Spectrophotometric Methods For Determination Of Proteins
Objectives

To Learn Different Method Of proteins determination

In this Lab you will using the following spectrophotometric methods:


Chemical reagents are added to the protein solutions to develop a color whose intensity is measured in a spectrophotometer.


Relies on direct spectrophotometric measurement
Importance of determining concentration of protein

- Protein assays are one of the most widely used methods in life science research.
- Estimation of protein concentration is necessary in cell biology, molecular biology, and other research applications.
- Is necessary before processing protein samples for isolation, protein purification, separation, and analysis.
Important Terms

**Assays**

**Qualitative assays**
Determine if specific substance is there or not, by color or some other quality

**Quantitative assays**
Determine the concentration of a substance

**Specificity and sensitivity**

**Sensitivity** of an assay is a measure of how little of the analyte the method can detect

**Specificity** of an assay relates to how good the assay is in discriminating between the requested analyte and interfering substances
The determination of protein concentration

Spectrophotometric and colorimetric methods

- Used for routine estimation, most of them are **colorimetric**
- Where a portion of the protein solution is reacted with a reagent that produces a coloured product.
- However, none of these methods is **absolute**,...

Acid hydrolyse a portion of the sample

- And then carry out amino acid analysis on the hydrolysate
- A truly accurate method
- However, this is expensive and relatively time-consuming, particularly if multiple samples are to be analysed.
Spectrophotometric method for determining the protein concentration

There are a wide variety of protein assays available, but each assay has its own advantages and limitations.

The factors that you should consider:
• Sensitivity
• The presence of interfering substance
• Time available of the assay
1-Bicinchoninic acid method

• The mechanism of color formation for the BCA assay is similar to that of the Lowry protein assay.

• Both BCA assay and Lowry assay are based on the conversion of Cu$^{2+}$ to Cu$^{1+}$ under alkaline conditions.

• In BCA the color develops in a single step but Lowry method in two steps

• In general, this method has a high sensitivity (1 µg)
1-Bicinchoninic acid method

**Principle:**
- Cu$^{+2}$ form a complex with nitrogen of the peptide bond under alkaline conditions producing Cu$^{+}$(the Cu$^{++}$ was reduced to Cu$^{+}$)
- This Cu$^{+}$ will then chelated by BCA to produce a copper-BCA complex with maximum absorbance 562 nm
1-Bicinchoninic acid method

Protein + Cu^{II} → Cu^{I} + 2 Bicinchoninic Acid (BCA) → Cu^{I}(BCA)_2 Complex
2-Bradford assay

- Very fast (15 min)
- Accurate
- Highly sensitive (1 µg protein can be detected)
- The amount of this coloured product is then measured spectrophotometrically and the amount of colour related to the amount of protein present by appropriate calibration.
- Disadvantages: Coomassie (Bradford) Protein Assay produces a nonlinear standard curve. Why?
2-Bradford assay

• Principle:
  • Coomassie brilliant blue G-250 bind to protein (binds particularly to basic and aromatic amino acids residues) in acidic solution
  • Make a complex which will shift the wavelength of maximum absorbance 465 to 595 nm.
  • This complex stabilized by hydrophobic and ionic interaction
Bradford assay

- Bradford reagent alone – maximum absorbance at (465 nm)
- Bradford reagent and protein maximum absorbance at (595 nm)
## Bradford assay-Method

**A- Set up 9 tubes and label them as follows:**

<table>
<thead>
<tr>
<th>Tube</th>
<th>Bovine Serum Albumin (BSA) (150µg/ml)</th>
<th>Distilled Water</th>
<th>Unknown</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(blank)</td>
<td>-</td>
<td>1 ml</td>
<td>-</td>
<td>Blank</td>
</tr>
<tr>
<td>A</td>
<td>0.07 ml</td>
<td>0.93 ml</td>
<td>-</td>
<td>10.5</td>
</tr>
<tr>
<td>B</td>
<td>0.13 ml</td>
<td>0.87 ml</td>
<td>-</td>
<td>19.5</td>
</tr>
<tr>
<td>C</td>
<td>0.26 ml</td>
<td>0.74 ml</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>D</td>
<td>0.4 ml</td>
<td>0.6 ml</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>E</td>
<td>0.66 ml</td>
<td>0.34 ml</td>
<td>-</td>
<td>99</td>
</tr>
<tr>
<td>F</td>
<td>1 ml</td>
<td>-</td>
<td>-</td>
<td>150</td>
</tr>
<tr>
<td>G</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
<td>?</td>
</tr>
<tr>
<td>H</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
<td>?</td>
</tr>
</tbody>
</table>

Add 5ml of Bradford reagent to each tube [blank – H].
C- Mix and Incubate at room temperature for 5 min.
D- Measure the absorbance at 595 nm.
**Bradford assay-Results**

<table>
<thead>
<tr>
<th>Tube</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance at 595 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(blank)</td>
<td>Blank</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
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<tr>
<td>F</td>
<td>150</td>
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</tr>
<tr>
<td>G</td>
<td>..................</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>..................</td>
<td></td>
</tr>
</tbody>
</table>
3-Warburg christian  (A280/A260)

- Relies on direct spectrophotometric measurement.
- Fast
- Semiquantitative analysis

**Principle:**
- Proteins can absorb light at 280 ultraviolet
- This is because proteins contain aromatic amino acids tyrosine and tryptophan.
- The amount of these residues vary greatly from protein to protein so this method is semiquantitative.
Warburg christian (A280/A260)

- Nucleic acid interfere with this method.
- So to solve this problem, we will measure the absorbance at 280 then we measure at 260
- Calculate A280/A260 ratio,
- then from a specific table we can get the correction factor
- A280 x correction factor
- A protein solution that has a high A280/A260 ratio: Less contaminated by DNA
- Or by another way:
- [groves formula]:
- Protein concentration [mg/ml]=\[1.55 \times A280\]-[0.76 \times A260]
Warburg christian (A280/A260)

- Calculate the protein concentration in the unknown from the following equations:

\[
A_{280} = \ldots \ldots \ldots \ldots \\
A_{260} = \ldots \ldots \ldots \ldots \\
A_{280}/A_{260} = \ldots \ldots \ldots \ldots \\
\text{Correction factor} = \ldots \ldots \ldots \ldots \\
A_{280} \times \text{correction factor} = \ldots \ldots \text{mg/ml protein} \\
\text{Unknown concentration} = \ldots \ldots \ldots \ldots \text{mg/ml}
\]

2-or [groves formula]:

Protein concentration [mg/ml] = [1.55 X A280] - [0.76 X A260]
Warburg christian
(A280/A260)

-A protein solution that has a high A280/A260 ratio: Less contaminated by DNA.

[It shows a lower absorbance at 260nm comparing to absorbance at 280nm].

-A protein solution that has a low A280/A260 ratio: Highly contaminated by DNA.

[It shows a higher absorbance at 260nm comparing to absorbance at 280nm].
Summary

- Protein assay is important in many aspects
- There are Many Methods for protein determination, each had it own advantages and disadvantages

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>ABSORPTION</th>
<th>MECHANISM</th>
<th>reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV absorption</td>
<td>280 nm</td>
<td>Tyrosine and tryptophan absorption</td>
<td>No reagent</td>
</tr>
<tr>
<td>Bicinchnonic acid</td>
<td>562 nm</td>
<td>copper reduction (Cu²⁺ to Cu¹⁺), BCA reaction with Cu¹⁺</td>
<td>BCA</td>
</tr>
<tr>
<td>Bradford or Coomassie brilliant blue</td>
<td>595 nm</td>
<td>complex formation between Coomassie brilliant blue dye and proteins</td>
<td>Coomassie brilliant blue</td>
</tr>
</tbody>
</table>