Protein analysis

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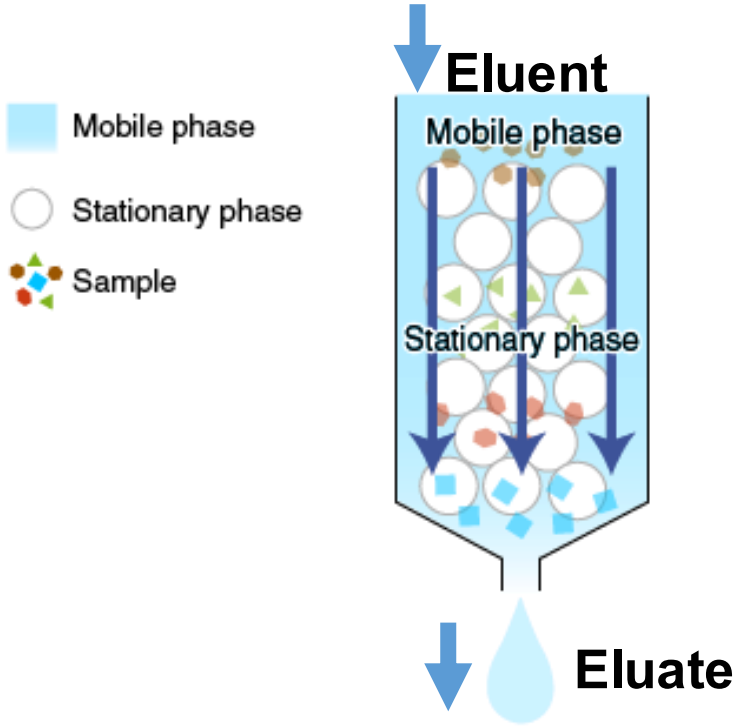
Part I: Protein purification

Bases of protein separation

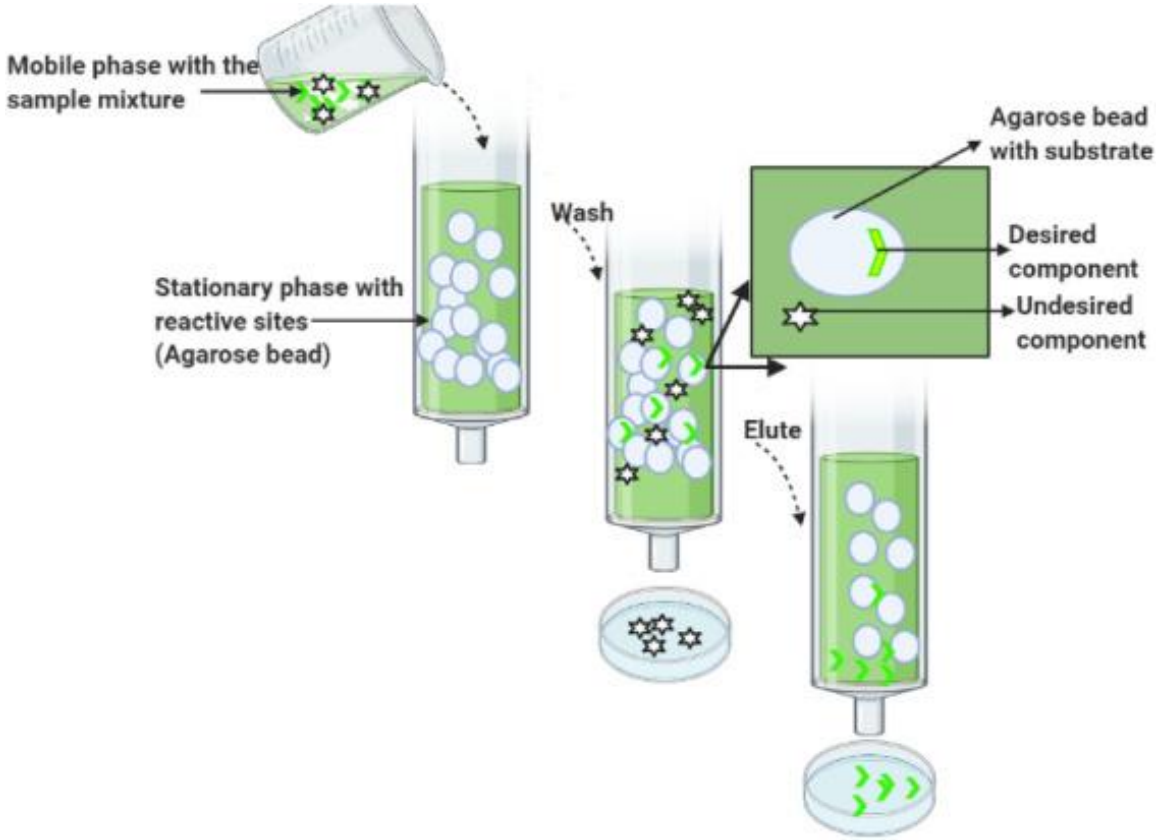
- Proteins can be purified using chromatography techniques on the basis of:

Size	Charge	Specific binding affinity	Hydrophobicity



Basis of charomatography

- A chromatography column containing a stationary phase (usually beads with a specific or treated surface) is prepared.
- The sample containing mixed proteins, for example, is added.
- The proteins bind to the bead surface.
- Unbound proteins are washed out of the column.
- Elution is carried out where an eluent (a solvent) is added to the column to elute (release) the proteins that are bound to the beads.
- The eluate containing the proteins of interest is collected and analyzed further.



Type of chromatography techniques

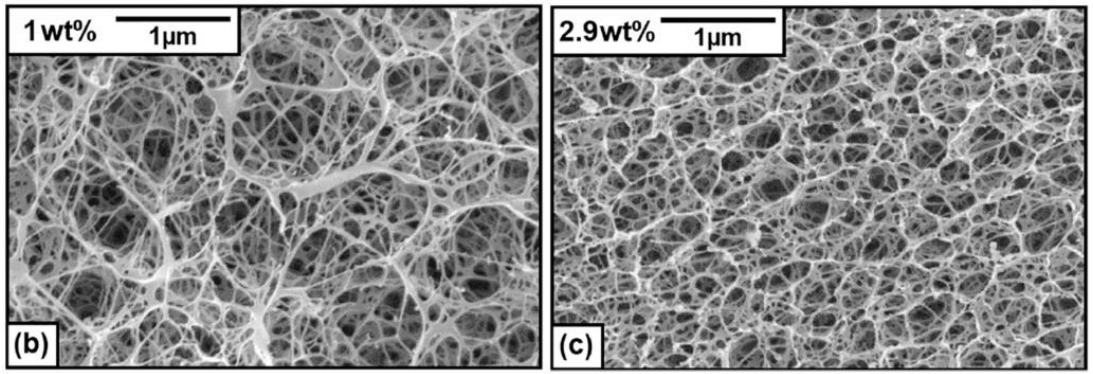
Type	Purpose
Size exclusion chromatography (AKA molecular sieve filtration chromatography/gel)	Separation of proteins based on size Larger proteins come out first and smaller proteins later
Ion-exchange chromatography A. Anionic exchange chromatography B. Cationic exchange chromatography	Separation of proteins based on isoelectric point A. Highly negatively charged proteins with lower PI come out later B. Highly positively charged proteins with higher PI come out later
Affinity chromatography	Separation of proteins based on their specific interaction Bound proteins are eluted by competitive binding of a free competitor added to the eluent or by reducing the non-covalent interactions
Reverse phase chromatography	Separation of proteins based on their hydrophobicity, where non-polar proteins come out later

Part II: Protein analysis

Gel electrophoresis

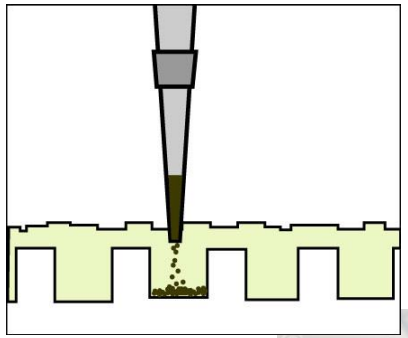
- A molecule with a net charge moves in an electric field
- This phenomenon, termed electrophoresis, offers a powerful means of separating proteins.
- In gel electrophoresis, proteins are separated as they move through a gel, which serves as a molecular sieve.

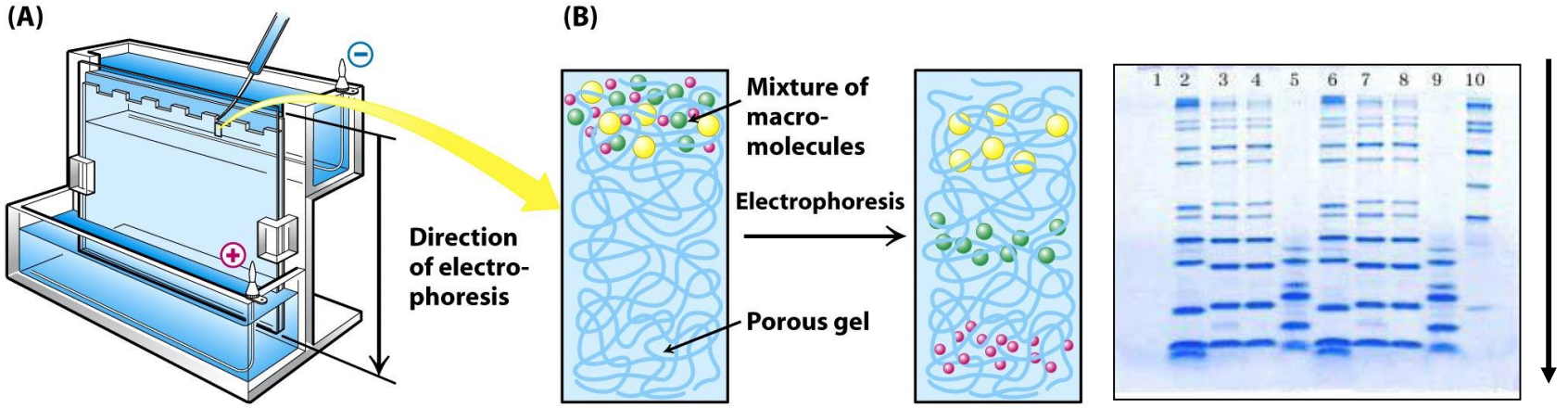
https://www.youtube.com/watch?v=i_6y6Z5UvwE



The process

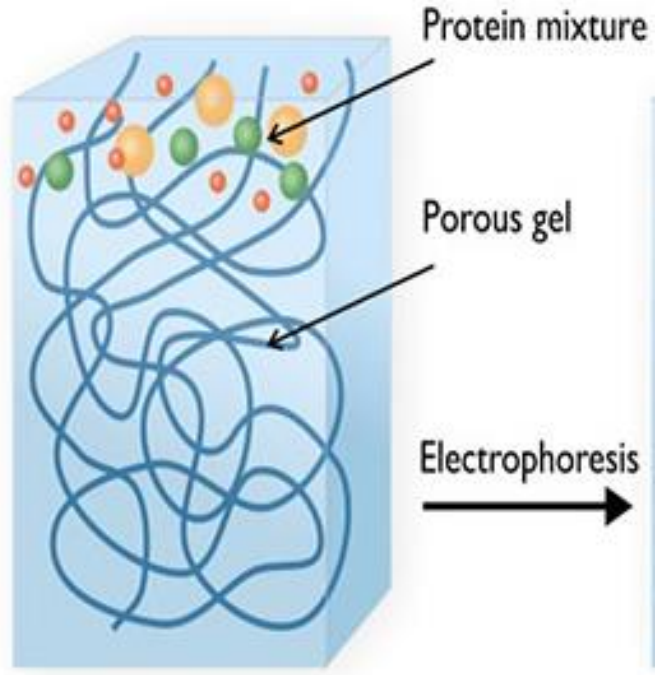
- The most commonly used protein electrophoresis technique is termed sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE).
- It is performed in a thin, vertical gel.
- The top of the gel consists of wells onto which samples are loaded.

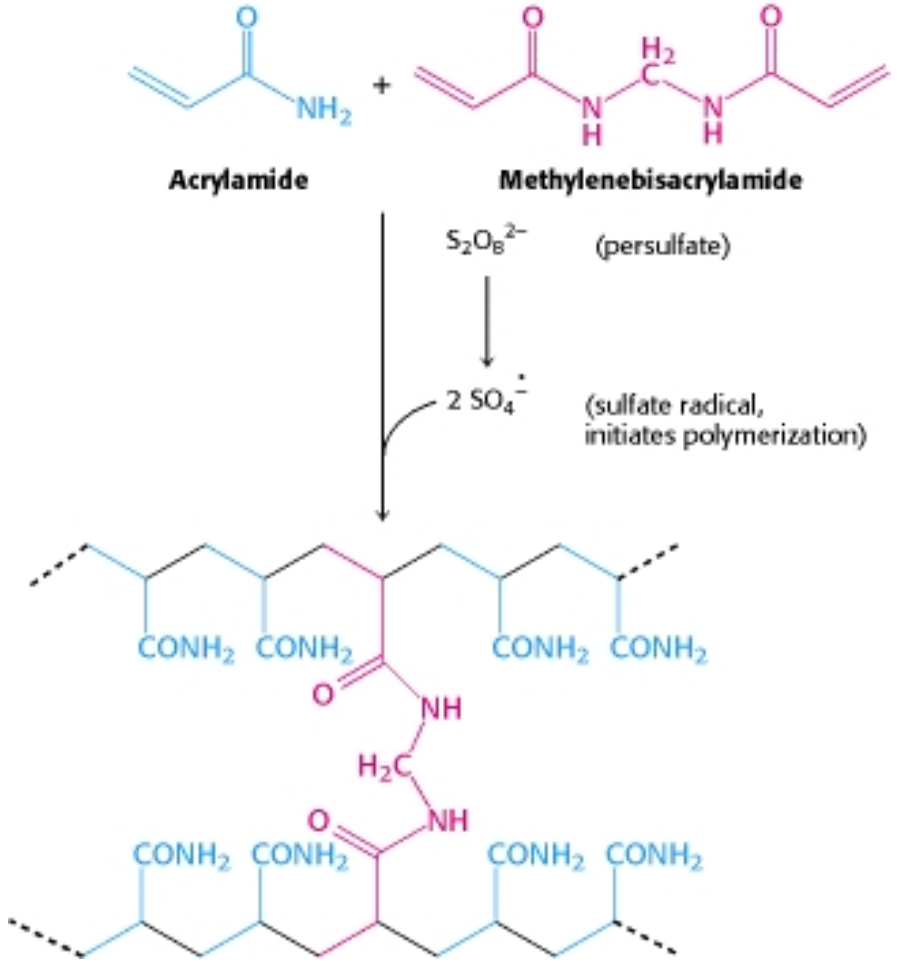




Formation of the gel

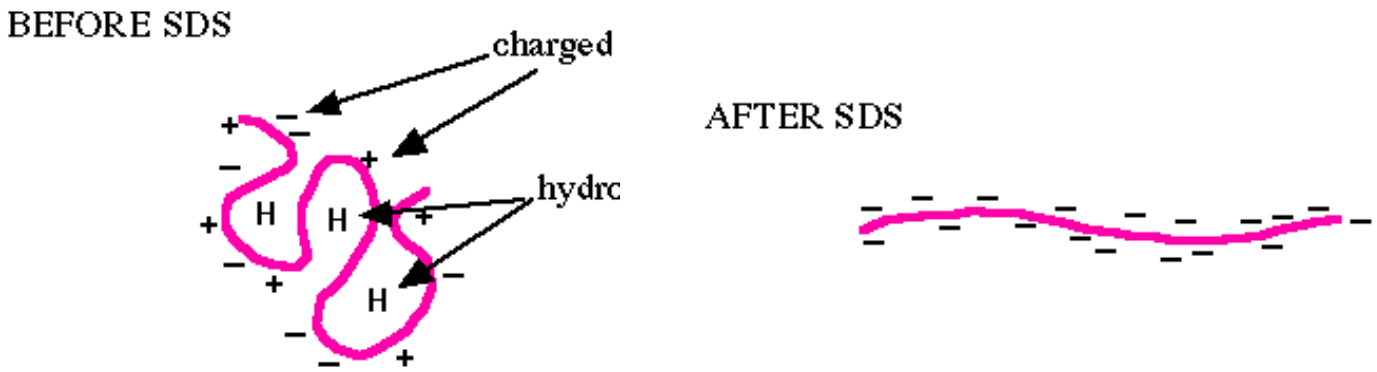
- The gel is made of a material known as polyacrylamide, which is formed by the polymerization of acrylamide and cross-linked by methylene-bisacrylamide.





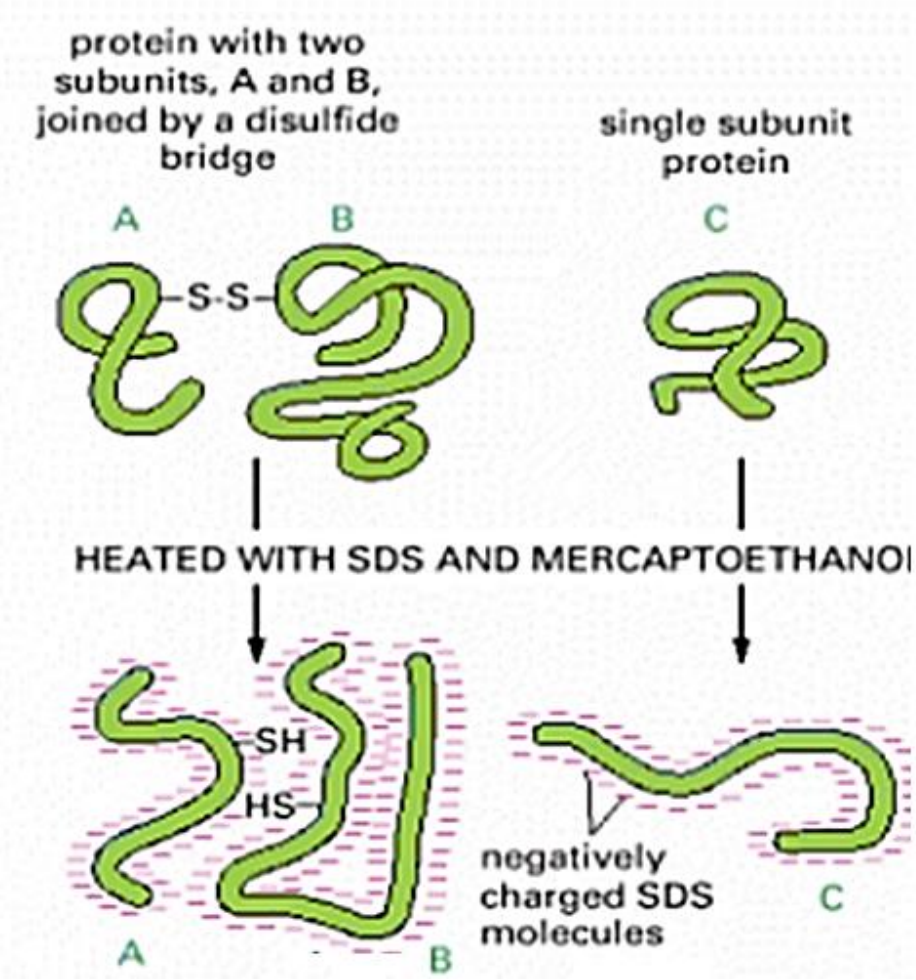
Purpose of SDS

- This technique utilizes a negatively charged detergent dodecyl sulfate) to denature and solubilize proteins (c condition).
  - Otherwise, non-denaturing condition or native condition w maintain their original structure and shape and are separat charge, size, and shape.
- SDS makes proteins have a uniform negative charge.



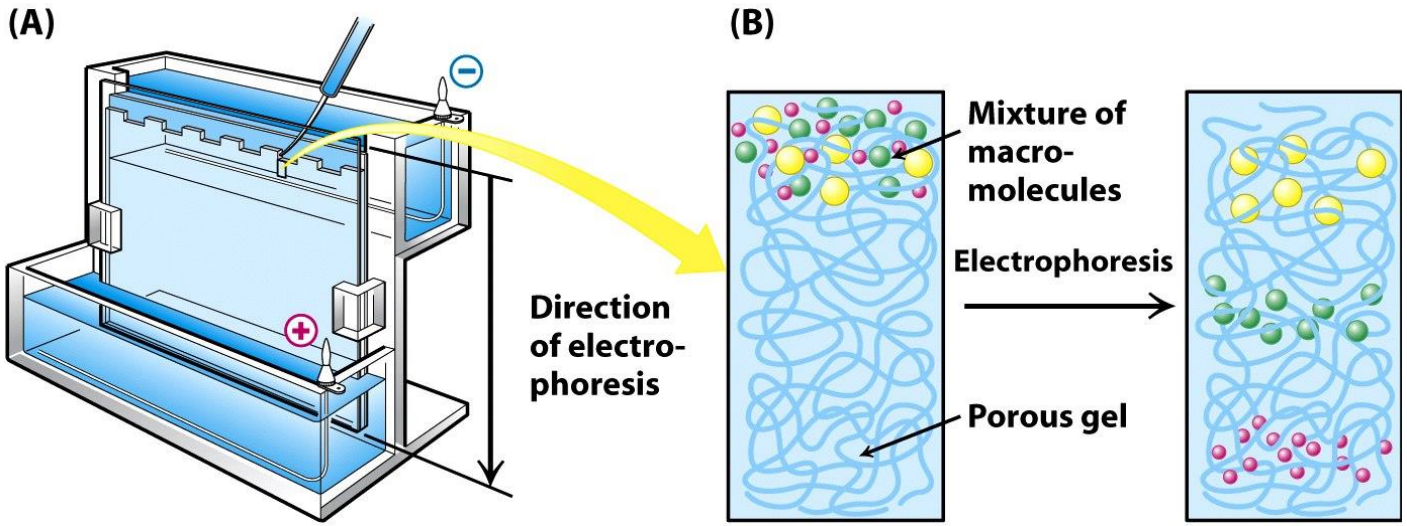
Purpose of reducing agents

- The mixture of proteins is also treated with reducing agents like β-mercaptoethanol or dithiothreitol to reduce disulfide bonds (reducing condition).
  - Otherwise, non-reducing condition



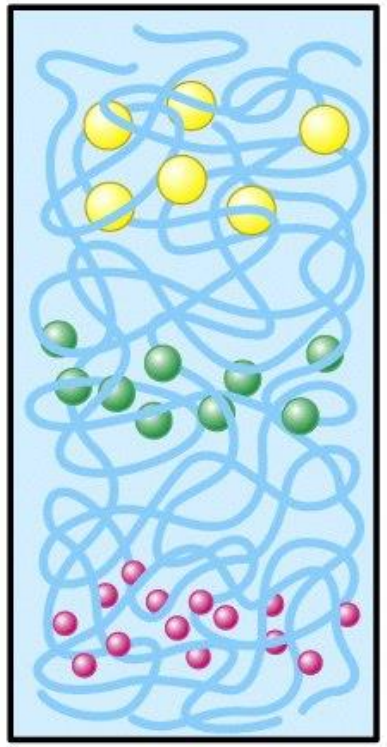
Migration of proteins

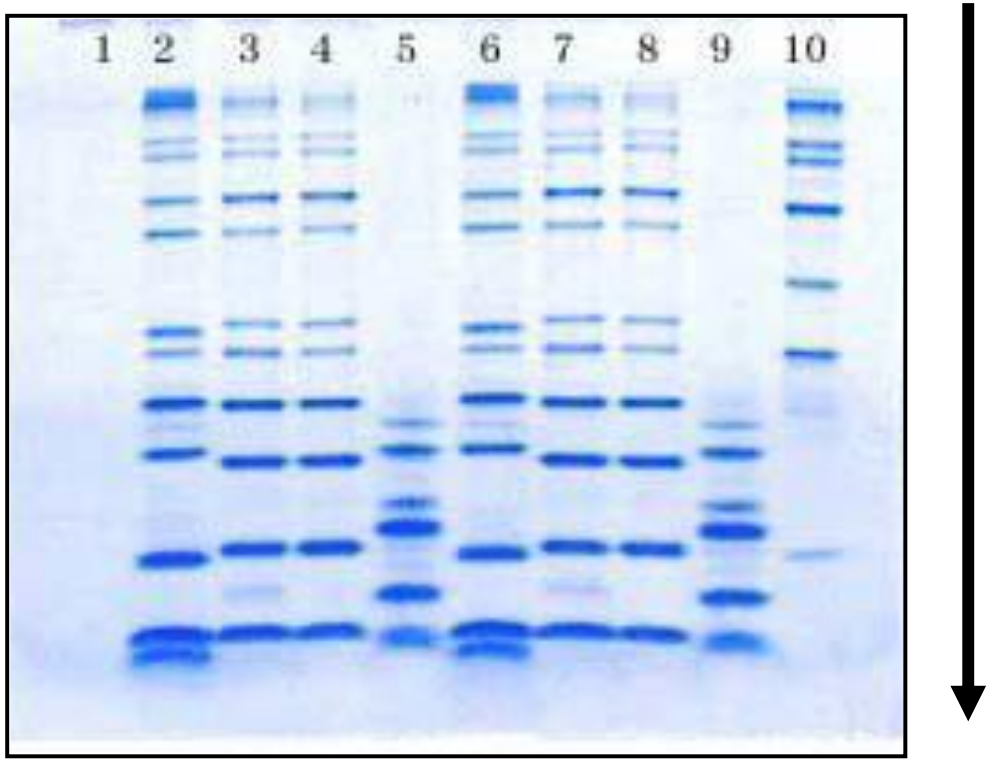
- When an electrical voltage is applied between the upper and lower ends of the gel, all proteins move in one direction towards the anode (positive) according to size only.
- The direction of movement is from top to bottom.
- Whereas smaller molecules move readily through the gel, larger molecules are slower.



Then…

- Once a gel has been "run", the separated proteins are stained to reveal their positions and they appear as bands.

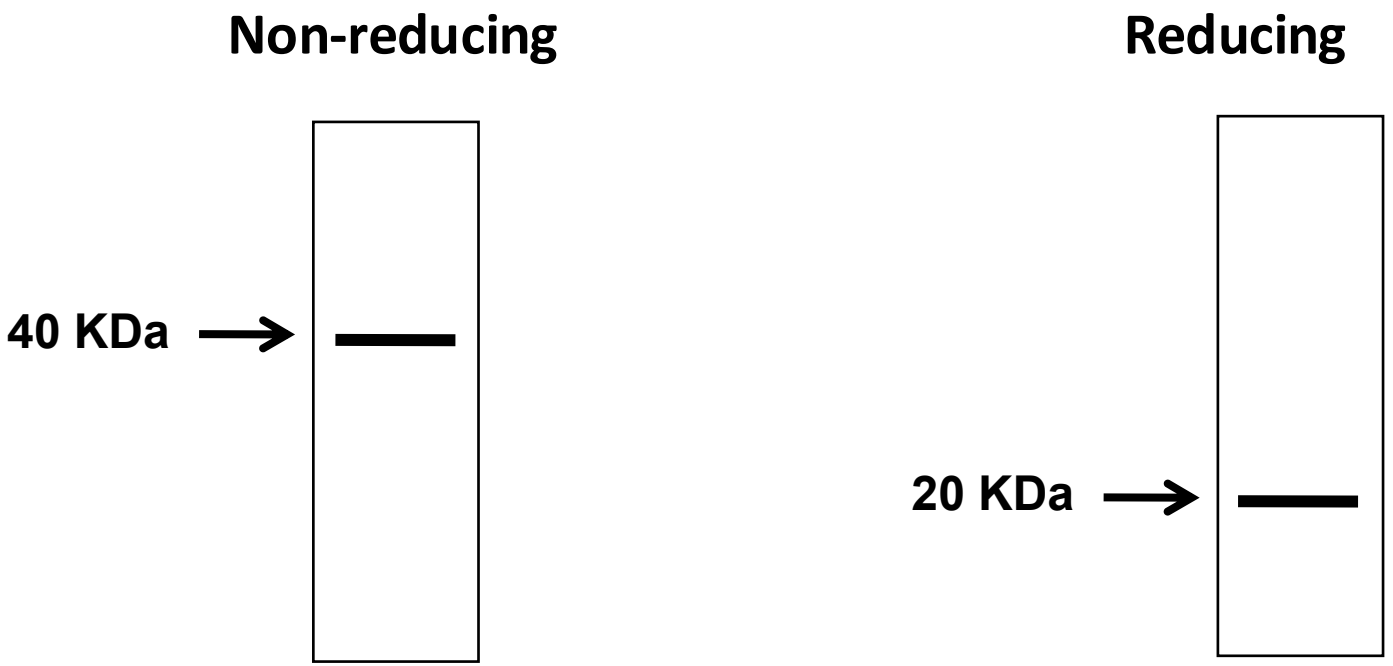




Questions

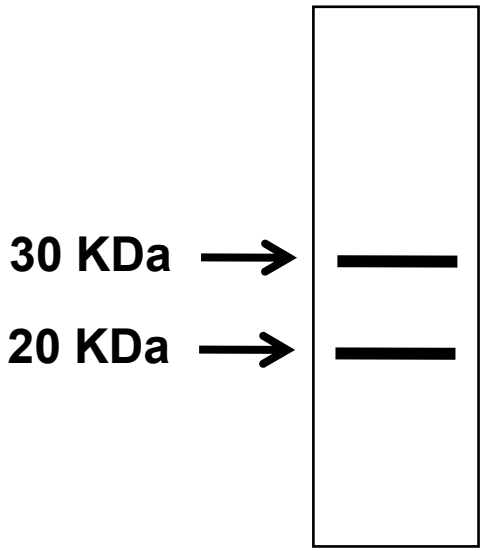
- Describe the protein's structure based on the following results of SDS-PAGE:
  - Under non-reducing conditions, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as a one 20-KDa band.
  - Under non-reducing conditions, a protein exists as two bands, 30 KDa and 20 KDa. Under reducing conditions, the protein also exists as two bands, 15 KDa and 10 KDa.
  - Under non-reducing conditions, a protein exists as two bands, 40 KDa and 20 KDa. Under reducing conditions, the protein exists as a one band of 20 KDa.

Under non-reducing, denaturing conditions, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as a one 20-KDa band.

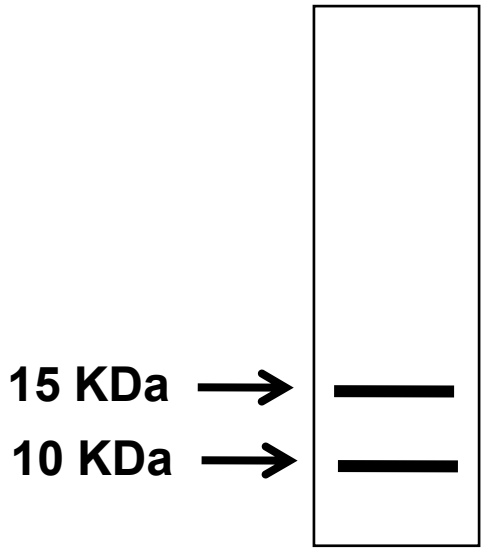


Under non-reducing, denaturing conditions, a protein exists as two bands, 30 KDa and 20 KDa. Under reducing conditions, the protein exists as two bands, 15 KDa and 10 Kda.

Non-reducing

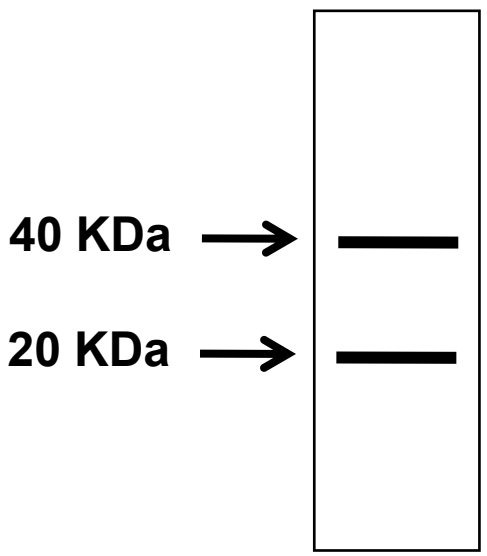


Reducing

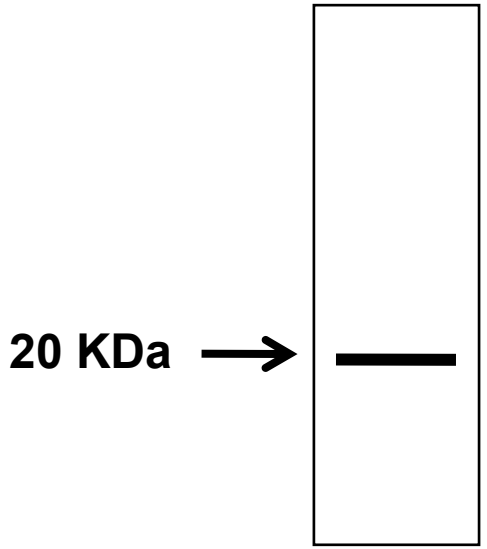


Under non-reducing, denaturing conditions, a protein exists as two bands, 40 KDa and 20 KDa. Under reducing conditions, the protein exists as a one band of 20 KDa.

Non-reducing



Reducing

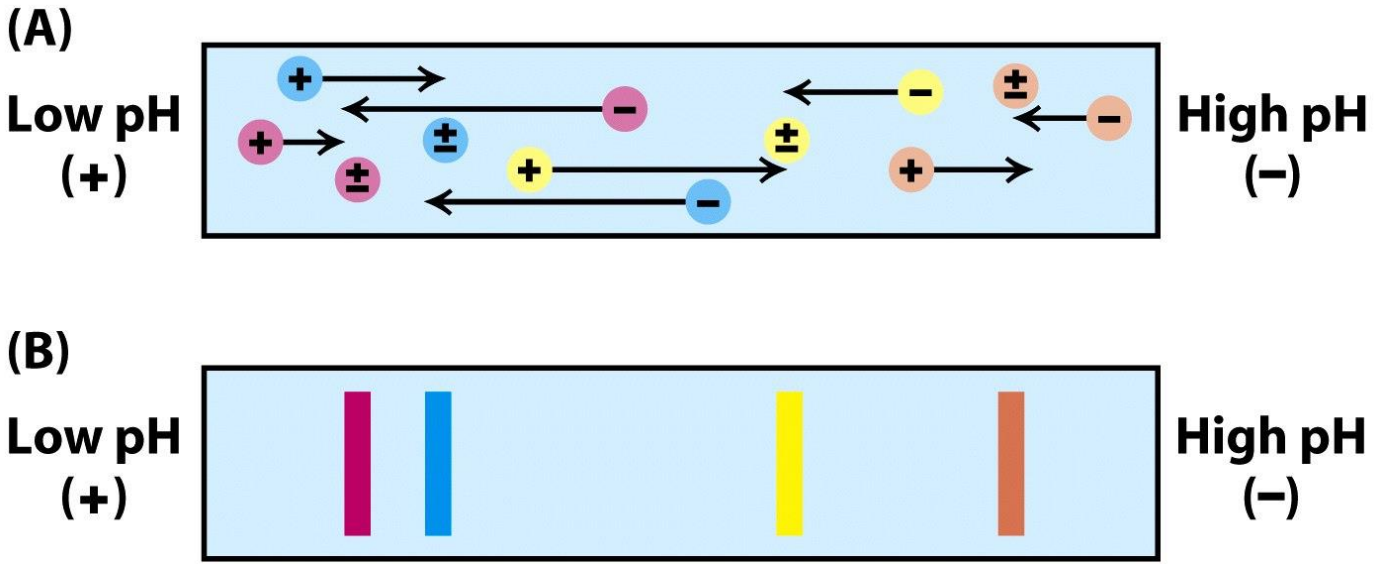


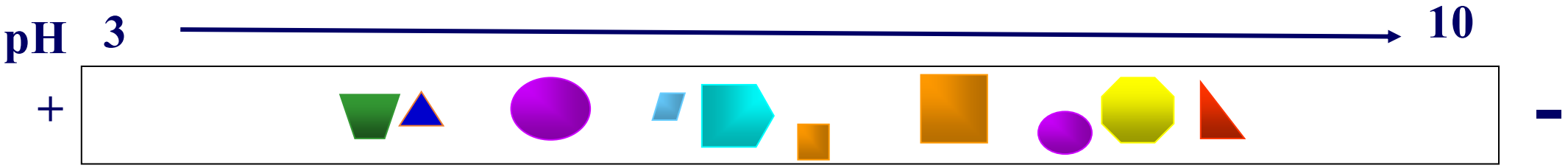
Think!!

- How would the globular proteins that we studied appear in SDS-PAGE gels under reducing and non-reducing conditions?

Isoelectric focusing

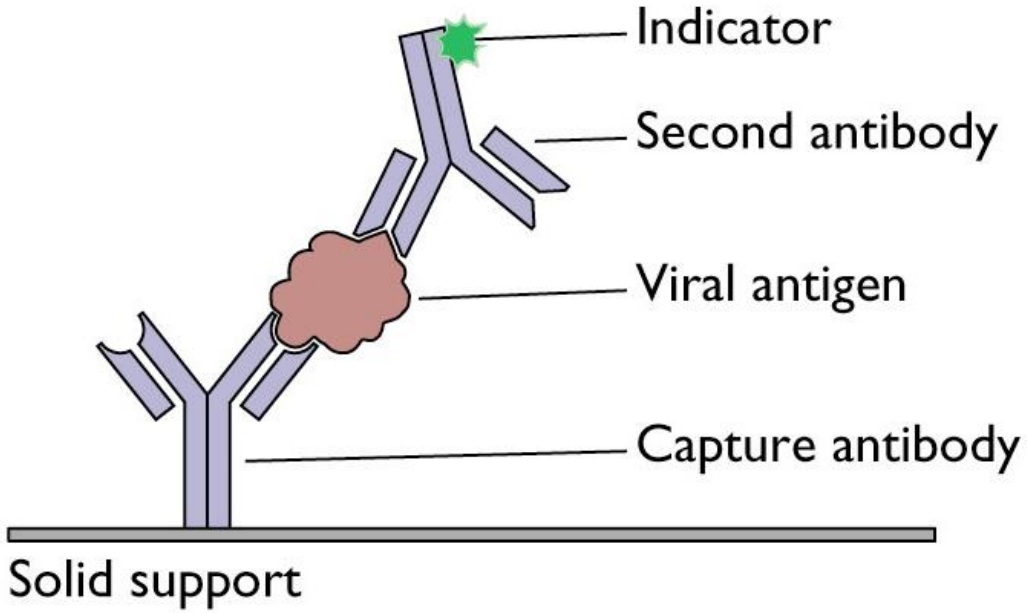
- A gel is prepared with a pH gradient.
- As proteins migrate through the gel, they encounter regions of different pH, so the charge on the protein changes.
- Eventually each protein reaches the point at which it has no net charge—its isoelectric point—and no longer migrates.
- Each protein remains at the position on the gel corresponding to its pI, allowing for separation of proteins.



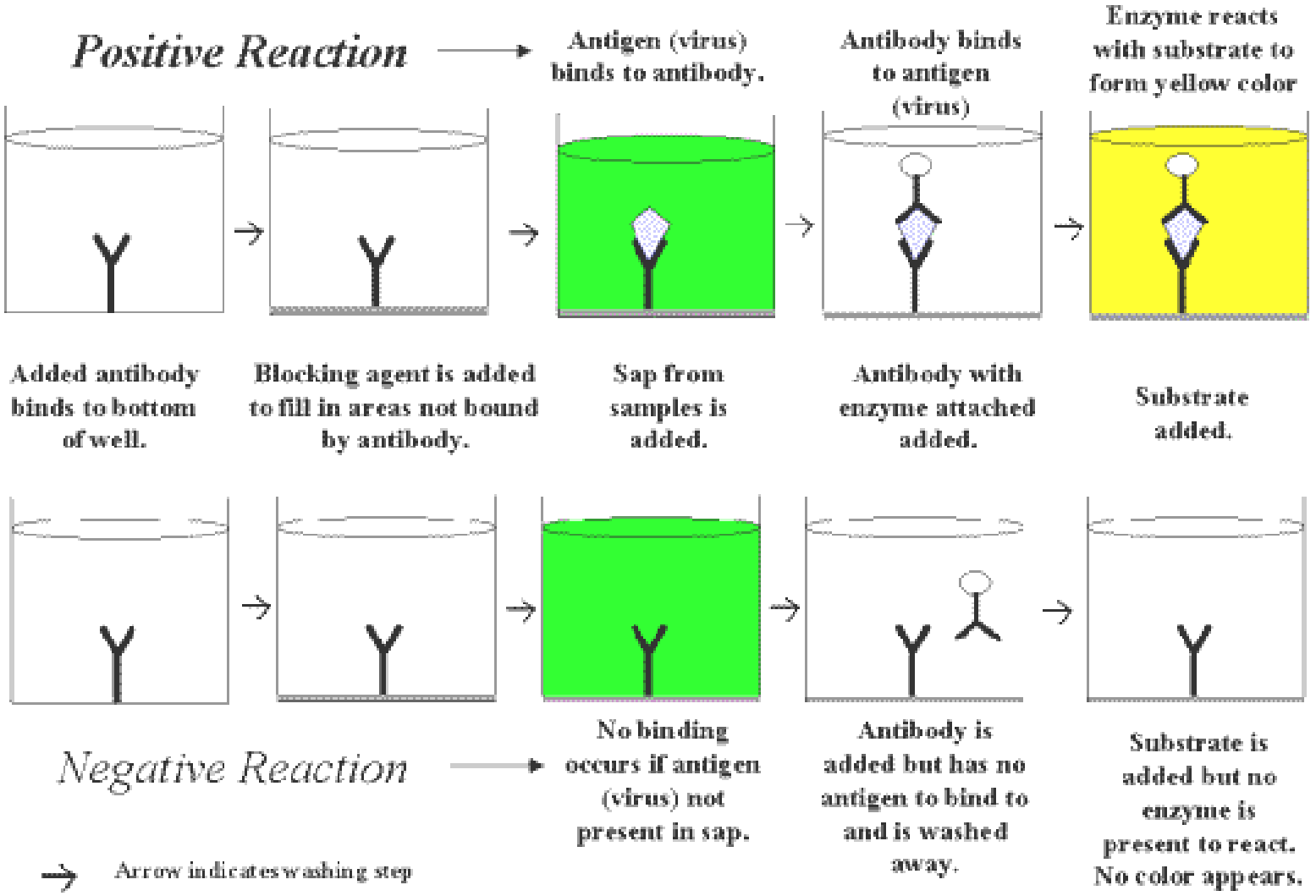

pH
3
10
+
-

ELISA

- Enzyme-linked immunosorbent assay
- Same concept as immunoblotting but rapid, convenient, and sensitive (less than nanogram (10-9 g) of a protein)
- http://www.genscript.com/gsfiles/flash/protein_a_elisa_protocol.swf



ELISA

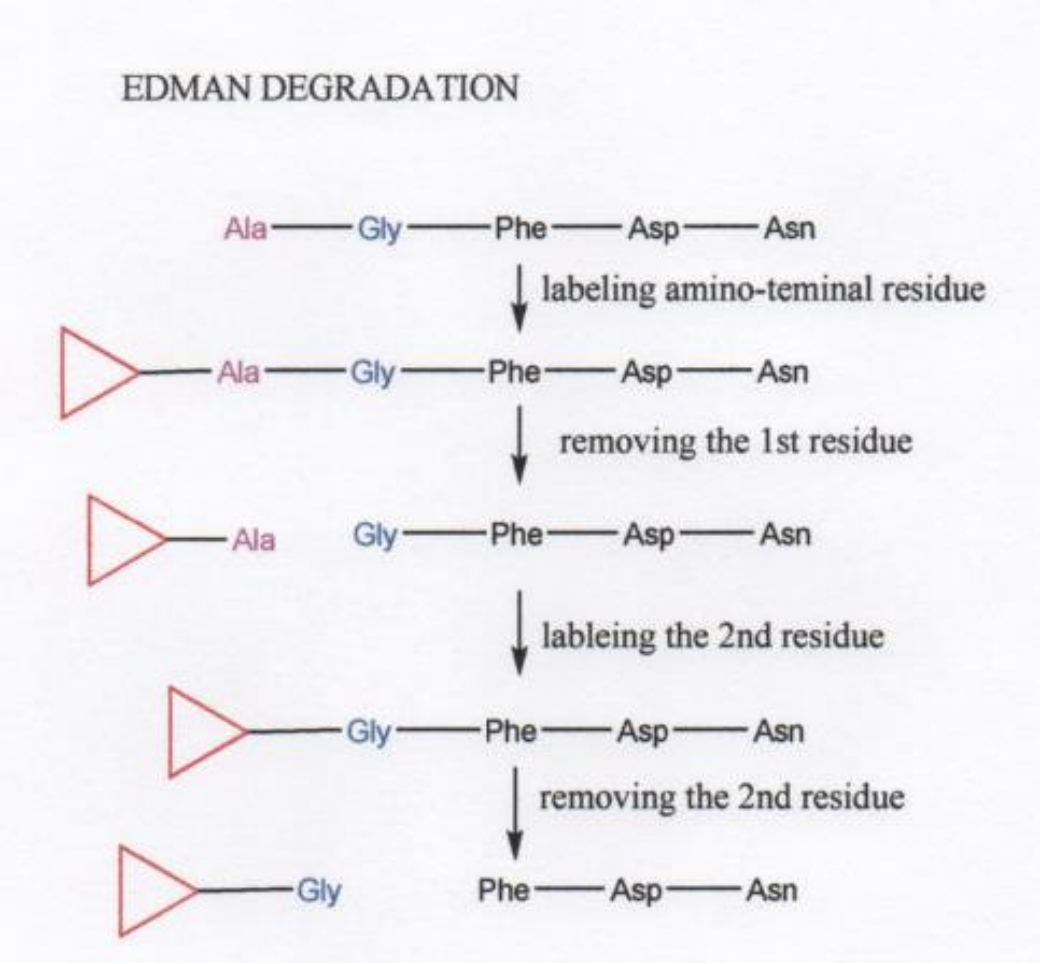

Positive Reaction
Antigen (virus) binds to antibody.
Antibody binds to antigen (virus)
Enzyme reacts with substrate to form yellow color
Added antibody binds to bottom of well.
Blocking agent is added to fill in areas not bound by antibody.
Sap from samples is added.
Antibody with enzyme attached added.
Substrate added.
Negative Reaction
No binding occurs if antigen (virus) not present in sap.
Antibody is added but has no antigen to bind to and is washed away.
Substrate is added but no enzyme is present to react. No color appears.
Arrow indicates washing step

Protein sequencing

- Protein sequencing is basically the process of knowing the amino sequence of a protein or a peptide.
- One technique is known as Edman Degradation.
- This procedure involves a step-by-step cleavage of the N-terminal residue of a peptide, allowing for the identification of each cleaved residue.

Procedure

- This method utilizes phenylisothiocyanate (PITC) to react with the N-terminal residue.
- The resultant amino acid is hydrolyzed, liberated from the peptide, and identified by chromatographic procedures.



Advantage

- Since the remainder of the peptide is intact, the entire sequence of reactions can be repeated over and over to obtain the sequences of the peptide.

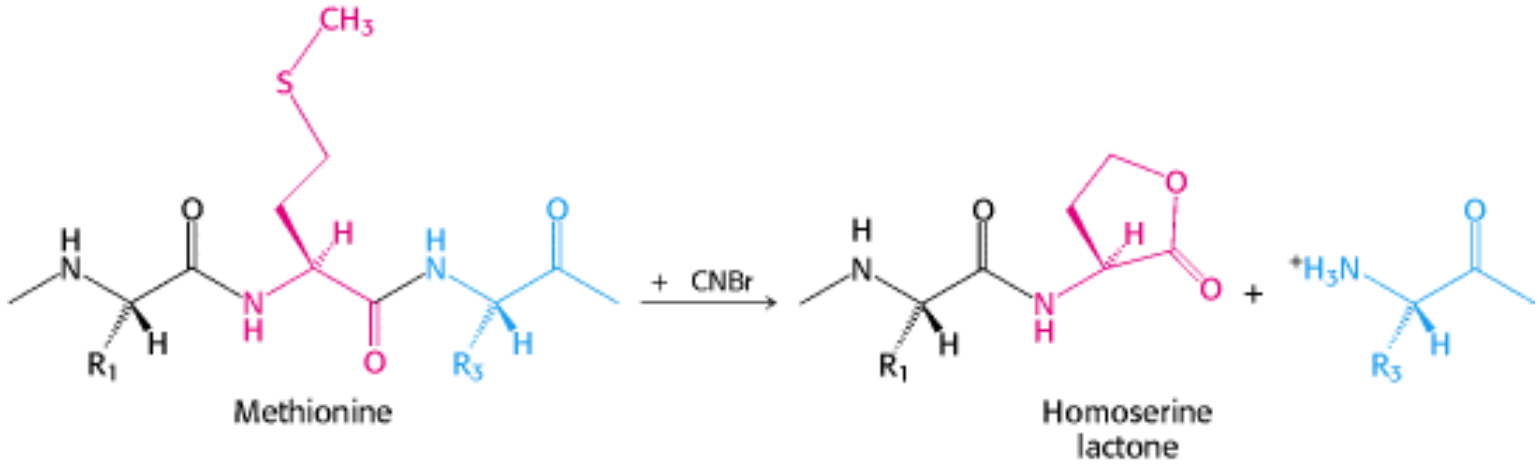
- The Edman degradation technique does not allow peptides more than 50 residues to be sequenced.

Cleavage methods

- It is possible to sequence whole proteins by cleaving them into smaller peptides.
- This is facilitated by three methods:
  - Chemical digestion
  - Endopeptidases
  - Exopeptidases

Chemical digestion

- The most commonly utilized chemical reagent that cleaves peptide bonds by recognition of specific amino acid residues is cyanogen bromide (CNBr).
- This reagent causes specific cleavage at the C-terminal side of methionine residues.
- A protein that has 10 methionine residues will usually yield 11 peptides on cleavage with CNBr.



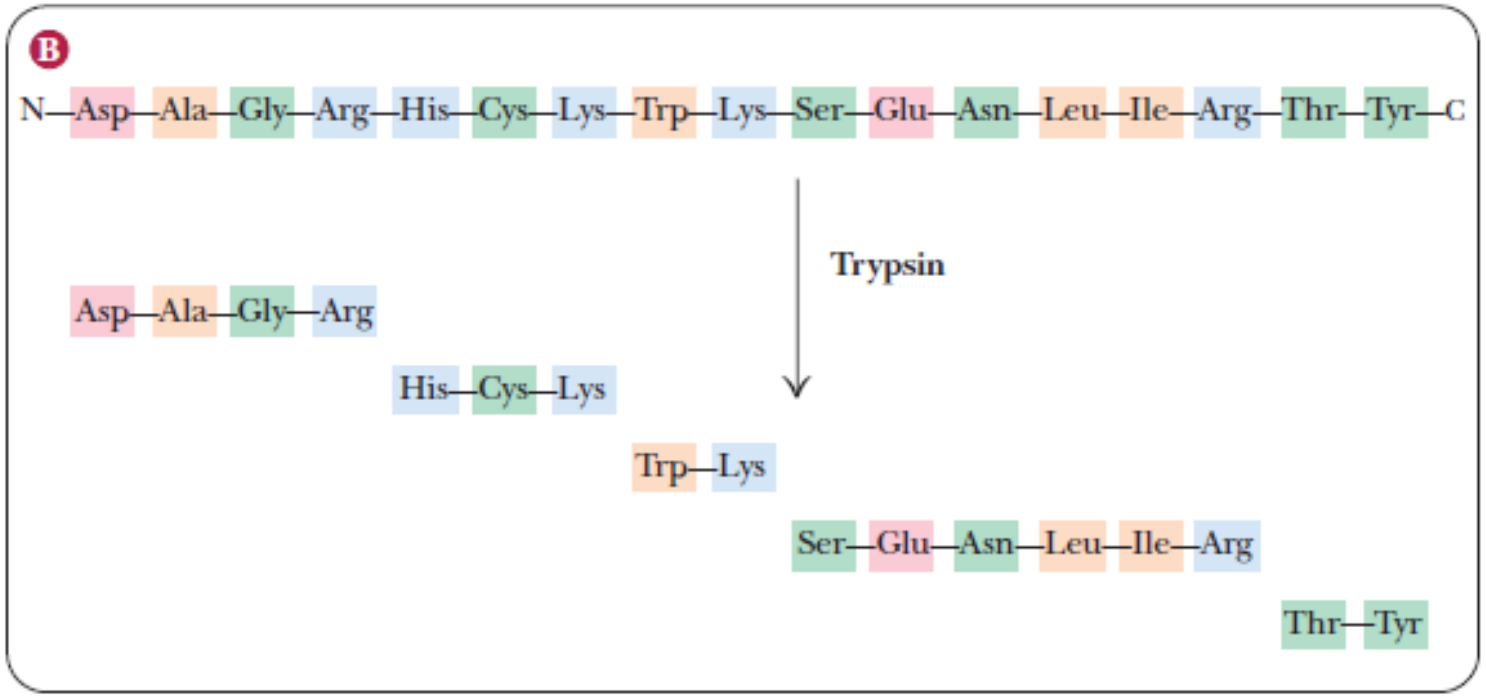
Endopeptidases

- These are enzymes that cleave at specific sites within the primary sequence of proteins.
- The resultant smaller peptides can be chromatographically separated and subjected to Edman degradation sequencing reactions.

Example

- Trypsin cleaves polypeptide chains on the carboxyl side of arginine and lysine residues.
- A protein that contains 9 lysine and 7 arginine residues will usually yield 17 peptides on digestion with trypsin.

Another example



Other examples

Enzyme	Specificity
Trypsin	peptide bond C-terminal to Arg or Lys, but not if next to Pro
Chymotrypsin	peptide bond C-terminal to Phe, Tyr, or Trp, but not if next to Pro
Elastase	peptide bond C-terminal to Ala, Gly, Ser, or Val, but not if next to Pro
Pepsin	peptide bond N-terminal to Leu, Phe, Trp, or Tyr, but not if it follows Pro

Exopeptidases

- These are enzymes that cleave amino acids starting at the end of the peptide.
- There are two types:
  - Aminopeptidases that cleave at the N-terminus
  - Carboxypeptidases that cleave at the C-terminus

Homework

Chymotrypsin	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe
Cyanogen bromide	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe—His—Met
Chymotrypsin	His—Met—Thr—Met—Ala—Trp
Cyanogen bromide	Thr—Met
Cyanogen bromide	Ala—Trp—Val—Lys—COO^-
Chymotrypsin	Val—Lys—COO^-
Overall sequence	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe—His—Met—Thr—Met—Ala—Trp—Val—Lys—COO^-

A sample of an unknown peptide was divided into two aliquots. One aliquot was treated with trypsin; the other was treated with cyanogen bromide. Given the following sequences (N-terminal to C-terminal) of the resulting fragments, deduce the sequence of the original peptide.

Trypsin treatment

Asn—Thr—Trp—Met—Ile—Lys

Gly—Tyr—Met—Gln—Phe

Val—Leu—Gly—Met—Ser—Arg

Cyanogen bromide treatment

Gln—Phe

Val—Leu—Gly—Met

Ile—Lys—Gly—Tyr—Met

Ser—Arg—Asn—Thr—Trp—Met

A sample of a peptide of unknown sequence was treated with trypsin; another sample of the same peptide was treated with chymotrypsin. The sequences (N-terminal to C-terminal) of the smaller peptides produced by trypsin digestion were as follows:

Met—Val—Ser—Thr—Lys

Val—Ile—Trp—Thr—Leu—Met—Ile

Leu—Phe—Asn—Glu—Ser—Arg

The sequences of the smaller peptides produced by chymotrypsin digestion were as follows:

Asn—Glu—Ser—Arg—Val—Ile—Trp

Thr—Leu—Met—Ile

Met—Val—Ser—Thr—Lys—Leu—Phe

Deduce the sequence of the original peptide.