Protein Separation and Enzyme Kinetics

University of California, Irvine
Department of Chemical and Biomolecular Engineering
CBE 140B

Introduction

Proteins, the natural polymers, are the building blocks of all living things. They are heat sensitive and m must maintain a specific conformation (3D structure, presence of subunits, coenzymes, etc.) to retain their functionalities. Their natural physiological environment (pH, temperature, salt contents and other necessary conditions) define when they exhibit highest activity and stability. Current technology allows the isolation and reproduction of important proteins that have applications such as medical treatment, drug delivery, and tissue engineering. In this experiment, you will have a chance to practice and learn basic protein isolation, purification, and characterization techniques. Table 1 lists common separation processes used in the chemical industry. Some of these processes must be modified before being applied to protein separation in order to minimize the chance of denaturation.

Table 1. Separation processes based on physical/chemical properties

Physical/chemical property	Separation process
Size	Filtration, microfiltration, ultrafiltration, dialysis, gas separation, gel permeation chromatography
Vapor pressure	Distillation, membrane distillation
Freezing Point	Crystallization
Affinity	Extraction, adsorption, absorption, reverse osmosis, gas separation, pervaporation, affinity chromatography
Charge	Ion exchange, electrodialysis, electrophoresis, diffusion dialysis
Density	Centrifugation
Chemical nature	Complexation, carrier mediated transport

Experiment A (Day 1)

Objective: To extract total whole cell protein from bacterial cells and perform affinity chromatography.

Background

Escherichia coli (*E. coli*) is a model host to express recombinant proteins due to the simple machinery in its metabolic pathway and fast doubling time. *E. Coli* bacteria normally live in the intestines of people and animals. Most *E. coli* are harmless and are an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic, meaning they can cause illness, either diarrhea or other symptoms when outside of the intestinal tract. The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food, or through contact with animals or persons.[1] The *E. coli* used in this lab is an engineered stain called XL1 Blue containing a small circular piece of DNA, or a plasmid, which encodes for the protein β-galactosidase with a six-histidine tag, under the lacZ promoter. *E. Coli* bacteria are unicellular prokaryote. Most recombinant proteins are designed to express and secrete into the cytosol. In order to extract the cytosolic protein, the bacterial cells can first be resuspended in a buffer before having their cell membranes ruptured mechanically. The cytosolic proteins are then released into the solution. The cell debris can be removed centrifugation. In this exercise, cells will be lysed via sonication.

Procedure

1. Decant liquid from cell pellet into a separate container (do not discard) until ~1 mL remains. Resuspend cell pellet by shaking. (Do step 10 with a small sample of unlysed cells.)

2. Transfer the resuspended cells to a 1.5 mL microcentrifuge tube. Keep on ice.

3. Clean sonicator tip with 70% ethanol and a kimwipe.

4. Set sonicator to no more than 50% power.

5. Put on ear protection.

6. Turn on sonicator and submerge tip into cell suspension.

7. Sonicate sample for 10-30 s. (Do not let sample warm up too much.)

8. Cool sample on ice for 1 min.

9. Repeat steps 6 and 7 twice more. Once finished, clean sonicator tip with 70% ethanol and a kimwipe.

10. Aliquot 48 μL of the whole cell lysate into a new microcentrifuge tube. Add 12 μL of pink 5x SDS-PAGE loading buffer to this tube. When all SDS-PAGE samples from day 1 are ready, boil at 95 °C for 5 minutes. Save for SDS-PAGE analysis on day 2.

11. Use the centrifuge to spin lysate at max speed (12500 RPM) for 3 min. Ensure each sample to be centrifuged is balanced by a tube of water of identical weight in the opposite position. Collect the supernatant from the pellet. This is the clarified lysate. The pellet, containing lysed cell debris, can be discarded. (Repeat step ten with a sample of the clarified lysate.)

12. Combine the clarified lysate with the decanted liquid from step 1 and mix well. Store on ice.

13. Pipet 0.5 mL of the solution from step 12 into a new microcentrifuge tube. Determine the protein concentration in this solution using the Bradford assay. With the remainder of the solution from step 12, you may simultaneously continue to step 14.

13.A. The Bradford assay is done by observing the absorbance at 595 nm, indicative of protein binding with Coomassie dye. In constructing a calibration curve, be aware that the relationship between absorbance and protein concentration is only linear for a certain range of concentrations.

13.B. For each trial, mix 980 μL of Bradford reagent with 20 μL of either a standard solution (0.1, 0.5, 1, 5, 10, 15, 20 mg mL^{-1} of Bovine Serum Albumin, BSA) or an unknown sample. Mix by using clean parafilm, inverting, and allow binding to occur for at least 5 minutes and no more than one hour. As absorbance may change over time, measure all samples promptly, taking note of incubation time.

14. The next several steps will detail how to separate histidine-tagged β-galactosidase from other proteins by using affinity chromatography. When operating the column, control the flow rate to not increase beyond one mL per five seconds. Ensure the water level never descends below the top of the beads, and always loosely cover the column with its lid to avoid contamination from the air. A column volume (CV) refers to the volume of the packed bed of affinity resin beads (~5 mL).

15. Pass through the column 8 CVs of elution buffer to wash off residual proteins. (Repeat step 10 with a sample of the resulting flowthrough.)

16. After draining as much elution buffer as possible (without drying beads), wash with 10 CVs of binding buffer. (Repeat step 10 with a sample of the resulting flowthrough.)

17. Apply the protein sample to the column from step 12. (Repeat step 10 with a sample of the resulting flowthrough.)

18. Reapply the flowthrough from step 17 back through the column. After passing through a second time, collect a sample to repeat step 10.

18. Wash column with 10 CVs of binding buffer. (Repeat step 10 with a sample of this flowthrough.)

19. Wash column with 10 CVs of wash buffer. (Repeat step 10 with a sample of this flowthrough.)

20. Elute out bound protein with 5 CVs of elution buffer. Collect the flowthrough in batches of 1 CV at a time. Repeat step 10 with a sample from each volume of eluent. Combine all five CVs of eluent together.

21. Repeat Bradford assay, step 13, to find protein concentration of eluent.

22. Label eluent and other samples with team number, date, and contents. Give to TA to store in fridge.

Experiment B (Day 1)

Objective: Characterize the kinetics of β-galactosidase

Background

The enzyme β-galactosidase typically cleaves lactose, a disaccharide, into two simple sugars, glucose and galactose. Typically, organisms prefer to use glucose as a food source, but if limiting, the lacZ operon will activate, producing the enzyme β-galactosidase, which can allow lactose to be used as a secondary food source. In this experiment, specially designed sugars will be digested as opposed to lactose, resulting in products that absorb certain wavelengths of light, allowing for ease of characterization.

Enzymes are typically assumed to follow mass-action kinetics:

$$[E] + [S] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [ES] \xrightarrow{k_2} [E] + [P]$$

Where [E] is enzyme concentration, [S] is substrate concentration, [P] is product concentration, and [ES] is the complex of substrate bound to enzyme. With the proper assumptions, the rate of reaction can be expressed in the following way:

$$r_P = -r_S = \frac{k_2[E][S]}{K_M + [S]}$$

Where the Michaelis-Menten constant, K_M, represents:

$$K_M = \frac{k_2 + k_{-1}}{k_1}$$

Procedure

1. Produce a total of 1 mL by mixing the eluent from day 1 with 250 mM Tris HCl + 1 mM MgCl buffer. The ratio of eluent to buffer should be optimized to obtain the desired enzyme activity---the first trial should be conducted using 1 mL of pure eluent. Store eluent on ice when not in use.

2. Preheat to desired temperature (nominally 37 ^{o}C).

3. Add eluent to semi-micro cuvette. Start spectrophotometer at 420 nm, blank to eluent solution.

4. Add 20 μL of 4 mg mL^{-1} ONPG solution. Promptly cover with parafilm, invert to mix, and collect absorbance over time until sufficient kinetic data is collected.

5. Conduct additional trials, varying enzyme concentration, ONPG concentration, T, and/or pH.

Experiment D (Day 1 and 2)

Objective: To separate proteins based on size using gel matrix (SDS-PAGE)

Background

A common method for separating proteins for analyzing the total protein size components is the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS is an anionic detergent and denatures the sample proteins, which also binds with proteins in proportion to their relative molecular mass. Electrical current is applied across the polyacrylamide gel matrix during electrophoresis. All SDS-bound negatively charged, denatured proteins migrate towards the cathode. Smaller molecules migrate faster than larger molecules in the polyacrylamide matrix. The charge-to-mass ratio is nearly the same among all denatured proteins and the separation is dependent mainly on size. In this lab, a pre-cast gel with 4 to 12% gradient polyacrylamide (4% at the top and 12% at the bottom) is used.

Procedure

1. At the end of Day 1, give all labeled SDS-PAGE samples to TA to store at -20 °C.

2. At the beginning of day 2, take SDS-PAGE samples out from freezer.

3. Place one cassette clamp in the left chamber of the electrophoresis tank with the anode connector aligned to the center.

4. Fill the left chamber with 360 mL DI water and 40 mL of 10x running buffer. Stir slightly with a glass rod.

5. Remove a 4-12% gradient acrylamide gel cassette from its plastic bag. Rinse the cassette with DI water.

6. Find the bottom of each of the 15 wells and label 1 to 15 below the wells.

7. Remove the gel comb by sliding the comb up one side at a time (be gentle and not to damage the wells when removing the comb, or samples may leak from one well to the other).

8. Remove the white sticker at the lower portion of the cassette.

9. Place the gel cassette into the left chamber with the wells facing towards you. Hold the cassette in a raised position and close the clamp by moving the cam handle forward to secure the cassette.

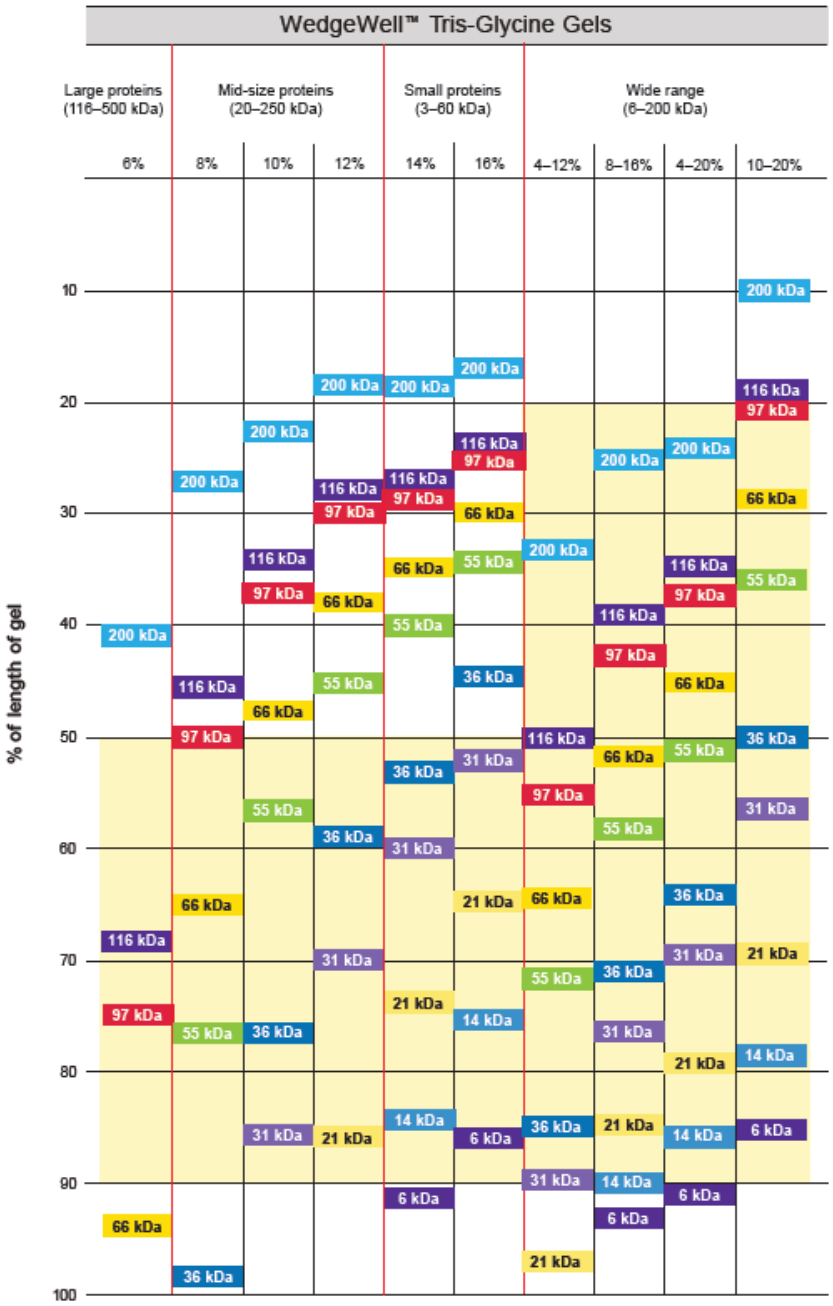

Figure 1. Expecting protein pattern on polyacrylamide gel with various compositions.

10. Fill a plastic disposable pipet with the 1x running buffer in the tank. Dispense buffer on the top of the wells and rinse the wells 3 times with the 1x running buffer. Be sure to displace all air bubbles from the cassette wells as they will affect sample running.

11. Make sure that the wells are filled with 1X running buffer and load your samples as assigned below (or as you prefer) using the smallest size tip on a 20 µL pipettor. Pipet from the top of the samples after being well shaken. If white pellets are seen at the bottom of the tube after centrifugation, do not disturb pellets. Load 30 µL total volume by slowly pipetting 15 µL of the sample. Be sure not to allow volume to spill into the reservoir or between lanes of the gel.

12. Align the electrical probes and place the lid on the electrophoresis tank.

13. With the power off, connect the electrode cords to power supply {red to (+) jack, black to (–) jack}.

14. Turn on the power of the power supply. Set the voltage to 200 V and the time to 40 minutes.

15. Start the cycle.

16. Turn off power at the end of the cycle or when pink dye front is close to the gel end (time permitting, continue to run the gel until the dye front reaches the bottom of the gel).

17. Remove tank lid. Release the clamp holder and take the cassette out.

18. Insert a gel knife into the cassette on the side. Force open the clear plastic cassette.

19. Cut along the original sticker area and remove the bottom of the gel.

20. Fill a tip box with DI water. Place the gel into the tip box, taking care not to rip the gel.

21. Change water by pressing the gel against the bottom of the box and pour out the rinse water. Add new water and lightly shake for 5 minutes. Repeat 3 times.

22. Remove water, add Imperial protein stain until the gel is covered. Close the box lid and place the box in a plastic bag.

23. Lightly shake the gel for at least 20 minutes, ideally ~45 minutes. The longer the gel stains, the darker the protein bands will be.

24. Pour used gel stain into a bottle labeled for reuse. Rinse the gel with DI water 3 times.

25. Cover gel with excess DI water. Add a Kimwipe into the water.

26. Shake the gel for at least 30 minutes, until individual protein bands are visible. Destaining removes background color from the gel as a result of nonspecific binding.

27. Scan or take a picture of the gel, analyzing the image using a software package like imageJ to quantify protein band relative intensity.

Suggested SDS-PAGE Lanes:
1. 5 µL protein ladder
2. 30 µL unlysed whole cells
3. 30 µL whole cell lysate
4. 30 µL clarified lysate
5. 30 µL elution buffer wash
6. 30 µL bind buffer wash
7. 30 µL flowthrough 1
8. 30 µL flowthrough 2
9. 30 µL binding buffer wash
10. 30 µL wash buffer wash
11. 30 µL eluent CV 1
12. 30 µL eluent CV 2
13. 30 µL eluent CV 3
14. 30 µL eluent CV 4
15. 5 µL protein ladder

Buffer Recipes:

Column binding buffer: 50 mM tris, 100 mM NaCl, 5 mM imidazole, 0.1 mM EDTA, 1 mM PMSF
Column wash buffer: 50 mM tris, 300 mM NaCl, 10 mM imidazole, 0.1 mM EDTA, 1 mM PMSF
Column elute buffer: 50 mM tris, 50 mM NaCl, 300 mM imidazole, 0.1 mM EDTA, 1 mM PMSF

Bradford reagent: 25 mg of Coomassie Brilliant Blue G-250, 25 mL methanol, 50 mL 85% phosphoric acid. Add mixture slowly to 425 mL DI water. Store at 4 ^{o}C.