

## General Concepts about Protein Purification

### Initial Considerations:

- Why do you want the protein (ie. what is your project all about)?
- How much protein do you need (ng, ug, mg, g)?
- How homogenous or pure?
- Need active or inactive form?
- Cost of preparation in \$?
- Cost in time and effort (these last two sometimes are a balancing act)?

### How are you going to Assay for protein?

- Determine concentration: Colorimetric or direct spectrophotometric. Need to take into consideration buffer complications for former and purity index for latter.
- SDS-PAGE: used to determine homogeneity at each step and MWT of pure protein.
- Enzyme Assays:
  - Going to look at either disappearance of [S] or formation of [P].
  - Might need to come up with a coupled enzyme assay, if neither S or P provides you with a good spectral handle.

## **Where do you get information for protocols and procedures?**

- **Lab (in house) protocols (remember they are not written in stone).**
- **The Literature:**  
**Review Articles (Annual Reviews of.....; The Proteins; The Enzymes).**  
**Research Publications (Biochemistry, JBC, JMB, Protein Structure, etc.).**

## **Literature Sources for Methods:**

- **Methods in Enzymology.**
- **Current Protocols in Protein Science.**
- **Molecular Cloning (the Maniatis book).**
- **Catalogs.**
- **Word of mouth.**

## **How to Prepare for a Protein Purification Prep:**

- **Know the procedure very well (flow chart).**
- **Know what materials are needed:**
  1. **sonicators.**
  2. **columns and equilibrated resins.**
  3. **buffers (pH and I).**
  4. **SDS-PAGE gels and all buffers.**
  5. **Chemicals (salts, etc.).**
  6. **centrifuges and rotors (pre-cooled).**
  7. **spectrophotometers.**
- **Amass the materials, reserve equipment (and make sure you use it when you are supposed to), set aside the time, and most importantly: DO IT!**

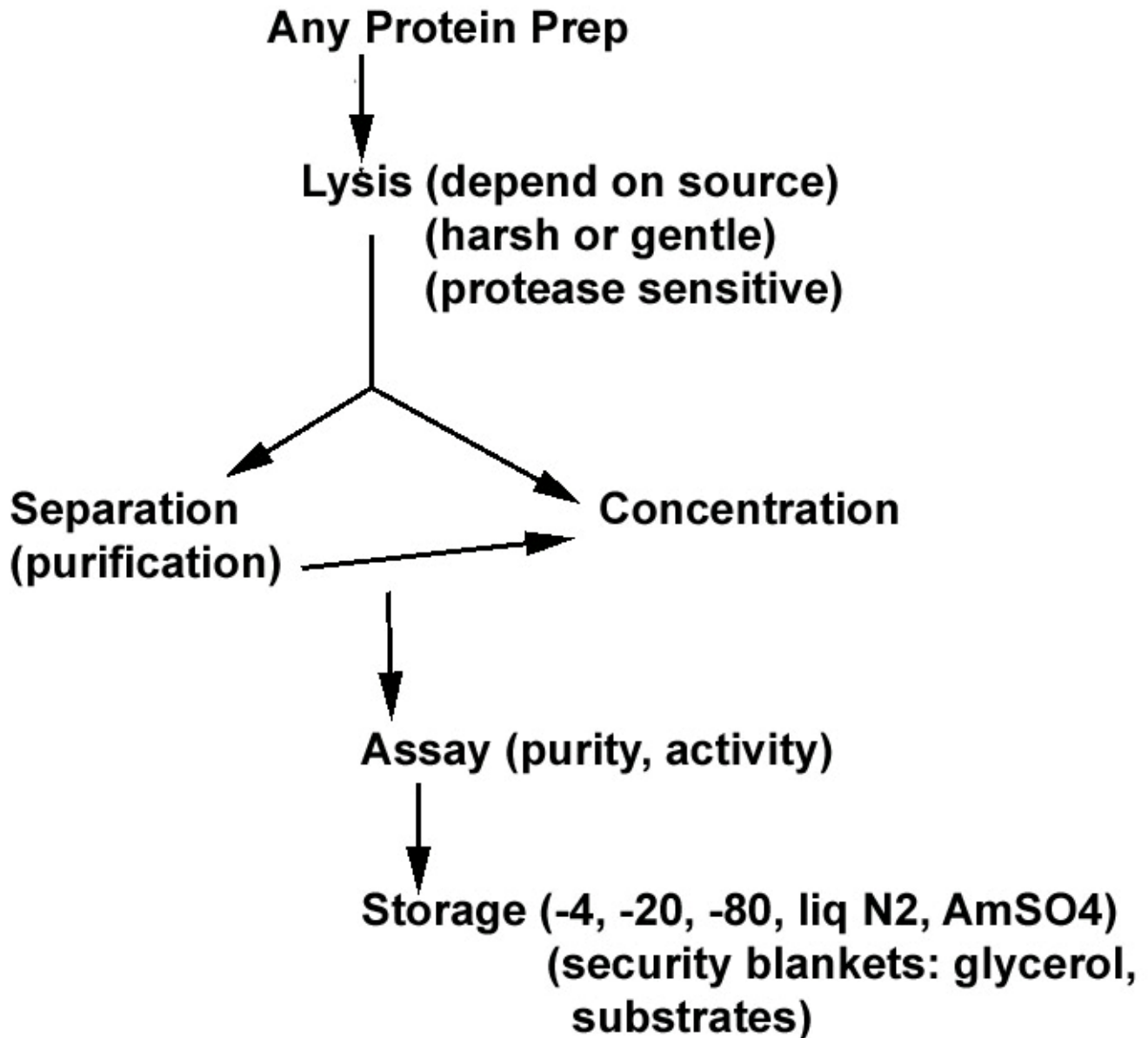
- **sometimes best to do a small scale prep 1<sup>st</sup>, then scale up.**

### **Sources of Proteins:**

- **Eukaryotic:**
  1. **usually larger scale preps because more starting material.**
  2. **cellular compartmentalization, know how to isolate particular organelles if necessary.**
- **Prokaryotic: Native or Recombinant.**
  1. **Native proteins: expressed constitutively by the organism, however gene expression might be regulated by components in growth medium. (Examples: beta-galactosidase and alkaline phosphatase (AP)).**
  2. **Recombinant Proteins:**
    - **Expressed from a plasmid that has been transformed into host cell.**
    - **How stable is plasmid?**
    - **Ease of transformation?**
    - **How compatible is the plasmid and host.**
    - **Will host express intact protein (important when cofactors are involved.**
    - **Stability of expressed protein?**
    - **Ease of gene manipulation?**

**Recombinant technology has greatly simplified the ability to study structure function relationships in proteins and enzymes by allowing for site-directed mutagenesis of specific amino acids that may play a significant role.**

Purification of recombinant proteins has also been simplified via use of “Tags”, however we have discussed complications due to this issue.



## Purification and Characterization of *E. coli* Alkaline Phosphatase (AP)

- AP is a phosphate hydrolase: catalyzes hydrolysis of phosphate monoesters.

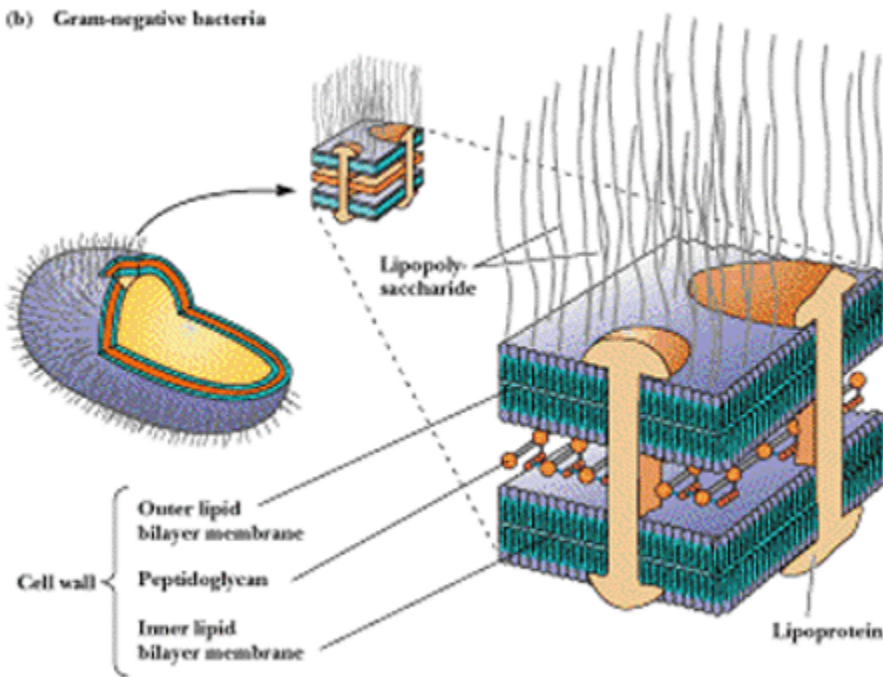


- **Physiological Role:** Cleave phosphoryl groups from a wide variety of phosphorylated compounds, providing the cell with inorganic phosphate.
- *E. coli* periplasmic enzyme, ideally located for its function.

*(Why is it important to provide the cell with  $P_i$  ?).*

- A homodimer, containing both Zn and Mg metals, an important consideration during purification. We will discuss the role of the metals in following classes.

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**AP resides in the periplasmic space, between the outer and inner lipid bilayers. The protein is synthesized in the cytoplasm. In order to translocate across the Inner Membrane, what event must happen?**

**In this experiment, we want to isolate the proteins in the periplasmic space from those in the cytoplasm. In order to do that Lysozyme is added to the cells, which then degrades the Peptidoglycan meshwork. This should suggest at least two things about the Outer Lipid Membrane!**

**The cytoplasm, with its intact membrane (Inner) is called a Spheroplast. Why do things not “leak” out of the cytoplasm? Gentle treatment is a must!**

## AP Prep

**Lysis (gentle with lysozyme)**



**Dialysis**

**Heat Denaturation (purification)**



**AmSO<sub>4</sub> precipitation (concentration)**



**Dialysis**

**DE52 column (purification, concentration)**



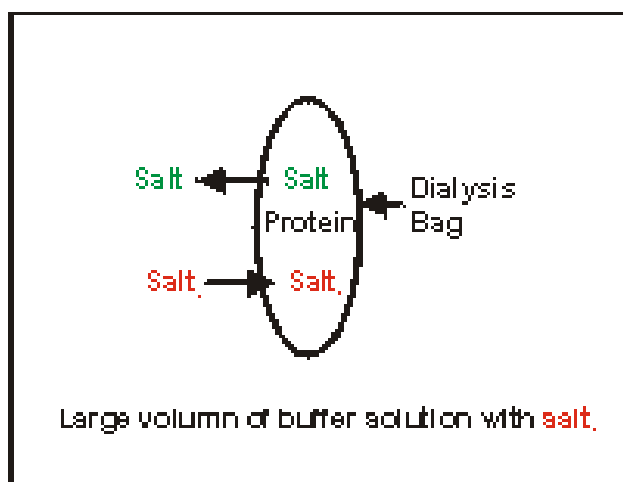
**Assays (SDS-PAGE, Activity)**

### Lysis Step (Stage 1):

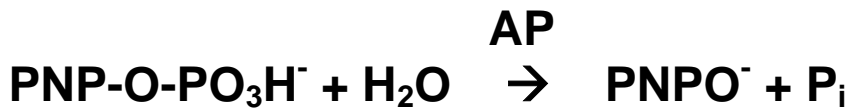
- Lysozyme is added to degrade peptidoglycan mesh.
- Why is this done in an isotonic buffer?
- Why is EDTA added? Are there any potential problems using EDTA with respect to AP?
- Why is  $\text{MgSO}_4$  added? Why  $[\text{MgSO}_4] \gg [\text{EDTA}]$ ?
- Why must you be gentle with each step?
- Suppose you were not gentle, how would you know you had lysed the spheroplasts (ie. you would look for the presence of what)?

After centrifugation, save an aliquot ( $\approx 0.5$  mL) for activity, Bradford, and SDS-PAGE assays.

### Why is dialysis necessary?

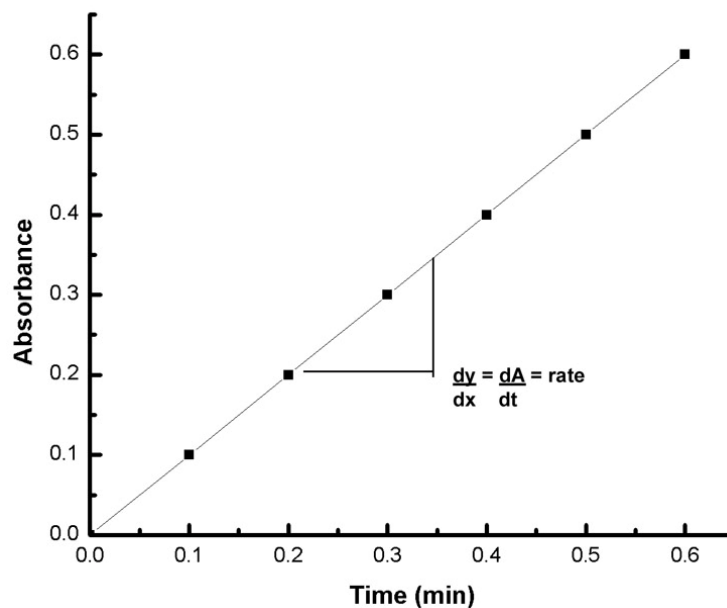


**Enzyme Activity Assay: Determination of the AMOUNT of enzyme present during each stage of the purification.**



Monitor the rate of  $\text{PNPO}^-$  produced per minute by following the reaction at \_\_\_\_\_ nm?

Follow reaction using Enzyme Kinetics mode on Cary, where you can input wavelength and extinction coefficient for  $\text{PNPO}^-$ .



We want to convert  $dA/dt$  to  $d[\text{PNPO}^-]/dt$ . What must we do?

Now you have  $\mu\text{M PNPO}^-/\text{min}$  IN THE ASSAY. In order to determine HOW MUCH enzyme is present, we want to know

**the micromoles of AP present in the TOTAL volume of the Stage 1 enzyme. How are you going to do this?**

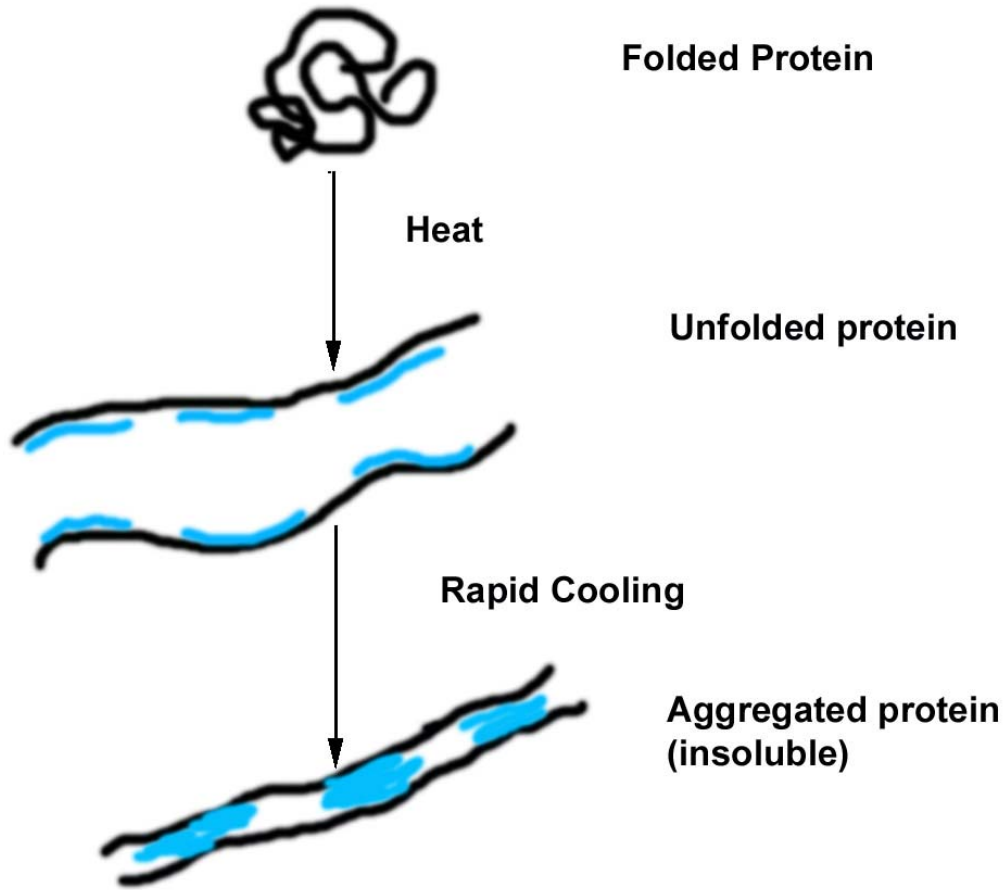
**BY DEFINITION: 1 Unit of enzyme activity is the amount of enzyme necessary to produce 1  $\mu\text{mol P/min}$ . So, you want to express the amount of enzyme present in terms of UNITS!**

## Stage 2 and Stage 3 Steps: Differential Solubility

Techniques employing differential solubility attempt to purify proteins by making some proteins precipitate or aggregate, while others remain in solution.

- When  $\text{pH} \equiv \text{pI}$ , some proteins will aggregate.
- Proteins can be made to precipitate or aggregate by denaturing with heat.
- Salting proteins out using  $\text{AmSO}_4$ 
  1.  $\text{AmSO}_4 = (\text{NH}_4)_2\text{SO}_4 \rightarrow 2 \text{NH}_4^+ + \text{SO}_4^{-2}$
  2. Expressed as %saturation (see Table handed out in class).
  3. Dramatically increases ionic strength.
  4. At high  $[\text{AmSO}_4]$ , charges on protein are neutralized.
  5. Proteins begin to aggregate = precipitation.
  6. Enzymes in precipitate are in NATIVE conformation, so they maintain activity!
  7. Resolubilize using low I buffer (dissolving pellet).

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**Heat Denaturation:**



**What might happen if you cooled sample down slowly?**

**Ppt is centrifuged out of solution. Careful with pellet, tends to break up easily!**

**Have you purified or concentrated protein in this step?**

### **AmSO<sub>4</sub> Fractionation:**

- **Sequential fractionation (or “cuts”).**
  1. **Weigh out appropriate amt of salt to give target %solution (percentage of saturation).**
  2. **Add AmSO<sub>4</sub> salt and stir, allow to sit, then centrifuge solution.**
  3. **Precipitated proteins in pellet.**
  4. **Increase amt of salt added, and repeat for 2<sup>nd</sup> %solution.**
  5. **Pellet can be resuspended using low I buffer.**
  6. **Aggregated proteins maintain enzymatic activity, so are not denatured by this treatment.**
- **In this experiment what percent solution do you have (consult table)? This technique is referred to as a BATCH CUT, where virtually all proteins are precipitated from solution.**
- **Are you purifying or concentrating?**

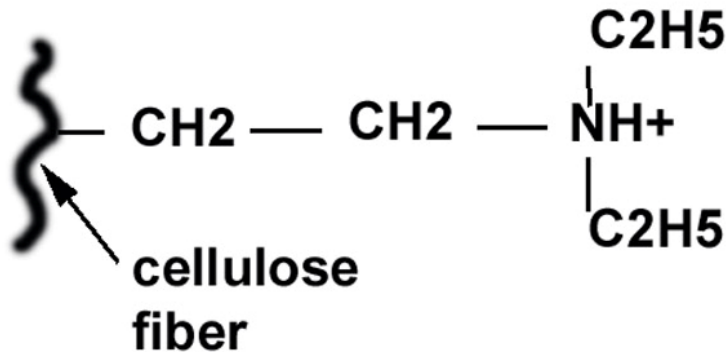
### **Other Chemical Methods (rarely used anymore):**

- **EtOH precipitation.**
- **Acetone precipitation (very flammable, toxic, and volatile, respirator with org. fume pads).**

**Both methods involve adding organic solvents to H<sub>2</sub>O decreasing polarity of the solvent, which generally increases Temp of solution. Precipitation of proteins at a given % is T sensitive, so you have to wait till T comes back down to some standard value (a rock salt water bath temp, ~ - 4° C).**

***These are very dangerous and toxic methods! Difficult to get rid of Acetone waste!***

## Ion Exchange Chromatography using DEAE-cellulose (Stage 4)



**DE-32:** dehydrated powder, need to rehydrate.

**DE-52:** hydrated resin, designed to be put in buffer and use immediately.

### Resin Preparation:

- 50 – 100 vol buffer/1 vol resin.
- 0.1 N NaOH treatment for 5 – 10 min.
- 0.1 N HCl treatment for 5 – 10 min.
- 0.5 M TrisHCl pH 7.4 (2-3X)
- 0.05 M TrisHCl pH 7.4 (2-3X)
- 0.005 M TrisHCl pH 7.4 + 5 mM  $\text{MgCl}_2$
- check pH and conductivity of buffer.

### Two Ways to Equilibrate Resin:

- Batch equilibration: in beaker with a lot of buffer.
- On column using either gravity feed or hydrostatic pump: slow and tedious.

## Today's Column:

- Equilibrate column with low I buffer (Buffer A) (what should you check before using?).
- Load protein sample.
- Rinse column with low I buffer (Why?).
- Elute with high I buffer. Is this a linear or step gradient method of eluting protein? Are you going to get any significant purification this way?

How would you improve method to increase purification? Why are you not doing this in this experiment?

- Do a spot plate assay for each fraction coming off column ( same ratio of PNPP/buffer you have been using for activity assay; 50 uL assay solution/well; add 10 uL fraction).
- Isolate fractions with maximal activity, combine, and do activity assay on this combined fraction.
- Save protein for Bradford and SDS-PAGE as S4.

**Bradford Assay:** for S1, S2, S3, and S4 want to determine  $[\text{protein}]_{\text{total}}$  in mg/mL. Knowing volume of the sample at that stage you can calculate total mg of protein at that stage.

## **Combining Activity and Bradford Assay Data:**

- **Activity assays tell you how many UNITS of enzyme you have during each step. Are you losing UNITS as you go through purification?**
- **% yield is a measure of how much enzyme you have at the end of the prep compared to what you started with in Stage 1 (= crude cell lysate).**
- **Bradford assay tells you, after the appropriate math, how many mg of protein you have at each step.**
- **Combining the two assay data:**

$$\text{Specific Activity} = \text{Units/mg total protein}$$

**Now, if you assume you are NOT losing enzyme, what should UNITS do as you go through prep?**

**If you are purifying protein during prep, what should mg total protein do?**

**What should Specific Activity do?**

**Specific Activity is used as a measure of PURIFICATION!**