Protein determination and concentration

Protein Production for Biophysical and Biochemical Studies

November 2014
Protein Quantification

- **Requirements:**
  - Fast and easy
  - Linear over a broad range
  - Low cost
  - Minimal interferents
Bradford assay

- Coomassie Blue changes color from brown to blue in presence of proteins
- Absorbance at 595 nm is measured to quantify protein amounts
- Ratio with absorbance at 450 nm is more exact
Bradford assay

- **Advantages:**
  - Simple
  - No need for an additional tag / chromophore
  - Compatible with reducing agents, chaotropic and chelating agents, metals

- **Disadvantages:**
  - A standard curve is needed (same buffer!)
  - Linear over a short range of concentrations
  - Dependence on MW of proteins
  - Not exact!
  - Interference by detergents
## Data Example

1. Measure known concentrations of BSA in duplicates

<table>
<thead>
<tr>
<th>[BSA] (µg/ml)</th>
<th>Absorbance at 595nm</th>
<th>Absorbance at 450nm</th>
<th>Ratio ($A_{595}/A_{450}$)</th>
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<tbody>
<tr>
<td>0</td>
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2. Compile calibration curve:

\[ y = 0.0159x + 0.5471 \]

\[ R^2 = 0.9985 \]
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2. Compile calibration curve:

3. Measure protein concentration

Example: Absorbance ratio: 0.8
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2. Compile calibration curve:

3. Measure protein concentration
   Example: Absorbance ratio: 0.8

4. Determine protein concentration
   Concentration: ~15µg/ml
Lowry assay

- Relies on color change in added solution
- Copper ions bind peptidic bonds (alkali conditions)
- Aromatic residues also take part
Lowry assay

- Advantages:
  - Simple
  - Compatible with detergents
  - Very precise
  - Compatible with many interferents after protein precipitation with TCA (Peterson)

- Disadvantages:
  - Standard curve needed
  - Aromatic residues
  - Lamp with near IR (750nm)
  - Interference: reducing agents and others
Absorbance at 280nm

- Aromatic side chains absorb light at 280nm
- Using Beer-Lambert law we can determine the concentration:
  \[ A = \varepsilon \times l \times C \]
  \[ \varepsilon = 5690 \times n_{Trp} + 1280 \times n_{Tyr} \]
  \[ l = 1 \text{ cm} \text{ (usually)} \]

- Absorbance should be between 0.1 and 1
Absorbance at 280nm

- **Advantages:**
  - Very precise for purified proteins
  - Non-disruptive
  - Additional info: pureness, aggregates

- **Disadvantages:**
  - Need aromatic residues
  - Different chromophores can interrupt nucleic acid, detergents, cofactors, phenolic compounds, pigments, reducing agents, etc.
  - Problematic for a mixture of proteins
Data Example

- Peak at 220nm cannot be quantified

\[ A = \epsilon \times l \times C \]
Data Example

Abs\(_{(280\text{nm})}\) = 0.45

\[ \varepsilon = 6970 \text{ M}^{-1}\text{cm}^{-1} \]

\[ l = 1\text{ cm} \]

\[ C = \frac{A}{\varepsilon \times l} \]

\[ C = \frac{0.45}{(6970 \times 1)} \]

\[ C = 64.5\mu\text{M} \]

- Peak at 220nm cannot be quantified
Fluorescence

- Advantages:
  - Compatible with reducing agents, detergents and nucleic acids
  - Very sensitive

- Disadvantages:
  - Tag needed
  - Need a fluorimeter
  - Costly
  - Linear only over a short range
  - Calibration curve needed
Protein concentration methods

- Non-denaturative methods
- Denaturative methods
Ultrafiltration

- Based on size exclusion and centrifugation
- Membrane: Polyethersulfone, cellulose triacetate
- Advantages:
  - Easy use
  - First choice
  - Widespread in Industry

- Disadvantages:
  - Slow concentration with viscous buffers (glycerol, etc)
  - Aggregation by over-concentrating
Ultrafiltration

- Based on size exclusion and centrifugation

Examples of ultrafiltration tubes
Lyophilization

- Freeze-dry samples to powder
- Resuspend in smaller volume

The process

The instrument

Protein concentration
Lyophilization

- Advantages:
  - No over-concentrating
  - Keeps the protein stable
  - Good choice when conditions are known

- Disadvantages:
  - Conditions are hard to find
  - Not suitable for all buffers (glycerol)
  - Salts and additives will get concentrated too
  - Takes overnight
  - Dangerous for unstable proteins
Ammonium Sulfate Precipitation

- \((\text{NH}_4)_2\text{SO}_4\) is added to protein sample
- Different proteins precipitate at different IS

- Purification: precipitating undesired proteins
- Concentrating: precipitate desired protein, then resuspend in lower volume
Ammonium Sulfate Precipitation

- **Advantages:**
  - Easy to perform
  - Keeps the protein stable in most cases
  - Good for concentrating proteins before GF
  - Eliminates some interferents

- **Disadvantages:**
  - Time
  - High salt / needs buffer exchange

- Tip: \((\text{NH}_4)_2\text{SO}_4\) takes up volume, use calculator
Ammonium Sulfate Precipitation

- **Calculator example:**

**Ammonium Sulfate Calculator:**

Ammonium Sulfate Precipitation is a simple and effective means of fractionating proteins. It is based on the fact that at high salt concentrations the natural tendency of proteins not to aggregate is overcome, since the surface charges are neutralized. Charge neutralization means that proteins will tend to bind together, form large complexes and hence are easy to precipitate out by mild centrifugation. Since each protein will start to aggregate at a characteristic salt concentration, this approach provides a simple way of enriching for particular proteins in a mixture, and is used, for example, to isolate immunoglobulins from sera. It is quite easy to bring a preparation to 50% saturated by simply adding an equal volume of saturated Ammonium Sulfate. It is more of a problem to make solutions of higher percentage saturation as you may have to add very large volumes of saturated Ammonium Sulfate. It is usually easier to add solid Ammonium Sulfate to your sample, but calculating how much to add is rather time consuming. The program below calculates how much solid Ammonium Sulfate you need to add to a specific volume of a solution to get a specific percentage saturation at a specific temperature.

Select temperature at which you are working (25°C is default):

- 0°C
- 4°C
- 10°C
- 20°C
- 25°C

Enter starting volume of solution in ml:

Enter desired percentage saturation of Ammonium Sulfate:

Press to Calculate

**An Ammonium Sulfate Precipitation Protocol:** You can either mix your protein preparation with 100% Ammonium Sulfate solution, or you can add solid Ammonium Sulfate to make the desired percentage concentration using the program above. A typical protocol is as below:
PEG Precipitation

- Polyethylene-glycol is a large polymer
- Generally inert, no protein interactions
- Protein will concentrate in areas of solvent without polymer – steric exclusion
On-Column Concentration

- Load dilute protein on column: (IEX, HIC, affinity)
- Elute up-flow at very low flow-rate in smaller volume than loading volume

- Advantage: Efficient
- Disadvantage: Over-concentrating, time, availability, need of a tag
Denaturative concentration methods

- Eliminate interferences before electrophoresis or protein determination
- Proteins get irreversible denatured and lose activity!
  - TCA-DOC: For precipitation of low concentration proteins
  - Normal TCA: Eliminates TCA soluble interferences
  - Acetone: Eliminates acetone soluble interferences
  - Ethanol: Removal of GuHCl before PAGE-SDS
  - Chloroform Methanol: Removal of salt and detergents
  - Acidified Acetone/Methanol: Removal of acetone and methanol soluble interferences (SDS before IEF)
Summary

- Each protein is unique!
  - What suits one protein might not suit another
- Method of choice depends on availability, time, cost
- Be careful of over-concentrating