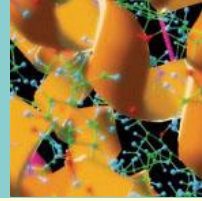

Proteomics

Protein purification and characterization techniques

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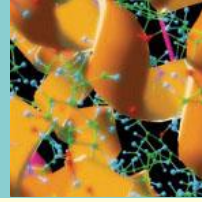
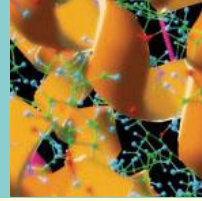


Proteomics

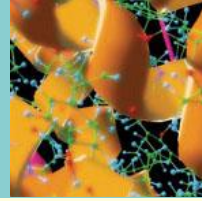
Understanding Proteins in the Postgenomic Era.

Completion of the Human Genome



- Draft sequences published in 2001.
- Approximately 35,000 genes.
- Genes account for 2% of genome sequence.
- Genes encode proteins.

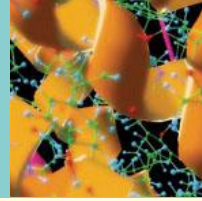
Proteomics



- Humans have 100,000 or more individual proteins.

- Proteomics is the study of all the proteins found in an organism.

Proteome Complexity



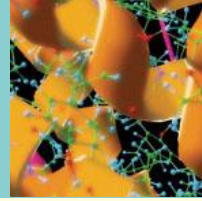
GENOME

- 4 nucleotides.
- Double helix.
- Same in all cells.

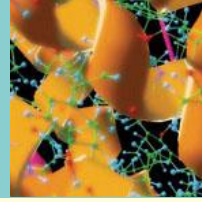
PROTEOME

- 20 amino acids.
- Each protein has unique 3D shape.
- Differs with cell type.

Types of Proteomics Research



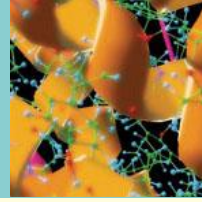
- Protein profiling.
- Predicting protein structure.
- Protein networks.



Protein Profiling

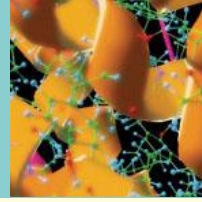
Determination of the proteins that make up a given proteome.

Challenges of Protein Profiling



- Proteomes vary by cell type.
- Proteomes vary by stage of cell development.
- Some proteins abundant, others very rare.
- Some biologically important proteins are tiny and difficult to detect.

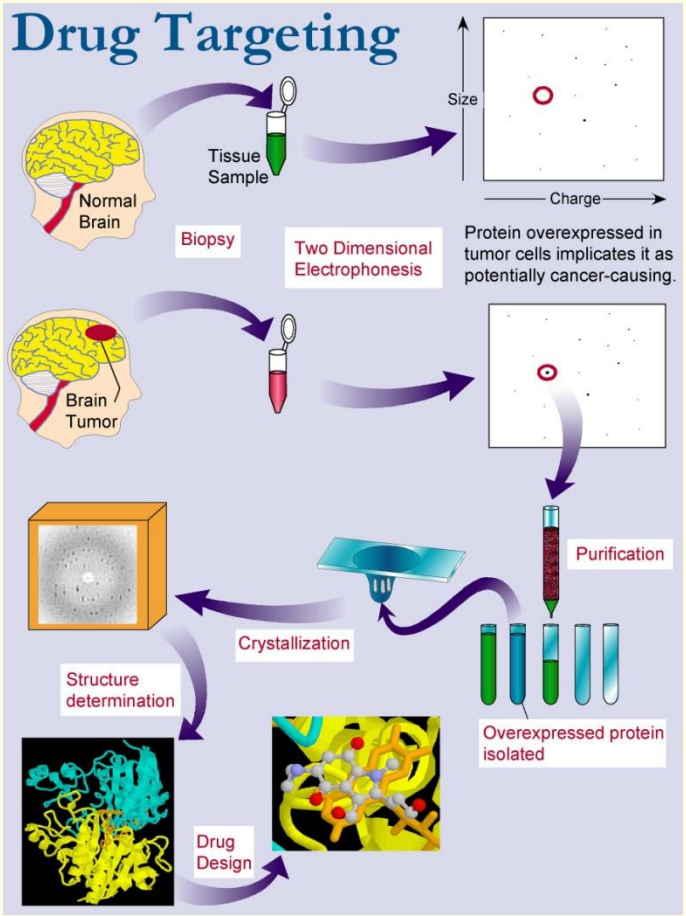
Protein Profiling Techniques



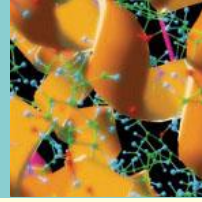
- Two-dimensional gel electrophoresis.
- Chemical protein sequencing.
- Protein sequencing by mass spectrometry.

Advantages of Protein Profiling

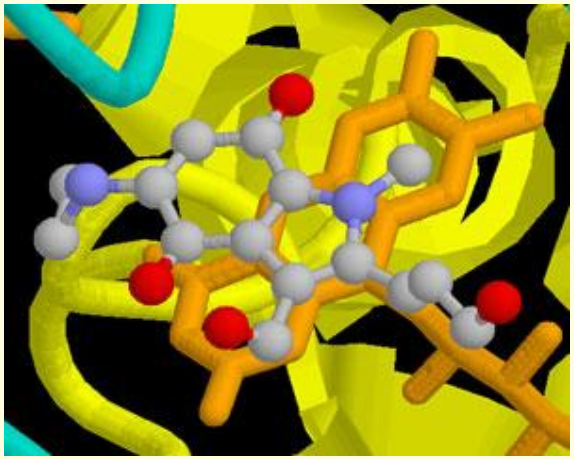
- Identify how genome is utilized in different tissues.
- Compare healthy and diseased tissue to find protein culprit.
- Identify targets for rationally designed drugs.



Rational Drug Design

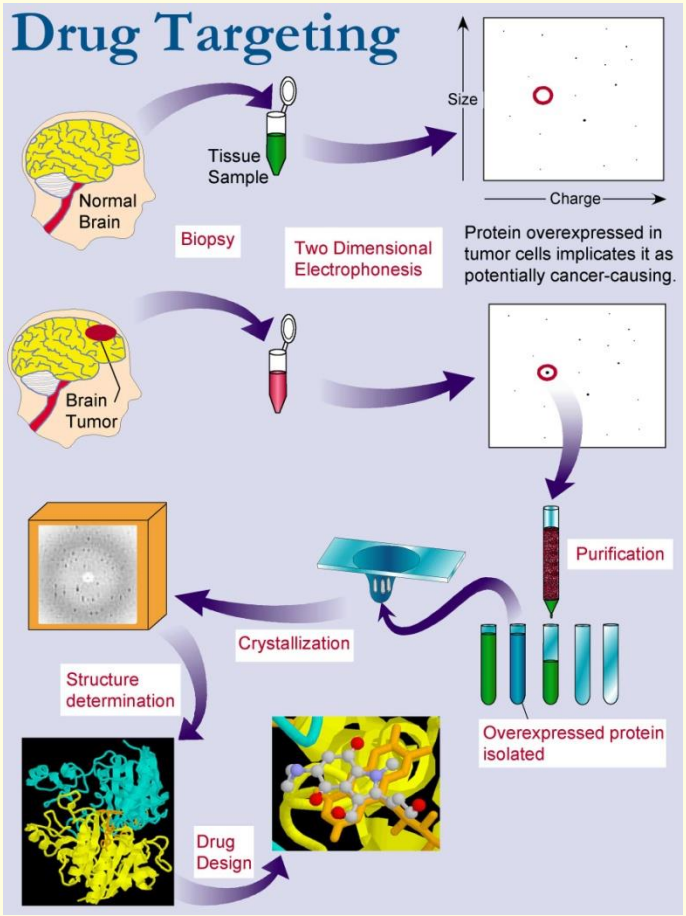


- Identify protein that is causative agent of disease.
- Custom shape a drug to alter function of disease-causing protein.
- Specifically targeted molecules may have fewer side effects.



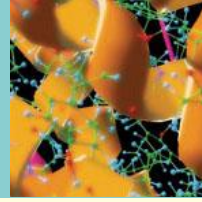
Rational Drug Design

- Compare proteome of healthy and cancerous tissue.
- Identify protein linked to onset of cancer.
- Determine 3-D shape.
- Design drug to alter protein function.



Protein Structure Prediction

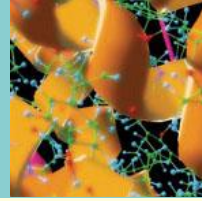
- Accurate determination of the three-dimensional shape of a protein from its amino acid sequence.



Protein Structure Determination Techniques

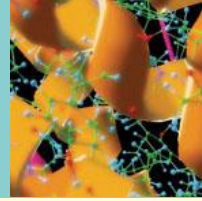
- X-ray crystallography reliable but slow, not all proteins crystallize.
- Current computer structure-prediction programs not reliable for all proteins.
- Proteomics scientists working on more sophisticated prediction algorithms to take advantage of genomic data.

Advantages of Protein Structure Prediction



- Can be used for any protein whose amino acid sequence is known.
- Speed – much faster than crystallography.
- Understand (structure dependent) function of proteins.
- Protein structure needed for drug design.

Protein Networks



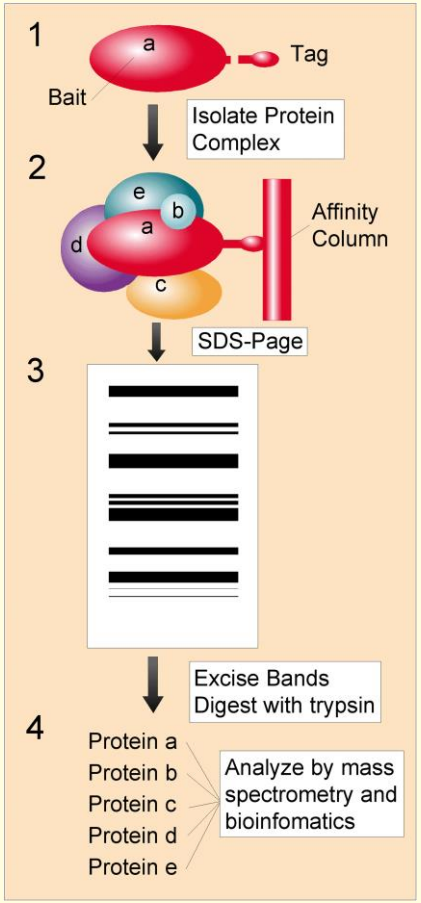
- Most proteins interact with more than one other protein in the cell.

- Many proteins may have multiple tasks in a cell.

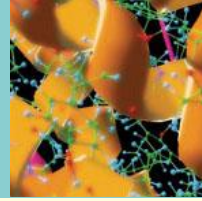
Studying Protein Networks: Complex Isolation

- Cell produces tagged “Bait” proteins.
- Cell contents poured over tag affinity column to capture complexes.
- Proteins in each cluster are identified.

- Has been witnessed that ~80% of proteins interact with each other.

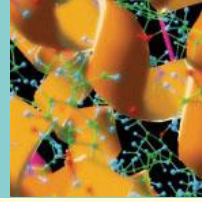


Advantages of Understanding Protein Complexes



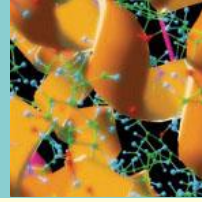
- Understand how proteins work together in metabolic pathways.
- Understand regulatory networks.
- Predict side effects of designed drugs.

The Future of Proteomics



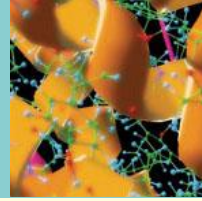
- Complement to genome data.
- Future of field depends on technological advances that will allow rapid analysis of thousands of unique proteins.
- Great potential for medical advances.
  - Disease diagnostics.
  - Rationally designed drugs.

How can proteins be extracted from cells?



- Many steps/techniques are needed to extract and separate protein of interest from many contaminants
- Separation techniques – size, charge and polarity
- Before purification begins, protein must be released from cell by **homogenization**

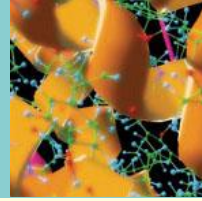
Isolation of Proteins from Cells



Many different proteins exists within one cell

- Many steps needed to extract protein of interest, and separate from many contaminants
- Before purification begins, protein must be released from cell by **homogenization**

What are different ways of homogenization of cells?



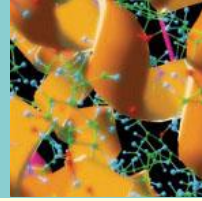
- Grinding tissue in a **blender** with a suitable buffer
- Releases soluble proteins and various subcellular organelles

- **Potter-Elvejhem homogenizer** – A thick walled test tube with a tight fitting plunger
- Breaks open cells – organelles intact

- **Sonication** – Sound waves to break open cells

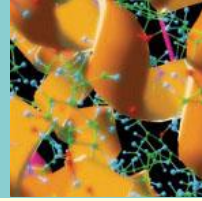
- Continuous **freezing and thawing** – Ruptures cells

Some important concepts for sample preparation



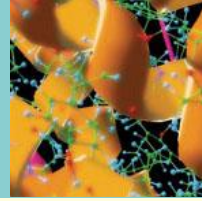
1. A good sample preparation is the key to good result.
2. The protein composition of the cell lysate or tissue must be reflected in the patterns of 2-DE.
3. Avoid protein contamination from environment.
4. Co-analytical modification (CAM) must be avoided. (pre-purification sometimes leads to CAM)
5. Highly selective procedure for tissue analysis (Laser capture micro dissection, LCM)

Some important concepts for sample preparation



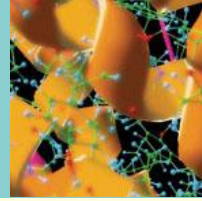
6. Treatment of sample must be kept to a minimum to avoid sample loss.
7. Keep sample as cold as possible.
8. Shorten processing time as short as possible.
9. Removal of salts
10. Minimized the unwanted processing, eg proteolytic degradation, chemical modification.

Frequently applied treatments



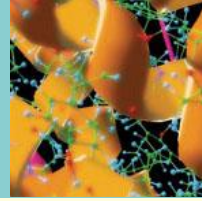
1. Cell washing
2. Cell disruption
3. Removal of contaminant
4. Microdialysis
5. Electrophretic desalting
6. Precipitation methods
7. For very hydrophobic protein

1. Cell washing



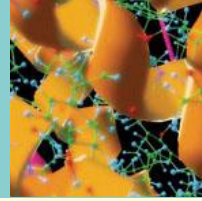
- To remove contaminant material.
- Frequent used buffer
  - PBS: phosphate buffer saline, sodium chloride, 145 mM (0.85%) in phosphate buffer, 150 mM pH7.2
  - Tris buffer sucrose (10mM Tris, 250 mM sucrose, pH 7,2)
- Enough osmoticum to avoid cell lysis

2. Cell disruption



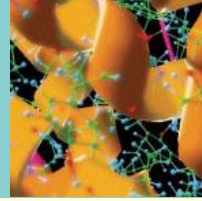
- Gentle lysis method
  1. Osmotic lysis (cultured cells)
     - Suspend cells in hypoosmotic solution.
  2. Repeated freezing and thawing (bacteria)
     - Freeze using liquid nitrogen
  3. Detergent lysis (yeast and fungi)
     - Lysis buffer (containing urea and detergent)
     - SDS (have to be removed before IEF)
  4. Enzymatic lysis (plant, bacteria, fungi)
     - Lysomzyme (bacteria)
     - Cellulose and pectinase (plant)
     - Lyticase (yeast)

2. Cell disruption (continued)



- Vigorous lysis method
  1. Sonication probe (cell suspension)
     - Avoid overheat, cool on ice between burst.
  2. French pressure (microorganism with cell wall)
     - Cells are lysed by shear force.
  3. Mortar and pestle (solid tissue, microorganism)
     - Grind solid tissue to fine powder with liquid nitrogen.
  4. Sample grinding kit (for small amount of sample)
     - For precious sample.
  5. Glass bead (cell suspension, microorganism)
     - Using abrasive vortexed bead to break cell walls.

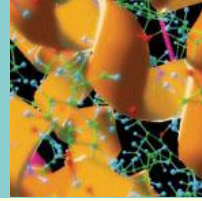
2. Cell disruption (continued)



- Key variable for successful extraction from crude material

1. The method of cell lysis
2. The control of pH
3. The control of temperature
4. Avoidance of proteolytic degradation

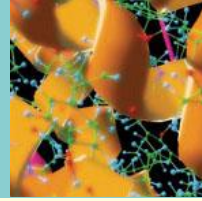
3. Removal of contaminants



- Major type of contaminants:

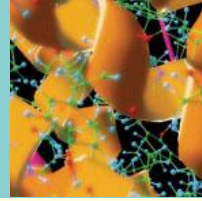
1. DNA/RNA
2. Lipids
3. polysaccharides
4. Solid material
5. Salt

DNA/RNA contaminant



- DNA/RNA can be stained by silver staining.
- They cause horizontal streaking at the acidic part of the gel.
- They precipitate with the proteins when sample applying at basic end of IEF gel
- How to remove:
  1. precipitation of proteins
  2. DNase/RNase treatment
  3. sonication (mechanical breakage)
  4. DNA/RNA extraction method (phenol/chroloform)

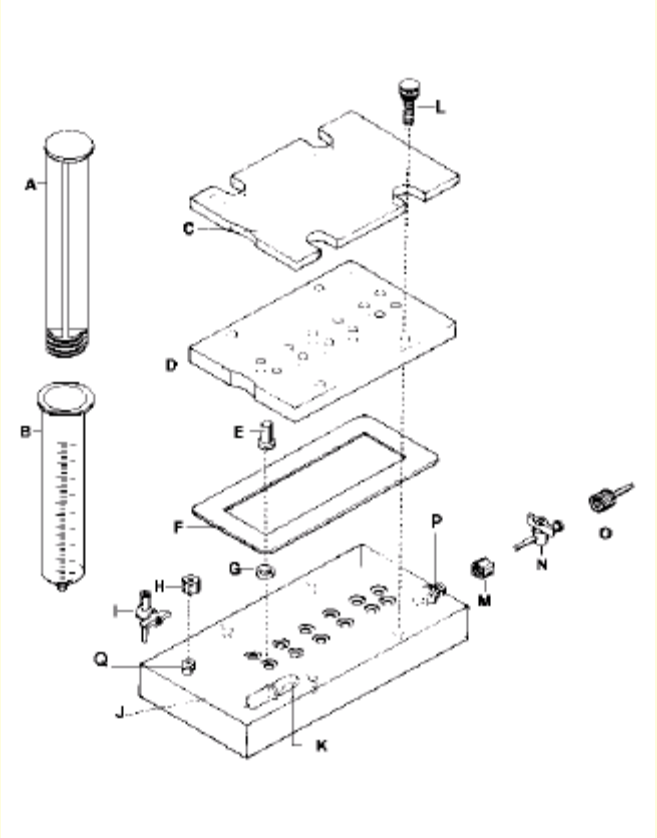
Removal of other contaminants



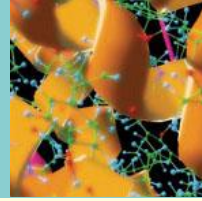
- Removal of lipids:
  - >2% detergent
  - Precipitation
- Removal of polysaccharides:
  - Enzymatic procedure
  - Precipitation
- Removal of solid material
  - Centrifugation
- Removal of salts
  - Microdialysis
  - Precipitation

4. Microdialysis

- Specially design for small volume samples
- Membrane cut-off is about 8000 Da
- Drawbacks:
  - 1. Time consuming (some protease might be active and digest proteins during the dialysis)
  - 2. Some proteins precipitation after dialsis.



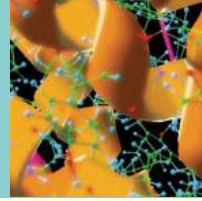
5. Electrophoretic desalting



- There are some case where the sample must not be dialysed. (halobacteria lysate)
- Some proteins will gel if desalted. (Bovine vitreous proteins)

Solution for above: low voltage (100V) for 5 hours before IEF running. (A. Gorg, 1995)

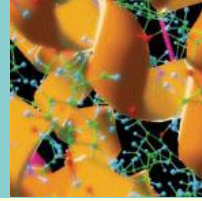
6. Precipitation methods.



- The reasons for applying protein precipitation procedure:

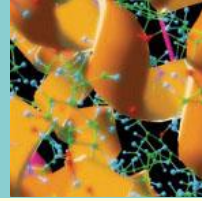
    1. Concentrate low concentrated protein samples.
    2. Removal of several disturbing material at the same time.
    3. Inhibition of protease activity.

6. Five precipitation methods.



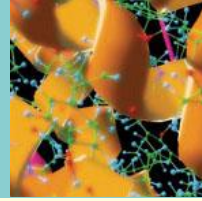
1. Ammonium sulfate precipitation
2. TCA precipitation
3. Acetone precipitation
4. TCA/Acetone precipitation
5. Ammonium acetate/method following phenol extraction

Ammonium sulfate precipitation



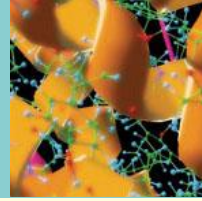
- Proteins tend to aggregate in high concentration of salt (salting out)
  - Add Ammonium sulfate slowly into solution and stir for 10-30 mins
  - Harvest protein by centrifugation.
- Limitation
  - Some proteins are soluble at high salt conc.
  - Ammonium sulfate seriously affect IEF.

TCA precipitation



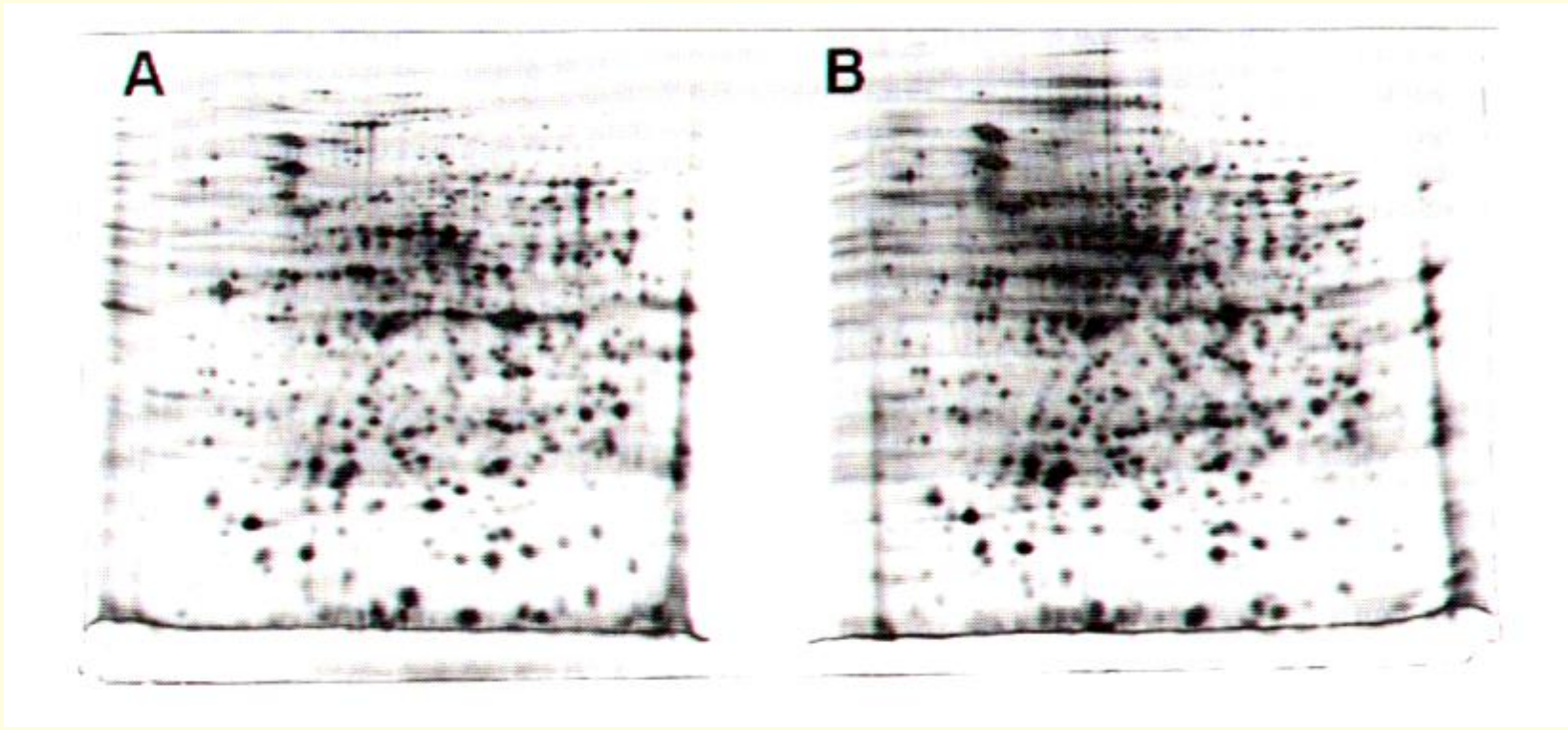
- Trichloroacetic acid is a very affective protein precipitant.
  - Add TCA to extract to final conc.10-20%.
  - Add 10-20% TCA directly to tissue or cells.
  - Harvest protein by centrifugation.
  - Wash access TCA by ethanol or acetone.
- Limitation
  - Sometimes the pellet is hard to redissolve.
  - TCA must remove complete. (affecting IEF)
  - Some degradation or modification of protein occurs

Acetone precipitation



- The most common organic solvent used to precipitated proteins, lipid and detergent remain in solution.
  - Add at least 3 vol. of ice-cold acetone into extract.
  - Stand on ice for at least 2 hours.
  - Harvest protein by centrifugation.
  - Remove access acetone by air drying.
- Limitation
  - Sometimes the pellet is hard to redissolve.
  - Some proteins would not precipitate.
  - DNA/RNA and glycan also precipitate.

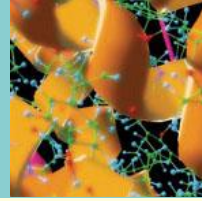
Example, Acetone precipitation



With Acetone precipitation

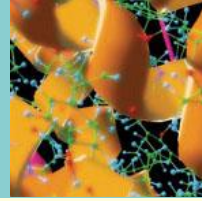
Crude extract by lysis buffer

TCA/acetone precipitation



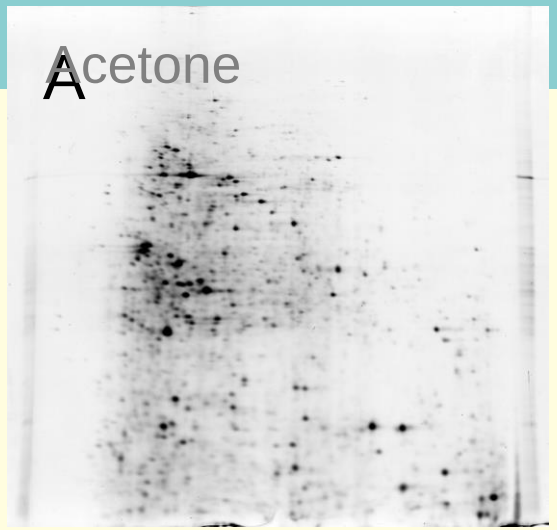
- The method is more active than TCA or acetone alone. Most commonly used in 2-DE.
  - Suspension samples in 10% TCA/Acetone with 0.07% 2-mercaptoethanol or 20mM DTT.
  - Stand on -20C for at least 45mins.
  - Harvest protein by centrifugation.
  - Wash the pellet by acetone with0.07% 2-mercaptoethanol or 20mM DTT.
  - Remove access acetone by air dry.
- Limitation
  - Sometimes the pellet is hard to redissolve.
  - TCA must remove complete. (affecting IEF)
  - Some degradation or modification of protein occurs

Precipitation with ammonium acetate in methanol following phenol extraction

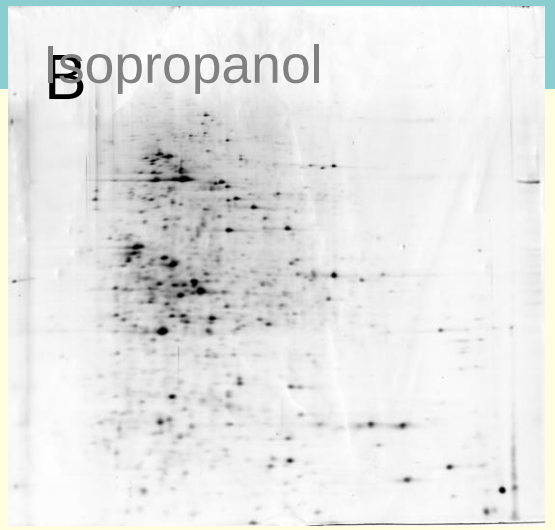


- The method is more suitable for plant sample with high level of interfering substance
  - Proteins are extracted into buffer saturated phenol.
  - Precipitated by ammonium acetate/methanol.
  - Harvest protein by centrifugation.
  - Wash with ammonium acetate/methanol followed by acetone.

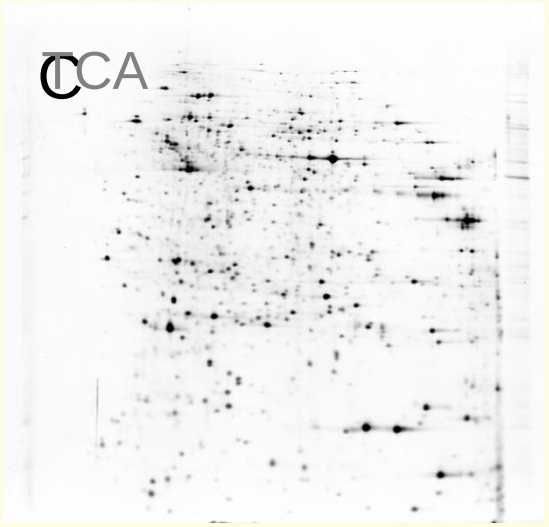
- Limitation
  - Complicated.
  - Time consuming.



Σ 823 spots



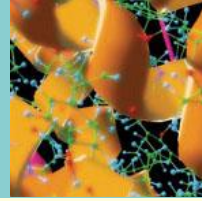
Σ 757 spots



Σ 969 spots

Σ 899 spots

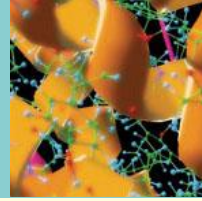
7. For very hydrophobic proteins



Membrane proteins do not easily go into solution. A lot of optimization work is required.

1. Thiourea procedure
2. SDS procedure
3. New zwitterionic detergent and sulfobetains

Thiourea procedure



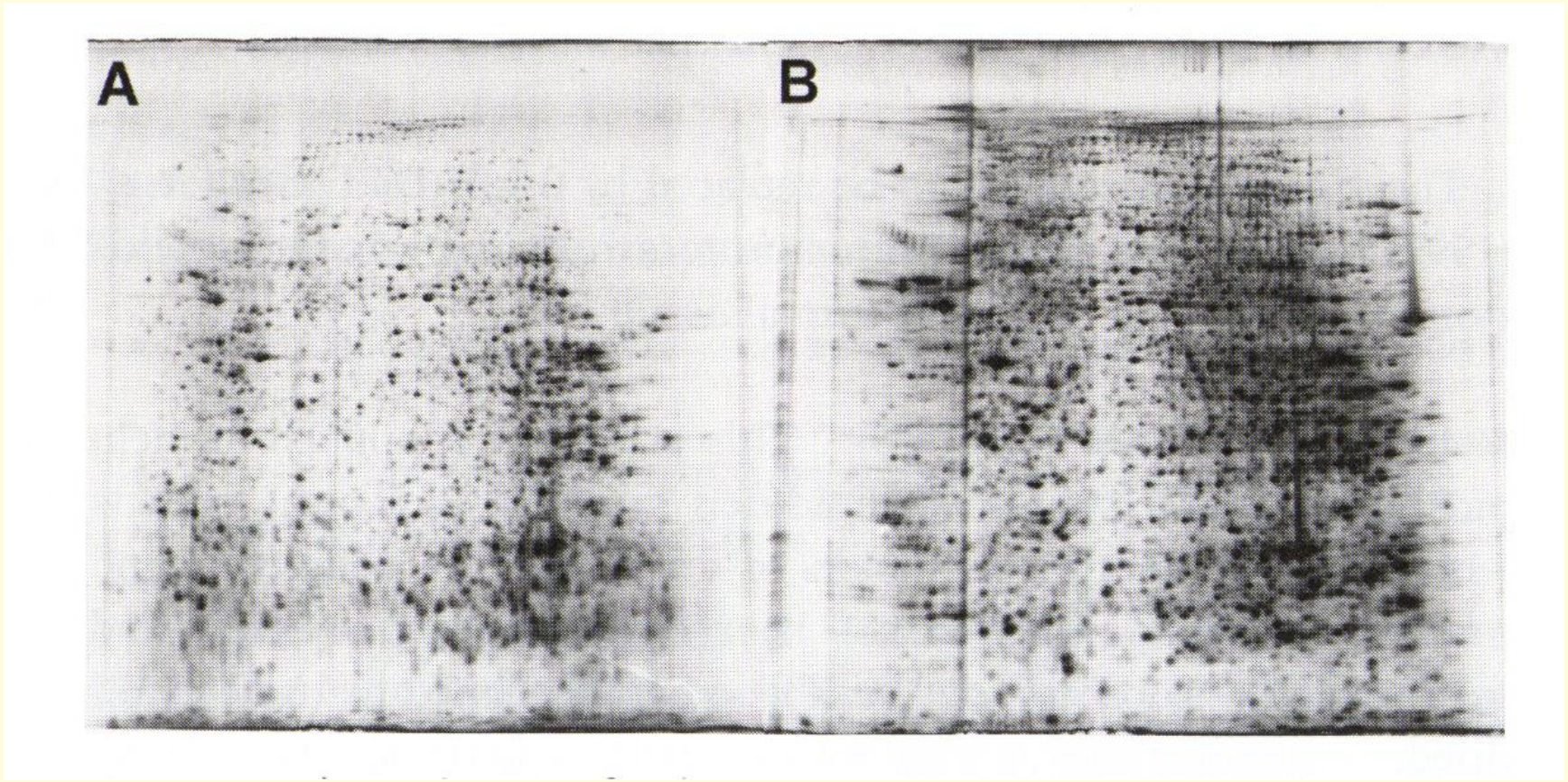
7M urea + 2M thiourea (Rabilloud, 1998)

Pros: Increase spot number considerably.

Cons: Causing artifact spots.

Causing vertical streaking at acidic area.

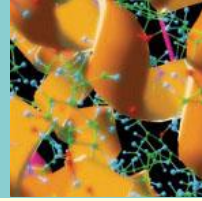
Example, thiourea procedure



Lysis buffer, 8M urea

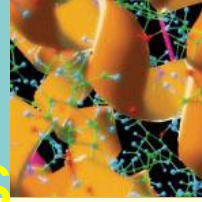
Lysis buffer, 7M urea+ 2M thioure

SDS procedure



- For emergency case.
- Up to 2% SDS can be used.
- Have to dilute SDS samples at least 20 fold with urea an a non or zwitterionic detergent containing solutions.
- The major reasons for using SDS:
    1. Formation of oligomers can be prevented
    2. Dissolved tough cell walls samples (with boiling)
    3. Dissolved very hydrophobic proteins

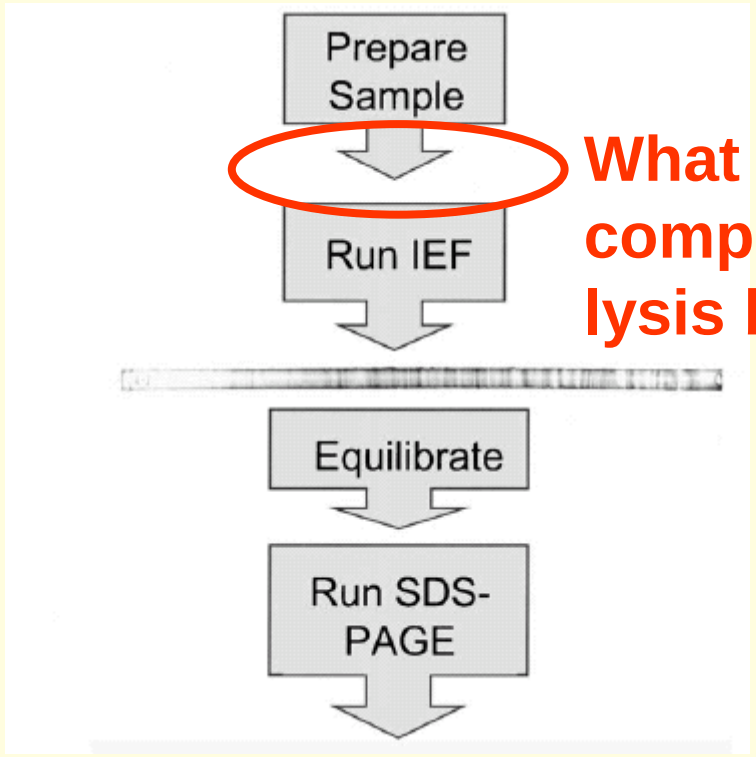
New zwitterionic detegent and sulfobetains



Three major types of detergent

1. Non ionic detergent
   - Triton x-100, Tween 20, Brij-35
2. Ionic detergent
   - SDS, CTAB, Digitonin
3. Zwittergent
   - CHAPS, CHAPSO, Zwittergent 3-08, 3-10, 3-12…

Now, we are ready to dissolve protein samples in IEF lysis buffer

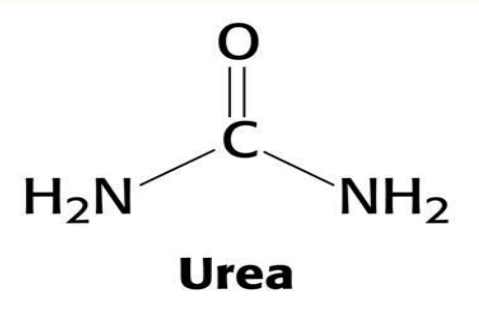


What is the composition in IEF lysis buffer?

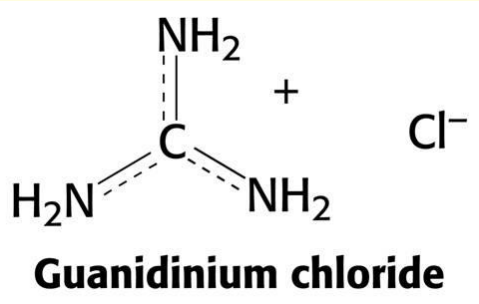
2-DE are in denaturing condition

Three components must present in 2-DE denaturing condition (namely, in IEF lysis buffer)

1. Urea (often > 7M)
2. Reductant (DTT used most widely)
3. Non-ionic or zwitterionic detergent
4. Dye

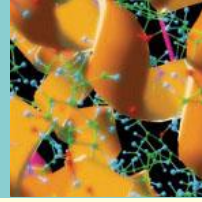


Urea



Guanidinium chloride

why not using native condition

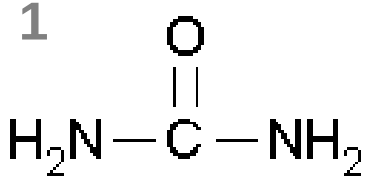


1. Under native condition, a great part of proteins exists in several conformations. This leads to more complex 2-DE patterns.
2. Native protein complexes sometimes too big to enter the gel.
3. Reduction of protein-protein interactions.
4. For match the theoretical pI and MW, all proteins should not have 3D structure or quanternary structure.

Composition of standard lysis buffer (for IEF)

1. 9M urea
2. 4% CHAPS
3. 1% DTT
4. 0.8% carrier ampholyte
5. 0.02% bromophenol blue.

1



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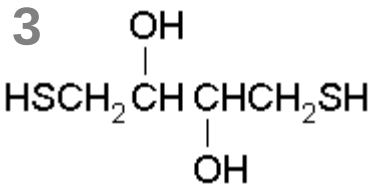
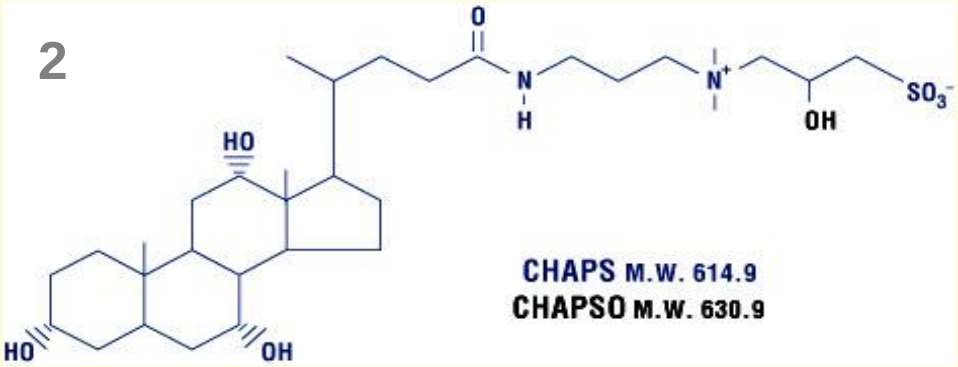
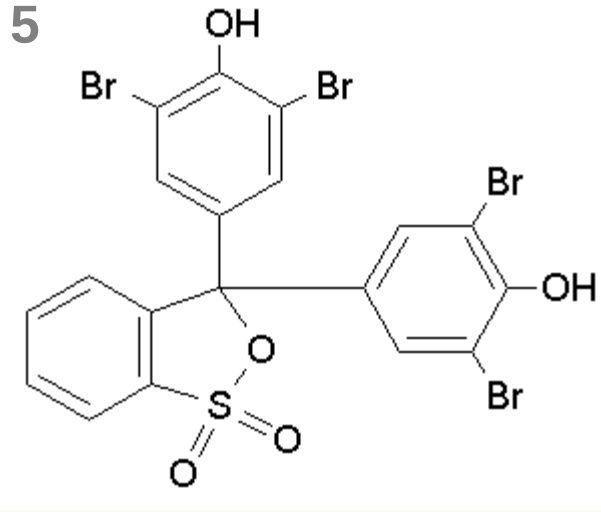


2

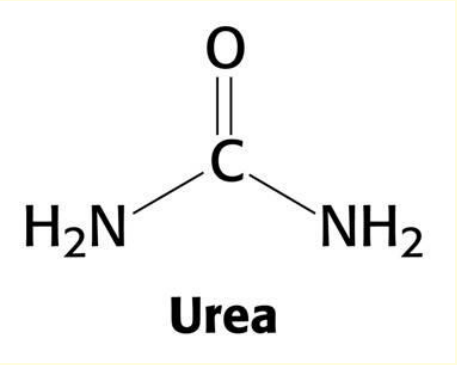


5

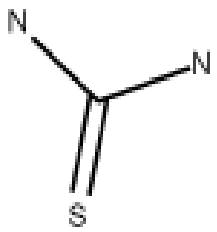


Functions of denaturant (Urea)

1. To convert proteins into single conformation by canceling 2nd and 3rd structure.
2. To keep hydrophobic proteins into solution.
3. To avoid protein-protein interaction.
4. Thio urea: for very hydrophobic proteins only.

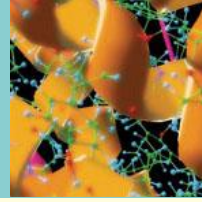


Urea



Thiourea

Beware when using urea



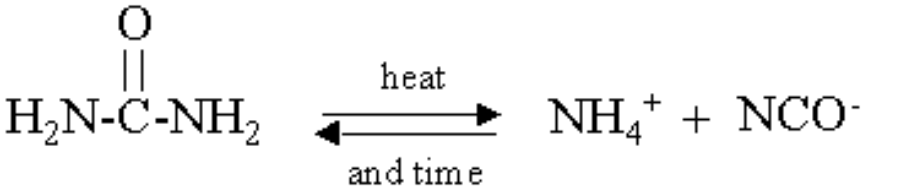
1. The purity of urea is very critical

2. Isocyanate impurities and heating will cause carbamylation of the proteins.

3. It does not seem to make a difference what grade of urea is used because, urea + heat + protein = carbamylation.

Carbamylation of proteins

Decomposition of Urea



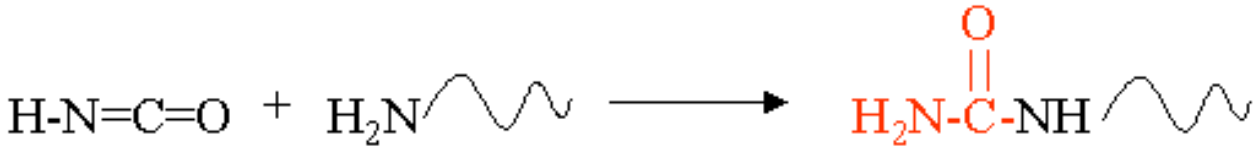
Urea

Ammonium Cyanate

IonSource.Com

Carbamylation of Proteins

(amino terminus of a peptide used as an example)

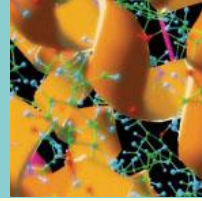


Isocyanic Acid

Peptide Amino Terminus
(or side chain of Lys or Arg)

Carbamylated Peptide or Protein

Results of Carbamylation

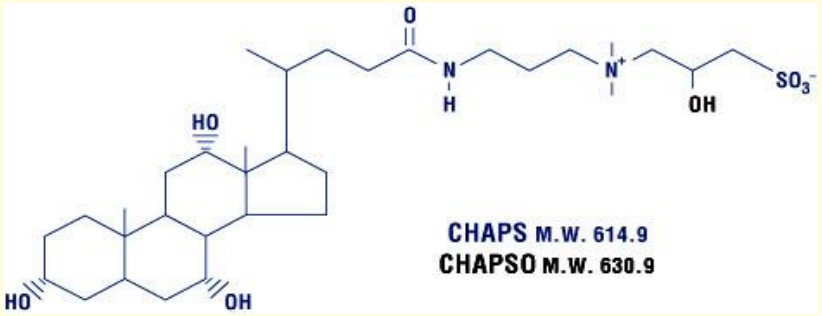


Amino Acid	Residue Composition	Residue Monoisotopic Mass	Delta Mass
Lysine	$C_6H_{12}N_2O$	128.09496	0
Carbamyl Lysine	$C_7H_{13}N_3O_2$	171.10078	43.00582
Carbamylation	*NHCO	43.00582	-

*Note: A proton is lost from the amino group on the protein during carbamylation and thus the change in composition is NHCO.

Functions of detergent (CHAPS)

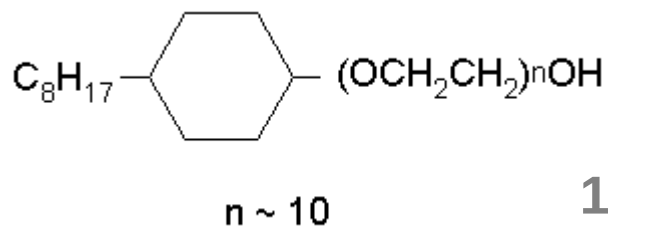
- To Combine all the advantages of polar, sulfobetaine-containing detergents and hydrophobic, bile salt, anionic detergents into a single molecule with superior membrane protein solubilization properties
- Non-denaturing
- Able to disrupt nonspecific protein interactions
- Less protein aggregation than non-ionic detergents
- Electrically neutral
- Easily removed by dialysis



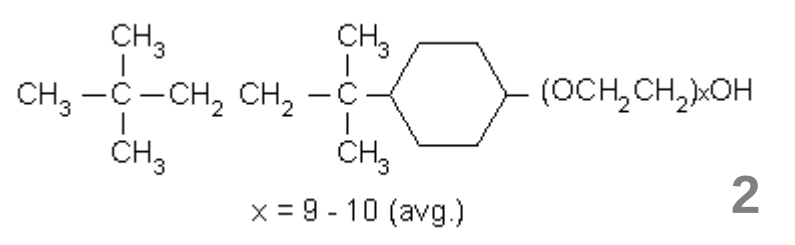
Other detergents

1. **Triton X-100**

(not easily remove and interfering MS)

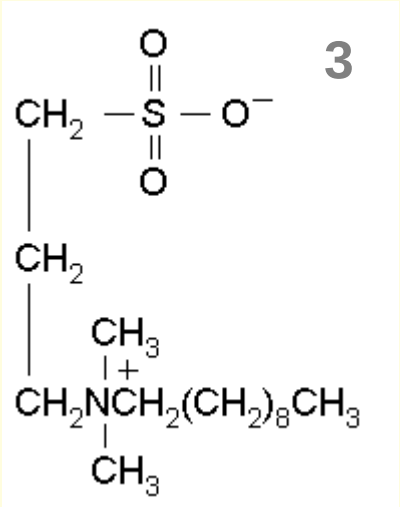


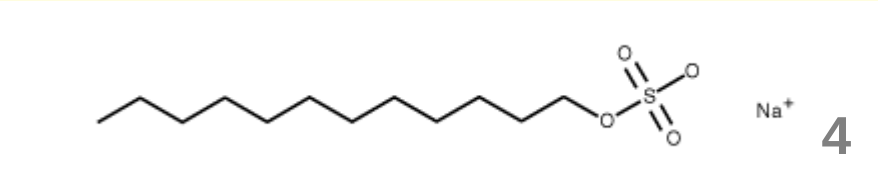
2. **Nonidet NP-40**



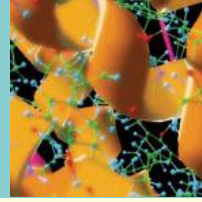
3. **SB3-10**

4. **SDS**





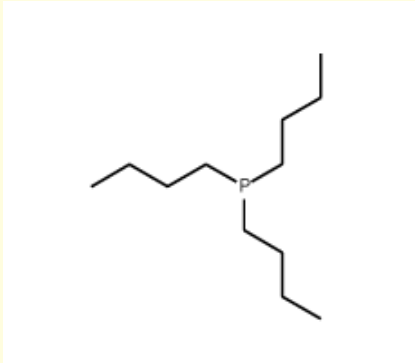
Functions of reductant



- To prevent different oxidation steps of proteins.
- 2-mercaptoethanol should not be used because its buffering effect above pH 8.
- Keratin contamination might from 2-mercaptoethanol.
- DTT (dithiothreitol) or DTE (dithioerythritol) are used widely.
- DTT and DTE ionized above pH8. They m[redacted] anode during IEF in basic pH gradient.
- It leads to horizonal streaking at basic area[redacted]

Other reduction methods

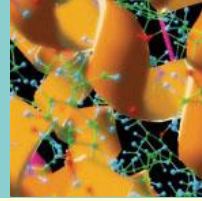
- TBP (tributylphosphine): very unstable.



- An alternative way to adequate and reproducible 2-DE patterns in basic area:

1. Addition of higher amount of DTT to the gel
2. Addition of more DTT to a cathodal paper strip.

Function of carrier ampholyte

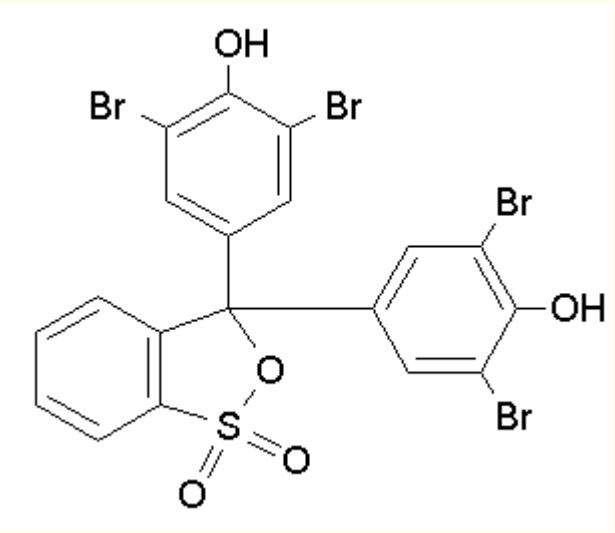


They do not disturb IEF like buffer addition, because they become uncharged when migrating to their pI.

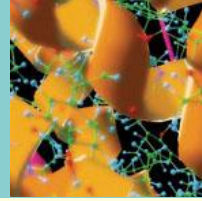
1. To generate pH gradients
2. To substituting ionic buffer
3. To improve the solubility of protein
4. Dedicated pH intervals, prepared for the addition to immobilized pH gradients, are called IPG buffer.

Function of dyes

- To visualize the sample solution
- To monitor the 2-DE running condition.
- Bromophenol blue is interchangeable with Orange G.



Other considerations

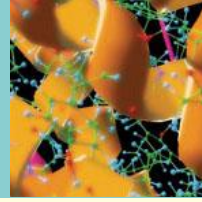


- Protease inhibitors

  - 1. Some proteases are also active in presence of urea and detergent.
  - 2. PMSF is frequently used (8mM), toxic and short half-life.
  - 3. Pefabloc (AEBSF) can also be used but modified proteins.
  - 4. NO complete insurance against protease activity
  - 5. Boiling sample in SDS buffer for a few seconds can inactive protease.
  - 6. Precipitate proteins with TCA/acetone at -20C might inactivation protease activity.

Protease inhibitor	Effective against:	Limitations
PMSF (Phenylmethylsulfonyl fluoride) Most commonly used inhibitor. *Use at concentrations up to 1 mM.*	PMSF is an irreversible inhibitor that inactivates: • serine proteases • some cysteine proteases	PMSF rapidly becomes inactive in aqueous solutions: Prepare just prior to use. PMSF may be less effective in the presence of thiol reagents such as DTT or 2-mercapto-ethanol. This limitation can be overcome by disrupting the sample into PMSF-containing solution lacking thiol reagents. Thiol reagents can be added at a later stage. PMSF is very toxic.
AEBSF (Aminoethyl benzylsulfonyl fluoride or Pefabloc SC Serine Protease Inhibitor) *Use at concentrations up to 4 mM.*	AEBSF is similar to PMSF in its inhibitory activity, but is more soluble and less toxic.	AEBSF-induced modifications can potentially alter the pI of a protein.
1 mM EDTA or 1 mM EGTA *Generally used at 1 mM.*	These compounds inhibit metalloproteases by chelating free metal ions required for activity.	
Peptide protease inhibitors (e.g. leupeptin, pepstatin, aprotinin, bestatin) • reversible inhibitors • active in the presence of DTT • active at low concentrations under a variety of conditions *Use at 2–20 µg/ml.*	Leupeptin inhibits many serine and cysteine proteases. Pepstatin inhibits aspartyl proteases (e.g. acidic proteases such as pepsin) Aprotinin inhibits many serine proteases. Bestatin inhibits aminopeptidases.	Peptide protease inhibitors are: • expensive. • small peptides and thus may appear on the 2-D map, depending on the size range separated by the second-dimension gel. Pepstatin does not inhibit any proteases that are active at pH 9.
TLCK, TPCK (Tosyl lysine chloromethyl ketone, tosyl phenylalanine chloromethyl ketone) *Use at 0.1–0.5 mM.*	These similar compounds irreversibly inhibit many serine and cysteine proteases.	
Benzamidine *Use at 1–3 mM.*	Benzamidine inhibits serine proteases.	

Other considerations



- Alkaline condition

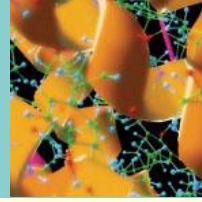
Tris base (40mM) or spermidine (25mM) sometimes add to lysis buffer to maximize protein extraction.

– Pros:

1. They can also precipitate DNA/RNA.
2. They keep proteasse activity low.

– Cons:

1. Precipitation of basic protein.
2. Ionic contamination is to high.



Before runninng IEF, you should…

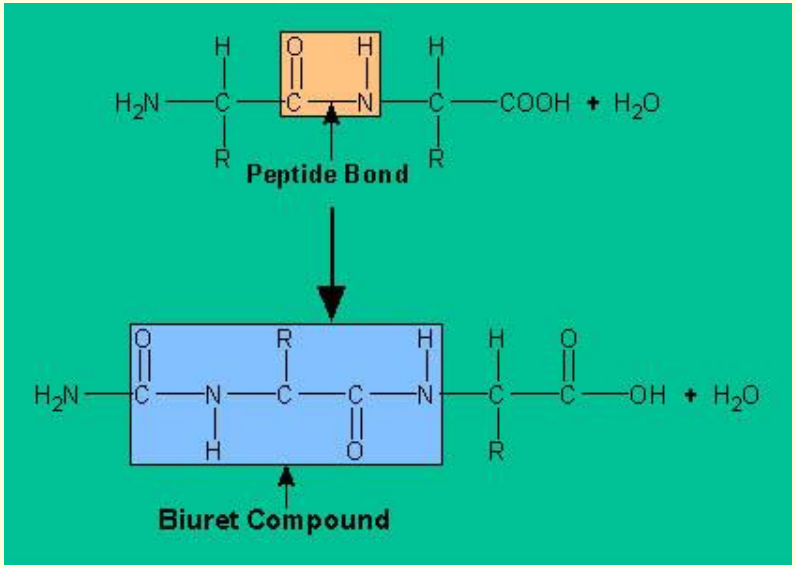
- Measure the protein conc. in your samples.

– Widely used protein assay methods

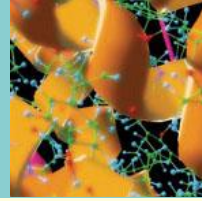
1. Biuret
2. Lowry methods.
3. Bradford methods.
4. UV methods.
5. Special methods
6. Other commercial methods.
    1. BCA assay (bicinchoninic acid assay, Pierce)
    2. DC protein assay (detergent compatible, Bio-rad)
    3. DC/RC protein assay (detergent/reducing agent

1. Biuret method

- Principle: The reactivity of the peptide bonds with the copper [II] ions under alkaline conditions to form purple biuret complex.
- Interfering substance: Ammonium sulfate, Tris, etc.
- Sensitivity: >mg



A white, crystalline, nitrogenous substance, C2O2N3H5, formed by heating urea. It is intermediate between urea and cyanuric acid.

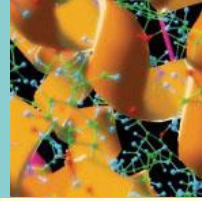


2. Lowry method

- Principle: The reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids (Try, Try).

- Interfering substance: amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids, ammonium ions, zwitterionic buffers, nonionic buffers and thiol compounds.

- Sensitivity: > 0.1 mg

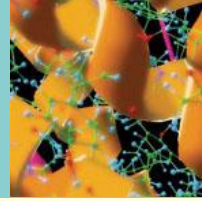


3. Bradford method

- Principle: The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie® dye binds primarily with basic and aromatic side chains. The interaction with arginine is very strong and less strong with histidine, lysine, tyrosine, tryptophan, and phenylalanine. About 1.5 to 3 molecules of dye bind per positive charge on the protein.

- Interfering substance: amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids, ammonium ions, zwitterionic buffers, nonionic buffers and thiol compounds.

- Sensitivity: >10 -100 ug

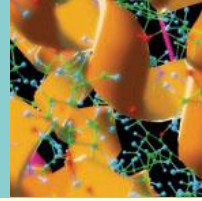


4. UV methods

- Principle: The aromatic groups (Phe, Tyr, Trp) and the peptide bonds have maximum UV absorbance around 280nm and 200nm. 280nm was used most frequently.

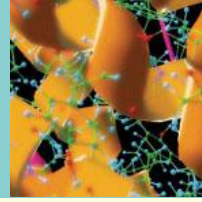
- Interfering substance: anything containing

- Sensitivity: >mg



5. Special methods

- Principle: Some proteins contain functional groups, eg: Heme in peroidase, hemoglobin and transferrin can be detected at 403nm, Cd2+ in some phytochelatins.
- Interfering substance: similar functional groups.
- Sensitivity: various



6. Commercial methods

A. BCA assay (bicinchoninic acid assay, Pierce)

This process is a two-step reaction.

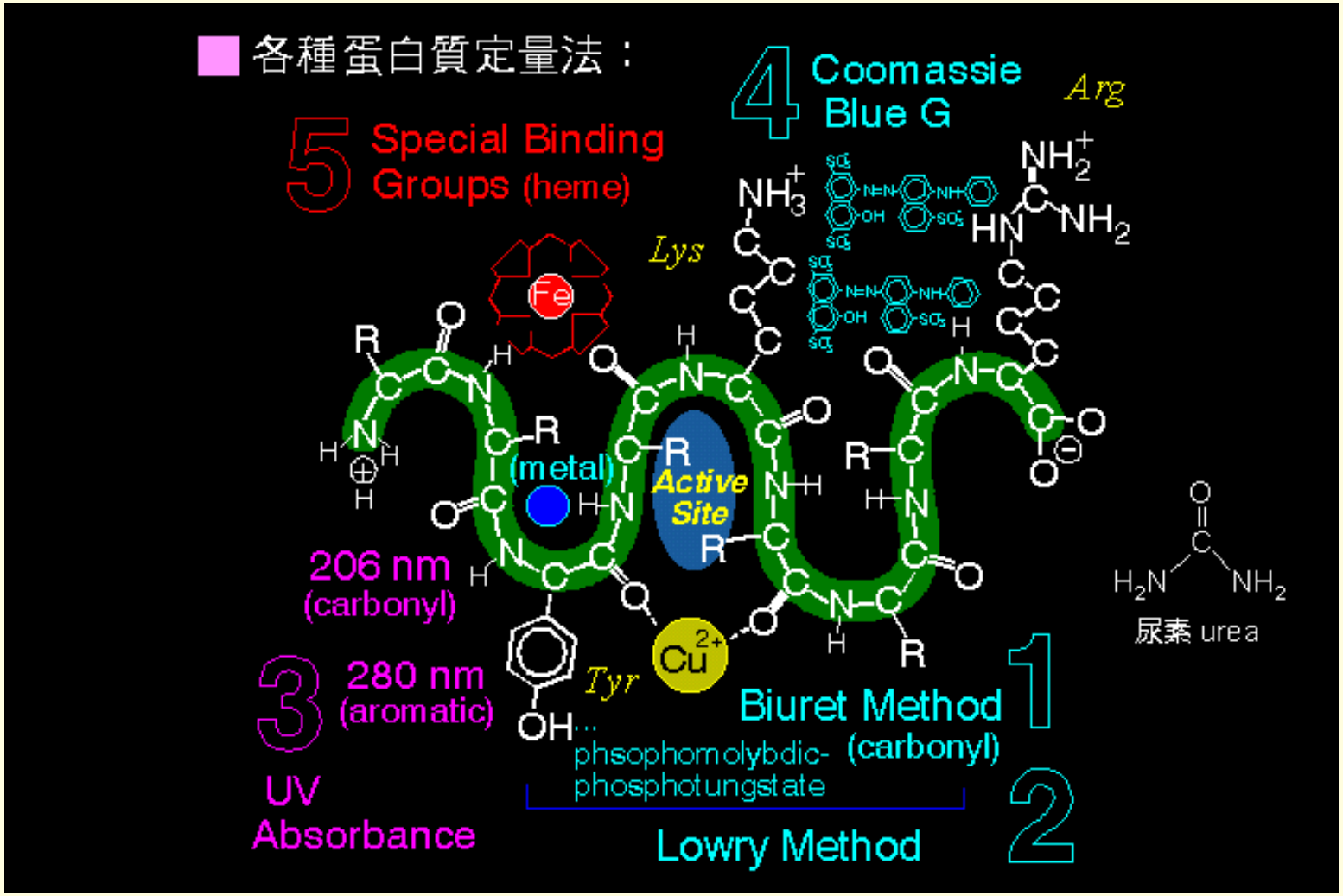
Protein + Cu^{2+} + OH^- → Cu^{1+}

Cu^{1+} + 2 BCA → Cu^{1+}/BCA chromophore (562 nm).

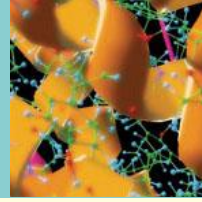
B. DC protein assay (detergent compatible, Bio-rad)

C. DC/RC protein assay (detergent/reducing agent compatible, Bio-rad)

Summary of protein quantitation methods



Working with proteins



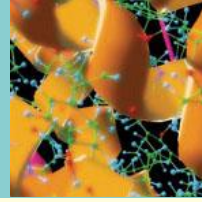
Classical methods for separating proteins take advantage of properties that vary from one protein to the next

1. Crude extract (tissues or microbial cells)

2. Separation and purification of individual components

3. Protein characterization (molecular mass, amino acid composition and sequence)

Purification techniques



1. **based on molecular size**
   - dialysis and ultrafiltration
   - density gradient centrifugation
   - size-exclusion chromatography)

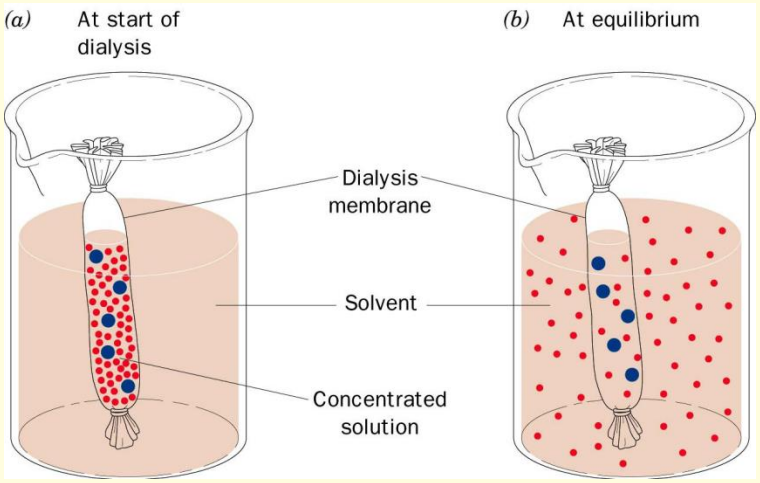
2. **based on solubility of proteins**
   - izoelectric precipitation
   - salting out

3. **based on electric charge**
   - ion-exchange chromatography
   - electrophoresis

1. Separation procedures based on molecular

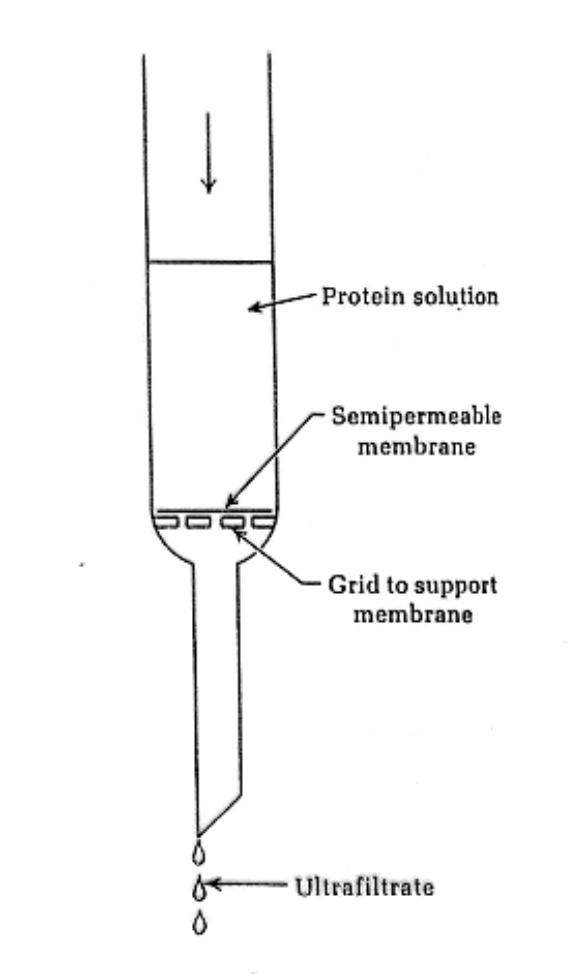
Dialysis and ultrafiltration

Procedures, that separate proteins from small solutes.



Membrane enclosing the protein solution is semipermeable, allows the exchange water and small solutes (glucose, salts) pass through the membrane freely but protein do not.

orce



How We Get Proteins Out of Cells

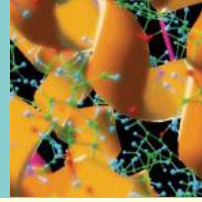
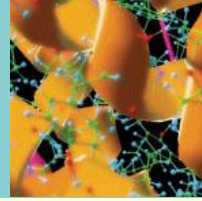


Table 5.1

Example of a Protein Purification Scheme: Purification of the Enzyme Xanthine Dehydrogenase from a Fungus

Fraction	Volume (mL)	Total Protein (mg)	Total Activity	Specific Activity	Percent Recovery
1. Crude extract	3,800	22,800	2,460	0.108	100
2. Salt precipitate	165	2,800	1,190	0.425	48
3. Ion-exchange chromatography	65	100	720	7.2	29
4. Molecular-sieve chromatography	40	14.5	555	38.3	23
5. Immunoaffinity chromatography	6	1.8	275	152.108	11

What is Salting out?

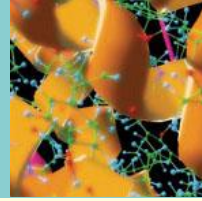


- **Ammonium sulfate (NH_4SO_4) -** used to "salt out"

- Takes away water by interacting with proteins
- makes protein **less soluble because hydrophobic interactions increases** among proteins

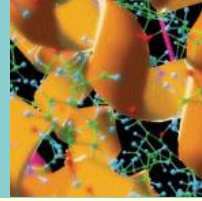
- Addition of salt – increases saturation
- Different set of proteins **precipitate**
- **Centrifuge and save the set of proteins**

Salting Out



- After Proteins solubilized, they can be purified based on solubility (usually dependent on overall charge, ionic strength, polarity
- Ammonium sulfate (NH_4SO_4) commonly used to “salt out”
- Takes away water by interacting with it, makes protein less soluble because hydrophobic interactions among proteins increases
- Different aliquots taken as function of salt concentration to get closer to desired protein sample of interest (**30, 40, 50, 75% increments**)
- One fraction has protein of interest

Salting out



- **Neutral salts influence the solubility of globular proteins.**

- **Hhydrophilic amino acid interact with the molecules of H_2O, allow proteins to form hydrogen bonds with the surrounding water molecules.**

- **Increasing salt concentrationn: attracted of the water molecules by the salt ions, which decreases the number of water molecules available to interact with protein. Increasing ionic strength decrease solubility of a protein.**

- **In general:**

a) small proteins more soluble than large proteins
b) the larger the number of charged side chains, the more soluble the protein
c) proteins usually least soluble at their isoelectric points.

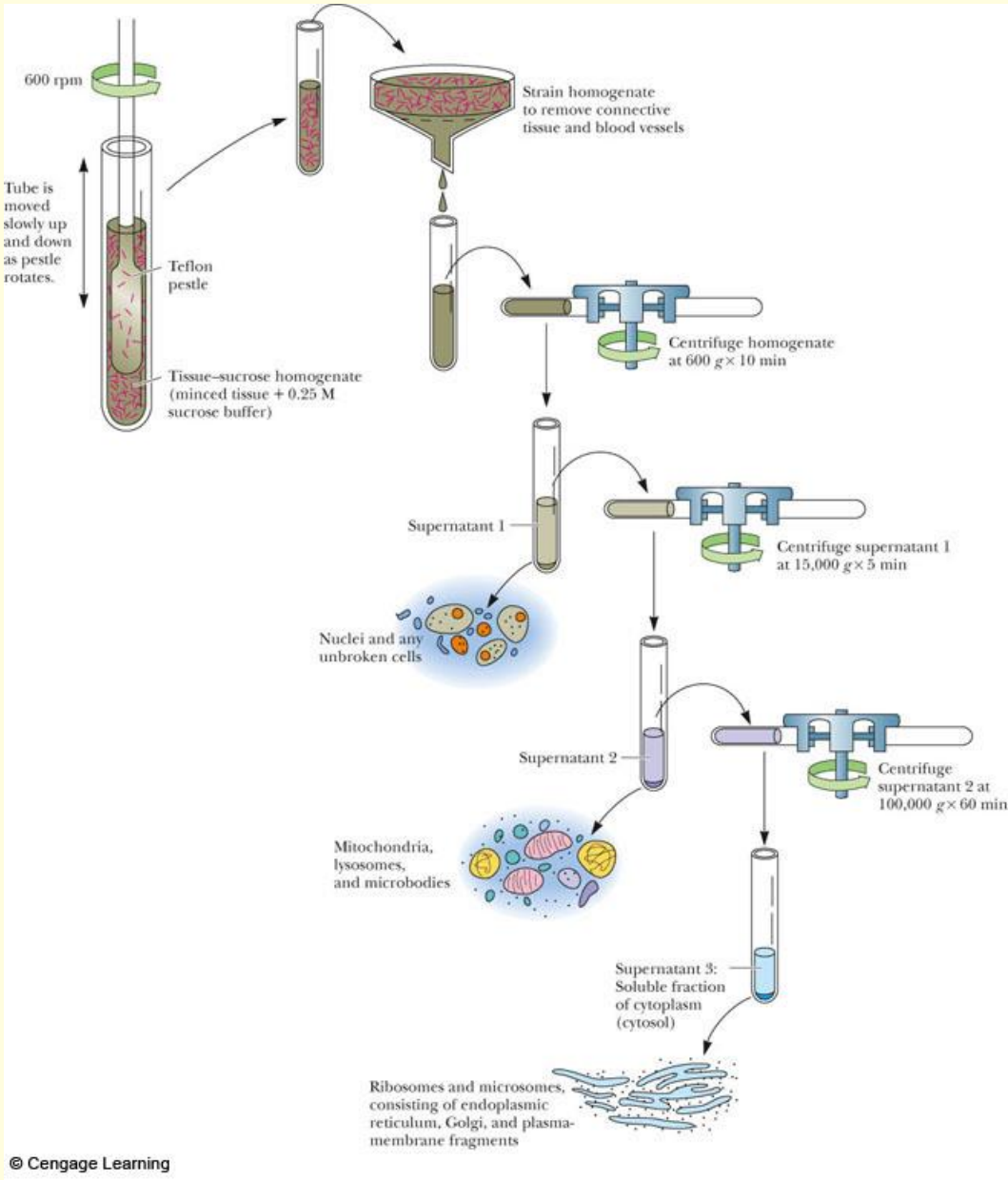
- **Sufficiently high ionic strength completely precipitate a protein from solution.**

- **Divalent salts [$MgCl_2$, $(NH_4)SO_4$] are far more effective than monovalent (NaCl)**

Differential Centrifugation

- Sample is spun, after lysis, to separate unbroken cells, nuclei, other organelles and particles not soluble in buffer used

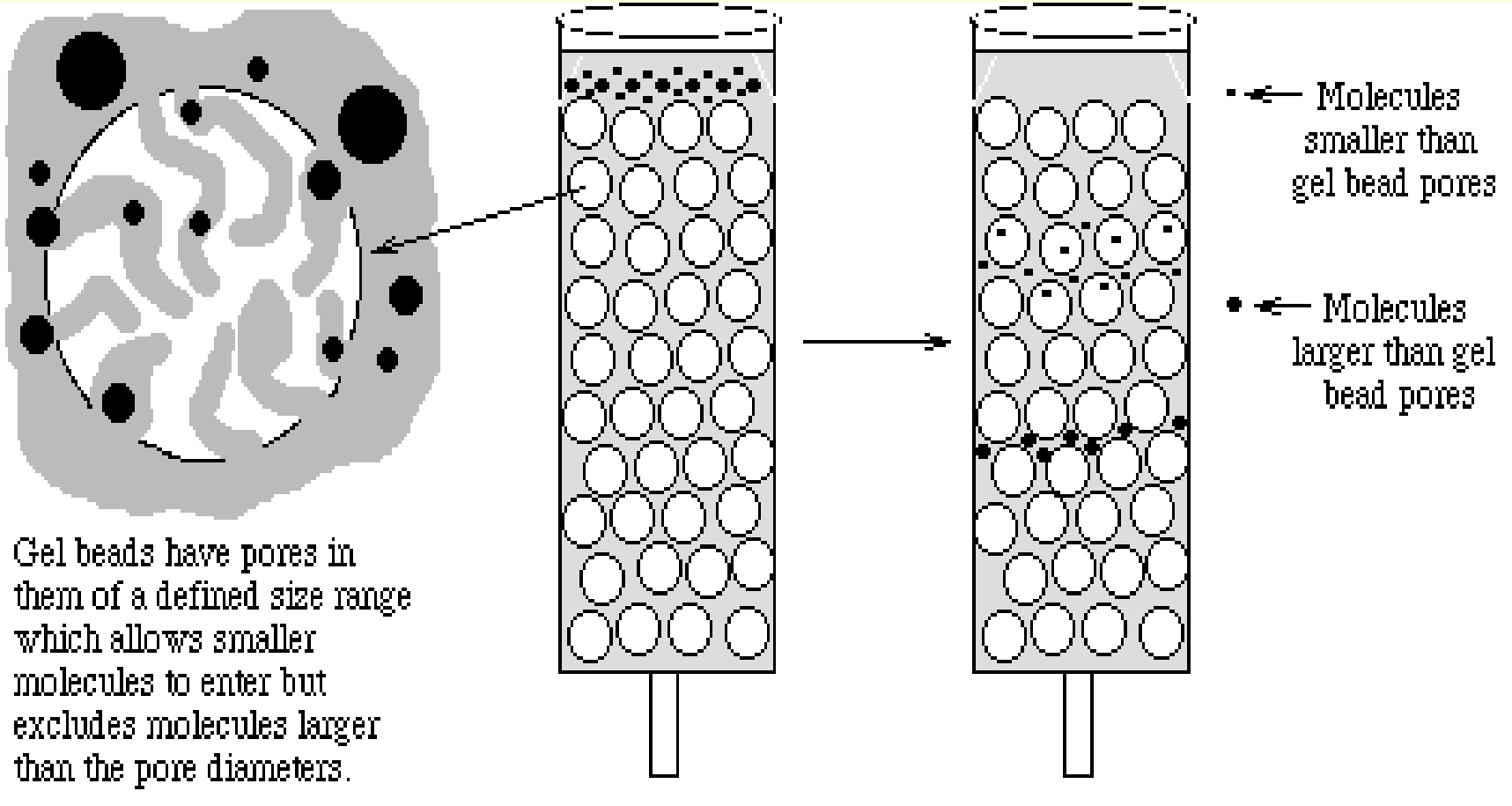
- Different speeds of spin allow for particle separation



B. Gel Filtration Chromatography

- Also called Gel Permeation Chromatography.
- Separates protein molecules according to their molecular **size.**
- The solution is inserted to the top of a specialized column.
- This column consists of specialized porous beads.
- Small molecules of protein enter the beads while large molecules can’t and stay in the space between the beads.
- Therefore, large molecules flow more rapidly through the column and emerge first from the bottom of the column.
- Advantage: larger quantities of proteins can be separated.
- Disadvantage: Lower resolution.

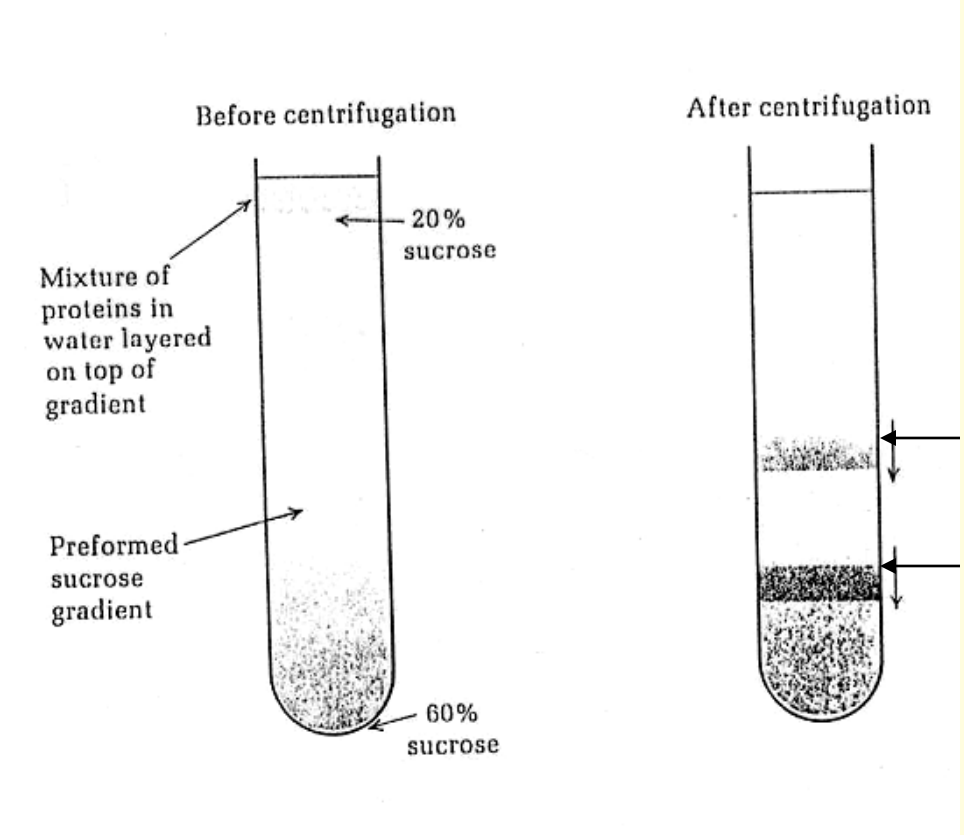
B. Gel Filtration Chromatography



Density gradient (zonal) centrifugation

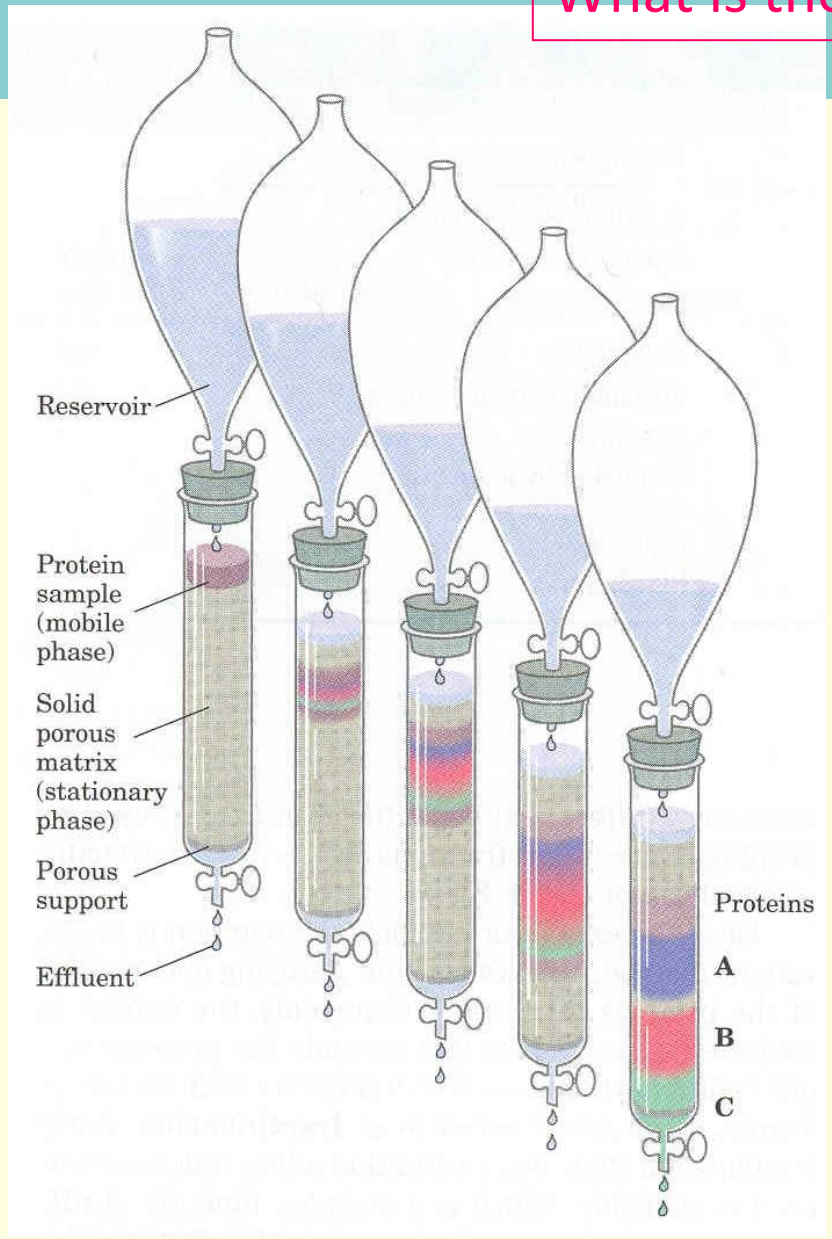
- **method for separation mixtures of proteins by centrifugation**
- **proteins in solution tend to sediment at high centrifugal fields**
- **in continuous density gradient of sucrose macromolecule sediment down at its own rate**
- **the rate of sedimentation is determined by weight, density and shape of macromolecule**

Test tube with sucrose gradient



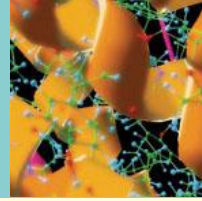
Separated and concentrated protein

What is the columne chromatography



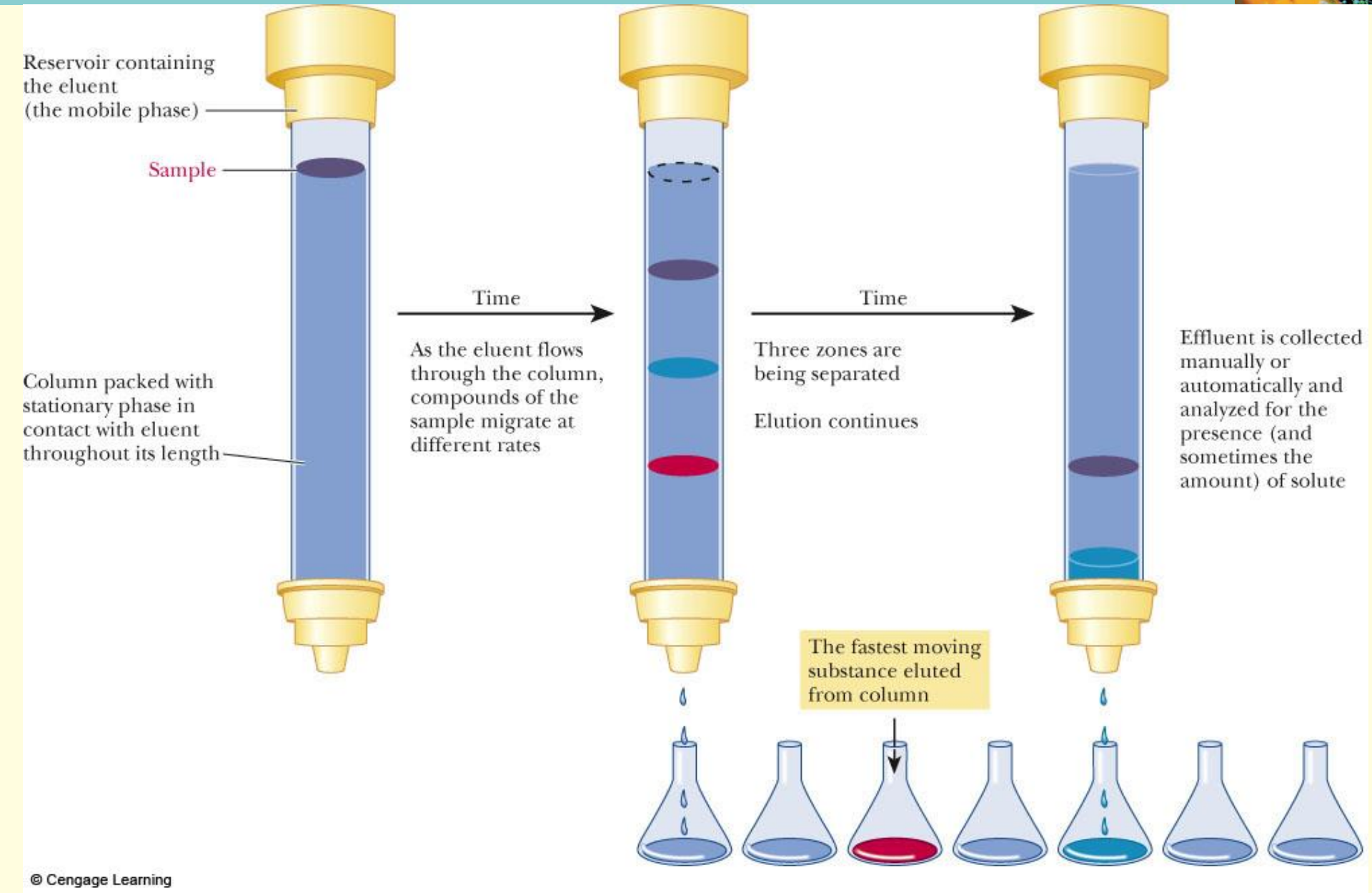
- Chromatographic column (plastic or glass) include a solid, porous material (matrix) supported inside – *stationary phase.*
- A solution – *the mobile phase* - flows through the matrix (stationary phase).
- The solution that pass out of the bottom is constantly replaced from a reservoir.
- The protein solution migrates through column.
- They are retarded to different degrees by their interactions with the matrix material.

Column Chromatography

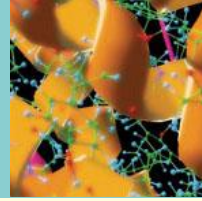


- Basis of Chromatography
  - Different compounds distribute themselves to a varying extent between different phases
- Interact/distribute themselves
- In different phases
- **2 phases:**
  - Stationary: samples interacts with this phase
  - Mobile: Flows over the stationary phase and carries along with it the sample to be separated

Column Chromatography



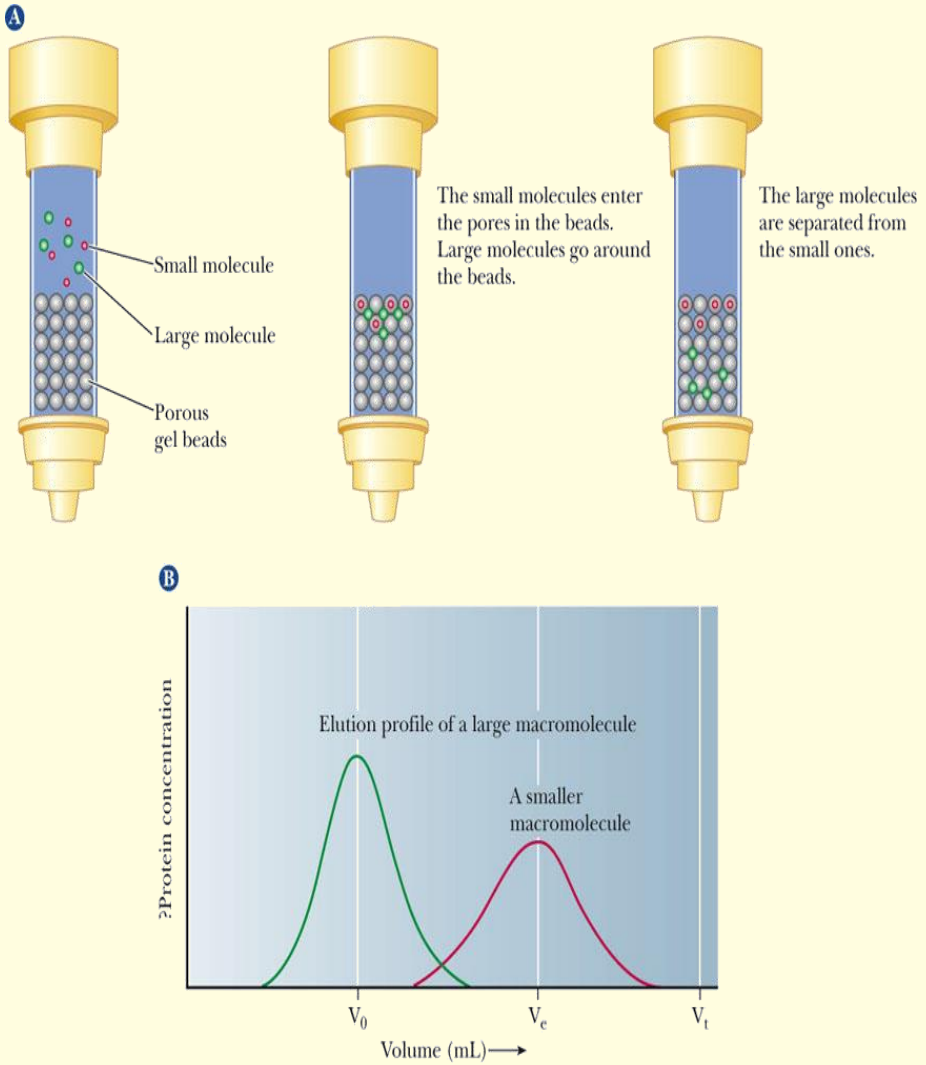
Size-Exclusion/Gel-Filtration



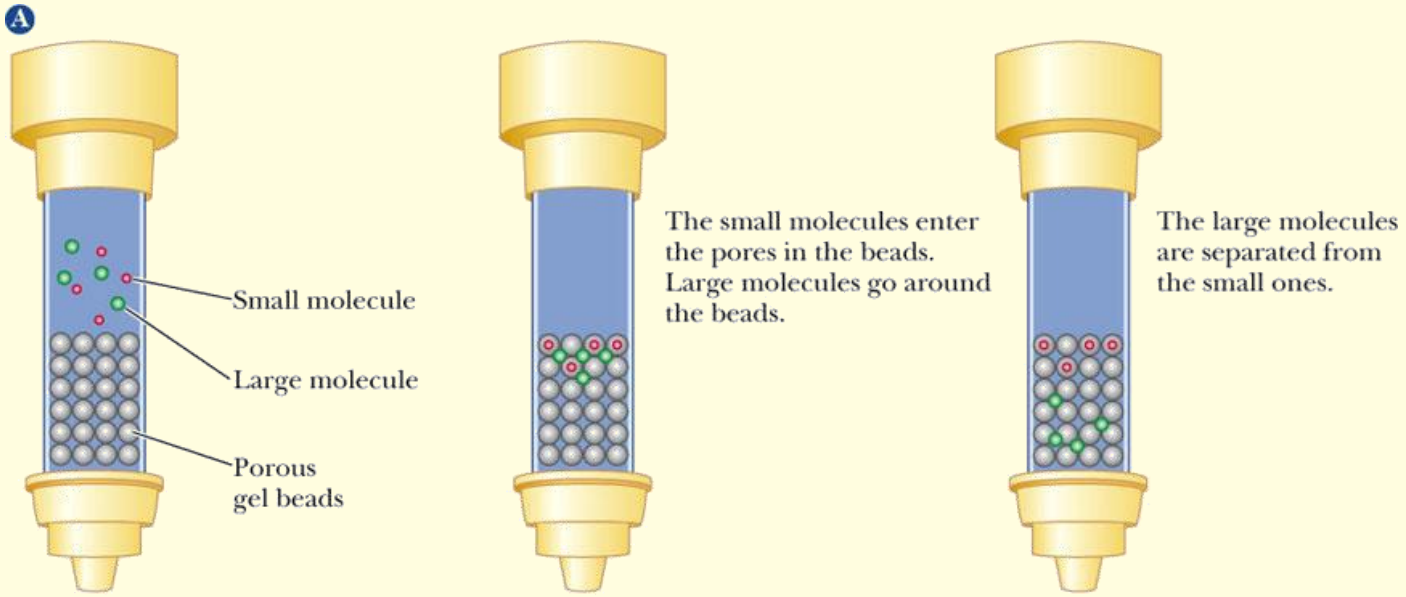
- Separates molecules based on size.
- Stationary phase composed of cross-linked gel particles.
- Extent of cross-linking can be controlled to determine pore size
- Smaller molecules enter the pores and are delayed in elution time. Larger molecules do not enter and elute from column before smaller ones.

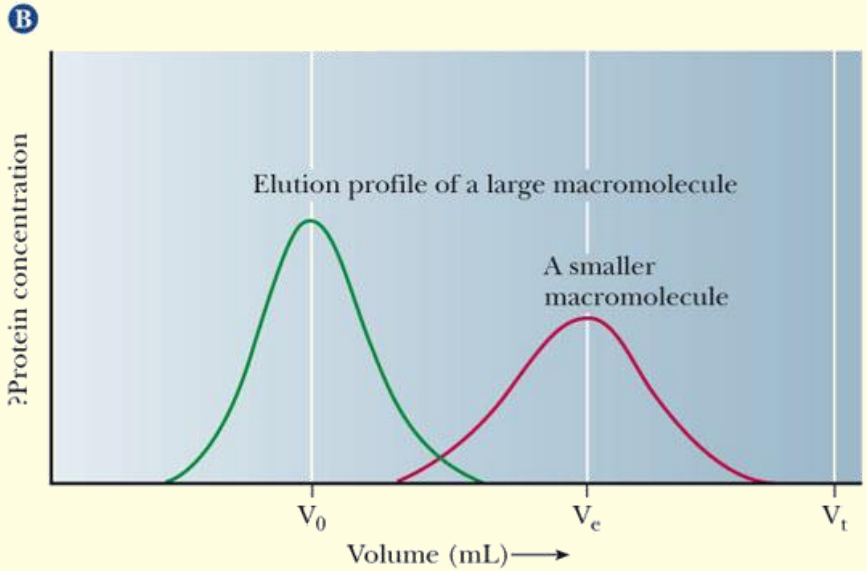
What is Size-exclusion/Gel-filtration chromatography?

- Separates molecules based on **size (molecular weight).**
- **Stationary phase** composed of **cross-linked gel particles (Beads)**.
- Two polymers – Carbohydrate polymer such as **dextran (Sephadex)** or **agarose (Sepharose)**
- **Polyacrylamide (Bio-Gel)**



Size Exclusion/Gel-filtration (Cont'd)

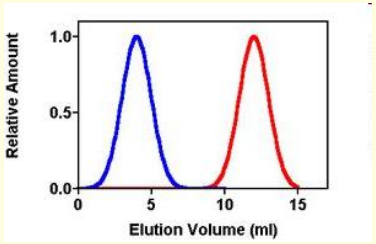


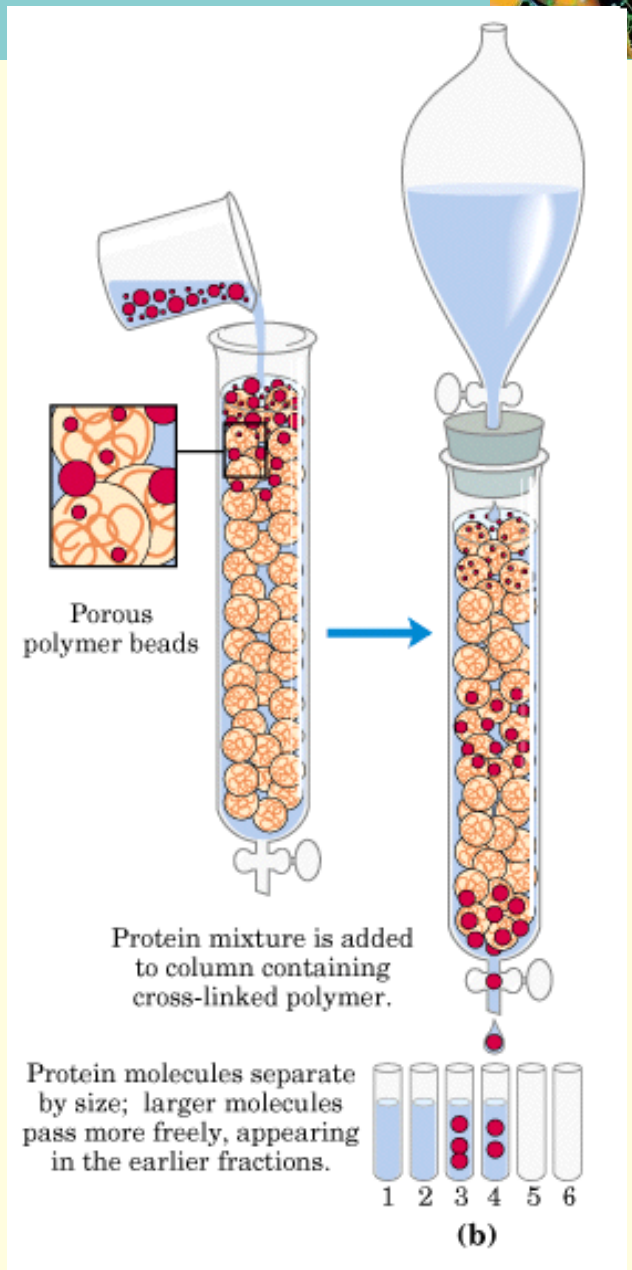


Size exclusion chromatography (gel filtration)

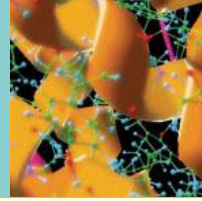
Method uses porous particles to separate molecules of different size

- mixture of proteins dissolved in suitable buffer, is allowed to flow by gravity down a column
- column is packed with beads of inert polymeric material (polysacchride agarose derivative, polyacrylamide derivative), Sephadex, Sephacryl
- very large molecules cannot penetrate into the pores of the beads, the small molecules enter the pores
- large molecules are excluded and small proteins are retarded





Advantages of Size-exclusion/Gel-filtration chromatography

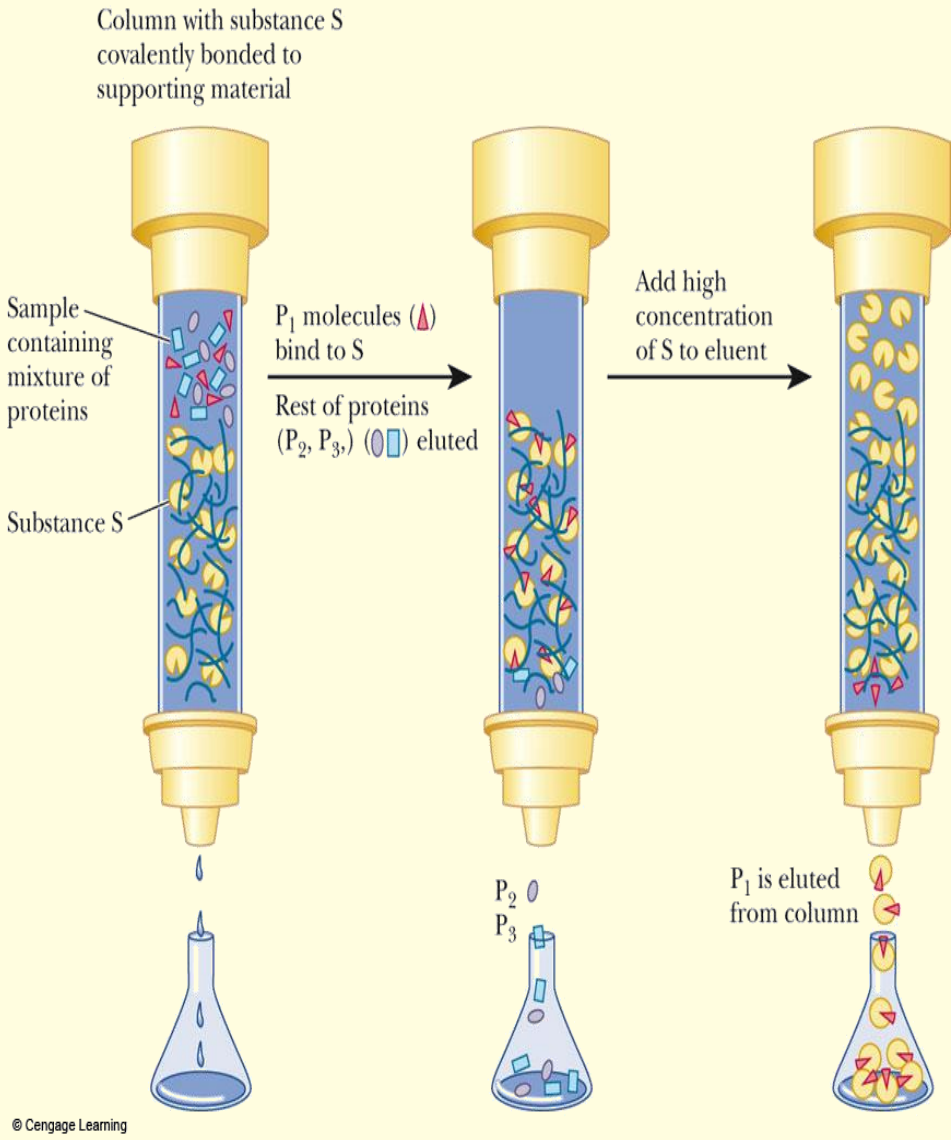


- Separate molecules based on size

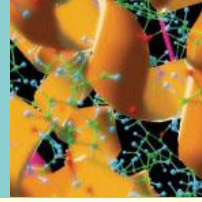
- Estimate molecular weight by comparing sample with a set of standards

What is Affinity Chromatography?

- Uses **specific binding properties** of molecules/proteins
- **Stationary phase** has a polymer that is covalently linked to a compound called a **ligand**
- **Ligands** bind to desired protein or vice versa
- Proteins that do not bind to ligand elute out



What is Affinity Chromatography?

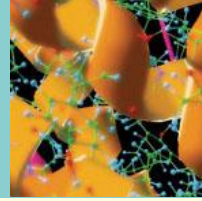


- Bound protein can be eluted from column by adding **high concentrations of ligand in soluble/mobile form.**

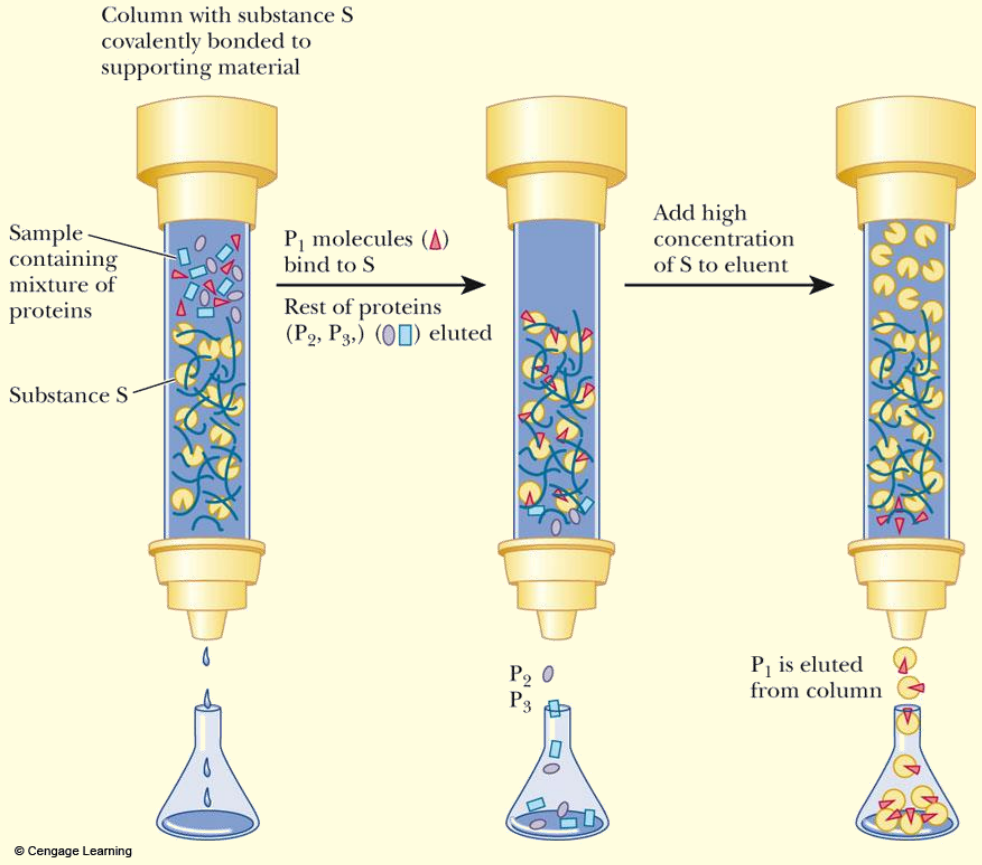
- Competition – **proteins bound to ligand in column will bind to mobile ligands**

- Recovered from column – Produces **pure proteins**

Affinity Chromatography

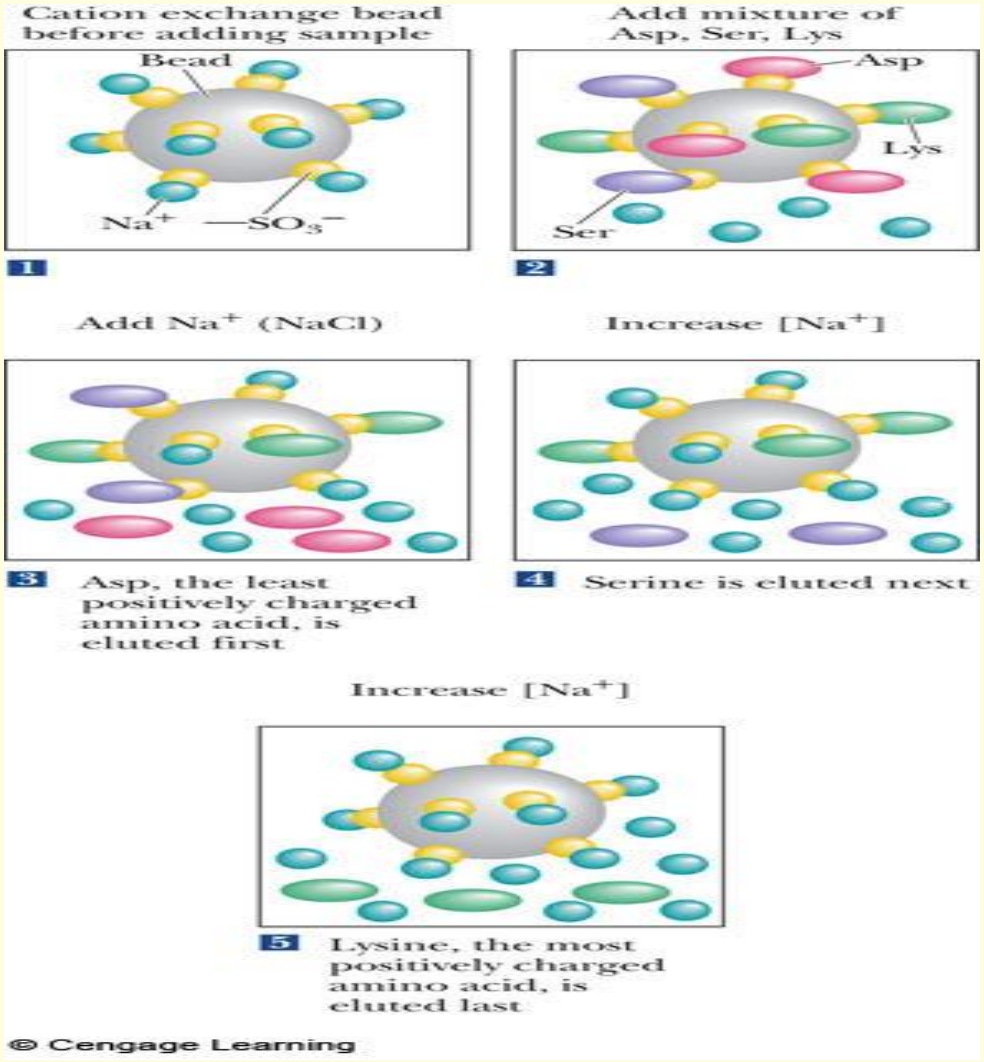


- Uses specific binding properties of molecules/proteins
- Stationary phase has a polymer that can be covalently linked to a compound called a **ligand** that specifically binds to protein



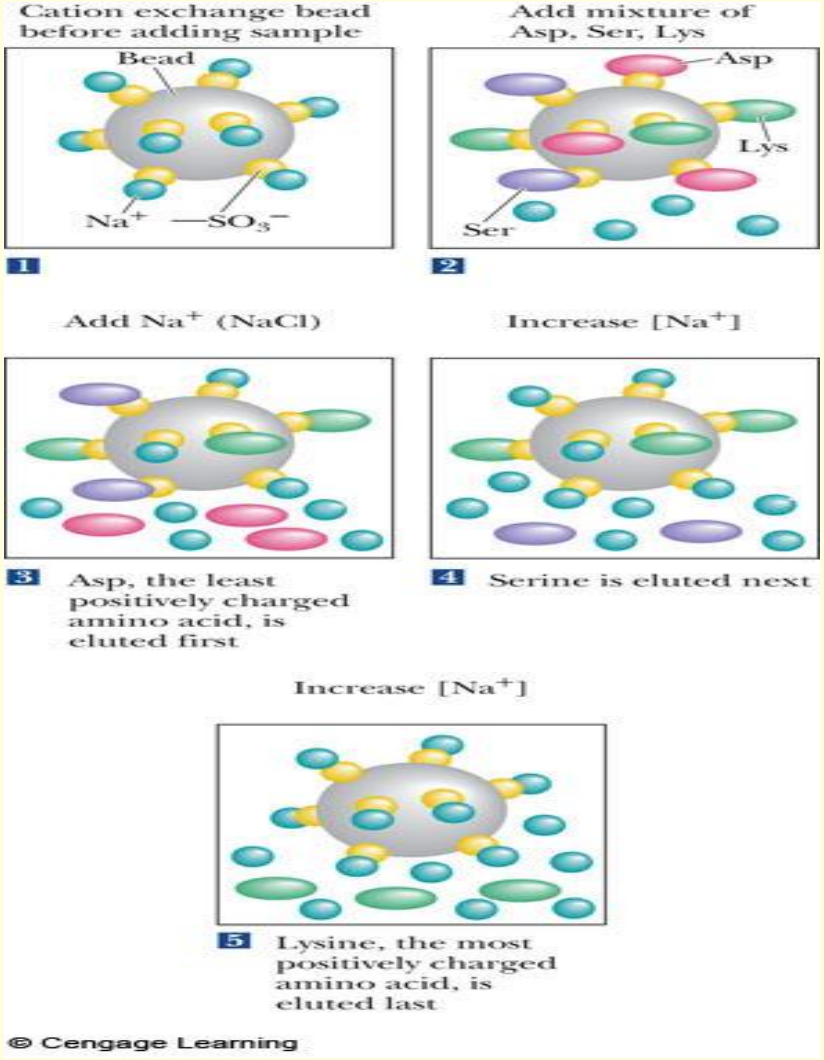
What is Ion-exchange chromatography?

- Interaction based on overall charge (less specific than affinity)
- Cation exchanger – negatively charged resin – bound to Na+ or K+ ions
- Anion exchanger – positively charged resin – bound to Cl- ions
- Figure 5.7 A and B

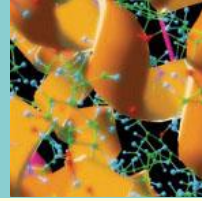


What is Ion-exchange chromatography?

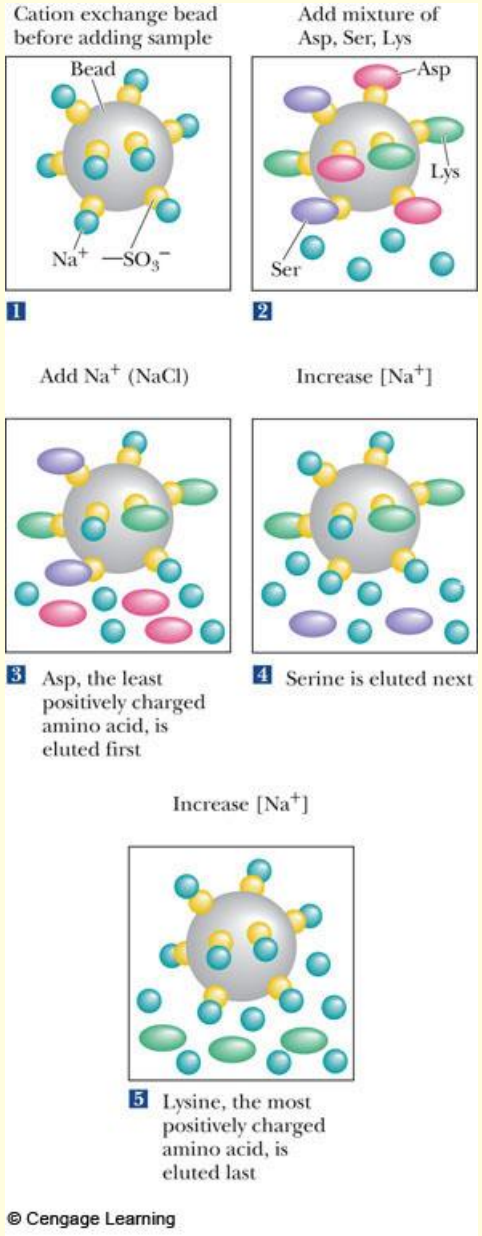
- Column is equilibrated with buffer of suitable pH and ionic strength
- Exchange resin is bound to counterions
- Proteins – net charge opposite to that of exchanger stick to column
- No net charge or same charge elute - first

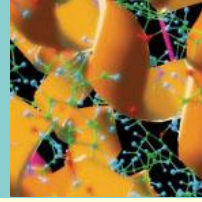


Ion Exchange



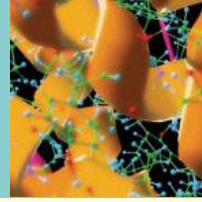
- Interaction based on overall charge (less specific than affinity)
- Cation exchange
- Anion exchange





C. Ion Exchange Chromatography

- Separates protein molecules according to their molecular **charge.**
- In this technique, the beads of the column have a specific charge on them. This is a result of a molecule that is attached to these beads.
- The beads might be +ve charged by attaching them to DEAE (diethylaminoethyl) cellulose or −ve charged by attaching them to carboxymethyl cellulose.
- The beads in the column depend on the protein that you want to purify.
- If the protein is −ve charged then you have to use positively charged beads and vice versa.

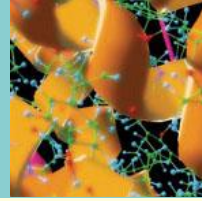


C. Ion Exchange Chromatography

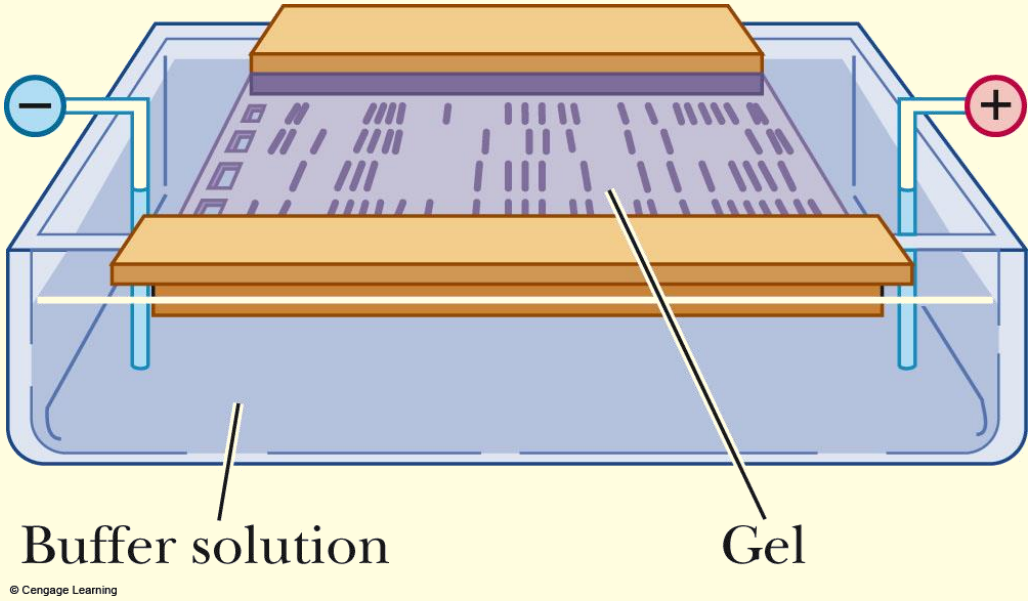
How does it work?

- For example, if the protein of interest is negatively charged, then you will use a DEAE-cellulose column.
- The protein will bind to the positively charged beads.
- This protein that is attached to the beads can be released by increasing the concentration of NaCl (or other salt).
- The Na^+ ions (or other cation) will compete and bind to the beads in the column instead of the protein.
- Proteins that are highly positively charged will emerge first because they will be repelled by the beads.

Electrophoresis



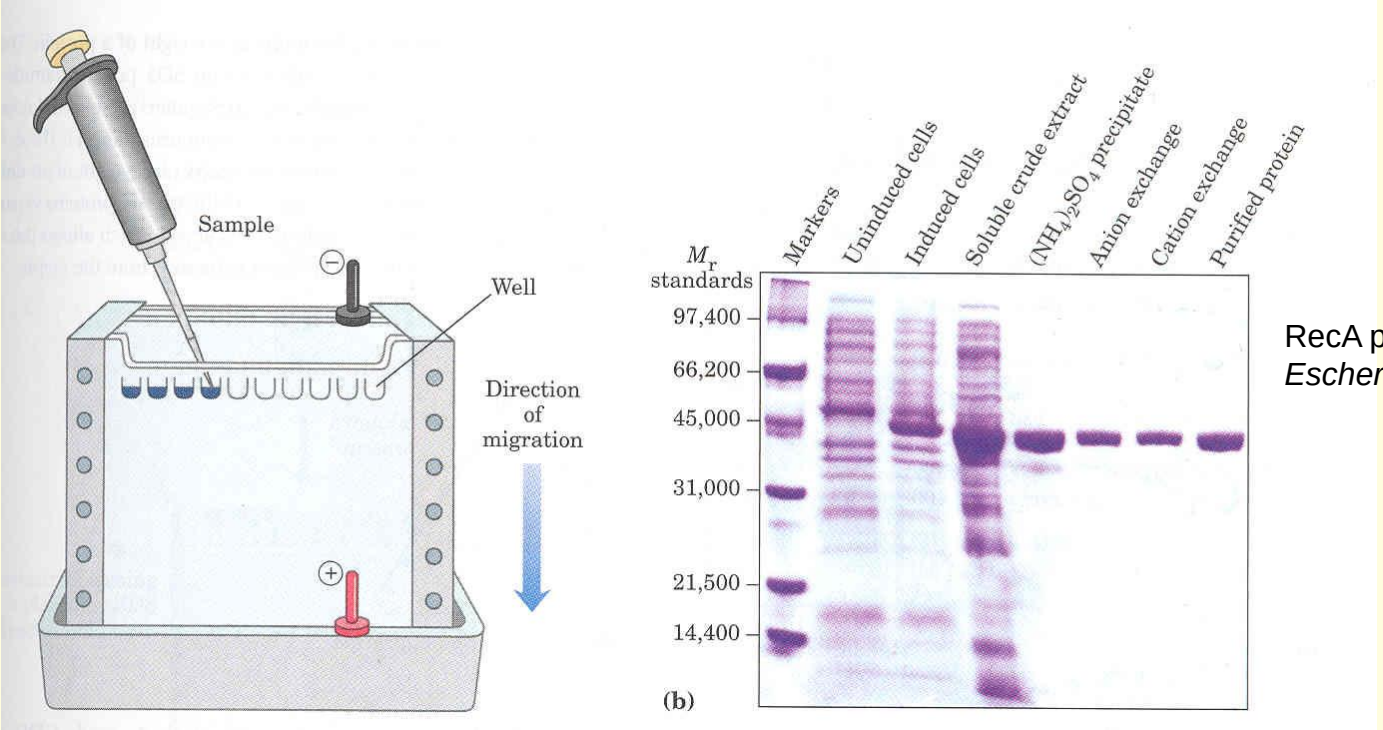
- **Electrophoresis**- charged particles migrate in electric field toward opposite charge
- Proteins have different mobility:
  - Charge
  - Size
  - Shape



- Agarose used as matrix for nucleic acids
- Polyacrylamide used mostly for proteins

Gel electrophoresis

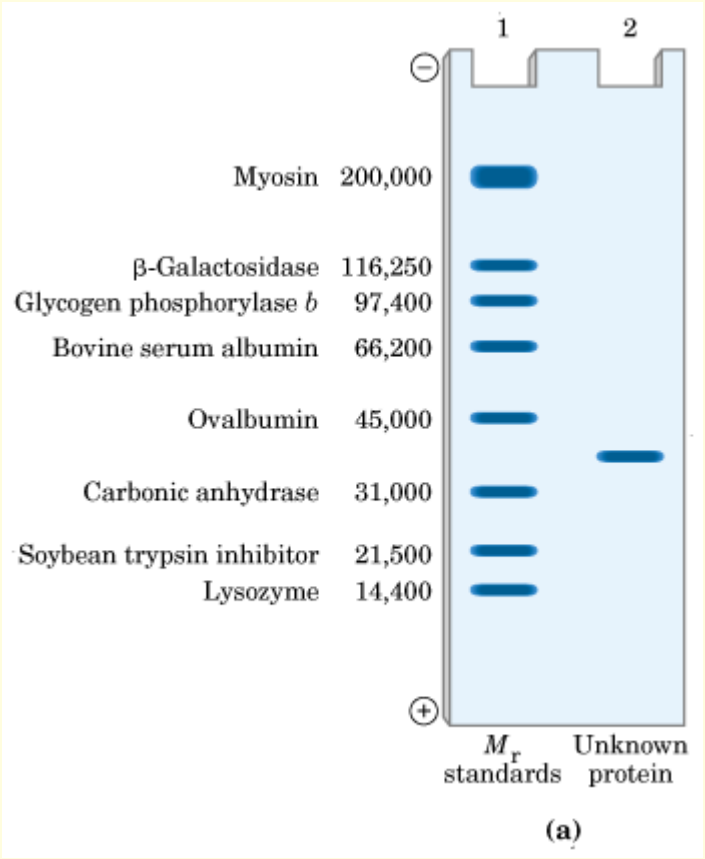
- Gel electrophoresis is a method that separates macromolecules (proteins, nucleic acids) on the basis of size, and electric charge.
- Polyacryl amide or agarose gels are stabilizing media.
- **SDS** (sodium dodecyl sulfate) – ionic surfactant, anionic substance.
- Anions of SDS bind to peptide chain and protein is negatively charged, moves to anode.

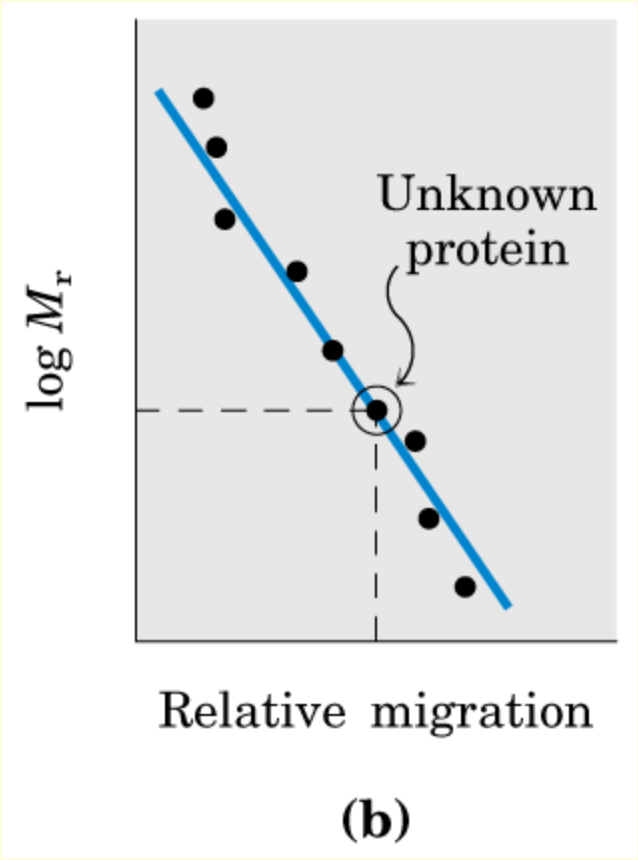


RecA protein of *Escherichia coli*

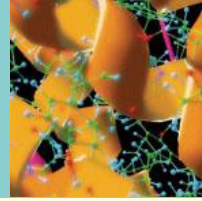
Estimating protein molecular weight from SDS gel electrophoresis

a) Diagram of a stained SDS gel: standards of known molecular weight (lane 1) and pure protein of unknown M.W. in lane 2
b) "standard curve" (calibration) to relate M.W. to mobility on THIS GEL





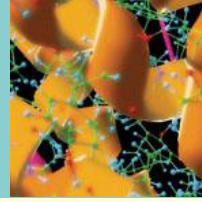
Differences between agarose and polyacrylamide gels



- Agarose - **matrix for nucleic acids**
- Charge, size, shape
- Agarose matrix has more resistance towards larger molecules than smaller
- Small DNA move faster than large DNA

- Polyacrylamide - **proteins**
- Charge, size, shape
- Treated with detergent (SDS) **sodium dodecyl sulfate – gains –ve charge**
- Random coil – shape
- Polyacrylamide has more resistance towards larger molecules than smaller
- Small proteins move faster than large proteins

Electrophoresis (Cont'd)

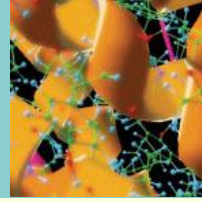


- Polyacrylamide has more resistance towards larger molecules than smaller

- Protein is treated with detergent (SDS) sodium dodecyl sulfate

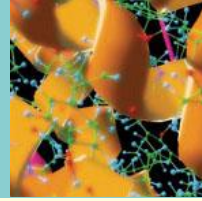
- Smaller proteins move through faster (charge and shape usually similar)

What is Isolectric focusing?



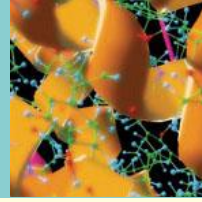
- Gel is prepared with **pH gradient that parallels electric-field**
- **Charge** on the **protein changes as it migrates across pH**
- When it gets to **pI, has no charge and stops**
- **Separated and identified** on differing isoelectric pts. (pI)

Isoelectric Focusing



- **Isolectric focusing-** based on differing isoelectric pts. (pI) of proteins
- Gel is prepared with pH gradient that parallels electric-field. What does this do?
  - Charge on the protein changes as it migrates.
  - When it gets to pI, has no charge and stops

What is Isolectric focusing?



- Gel is prepared with **pH gradient that parallels electric-field**
- **Charge** on the **protein changes as it migrates across pH**
- When it gets to **pI, has no charge and stops**
- **Separated and identified** on differing isoelectric pts. (pI)

2. Separation procedures based on solubilit

Isoelectric precipitation

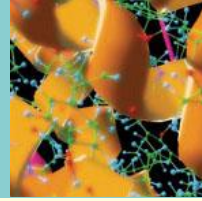
- **Protein itself can be either positively or negatively charged overall due to the terminal amine -NH_2 and carboxyl (-COOH) groups and the groups on the side chain.**

- **Protein is positively charged at low pH and negatively charged at high pH. The intermediate pH at which a protein molecule has a net charge of zero is called the *isoelectric point* of that protein - *pI***

- **Protein is the least soluble when the pH of the solution is at its isoelectric point.**

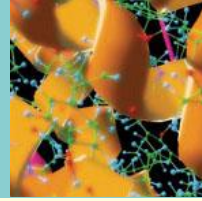
- **Different proteins have different pI values and can be separated by *isoelectric precipitation***

What is two-dimensional gel electrophoresis?



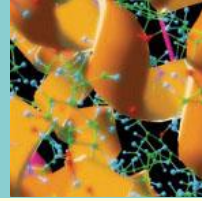
- Isoelectric focussing in one dimension and SDS-PAGE running at 90 degree angle to the first

How is 1˚ structure determined?



1) Determine which amino acids are present and in what proportions (amino acid analyzer)
2) Specific reagents - determine the N- and C- termini of the sequence
3) Cleave - determine the sequence of smaller peptide fragments (most proteins > 100 a.a)
4) Some type of cleavage into smaller units necessary

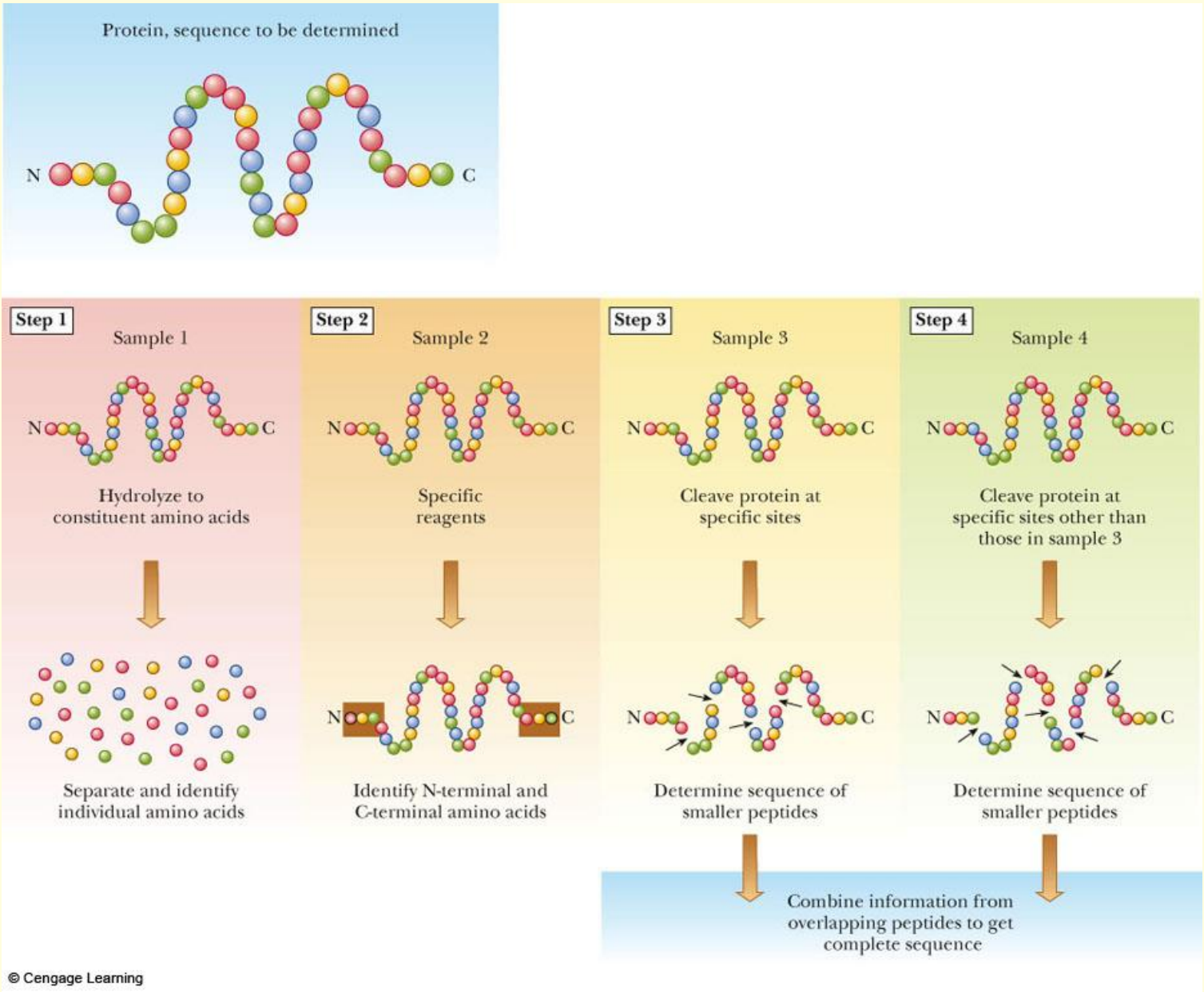
Primary Structure Determination



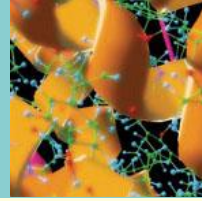
How is 1˚ structure determined?

1) Determine which amino acids are present (amino acid analysis)
2) Determine the N- and C- termini of the sequence (a.a sequencing)
3) Determine the sequence of smaller peptide fragments (most proteins > 100 a.a)
4) Some type of cleavage into smaller units necessary

Primary Structure Determination



What is Edman degradation?

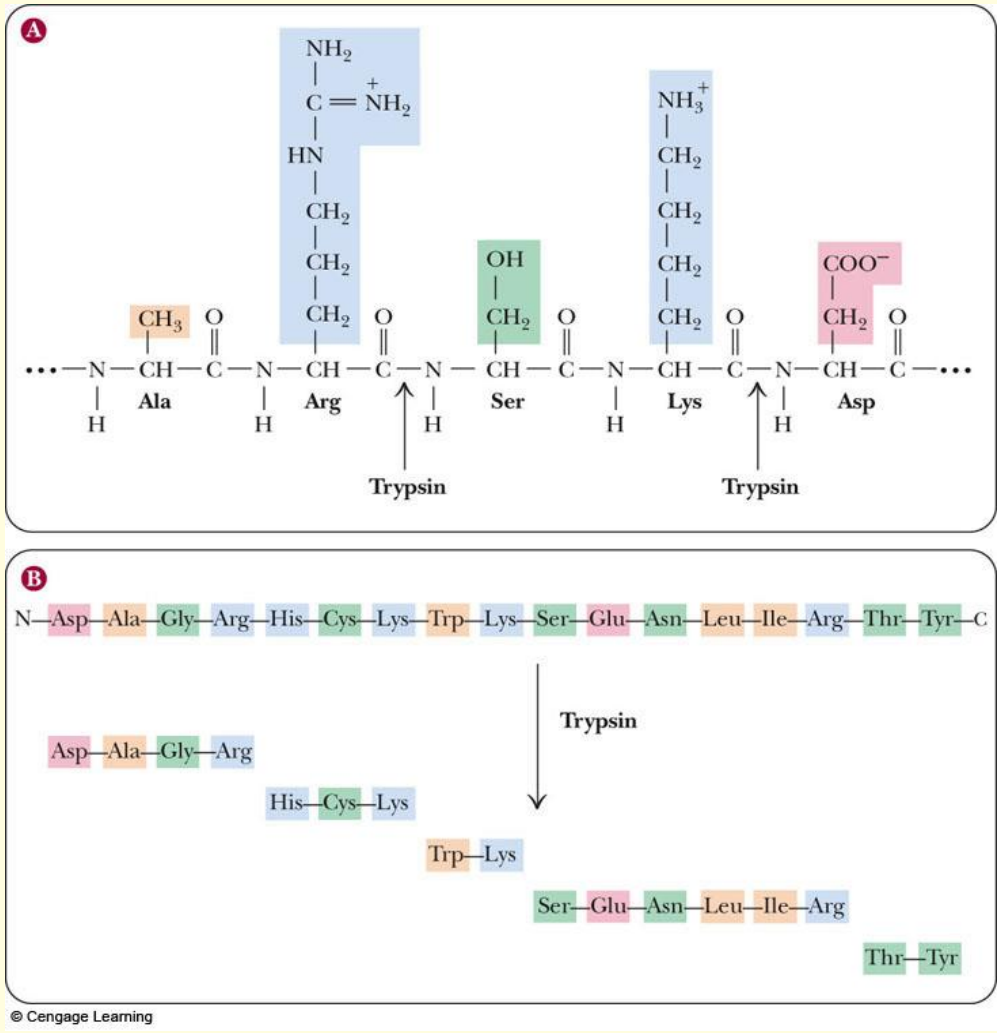


- **Cleaving** of each amino acid in sequence followed by their **subsequent identification and removal**
- Becomes difficult with **increase** in number of amino acids
- Amino acid **sequencing - Cleave long chains into smaller fragments**

Protein Cleavage

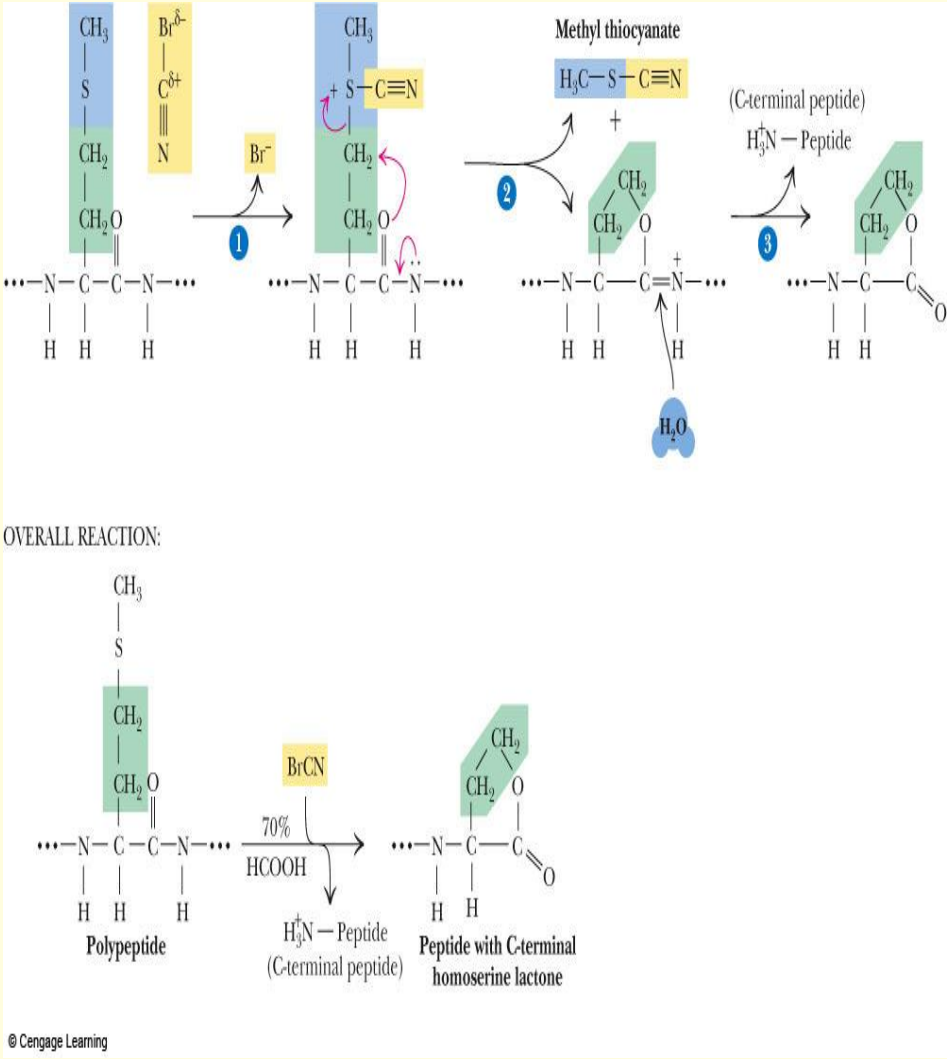
- **Enzymes or Chemical reagents**
- **Trypsin-** Cleaves @ C-terminal of (+) charged side chains/R-groups
- **Chymotrypsin-** Cleaves @ C-terminal of aromatics

Figure 5.17

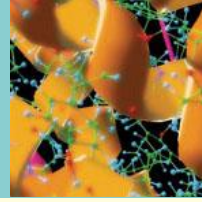


Cleavage by Chemical reagent

- **Cyanogen bromide**
- Cleaves @ C-terminal of **INTERNAL** methionines
- **Sulfur of methionine** reacts with **carbon of cyanogen bromide** to produce a **homoserine lactone at C-terminal end** of fragment



Protein Cleavage



Protein cleaved at specific sites by:

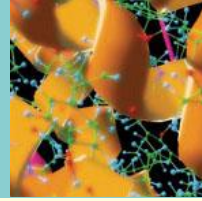
1) **Enzymes-** Trypsin, Chymotrypsin
2) **Chemical reagents-** Cyanogen bromide

Enzymes:

Trypsin- Cleaves @ C-terminal of (+) charged side chains

Chymotrypsin- Cleaves @ C-terminal of aromatics

Determining Protein Sequence

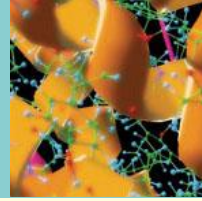


After cleavage, mixture of peptide fragments produced.

- Can be separated by HPLC or other chromatographic techniques
- Use different cleavage reagents to help in 1˚ determination

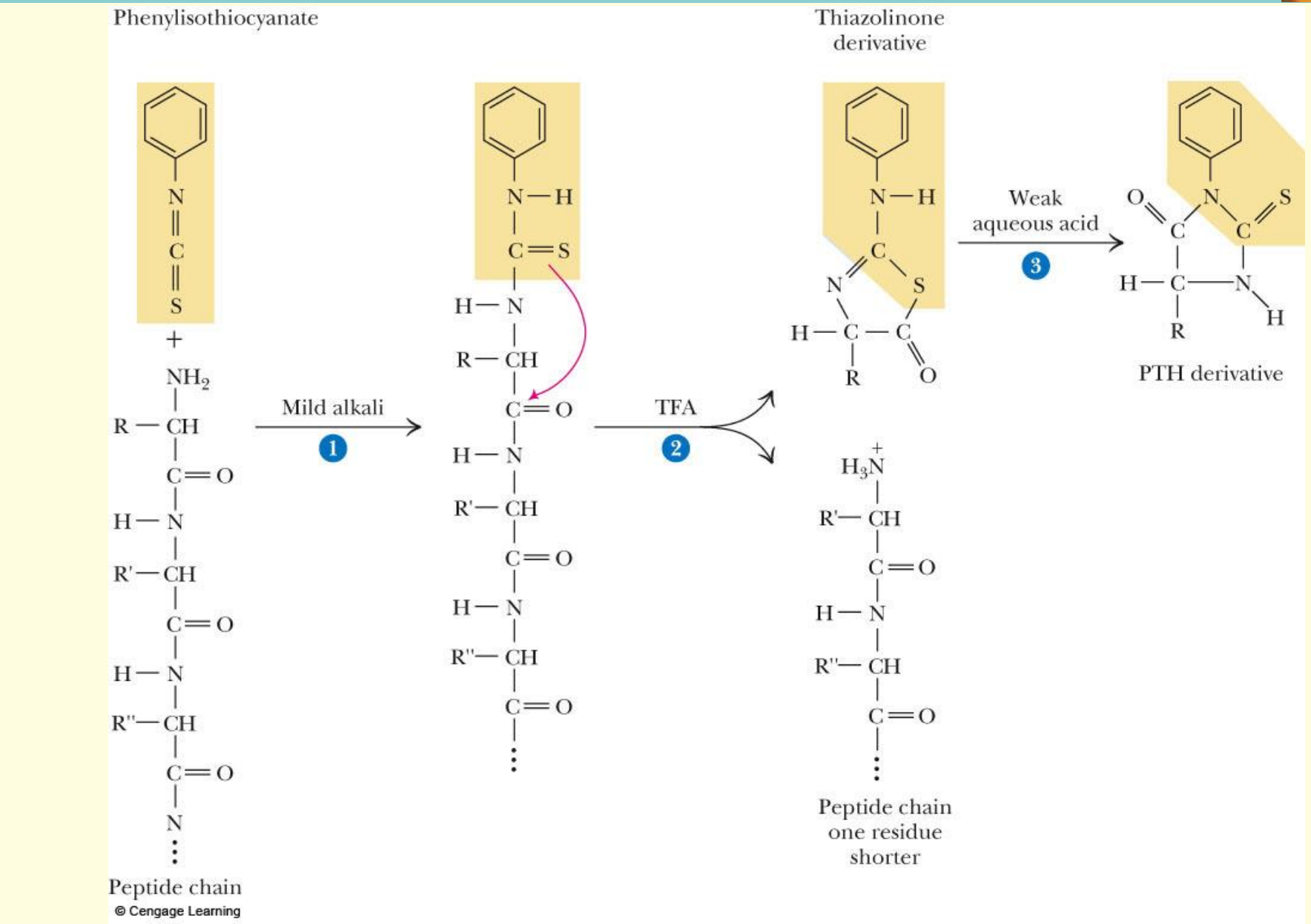
Chymotrypsin	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe
Cyanogen bromide	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe—His—Met
Chymotrypsin	His—Met—Thr—Met—Ala—Trp
Cyanogen bromide	Thr—Met
Cyanogen bromide	Ala—Trp—Val—Lys—COO^-
Chymotrypsin	Val—Lys—COO^-
Overall sequence	$H_3\overset{+}{N}$—Leu—Asn—Asp—Phe—His—Met—Thr—Met—Ala—Trp—Val—Lys—COO^-

Peptide Sequencing

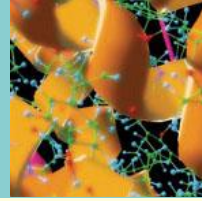


- Can be accomplished by Edman Degradation
- Relatively short sequences (30-40 amino acids) can be determined quickly
- So efficient, today N-/C-terminal residues usually not done by enzymatic/chemical cleavage

Peptide Sequencing

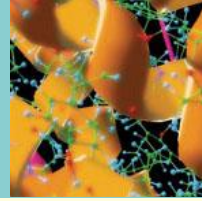


3D Structure Determination



- X Ray Diffraction
  - need to crystalize (Difficult)
- NMR
  - small proteins 25kD
- Electron Micrograph
  - poor resolution

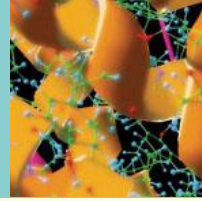
X-ray Crystallography



X ray diffraction

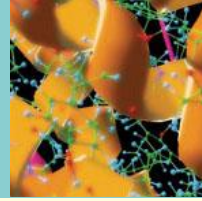
- beam of x rays directed at protein
- beam is diffracted by electrons of atoms in protein
- these beams hit a film detector
- computer analysis to create electron density map

NMR
Nuclear Magnetic Resonance



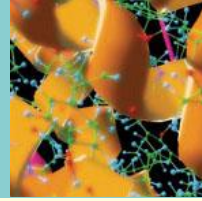
- apply magnetic field to protein
- atomic nuclei spin - create their own magnetic field
- emit radiation

Electron Micrograph



Any of a class of microscopes that use electrons rather than visible light to produce magnified images, especially of objects having dimensions smaller than the wavelengths of visible light, with linear magnification approaching or exceeding a million (10^6).

Methods of Stabilization of Proteins



Correct pH

Maintain temperatures (usually low)

Minimize processing times

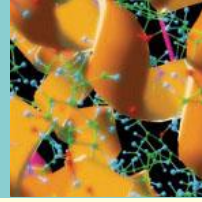
Minimize agitation

Minimize denaturing chemicals

Add protease inhibitors

Add reducing agents (Oxidation can cause inactivation typically intracellular proteins)

Examples of Stabilizers



A. These reduce free water levels by hydrogen bonding with H_2O

Glycerol

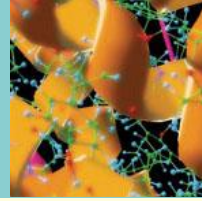
Sugar

Polyethene Glycol

B. BSA Bovine serum albumin

added to proteins which are at LOW concentration

Storage of Proteins



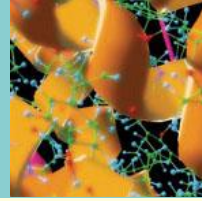
Similar conditions apply as with Stability

Freezing (and thaw) is typically OK

How long?

Concentration of contaminants

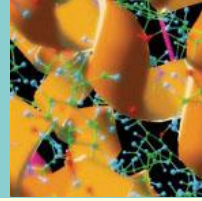
Lyophilization



Drying of protein

- Freeze protein
- Increase temperature
- Apply vaccum
- Remove water vapor

Mass Spectrometry



Molecular Mass determination

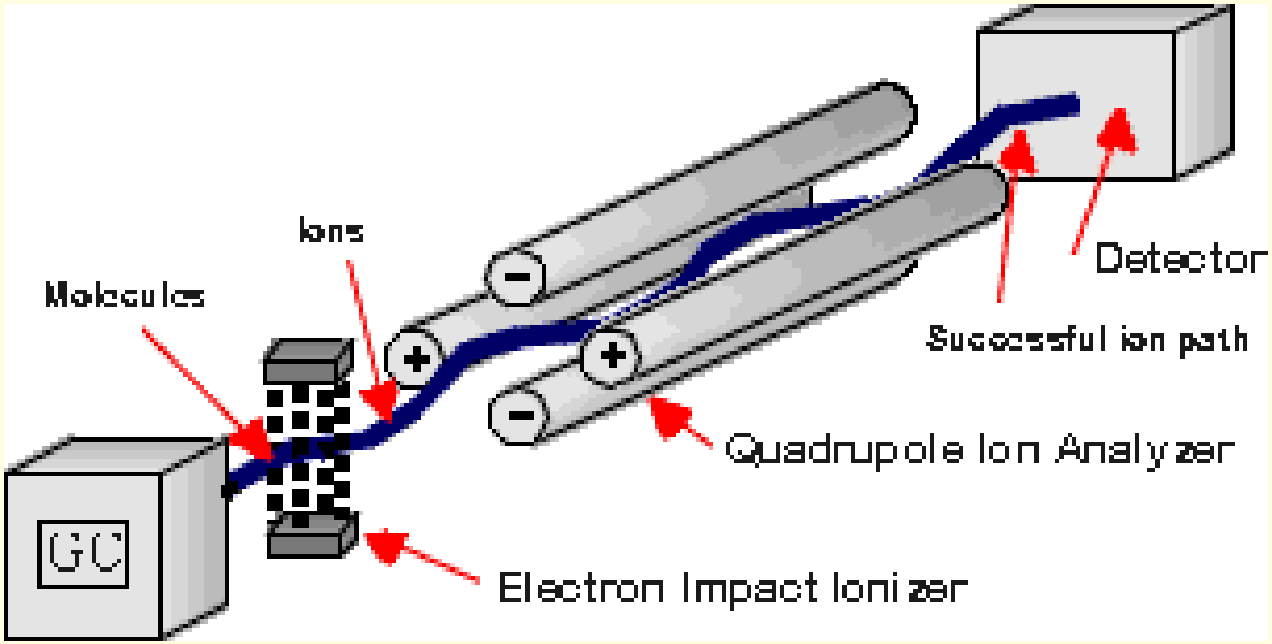
More accurate than SDS PAGE

Less protein needed for analysis than SDS PAGE

MALDI MS

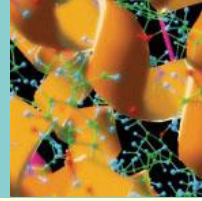
matrix-assisted laser desorption and ionization

- mix protein with matrix (absorb UV rad)
- bombard mixture with UV photons
- matrix absorbs UV - flight into gas phase
- protein becomes ionized
- electric field pulls protein through analyzer tube



http://www.chem.arizona.edu/massspec/intro_html/intro.html

Inclusion Bodies

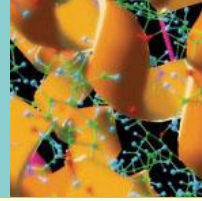


Define:

Insoluble protein (precipitates) and RNA aggregates

Dense granular structures

Why?



A. Incorrect disulfide bond formation

B. Too much protein produced

C. Incorrect folding

As protein synthesized -

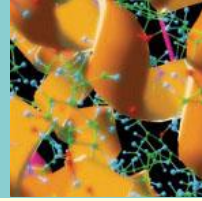
intermediate folding conformations

Native structure is achieved

In the intermediate conformation,
hydrophobic patches may be exposed (normally inside)

At high concentrations, these regions associate with each other before native formation can be formed.

What do we do from here?



A. Fix misfolding

- Dissociate polypeptides
- Solubilize (SDS, urea, guanidine hydrochloride)
- Renature

B. Prevent misfolding

- Lower Temperature
  - Hydrophobic interactions decrease at lower temps
- Co Express Chaperones
  - Proteins which expend energy to maintain the partially folded proteins in a soluble state

Thank you for your attention