Lecture 20: Analysis of Enzyme Inhibition & Protein Purification

Reading in Campbell: Chapter 6.7, chapter 5

20.1 Summary of Analysis of Inhibition

A competitive inhibitor binds to the substrate-binding pocket of E with a dissociation constant of K_I. By competing with substrate for binding to the same site in E, a competitive inhibitor effectively increases the KM of the enzyme-catalyzed reaction. Because it does *not* bind to the ES complex, a competitive inhibitor does *not* affect VMAX. α, the degree to which a competitive inhibitor increases KM, is obtained from the ratio of the slopes (slope (+ inhibitor) / slope (– inhibitor)) on a double reciprocal plot. From α, K_I can be calculated if the inhibitor concentration is known.

$$K_I = \frac{[E][I]}{[EI]}$$

$$\frac{1}{v} = \frac{\alpha K_m}{V_{MAX}} \frac{1}{[S]} + \frac{1}{V_{MAX}}$$

$$\alpha = 1 + \frac{[I]}{K_I}$$

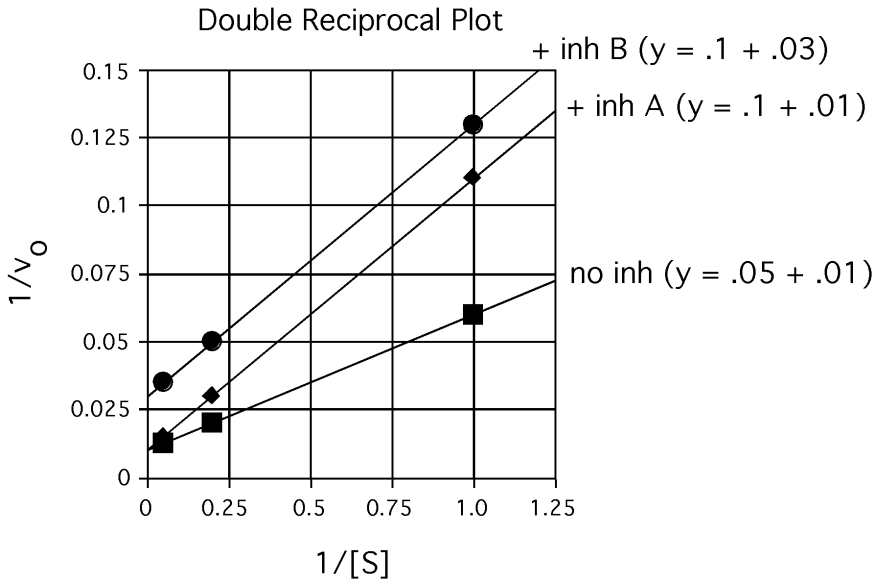
A noncompetitive inhibitor binds to *both* E and ES at a site *distinct* from the substrate binding site. Although a noncompetitive inhibitor usually binds to E and ES at the same site, it does so with differing affinities. It binds E with a dissociation constant of K_I and ES with a dissociation constant of $K_{I'}$. Binding of a noncompetitive inhibitor to E and ES allosterically inhibits both substrate binding and catalysis, respectively. Thus *both* KM and VMAX are affected. α, the degree to which a noncompetitive inhibitor increases KM, is obtained from the ratio of the slopes (just like for a competitive inhibitor). α', the degree to which a noncompetitive inhibitor decreases VMAX, is obtained from the ratio of the y-ints (y-int (+ inhibitor) / y-int (- inhibitor)). K_I and $K_{I'}$ can be calculated from α and α', respectively, if the inhibitor concentration is known.

$$K_I = \frac{[E][I]}{[EI]} \qquad K_{I'} = \frac{[ES][I]}{[ESI]}$$

$$\frac{1}{v} = \frac{\alpha K_m}{V_{MAX}} \frac{1}{[S]} + \frac{\alpha'}{V_{MAX}}$$

$$\alpha = 1 + \frac{[I]}{K_I} \qquad \alpha' = 1 + \frac{[I]}{K_{I'}}$$

Sample Analysis:



20.2 Protein Purification

Reading: Campbell Chapter 5

Key Terms:

- **Specific Activity**
- **Fold purification**
- **Purification Scheme**
- **Chromatographic Steps**

Concept of a Purification Scheme:

A purification scheme usually begins with a crude mixture of cellular proteins, referred to as a *lysate*. The lysate is treated in a series of physical steps or processes. Each step separates a mixture of proteins into two or more fractions. Fractions that contain the protein or enzyme of interest are retained for the next step of the purification scheme while the other fraction(s) are discarded until the protein is deemed to be pure. The entire sequential process is referred to as a purification scheme.

The actual separation steps are based on different physical/chemical properties of proteins:

- Solubility in different salts (ammonium sulfate)
- Size
- Charge
- Hydrophobicity
- Binding to Specific Ligands

How to monitor purity: It is essential to have some method to evaluate fold-purification or fold-enrichment, otherwise how do you know whether a specific step in the purification scheme has been successful in increasing the purity of the desired protein?

Amount of target enzyme: It is essential to have some method of determining the amount of the desired enzyme at any given step of the purification scheme. Consequently, the most critical step in any purification scheme is to develop a suitable assay for the enzyme that is being purified.

Measuring purity during purification: The purity of the enzyme during the purification scheme is generally monitored by measuring the specific activity. Specific activity is defined as:

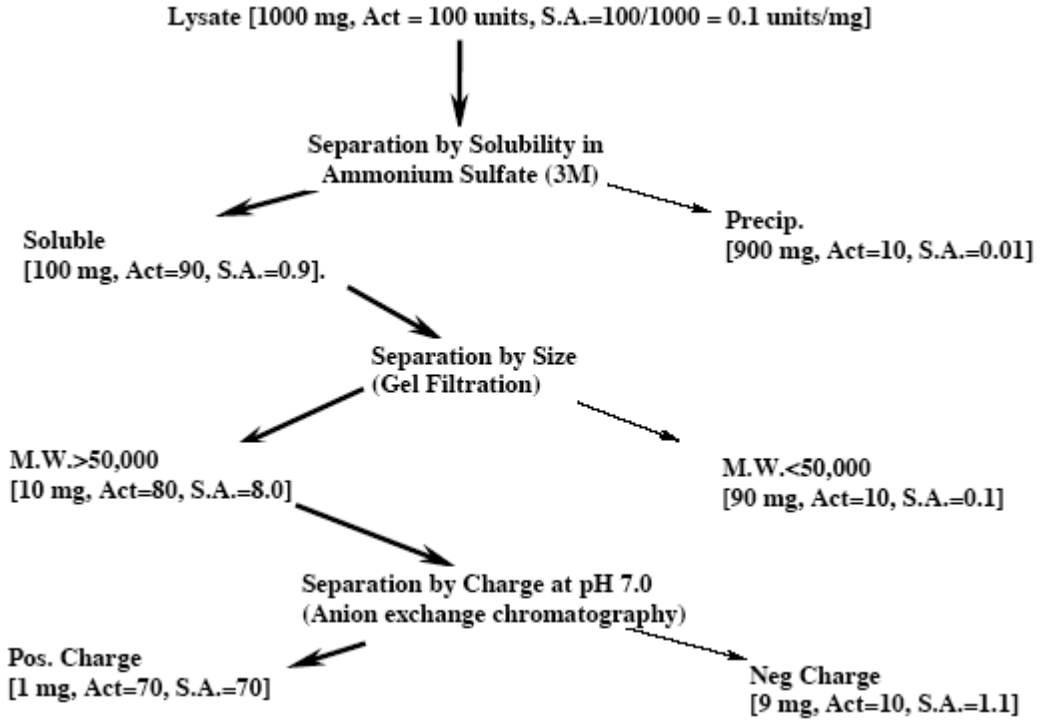
Specific Activity: The units of activity (eg, V_{MAX} in the case of an enzyme catalyzed reaction) for the desired enzyme divided by the total amount of *all* protein species in the sample. Typical units would be μmol/sec/(mg total protein), where the μmol/sec refers to the amount of product produced (μmol)/unit time. As the purification scheme progresses, the specific activity should *increase*, reaching a maximum for the pure enzyme.

Example Purification Scheme:

The following scheme is an example of purification of a large (i.e. > 50,000 Da) protein that is soluble in 3M ammonium sulfate and has a positive charge at pH=7.0.

1. Initially, we begin with 1 gram of total protein which contains 100 units of activity, giving an initial specific activity of 0.1 units/mg.

2. The final yield is 1 mg of purified material with a specific activity of 70 units/mg. Since the total activity of the final material was 70 units, the yield of this purification scheme is 70% (70 units/100 units).



Example purification scheme: Garden beets were placed into a Waring blender to produce an initial lysate for the purification of rubisco, an important plant enzyme. The following data were obtained for each step of the purification:

Sample	Units of Activity μmol product/sec	Total Protein (mg)	Specific Activity μmol product/sec/mg
1. Crude lysate	50,000 μmol/sec	5,000 mg	10
2. After separation by solubility in ammonium sulfate.	45,000 μmol/sec	900 mg	50
3. After separation by size.	40,000 μmol/sec	800 mg	50
4. After separation by charge.	30,000 μmol/sec	10 mg	3000

i) Calculate the net % yield:

ii) Calculate the increase in purity:

iii) Which step in the above purification scheme was a complete waste of time? How do you know?

iv) Is the protein pure after the last step?

Evaluating Final Purity:

After the protein is pure, its purity can be monitored by:

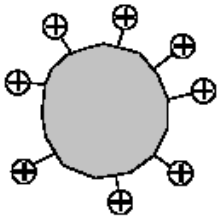
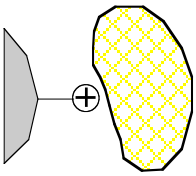
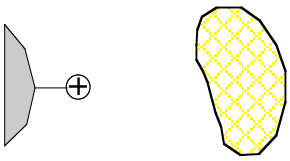
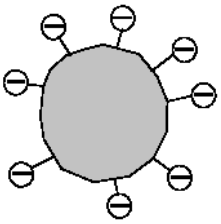
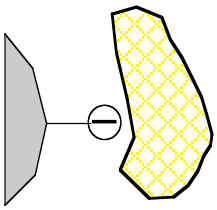
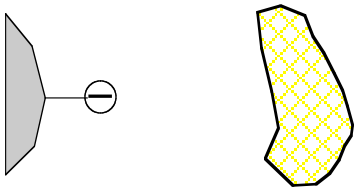
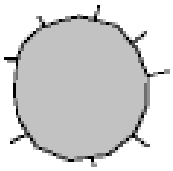
a) SDS-page gel electrophoresis.
b) Mass spectrometry.
c) Amino terminal sequencing.

Separation using Column Chromatography:

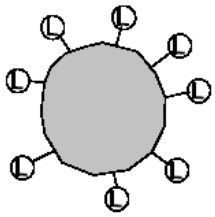
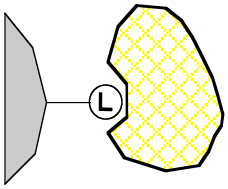
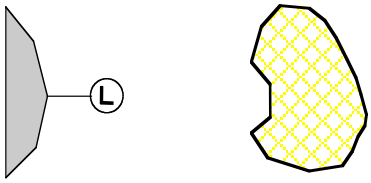
In most cases chromatography is performed in long glass tubes filled with a matrix or resin (particle size similar to a fine sand) that is completely immersed in a buffered salt solution. The mixture of proteins is added to the top of this column and buffer is allowed to flow through the column. As the buffer flows through the column the mixture of proteins is drawn down through the column and interacts with the matrix or resin. The actual mode of separation depends on the nature of the resin. Usually several different chromatographic steps are performed with different resins during a purification scheme. The actual order of separation methods will depend on the protein being purified.

Separation by Binding:

- Anion exchange
- Cation exchange
- Hydrophobicity
- Affinity chromatography

Type	Type of Resin	Principal of Separation	How to Elute the Protein
Anion Exchange *Separation by charge.*	• Beads with a **positive** charge 	Protein sticks to resin because of: • Overall **negative** charge (*anions*) • Proteins have patches of negative charge 	• Increase salt concentration to weaken electrostatic interaction. • Change of pH to pH < pI (protein becomes positively charged) 
Cation Exchange *Separation by charge.*	• Beads with a **negative** charge 	Protein stick to resin because of: • Overall **positive** charge (*cations*) • Proteins have patches of positive charge 	• Increase salt concentration to weaken electrostatic interactions. • Change of pH to pH > pI (protein becomes negatively charged) 
Hydro-phobic Chromato-graphy *Separation by hydro-phobicity*	•Beads with a **hydrophobic** group 	Protein sticks to resin in high salt because of: • **Hydrophobic** patches on surface (salts like ammonium sulfate remove water ordered around hydrophobic patches on protein surface and expose them for interaction with the resin)	• Decrease salt concentration to weaken hydrophobic interactions (hydrophobic patches are once again surrounded by water)

<table>
<tr>
<td rowspan="2">Affinity Chromato-graphy

Separation by affinity.</td>
<td>• Beads with a ligand:
  </td>
<td>Protein stick to resin because of:
• Binding site for ligand
  </td>
<td>• Excess ligand
• Change in pH, salt, solvent to weaken protein-ligand interaction.
  </td>
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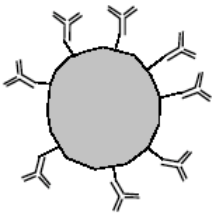
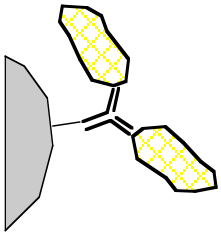
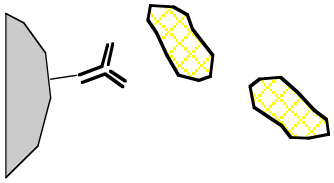
<td>• or Antibody
  </td>
<td>Protein stick to resin because of:
• Binding to antibody
  </td>
<td>• Changes in solution conditions (pH, salt, solvent) to weaken protein-antibody interaction.
  </td>
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<tr>
<td>Gel Filtration:

Separation by size</td>
<td>  </td>
<td>• Proteins don't "stick".

• Small proteins enter the interior of the beads, and therefore take longer to wash off of the column.</td>
<td>• Simply washing the column with buffer will eventually wash the proteins out of the column.

• Smaller proteins elute last.</td>
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