Protein Purification and Characterization Techniques

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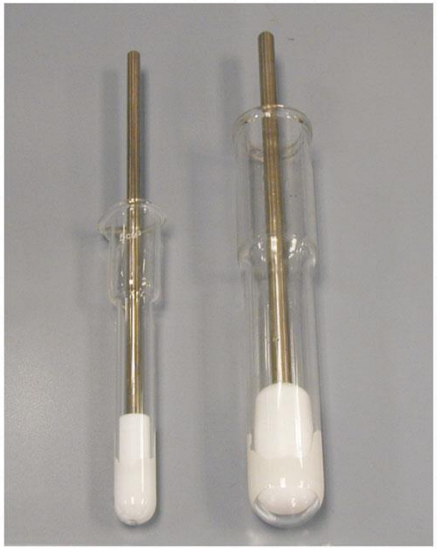
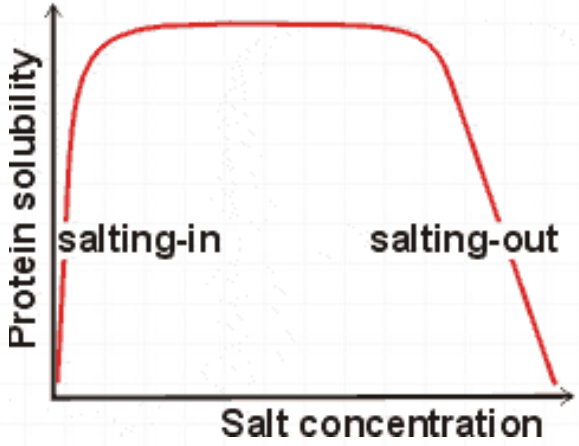
Extracting Pure Proteins from Cells

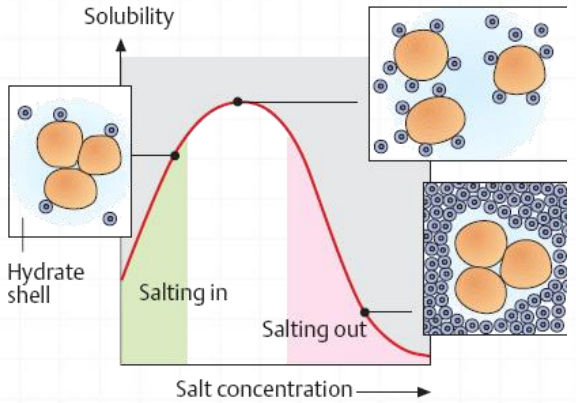
- Purification techniques focus mainly on size & charge
- The first step is **homogenization** (grinding, Potter–Elvejhem homogenizer, sonication, freezing and thawing, detergents)
- **Differential centrifugation (**600 *g:* unbroken cells & nuclei; 15,000 *g:* mitochondria; 100,000 *g:* ribosomes and membrane fragments



Salting in & out

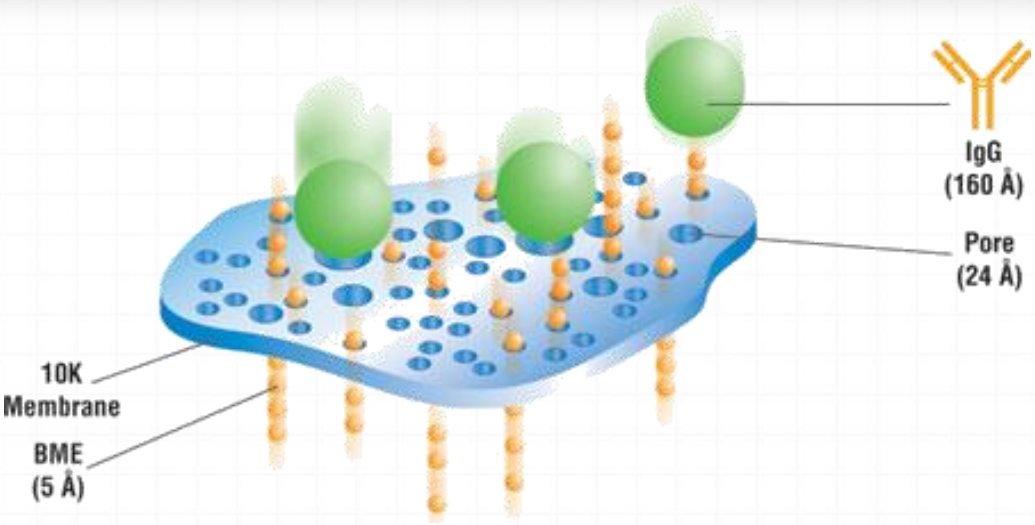
- Are proteins soluble? If yes, to which limit?
- Salt stabilizes the various charged groups on a protein molecule and enhance the polarity of water and increases the ionic strength, thus attracting protein into the solution and enhancing the solubility of protein
- Ammonium sulfate is the most common reagent to use at this step
- This technique is important but results are crude

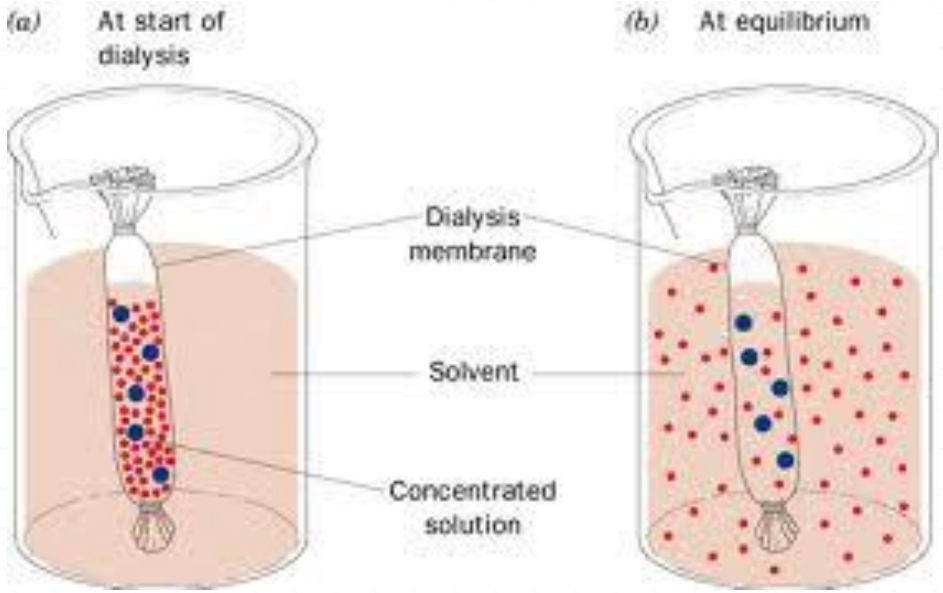


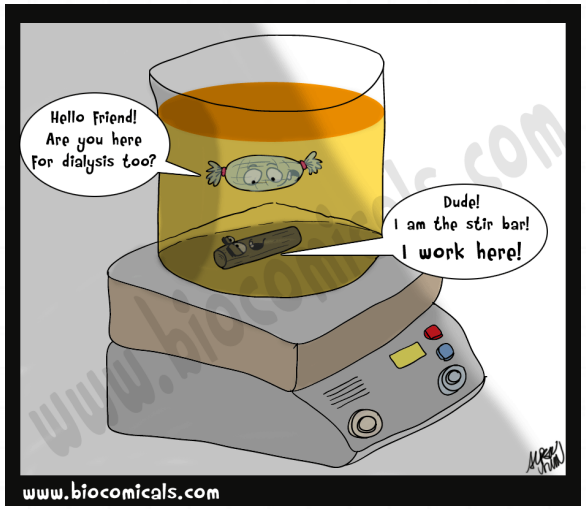


Dialysis

- Principle of diffusion
- Concept of MW cut-off
- Pure vs. crude

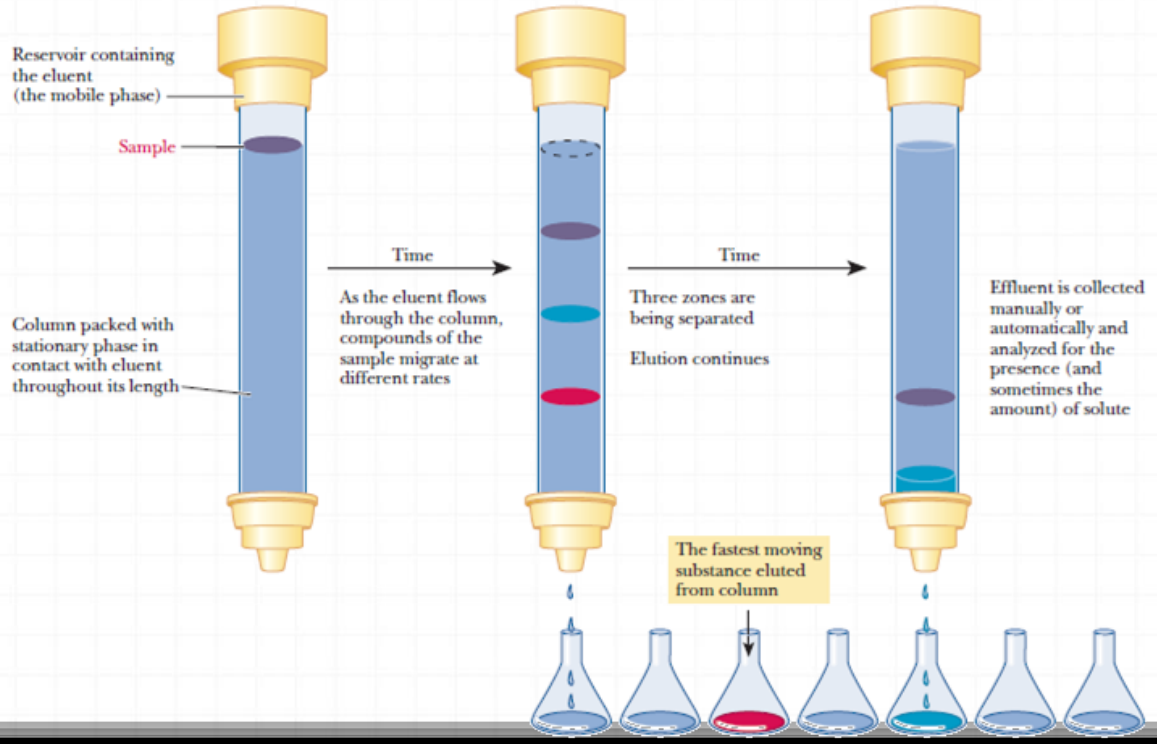






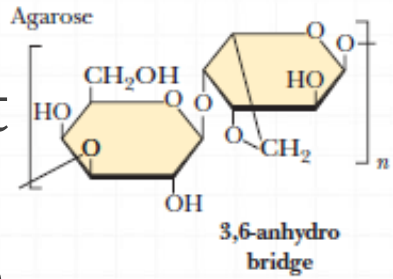
Column Chromatography

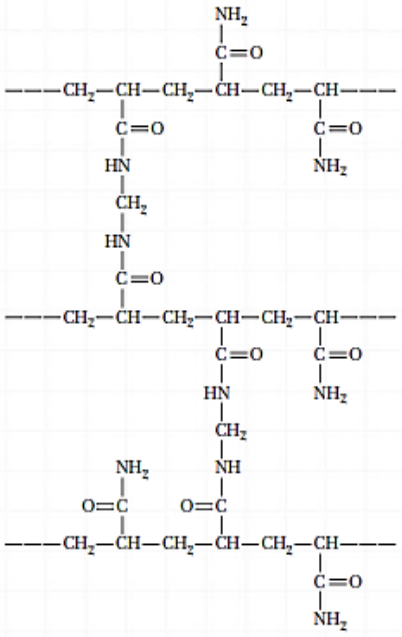
- Greek *chroma,* "color," and *graphein,* "to write"
- Is it just for colourful proteins?
- Chromatography is based on two phases: stationary & mobile
- Washing or Elution?
- What are the different kinds?



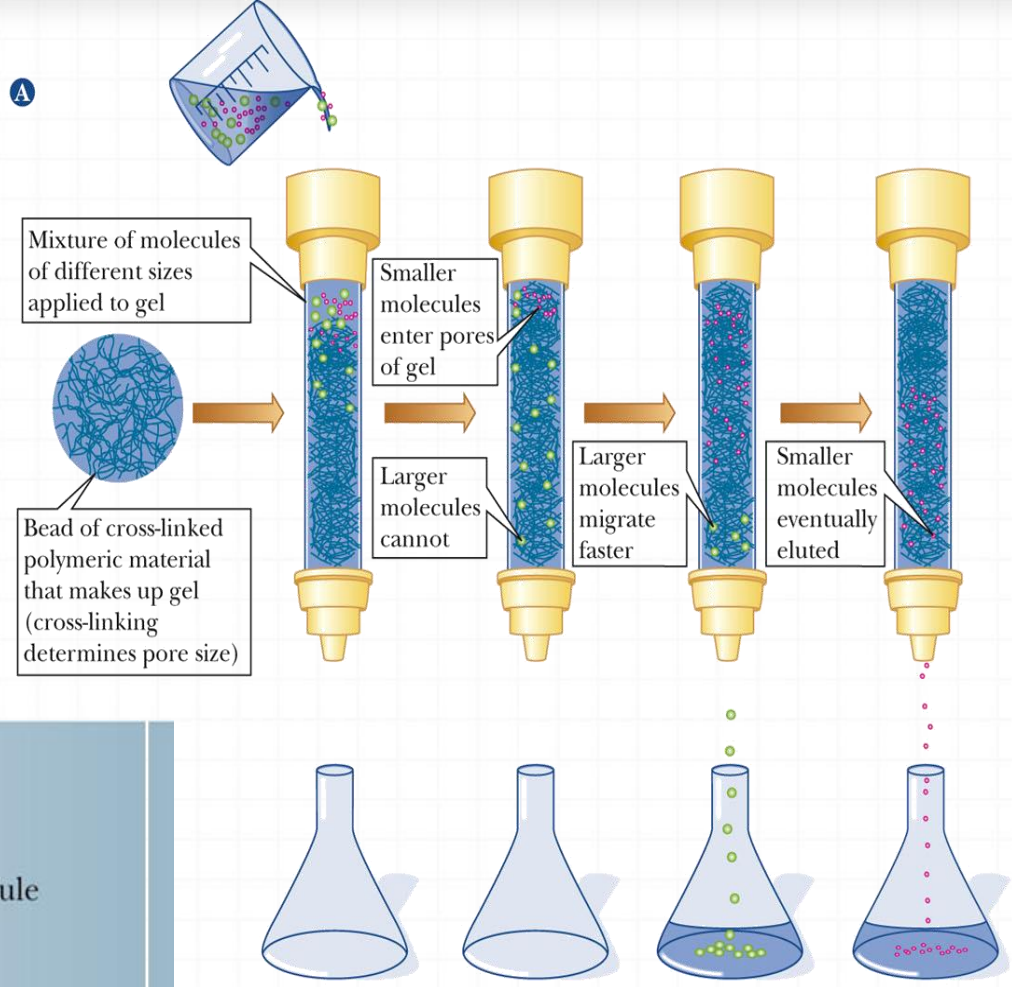
Size-exclusion chromatography
Gel-filtration chromatography

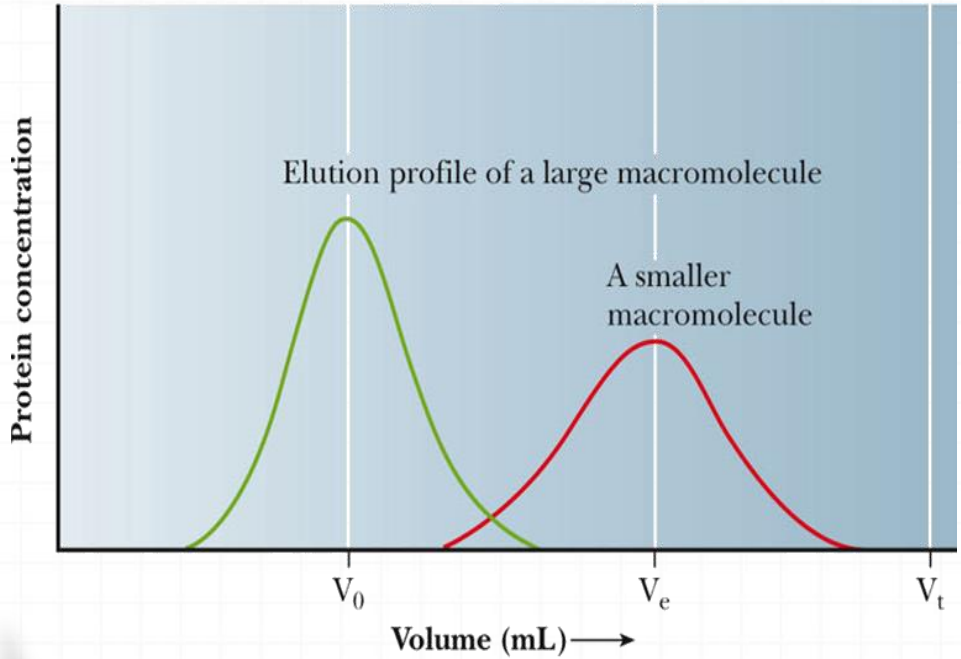
- Separation on the basis of size (MW)
- Stationary (cross-linked gel particles): consist of one of two kinds of polymers; the 1st is a carb. polymer (ex. **dextran** or **agarose);** often referred to by Sephadex and Sepharose. The 2nd is based on **polyacrylamide** (Bio-Gel)
- Extent of crosslinking & pore size (exclusion limit)
- Convenient & MW estimate
- Each gel has range of sizes that separate linearly with the log of the molecular weight





Molecular-sieve chromatography

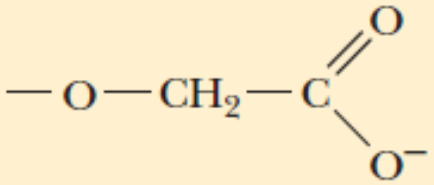




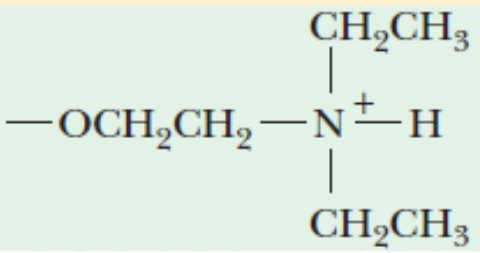
Ion-exchange chromatography

- Interaction based on net charge & is less specific
- Resin is either negatively charged (**cation exchanger) or** positively charged (**anion exchanger)**
- Buffer equilibration, exchange resin is bound to counter-ions. A cation-exchange resin is usually bound to Na+ or K+ ions, and an anion exchanger is usually bound to Cl^- ions
- Proteins mixture loading
- Elution (higher salt concentration)

Weakly acidic: carboxymethyl (CM) cellulose



Weakly basic: diethylaminoethyl (DEAE) cellulose

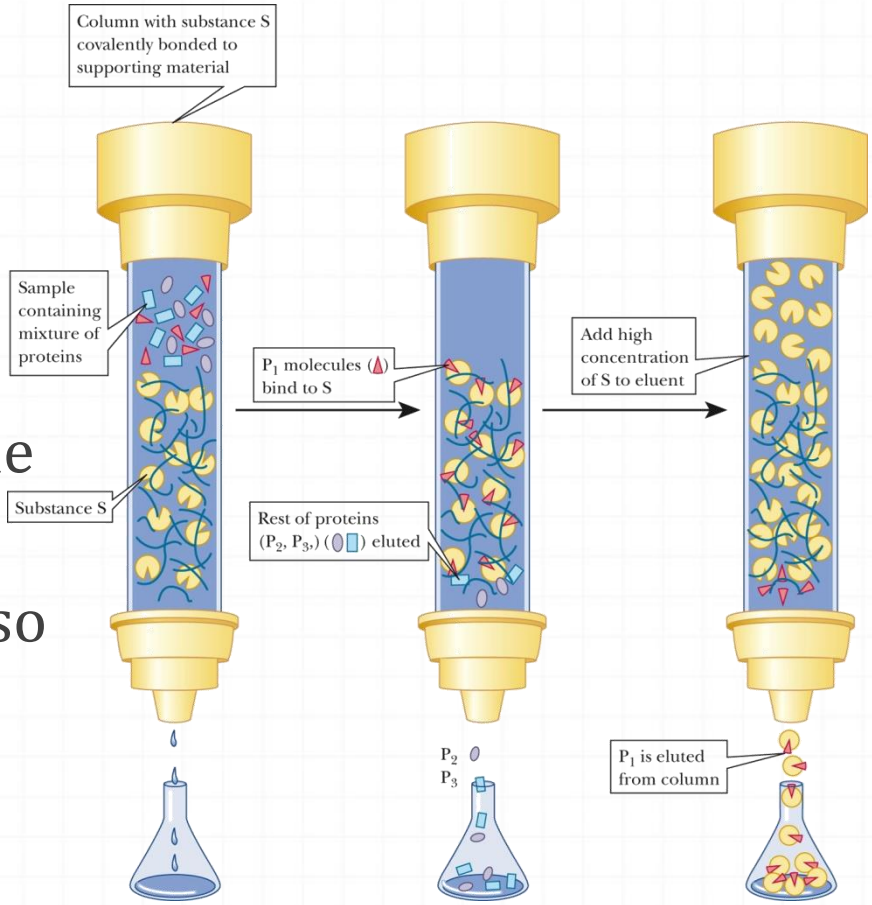


Problem

- You have 5 different proteins (#1, #2, #3, #4, and #5), with different isoelectric points (pIs).
  - 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?

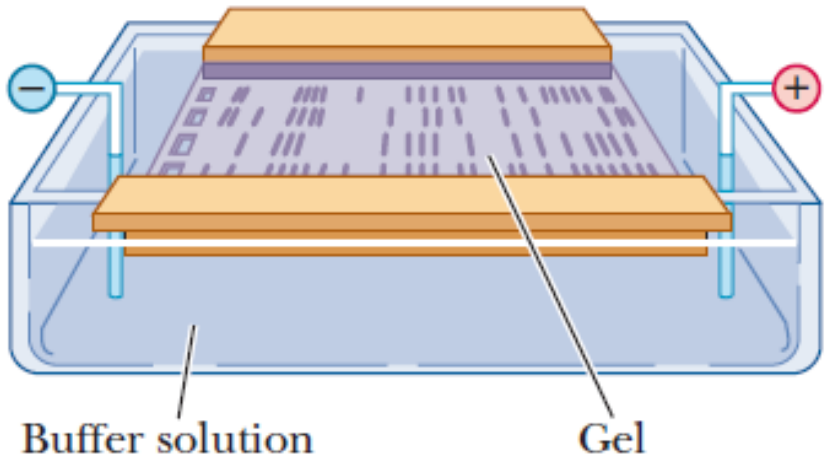
Affinity chromatography

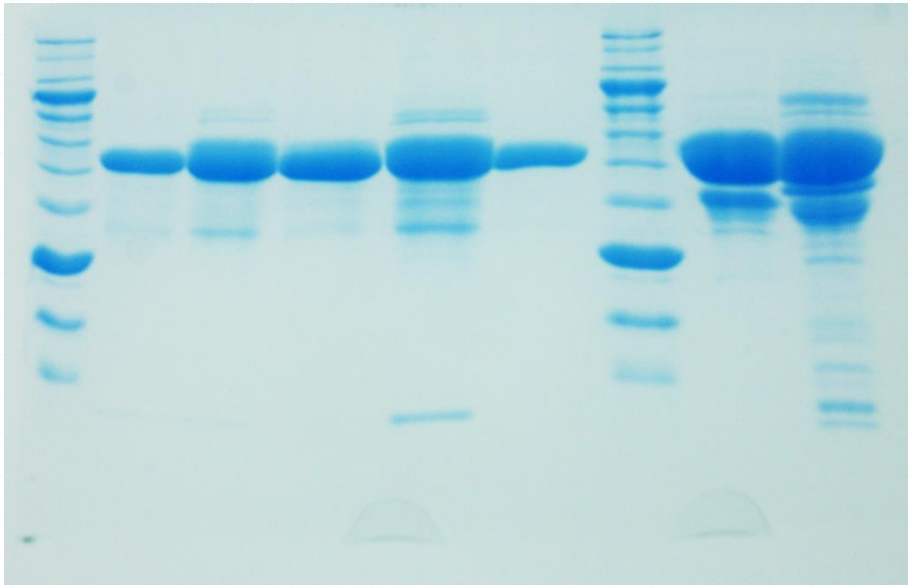
- It has specific binding properties
- The polymer (stationary) is covalently linked to a *ligand* that binds specifically to the desired protein
- The bound protein can be eluted by adding high conc. of the soluble ligand
- Protein–ligand interaction can also be disrupted with a change in pH or ionic strength
- Convenient & products are very pure (Antigen-antibody, His-tag, GST-Tag)



Electrophoresis

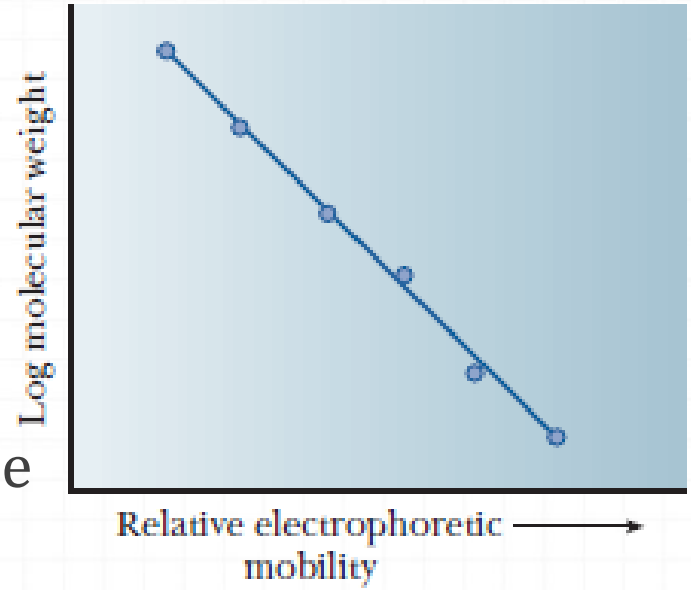
- Based on the motion of charged particles in an electric field
- Macromolecules have differing mobilities based on their charge, shape, and size
- The most common medium is a polymer of agarose or acrylamide





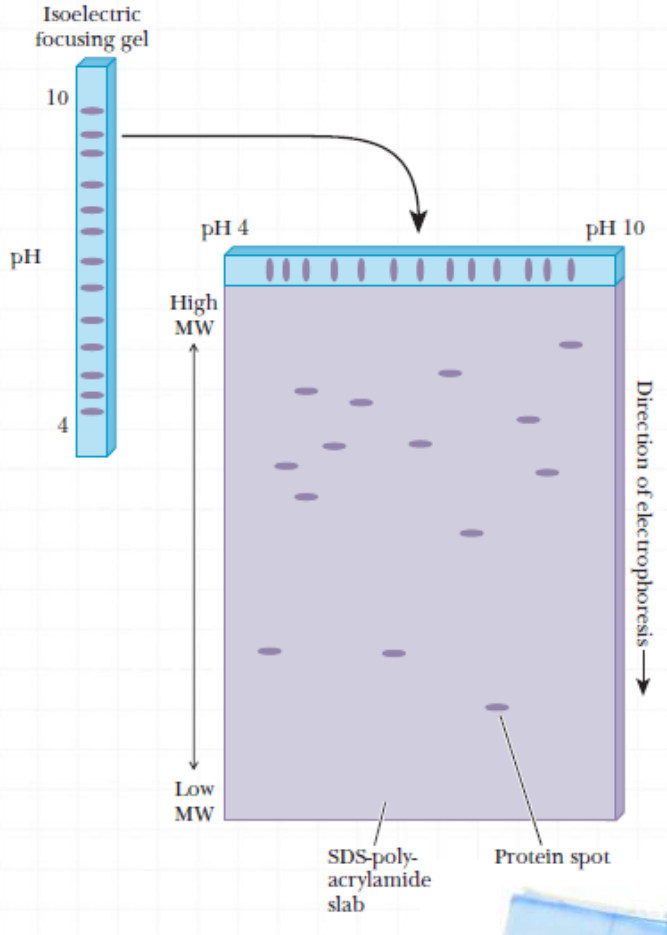
Agarose or PAGE?

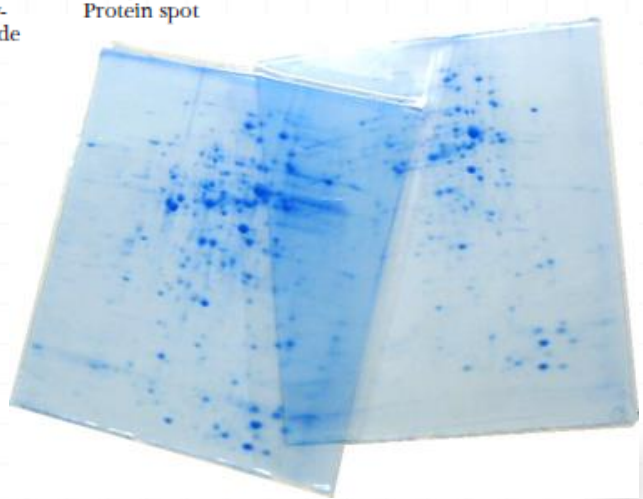
- Agarose (nucleic acids), PAGE (proteins)
- In PAGE: SDS or NO-SDS, detergent, $CH_3(CH_2)_{10}CH_2OSO_3Na^+$
- SDS completely denatures proteins (multi-subunit proteins)
- Acrylamide offers higher resistance to large molecules
- Shape and charge are approximately the same (sizes is the determining factor)
- Acrylamide without the SDS (**native gel):** study proteins in their native conformation (mobility is not an indication of size)



Isoelectric focusing

- Proteins have different isoelectric points
- Gel prepared with a pH gradient parallel to electric-field gradient
- Two-dimensional gel electrophoresis (2-D gels)





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.

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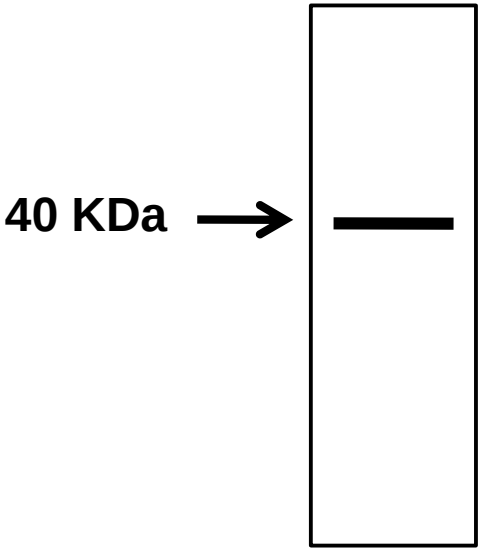
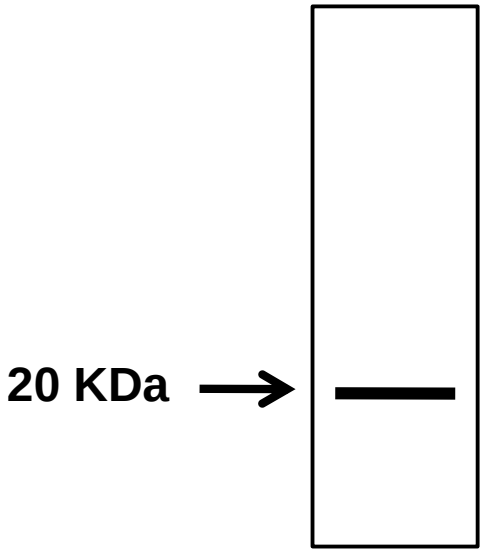
Non-reducing



Reducing



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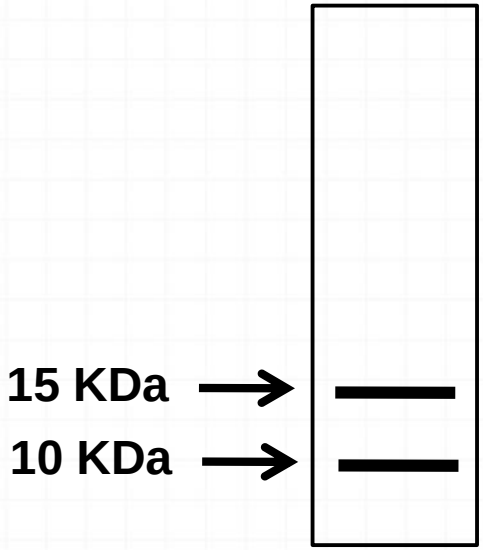
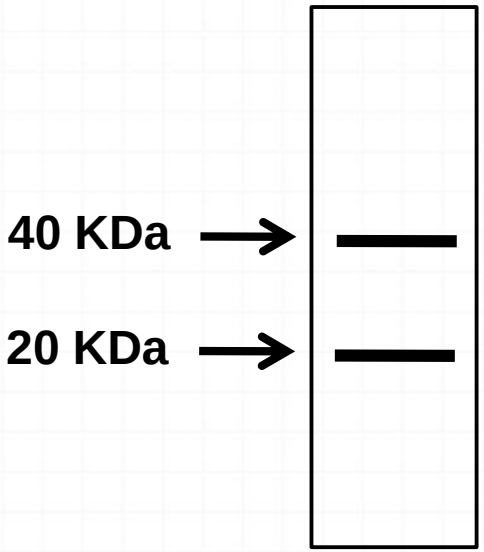
Non-reducing

Reducing

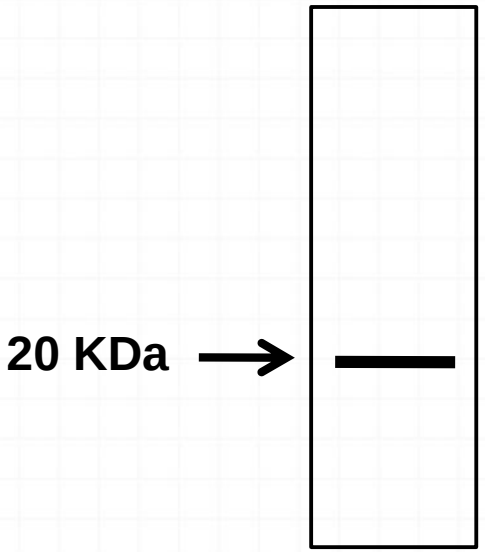


Under non- reducing condition, a protein exists as two bands, 40 KDa and 20 KDa. Under reducing conditions, the protein exists as one bands of 20 KDa.

Non-reducing

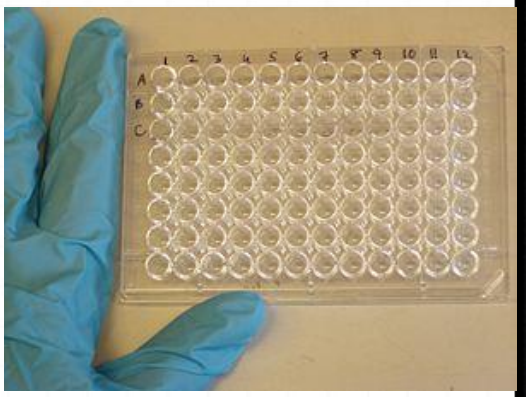


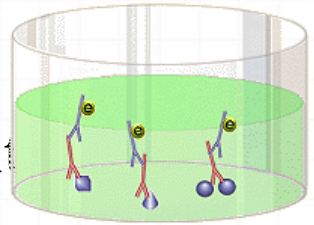
Reducing



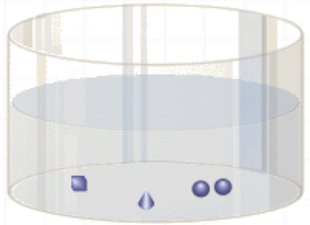
Immunoassays - ELISA

- Enzyme-Linked Immunosorbent Assay
- Detect & quantify substances (peptides, proteins, antibodies & hormones)
- Usually done in 96-well plates
- Rapid, convenient, and sensitive (10^{-9} g)
- Apllication:
- Screening (HIV, Hepatitis B&C)
- Detecting food allergens, such as milk, peanu walnuts, almonds, and eggs
- Hormones (HCG, LH, TSH, T3, T4)

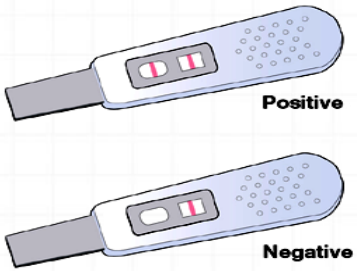


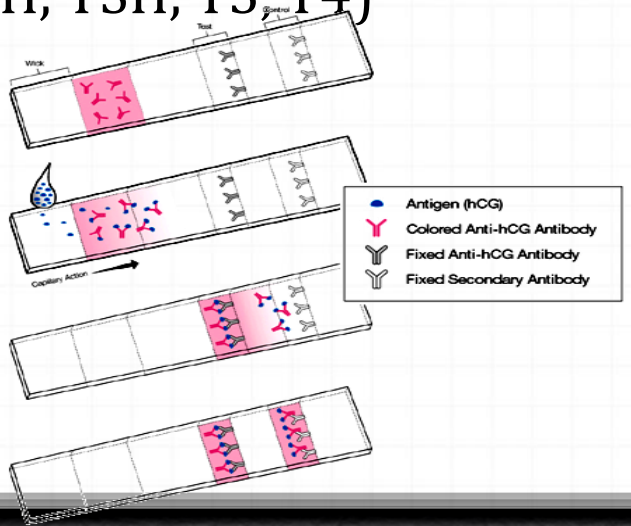


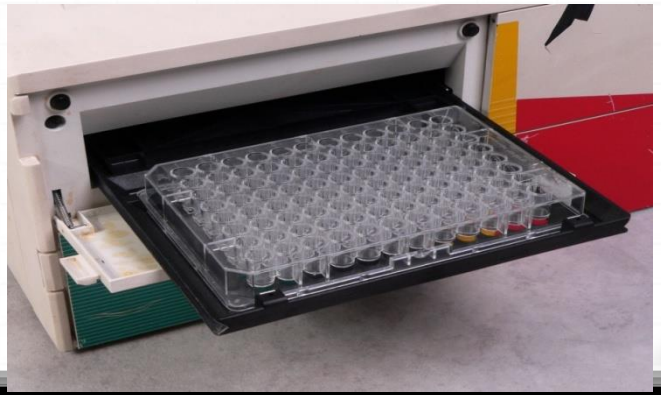
(Green, positive)



(No color, negative)





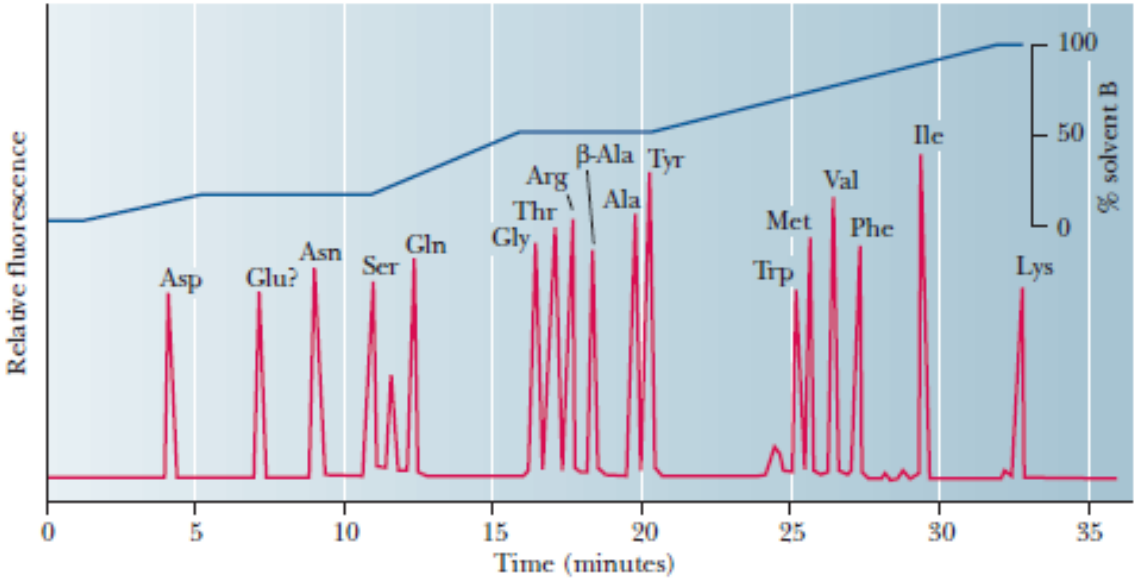


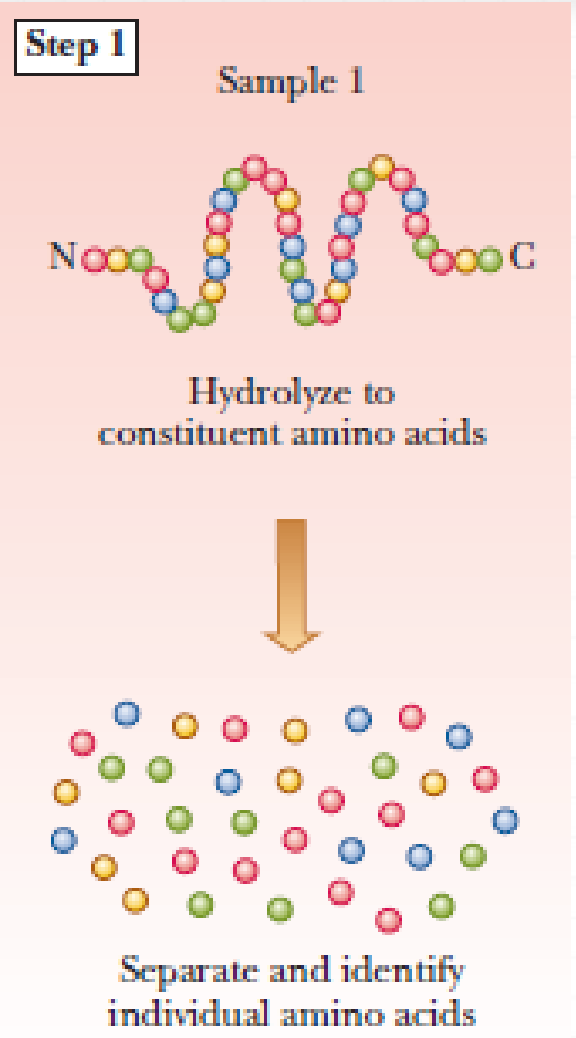
Protein sequencing

- Protein sequencing is basically the process of knowing the amino sequence of a protein o 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.

Protein sequencing - Edman Method

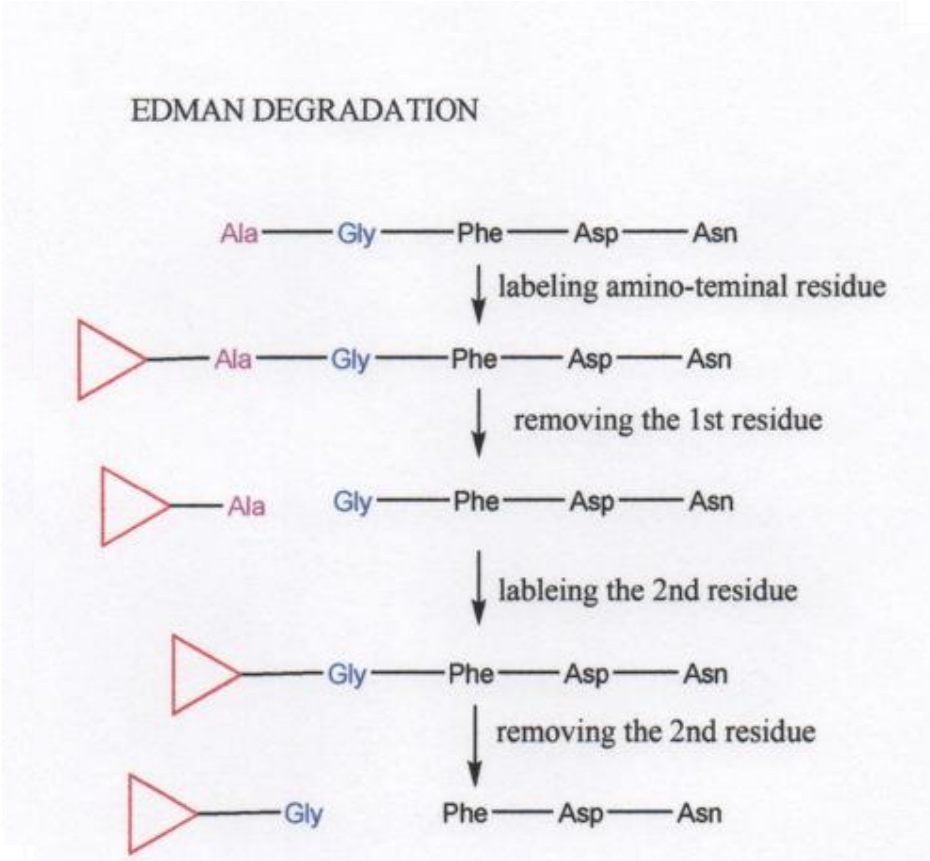
- how much and which amino acids are involved
- **Hydrolysis** (heating + HCl) **& Separation** (ion-exchange chromatography or by **high performance liquid chromatography, HPLC)**





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.

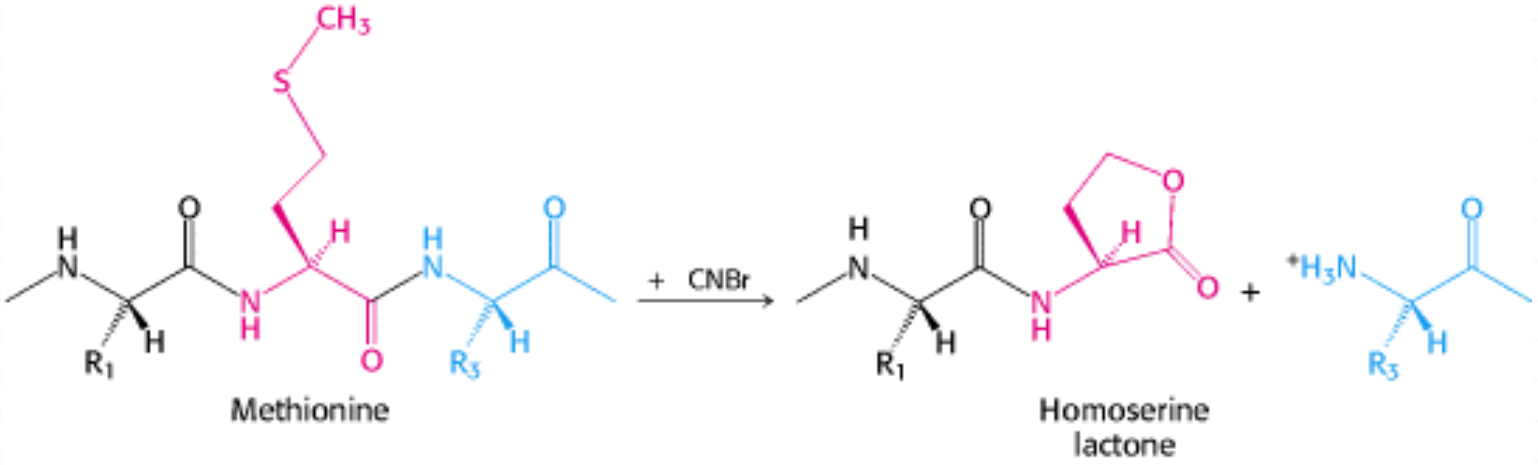
- The Edman degradation technique does not allow peptides more than 50 residues to be sequenced.

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.



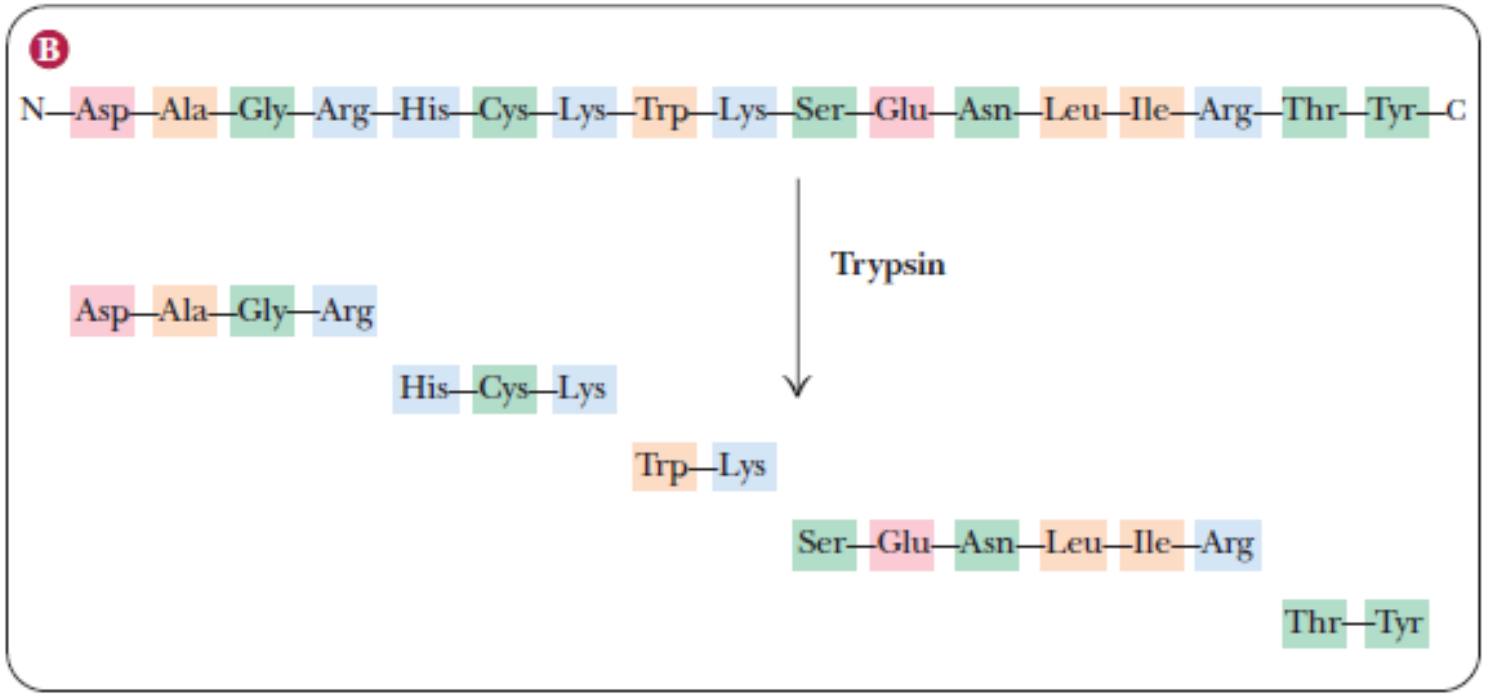
Endopeptidases

- 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 R, K, but not if next to P
Chymotrypsin	peptide bond C-terminal to F, Y, W but not if next to P
Elastase	peptide bond C-terminal to A, G, S, V, but not if next to P
Pepsin	peptide bond N-terminal to L, F, W, Y, but not when next to P

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

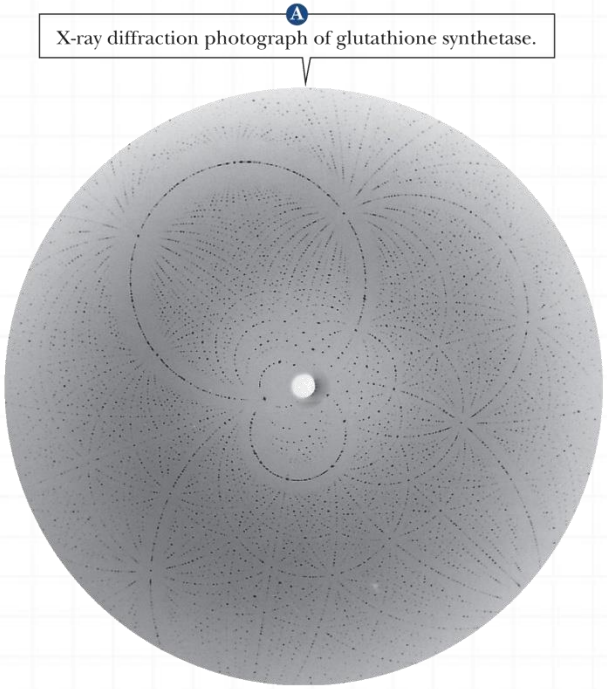
Protein sequencing – prediction from DNA & RNA

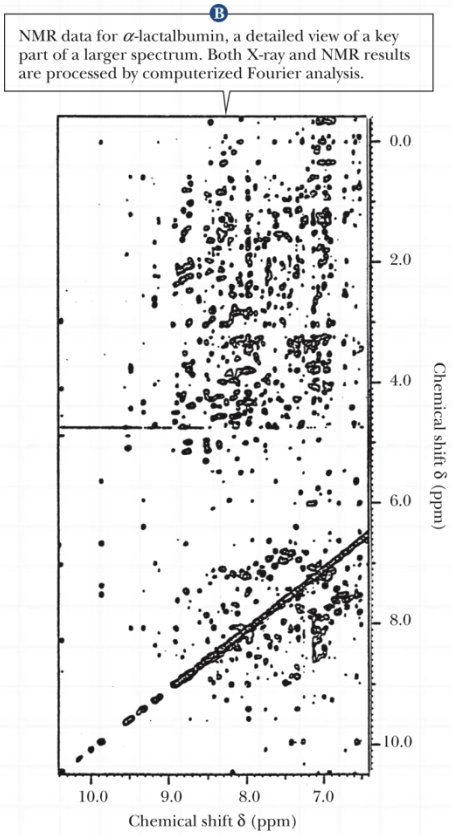
- If the sequence of the gene is known, this is very easy
- If the sequence of the gene is unknown (newly isolated proteins)? Sequence a short segment, complementary RNA, isolate mRNA, PCR, gene sequencing

Determination of 3°Structure

- **X-ray crystallography**
  - uses a perfect crystal; that is, one in which all individual protein molecules have the same 3D structure and orientation
  - exposure to a beam of x-rays gives a series diffraction patterns
  - information on molecular coordinates is extracted by a mathematical analysis called a Fourier series
- **2-D Nuclear magnetic resonance**
  - can be done on protein samples in aqueous solution

X-Ray and NMR Data





- High resolution method to determine 3˚ structure of proteins (from crystal)
- Diffraction pattern produced by electrons scattering X-rays
- Series of patterns taken at different angles gives structural information

- Determines solution structure
- Structural info. Gained from determining distances between nuclei that aid in structure determination
- Results are independent of X-ray crystallography