Proteins

Why are proteins analysed?

To test for:

- Purity – has the protein of interest been separated from other proteins?
- Quantity – how much protein has been purified?
- Activity – has purification or separation from other proteins maintained the activity of the protein of interest?

How are proteins analysed?

Electrophoresis analyses

- Analyse the relative amounts of protein present
    - Native electrophoresis
        - Agarose
        - PAGE
    - Denaturing SDS-PAGE
    - 2-D gel electrophoresis

Quantitative protein assays

- Measures the total concentration of protein, not just the protein of interest
    - UV spectrophotometry
    - Biuret Assay
    - Bradford Assay
    - Lowry Assay

Activity assays

- Measures the activity of the protein of interest

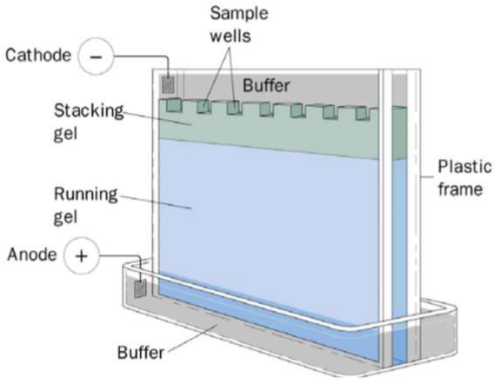
Using gel electrophoresis to separate proteins

Vertical acrylamide gel electrophoresis

- Analytical method to separate and visualise proteins
- Used to:
    - Estimate the number of proteins within a mixture
    - Determine properties such as approximate molecular weight and isoelectric point (2-D gels)
    - Determine purity of a protein preparation (i.e. efficacy of purification process)

Gel is made of polyacrylamide → polyacrylamide gel electrophoresis (PAGE)

- Contains crosslinked acrylamide (polymer)
    - Forms a matrix
    - Stacking gel: low percentage of acrylamide which allows concentration of the protein sample
    - Resolving/running gel: higher percentage of acrylamide to separate the proteins in the sample
    - Acts like a molecular sieve → proteins move in proportion to their charge-to-mass ratio
- An electric field causes the proteins to move down the gel
    - Small proteins → little resistance → move faster
    - Large proteins → greater resistance → move slower



Polyacrylamide + denaturing gel electrophoresis: SDS-PAGE

To denature the proteins:

1. Mix in a denaturation buffer containing:
    a. Buffer (often Tris)
    b. Sodium dodecyl sulphate (SDS)
        i. Anionic detergent
        ii. Denatures protein
        iii. One SDS binds to one amino acid (binding proportional to molecular mass of protein)
    c. A reducing agent (e.g. DTT or β-Me)
        i. Reduces any disulphide bonds present between and within subunits → breaks quaternary structure of protein
    d. Sucrose or glycerol
        i. Gives the protein sample density of that it settles into well of the gel
    e. Dye (e.g. bromophenol blue)
        i. To see the sample – helps with loading sample onto gel
        ii. To monitor the gel
2. Boil the sample mixture at 100°C for 5-10 min

Results in linear polypeptides with a net negative charge so separation is according to their size only.

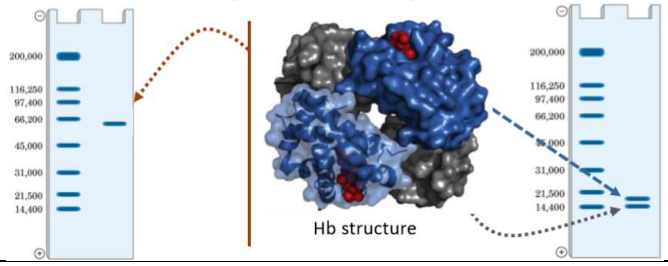
Separation according to size depends on the concentration of acrylamide. Separation range may be changed by adjusting the polyacrylamide concentration.

To visualise the proteins, stain with either:

- Coomassie Brilliant blue dye
    - Binds to protein to form a blue dye-protein complex
    - Detects ~50 ng proteins in a band
- Silver stain
    - Proteins bind to silver ions which can then be reduced under specific conditions to build up a visible image
    - Detects ~1 ng protein in a band (i.e. very sensitive)

Difference between native gel electrophoresis and denaturing gel electrophoresis
Haemoglobin has 2 alpha and 2 beta chains (alpha chain is smaller than the beta chain)

- Native: Hb will run as a single 63,200 Da band
- Denaturing: Hb will run as two bands: 15,500 Da and 16,100 Da



SDS-PAGE can be used to:

- Estimate the relative molecular weight
    - Size of proteins can be approximated according to their relative positions within the gel
    - Need to run a protein marker → calibration curve (±10% accuracy)
- Identify specific proteins
    - Immunoblot/Western blotting

Western blotting

Used to identify protein of interest and to make sure it is not a different protein with a similar molecular weight

Need to:

- Transfer proteins from SDS-PAGE gel to a nitrocellulose membrane
- Treat the membrane with antibody

Antibody

Immunoglobulins produced in response to antigen

Hypervariable region (N-terminal) recognises and binds to specific epitope

- An epitope is a site on an antigen that is recognised by an antibody and is normally about 6 amino acids in size

Constant region (C-terminal) is species specific

1. Transfer proteins on SDS-PAGE onto nitrocellulose membrane using electric blotting
    a. Wet transfer: fibre pad, filter paper, membrane, gel, filter paper, fibre pad
    b. Semi-dry transfer: filter paper, membrane, gel, filter paper
2. Incubate in non-specific protein (e.g. 10% BSA or 5% non-fat dry milk solution) then wash
    a. To block unoccupied hydrophobic sites which the antibody might bind to
3. Incubate in antibody with N-terminal which is specific to the protein of interest (primary antibody) then wash off excess antibodies
4. Incubate in antibody with N-terminal which is specific to the C-terminal of the primary antibody (secondary antibody)
    a. C-terminal of secondary antibody has an enzyme complexed to it → add a substrate which will bind to enzyme → produces a product that is coloured

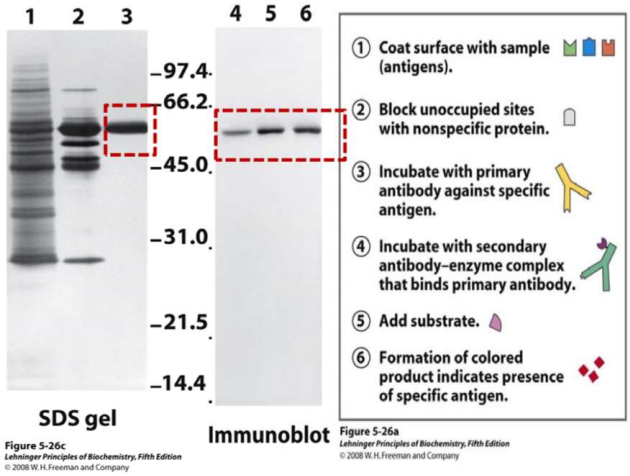


Figure 5-26c Lehninger Principles of Biochemistry, Fifth Edition © 2008 W. H. Freeman and Company

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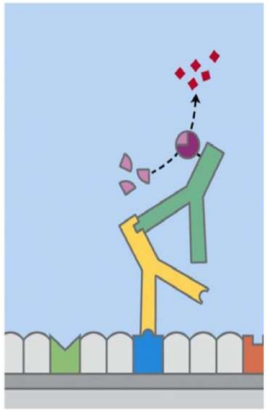
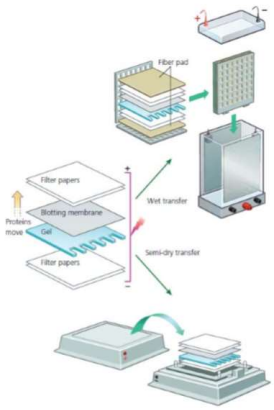


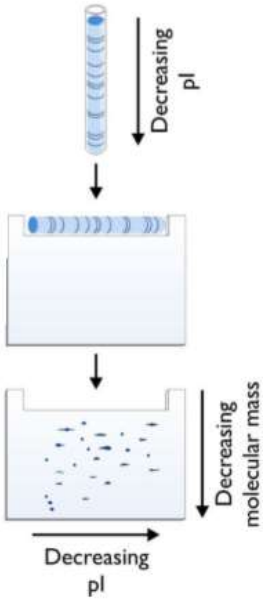


2-D gel electrophoresis

1-D gels have limited ability to resolve proteins in a complex mixture (e.g. SDS-PAGE resolves about 50 – 100 bands)

2-D gels can improve resolution

- First dimension: isoelectric focusing (IEF)
    - Separates molecules according to their isoelectric point
    - High resolution: able to separate proteins that differ in pI by as little as 0.01 of a pH unit
- Second dimension: SDS-PAGE
    - Separates molecules according to their size



Advantages:

- Sensitive
- Separates proteins with identical Mr but different pI
- Separates proteins with same pI but different Mr