



Protein analysis

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Resources

This lecture

Campbell and Farrell's Biochemistry, Chapters 5

Bases of protein separation

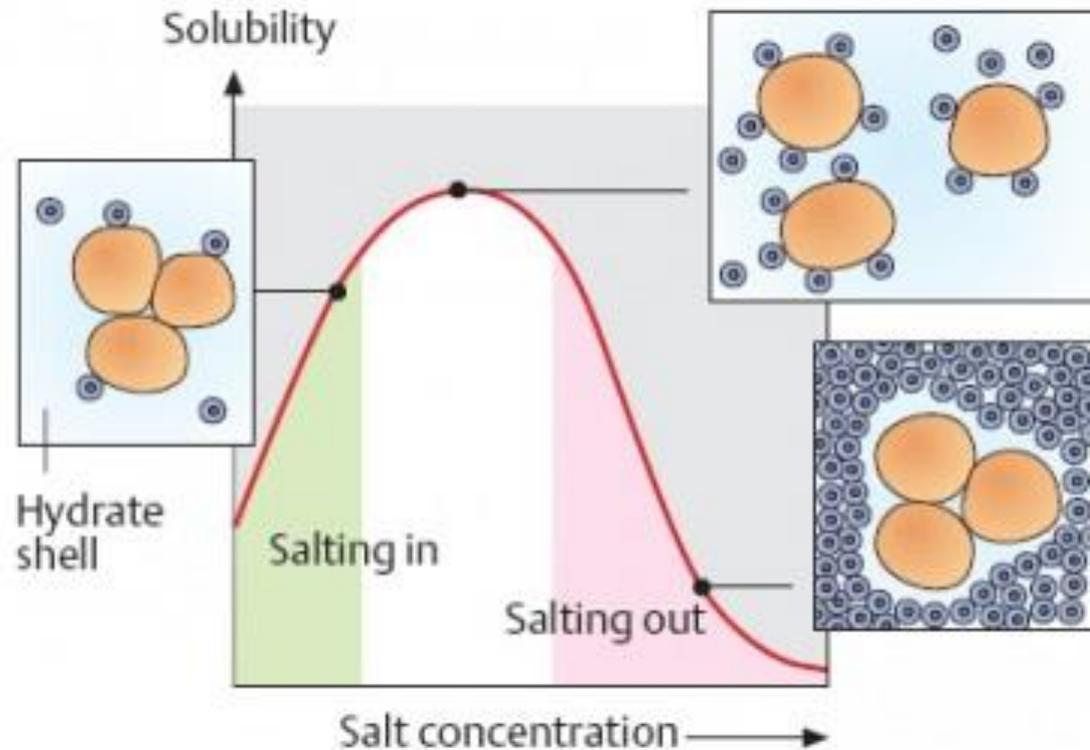


- Proteins can be purified on the basis
 - Solubility
 - Size
 - Charge
 - specific binding affinity

Salting in...salting out



- Proteins are poorly soluble in pure water, but their solubility increases as the ionic strength increases.
 - salting in
- At very high concentrations of salt, most proteins become less soluble.
 - salting out
- Why?



Why?

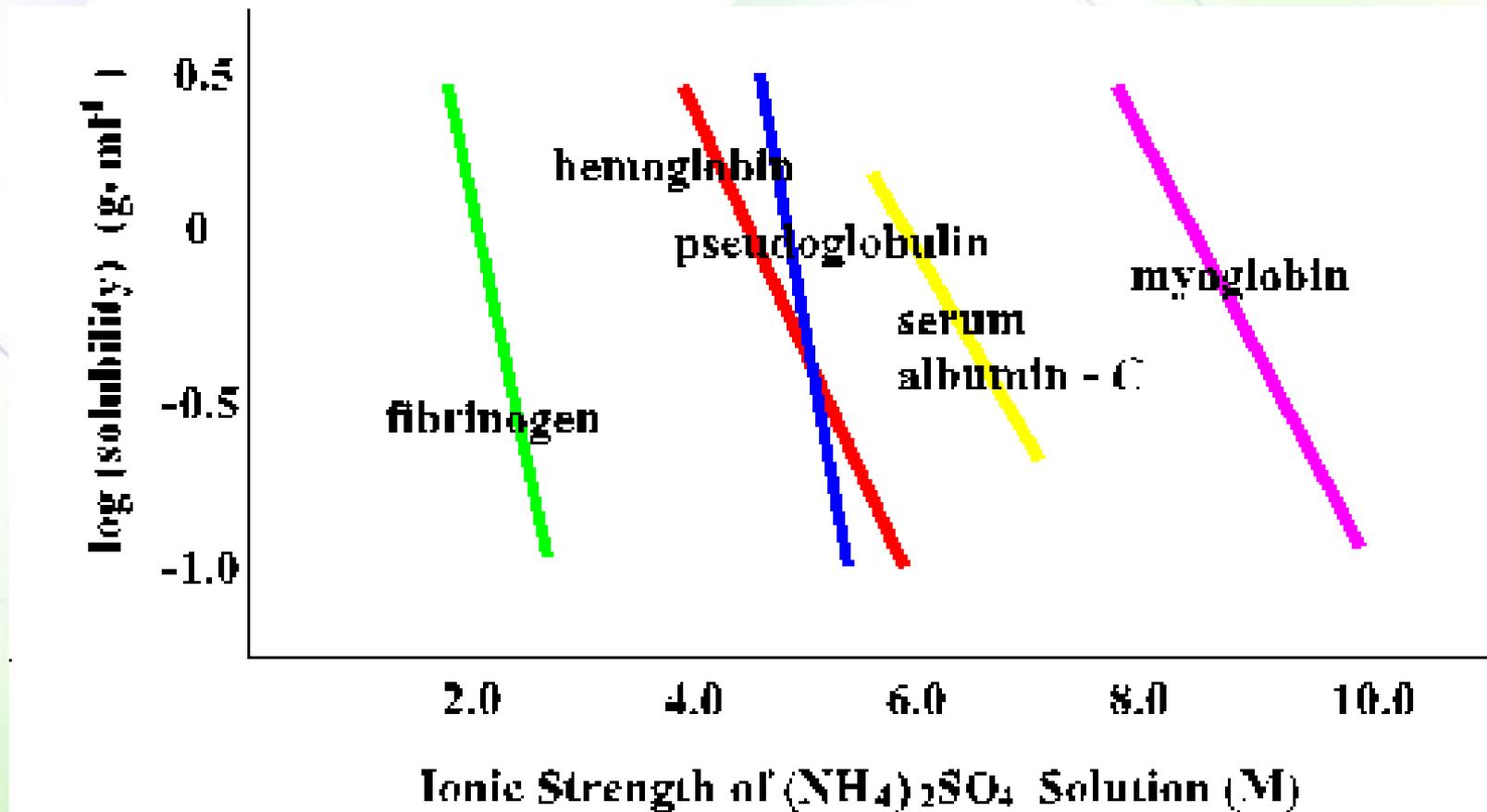


- When salt, like ammonium sulfate, is added to a protein solution, some of the water is taken away from the protein to make ion–dipole bonds with the ions.
- With less water available to hydrate the proteins, they begin to interact with each other through hydrophobic bonds.
- At a defined amount of ammonium sulfate, a precipitate that contains some proteins forms. These proteins can then be separated.
- When more salt is added, a different set of proteins, precipitates.
- And so on.

Uses of salting out



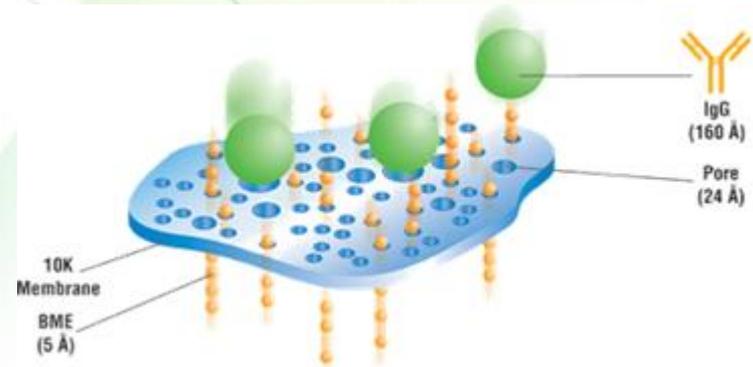
- Salting out can be used to fractionate proteins



Dialysis

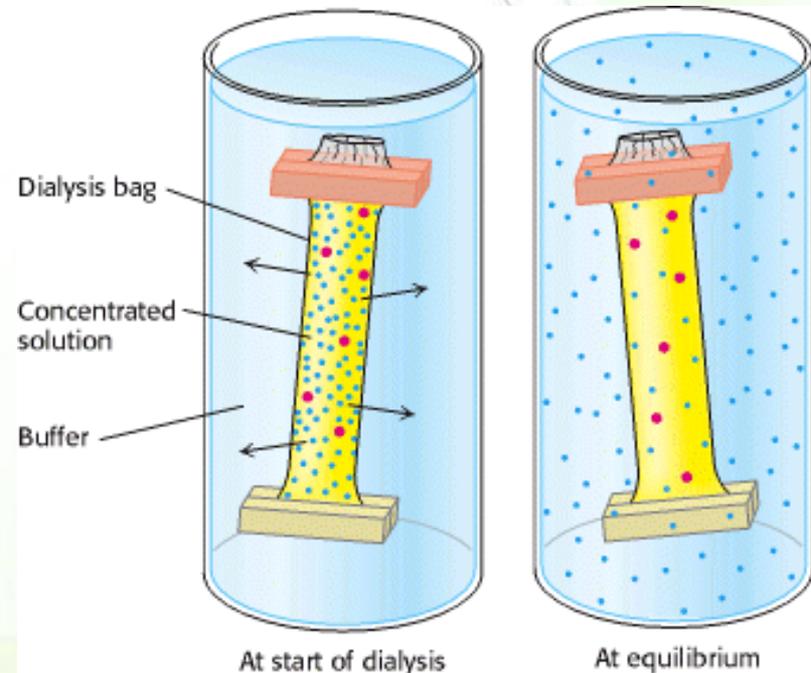


- Proteins can be separated from small molecules by dialysis through a semi-permeable membrane.



Disadvantage of dialysis

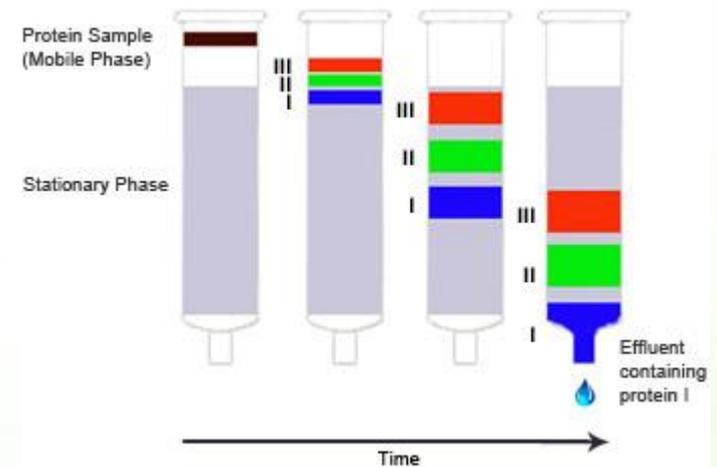
1. Large number of larger proteins will still exist.
2. Smaller proteins of significance are lost.



Chromatography techniques



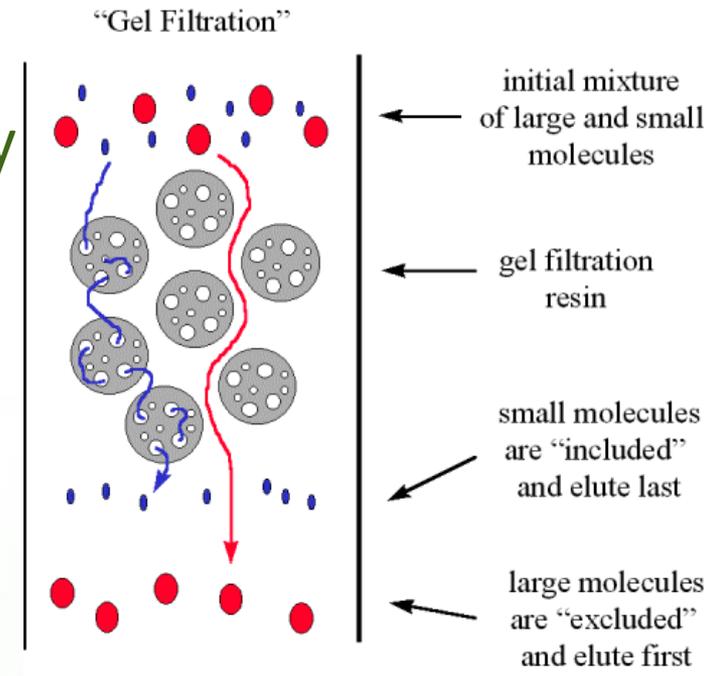
- Separation of molecules present in a liquid or gaseous environment (**mobile phase**) via passing through a column (**long tube**) that contains an immobile phase (**stationary phase**)
- Types:
 - Gel filtration
 - Ion-exchange chromatography
 - Affinity chromatography

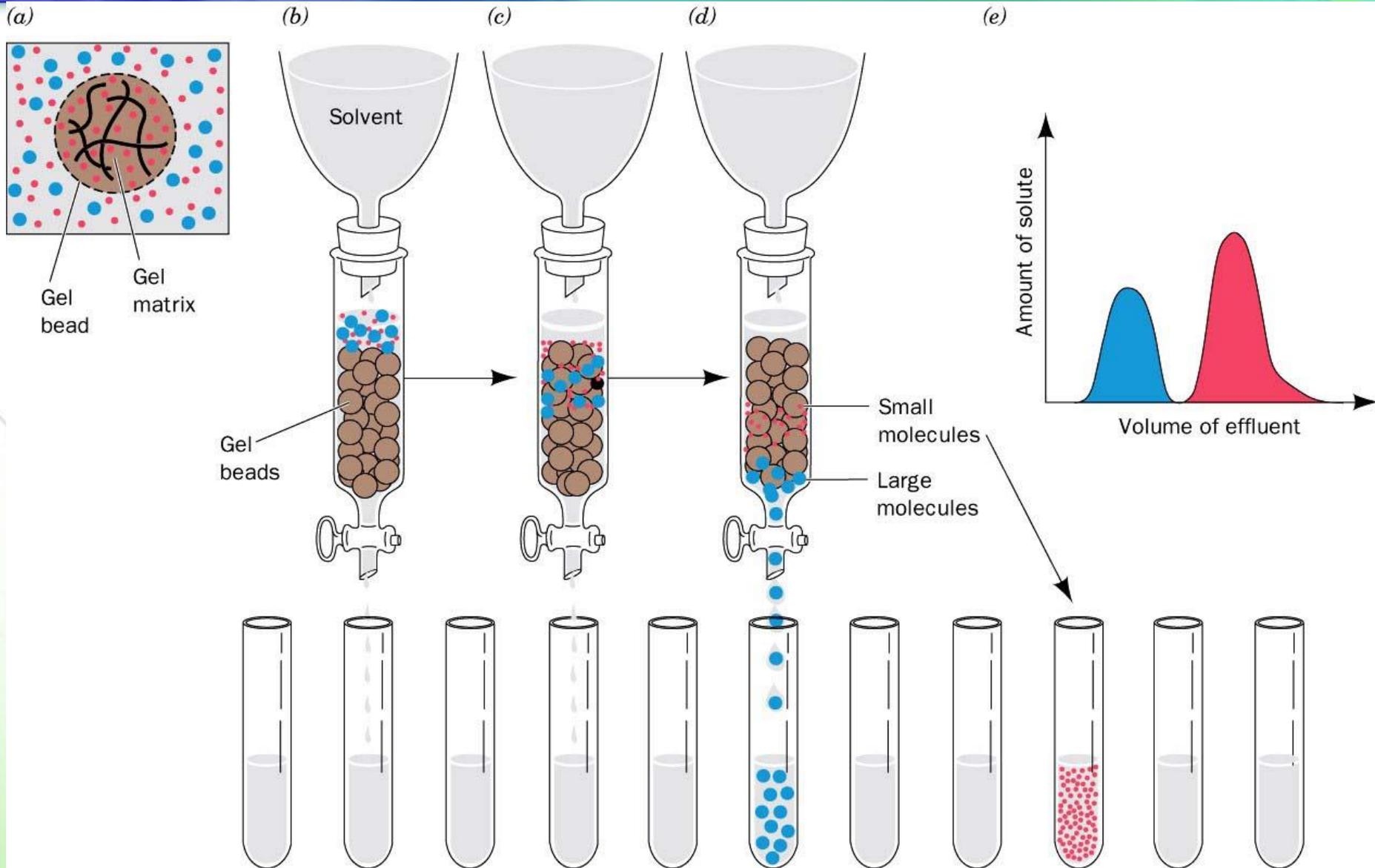


Gel-filtration chromatography



- Separations on the basis of size
 - Size-exclusion chromatography
 - Molecular sieve chromatography
- The stationary phase: porous beads
- Large molecules flow more rapidly and come out first
- Smaller molecules take a longer time in the column and exit late

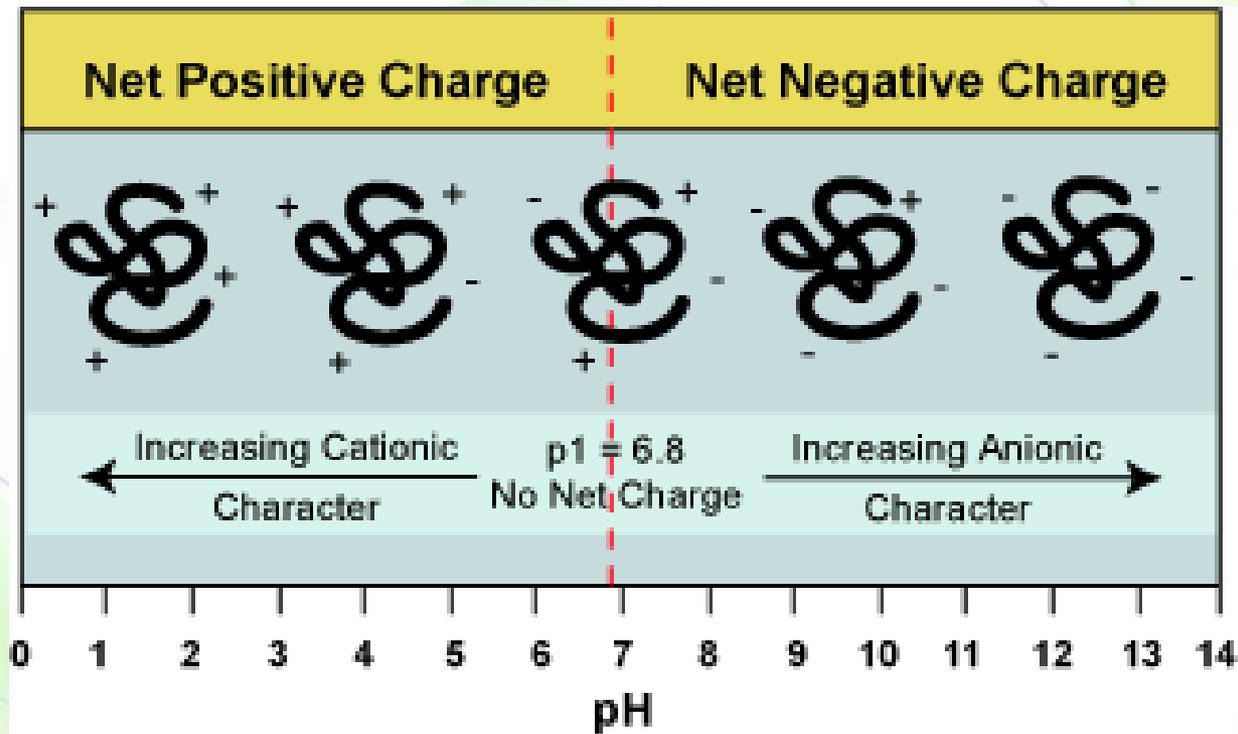




Ion-Exchange Chromatography



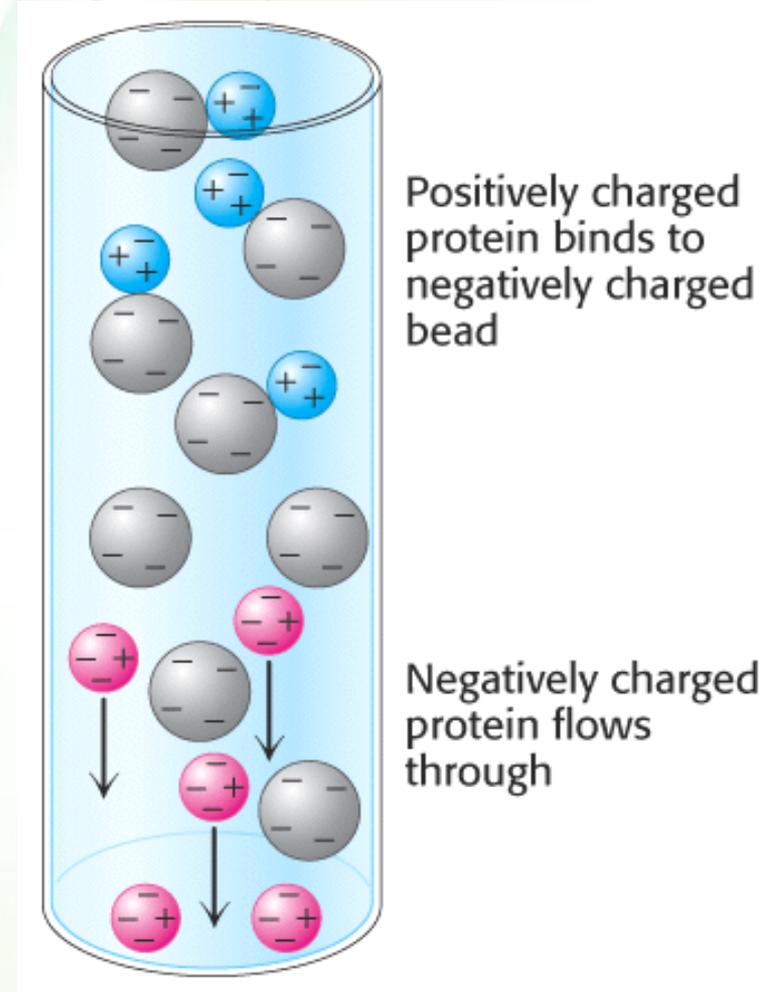
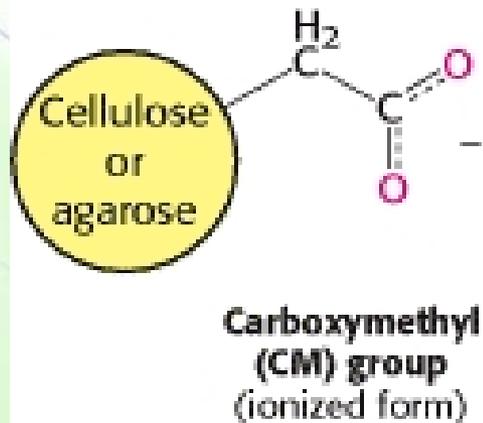
- Proteins have different pI 's (and net charges at various pH 's).



Cationic-exchange chromatography



- The beads are negatively-charged.
- Proteins that have a net positive charge will tend to emerge first, followed by those having a higher positive charge density.



Elution

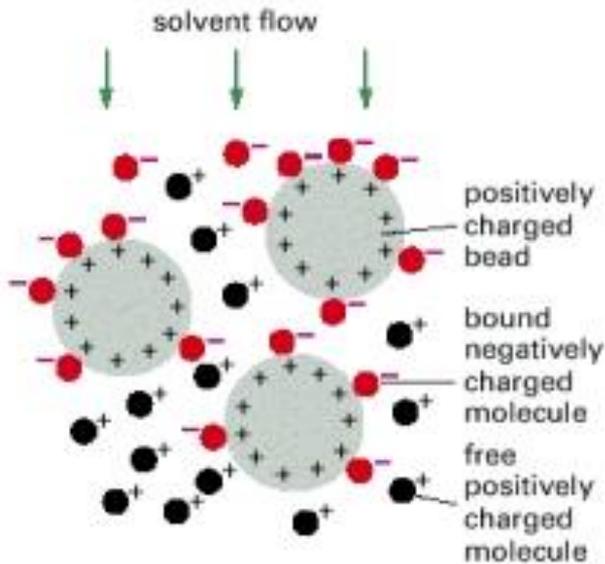


- A positively charged protein bound to such a column can then be eluted (released) by adding increasing concentrations of sodium chloride. Why?
 - Because sodium ions compete with positively charged groups on the protein for binding to the column.

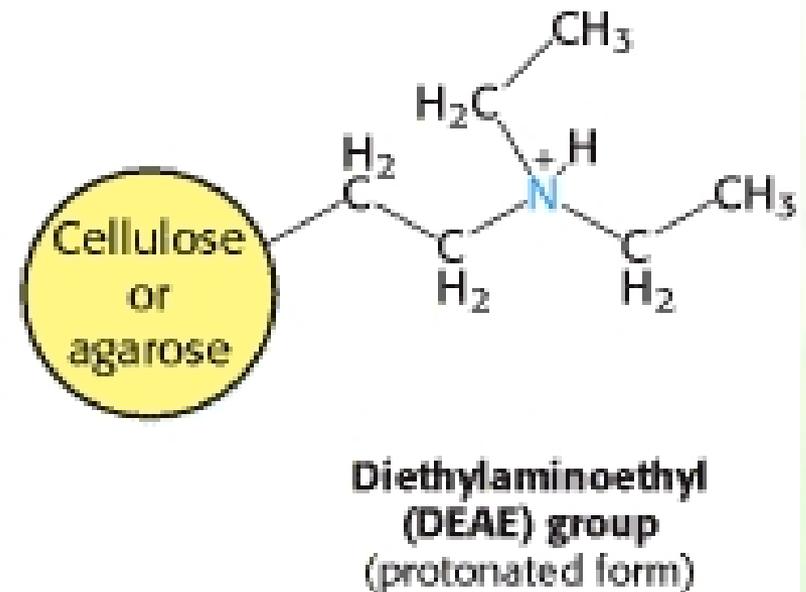
Anionic-exchange chromatography



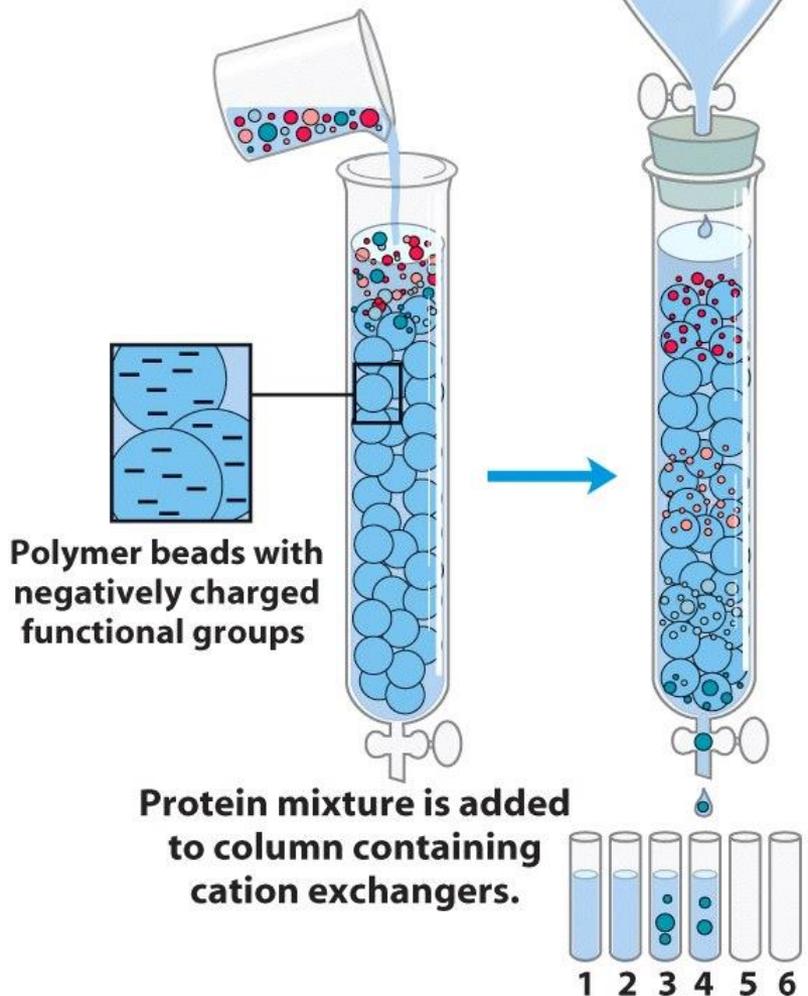
- The beads are positively-charged.
 - Diethylaminoethyl-cellulose column
- Negatively charged proteins are separated.



(A) ION-EXCHANGE CHROMATOGRAPHY



- Large net positive charge
- Net positive charge
- Net negative charge
- Large net negative charge



Polymer beads with negatively charged functional groups

Protein mixture is added to column containing cation exchangers.

Proteins move through the column at rates determined by their net charge at the pH being used. With cation exchangers, proteins with a more negative net charge move faster and elute earlier.

Problem

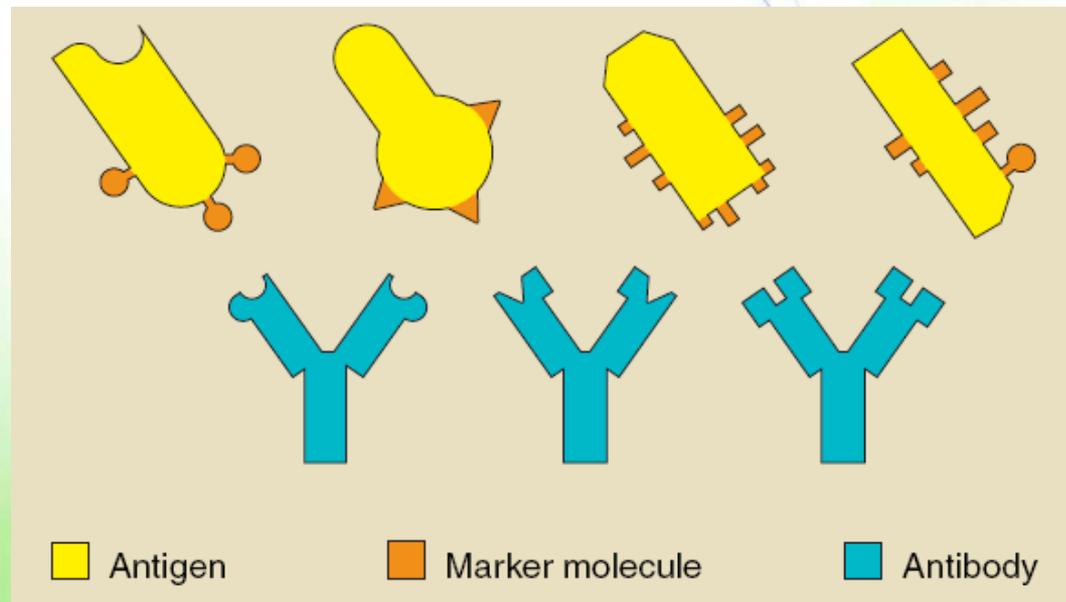
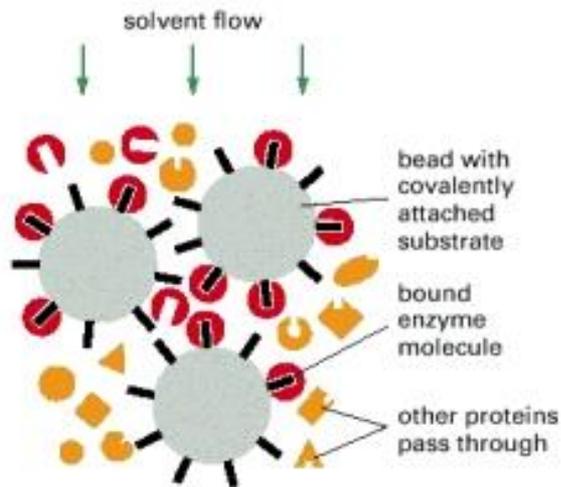


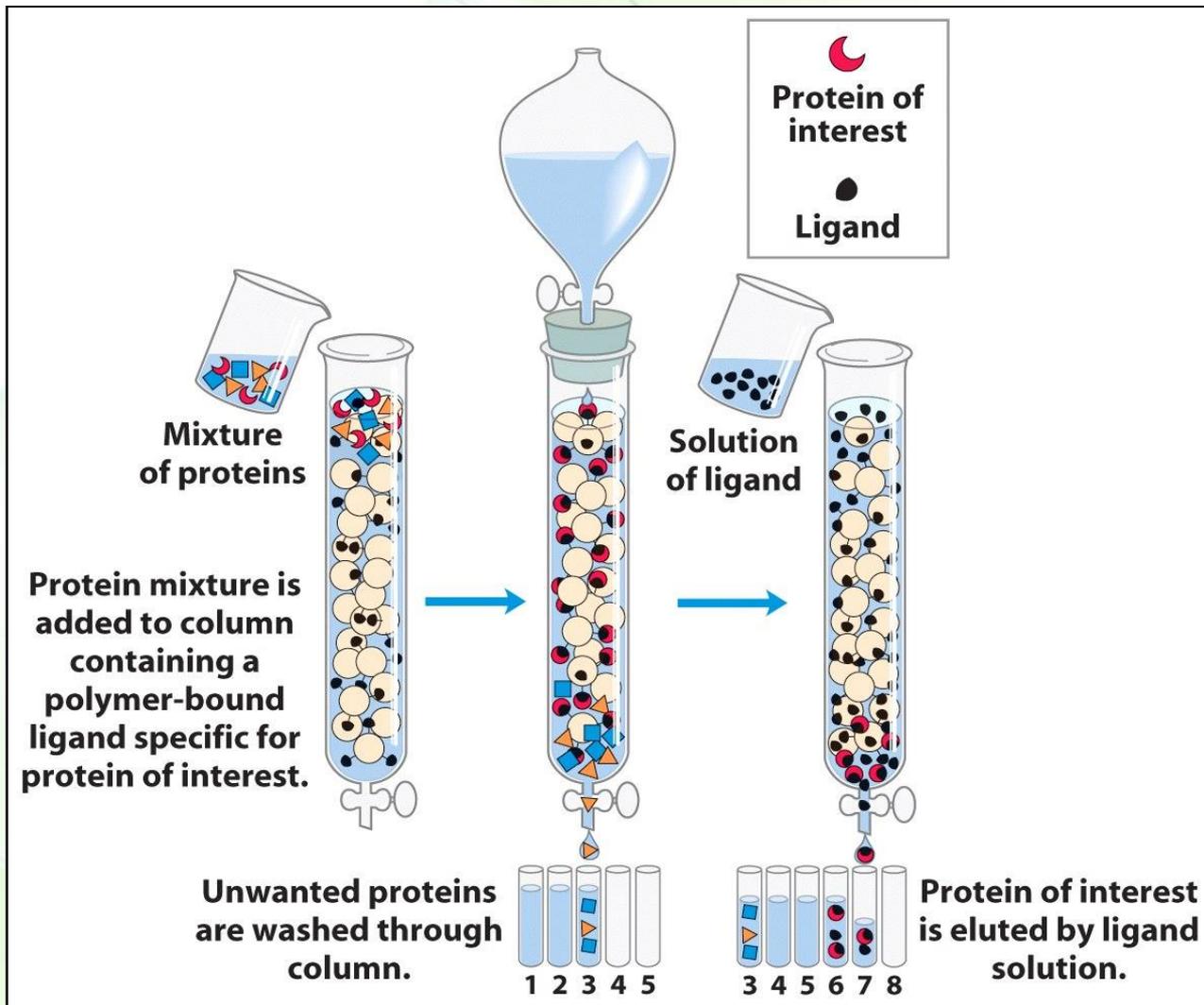
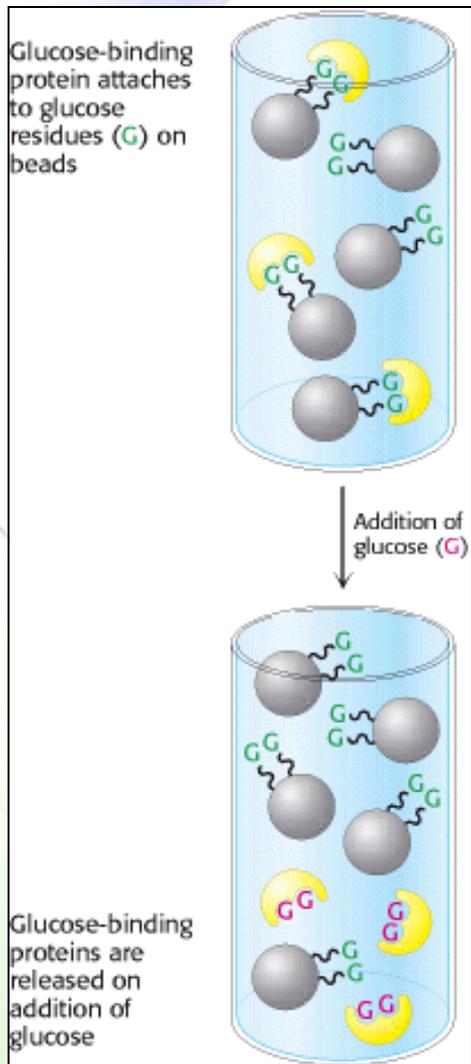
- You have 5 different proteins (#1, #2, #3, #4, and #5), with different isoelectric points (pI's).
 - pI#5 = 2.3
 - pI#4 = 4.7
 - pI#1 = 7.2
 - pI#2 = 9.1
 - pI#3 = 12.1
- Starting the column at pH 6.5, the sample is added and then washed to remove unbound molecules. What is the order of protein elution in a
 - Cationic-exchange chromatography?
 - An anionic exchange chromatography?

Affinity Chromatography



- Affinity: strength of binding between two molecules
- Affinity chromatography takes advantage of the high affinity of many proteins for specific chemical groups or other proteins (antibodies).
- Affinity chromatography is most effective when the interaction of the protein and the molecule that is used as the bait (trap) is highly specific.



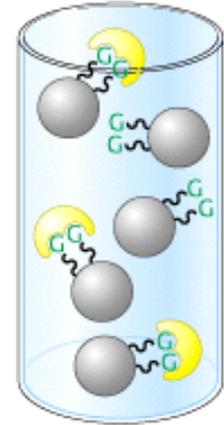


Example



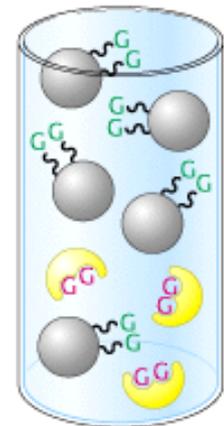
- The plant protein concanavalin A, which binds to glucose with high affinity, can be purified by passing a protein mixture through a column of beads attached to glucose residues.
- Concanavalin A, but not other proteins, binds to the beads.
- The bound concanavalin A can then be released by adding a concentrated solution of glucose.

Glucose-binding protein attaches to glucose residues (G) on beads



Addition of glucose (G)

Glucose-binding proteins are released on addition of glucose



What kind of proteins would a concanavalin A-linked bead separate?

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.

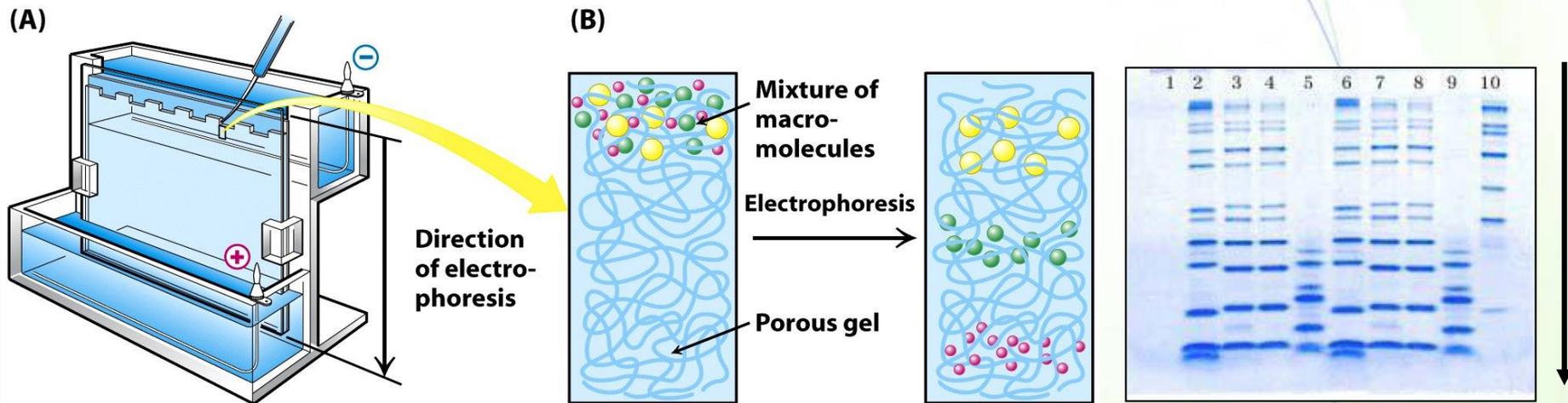
<http://course1.winona.edu/sberg/ANIMTNS/FlashAnimations/SDSgel/sdsGelAdvanced.swf>

http://sdspagestudy.blogspot.com/2009_10_01_archive.html

The process



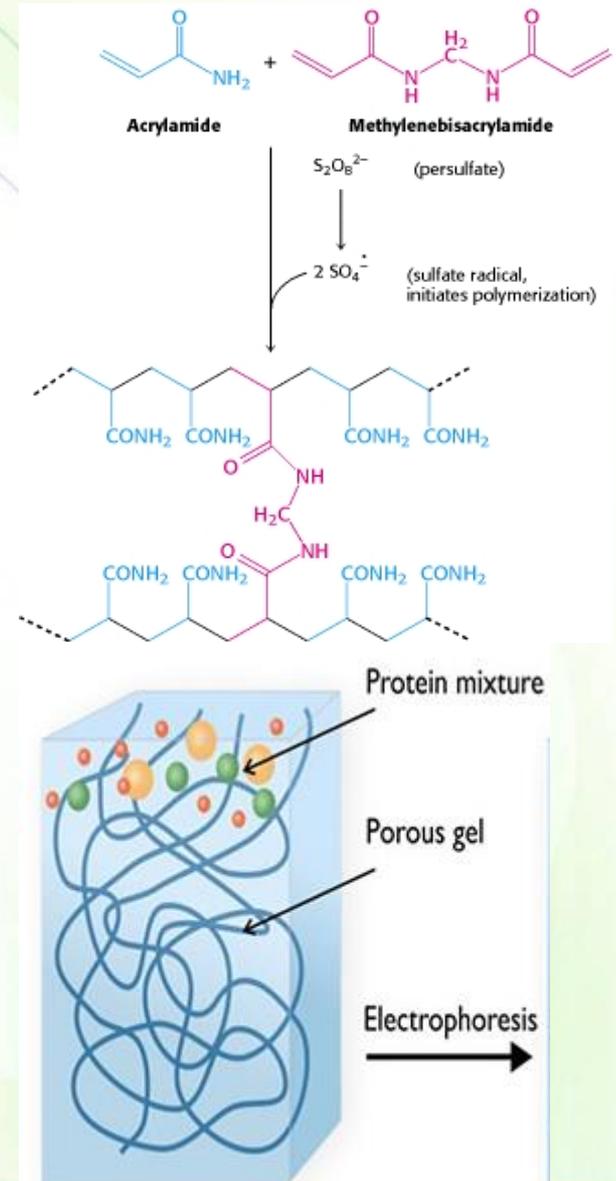
- The most commonly used protein electrophoresis technique is termed SDS polyacrylamide gel electrophoresis (SDS-PAGE).
- It is performed in a thin, vertical gel.
- The top of the gel consists of wells onto which samples are loaded.



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 methylenebisacrylamide.



Purpose of SDS

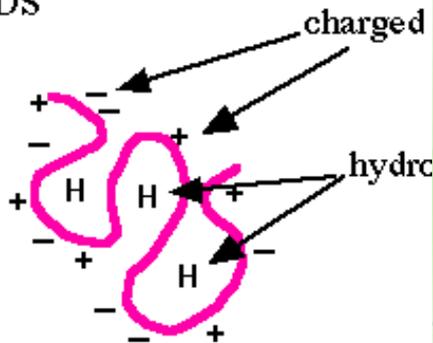


- This technique utilizes a negatively charged detergent (sodium dodecyl sulfate) to denature and solubilize proteins (**denaturing condition**).
 - Otherwise, non-denaturing condition or native condition where proteins maintain their original structure and shape and are separated based on charge, size,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



Sodium dodecyl sulfate (SDS)

BEFORE SDS



AFTER SDS

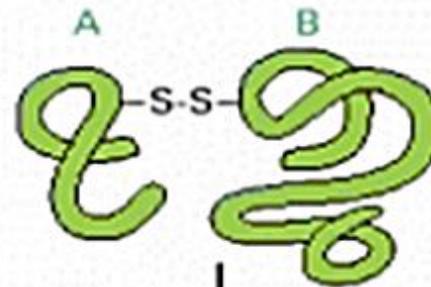


Purpose of reducing agents



- 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

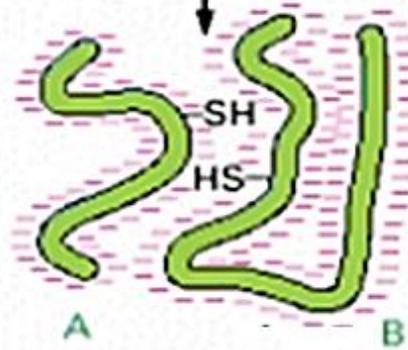
protein with two subunits, A and B, joined by a disulfide bridge



single subunit protein



HEATED WITH SDS AND MERCAPTOETHANOL



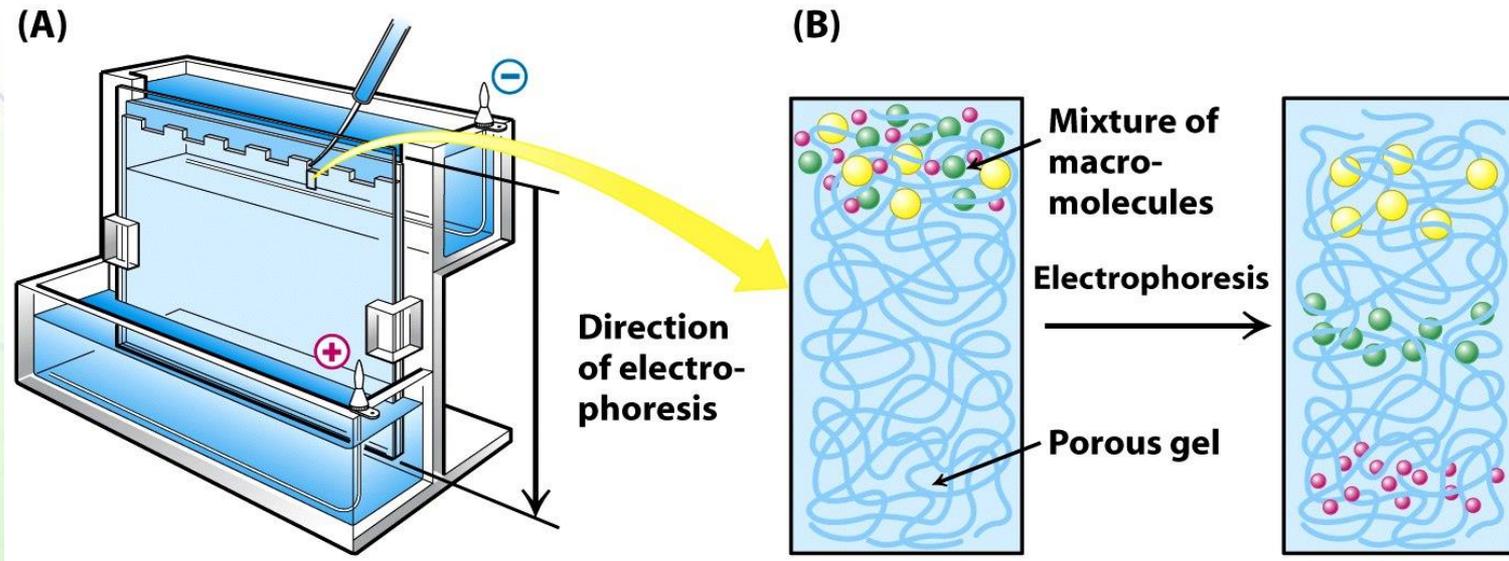
negatively charged SDS molecules

C

Migration of proteins



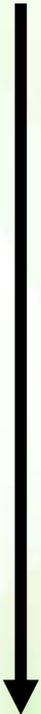
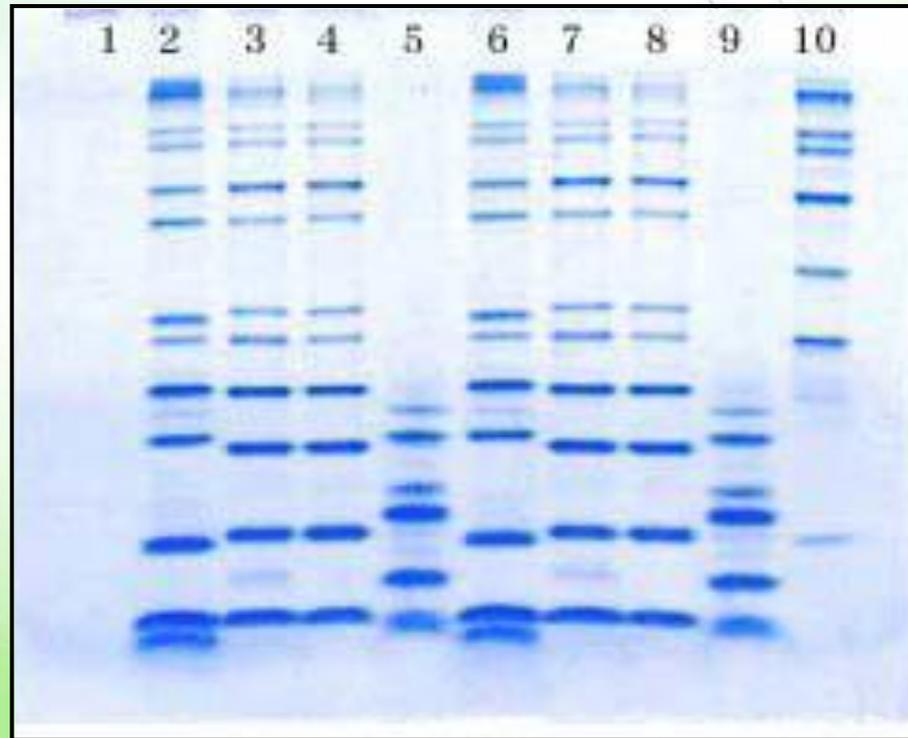
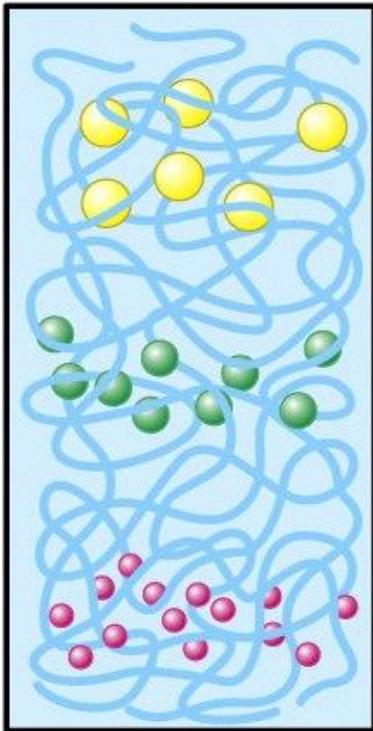
- When an electrical voltage is applied between the upper and lower ends of the gel, all proteins move in one direction towards the anode (positive) according to size only.
- The direction of movement is from top to bottom.
- Whereas smaller molecules move readily through the gel, larger molecules are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



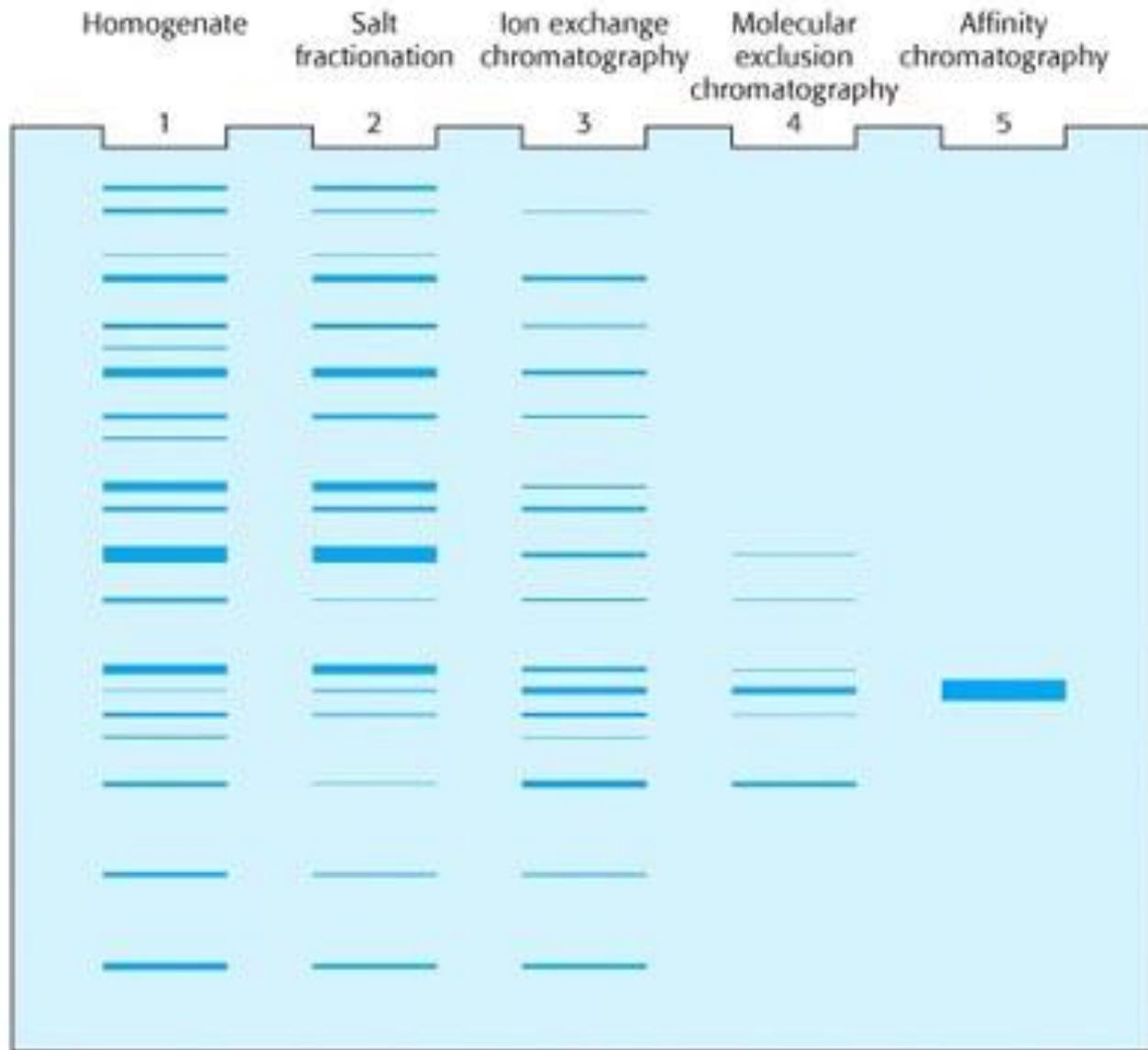
Then...



- Once a gel has been "run", proteins are stained to reveal the positions of the proteins that appear as bands.



Electrophoretic analysis of protein purification



Questions

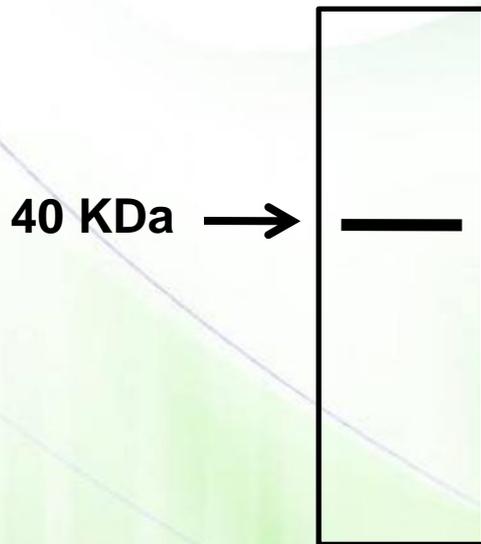


- Describe the protein's structure based on the following results of SDS-PAGE:
 1. Under non-reducing condition, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as two 20-KDa bands.
 2. Under non- reducing condition, a protein exists as two bands, 30 KDa and 20 KDa.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

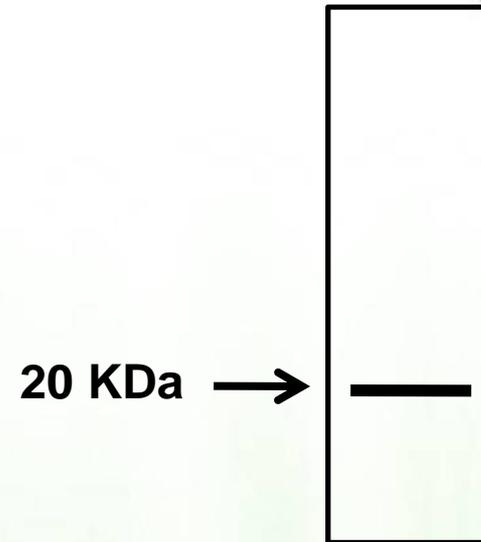


Under non-reducing, denaturing conditions, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as two 20-KDa bands.

Non-reducing



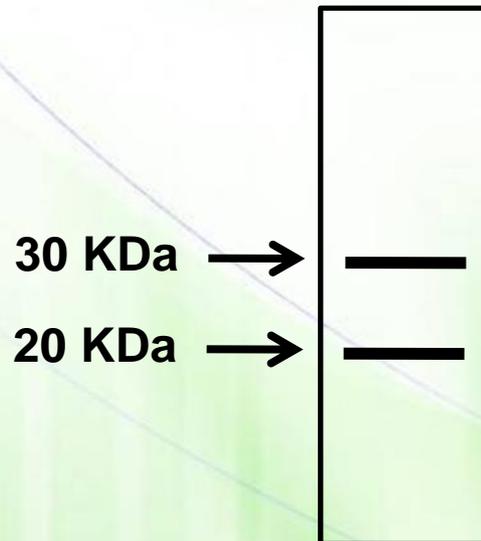
Reducing



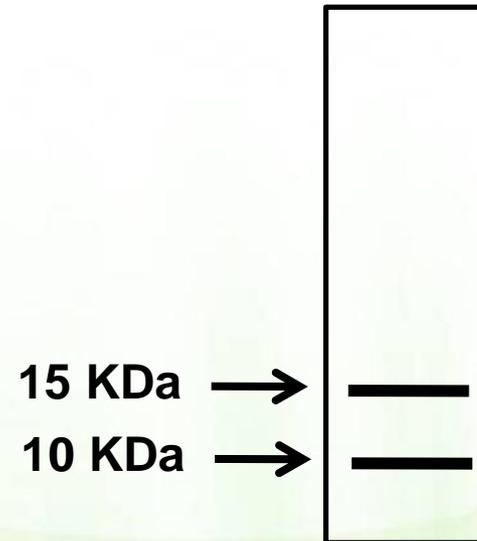


Under non-reducing, denaturing conditions, a protein exists as two bands, 30 KDa and 20 KDa. Under reducing conditions, the protein exists as two bands, 15 KDa and 10 KDa.

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 one bands of 20 KDa.

Non-reducing



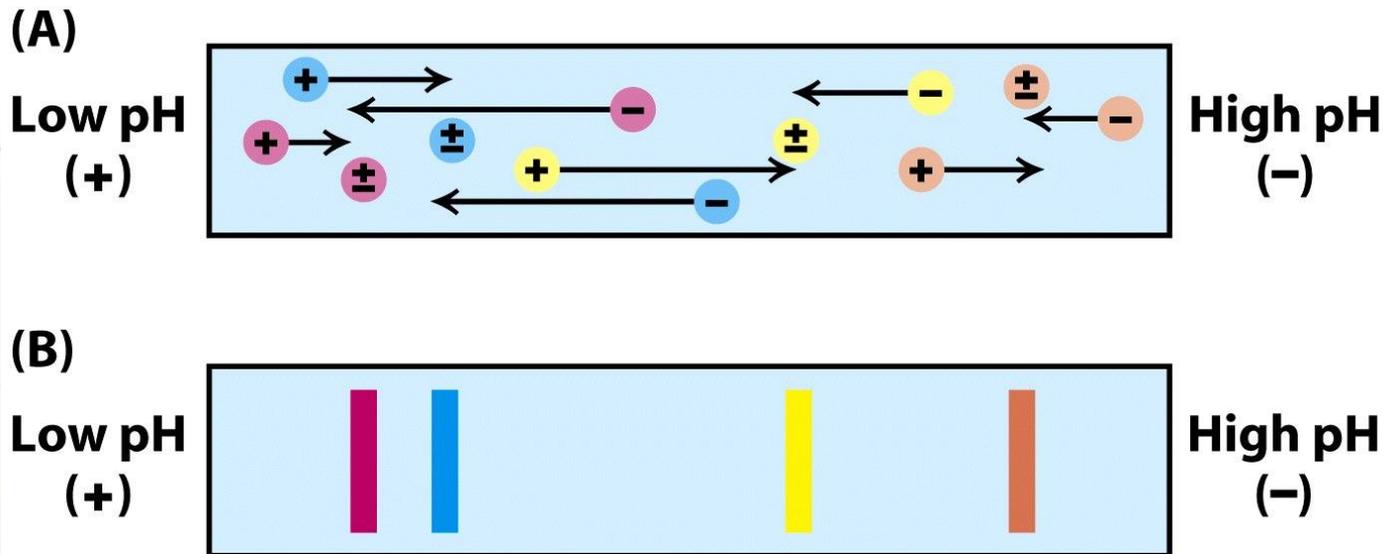
Reducing



Isoelectric focusing



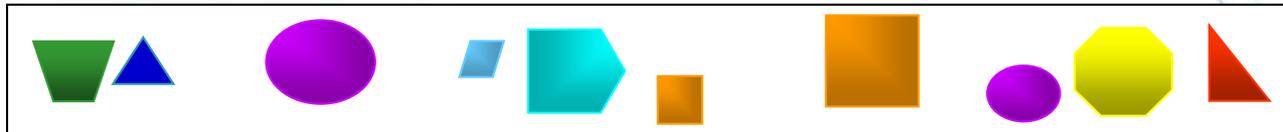
- A gel is prepared with a pH gradient.
- As proteins migrate through the gel, they encounter regions of different pH, so the charge on the protein changes.
- Eventually each protein reaches the point at which it has no net charge—its isoelectric point—and no longer migrates.
- Each protein remains at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





pH 3  10

+



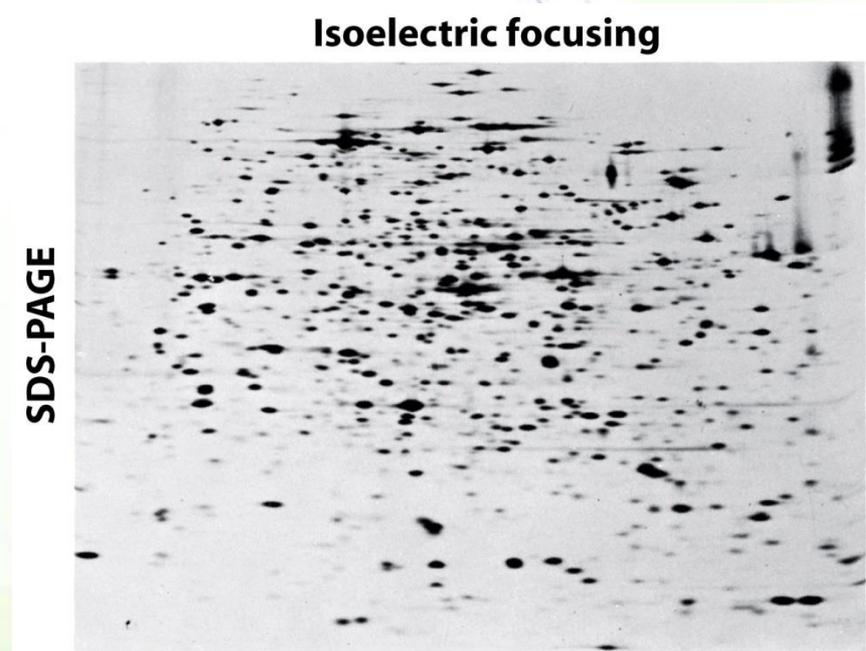
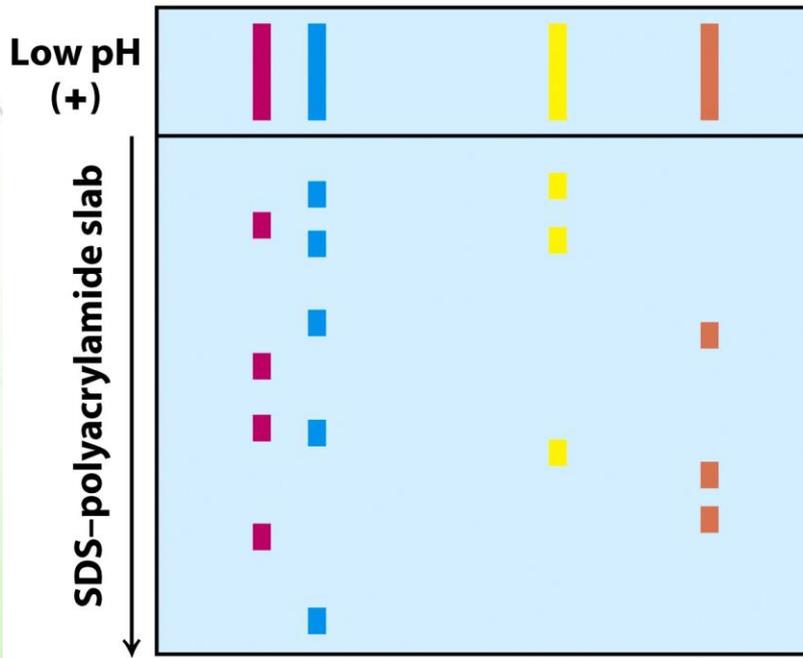
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Two-dimensional gel electrophoresis



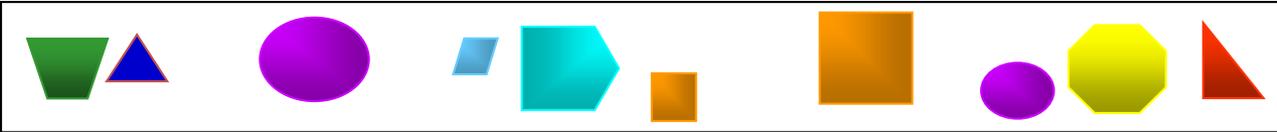
(2D-PAGE)

- In 2D-PAGE, proteins are separated by, first, isoelectric focusing, then through an SDS-PAGE.
- Thus, proteins are separated based on both charge and size.



pH 3  10

+



-

High
MW

Low
MW



2D SDS-PAGE animation



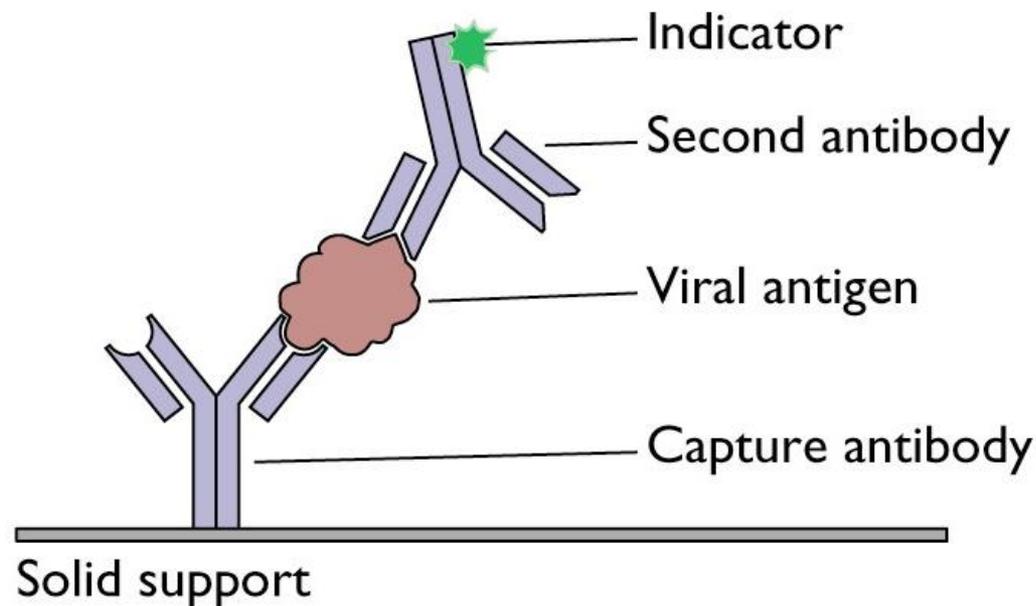
<http://highered.mheducation.com/sites/dl/free/0072835125/126997/animation43.html>

ELISA

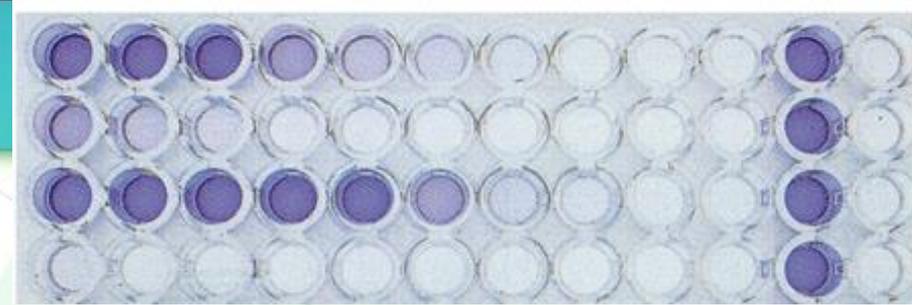


- Enzyme-linked immunosorbent assay
- Same concept as immunoblotting but rapid, convenient, and sensitive (less than nanogram (10^{-9} g) of a protein)

http://www.genscript.com/gsfiles/flash/protein_a_elisa_protocol.swf



ELISA

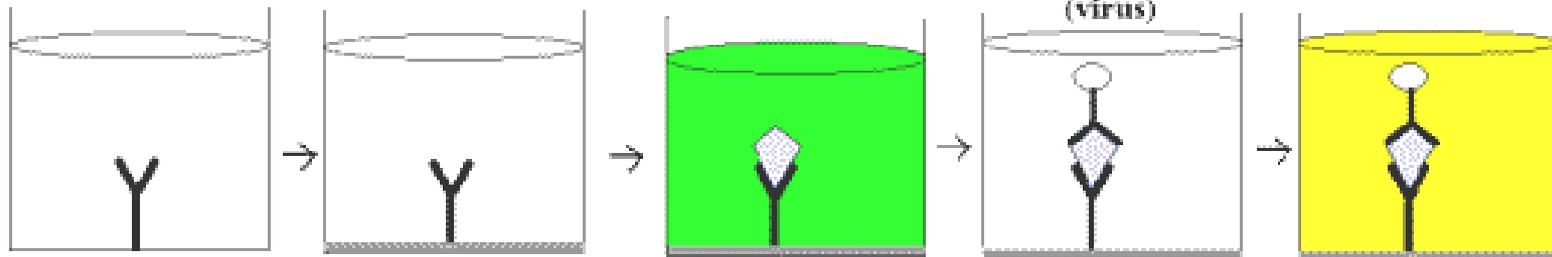


Positive Reaction

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.

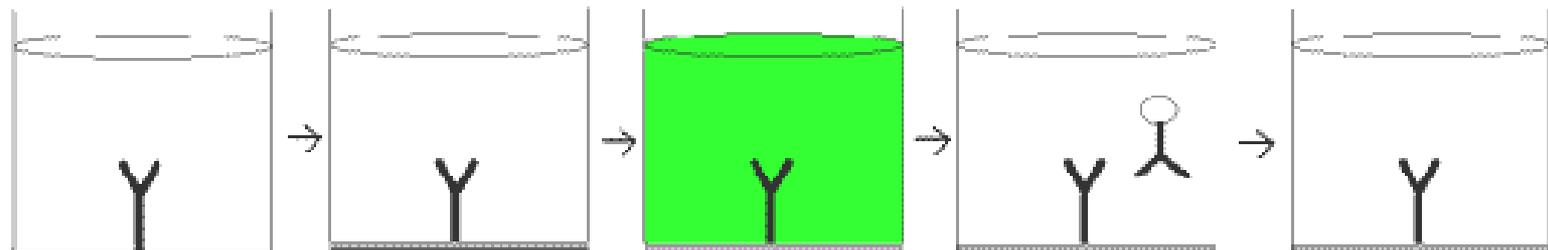
Substrate added.

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



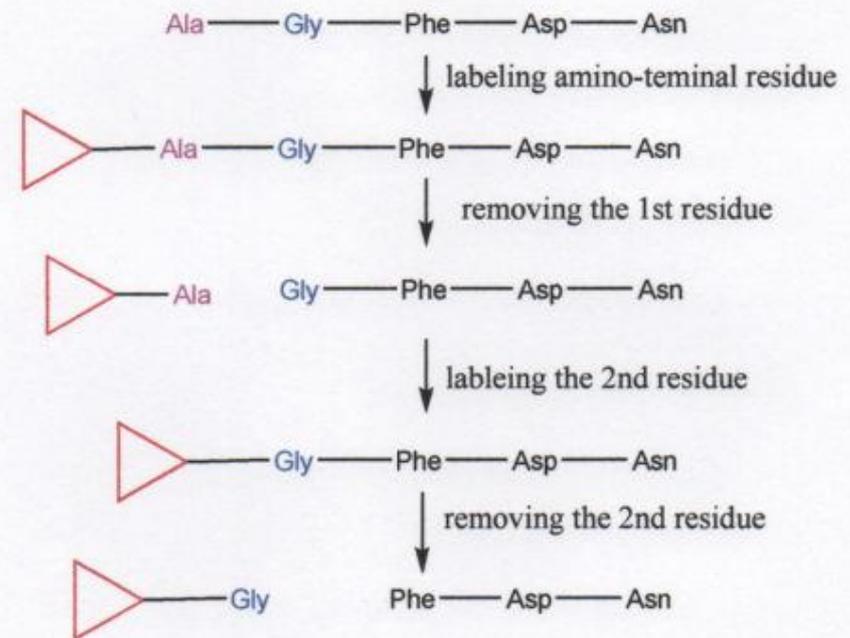
- Protein sequencing is basically the process of knowing the amino sequence of a protein or a peptide.
- One technique is known as Edman Degradation.
- This procedure involves a step-by-step cleavage of the N-terminal residue of a peptide, allowing for the identification of each cleaved residue.

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.

EDMAN DEGRADATION



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

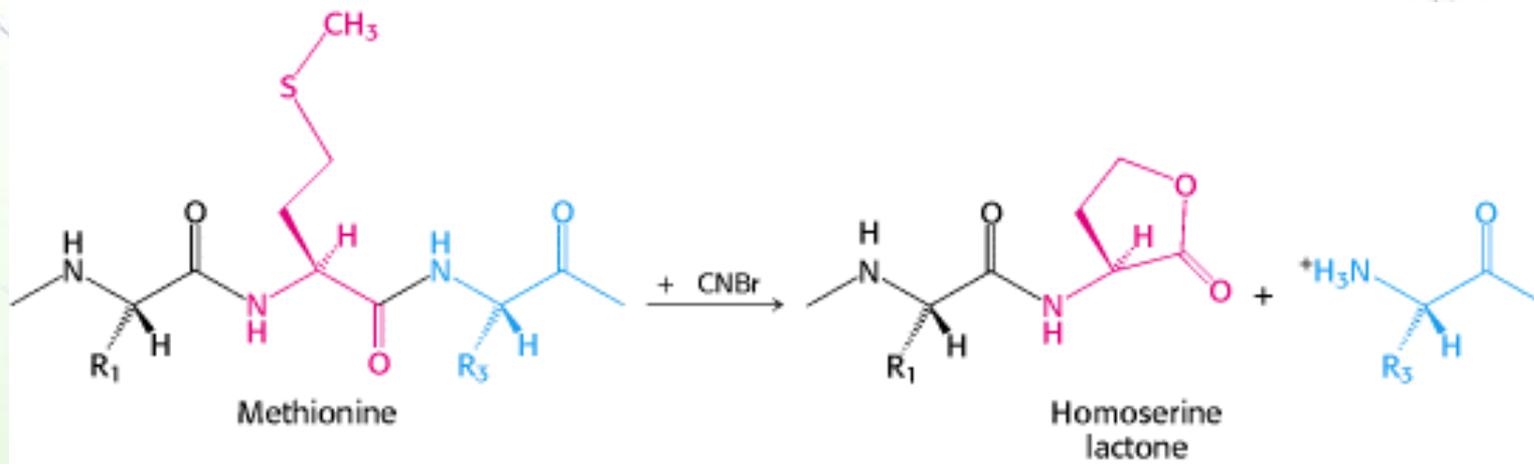


- It is possible to sequence whole proteins by cleaving them into smaller peptides.
- This is facilitated by three methods:
 - Chemical digestion
 - Endopeptidases
 - Exopeptidases

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

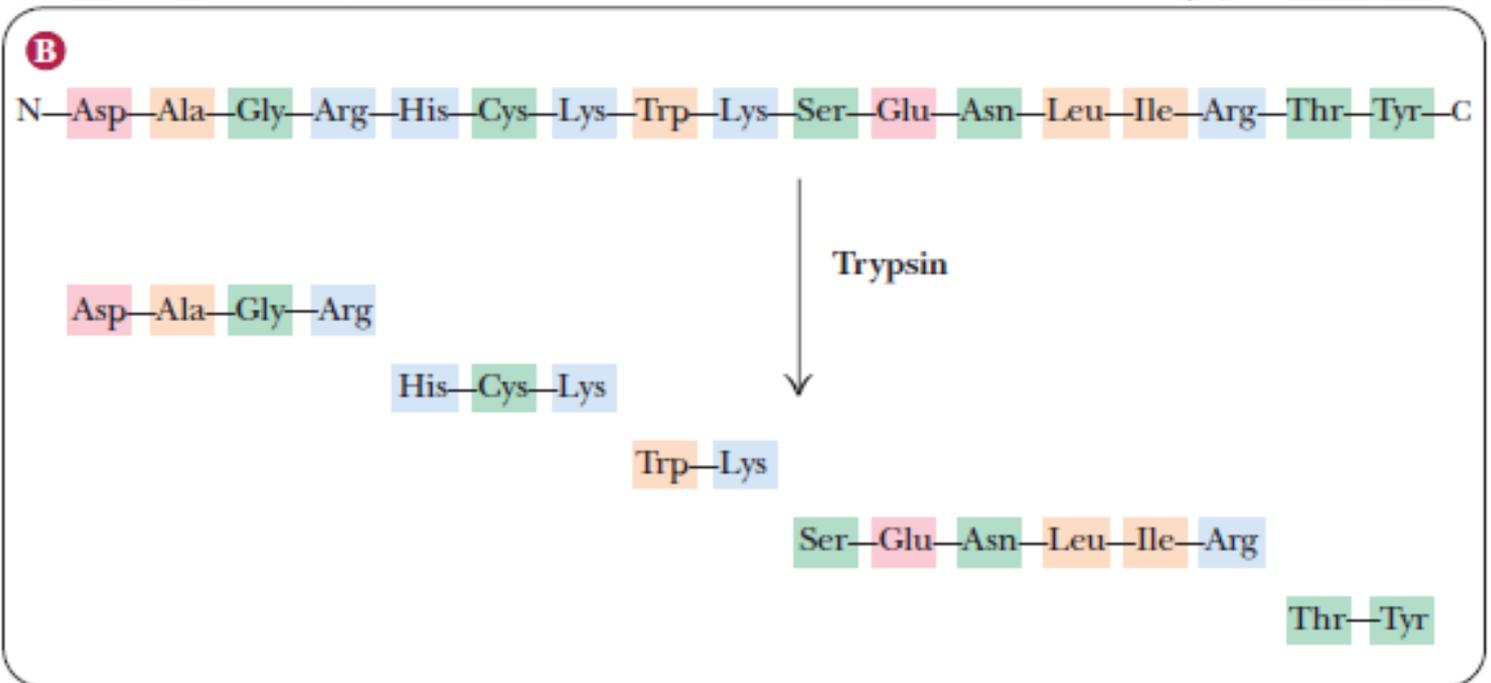


- These are enzymes that cleave at specific sites within the primary sequence of proteins.
- The resultant smaller peptides can be chromatographically separated and subjected to Edman degradation sequencing reactions.

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 next to Pro |

Exopeptidases

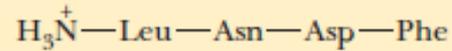


- These are enzymes that cleave amino acids starting at the end of the peptide.
- There are two types:
 - Aminopeptidases that cleave at the N-terminus
 - Carboxypeptidases that cleave at the C-terminus

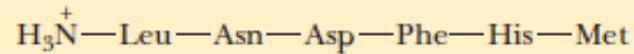
Homework



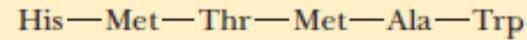
Chymotrypsin



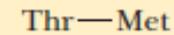
Cyanogen bromide



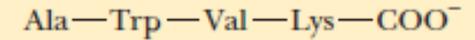
Chymotrypsin



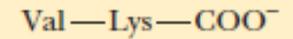
Cyanogen bromide



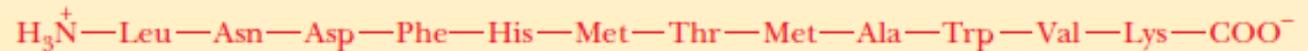
Cyanogen bromide



Chymotrypsin



Overall sequence



- Do questions 45 and 46 (9th edition)



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

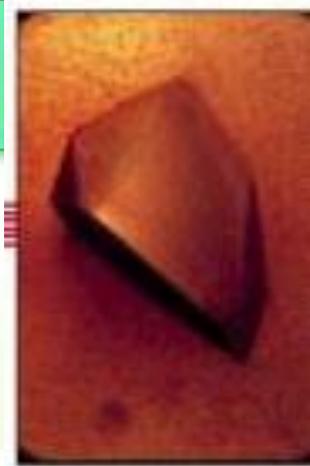
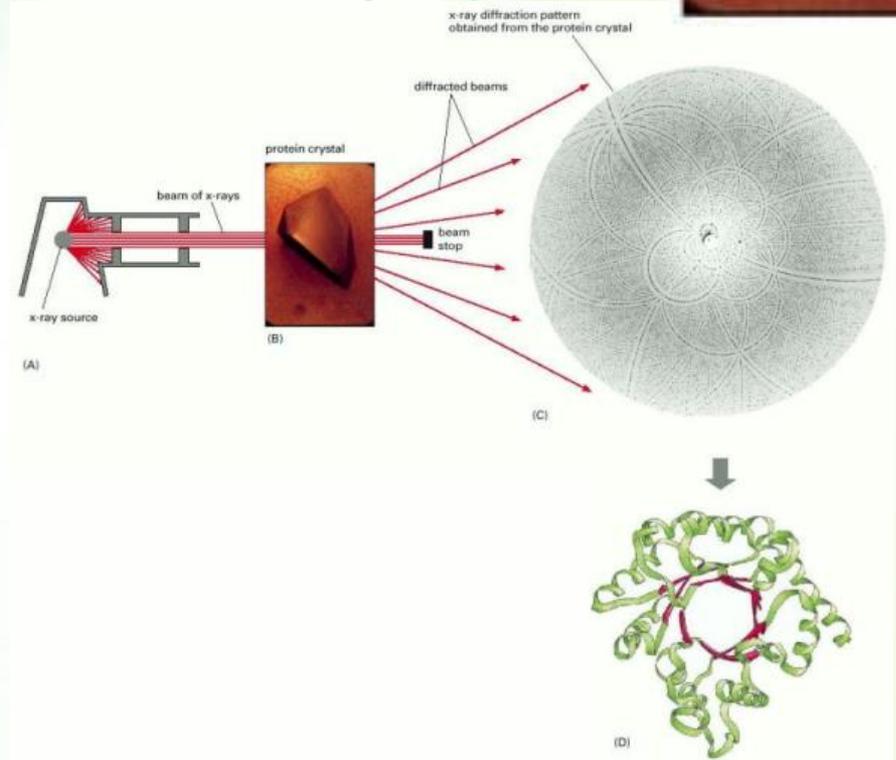
Thr—Leu—Met—Ile

Met—Val—Ser—Thr—Lys—Leu—Phe

Deduce the sequence of the original peptide.

Crystallography

- X-ray crystallography is used to determine the three-dimensional structure of proteins.
- A protein must first be turned into a crystal before being exposed to x-rays, which are scattered by the electrons of the molecule.



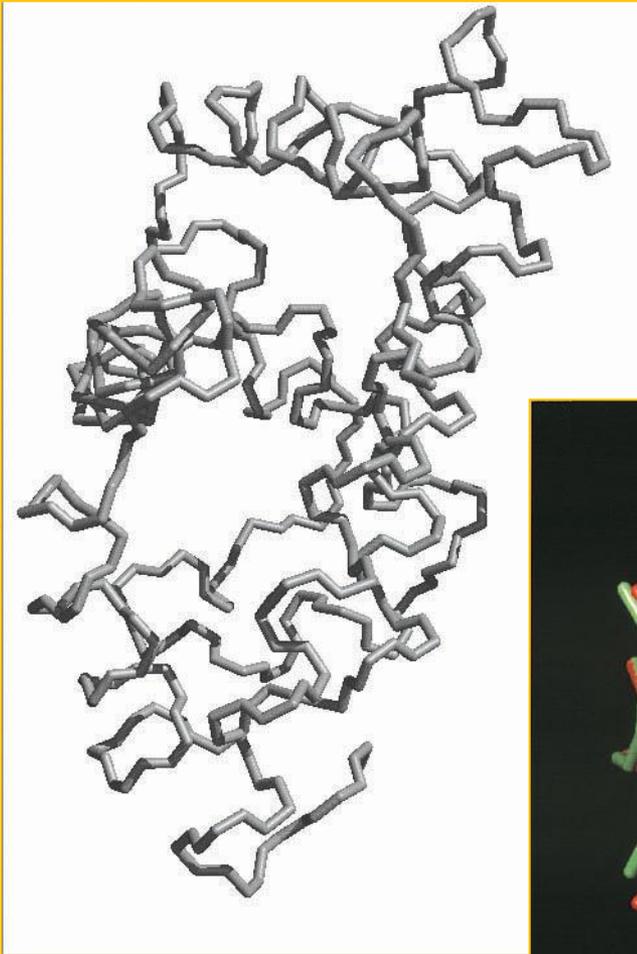
<http://www.dnatube.com/video/279/Protein-Structure-Revealed-xray-crystallography>

Nuclear magnetic resonance (NMR) spectroscopy

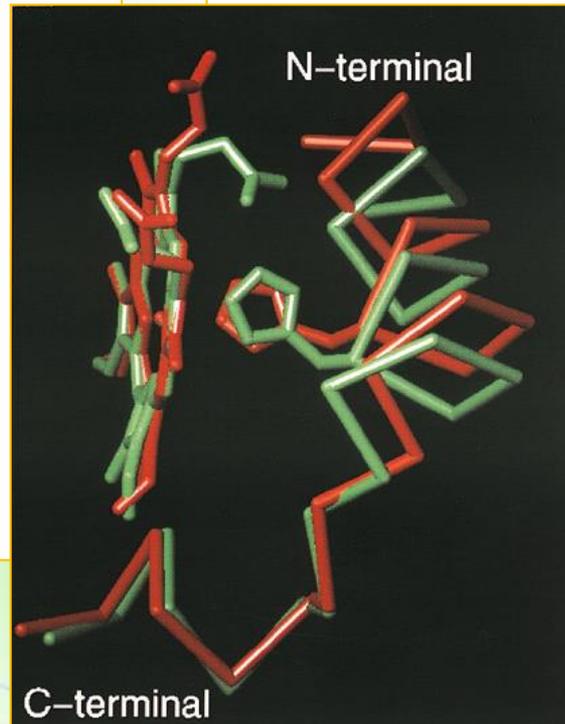
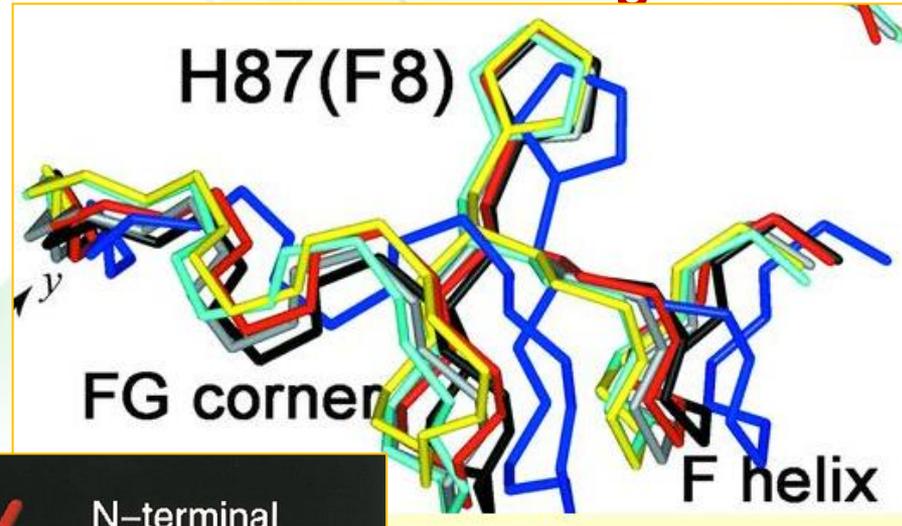


- Nuclear magnetic resonance (NMR) spectroscopy reveals the structure and dynamics of proteins in solution (not crystals).
- This is important with protein binding to other molecules like enzymes to their substrates, receptors to their ligands, etc.

Myoglobin



Hemoglobin



Myoglobin and Hemoglobin