CHEM525 Experiment 6
Protein Purification

Outline:

Lyse and centrifuge the cells
Purify the LDH protein using FPLC
Locate the protein in the column fractions
Pool and dialyze the fractions

Purpose and Theory:

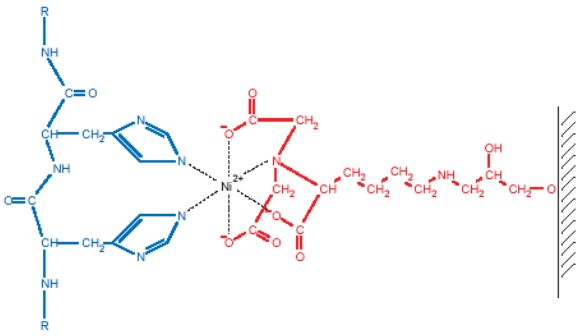
This is a VERY full week, so make sure that you come into lab knowing what you have to get done and do it efficiently! First, we are going to break open our IPTG induced cells and spin them down to separate the protein from the insoluble cell parts, like the cell membrane and organelles, which will form a pellet at the bottom of the centrifuge tube. Next, we'll run this supernatant through a protein purification column (either metal affinity or ion exchange) and elute the protein in fractions.

New Techniques:

Sonication

To break open a cell without damaging its contents, we use a technique called sonication. This uses ultrasonic vibrations to break the bacterial cell wall and membrane, whcih releases all of the contents of the cell, including the protein. Then we can spin down these "pieces of cell" to separate the insoluble organelles and pieces of the cell wall and lipid membrane from the soluble proteins.

Affinity Chromatography



Remember when we discussed cloning and how it can be used to introduce a poly-histidine tag (which we actually already had)? Well, we're finally going to get to use it.

The active groups on the Ni-NTA resin that is in your column are shown above. The NTA (nitrilotriacetic acid) chelates the Ni^{2+} in four of its six preferred positions. The other two sites are then free to interact with two histidine residues from your protein tag, completing an octahedral geometry, as shown above. This means that the tagged protein binds, but other proteins will flow through the column, unless they just happen to have a series of available histidines, which is very rare (but this does happen, so be careful if you ever use this in the future!). We then use imidazole to compete off the histidines, thereby eluting the protein.

Ion-exchange Chromatography

In your bioinformatics activity, you determined the theoretical pI of lactate dehydrogenase based on the amino acid sequence. This is very useful in protein purification because it allows us to predict the net charge on our protein. If pH < pI, then the protein will have a net positive charge. Conversely, lactate dehydrogenase will have a net negative charge if the pH > pI. Hopefully, you determined that our protein has a pI of ~5.5. Since the buffers that we prepared last week are pH 8.0, our protein will have a net negative charge. If we pass our protein through a column that has a positive charge, it should interact quite strongly. We can use a gradient of increasing salt concentration (we will use NaCl) to elute our enzyme.

FPLC (Fast Protein Liquid Chromatography)

50 years ago, using chromatography to purify proteins was a slow process that relied on gravity to force solutions through the column. In addition to being slow, this was also a process that was quite susceptible to mistakes (adding the wrong buffer, letting the column run dry, etc.). In this lab, we have the advantage of using sophisticated instruments that automate the process. In the simplest sense, FPLCs are just two pumps and a fraction collector that work together to pass two or more solutions through a column and collect the flow through in small fractions. The instrument we will work with is a bit more complex – it will be thoroughly explained in lab.

Procedure

1. **Retrieve you buffers – keep them cold at all times**

2. **Cell Lysis:**
    a. During the downtime in the lysis process, you should be working on part 4.
    b. Find your cell pellet (This is the total cell mass that was harvested from your 1L of LB induced with 400 μM IPTG for 4 hours at 37°C.)
    c. Resuspend your cells pellet in 15 mL of lysis buffer. We will be combining samples from all groups for the lysis.
    d. Using the sonicator, lyse the pooled cells. The cells are broken up by sonication, which uses ultrasonic waves to break open the bacteria. The process takes 12 minutes, consisting of 16 cycles of 15 seconds on, 30 seconds off. The off cycles allow the cells to cool between sonication bursts.

3. **Clarifying by centrifugation:**
    a. Procure a 30 mL centrifuge tube.
    b. Divide the cells into four equal batches (one per group).
    c. Balance your tube with another group. These need to be exactly the same mass. You can adjust the mass by adding lysis buffer to one of the samles.
    d. In the SS34 rotor, place your centrifuge tube opposite the other group.
    e. Centrifuge for 15 minutes at 12,000 RPM.
    f. Decant – the supernatant contains the LDH protein.
    g. Take a 50 μL sample of the cell lysate. You will use this next week.

4. **FPLC preparation:**
    a. Place line A into your lysis buffer and line B into your Buffer B.
    b. Run the pump-wash program. This equilibrates the instrument with your buffers.
    c. Plumb your column (Metal Affinity or Q-column) onto the FPLC and make sure that the connections are tight.
    d. Flow Buffer A through your column until it

5. **Chromatography:**
    a. With the help of the instructor, load the cell lysate into the 50 mL superloop and connect it to the FPLC.
    b. At a rate of 1 mL/min, pass all of the supernatant through the column (8 mL fractions).
    c. Wash the column with Buffer A until the absorbance reaches baseline.
    d. Elution (2 mL fractions):
        i. Ion exchange – run a linear gradient from 0 → 100% B over 40 minutes.
        ii. Affinity – run a step gradient.
            1. Wash the column with 16 % B (40 mM Imidazole) for 20 mL
            2. Wash the column with 100% B (250 mM Imidazole) for 20 mL.

6. **Preparation of Gel Samples for Next Week:**
    a. Transfer 50 μL of each fraction that contains protein and into a labelled 1.5 mL tube.
    b. Along with the sample from 3g, place these into a rack and store at -20 °C.