Enzymatic Reaction and Protein Separation

CBEMS 140B
Winter 2017

UC Irvine
Department of Chemical Engineering
and Materials Science

Version 2

Introduction

Proteins, the natural polymers, are the building blocks of all living things. They are heat sensitive and have to maintain a specific conformation (3D structure, presence of subunits, coenzymes, etc) to retain their functionalities. Their natural physiological environment defines the pH, temperature, salt contents and other necessary conditions when they can be preserved and execute their roles in the body. Current technology allows the isolation and reproduction of important proteins that have applications in medical treatment, drug delivery, tissue engineering, etc. In this experiment, you will have a chance to practice and learn basic protein isolation, purification and characterization techniques. Table 1 listed common separation processes used in the chemical industry. Some of these processes have to be modified before applying 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.

Background

Escherichia coli (*E. coli*) is a model host to express recombinant protein 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 actually are an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic, meaning they can cause illness, either diarrhea or illness 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 with genotype *rec*A1 *end*A1 *gry*A96 *thi*-1 hsdR17 *sup*E44 *rel*A1 *lac*.

E. Coli bacteria are unicellular prokaryote. For easy extraction, most recombinant protein are designed to express and secrete into the cytosol. In order to extract the cytosolic protein, the bacteria

cells can first be resuspended into a protein buffer and then have their cell membranes ruptured mechanically. The cytosolic protein is then leaked into the buffer. The cell debris can be removed by membrane filtration or centrifugation. In this exercise, a commercially available cell rupture kit, OmniLyse (Claremont BioSolutions), will be used.

Procedure

1. Resuspend cell pellets in 1000ul of 1xPBS (pH 7.4).
2. Blank the spectrophotometer with 1xPBS at 600nm and measure the optical density (OD) of the cell suspension at 600nm.
3. If 0.5<OD<3, the OD is within the calibration linear range. OD_{600} of 1.0 = 8 x 10^8 cells/ml.
4. If OD>1.0, dilute cell suspension to prepare 1ml of OD=1 cell suspension in a new microcentrifuge tube.
5. Prime the Omnilyse cartridge with 0.5-1.0 mL of 1X PBS (pH 7.4).
6. Attach the cartridge to the battery back with the blue mating connectors by lining up the RED arrows.
7. With the provided syringes, fill it up with 1X PBS (pH 7.4). Turn on the battery pack and slowly expel the buffer into a microcentrifuge tube. Repeat dispensing and withdrawing into the cartridge about 4-5 times. Expel all the fluid and turn off the battery pack.
8. Insert the outlet of the cartridge into 1ml of cell suspension (OD=1) and draw the suspension into the Omnilyse cartridge with the attached syringe (can use the same one as in step 3 if clean), displacing the air within the cartridge. **STOP ONCE THE AIR IS DISPLACED! You should still have cell suspension in your microcentrifuge tube.**
9. Turn on the battery pack and slowly draw the majority of the remaining sample through the cartridge such that the cartridge remains filled with the suspension AT ALL TIMES. Reverse direction to dispense the majority of the same through the cartridge into the microcentrifuge tube. Continue dispensing and withdrawing for at least 5 minutes, depending upon ease of lysis and desired lysis efficiency.
10. After lysing is done, fully expel the suspension from the cartridge and displace with air.
11. Turn off the battery pack and discard the cartridge and syringe into the biohazardous bin. Chill the lysate on ice and save the lysate for Experiment B.
12. Aliquot 48ul of the lysate into a new microcentrifuge tube. Add 12ul of pink 5x SDS-PAGE loading buffer to this tube. When samples from Experiment B & C are ready, boil tube at 95°C for 5 minutes. Save it for SDS-PAGE analysis (Experiment D).

Experiment B (Day 1)

Objective

- To destroy background protein using enzymatic reaction

Background

In our stomach, there is a very powerful enzyme called pepsin, which breaks down proteins into smaller peptides before food can be absorbed by the body. Pepsin is a protease which accelerates peptide bond hydrolysis. It preferentially cleaves C-terminal to the Phe and Leu residues and to a lesser extent the Glu linkages, but does not cleave at Val, Ala, or Gly. Therefore, pepsin does not digest collagen due to its embedded backbone and also its high content of glycine. In this exercise, we will study the specificity of pepsin (34.6 kDa) by adding pepsin to a mixture of native collagen and the bacterial whole cell lysate prepared in exercise A.

Procedure

1. Prepare 48ul of 0.2mg/ml of native collagen in water. Safe this sample for SDS-PAGE analysis.
2. Prepare 48ul of 0.2mg/ml of pepsin in water. Safe this sample for SDS-PAGE analysis
3. Add 0.2 mg/ml of native collagen, and 0.2mg/ml of pepsin into 142ul of chilled whole cell lysate. Pipet up and down gently to mix on ice.
4. Aliquot this tube into 3 tubes of ~48ul reaction tube on ice. Label them Tube 1 to 3.
5. Incubate Tube 1 in the water bath at 37°C.
6. Lower the pH of Tube 2 to pH 1 by adding 2.5 ul of 0.2N HCl and leave on ice.
7. Lower the pH of Tube 3 to pH 1 by adding 2.5 ul of 0.2N HCl and incubate in the water bath at 37°C.
8. Let tubes react for 10 mins.
9. Add 12ul of pink 5x SDS-PAGE loading buffer to the collagen tube, the pepsin tube and Tube 1 to 3. Add 1N NaOH to low pH samples until the sample tube stays pink.
10. When samples from Experiment C are ready, boil sample tubes at 95°C for 5 minutes. Save them for SDS-PAGE analysis.

Experiment C (Day 1)

Objective

- To investigate the Michaelis constant, K_M, of pepsin

Background

Most single substrate enzymatic reaction can be described by Michaelis-Menten kinetics. In their model, the Michaelis constant is defined by:

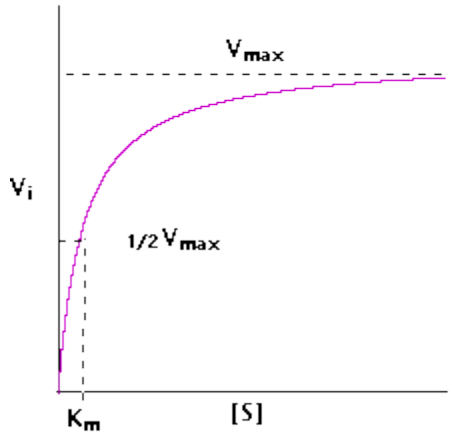
$$K_M \equiv \frac{k_2 + k_{-1}}{k_1}$$

when the enzymatic reaction is described as:

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

where *E* is the enzyme, *S* is the substrate, *ES* is the enzyme-substrate complex and *P* is the product.

And K_m can be determined graphically:



where V_i is the initial velocity, V_{max} is the maximum velocity and [S] is the substrate concentration.

Procedure

1. Aliquot 3 tubes of 47ul whole cell lysates using microcentrifuge tubes. Label them Tube 4 to 6.
2. Dilute and make appropriate pepsin stock solutions for step 3 to 5 respectively:
3. Add 20 ug/ml of pepsin to Tube 4. Pipet up and down gently to mix.
4. Add 2 ug/ml of pepsin to Tube 5. Pipet up and down gently to mix.
5. Add 0.2 ug/ml of pepsin to Tube 6. Pipet up and down gently to mix.
6. Lower the pH of these tubes to pH 1 by adding 2 ul of 0.2N HCl and incubate at 37°C.
7. Let tubes react for 10 mins.
8. Add 12 ul of pink 5x SDS-PAGE loading buffer to each tube. Add 1N NaOH until the sample tube stays pink.
9. Boil sample tubes and those from Expt A & B at 95°C for 5 minutes. Save them for SDS-PAGE analysis (Experiment D).
10. Separate proteins using SDS-PAGE as described in Experiment D.
11. Scan the SDS-PAGE gel and analyze the image.
12. Pick an *E. Coli* protein band of a particular protein size and calculate band area using a computer software such as Image J.
13. Plot –d[S]/dt vs [S] to determine K_m.

Experiment D (Day 1 and 2)

Objective

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

Background

A very 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 the SDS molecules in proportion to its relative molecular mass. Electrical current is applied across the polyacrylamide gel matrix during electrophoresis. All SDS bind negatively charged denatured protein will migrate towards the cathode. Smaller molecules migrate faster than larger molecules in the matrix because larger molecules are more restrained. 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. The migration pattern is expected as shown below:

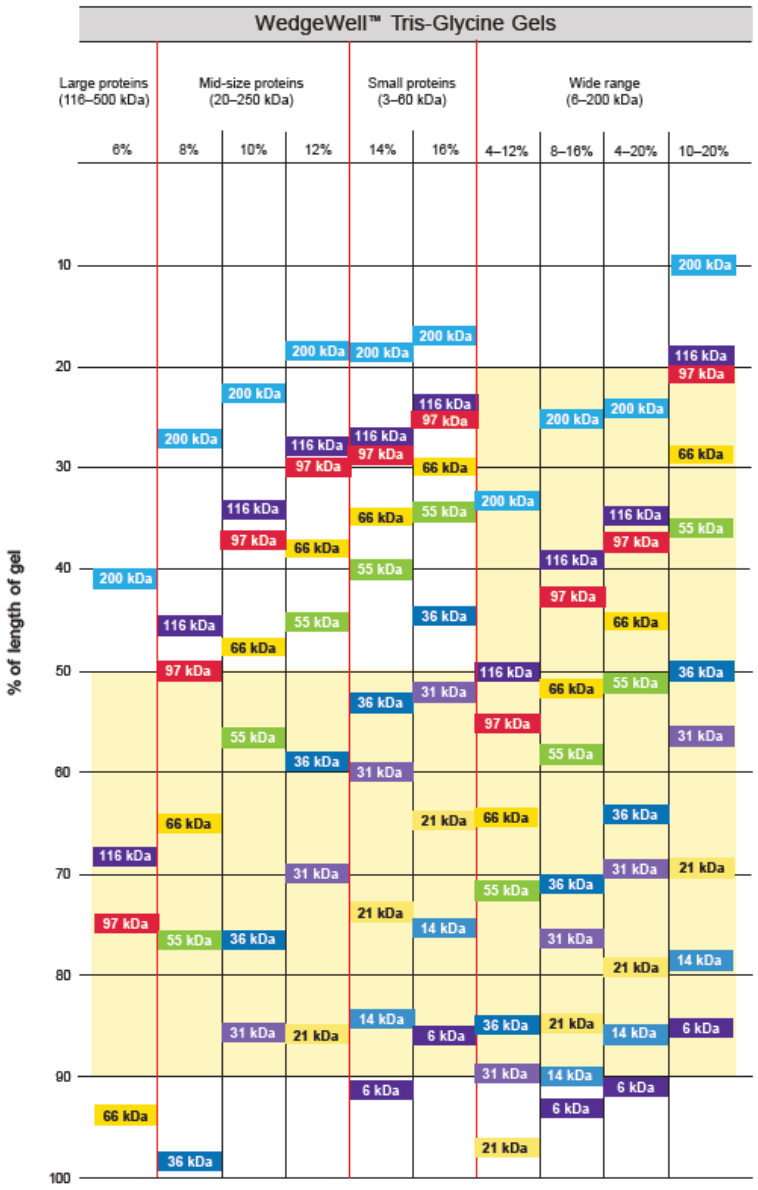


Fig 1. Expecting protein pattern on polyacrylamide gel with various compositions.

Procedure

1. At the end of Day 1, freeze all SDS-PAGE samples at -20°C
2. At the beginning of day 2, take SDS-PAGE samples out from freezer.
3. Centrifuge tubes at 9,000 g for 10 mins with balanced loading.
4. Place one cassette clamp in the left chamber of the electrophoresis tank with the anode connector aligned to the center.
5. Fill the left chamber with 360 ml DI water and 40 ml of 10x running buffer. Stir slightly with a glass rod.

6. Remove a 4-12% gradient acrylamide gel cassette from its plastic bag. Rinse the cassette with DI water.
7. Fine the bottom of each of the 10 wells and label 1 to 10 below the wells.
8. 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).
9. Remove the white sticker at the lower portion of the cassette.
10. 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.
11. 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.
12. Make sure that the wells are completely filled with 1X running buffer and load your samples as assigned below using the smallest tip on a 20ul pipetor. Pipet from the top of the samples inside the microcentrifuge tubes. If white pellets are seen at the bottom of the tube after centrifugation, do not disturb pellets. Load 30ul total volume by pipetting and loading 15 ul twice.
13. Align the connectors and place the lid on the electrophoresis tank.
14. With the power off, connect the electrode cords to power supply {red to (+) jack, black to (–) jack}.
15. Turn on the power of the power supply. Set the voltage to 200V and the time to 40 mins.
16. Start the cycle.
17. Turn off power at the end of the cycle or when pink dye front are close to the gel end. 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. Carefully flip the gel into the tip box.
21. Change water by pressing the gel against the bottom of the box and pour out the rinse water. Add new water and 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. Shake the gel for 45 mins.
24. Pour used gel stain into the reuse bottle. Rinse the gel with DI water 3 times.
25. Cover gel with DI water. Add a tided up Kimwipe into the water.
26. Shake the gel for 30 minutes or until individual protein bands are visible.
27. Scan the gel and analyze the image.

SDS-PAGE Lane:

1. 5ul Protein Ladder
2. 30ul 1x *E. Coli* whole cell lysate
3. 30ul Pepsin digestion at pH 7.4 at 37°C with collagen (Tube 1)
4. 30ul Pepsin digestion at pH 1 on ice with collagen (Tube 2)
5. 30ul Pepsin digestion at pH 1 at 37°C with collagen (Tube 3)
6. 30ul Pepsin digestion at pH 1 at 37°C (20ug/ml pepsin) (Tube 4)
7. 30ul Pepsin digestion at pH 1 at 37°C (2ug/ml pepsin) (Tube 5)
8. 30ul Pepsin digestion at pH 1 at 37°C (2ug/ml pepsin) (Tube 6)
9. 30ul 0.2 mg/ml pepsin
10. 30ul 0.2 mg/ml native collagen

Reference

[1] Centers for Disease Control and Prevention, "E. Coli," 6 November 2015. [Online]. Available: https://www.cdc.gov/ecoli/general/index.html.