Lecture 10
Protein and Peptide Chemistry
Margaret A. Daugherty
Fall 2004

Purification: Step 1

• Cells: Break them open!

Crude Extract
Total contents of cell

Big Problem: Crude extract is not the “natural” environment
If you break open a cell, proteins are exposed to other proteins that “specialize” in protein degradation.
PROTEASES! ==> proteins which destroy other proteins by cleaving peptide bonds

Factors in Stabilizing Proteins During Purification

• pH
  - Requires the use of a buffer to maintain the pH in a physiological range (~7.0) where the protein will not denature

• Temperature
  - Thermal stability of proteins varies. Many proteins irreversibly denature (lose 3rd structure) at higher temperatures. A

• Degradative enzymes
  - Proteases chew up proteins. Nucleases chew up nucleic acids. These can be "controlled" by pH, temperature and inhibitors.
  (Temperature is a factor - proteases less active at 4C)

• Adsorption to surfaces
  - Fewer steps the better! The air-liquid interface is a prime spot for denaturation.

Purification: Step 2
getting rid of “cellular debris”

Crude extract -------> contains your protein of interest

+ Other Proteins, Nucleic acids,
Polysaccharides, Lipids, Cellular membranes,
Organelles..., 

Separate “cellular debris” from soluble molecules
CENTRIFUGATION

Separation method that involves applying a centrifugal force to a particle. Separation based on mass of particle.

\[ F = m \omega^2 r \]

- \( m \) = mass of object
- \( r \) = distance from center of rotation
- \( \omega \) = rotor speed (radians/sec)

Two objects of differing mass will experience different centrifugal forces at the same rotor speed.

How do we know where our protein is? protein assay

Most important piece of any purification protocol!

- Spectroscopic
- Functional Assay
  - Enzyme activity
  - DNA binding
  - Substrate binding
- Immunoassay
  - Elisa

Ammonium Sulfate Precipitation

In general, lower solubility at high salt concentrations.

"Salting out" of proteins. Specific control of the concentration can selectively precipitate some and leave others in solution.

An example:

Increasing (NH₄)₂SO₄
Dialysis

- A procedure for exchanging the solvent around a protein;
- Semi-permeable membrane (dialysis bag) contains protein --> suspend in a larger volume of buffered solution;
- Membrane is permeable to small solutes, but not to proteins;
- Buffers & salts exchange until an equilibrium is established between the inside & outside of the membrane.

Important because many purification techniques involve changing buffer conditions (pH & salt)

Column Chromatography

- Most powerful of the fractionation methods;
- Separates components of a mixture based on:
  - Size
  - Charge
  - Binding affinity

Size Exclusion Chromatography

Principles of Column Chromatography

- Reservoir with buffer (mobile phase) of interest
- Porous resin (solid phase) with certain chemical properties
- Protein applied in mobile phase
- Effluent: what comes out of the column
**Ion-exchange Chromatography**

- **Resins used:**
  - **Polaranionic:** used to separate positively charged molecules (e.g., cation-exchange chromatography!)
  - **Polycationic:** used to separate negatively charged molecules (e.g., anion-exchange chromatography!)

**Affinity Chromatography**

- **S. nuclease purification**

**Protein Purification**

1. **Lyse cells**
2. **Separate out cellular debris**
   - **Centrifugation**
3. **Purify based on**
   - **Size:** Size exclusion chromatography
   - **Solubility:** Ammonium Sulfate Precipitation
   - **Charge:** Ion exchange chromatography
   - **Binding ability:** Affinity chromatography
4. **Follow purification**
   - **Spectroscopic Assays:** UV/Vis spectroscopy
   - **Functional Assays:** Based on biological function
   - **Immunoassays:** ELISA
PROTEIN CHARACTERIZATION

What is the amino acid content?
What is the amino acid sequence?
What is the molecular weight?
What is its pI?
Does the protein self-associate (have quaternary structure?)
   If so, what is association stoichiometry?
   (monomer-dimer, monomer-trimer....)
   What is the association equilibrium constant?
   What is the shape of the molecule?

Electrophoresis:
analytical method to characterize proteins

- Separates components of a mixture based upon their charge and/or size
  - Paper electrophoresis
  - Polyacrylamide gel electrophoresis (PAGE)
  - SDS-PAGE
  - Isoelectric focusing (IEF)
  - 2D Gel Electrophoresis

PAGE: polyacrylamide gel electrophoresis

- Cross-linked acrylamide gels act as a molecular sieve
- An electric field is used to separate the proteins;
- Migration ∝ charge-to-mass ratio
  - Electrophoretic mobility:
  - Small molecules >> large molecules (with the same charge density)
- pH of the buffer and protein mixture is high (~9) so that the proteins carry a net-negative charge;
- Molecules of similar size and charge move through the gel as a band.

Visualization

- Most frequently, Coomasie Brilliant Blue or Silver stain is used;
- These stains bind to the proteins, not the gel.
- Can use this to monitor protein purification - # of bands should decrease with each purification step.

Problem: Molecules contain disulfides; Not all molecules have the same charge to mass ratio under near native conditions.
Determining Molecular Weights

SDS-PAGE

- Disulfides are reduced with β-mercaptoethanol;
- All secondary, tertiary & quaternary structure is lost.
- All proteins assume a rod like shape with a uniform charge/mass ratio;

Identification of MW of an unknown protein

SDS both denatures & binds the protein. Net result - protein is negatively charged.

Amino Acid Analysis

1. **Hydrolyze peptide bonds**: HCl/110°C/24hrs
   - Harsh: trp destroyed/ser,thr,tyr often degraded
   - asn & gln hydrolyzed to asp & glu --> glx/asx content

2. **Separation by cation-exchange chromatography**;
   - Detection with fluorophores (10⁻¹⁸ M; attamolar!)

3. **Quantitation of amino acids**;
   - Areas under peaks proportional to concentration

Protein Fragmentation

- Removal of disulfides:
  - mercaptoethanol, other means
- Chemical
  - Cyanogen bromide (CNBr): cleaves after Met
- Proteases
  - Trypsin: cleaves after Arg, Lys (basic)
  - Chymotrypsin: cleaves after Tyr, Phe, Trp (aromatics)
  - Staph protease: cleaves after Asp, Glu (acidic)
  - Carboxypeptidase A*: cleaves successive AAs from the C-term of peptides; no cleavage of Lys, Arg
  - Carboxypeptidase B*: cleaves Arg, Lys from C-term
  - Aminopeptidase: cleaves successive AAs from the N-term of peptides

* can not cleave proline!
**Fragmentation Strategies**

- If you have an unknown sequence:
- Three agents can be used to produce an overlapping set of peptides;
- If each peptide sequence is determined, the entire protein sequence can be reassembled.

*Chymotrypsin digestion produces:*

\[
\text{Met-Ala-Phe-Val-Arg-Ile-Glu-Trp-His-Met-Cys-Lys}
\]

*Trypsin digestion produces:*

\[
\text{Met-Ala-Phe-Val-Arg-Ile-Glu-Trp-His-Met-Cys-Lys}
\]

*CNBr digestion produces:*

\[
\text{Met-Ala-Phe-Val-Arg-Ile-Glu-Trp-His-Met-Cys-Lys}
\]

The original protein sequence was:

\[
\text{Met-Ala-Phe-Val-Arg-Ile-Glu-Trp-His-Met-Cys-Lys}
\]

---

**Protein Sequencing**

**Edman degradation**

1) N terminal AA reacts with phenylisothiocyanate;
2) Derivatized AA is cleaved in acid;
3) Derivatized AA rearranges to yield phenylthiohydantoin (PTH) derivative:
   a). Identifiable AA (chromatographic methods)
   b). Doesn’t affect rest of chain
4) Repeat.

Good for ~ 50 AA polypeptides
High efficiency

---

**Review**

What properties are used in purifying and characterizing a protein?
What is a crude extract?
What factors are used to stabilize a protein during purification?
What would you use (NH₄)₂SO₄ precipitation?
What is the basis of:
- size exclusion chromatography?
- ion exchange chromatography?

What is the basis of centrifugation?
How do you determine MW by electrophoresis?
What is used for protein proteolysis?
What is the Edman degradation? Why is it used?

---

**Summary**

<table>
<thead>
<tr>
<th>Protein Characteristic</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charge</td>
<td>Ion exchange column</td>
</tr>
<tr>
<td>Polarity</td>
<td>Hydrophobic interactions</td>
</tr>
<tr>
<td>Size</td>
<td>Gel-filtration column</td>
</tr>
<tr>
<td></td>
<td>SDS-PAGE</td>
</tr>
<tr>
<td></td>
<td>Ultracentrifuge</td>
</tr>
<tr>
<td>Binding specificity</td>
<td>Affinity chromatography</td>
</tr>
<tr>
<td>Charge + size</td>
<td>2D electrophoresis</td>
</tr>
<tr>
<td>Composition</td>
<td>Amino acid analysis (AAA)</td>
</tr>
<tr>
<td></td>
<td>Protein sequencing</td>
</tr>
</tbody>
</table>