Isolation of Alkaline Phosphatase from *E. coli*

 Alkaline phosphatase is an enzyme that catalyzes the hydrolysis of phosphate-containing compounds. The enzyme is a dimeric protein that requires Zn2+; its activity is further enhanced by the presence of Mg2+. This enzyme will be isolated from *E. coli*. The strain of *E. coli* that will be used is K12. This strain was originally isolated from the stool of a malaria patient in 1921. K12 has been genetically modified so that production of alkaline phosphatase is unregulated, with the result that it is produced in large amounts. (Many other genetically modified strains of this strain have been prepared.)

Figure . Gram negative bacterial cell wall. Figure by Jeff Dahl from Wikipedia.

 *E. coli* is a Gram negative bacteria; such bacteria have two cell walls, Figure 1, separated by a periplasmic space, Figure 2. (Peri- means all around, or enclosing.) The alkaline phosphatase will be isolated from the periplasmic space because, unlike within the cell, the periplasmic space does not contain

 

Figure . Enlarged view of the cell wall of Gram negative bacteria. Figure by Jeff Dahl from Wikipedia.

many proteins, making purification of the enzyme easier. The alkaline phosphatase enzyme is associated with the surface of the inner membrane.[[1]](#endnote-1) To free the enzyme, the peptidoglycan has to be broken into smaller pieces. This is done using lysozyme, which hydrolyzes peptidoglycan. When the outer membrane is removed, what remains is called a spheroplast. This remnant of the cell is fragile, and must be handled gently to avoid spilling its contents into solution.

# Solutions

**sterile LB (Luria-Bertani) media for liquid culture**

To make 1 L, combine 25 g dehydrated LB Brotha and 950 mL deionized H2O in a 1 L Erlenmeyer flask. Cover with an inverted beaker. Sterilize by boiling the solution for a few minutes.

## Stock solution of the *E. coli* culture (prepared by instructor)

 A culture of this bacteria is stored at -80°C in a solution that is 50% glycerol. Transfer 1 mL of the thawed culture to a 40 mL of LB broth in a 50 mL centrifuge tube. Keep covered. Store overnight at 37°C (A couple of days may be necessary at room temperature). The solution should be shaken occasionally to provide oxygen to the cells (not essential, as *E. coli* is an adaptive anaerobe.) (The organisms can be grown at room temperature, just not as quickly.)

## Students’ solutions of the *E. coli* culture (prepared by students the week before the lab)

 Each pair of students will transfer 10 mL of LB broth to a labeled (with, e.g., initials) 15 mL centrifuge tube. Keep covered with the lid so that bacteria in the air do not contaminate the solution. To the solution add 0.1 to 0.5 mL of the stock solution of *E. coli*. Mix, then leave in the storage area provided. Over the next week the *E. coli* will reproduce, causing the contents to become cloudy. (An absorbance at 550 nm of from 0.8 to 1.8 would be appropriate.)

## Solutions

Lysozyme, 10 mg/mL

Place 1.5 mL of deionized water in a 2 mL disposable plastic centrifuge tube; add 15 mg of lysozyme. Shake until the solid has all dissolved. Prepare a second tube the same way. Label the tubes “lysozyme”. (Store at 4°C for up to 4 days.)

DNAse I, 5 mg/mL (10,000 U/mL)

Place 1.5 mL of deionized water in a 2 mL disposable plastic centrifuge tube; add 7.5 mg of DNAse I. Shake until the solid has all dissolved. Label the tubes “DNAse I”. Store on ice during the class period. (Store at 4°C for up to 4 days.)

100 mL 0.1 M EDTA

To a small plastic bottle with lid, add 100 mL of deionized water and 3.72 g of the disodium salt of EDTA (ethylenediaminetetraacetic acid). Shake for a few minutes until the solid is completely dissolved. Label the bottle “0.1 M Na2EDTA”.

100 mL 1 M MgSO4

To a small plastic bottle with lid, add 100 mL of deionized water and either 2.5 g of MgSO4·7H2O or 1.2 g of MgSO4. Shake for a minute until the solid is completely dissolved. Label the bottle “1 M MgSO4”.

1 L 0.2 M Tris HCl buffer, pH 8.0

To a 1 L volumetric flask (accuracy isn’t important) add 16.1 g “Trizma Base” and 10.6 g “Trizma HCl”. Dilute to the mark with deionized water. Label the bottle “0.2 M Tris buffer, pH 8”.

1 L 50X stock dialysis solution (0.5 M Tris HCl buffer, pH 7.4, 0.5 M MgSO4)

To a 1000 mL Erlenmeyer flask add 53.7 g “Trizma Base”, 8.8 g “Trizma HCl”, and either 123 g of MgSO4·7H2O or 60.2 g of MgSO4. Dilute to the 1000 mL mark with deionized water. Label the bottle “50X dialysis solution”.

# Procedure

## Day 1

Cool 100 ml H2O on ice.

## Isolate *E. coli* cells

 Fill a 50 mL centrifuge tube with about 40 mL of the cloudy broth containing *E. coli* K12. Transfer the broth to 3 15 mL centrifuge tubes, putting about 12 mL in each. Stopper with cork corks (the lids are too large to fit in the centrifuge). Label the tubes with your initials. Centrifuge the 15 mL tubes at 12,000 gmax at 4°C for 15 minutes (*g* is the acceleration due to gravity).

The angular velocity in rpm that gives a particular *g*max depends on the radius of the centrifuge’s rotor (the spinning element).
$$v=\frac{9.8\frac{m}{s} ×60\frac{s}{min}}{.01\frac{m}{cm}× 2π}\sqrt{\frac{g\_{max}}{radius}}$$

This simplifies to

$$v, in rpm=299\sqrt{\frac{g\_{max}}{radius, cm}}$$

(The temperature control prevents heat build-up.) The cells will form a pellet at the bottom of the tube. Discard the supernatant into the container marked “Waste E. coli solution”.

## Osmotic shock of bacterial cells

## Shock buffer: 20% sucrose, 1 mM EDTA, 0.3 M Tris-HCl pH 8.1

Resuspend the pellet in 25 ml shock buffer.

Incubate @ room temperature for 10 minutes.

Centrifuge as above.

Decant the supernatant.(the protein is still in the shriveled-up cells).

Resuspend the pellet in the residual liquid.

Add 25 ml of ice cold H2O.

Incubate on ice for 10 minutes.

Centrifuge for 10 min.

Decant and save the supernatant (could filter through a 0.45μm filter). The protein is in the supernatant!

Rinse the 50 mL tube and leave at the front desk. Add 4 mL of pH 8.0 0.2 M tris-HCl buffer to each tube, and shake to suspend the cells. Combine the suspensions into one tube, using a little more buffer to help with the transfer. Centrifuge again, decant the buffer, and resuspend the cells in 10 mL of the buffer solutions.

## Remove spheroplasts

To the cell suspension in buffer add 100 µL of the 10 mg/mL lysozyme solution. This is to break the outer cell wall. To prevent breaking the now-fragile inner cell wall, handle the solution gently.

Add 100 µL of the 5 mg/mL DNAse I solution. This is to break up any DNA that may have been released if some of the spheroplasts rupture. (Breaking up the DNA makes the solution less viscous, which speeds up the centrifugation step.) Give the enzymes time to work by incubating the solution for two minutes, which is done by placing the centrifuge tube in a beaker of lukewarm water.

Add 100 µL of 0.1 M Na2EDTA. EDTA is a chelating ligand that coordinates to metal ions. In this case, Ca2+ bind to the cell wall, making the wall stronger. Removing it makes it easier for the lysozyme to cleave the cell wall. Very gently mix the solution by swirling. Incubate for another 10 minutes.

 

Figure . EDTA is a chelating ligand.

Add 100 µL of 1 M MgSO4 and gently swirl the solution. The Mg2+ combines with the excess EDTA, so it can’t interact with other metals. (Lysozyme contains Zn2+; the excess EDTA2- would eventually remove that necessary ion from the enzyme.) Incubate the solution for 20 minutes.

Centrifuge the solution for 20 minutes in the refrigerated high speed centrifuge going at maximum speed. The spheroplasts will go to the bottom of the tube, leaving the enzyme in solution. The centrifuge is set at 4°C so that the sample does not heat up during the separation. A force of around 12,000 *g* is desirable. Calculate the maximum *g* force on the centrifuge tube using the following formula.

$$v, in rpm=299\sqrt{\frac{g\_{max}}{radius, cm}}$$

*Maximum g force: \_\_\_\_\_\_\_\_\_\_\_*

In fact, our centrifuge will not provide 12,000 *g*, but the separation works well anyway. The centrifuge will hold everyone’s tube at one time.

While the centrifuge is running, cut off an 11 cm length of dialysis tubing from the roll of tubing. Soak it in deionized water for 20 minutes (a 150 mL beaker should work for this).

When the 20 minutes are over, the centrifuge will slowly slow down, so as not to resuspend the solid at the bottom of the tube.

 Carefully decant (pour) the supernatant into a clean 50 mL centrifuge tube, leaving the solid plug of cell debris behind. Record the volume of the solution.

*Volume of enzyme solution: \_\_\_\_\_\_\_\_\_\_\_\_*

Dispose of the solid plug by suspending it in a little water, perhaps with shaking or stirring, and emptying the suspension into the container labeled *E. coli* waste.

## Remove small molecules

 Dialysis reduces the concentration of salts and nonpolar substances in a solution. The dialysis tubing we will use has a molecular weight cut-off of 12,000-14,000, so it will retain the alkaline phosphatase. Rinse the dialysis tubing with deionized water. Obtain two clamps for the dialysis tubing: one with a piece of string attached, and one without the string. Label a piece of labeling tape with your names, and attach the tape to the end of the string. Clamp one end of the dialysis tubing with a clamp. Use a plastic disposable pipette to transfer the supernatant from centrifuging into the dialysis tubing. Clamp the other end of the tubing. (Leave lots of space in the dialysis tubing for expansion; if the tubing looks like a sausage, not enough space is available for expansion.) Place the clamped, labeled, dialysis tube in the large beaker at the front of the room. This beaker contains a solution that is salty, so that not much water will diffuse into the dialysis tubing. The tubing should be completely submerged within this salt solution.

## Day 2

Alkaline phosphatase is relatively stable at high temperatures. This is used to purify the enzyme: A solution containing the enzyme is heated to nearly 80°C, which denatures other proteins, causing them to precipitate.

Some proteins will precipitate from solution when salt is added. This will be used to further purify the enzyme: ammonium sulfate will be added, then the protein will be isolated as a solid, which will be re-dissolved. The solution will be dialyzed again to remove remaining ammonium sulfate.

## Procedure

**Heat denaturing:**

Start heating water in a 500 mL beaker containing about 300 mL of tap water.

Transfer the solution in the dialysis tubing to 15 mL centrifuge tubes (use as many as necessary to hold your solution). Squeeze the dialysis tubing to remove the last bit of solution. Use a plastic pipet to transfer a half mL of the solution to a tiny plastic tube labeled “dialyzed”; this solution will be analyzed for alkaline phosphatase activity. Also, ensure that the centrifuge tubes contain the same amount of solution (this may help with balancing the centrifuge).

When you get a chance, start soaking a 10 cm long piece of dialysis tubing in deionized water. This is used in the last step of the procedure.

Place the lid on the centrifuge tube loosely (it can be tightened once the tube has warmed up), and put the solution in a water bath that is between 75 and 80°C. Leave the tube in the solution for 15 minutes. While waiting, perform the analysis of the “dialyzed” sample by following the “Assay of Alkaline Phosphatase” directions.

Cool the tubes under running water (or in ice-water).

Centrifuge the tubes with the centrifuge going at top speed (set at 4900 rpm) for 15 minutes.

Transfer the supernatant to a 15 mL centrifuge tube (or use a 50 mL tube if more than 15 mL of solution are present). Do this carefully so as not to disturb the pellet of denatured protein. Again, use a plastic pipet to transfer a half mL of the solution to a tiny plastic tube labeled “heated”; this solution will also be analyzed later for alkaline phosphatase activity.

**Salt-precipitation:**

Estimate the volume of enzyme solution in the centrifuge tubes. Weight out solid ammonium sulfate, obtaining 0.603 g of salt for every mL of enzyme solution. Put the lid on the tube and gently shake it to dissolve all the salt. (If using a 50 mL centrifuge tube, after shaking, transfer the solution to 15 mL tubes.) Let the solution sit for 15 minutes so that precipitation can occur. While waiting, perform the analysis of the “heated” sample.

Centrifuge the solution for 30 minutes at high speed. Proteins do not usually require so long a centrifuge time, but the added salt makes the solution denser, which slows how fast the solid settles.

Carefully decant the liquid, which may be discarded. The tiny amount of solid in the tube is the enzyme. (The solution may have some white solid floating on it; this is probably some lipid material.)

Add 0.5 mL of 0.010 M tris buffer, pH 7.4, that contains 0.010 M MgSO4 (*i.e.*, the dialysis buffer) to the pellet in the centrifuge tube. Gently re-suspend the pellet. Transfer the solution to a dialysis tube. Wash the centrifuge three more times with 0.5 mL of the buffer solution to complete transfer of the enzyme to the tubing.

Label the dialysis tubing and leave it in the beaker of dialysis buffer at the front of the lab.

## Assay of Alkaline Phosphatase

Solutions:

1 L 0.2 M tris buffer, pH 8.0

10 mL 0.200 *M* *p*-nitrophenyl phosphate di(tris)

Place 0.0461 g of p-nitrophenyl phosphate di(tris) in a 10 mL volumetric flask. Dilute to the mark with deionized water. Store on ice.

500 mL 0.050 M p-nitrophenol (139.11 g/mol) in 0.2 M tris buffer, pH 8.0.

0.0035 g p-nitrophenol is dissolved in a 500 mL volumetric flask with the pH 8 buffer. Transfer the solution to a plastic container. Label the container “Stock 0.050 M p-nitrophenol in pH 8 buffer”.

100 mL 0.000050 M p-nitrophenol (139.11 g/mol) in 0.2 M tris buffer, pH 8.0.

Dilute 1 mL of the 0.050 M p-nitrophenol stock solution to 1 L volumetric flask. Dilute to the mark with in the pH 8 buffer. Transfer the solution to a plastic container labeled “0.000050 M p-nitrophenol in pH 8 buffer”.

From:

Bessey, O.A., Lowry O.H. and Brock M.J.:(1946) J.Biol. Chem. 164 321

Described at <http://www.worthington-biochem.com/bap/assay.html> under “Assay (E. coli Enzyme)”.

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1. Identification of a Regulated Alkaline Phosphatase, a Cell Surface-Associated Lipoprotein, in *Mycobacterium smegmatis.*  Kriakov, J.; Lee, S. h.; William R. Jacobs, W. R., Jr. *J. Bacteriol.* **2003** *185*, 4983-4991. [↑](#endnote-ref-1)