RISE Program Workshop in Protein Purification

Objectives: The purpose of this workshop is to introduce students to the principles and practice of protein purification. Each afternoon session will consist of a practical lab component mixed with discussions. Worksheets are provided each day with information on the techniques to be covered and practice questions for participants to test their knowledge of concepts. Participants will work in teams to complete the experiments outlined on the worksheet, and results from each team will be compared at the end of each project. At the end of this one-week workshop, participants should be able to design and implement a purification protocol for any soluble protein based on first principles and be proficient in the following techniques:

- Cell lysis and fractionation
- Periplasmic protein prep
- Ammonium sulfate and polyethyleneimine precipitation
- Nickel affinity chromatography
- Ion exchange chromatography
- Gravity and Fast Protein Liquid Chromatography (FPLC) operations
- Protein quantitation by Bradford assay and absorbance spectroscopy
- Protein desalting, concentration and storage
- SDS Polyacrylamide gel electrophoresis

Materials: Participants will be provided with cell pellets containing the overexpressed protein of interest. Cells, buffers, media and reagents will be provided by the Yukl lab.

Schedule: Our first meeting will be at 11:00 am on Monday the 22nd in chemistry room 205 for an explanation of some of the techniques we will be using. Lab sessions will be held in the Yukl lab (W364 of the chemistry building) from 1 pm each afternoon until completion of the experiments.

Monday 6/22 Begin His-tagged protein purification. Cell lysis, fractionation, gravity flow affinity chromatography and dialysis. Analysis of purified protein by SDS-PAGE.

Tuesday 6/23 UV-vis spectroscopy and Bradford assay. Discussion of results. Desalting and storage of protein.

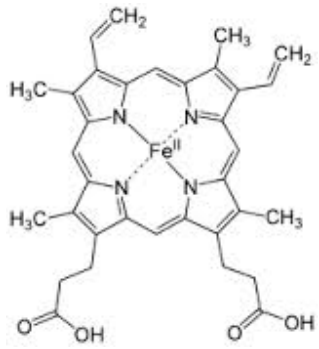
Wednesday 6/24 Begin periplasmic protein purification. PEI and ammonium sulfate precipitation. Design purification protocol on 1st principles.

Thursday 6/25 Ion exchange chromatography of solubilized pellets. SDS-PAGE. Begin size exclusion chromatography.

Friday 6/26 Analysis of purified protein by SDS-PAGE and Bradford assay. Comparison and discussion of untagged protein purification results.

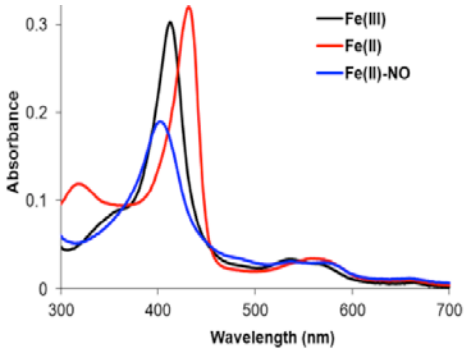
6/22/15 Purification of *Legionella pneumophila* H-NOX by Ni affinity chromatography

Background: The H-NOX's are a family of bacterial heme proteins responsible for sensing environmental conditions. The presence of heme bound to these proteins causes them to be intensely colored, just like your own hemoglobin. NO and O_2 bind to the H-NOX heme iron, causing conformational changes that effect the interaction of H-NOX with downstream signaling proteins. Binding of various ligands to the heme iron also alters the electronic properties of the heme, which can be detected by visible absorbance spectroscopy.



Structure of heme-b

Expression: *E. coli* cells overexpressing H-NOX bearing a 6-His tag at its N-terminus were grown to OD_{600} = 0.3-0.4. Hemin was added to the culture and expression was induced with IPTG. Cells were grown at 18° C overnight, collected by centrifugation and stored in a -20° C freezer. This is your starting material.



Absorbance spectra of H-NOX

Physical Parameters (Uniprot Acc # Q8EF49)
MW = 22.9 kD
Theoretical pI = 6.98
Experimental ε_{412} = 110,000 $M^{-1}cm^{-1}$

Procedure:

- Resuspend cell pellet with 50 mL of lysis/equilibration buffer (50 mM phosphate pH 7.5, 150 mM NaCl, 20 mM imidazole) ***Save a 20 uL aliquot for SDS-PAGE**
- Transfer cells to metal beaker on ice, sonicate using preset #1 at 60% amplitude. Repeat 3x, allowing ~1 minute between sonication cycles to prevent overheating.
- Give lysate to Dr. Yukl for centrifugation at 20,000 x g for 20 min. at 4° C
- Meanwhile, deliver 2 mL of Ni-NTA resin (a 50% slurry with 20% ethanol) to a small glass column. Wash 3x with 10 mL of lysis/equilibration buffer
- Take cleared lysate ***Save a 20 uL aliquot for SDS-PAGE**, and transfer it and your equilibrated resin to a 50 mL centrifuge tube. Affix to shaker in 4° C cold room and shake for 30 minutes.

Why is 20 mM imidazole included in the lysis/equilibration buffer?

Why are all of the steps done at 4° C?

What is happening while the lysate is incubating with the resin?

- Still in the cold room, carefully pour the lysate + resin back into your column. Collect the flow-through and save for SDS-PAGE. Use the stopcock to stop the flow as it reaches the top of the resin bed.
- Wash the resin 4x with 10 mL lysis/equilibration buffer. Collect washes for SDS-PAGE. **Try to mimimize disturbance of the resin bed by using a transfer pipette**
- With the stopcock closed, add 1 mL of elution buffer (same as lysis/equilibration buffer but with 250 mM imidazole). Open the stopcock and collect the eluent, closing it again before the resin dries. Repeat, collecting separate 1 mL fractions until no more color is observed in the eluent.

What is the purpose of NaCl in the lysis/equilibration and elution buffers?

What in the elution buffer causes H-NOX to come off the resin? Why?

SDS-PAGE:

- Combine 11 uL of each sample with 4 uL sample dye and 1 uL 1 M DTT. Mix well
- Carefully pipet 7 uL of the protein standard in lane 1 followed by 12 uL of each sample in the remaining lanes. ***Keep track of which samples are in which lanes***

- Run the gel at 200 V for 35 minutes.
- When gel is finished running, remove from plastic case, rinse with dI H_2O and add enough SafeStain dye to cover the gel. Shake at room temperature for 1 hr.
- Pour off dye into waste container, rinse with dI H_2O and cover gel with dI H_2O. Shake at room temperature overnight

What is the purpose of DTT in the SDS-PAGE samples? What is the purpose of SDS?

Cleanup and Storage

- Store purified protein fractions on ice, in a closed Styrofoam box in the cold room overnight
- Wash resin with 10 mL elution buffer, 2 x 10 mL of dI H_2O and 2 x 10 mL of 20% ethanol. Use 20 % ethanol to transfer resin to a 15 mL centrifuge tube for storage

6/23/15 Characterization of Purified H-NOX

Affix a picture of your gel . Comment on the purity of your prep and how it might be improved.

Buffer exchange and protein storage

- Combine the purest and most concentrated eluent fractions and apply by syringe to desalting columns pre-equilibrated in storage buffer (50 mM HEPES pH 7.5, 300 mM NaCl). Elute with storage buffer using FPLC to collect 1.0 mL fractions. Combine fractions containing protein.

Why is glycerol added to the protein?

How is the desalting step similar to size-exclusion chromatography? In this case, what is being excluded from the beads and what is able to diffuse in and out?

Describe another method of buffer exchange

Absorption Spectroscopy:

- Pipet 490 uL of storage buffer into a quartz cuvette. Measure the baseline absorbance from 200 – 800 nm.
- Add 10 uL of purified H-NOX and scan again.

Abs 412 nm = *[H-NOX] based on* ε_{412} = 110,000 $M^{-1}cm^{-1}$ =

Bradford Assay:

- Pipet 15 uL of BSA standards from 0 – 1.0 mg/mL and an appropriate dilution of your purified protein into labeled disposable cuvettes. ***Make sure that the H-NOX concentration is within the range of standards. Use the MW of the protein and the concentration determined from heme absorption to estimate the protein concentration in mg/mL***

- Add 1.5 mL Bradford reagent to each standard and sample and mix well
- Allow the samples to sit for at least 10 minutes prior to measuring absorbance at 595 nm.

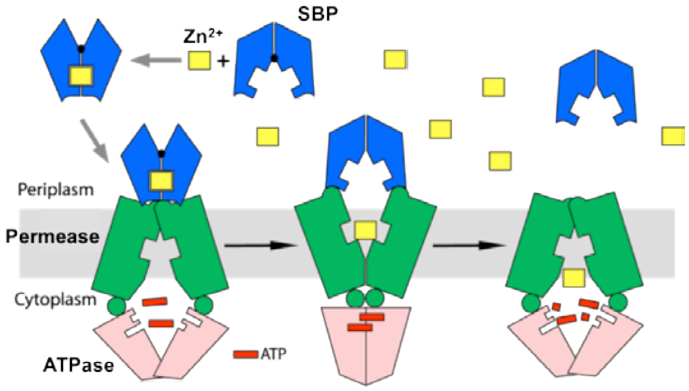
What is the R^2 for your calibration curve? *What is the concentration of H-NOX?*

How do the concentrations measured by each method compare? Speculate on the source of any observed difference and keep track of dilutions.

- Concentrate the protein to ~ 25 mg/mL, aliquot into 25-50 µL fractions and store at -80° C

6/24/15 Periplasmic Protein Purification of *Paracoccus denitrificans* Pden1597

Background: ATP binding cassette (ABC) transporters allow bacteria to acquire specific nutrients from the environment. In gram-negative bacteria, a soluble periplasmic protein called a solute binding protein (SBP) binds to a specific nutrient and delivers it to a transmembrane permease for translocation into the cytoplasm. This process is powered by ATP hydrolysis by an associated ATPase. Based on sequence homology, Pden1597 is a SBP for the ABC transporter specific for the essential element Zn in *P. denitrificans.* Since this protein is periplasmic, there is no need to lyse the cells. Rather an osmotic shock protocol is used to gently isolate the periplasmic proteins without disrupting the cytoplasmic membrane. This decreases the number of host proteins in our starting mixture and also allows for the correct formation of disulfide bonds that would otherwise not form in the reducing environment of the cytoplasm.



Schematic of Zn import by an ABC transporter

Physical Parameters (Uniprot Acc # A1B2F3)
MW = 29.7 kD
Theoretical pI = 4.77
Calculated ε_{280} = 20,525 $M^{-1}cm^{-1}$

Procedure:

- Resuspend cells at 5 mL/g wet weight cells in 50 mM phosphate pH 8.0, 0.5 M sucrose and 0.67 mM EDTA. ***Save a 20 uL aliquot for SDS-PAGE**. Add lysozyme to 7.5 mg/g wet weight cells. Incubate at 30° for 15 min
- Add an equal volume of dI water and incubate for a further 45 min as above
- Add $MgCl_2$ to 0.5 mM and NaCl to 300 mM and give cells to Dr. Yukl to centrifuge

What is the purpose of lysozyme?

What is the purpose of adding dI H_2O?

- Collect the periplasmic extract ***Save a 20 uL aliquot for SDS-PAGE**, and add polyethyleneimine (PEI) to 0.5% and incubate on ice 10 min. Give to Dr. Yukl to centrifuge
- Collect supernatant and add ammonium sulfate to 60% saturation. Ensure that all AS is dissolved and incubate on ice for 30 min. Give to Dr. Yukl to centrifuge.

Why does a precipitate form after adding PEI? What is in that precipitate?

Why does a precipitate form after adding Ammonium sulfate? What is in that precipitate?

Purification protocol from 1st principles

- In Uniprot, search for the accession # P00698 and answer the following questions:

What is the putative function of this protein?

Where does this protein localize?

Are there post-translational modifications? If so, what are they?

What are the pI and MW of this protein?

Given what you know, devise a detailed protocol for the isolation of this protein, starting with protein expression. Include one or more methods to assess the purity and quality of your preparation. Feel free to include pictures.

6/25/15 Anion exchange purification of Pden1597 and purity assessment

- Dissolve AS pellets in a minimum (1-2 mL each) of 20 mM tris pH 8.0. Transfer to new tube, dilute to 50 mL and give to Dr. Yukl to centrifuge out remaining solids.
- Take supernatant ***Save a 20 uL aliquot for SDS-PAGE**. Load onto 5 mL Q-column pre-equilibrated with 20 mM tris pH 8.0 using the superloop
- Run the "Pd97_Qcol" method on the FPLC. This will go through the following steps:
    - Wash column with 5 cv 20 mM tris pH 8.0 (buffer A) at a flow rate of 3 mL/min.
    - Increase to 15 %B (20 mM tris pH 8.0 + 1 M NaCl) and run 3 cv, collecting fractions
    - Apply a gradient of 15 – 35 % B over 15 cv. Pd97 elutes ~25% B
    - Wash column with 5 cv 100% B and store in 20% EtOH

Why does Pd97 bind to the Q-column at pH 8.0? What would you predict it we ran the procedure at pH 4.0?

Why do we measure the absorbance at 280 nm during the run? Why do we measure the conductance?

SDS-PAGE:

- Identify the fractions likely containing Pd97. Combine 11 uL of each sample with 4 uL sample dye and 1 uL 1 M DTT. Mix well
- Carefully pipet 7 uL of the protein standard in lane 1 followed by 12 uL of each sample in the remaining lanes. ***Keep track of which samples are in which lanes***

Size Exclusion Chromatography:

- Combine peak fractions from the ion exchange chromatography step and concentrate to 500 µL
- Inject sample into 500 µL loop and run the SEC method using 20 mM tris pH 8.0, 150 mM NaCl as buffer A

Affix a picture of your chromatogram showing the trace of absorbance at 280 nm and a picture of your gels. Comment on the purity of your prep and how it might be improved.

6/26/15 Final preparation of Pd97

- Combine the fractions containing Pd97 at acceptable purity and concentration
- Add glycerol to a final 10% v/v

Absorption Spectroscopy:

- Prepare 2 quartz cuvettes, 1 containing 490 µL of 6 M guanidine HCl, the other containing 20 mM tris pH 8.0, 150 mM NaCl.
- Baseline the UV-vis instrument from 200 – 400 nm using the guanidine-containing cuvette. Add 10 µL of protein, mix well and measure absorbance.
- Determine the extinction coefficient at 288 nm using the following formula:
  ε_{280} = 5690 (W) + 1280 (Y) + 120 (C)

Abs 280 nm = *[Pd97] based on* ε_{280} =

- Now repeat the above procedure with the cuvette containing tris buffer. Use the concentration of Pd97 determined above to calculate the ε_{280} of the folded protein.

ε_{280} *Folded Pd97 =*

Bradford Assay:

- Pipet 15 uL of BSA standards from 0 – 1.0 mg/mL and an appropriate dilution of your purified protein into labeled disposable cuvettes. ***Make sure that the Pd97 concentration is within the range of standards. Use the MW of the protein and the concentration determined from 280 nm absorption to estimate the protein concentration in mg/mL***

- Add 1.5 mL Bradford reagent to each standard and sample and mix well
- Allow the samples to sit for at least 10 minutes prior to measuring absorbance at 595 nm.

What is the R^2 *for your calibration curve?* *What is the concentration of Pd97?*

How do the concentrations measured by each method compare? Speculate on the source of any observed difference and keep track of dilutions.

- Concentrate the protein to ~ 25 mg/mL and store at -80° C