Cellular Fractionation and Isolation of Enzymatic Activity

(adapted with permission from Sheri Miraglia, Ph.D., City College of San Francisco)

A. Objectives

Become familiar with

1. Targeting of proteins to different locations;
2. The correct use of centrifuges;
3. The correct use of micropipettors;
4. The correct disposal of all materials, tubes, cuvettes, etc.

B. Before coming to lab

1. Read this laboratory exercise and study Figure 6.5 on page 97 of Campbell et al. *Biology.*
2. Write or draw a concise protocol for this exercise
3. In your lab notebook, answer the four "Test your understanding" questions below.
4. In your lab notebook, make an argument for each of the following three hypotheses:
   a. The amylase will be found in the cytosol.
   b. The amylase will be found in the periplasm.
   c. The amylase will be found in the extracellular space
5. Also in your lab notebook, write down which of the above hypotheses you think is the most likely (this will be your initial hypothesis).

C. During lab

1. Work in groups of two. One group should, in addition to their experiment, set up a positive control for the Benedict's test.

2. Fractionate the *E.coli* suspension you are given (see Part E, Note 2) utilizing the clinical and high speed centrifuges.

3. Determine where the alpha amylase enzyme is located (is it secreted into the medium, secreted into the periplasmic space, or is it located within the bacterial cell?) Write your conclusion in your laboratory notebook. **The answer will count for 3 points towards your notebook grade.**

4. Remove all labels from all of your tubes and dispose of their contents in the containers provided. Place all the tubes, except the ones from the Benedict's test, in containers of disinfectant. Get a test tube brush from under the sink and scrub the tubes used for the Benedict's test with detergent to remove all traces of precipitate. Then rinse and invert in the wire baskets in the disposal area.

D. After lab

If you do not opt for writing a laboratory report on this exercise, write one paragraph in your lab notebook in which you evaluate your initial hypothesis in light of your results.
Do the take-home assignment on page 53 and turn it in at the beginning of the next lab period.

E. Background

Note 1: Experimental theory

Bacteria naturally produce many proteins that have a variety of uses in the cell. Some of these proteins remain inside the cell (cytoplasmic), while some are secreted either to the periplasm (the space in between the cell wall and the cell membrane) or to the outside of the cell. Generally speaking, a protein is sent to wherever it is needed, based on its function in the cell (a transmembrane receptor, for example, must be sent to the plasma membrane after it is synthesized in other parts of the cell). When new proteins are synthesized they are "outfitted" with signal sequences that direct their internal transport within the cell to their final destination. These signal sequences are then clipped off as a part of the post-translational processing of the protein.

Sometimes, as in the case of a transmembrane receptor protein, it is obvious where the protein will be located in the cell. However, at other times, it may not be so obvious. To determine where a protein is within the cell structure, you need to first fractionate (separate) the cell's contents and then assay (test) for the protein you are interested in. To determine if the protein is secreted, one must simply assay the media that the cells were suspended in. The isolation of periplasmic fractions involves removing the cells from the media and resuspending them in a buffered solution containing isotonic concentrations of sucrose. The solution is buffered with an amine containing molecule known to biologists as 'tris' which is present in most biological media to stabilize the pH. EDTA (ethylenediamine-tetraacetic acid) is then added which chelates (binds to) divalent cations such as calcium and magnesium that are required by many cellular proteases that would otherwise chew up the protein you are trying to isolate. EDTA also disrupts the cell wall, while the isotonic sucrose solution allows the plasma membrane to remain intact. The resulting products (cytoplasmic contents surrounded by the plasma membrane) are called spheroplasts. Cytoplasmic fractions can be isolated by resuspending the spheroplasts in cold water. The cells will swell in the hypotonic solution and burst, releasing their contents.

Test your understanding:

1. How are proteins targeted to a particular location?
2. How do you isolate the extracellular fraction?
3. How do you isolate the periplasmic fraction?
4. How do you isolate the cytoplasm?

The bacterial cells you will be given in this experiment have been growing overnight in culture media. They produce an enzyme called amylase which breaks down large starch molecules into smaller dimers, trimers, etc. of glucose. This enzyme is homologous to the amylase that is produced in human saliva. You may notice that if you hold a small piece of bread in your mouth without swallowing for a few minutes, it gradually becomes sweeter. This is due to the production of glucose as the amylase protein breaks down the starch. Your mission is to fractionate the cells and determine where the amylase that is produced is sent.

Note 2: Experimental method

Fill a 50 mL plastic centrifuge tube with 40 ml of bacterial suspension provided. You do not need to use aseptic technique (why not?). Balance your tube against that of another student and centrifuge for 5 minutes at 4000 r.p.m. in the clinical centrifuge (use the highest setting on the speed control knob to get 4000 r.p.m.).

Decant the medium that the bacteria were suspended in and save it (why?). Resuspend the pellet in 4 ml of ice cold 20% sucrose, which includes 10 mM tris-HCl (pH 7.5). Vortex the

suspension gently until there are no “chunks” of *E. coli* floating around. This time, your suspension will be cloudy.
For every 1 mL of sucrose solution you added, add 50 microliters (0.05 mL) of 0.25 M EDTA (pH 8) to your suspension. Leave this suspension on ice for ten minutes, vortexing gently twice during this period.
Centrifuge the mixture in the high speed centrifuge at 8000 r.p.m. for 5 minutes. Remember to balance the centrifuge.
Decant the supernatant and save (why?). Add 4 ml ice cold distilled water. Incubate on ice for 20 minutes, vortexing vigorously every 2.5 minutes for a total of 20 minutes. This suspension now corresponds to which fraction?
Determine the location of the alpha amylase enzyme within the original bacterial suspension using the Benedict’s test. Incubate 2.5 mL of 1% starch with 2.5 mL of each fraction you have collected that you think might contain amylase at 37° C for 10 minutes. Then pour 1 mL of the mixture into a clean 16 x 150 mm culture tube, add an approximately equal amount of Benedict’s reagent, and heat in boiling water for 5 minutes (refer to laboratory 2 to read up on the Benedict’s test).

F. Review questions

1. What is the function of amylase?

2. How does a cell know where to send a particular protein?

3. Where is the periplasm?

4. What is the tonicity of our *E. coli*?

5. What is the function of tris?

6. What are the two functions of EDTA?

7. How does EDTA work?

8. Define spheroplast

9. Why will *E. coli* spheroplasts burst when suspended in cold water?

10. What is the content of the pellet after centrifuging your original bacterial suspension for 5 minutes at 4000 r.p.m.?

11. Why do you have to save the supernatant from the above centrifugation step?

12. After centrifuging the first time for 5 minutes at 8000 r.p.m., what is the content of a) the pellet and b) the supernatant?

13. Why do we incubate our fractions with starch?

14. Which fraction contains sucrose? Does that invalidate results for that fraction?

15. Why do we need to keep everything cold?

16. In our second investigation of the model cell, we found that starch cannot pass through dialysis tubing. How is this finding relevant for today's investigation? Is there a contradiction?

17. Would you expect *E. coli* to digest starch? Why (not)?