Protein Structure Determination

Specific learning objectives

- Differential centrifugation used to prepare subcellular fractions or to isolate specific organelles.
- Purification methods are based on properties of proteins.
- Methods of Proteins Purification includes chromatography techniques.
- Protein denaturation and renaturation.

Introduction

- First step in protein purification is to break and open these cells, releasing their proteins into a solution called a crude extract.
- Differential centrifugation can be used to prepare subcellular fractions or to isolate specific organelles.
- Once the extract or organelle preparation is ready, various methods are available for purifying one or more of the proteins.

Differential Centrifugation

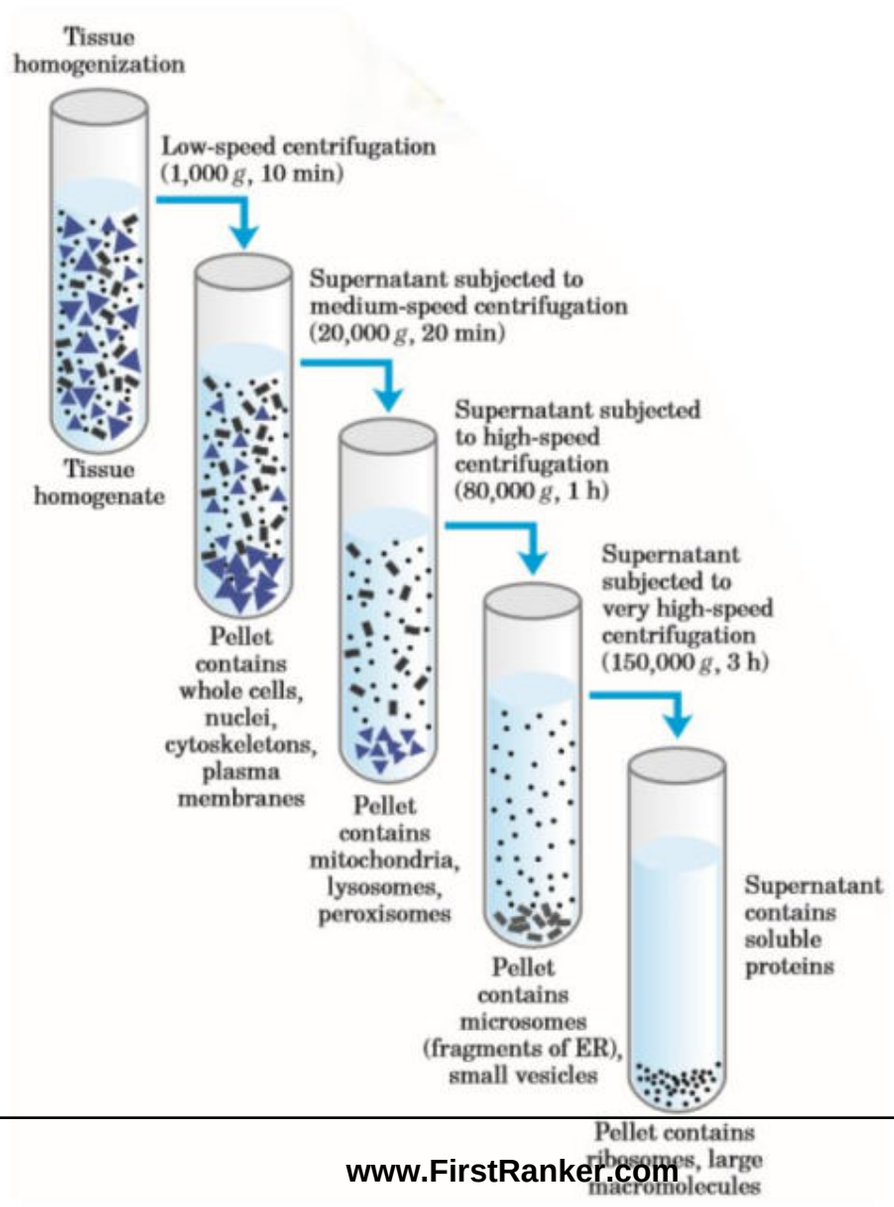


Fig1.8 a: Lehninger Principles of Biochemistry by David L Nelson

Methods of Proteins Purification

All purification techniques are based on:

- Stability
- Solubility
- Size
- Charge
- Binding affinity
- Hydrophobicity

Chromatographic techniques separate one protein from another based upon difference in their:

- Size (size exclusion chromatography),
- Charge (ion-exchange chromatography),
- Hydrophobicity (hydrophobic interaction chromatography),
- Ability to bind a specific ligand (affinity chromatography).

Dialysis

- Proteins separated from small molecules such as salt by dialysis through a semipermeable membrane with pores.

- Protein mixture is placed inside the dialysis bag, submerged in a buffer solution that devoid of small molecules to be separated away.

- Molecules dimensions significantly greater than the pore diameter are retained inside the dialysis bag.

Cont--

- Smaller molecules and ions capable of passing through the pores of the membrane diffuse down their concentration gradients and emerge in the solution outside the bag.

- Useful for removing a salt or other small molecule from a cell fractionate, but it will not distinguish between proteins effectively.

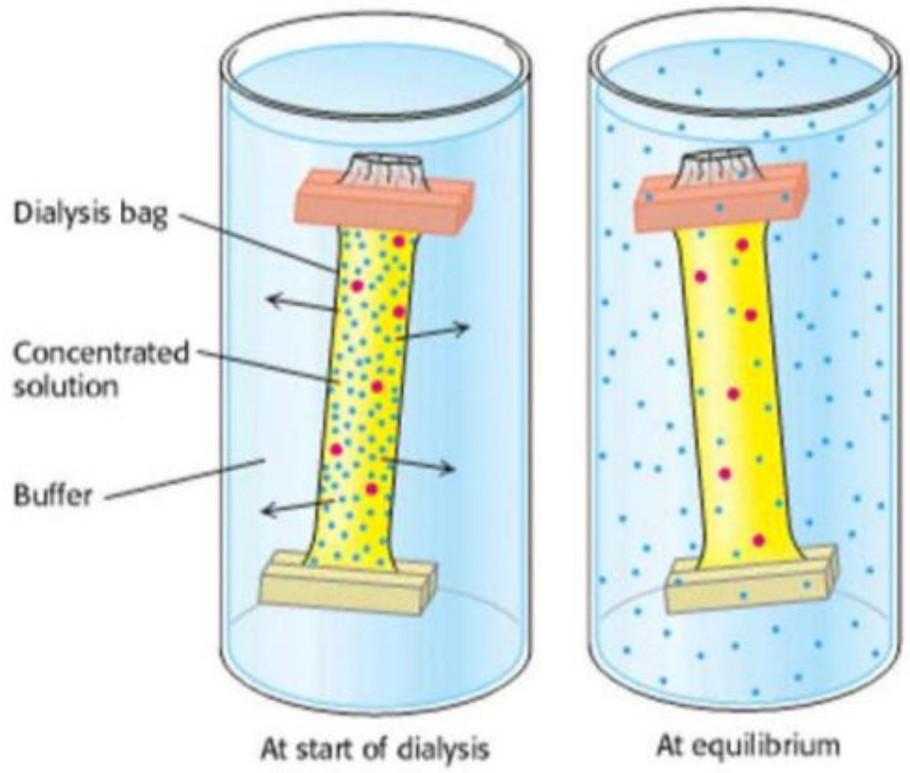


Fig. 3.2. Protein molecules (red) are retained within the dialysis bag, whereas small molecules (blue) diffuse into the surrounding medium. Biochemistry 7th edition by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer.

Hydrophobic Interaction Chromatography

- It separates proteins based on their tendency to associate with a stationary phase matrix coated with hydrophobic groups (eg, phenyl Sepharose, octyl Sephadex).

- Proteins with exposed hydrophobic surfaces adhere to the matrix via hydrophobic interactions enhanced by employing a mobile phase of high ionic strength.

Cont--

- After non-adherent proteins are washed away, polarity of the mobile phase is decreased by gradually lowering its salt concentration.

- If the interaction between protein and stationary phase is strong, ethanol or glycerol added to the mobile phase to decrease its polarity and weaken hydrophobic interactions.

Short hydrophobic-interaction chromatography video attached with email

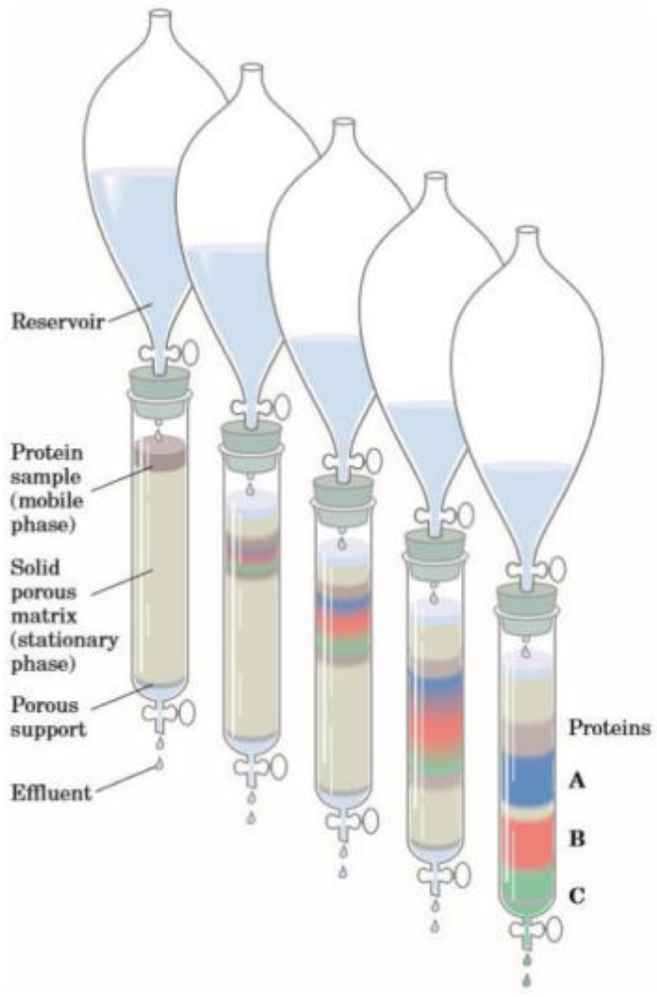
Column Chromatography

- Porous solid material with appropriate chemical properties (the stationary phase) held in a column, and a buffered solution (the mobile phase) pass through it.

- Protein-containing solution, layered on the top of the column, pass through the solid matrix as an ever-expanding band within the larger mobile phase.

- As the mobile-phase liquid emerges from the column, it is automatically collected in a series of small portions called fractions.

Column Chromatography



Column chromatography: The standard elements of a chromatographic column include a solid, porous material supported inside a column, generally made of plastic or glass. The solid material (matrix) makes up the stationary phase through which flows a solution, the mobile phase. The solution that passes out of the column at the bottom (the effluent) is constantly replaced by solution supplied from a reservoir at the top. The protein solution to be separated is layered on top of the column and allowed to percolate into the solid matrix. Additional solution is added on top. The protein solution forms a band within the mobile phase that is initially the depth of the protein solution applied to the column. As proteins migrate through the column, they are retarded to different degrees by their different interactions with the matrix material. The overall protein band thus widens as it moves through the column. Individual types of proteins (such as A, B, and C, shown in blue, red, and green) gradually separate from each other, forming bands within the broader protein band. Separation improves (resolution increases) as the length of the column increases. However, each individual protein band also broadens with time due to diffusional spreading, a process that decreases resolution. In this example, protein A is well separated from B and C, but diffusional spreading prevents complete separation of B and C under these conditions. **Fig 3.17: Lehninger Principles of Biochemistry by David L Nelson**

Short column chromatography video attached with email

High-Pressure Liquid Chromatography (HPLC)

- Column materials are very finely divided and, as a consequence, more interaction sites and greater resolving power.
- Column is made of finer material, high-pressure applied to the column to obtain adequate flow rates.
- Net result is both high resolution and rapid separation.

Cont--

- In a typical HPLC setup, a detector that monitors the absorbance of the eluate at a particular wavelength is placed after the column.
- In the sample HPLC elution profile, proteins are detected by setting the detector to 220 nm (the characteristic absorbance wavelength of the peptide bond).
- In a short span of 10 minutes, a number of sharp peaks representing individual proteins can be readily identified.

Cont--

Gel filtration by HPLC clearly defines the individual proteins because of its greater resolving power:

1. Thyroglobulin (669 kd),
2. Catalase (232 kd),
3. Bovine serum albumin (67 kd),
4. Ovalbumin (43 kd), and
5. Ribonuclease (13.4 kd).

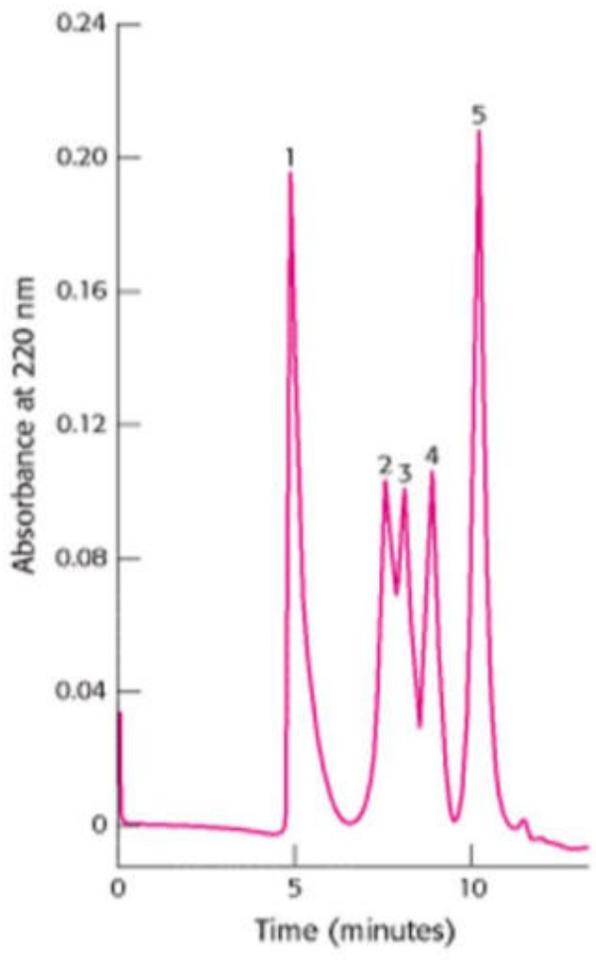


Fig. 4.6. High-Pressure Liquid Chromatography (HPLC): Biochemistry 7th edition by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer.

Ion-Exchange Chromatography

- In mobile phase, proteins with a net positive charge migrate through the matrix more slowly than those with a net negative charge, because the migration of the former is retarded more by interaction with the stationary phase.

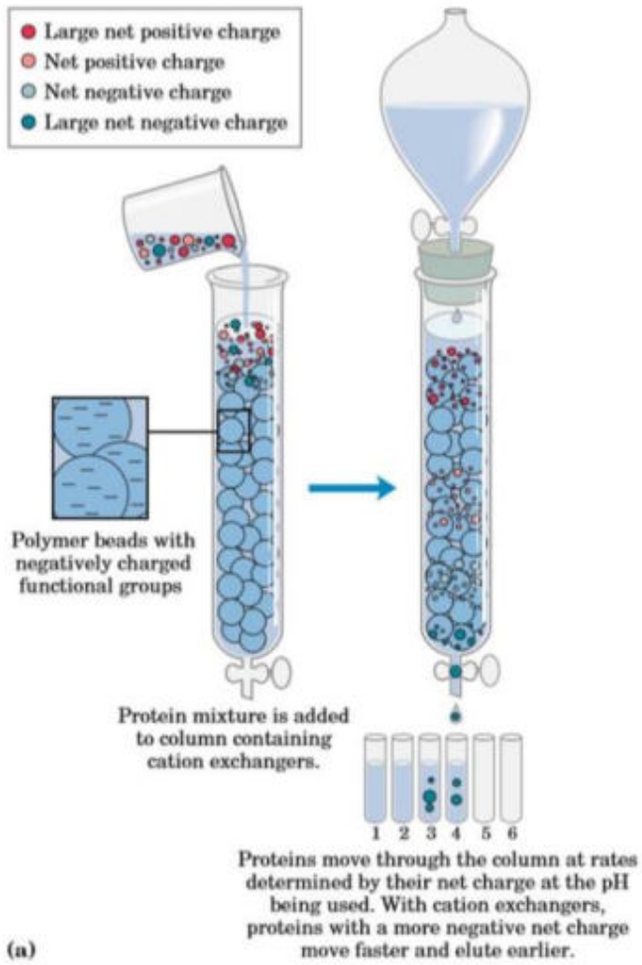
- Expansion of the protein band in mobile phase caused both by separation of proteins with different properties and by diffusional spreading.

Cont--

- As the length of the column increases, the resolution of two types of protein with different net charges generally improves.

- Rate at which the protein solution can flow through the column decreases with column.

Ion-Exchange Chromatography

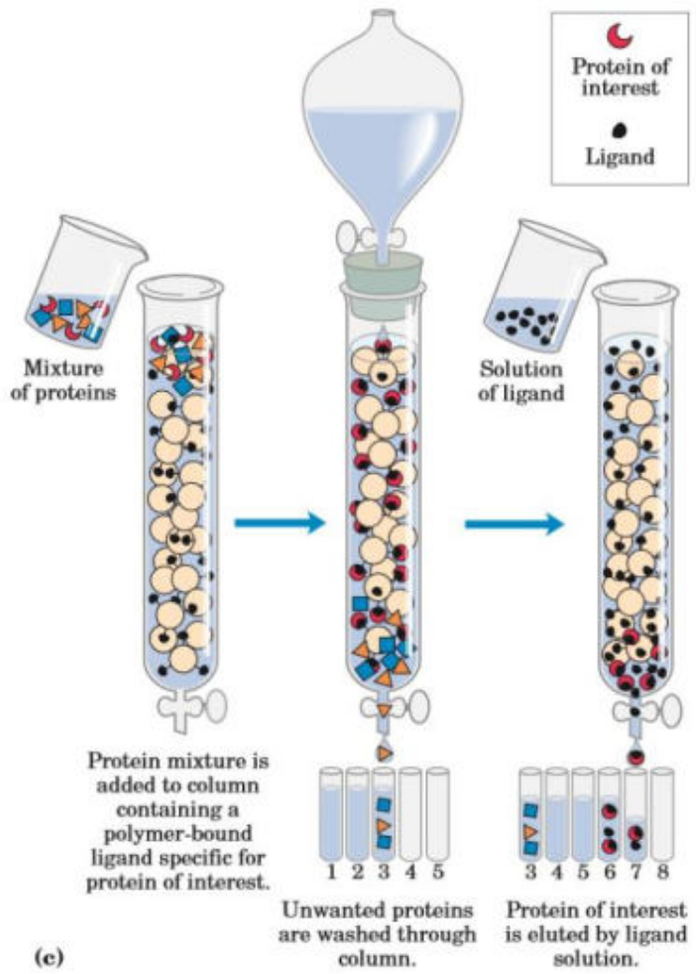


Ion-exchange chromatography exploits differences in the sign and magnitude of the net electric charges of proteins at a given pH. The column matrix is a synthetic polymer containing bound charged groups; those with bound anionic groups are called cation exchangers, and those with bound cationic groups are called anion exchangers. Ion-exchange chromatography on a cation exchanger is shown here. The affinity of each protein for the charged groups on the column is affected by the pH (which determines the ionization state of the molecule) and the concentration of competing free salt ions in the surrounding solution. Separation can be optimized by gradually changing the pH and/or salt concentration of the mobile phase so as to create a pH or salt gradient. Fig 3.18 a: Lehninger Principles of Biochemistry by David L Nelson. **Short Ion-exchange video attached with email**

Affinity Chromatography

- Based on binding affinity of a protein.
- Beads in column covalently attached to chemical group.
- Protein with affinity for this particular chemical group bind to the beads in the column, and its migration retarded.

Affinity Chromatography



Affinity chromatography separates proteins by their binding specificities. The proteins retained on the column are those that bind specifically to a ligand cross-linked to the beads. (In biochemistry, the term "ligand" is used to refer to a group or molecule that binds to a macromolecule such as a protein.) After proteins that do not bind to the ligand are washed through the column, the bound protein of particular interest is eluted (washed out of the column) by a solution containing free ligand. Fig 3.18 c: Lehninger Principles of Biochemistry by David L Nelson. **Short affinity-chromatography video attached with email**

Gel-Filtration/Molecular Exclusion Chromatography

- More-discriminating separations on the basis of size.

- Sample is applied to the top of a column consisting of porous beads made of an insoluble but highly hydrated polymer such as dextran or agarose (which are carbohydrates) or polyacrylamide.

- Sephadex, Sepharose, and Biogel are commonly used for preparations of these beads (0.1 mm).

- Small molecules can enter these beads, but large ones cannot.

Cont--

- Small molecules distributed in the aqueous solution both inside the beads and between them, whereas large molecules located only in solution between the beads.

- Large molecules flow more rapidly through this column and emerge first because a smaller volume is accessible to them.

- Molecules enter a bead will flow from the column at an intermediate position, and small molecules, which take a longer path, exit last.

Gel-Filtration Chromatography

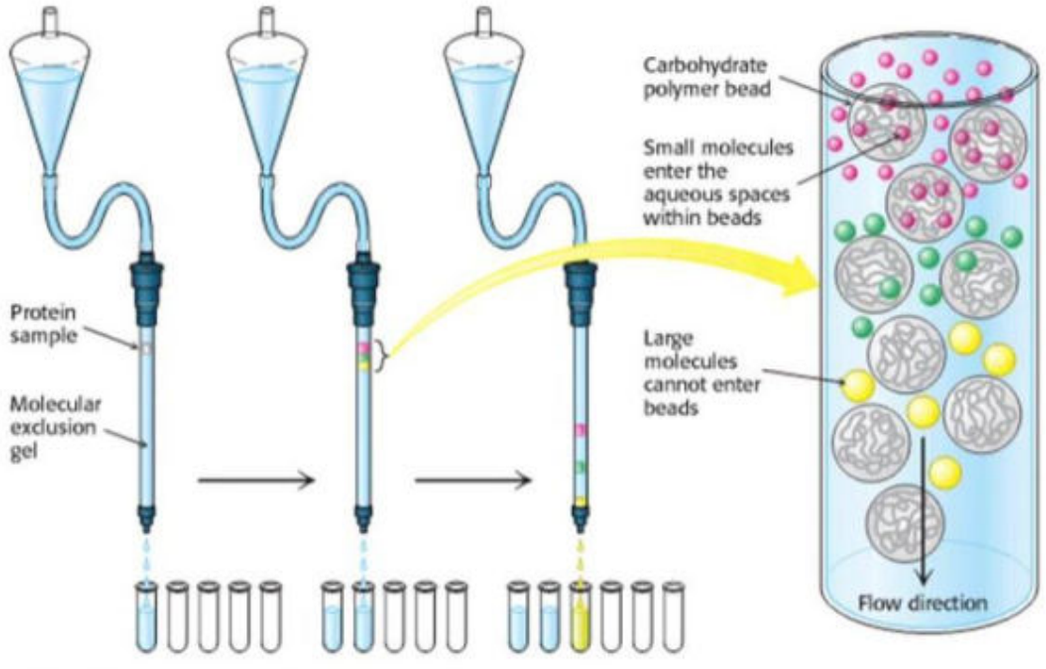


Figure 4.3. Gel Filtration Chromatography. A mixture of proteins in a small volume is applied to a column filled with porous beads. Because large proteins cannot enter the internal volume of the beads, they emerge sooner than do small ones. Fig. 4.3. Biochemistry 7th edition by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer.

Short gel-filtration chromatography video attached with email

Denaturation at the protein levels

At primary structure: Sequence of aa held together by covalent peptide bonds, is not disrupted by denaturation.

At secondary structure: Proteins lose all regular repeating patterns such as α-helices and β-pleated sheets and adopt a random coil shape.

Cont--

At tertiary structure: Disruption of covalent interactions between aa side chains (such as disulfide-bridges bet cysteine groups), non-covalent interactions between polar aa side-chains, van-der waals interactions between non-polar aa side chains.

At quaternary structure: Protein sub-units are dissociated and/or spatial arrangement of protein subunits is disrupted.

Cont--

- Native Protein denaturate in presence of reducing agent, alter pH, temp, ionic strength, and solubility.

- Denature protein causes loss of structure and function.

Various denaturants are:

1. Heat disrupts hydrogen bonds and hydrophobic interactions bet non-polar residues. Ex. Albumin in egg denature and coagulate during cooking.

1. Strong acids and bases disrupts the salt bridges formed in a protein structure. Ex. In digestive system, acidic gastric juices causes coagulation of milk by proteolytic enzyme renin.

Cont--

2. Urea disrupts the stabilizing hydrophobic interactions, thus freeing entire polypeptide from its folded conformation.

3. Reducing agents like guanidine hydrochloride (GdnHCL) or β-mercaptoethanol reduces the disulphide bonds to sulfhydryl group and breaks intra and interchain disulphide bonds.

4. Detergents (SDS) disrupts hydrophobic interactions.

Renaturation of Unfolded Proteins

- The Nobel prize in chemistry 1972 was divided to Christian B Anfinsen provided the first evidence that:

➢Aa sequence of a polypeptide chain contains all information required to fold the chain into its native, 3-D structure.

- Certain globular proteins denatured by heat, extremes of pH, or denaturing reagents will regain their native structure and their biological activity if returned to conditions in which the native conformation is stable. This process is called renaturation.

Renaturation of Unfolded, Denatured Ribonuclease

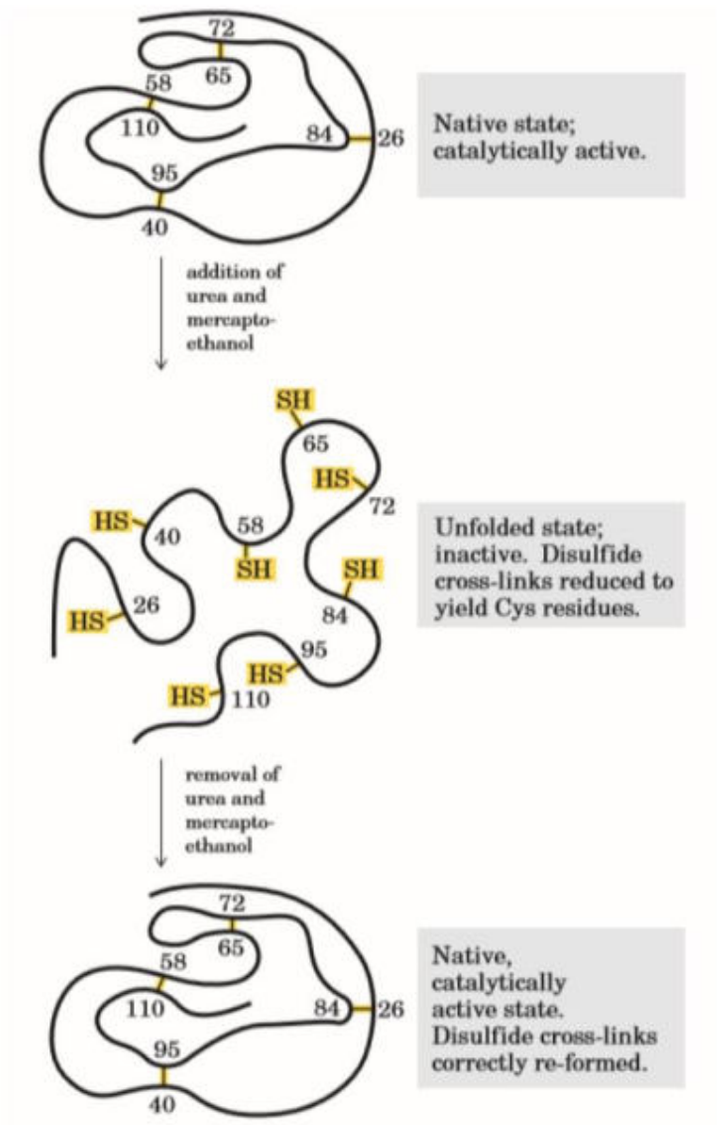


FIGURE 4-27 Renaturation of unfolded, denatured ribonuclease. Urea is used to denature ribonuclease, and mercaptoethanol ($HOCH_2CH_2SH$) to reduce and thus cleave the disulfide bonds to yield eight Cys residues. Renaturation involves reestablishment of the correct disulfide cross-links.

Lehninger Principles of Biochemistry by David L Nelson

Summary

- Proteins are separated and purified based on their properties.
- Proteins precipitated by the addition of certain salts.
- Chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties.
- These include ion-exchange, size-exclusion, affinity, and high-performance liquid chromatography.

Cont--

- The 3-D structure and the function of proteins destroyed by denaturation, which demonstrate the relationship between structure and function.
- Some denatured proteins can renature spontaneously to form biologically active protein.

Reference Books

1) Harper's Illustrated Biochemistry-30th edition.
2) Biochemistry 7th edition by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer.
3) Lehninger Principles of Biochemistry by David L Nelson.
4) Short you-tube video clips on types of chromatography.

Thank you