immediately require. Expression of the lac operon is repressed in the presence of the lac repressor protein. This regulatory protein, encoded by the lacI gene immediately 5' to the lac operon, is a constitutively expressed DNA binding protein that has a high affinity for the lac operator sequence. When the lac repressor binds the operator sequence, it blocks RNA polymerase from transcribing the lac operon. As stated earlier, transcription of the lac operon is increased in the presence of lactose. This phenomenon is due to the effect that allolactose (the inducer) has on the affinity of the lac repressor for the operator sequence (Fig. 7-2). As the lac repressor (LacI) binds allolactose, it loses its affinity for the operator sequence. RNA polymerase is then able to transcribe the lac operon to produce the proteins required for lactose metabolism. In the presence of lactose, expression of the lac operon can increase by up to as much as 1000-fold. Although allolactose is the natural inducer of the lac operon, it is not the only compound that will increase its level of expression. Isopropylthiogalactoside (IPTG, Fig. 7-3) is a gratuitous inducer of the lac operon. Although it is not a substrate for β-galactosidase, it too will complex with the lac repressor protein and lower its affinity for the operator sequence.

The regulation of the lac operon just described is a form of negative regulation: LacI represses expression of the operon in the absence of the inducer. It turns out, however, that transcription of the lac operon is also subject to positive regulation. Immediately 5' to the lac promoter is another regulatory DNA region called the CAP (catabolite gene activator protein) site. When CAP is bound at this site, transcription of the lac operon can be increased 20-fold to 50-fold. By itself, CAP has low affinity for the CAP site in the operon. The affinity of CAP for this site increases dramatically, however, when the protein binds cAMP (Fig. 7-2). Since the lac promoter by itself is relatively weak, the cAMP–CAP complex bound at the CAP site is required for significant expression of the lac operon. It is apparent, then, that the expression of the lac operon is closely tied to the level of intracellular cAMP.

Adenylate cyclase is the enzyme responsible for catalyzing the conversion of ATP to cAMP (Fig. 7-4). This enzyme is inactive in the cell when glucose levels are high. As a result, cAMP levels decrease in the cell when glucose is present. The net effect of this is that the cAMP–CAP complex is not able to form, and transcription of the lac operon is decreased. This will be the case even if lactose is also present. Adenylate cyclase is the enzyme responsible for catalyzing the conversion of ATP to cAMP (Fig. 7-4). This enzyme is inactive in the cell when glucose levels are high. As a result, cAMP levels decrease in the cell when glucose is present. The net effect of this is that the cAMP–CAP complex is not able to form, and transcription of the lac operon is decreased. This will be the case even if lactose is also present.
The ability of glucose to prevent expression of the lac operon even in the presence of its inducer is an example of catabolite repression. Since glucose and gluconate are the preferred carbon sources for E. coli, these compounds are very effective catabolite repressors of the lac operon. Other carbon sources, such as glycerol, acetate, and succinate, are less effective catabolite repressors. Since catabolite repression is due to the effect of high glucose levels on adenylate cyclase activity, the effect of catabolite repression can be overcome (reversed) in vivo through the addition of cAMP to the growth medium.

You will use p-nitrophenyl-β-galactopyranoside (ONPG), rather than lactose, as the substrate for the β-galactosidase assays performed in this experiment. β-galactosidase will hydrolyze the ONPG substrate to produce two products, one of which absorbs light at 420 nm under alkaline conditions. By monitoring the change in $A_{420}$ of a solution containing β-galactosidase and ONPG, you will be able to continuously measure the change in concentration of the product (p-nitrophenol, ONP) over time.

In this four-period experiment, you will study and characterize the kinetics of β-galactosidase at varying pH and temperature as well as in the presence of two different inhibitors of the enzyme. In these exercises, you will determine the $K_M$ and $V_{max}$ for the enzyme with respect to its ONPG substrate, as well as the inhibition constant ($K_i$) for the two inhibitors. In addition, the temperature studies will allow you to calculate the activation energy for the β-galactosidase catalyzed reaction. Finally, you will utilize a number of different E. coli strains in an in vivo β-galactosidase assay to demonstrate the intricate regulatory system of the lac operon and the concept of catabolite repression. Before you begin the experiment, review the section on enzymology in the Introduction to Section II.

### Supplies and Reagents

- 0.08 M sodium phosphate buffer, pH 7.7
- 0.08 M sodium phosphate buffer, pH 6.4
- 0.08 M sodium phosphate buffer, pH 6.8
- 0.08 M sodium phosphate buffer, pH 7.2
- 0.08 M sodium phosphate buffer, pH 8.0
- β-galactosidase solution (~10 units/milliliter in 0.08 M sodium phosphate buffer, pH 7.7, supplemented with 1 mg/milliliter bovine serum albumin)
- p-nitrophenyl-β-galactopyranoside solutions (2.5 mM and 10 mM)
- 13 × 100 mm glass test tubes
- 16 × 125 mm glass test tubes with caps (sterile)
- 1.5-ml plastic microcentrifuge tubes
- P-20, P-200, P-1000 Pipetmen with disposable tips
- methyl-β-D-galactopyranoside (MGP) solution (750 mM)
- methyl-β-D-thiogalactoside (MTG) solution (150 mM)
- isopropylthiogalactoside (IPTG) solution (200 mM)—filter-sterilized
- cAMP solution (100 mM)—filter sterilized
- 1 M Na$_2$CO$_3$
- 0.1% (wt/vol) sodium dodecyl sulfate (SDS) solution
- chloroform
- YT broth (1% wt/vol hactotryptone, 0.5% wt/vol yeast extract, 0.5% wt/vol NaCl)
- 20% (wt/vol) glucose solution—filter sterilized
- spectrophotometer to read absorbance at 280 nm, 420 nm, 600 nm, and 550 nm
- heating blocks or temperature-controlled water baths
- colorimeter tubes and quartz cuvettes
Protocol

Day 1: Determination of the Activity and Specific Activity of the β-Galactosidase Solution

1. Set up the following reactions in six 13-by-100-mm glass test tubes. *Keep the enzyme solution on ice and add it to the tubes below, which are at room temperature.*

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of 0.08 M Sodium Phosphate, pH 7.7 (ml)</th>
<th>Volume of 2.5 mM ONPG (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>4.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

2. Add 0.5 ml of sodium phosphate buffer, pH 7.7 to tube 1 (blank) and mix gently in a Vortex mixer. Blank your spectrophotometer to read zero absorbance at 420 nm against this solution.

3. Add 0.5 ml of undiluted β-galactosidase solution to tube 2. Mix gently with a Vortex mixer for 5 sec and place the solution in your spectrophotometer. Exactly 30 sec after the addition of the enzyme, record the A₄₂₀ of the solution in your notebook. Continue to record the A₄₂₀ value at 30-sec intervals until the A₄₂₀ value no longer changes (indicates that the reaction is complete). This final A₄₂₀ value will be used later to determine the activity of β-galactosidase in each reaction (ΔA₄₂₀max).

4. Prepare a twofold dilution of the enzyme by adding 250 μl of the β-galactosidase stock solution to 250 μl of 0.08 M sodium phosphate buffer, pH 7.7. Add this entire 0.5-ml solution to tube 3. Exactly 30 sec after the addition of the enzyme, record the A₄₂₀ of the solution in your notebook. Continue to record the A₄₂₀ value at 30-sec intervals. Unlike with tube 2, it is not necessary that this reaction is followed to completion. Rather, you are looking to obtain a set of A₄₂₀ values that are linear with respect to time over about 2 min.

5. Prepare a fivefold dilution of the enzyme by adding 100 μl of the β-galactosidase stock solution to 400 μl of 0.08 M sodium phosphate buffer, pH 7.7. Add this entire 0.5-ml solution to tube 4. Exactly 30 sec after the addition of the enzyme, record the A₄₂₀ of the solution in your notebook. Continue to record the A₄₂₀ value at 30-sec intervals. Unlike with tube 2, it is not necessary that this reaction is followed to completion. Rather, you are looking to obtain a set of A₄₂₀ values that are linear with respect to time over about 5 min.

6. Prepare a 10-fold dilution of the enzyme by adding 50 μl of the β-galactosidase stock solution to 450 μl of 0.08 M sodium phosphate buffer, pH 7.7. Add this entire 0.5-ml solution to tube 5. Exactly 30-sec after the addition of the enzyme, record the A₄₂₀ of the solution in your notebook. Continue to record the A₄₂₀ value at 30-sec intervals. Unlike with tube 2, it is not necessary that this reaction is followed to completion. Rather, you are looking to obtain a set of A₄₂₀ values that are linear with respect to time over about 5 min.

7. Prepare a 20-fold dilution of the enzyme by adding 25 μl of the β-galactosidase stock solution to 475 μl of 0.08 M sodium phosphate buffer, pH 7.7. Add this entire 0.5-ml solution to tube 6. Exactly 30 sec after the addition of the enzyme, record the A₄₂₀ of the solution in your notebook. Continue to record the A₄₂₀ value at 30-sec intervals. Unlike with tube 2, it is not necessary that this reaction is followed to completion. Rather, you are looking to obtain a set of A₄₂₀ values that are linear with respect to time over about 5 min.

8. Prepare a plot of A₄₂₀ versus time for the data obtained from the reactions in tubes 2 to 6. Plot the data from all five reactions on a single graph for comparison.

9. Draw a “best-fit” curve through each set of data points. For each curve, determine over what time frame the reaction kinetics appear linear (where ΔA₄₂₀/Δtime yields a straight line). Calculate the slope of the linear portion of each curve.

10. Use the following equation to determine the β-galactosidase activity (micromoles of ONPG hydrolyzed per minute/per milliliter of enzyme) in each reaction:

    \[
    \text{Activity} = \frac{[\Delta A_{420}/\Delta \text{time}][1.25/\Delta A_{420\max}]}{V_p}
    \]
where $1.25/\Delta A_{420\text{max}}$ is a conversion factor relating the number of micromoles of ONPG hydrolyzed to the change in absorbance at 420 nm, and $V_p$ is the total volume (in milliliters) of $\beta$-galactosidase solution present in each reaction. Taking the volumes of the stock $\beta$-galactosidase solution (undiluted) used in each reaction into consideration, what is the activity of the $\beta$-galactosidase in the stock solution of the enzyme? **NOTE:** You should be able to calculate several activity values for the stock $\beta$-galactosidase solution using the reactions in tubes 2 to 6. These values should be averaged and presented along with a deviation from the mean.

11. Obtain two 1.5-ml plastic microcentrifuge tubes. To one, add 0.5 ml of distilled water and 0.5 ml of the stock $\beta$-galactosidase solution. To the other, add 0.8 ml of distilled water and 0.2 ml of the stock $\beta$-galactosidase solution. Cap each tube and invert several times to mix.

12. Read the absorbance of both solutions in a quartz cuvette at 280 nm using water as a blank to zero your spectrophotometer. Record the $A_{280}$ value of both solutions in your notebook.

13. Based on these values, estimate the total protein concentration (in milligrams per milliliter) of the stock $\beta$-galactosidase solution using Beer’s law ($A = ecd$). Assume an extinction coefficient of 0.8 (mg/ml)$^{-1}$ cm$^{-1}$. This extinction coefficient is based on the average value of aromatic amino acids found in most proteins.

14. Calculate the specific activity of the stock $\beta$-galactosidase solution (micromoles of ONPG hydrolyzed per minute per milligram of protein) using the following equation:

$$\text{Specific activity} = \frac{\text{activity}}{\text{[protein]} \times \text{[mg/ml]}}$$

**Day 2: Determination of $K_M$ and $V_{max}$ and the Effect of Inhibitors on $\beta$-Galactosidase Activity**

**Determination of $K_M$ and $V_{max}$**

1. From the data obtained on Day 1, determine a dilution of the stock $\beta$-galactosidase solution that showed good linear kinetics over a 5-min period (where $\Delta A_{420}/\Delta \text{time}$ produced a straight line over 5 min). Use a dilution of the enzyme that gives a change in absorbance at 420 nm of about 0.1 per min. Prepare 9 ml of a dilute $\beta$-galactosidase solution by making the appropriate dilution of the $\beta$-galactosidase stock solution in sodium phosphate buffer, pH 7.7. This dilute $\beta$-galactosidase solution will be used in both of the assays described below. **Keep this solution on ice and add to the tubes below at room temperature when you are ready to perform the assay.**

2. Set up the following reactions in six 13-by-100 mm glass test tubes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of Phosphate Buffer, pH 7.7 (ml)</th>
<th>Volume of 2.5 mM ONPG (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.00</td>
<td>0.50</td>
</tr>
<tr>
<td>2</td>
<td>4.00</td>
<td>0.50</td>
</tr>
<tr>
<td>3</td>
<td>4.20</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>4.30</td>
<td>0.20</td>
</tr>
<tr>
<td>5</td>
<td>4.35</td>
<td>0.15</td>
</tr>
<tr>
<td>6</td>
<td>4.40</td>
<td>0.10</td>
</tr>
</tbody>
</table>

3. Add 0.5 ml of sodium phosphate buffer, pH 7.7, to tube 1 (blank) and mix by gently swirling. Blank your spectrophotometer to read zero absorbance at 420 nm against this solution.

4. Add 0.5 ml of the dilute $\beta$-galactosidase solution to tube 2. Mix gently with a Vortex mixer for 5 sec and place the solution in your spectrophotometer. Exactly 30 sec after the addition of the substrate, record the $A_{420}$ of the solution in your notebook. Continue to record the $A_{420}$ value at 30-sec intervals for 5 min.

5. Follow the exact same procedure as described in step 3 for tubes 3 to 6. Record the $A_{420}$ at 30-sec intervals for at least 5 min for each reaction.

6. Prepare a plot of $A_{420}$ versus time for the data obtained from the reactions in tubes 2 to 6. Record the $A_{420}$ at 30-sec intervals for at least 5 min for each reaction.

7. Calculate the slope of each line. If your enzyme dilution prepared in step 1 was correct, the $\Delta A_{420}$ per minute for each reaction should be linear for at least 5 min.
8. Use the following equation to determine the initial velocity of each \( \beta \)-galactosidase reaction (micromoles of ONPG hydrolyzed per minute):

\[
\text{Initial velocity } (V_0) \, (\text{micromoles ONPG/minute}) = \frac{(\Delta A_{420}/\Delta \text{time})(1.25/\Delta A_{420\text{max}})}{1000}
\]

Note how the slope of each line (initial velocity) changes with decreasing ONPG concentration.

9. Prepare a plot of \(1/V_0\) versus \(1/[\text{ONPG}]\) (a Lineweaver–Burk plot) using the data obtained from tubes 2 to 6. Do the data points “fit” to a straight line? From the \(x\)-intercept of this graph, can you determine the \(K_M\) (mM) for \( \beta \)-galactosidase with respect to ONPG? From the \(y\)-intercept of this graph, can you determine the \(V_{\text{max}}\) for the \( \beta \)-galactosidase solution (micromoles of ONPG hydrolyzed per minute)?

Effect of Inhibitors on \( \beta \)-Galactosidase Activity

1. Set up the following reactions in twelve 13-by-100-mm glass test tubes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of Phosphate Buffer, pH 7.7 (ml)</th>
<th>Volume of MGP (ml)</th>
<th>Volume of MTG (ml)</th>
<th>Volume of 2.5 mM ONPG (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.9</td>
<td>0.1</td>
<td>—</td>
<td>0.50</td>
</tr>
<tr>
<td>2</td>
<td>3.9</td>
<td>0.1</td>
<td>—</td>
<td>0.50</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>0.1</td>
<td>—</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
<td>0.1</td>
<td>—</td>
<td>0.20</td>
</tr>
<tr>
<td>5</td>
<td>4.25</td>
<td>0.1</td>
<td>—</td>
<td>0.15</td>
</tr>
<tr>
<td>6</td>
<td>4.30</td>
<td>0.1</td>
<td>—</td>
<td>0.10</td>
</tr>
<tr>
<td>7</td>
<td>3.9</td>
<td>—</td>
<td>0.1</td>
<td>0.50</td>
</tr>
<tr>
<td>8</td>
<td>3.9</td>
<td>—</td>
<td>0.1</td>
<td>0.50</td>
</tr>
<tr>
<td>9</td>
<td>4.1</td>
<td>—</td>
<td>0.1</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>4.2</td>
<td>—</td>
<td>0.1</td>
<td>0.20</td>
</tr>
<tr>
<td>11</td>
<td>4.25</td>
<td>—</td>
<td>0.1</td>
<td>0.15</td>
</tr>
<tr>
<td>12</td>
<td>4.3</td>
<td>—</td>
<td>0.1</td>
<td>0.10</td>
</tr>
</tbody>
</table>

2. Add 0.5 ml of sodium phosphate buffer, pH 7.7, to tube 1 (blank) and mix by gently swirling. Blank your spectrophotometer to read zero absorbance at 420 nm against this solution.

3. Add 0.5 ml of the dilute \( \beta \)-galactosidase solution to tube 2. Swirl gently for 5 sec and place the solution in your spectrophotometer. Exactly 30 sec after the addition of the substrate, record the \(A_{420}\) of the solution in your notebook. Continue to record the \(A_{420}\) value at 30-sec intervals for 5 min.

4. Follow the exact same procedure as described in step 3 for tubes 3 to 6. Record the \(A_{420}\) at 30-sec intervals for at least 5 min for each reaction.

5. Add 0.5 ml of sodium phosphate buffer, pH 7.7, to tube 7 (blank) and mix by gently swirling. Zero your spectrophotometer at 420 nm against this solution.

6. Add 0.5 ml of dilute \( \beta \)-galactosidase solution to tube 8. Swirl gently for 5 sec and place the solution in your spectrophotometer. Exactly 30 sec after the addition of the substrate, record the \(A_{420}\) of the solution in your notebook. Continue to record the \(A_{420}\) value at 30-sec intervals for 5 min.

7. Follow the exact same procedure as described in step 6 for tubes 9 to 12. Record the \(A_{420}\) at 30-sec intervals for at least 5 min for each reaction.

8. Prepare two plots of \(A_{420}\) versus time: one plot should include data obtained from the reactions in tubes 2 to 6, the other should include data obtained from the reactions in tubes 8 to 12. Plot the data from reactions containing the same inhibitor on a single graph for comparison.

9. Use the following equation to determine the initial velocity of each \( \beta \)-galactosidase reactions (micromoles of ONPG hydrolyzed per minute):

\[
\text{Initial velocity } (V_0) = \frac{(\Delta A_{420}/\Delta \text{time})(1.25/\Delta A_{420\text{max}})}{1000}
\]

10. Prepare a plot of \(1/V_0\) versus \(1/[\text{ONPG}]\) (a Lineweaver–Burk plot) using the data obtained from tubes 2 to 6. Compare the \(x\)-intercept and \(y\)-intercept of this graph with those obtained in the absence of inhibitor (to determine \(K_M\) and \(V_{\text{max}}\)). Are they the same? What type of inhibition does methyl-\( \beta \)-D-galactopyranoside display toward \( \beta \)-galactosidase? Can you calculate an inhibition constant \((K_I)\) for this inhibitor (remember that the methyl-\( \beta \)-D-galactopyranoside stock solution was at a concentration of 750 mM)?

11. Prepare a plot of \(1/V_0\) versus \(1/[\text{ONPG}]\) (a Lineweaver–Burk plot) using the data obtained...
from tubes 8 to 12. Compare the x-intercept and y-intercept of this graph with those obtained in the absence of inhibitor (to determine $K_M$ and $V_{max}$). Are they the same? What type of inhibition does methyl-$\beta$-D-thiogalactoside display toward $\beta$-galactosidase? Can you calculate an inhibition constant ($K_I$) for this inhibitor (remember that the methyl-$\beta$-D-thiogalactoside stock solution was at a concentration of 150 mM)?

12. Based on the $K_I$ values for methyl-$\beta$-D-galactopyranoside and methyl-$\beta$-D-thiogalactoside, which compound is the more potent inhibitor of $\beta$-galactosidase?

13. Based on the type(s) of inhibition displayed by these two inhibitors, can you determine the site on the $\beta$-galactosidase enzyme with which these two compounds interact? Explain. Would you expect to be able to reverse the effects of these inhibitors by changing the concentration of ONPG used in these assays? Explain.

Day 3: Effect of pH and Temperature on $\beta$-Galactosidase Activity

Effect of pH on $\beta$-Galactosidase Activity

1. Prepare two different dilutions of the stock $\beta$-galactosidase solution: the first dilution should be the same as that done for the experiments on Day 2 (one that gave a linear change in absorbance at 420 nm per minute of about 0.1). Designate this solution “$\beta$-gal 1.” The second solution should be twice as dilute as the first solution. Designate this solution “$\beta$-gal 2.” You will need a total of 1.0 ml of each dilute $\beta$-galactosidase solution for the assays described below.

2. Obtain 10 ml of sodium phosphate buffers ranging in pH from 6.4 to 8.0:

Buffer 1 = pH 6.4
Buffer 2 = pH 6.8
Buffer 3 = pH 7.2
Buffer 4 = pH 7.7
Buffer 5 = pH 8.0

3. Set up the following reactions in thirteen 13-by-100-mm glass test tubes. All volumes are given in milliliters:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Buffer 1</th>
<th>Buffer 2</th>
<th>Buffer 3</th>
<th>Buffer 4</th>
<th>Buffer 5</th>
<th>$\beta$-gal 1</th>
<th>$\beta$-gal 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>3.9</td>
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<tr>
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<td></td>
<td>3.9</td>
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<td>6</td>
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<td>3.9</td>
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<td>7</td>
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<td></td>
<td>3.9</td>
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<td>8</td>
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<td>12</td>
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<td></td>
<td></td>
<td>3.5</td>
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</tbody>
</table>

4. To tube 1 (blank), add 0.5 ml of 2.5 mM ONPG and 0.5 ml 1 M Na$_2$CO$_3$, mix, and use the solution to zero your spectrophotometer at 420 nm.

5. Add 0.5 ml of 2.5 mM ONPG to tube 2, mix gently with a Vortex mixer for 5 sec, and incubate at room temperature for 4 min.

6. After the 4 min incubation, stop the reaction by adding (and mixing in) 0.5 ml of 1 M Na$_2$CO$_3$. The addition of this weak base will raise the pH of the reaction enough to completely stop the reaction.

7. Perform steps 5 and 6 on tubes 3 to 7. Record the final $A_{420}$ of all these solutions in your notebook.

8. To tube 12 (blank), add 0.5 ml of 2.5 mM ONPG and 0.5 ml of 1 M Na$_2$CO$_3$, mix, and use the solution to zero your spectrophotometer at 420 nm.

9. Add 0.5 ml of 2.5 mM ONPG to tube 8, mix gently with a Vortex mixer for 5 sec, and incubate at room temperature for 4 min.

10. After the 4-min incubation, stop the reaction by adding (and mixing in) 0.5 ml of 1 M Na$_2$CO$_3$.

11. Perform steps 9 and 10 on tubes 9 to 11. Record the final $A_{420}$ of all these solutions in your notebook.

12. To tube 13, add 0.5 ml of the undiluted $\beta$-galactosidase stock solution. Add 0.5 ml of 2.5 mM ONPG, mix gently with a Vortex mixer for 5 sec, and incubate at room temperature for 10 min, or until the $A_{420}/\Delta$time is equal to zero (indicates that the reaction is complete).
13. Add 0.5 ml of 1 M Na₂CO₃, mix, and record the final $A_{420}$ value of this solution in your notebook. This value ($A_{420\text{max}}$) will be used to calculate the initial velocity of each of these β-galactosidase reactions. *It is necessary to calculate this new conversion factor since these fixed time-point assays are being stopped by increasing the pH.* Recall from Experiment 1 that the absorbance of a solute is strongly dependent on the pH and ionic strength of the solution in which it resides. Since the pH at which you are measuring the absorbance of these solutions is not the same as the pH at which you were measuring the absorbance of the solutions on Day 1, a new conversion factor must be calculated.

14. Calculate the initial velocity of each β-galactosidase reaction using the following equation (micromoles of ONPG hydrolyzed per minute):

$$V_0 = \frac{\Delta A_{420}}{4 \text{ min}} \left( 1.25 \frac{1}{\Delta A_{420\text{max}}} \right)$$

**NOTE:** Two dilutions of the enzyme were used at each pH because this is a fixed time-point assay. Rather than continuously measuring the change in absorbance at 420 nm over time, you stopped each reaction at 4 min by increasing the pH. If the enzyme dilution that showed linear kinetics over 4 min at pH 7.7 did not show linear kinetics at a different pH, the solution with a lower concentration of the enzyme (β-gal 2) will produce linear kinetics over 4 min. If β-gal 1 and β-gal 2 both displayed linear kinetics over 4 min at a particular pH, then the initial velocity of the solution containing β-gal 1 will be twice that of the solution containing β-gal 2.

15. Prepare a plot of $V_0$ versus pH based on the results of this assay. From this plot, can you determine the pH optimum for the reaction catalyzed by β-galactosidase?

16. Based on the pH optimum for β-galactosidase and your knowledge of the pKₐ values of the various amino acid side chains, can you hypothesize which amino acids might be present or important in the active site of the enzyme?

### Effect of Temperature on β-Galactosidase Activity

1. Prepare the following reactions in nine 13-by-100 mm glass test tubes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of Phosphate Buffer, pH 7.7 (ml)</th>
<th>Volume of 2.5 mM ONPG (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>3.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

2. Place tubes 2 and 6 in a 25°C water bath. Place tubes 3 and 7 in a 30°C water bath. Place tubes 4 and 8 in a 37°C water bath. Place tubes 5 and 9 in a 48°C water bath. Incubate all of these tubes at the indicated temperatures for 10 min.

3. Add and mix 0.1 ml of β-gal 1 to tubes 2 to 5. *Note the exact time that the dilute β-galactosidase solution was added to each of the tubes.* After exactly 4 min, add 0.5 ml of 1 M Na₂CO₃ to each of tubes 2 to 5.

4. Add and mix 0.1 ml of β-gal 2 to tubes 6 to 9. *Note the exact time that the dilute β-galactosidase solution was added to each of the tubes.* After exactly 4 min, add 0.5 ml of 1 M Na₂CO₃ to each of tubes 6 to 9.

5. To tube 1 (blank), add 0.5 ml of 1 M Na₂CO₃, mix, and use the solution to zero your spectrophotometer at 420 nm.

6. Measure and record the final $A_{420}$ values of the solutions in tubes 2 to 9 in your notebook.

7. Calculate the initial velocity of each β-galactosidase reaction using the following equation (micromoles of ONPG hydrolyzed per minute):

$$V_0 = \frac{\Delta A_{420}}{4 \text{ min}} \left( 1.25 \frac{1}{\Delta A_{420\text{max}}} \right)$$

**NOTE:** Two dilutions of the enzyme were used at each temperature because this is a fixed time-point assay. Rather than continuously measuring the change in absorbance at 420 nm over time, you stopped each reaction at 4 min by increasing the pH. If the enzyme dilution that showed linear kinetics over 4 min at a particular temperature did not show linear kinetics at a different temperature, the solution with a lower concentration of the enzyme (β-gal 2)
will produce linear kinetics over 4 min. If β-gal 1 and β-gal 2 both displayed linear kinetics over 4 min at a particular temperature, then the initial velocity of the solution containing β-gal 1 will be twice that of the solution containing β-gal 2.

8. Construct a plot of log $V_0$ versus 1/absolute temperature (°K). Determine the slope of the line through the linear portion of this curve. From this slope, determine the Arrhenius activation energy ($E_a$) for the reaction catalyzed by β-galactosidase using the following equation:

$$\text{Slope} = -\frac{E_a}{2.5R}$$

where $R$ is the gas constant (1.98 cal/deg.-mol).

9. What do you notice about the activity of the enzyme at higher temperatures? Explain what you think is happening to the enzyme at higher temperatures.

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**Day 4: Catabolite Repression and Regulation of the lac Operon in Vivo**

We suggest that steps 1 through 3 be performed by the instructor prior to the beginning of the experiment.

1. The night before the experiment, grow 5-ml overnight cultures of each of the following E. coli strains in YT broth with shaking at 37°C.

   - EM1: E. coli K-12 (lacI, cya, crp, thi)
   - EM1327: E. coli K-12 (lacI, cya, crp, thi)
   - EM1328: E. coli K-12 (lacI, Δcrp, crp, thi)
   - EM1329: E. coli K-12 (ΔlacI, cya, crp, thi)

Recall that the lacI, crp, and cya genes encode the LacI repressor protein, the catabolite gene activator protein, and adenylate cyclase, respectively.

2. The next morning (~3–4 hr before the experiment), dilute the cultures 1:10 to 1:200 in 50 ml of fresh YT broth. We have found that these strains display different growth rates. It is very important that you perform growth trials beforehand to determine the correct dilution for each strain to ensure that the cultures will grow to the same optical density prior to the beginning of the experiment.

3. Grow these cultures at 37°C with shaking to late log phase ($A_{600} \sim 1.0$). This will take approximately 3 to 4 hr.

4. Set up the following inoculations in 12 sterile (16 × 125 mm) glass test tubes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>YT Broth (ml)</th>
<th>IPTC (ml)</th>
<th>cAMP (ml)</th>
<th>20% Glucose (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>0.05</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>0.05</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>7</td>
<td>5.0</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>8</td>
<td>5.0</td>
<td>0.05</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>9</td>
<td>5.0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>10</td>
<td>5.0</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>11</td>
<td>5.0</td>
<td>0.05</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
<td>12</td>
<td>5.0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

5. Using sterile technique (consult the instructor), add 100 µl of late-log-phase strain EM1 culture to tubes 1 to 3. Add 100 µl of late-log-phase strain EM1327 culture to tubes 4 to 6. Add 100 µl of late-log-phase strain EM1328 culture to tubes 7 to 9. Add 100 µl of late-log-phase strain EM1329 culture to tubes 10 to 12.

6. Grow all of the cultures with shaking at 37°C for 2.5 to 3.0 hr.

7. Set up 12 16 × 125 mm glass test tubes (one for each culture) containing the following:

   - 3.75 ml of sodium phosphate buffer, pH 7.7
   - 50 µl of chloroform
   - 50 µl of 0.1% (wt/vol) sodium dodecyl sulfate (SDS) solution

Number the tubes 1 to 12. The reaction for each numbered culture will be done in the corresponding numbered reaction tube.

8. Add 0.25 ml of each culture to the corresponding reaction tube. Mix vigorously in a Vortex mixer for 5 sec. The chloroform and SDS will disrupt the integrity of (solubilize) the cell membrane, allowing the ONPG substrate to come into contact with the β-galactosidase present in the cell.

9. Add 0.5 ml of 10 mM ONPG to each reaction tube. Mix and incubate at room temperature
for 15 to 20 min or until a yellow color begins to appear (be sure that some yellow color begins to appear in at least some of the reaction tubes. If it does not appear after 20 min, continue the reaction until it does. If you see yellow color appear after 2 or 3 min, decrease the incubation time to 5 min). It is important that you note the exact time that the ONPG is added to each tube.

10. Stop each of the 12 reaction tubes by adding and mixing in 0.5 ml of 1 M Na₂CO₃. It is important that you note the exact time that the Na₂CO₃ is added to each tube to stop the reaction.

11. Prepare a blank solution in a large glass test tube containing the following:

3.75 ml of sodium phosphate buffer, pH 7.7
50 μl of chloroform
50 μl of 0.1% (wt/vol) SDS solution
0.25 ml of YT broth
0.5 ml of 10 mM ONPG
0.5 ml of 1M Na₂CO₃

Use this solution to zero your spectrophotometer at both 550 nm and 420 nm in the absorbance determination described below.

12. Measure the A₄₂₀ and A₅₅₀ of the reaction solutions in each of the 12 reaction tubes. Record these values in your notebook.

13. Measure the A₆₀₀ of each of the 12 cultures (not the reaction tubes). For the absorbance measurements taken at 600 nm, the spectrophotometer should be blanked to read zero absorbance of a solution containing only YT broth.

14. Calculate the activity of β-galactosidase (nanomoles of ONPG hydrolyzed per minute/A₆₀₀ of culture) using the following equation:

\[
\text{Activity} = \frac{A_{420} - (1.75 \times A_{550})}{A_{600} t} \cdot \frac{1}{0.0045} \cdot 5 \text{ ml}
\]

where \( t \) = time of reaction (min), 0.0045 = extinction coefficient (μM⁻¹ cm⁻¹) for ONP under these conditions, (1.75 \( \times \) A₅₅₀) = correction factor for the light scattering of the culture at 420 nm, and 5 ml = total reaction volume.

15. Based on your knowledge of the regulation of the lac operon and catabolite repression, discuss whether or not each strain displayed a level of β-galactosidase activity that was expected under the various growth conditions.

### Exercises

1. During the purification of β-galactosidase, a student found that 1 ml of a 1:10,000 dilution of crude extract gave A₄₂₀ readings as follows:

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>A₄₂₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.059</td>
</tr>
<tr>
<td>30</td>
<td>0.084</td>
</tr>
<tr>
<td>45</td>
<td>0.109</td>
</tr>
<tr>
<td>60</td>
<td>0.134</td>
</tr>
<tr>
<td>75</td>
<td>0.159</td>
</tr>
</tbody>
</table>

The total reaction volume was 10 ml, containing 8.0 ml of buffer (pH 7.5), 1.0 ml of 7.5 mM ONPG solution, and 1.0 ml of the diluted β-galactosidase solution. A standard 10-mM solution of ONP, pH 7.5, has an A₄₂₀ of 0.50 in the 1-cm pathlength colorimeter cuvettes used for these assays.

a. One unit of β-galactosidase under these conditions should correspond to what increase in A₄₂₀ per minute (1 unit = one micromole of ONPG hydrolyzed per minute)?

b. If the protein concentration of the undiluted crude extract was 13.3 mg/ml, what is the specific activity of the extract in units per milligram?

2. Should the presence of IPTG in the growth medium have any effect on the expression of the lac operon in the lacI⁻ strain? Explain.

3. Should the presence of cAMP in the growth medium have any effect on the expression of the lac operon in the lacI⁻ strain? Explain.

4. Should the presence of IPTG in the growth medium have any effect on the expression of the lac operon in the cya⁻ strain? Explain.

5. Should the presence of cAMP in the growth medium have any effect on the expression of the lac operon in the cya⁻ strain? Explain.

6. Can you think of a possible mutation in the promoter sequence of the lac operon that would
produce a strain with the same level of expression of the lac operon as the lacI– strain in the presence of IPTG and/or cAMP?

6. Explain how the activity of adenylate cyclase affects expression of the lac operon. Describe situations (growth conditions) when the activity of adenylate cyclase would be high and when it would be low.

7. Explain how a mutation in the lacY gene could affect the regulation of the lac operon.

8. Why was it necessary to calculate a new conversion factor relating the $\Delta A_{420}$ to the number of micromoles of ONPG hydrolyzed for the kinetics experiments done on Day 3, despite the fact that the concentration of ONPG stock solution was the same as that on Day 1 and Day 2?

REFERENCES


