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Biochemistry

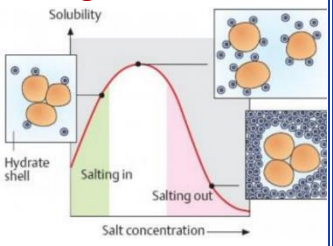
Protein purification

❖ Protein purification

- Proteins can be purified (separated) according to **solubility**, **size**, **charge** and **specific binding affinity**
- These techniques include:

♦ Salt fractionation

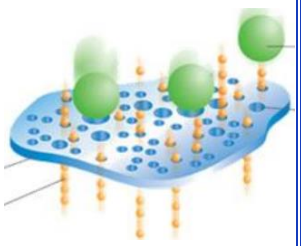
- Proteins are poorly soluble in pure water, but their solubility increases as the ionic strength increases
  - **Salting in:** The increase the concentration of ions in a solution, which increases protein solubility, due to the formation of more electrostatic interactions and H bonds forming the hydration shell



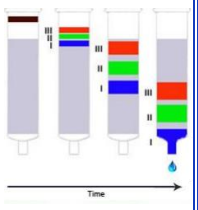
  - **Salting out:** At very high concentrations of salt, most proteins become less soluble, because when salt (like ammonium sulfate) is added to a protein solution, some water molecules are taken away from the protein to make ion dipole bonds with the ions, with less water available to hydrate the proteins they begin to interact with each other through hydrophobic bonds and form aggregates that precipitate down
- Each type of protein can precipitate at a specific salt concentration, allowing the fractionation of proteins

♦ Dialysis

- The separation of the protein sample from the small molecules (either ions or small proteins) through a semi-permeable membrane
  - The disadvantages of dialysis are that a large number of proteins will still exist and small proteins of significance are lost



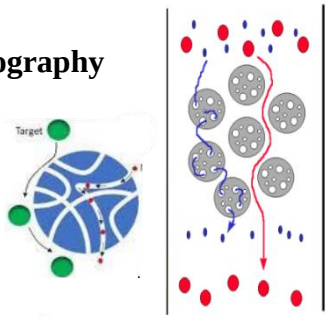
♦ Chromatography techniques

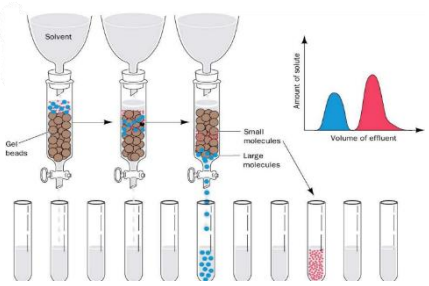


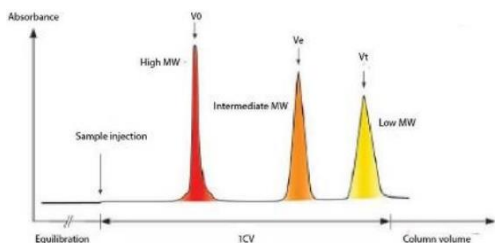
- Separation of molecules present in a liquid or gaseous environment (mobile phase) via passing through a column (long tube) that contains an immobile phase (stationary phase)
- There are 3 chromatography techniques:

1) Gel filtration

- Separations on the basis of **size**
- It is also called **Size-exclusion chromatography** and **Molecular sieve chromatography**
- The stationary phase: **porous beads**
  - **Smaller** molecules can be included into the pores of the beads and take a **longer time** in the column and exit **late**
  - **Large** molecules are excluded and flow **more rapidly** and come out **first**







2) Ion-Exchange Chromatography

- Proteins have different isoelectric points (pI's) and net **charges** at various pH conditions
  - At high pH, basic proteins appear isoelectric and acidic proteins appear negatively charged
  - At low pH, basic proteins appear positively charged and acidic proteins appear isoelectric

a) Anionic-exchange chromatography

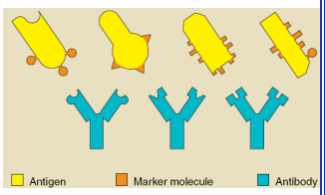
- The beads are **positively**-charged, such as Diethylaminoethyl-cellulose column
  - Negatively charged proteins are separated
- After the negatively charged, proteins are bound to the beads, proteins are **eluted** (released) by adding increasing concentrations of sodium chloride and so the chloride ions compete with negatively-charged groups on the protein for binding to the column

b) Cationic-exchange chromatography

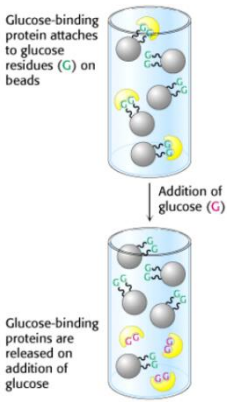
- The beads are **negatively**-charged
- Proteins that have a net positive charge will tend to emerge first, followed by those having a higher positive charge density
- You have 5 different proteins (#1, #2, #3, #4, and #5), with different isoelectric points (pI's). Starting the column at pH 6.5, the sample is added and, then, washed to remove unbound molecules.What is the order of protein elution in a:
  - Cationic-exchange chromatography?
  - Anionic exchange chromatography?

  pI#1 = 7.2 / pI#2 = 9.1 / pI#3 = 12.1 / pI#4 = 4.7 / pI#5 = 2.3

3) Affinity Chromatography

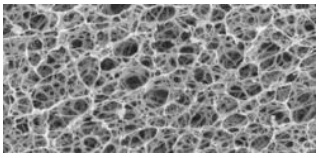


- **Affinity:** strength of binding between two molecules
- Affinity chromatography takes advantage of the **high affinity** of many proteins for specific chemical groups or other proteins (antibodies)
- Affinity chromatography is most effective when the interaction of the protein and the molecule that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



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

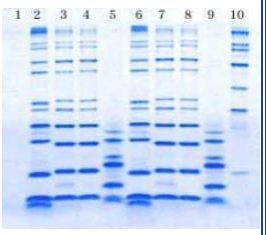
❖ Gel electrophoresis



- Electrophoresis: The phenomenon where a molecule with a net charge moves in an electric field
  - It offers a powerful means of separating proteins
  - In gel electrophoresis, proteins are separated according their **size** as they move through a gel, which serves as a molecular sieve
  - The gel is made of a material known as **polyacrylamide**, which is formed by the polymerization of acrylamide and crosslinked by methylene-bisacrylamide
- The **most commonly** used protein electrophoresis technique is sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE)
  - It is performed in a thin, vertical gel
  - The top of the gel consists of **wells** onto which samples are loaded
- This technique utilizes **SDS** as a negatively charged detergent to **denature and solubilize proteins**
  - SDS makes proteins have a **uniform negative structure**
  - If SDS is not used (non-denaturing condition or native condition) proteins maintain their original structure and shape and are separated based on charge, size, and shape

- Proteins are also treated with reducing agents like **β-mercaptoethanol** or **dithiothreitol** to reduce disulfide bonds (reducing condition)

- When an electrical voltage is applied between the upper and lower ends of the gel, all proteins move in one direction towards the anode (positive) **according to size only**

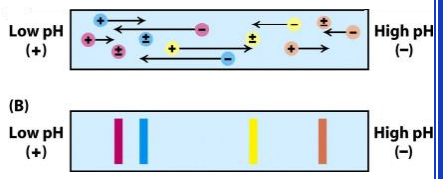


  - The direction of movement is from top to bottom
  - **Smaller molecules move readily** through the gel, larger molecules are slower
  - Once a gel has been run, proteins are stained to reveal the positions of the proteins that appear as bands

- Describe the protein's structure based on the following results of SDS-PAGE:
  - Under non-reducing condition, a protein exists as one 40-KDa band. Under reducing conditions, the protein exists as two 20-KDa bands
  - 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
  - Under non- reducing condition, a protein exists as two bands, 40 KDa and 20 KDa. Under reducing conditions, the protein exists as one bands of 20 KDa
  - Under non- reducing condition, a protein exists as one band, 50 KDa. Under reducing conditions, the protein exists as two bands of 10 KDa and 15 KDa

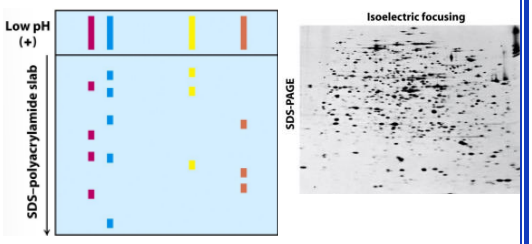
❖ Isoelectric focusing

- A gel is prepared with a **pH gradient**
- As proteins migrate through the gel, they encounter regions of different pH, so the charge changes
  - Positively charged proteins migrate toward the cathode and the negatively charged proteins migrate toward the anode
- Eventually each protein reaches the point at which it has no net charge **(isoelectric point)** and no longer migrates (**stop migrating**)
- Each protein remains at the position on the gel corresponding to its pI, allowing for separation of proteins



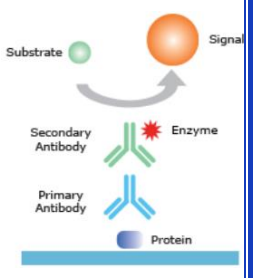
❖ Two-dimensional gel electrophoresis (2D-PAGE)

- In 2D-PAGE, proteins are separated by, first, **isoelectric focusing**, then through an **SDS-PAGE**
  - Thus, proteins are separated based on both **charge and size**
  - It has a higher resolution and purification power than other techniques

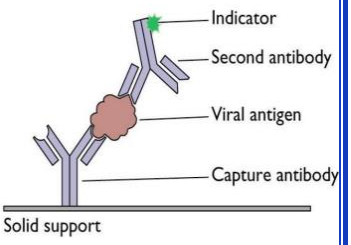


❖ Immunoblotting (Western blotting)

- Specific proteins are detected by **antibodies** following SDS-PAGE
  - Firstly, proteins are separated by gel-electrophoresis according to their size and then the bands and proteins are transferred into a membrane
  - 2 types of antibodies are added, **primary antibody** which binds the intended proteins **specifically** and **secondary antibody** which binds the primary one and has an enzyme on its Fc which coverts a substrate to signal with a certain color



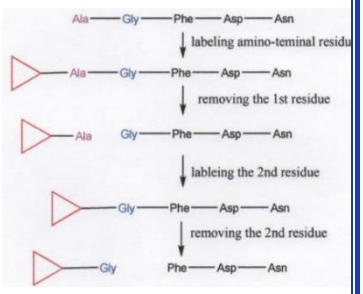
❖ ELISA (Enzyme-linked immunosorbent assay)



- It has the same concept as immunoblotting but **rapid, convenient, and sensitive** (can detect proteins less than a nanogram)
  - ELISA is the standard assay used in clinical laboratories because of its low cost, fast and automated
- The benefits of this method are to know of a **specific protein** exist or not, and to know the **amount** of the it (because the secondary antibody binds the antigen itself not the primary antibody)
  - The primary antibody is bound to a surface

❖ Protein sequencing

- Protein sequencing is basically the process of knowing the amino sequence of a protein or a peptide
- One technique is known as **Edman Degradation**
  - This procedure involves a step-by-step cleavage of the N-terminal residue of a peptide, allowing for the identification of each cleaved residue
  - This method utilizes **phenyl isothiocyanate (PITC)** to react with the N-terminal residue
  - The resultant amino acid is hydrolyzed, liberated from the peptide, and identified by chromatographic procedures
  - 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:** possible to sequence **whole proteins** by cleaving them into smaller peptides
- They include:

1) 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 with 10 methionine residues will usually yield 11 peptides with Met on the C-terminus

2) 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
- Trypsin which cleaves polypeptide chains on the **carboxyl side of arginine and lysine**
  - ✓ A protein that contains 9 lysine and 7 arginine residues will usually yield 17 peptides
- **Chymotrypsin** which cleaves polypeptide chains on the **C-terminal to Phe, Tyr, or Trp**, but not if next to Pro
- **Elastase** cleaves polypeptide chains on **C-terminal to Ala, Gly, Ser, or Val**, but not if next to Pro
- Pepsin cleaves polypeptide chains on **N-terminal to Leu, Phe, Trp, or Tyr**, but not if next to Pro

3) 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

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

    Trypsin treatment:
    Asn—Thr—Trp—Met—Ile—Lys
    Gly—Tyr—Met—Gln—Phe
    Val—Leu—Gly—Met—Ser—Arg

    Cyanogen bromide treatment
    Gln—Phe
    Val—Leu—Gly—Met
    Ile—Lys—Gly—Tyr—Met
    Ser—Arg—Asn—Thr—Trp—Met

  - A sample of a peptide of unknown sequence was treated with trypsin; another sample of the same peptide was treated with chymotrypsin. Given the following sequences (N-terminal to C-terminal) of the resulting fragments, deduce the sequence of the original peptide

    Trypsin treatment:
    Met—Val—Ser—Thr—Lys
    Val—Ile—Trp—Thr—Leu—Met—Ile
    Leu—Phe—Asn—Glu—Ser—Arg

    Chymotrypsin treatment
    Asn—Glu—Ser—Arg—Val—Ile—Trp
    Thr—Leu—Met—Ile
    Met—Val—Ser—Thr—Lys—Leu—Phe

❖ Crystallography

- X-ray crystallography is used to determine the **three-dimensional structure of proteins**
  - A protein must first be turned into a crystal (by removing water from it) before being exposed to X-rays, which are scattered by the electrons of the molecule
  - Important in the formation of drugs

❖ Nuclear magnetic resonance (NMR) spectroscopy

- It reveals the **structure and dynamics of proteins in solution** (not crystals)
- This is important with protein binding to other molecules like enzymes to their substrates, receptors to their ligands, etc
- Used to detect the conformational changes due to the binding of a protein to its ligand such as hemoglobin to O_2