Drug resistance & rational drug design:

- Error prone copying of vRNA to DNA introduces changes in the sequence of the viral RNA (mutations), leading to altered amino acids in the viral proteins.
- Changes in the residues that are involved in drug binding may reduce binding.
- The mutant virus is no longer inhibited from growing and will quickly overgrow the wild-type virus.
- A common mutation that arises in many HIV patients is changing Val82→Asn82 in HIV protease.
- The altered HIV protease can be inhibited with modified protease inhibitors.

http://www.andrew.cmu.edu/user/rule/jmol/hiv_prot_mut.html

Example Question: A double reciprocal plot is shown for wild-type enzyme, the mutant (Val82-Asn) enzyme, and the data acquired with 5 nM of the cyclohexane inhibitor (Note: Both the wild-type and mutant show the same activity in this example, typically the mutation would affect both $K_M$ and $k_{cat}$)

i) How much poorer does the drug bind to the mutant enzyme? $[K_I = [I]/(\alpha-1)]$

ii) What is the extent of inhibition for the wild-type and mutant enzymes if the concentration of the drug is 10 nM? $[Y = [L]/(K_0 + [L])]$

ii) How might you alter the existing inhibitor to be effective at binding to HIV protease with the Asn82 mutation?
Lecture 21: Protein Purification.

Goals:
- Calculate specific activity
- Calculate net yield

Protein purification:

How to monitor purity: It is essential to have some method to evaluate purity, otherwise how do you know whether a specific step in the purification scheme has been successful in increasing the purity of the desired protein? To do so, we need to obtain two experimental measurements:

i) Amount of target enzyme: It is essential to have some method of determining the amount of the enzyme you are trying to purify 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. This is usually done using enzymatic assays that produce colored products—allowing a spectrophotometric assay.

If you were trying to purify chymotrypsin from pancreatic tissue, what substrate should you use?

ii) Amount of total protein in the sample:

Simply measuring the absorbance at λ = 280 nm will often suffice.

The purity of the enzyme during the purification scheme is generally monitored by measuring the specific activity:

Specific Activity: The velocity of the 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) per unit time.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Separation method</th>
<th>Units of Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td></td>
<td>50</td>
<td>25</td>
<td>2.0</td>
</tr>
<tr>
<td>Step A</td>
<td>Size</td>
<td>50</td>
<td>16</td>
<td>3.1</td>
</tr>
<tr>
<td>Step B</td>
<td>Not spherical</td>
<td>50</td>
<td>12</td>
<td>4.2</td>
</tr>
<tr>
<td>Step C</td>
<td>Binding site for Ligand</td>
<td>40</td>
<td>5</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Net yield (final amount of target/starting amount of target): 40/50 = 80%

Increase in purity (final specific activity/initial specific activity): 8.0/2.0 = 4 fold increase in purity.
**Separation by Solubility:** High concentrations of ammonium sulfate can precipitate proteins by reducing the ability of water to interact with the protein. Different proteins have different levels of solubility at any given concentration of ammonium sulfate. The precipitated proteins usually remain active and can be redissolved in buffer.

**Separation using Column Chromatography:** There are four common methods of separation by column chromatography.

1. Gel Filtration – separation by size
2. Anion exchange – separation by charge
3. Cation exchange – separation by charge
4. Affinity chromatography – separation by affinity

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 to keep the proteins in their native (folded) form. 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.

**Effect of pH on net Charge of Protein – ion exchange.**

- **pI** (Isoelectric pH): pH at which proteins have no net charge:
  - Proteins become positively charged for pH < pI
  - Proteins have a negative charged if pH > pI.

Therefore the charge on a protein can be changed by altering the pH, increasing or decreasing the binding to ion exchange resins.
<table>
<thead>
<tr>
<th>Type</th>
<th>Type of Resin</th>
<th>Principal of Separation</th>
<th>How to Elute the Protein</th>
</tr>
</thead>
</table>
| Size Exclusion (gel filtration) Separation by size | ![Diagram](image1.png) | - Proteins don't "stick".  
  - Small proteins enter the interior of the beads, and therefore take longer to wash off of the column | - Simply washing the column with buffer will eventually wash the proteins out of the column.  
  - Smaller proteins elute last. |
| Anion Exchange Separation by charge. | - Beads with a positive charge     | Protein stick to resin because of:  
  - Overall negative charge (anions)  
  - Proteins have patches of negative charge | Wash, bound proteins may elute.  
  - 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 | Wash, bound proteins may elute.  
  - Increase salt concentration to weaken electrostatic interactions.  
  - Change of pH to pH > pI (protein becomes negatively charged) |
| Affinity Chromatography Separation by affinity, either ligand affinity, or antibody. | - Beads with a ligand:  
  - Common: Ni^{2+} ions attached to resin.  
  - or Antibody  
  - Protein has 6 His residues on N or C-terminus. "His-tag":  
  - or Antibody | Protein stick to resin because of:  
  - Binding site for ligand  
  - Excess ligand  
  - Change in pH, Salt, solvent to weaken protein-ligand interaction.  
  | Eluted with imidazole (similar to His sidechain).  
  | Changes in solution conditions (pH, Salt, solvent) to weaken protein-antibody interaction. |
Example – Purification of C

<table>
<thead>
<tr>
<th>Protein</th>
<th>[Ammonium Sulfate] that precipitates 50% of protein*</th>
<th># Residues (Mol Wt)</th>
<th>#Asp (pK_a=4.0)</th>
<th>#His (pK_a=6.0)</th>
<th>#Lys (pK_a=9.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0 M</td>
<td>120 (13,200 Da)</td>
<td>2</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>1.5 M</td>
<td>120 (13,200 Da)</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>4.0 M</td>
<td>120 (13,200 Da)</td>
<td>4</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>D</td>
<td>6.0 M</td>
<td>240 (26,400 Da)</td>
<td>2</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

**Step 1 – ppt using 3 M amm. Sulfate**
- Set concentration of salt (3M)
- Perform step
- Assay fractions (ppt & super.)
- Collect supernatant

**Step 2 – Size exclusion (gel filtration)**
- Run column
- Assay one fraction each peak
- Select all fractions with activity
- Collect samples that contain C.

*Alternative Step 2 – cation exchange @ pH=6.0*

\[ q_c = -4 + 1.5 + 12 = +9.5, \text{ will elute 2}\text{nd peak.} \]

\[ q_o = -2 + 0 + 10 = +8.0, \text{ would elute 1}\text{st peak} \]

**Evaluating Final Purity:**
After the protein is pure, its purity can be monitored by any of the following:

a) SDS-page gel electrophoresis.

b) Mass spectrometry.

c) Amino terminal sequencing.

d) Isoelectric focusing (separation by pI).

**SDS-polyacrylamide gel electrophoresis**
(separation of denatured proteins by size.)

a) In the presence of an electric field, proteins will migrate at a velocity that is proportional to their intrinsic charge-mass ratio, \(v=\frac{q}{M}\)

b) **Denaturation** and coating of proteins by SDS gives them a uniform charge-to-mass ratio.

c) Forcing the protein-SDS complexes through a polyacrylamide gel causes separation of the proteins according to size.

d) Gels are stained with a stain that is specific for protein.

e) Distance, \(d\), migrated down the gel during electrophoresis depends on molecular weight. Smaller proteins move faster.