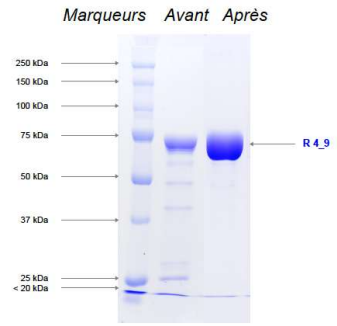
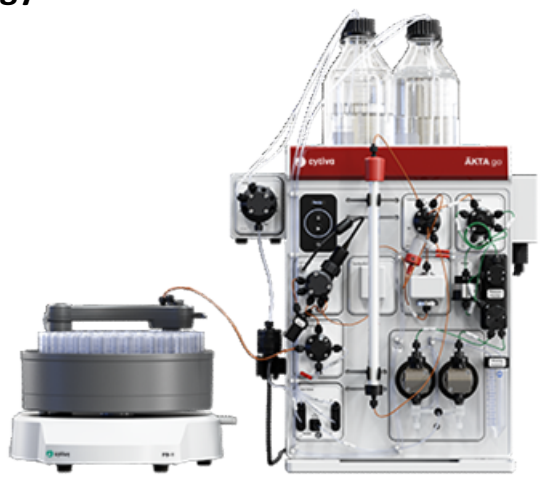
Separation/Purification of Biomacromolecules

Master I Applied Microbiology

University of Jijel

Pr H Ouled Haddar





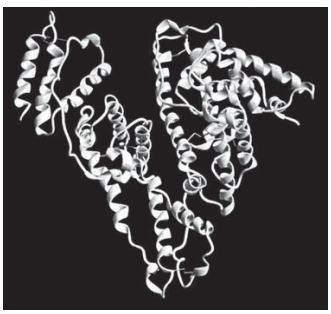
UE. Separation/Purification of Biomacromolecules

- **Reminder :** Structure and physico-chemical properties of biomacromolecules (BMM)
- **Chapter I :** Principal steps of biomacromolecule purification
- **Chapter II :** Clarification and extraction techniques
- **Chapter III** : Protein quantification and storage
- **Chapter IV :** Techniques of concentration of BMM
- **Chapter V :** Chromatography techniques
- **Chapter VI :** Electrophoresis techniques

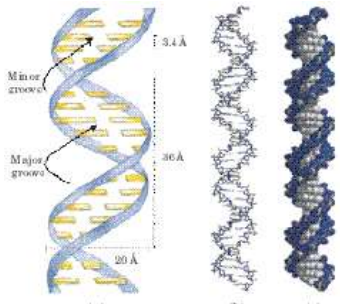
Reminder:

Structure and physico-chemical properties of biomacromolecules (BMM)

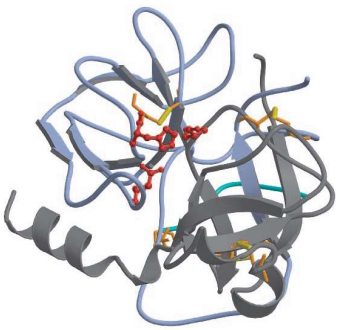
Reminder
Structure and physico-chemical properties of biomacromolecules (BMM): Proteins and nucleic acids



Human serum albumin. From He and Carter.

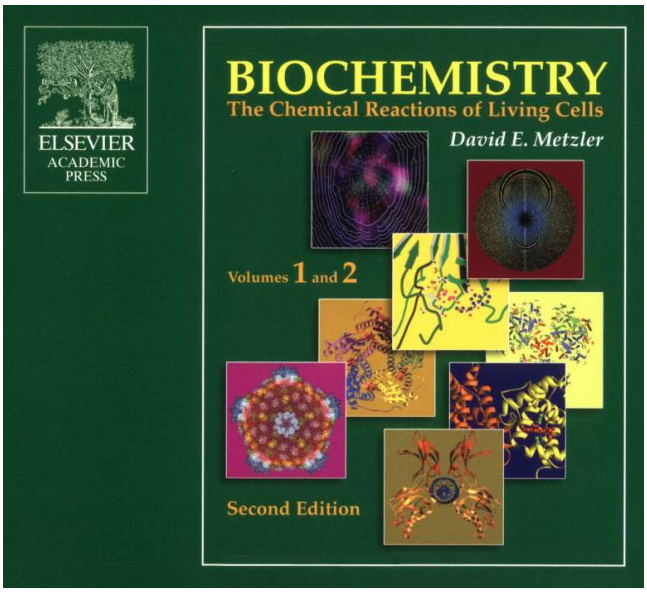


DNA



Chymotrypsin

Références

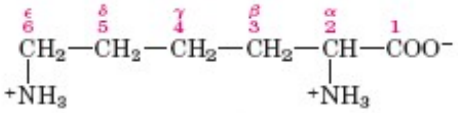


Lehninger, Principles of Biochemistry, Fourth Edition

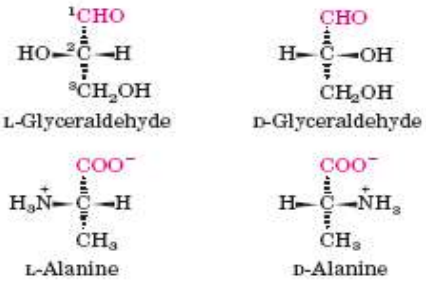
I. Proteins

Amino-acids

FIGURE 3-2 General structure of an amino acid. This structure is common to all but one of the α-amino acids. (Proline, a cyclic amino acid, is the exception.) The R group or side chain (red) attached to the α carbon (blue) is different in each amino acid.



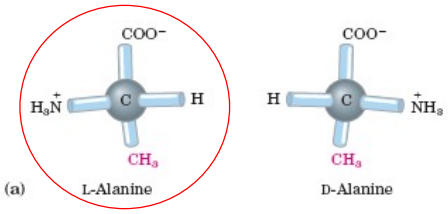
Lysine



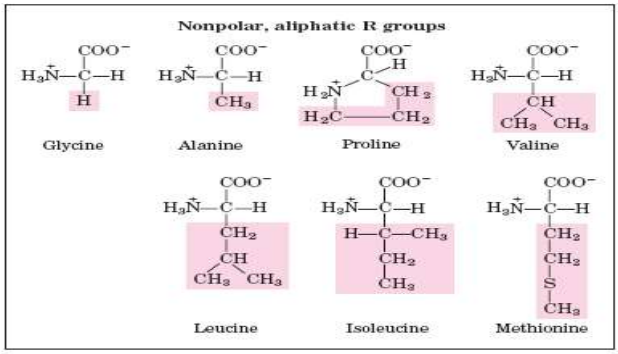
L-Glyceraldehyde D-Glyceraldehyde

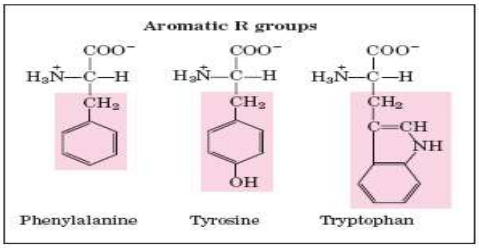
L-Alanine D-Alanine

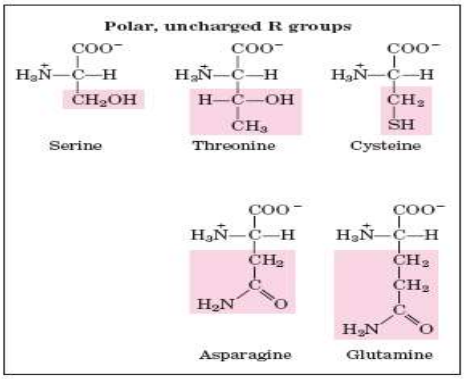
FIGURE 3-4 Steric relationship of the stereoisomers of alanine to the absolute configuration of L- and D-glyceraldehyde. In these perspective formulas, the carbons are lined up vertically, with the chiral atom in the center. The carbons in these molecules are numbered beginning with the terminal aldehyde or carboxyl carbon (red), 1 to 3 from top to bottom as shown. When presented in this way, the R group of the amino acid (in this case the methyl group of alanine) is always below the α carbon. L-Amino acids are those with the α-amino group on the left, and D-amino acids have the α-amino group on the right.

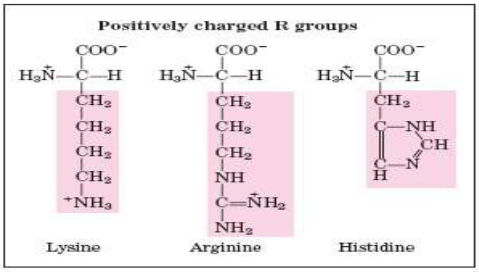


(a) L-Alanine D-Alanine









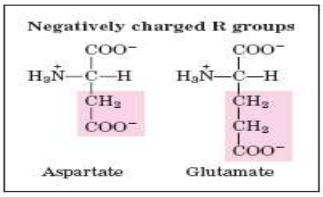


FIGURE 3-5 The 20 common amino acids of proteins. The structural formulas show the state of ionization that would predominate at pH 7.0. The unshaded portions are those common to all the amino acids; the portions shaded in red are the R groups. Although the R group of histidine is shown uncharged, its pK_a (see Table 3-1) is such that a small but significant fraction of these groups are positively charged at pH 7.0.

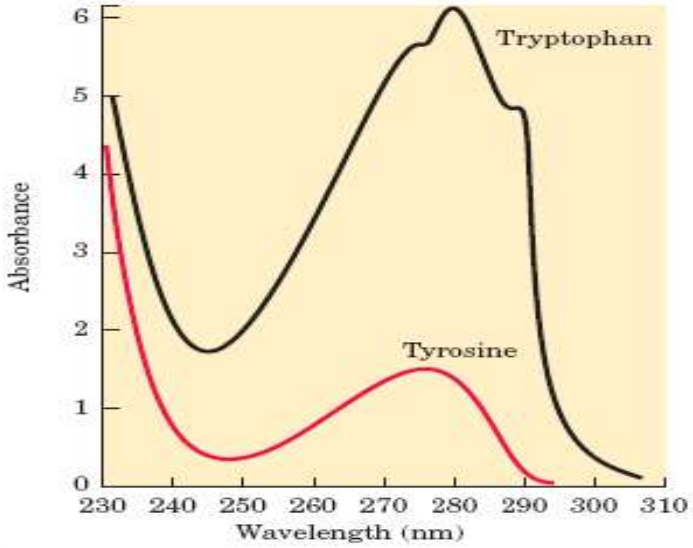
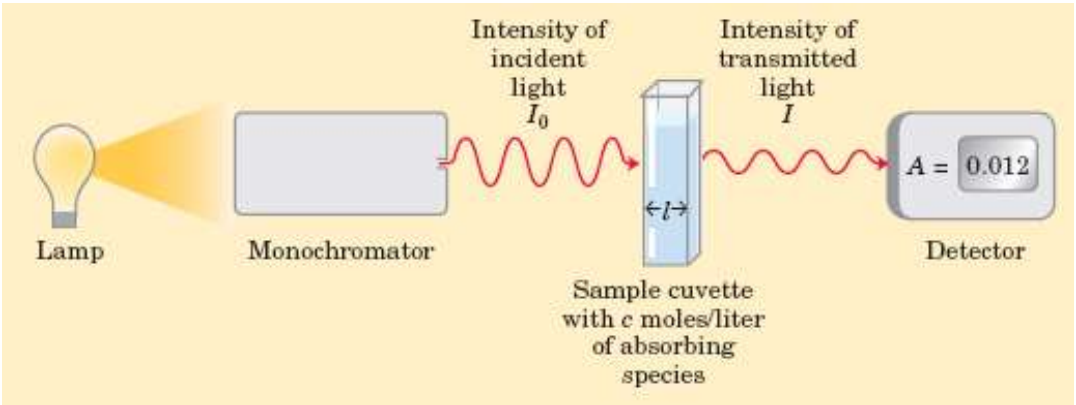


FIGURE 3–6 Absorption of ultraviolet light by aromatic amino acids. Comparison of the light absorption spectra of the aromatic amino acids tryptophan and tyrosine at pH 6.0. The amino acids are present in equimolar amounts (10^{-3} M) under identical conditions. The measured absorbance of tryptophan is as much as four times that of tyrosine. Note that the maximum light absorption for both tryptophan and tyrosine occurs near a wavelength of 280 nm. Light absorption by the third aromatic amino acid, phenylalanine (not shown), generally contributes little to the spectroscopic properties of proteins.

Absorption of Light by Molecules: The Lambert-Beer Law

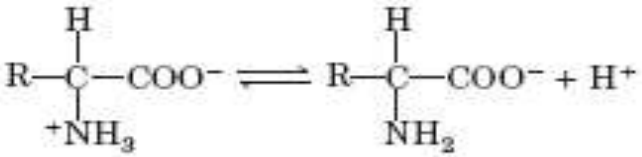


$$\log \frac{I_0}{I} = \varepsilon c l \qquad A = \epsilon c l$$

Beer's law

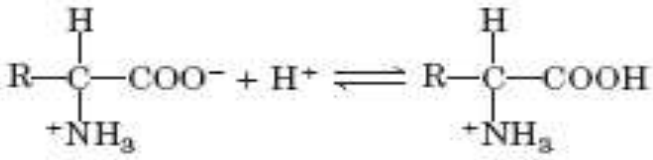
Amino Acids Can Act as Acids and Bases

When an amino acid is dissolved in water, it exists in solution as the dipolar ion, or **zwitterion** (German for "hybrid ion"), shown in Figure 3–9. A zwitterion can act as either an acid (proton donor):



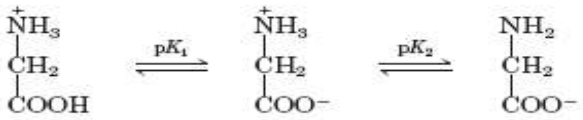
Zwitterion

or a base (proton acceptor):



Zwitterion

ampholytes



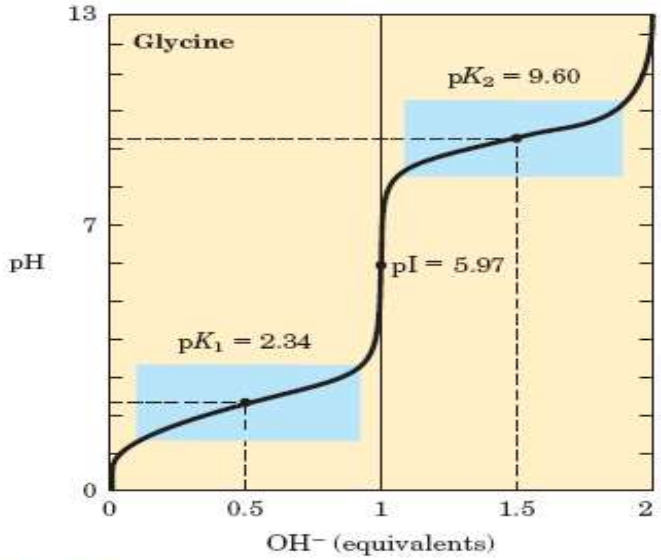


FIGURE 3–10 **Titration of an amino acid.** Shown here is the titration curve of 0.1 M glycine at 25 °C. The ionic species predominating at key points in the titration are shown above the graph. The shaded boxes, centered at about $pK_1 = 2.34$ and $pK_2 = 9.60$, indicate the regions of greatest buffering power.

Isoelectric pH

$$pI = \frac{1}{2}(pK_1 + pK_2) = \frac{1}{2}(2.34 + 9.60) = 5.97$$

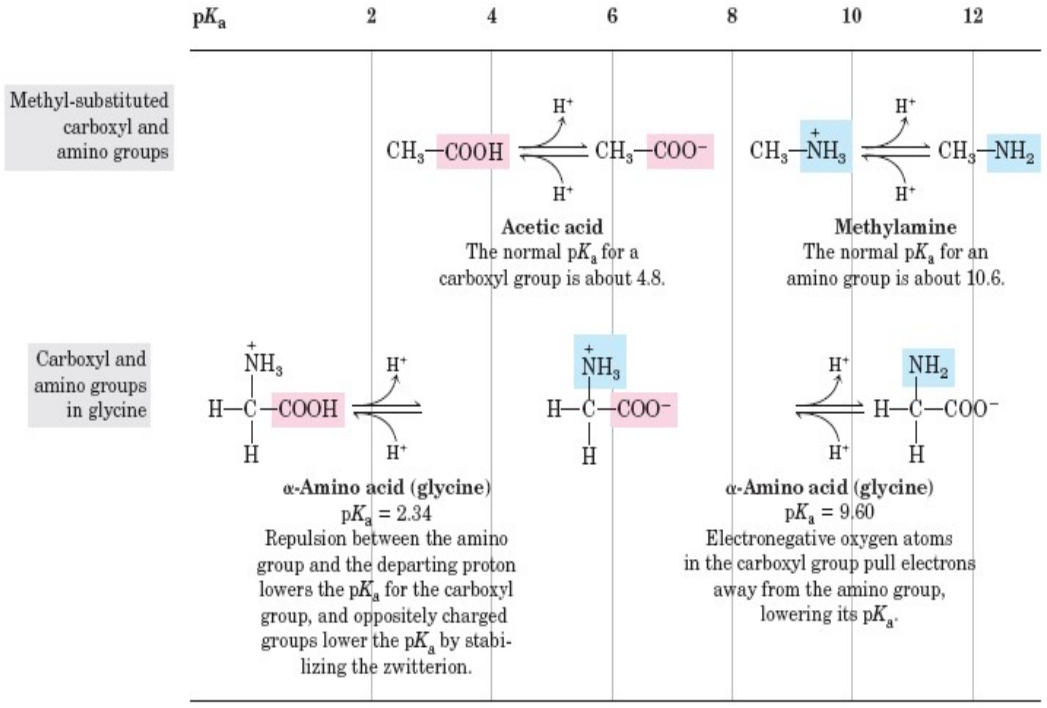


FIGURE 3-11 Effect of the chemical environment on pK_a. The pK_a values for the ionizable groups in glycine are lower than those for simple, methyl-substituted amino and carboxyl groups. These downward perturbations of pK_a are due to intramolecular interactions. Similar effects can be caused by chemical groups that happen to be positioned nearby—for example, in the active site of an enzyme.

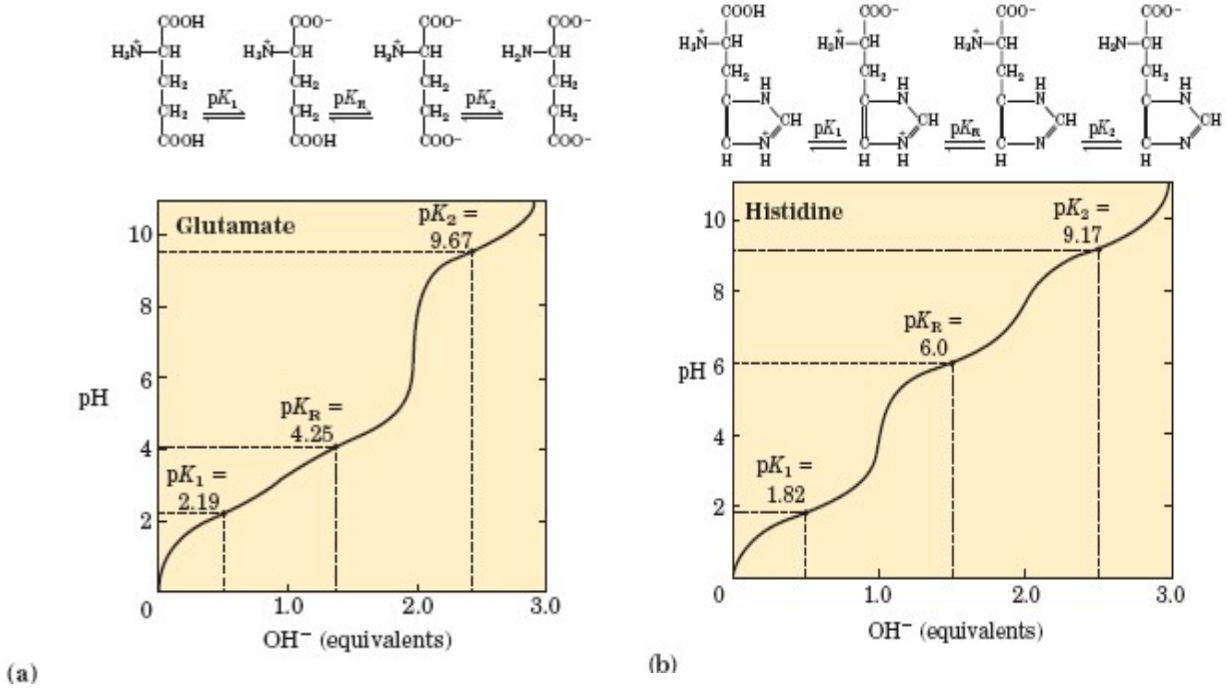


FIGURE 3-12 Titration curves for (a) glutamate and (b) histidine. The pK_a of the R group is designated here as pK_R.

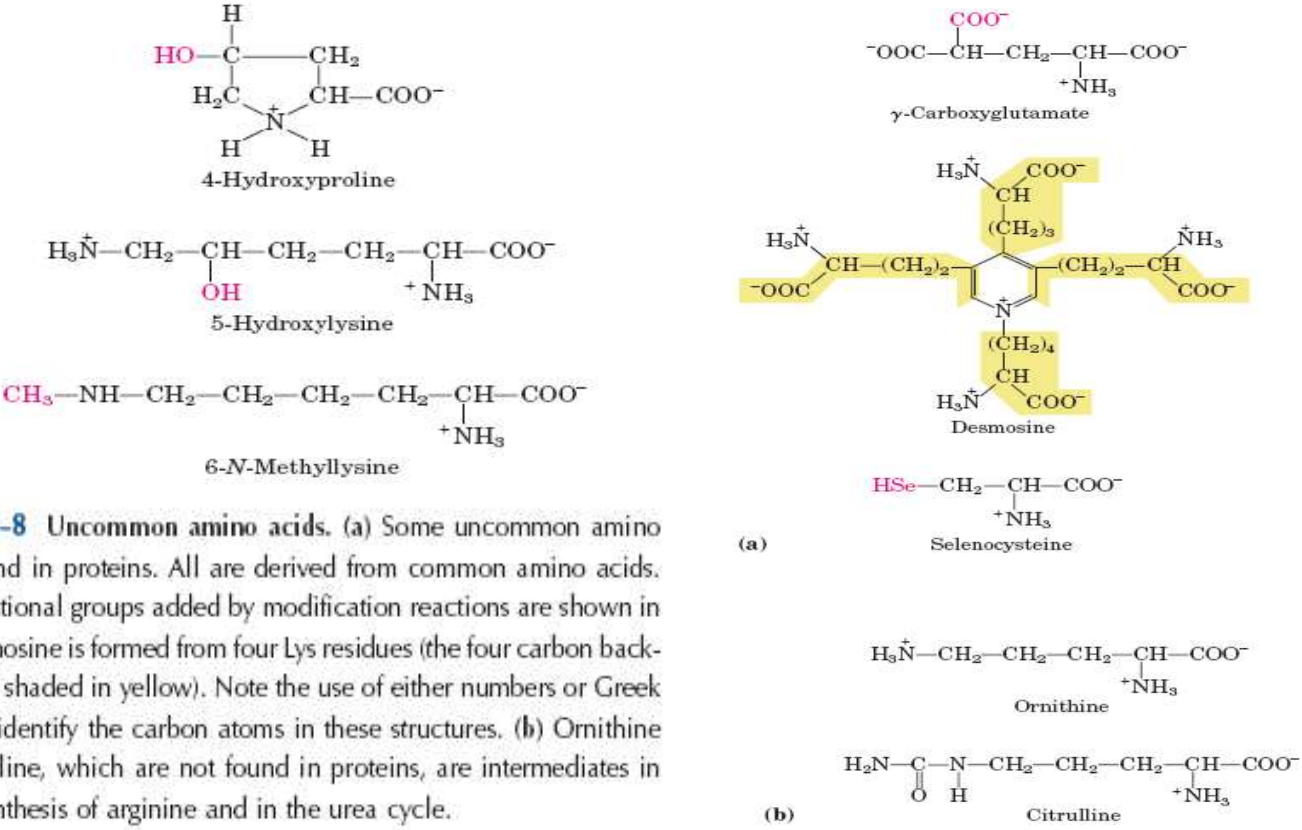


FIGURE 3–8 **Uncommon amino acids.** (a) Some uncommon amino acids found in proteins. All are derived from common amino acids. Extra functional groups added by modification reactions are shown in red. Desmosine is formed from four Lys residues (the four carbon backbones are shaded in yellow). Note the use of either numbers or Greek letters to identify the carbon atoms in these structures. (b) Ornithine and citrulline, which are not found in proteins, are intermediates in the biosynthesis of arginine and in the urea cycle.

Peptides and proteins

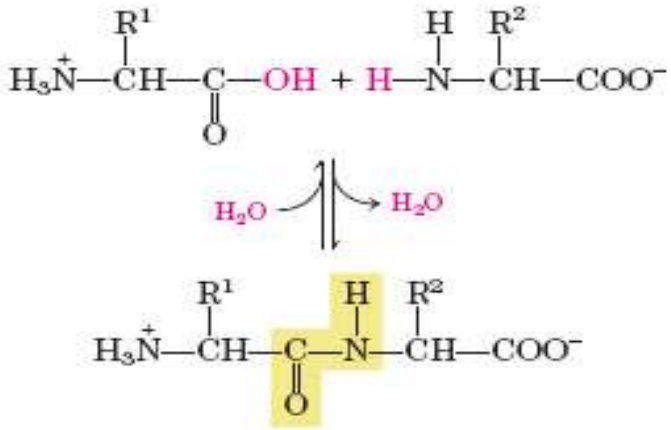


FIGURE 3–13 **Formation of a peptide bond by condensation.** The α-amino group of one amino acid (with R^2 group) acts as a nucleophile to displace the hydroxyl group of another amino acid (with R^1 group), forming a peptide bond (shaded in yellow). Amino groups are good nucleophiles, but the hydroxyl group is a poor leaving group and is not readily displaced. At physiological pH, the reaction shown does not occur to any appreciable extent.

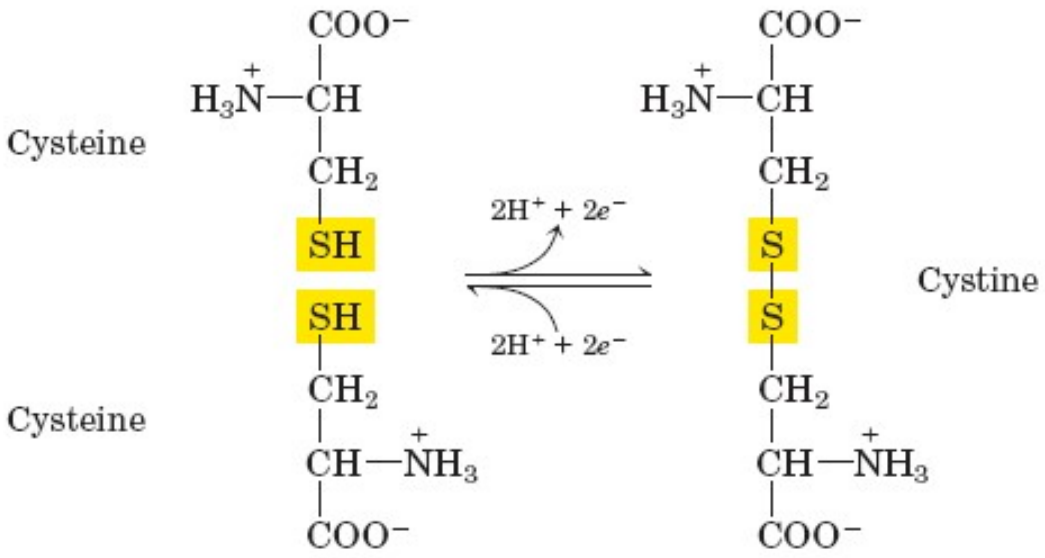


FIGURE 3–7 Reversible formation of a disulfide bond by the oxidation of two molecules of cysteine. Disulfide bonds between Cys residues stabilize the structures of many proteins.

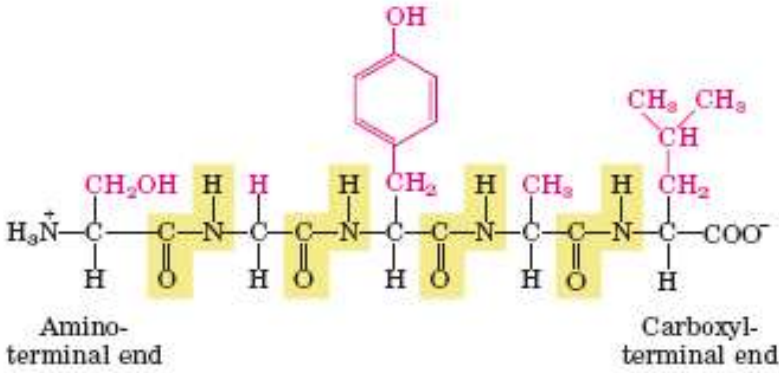


FIGURE 3–14 The pentapeptide serylglycyltyrosylalanylleucine, or Ser–Gly–Tyr–Ala–Leu. Peptides are named beginning with the amino-terminal residue, which by convention is placed at the left. The peptide bonds are shaded in yellow; the R groups are in red.

TABLE 3–4 Conjugated Proteins

Class	*Prosthetic group*	*Example*
Lipoproteins	Lipids	β_1-Lipoprotein of blood
Glycoproteins	Carbohydrates	Immunoglobulin G
Phosphoproteins	Phosphate groups	Casein of milk
Hemoproteins	Heme (iron porphyrin)	Hemoglobin
Flavoproteins	Flavin nucleotides	Succinate dehydrogenase
Metalloproteins	Iron	Ferritin
	Zinc	Alcohol dehydrogenase
	Calcium	Calmodulin
	Molybdenum	Dinitrogenase
	Copper	Plastocyanin

Secondary, tertiary and quaternary structure

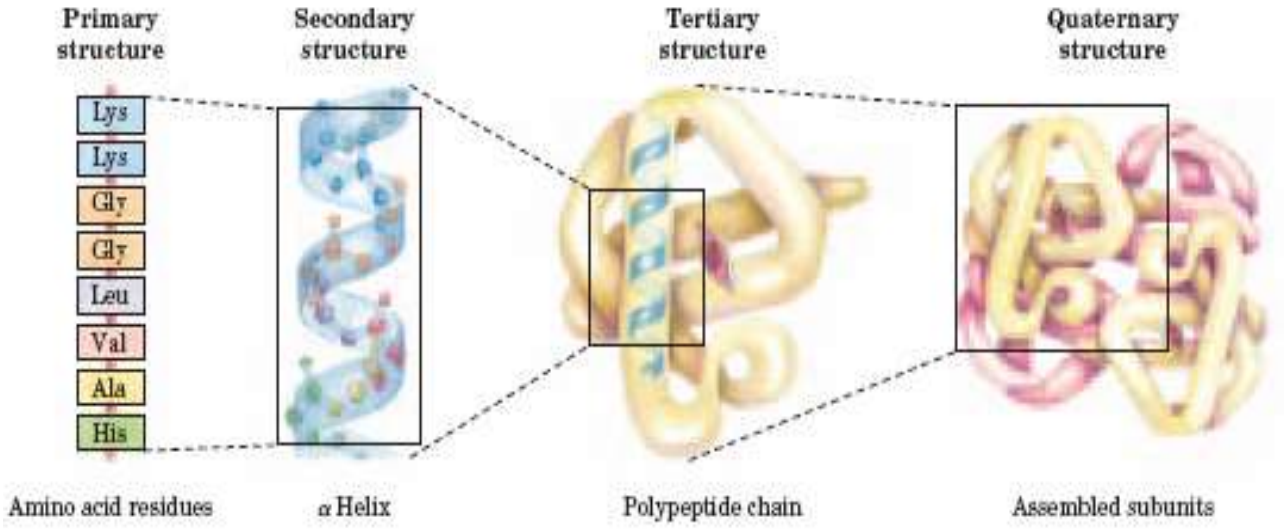
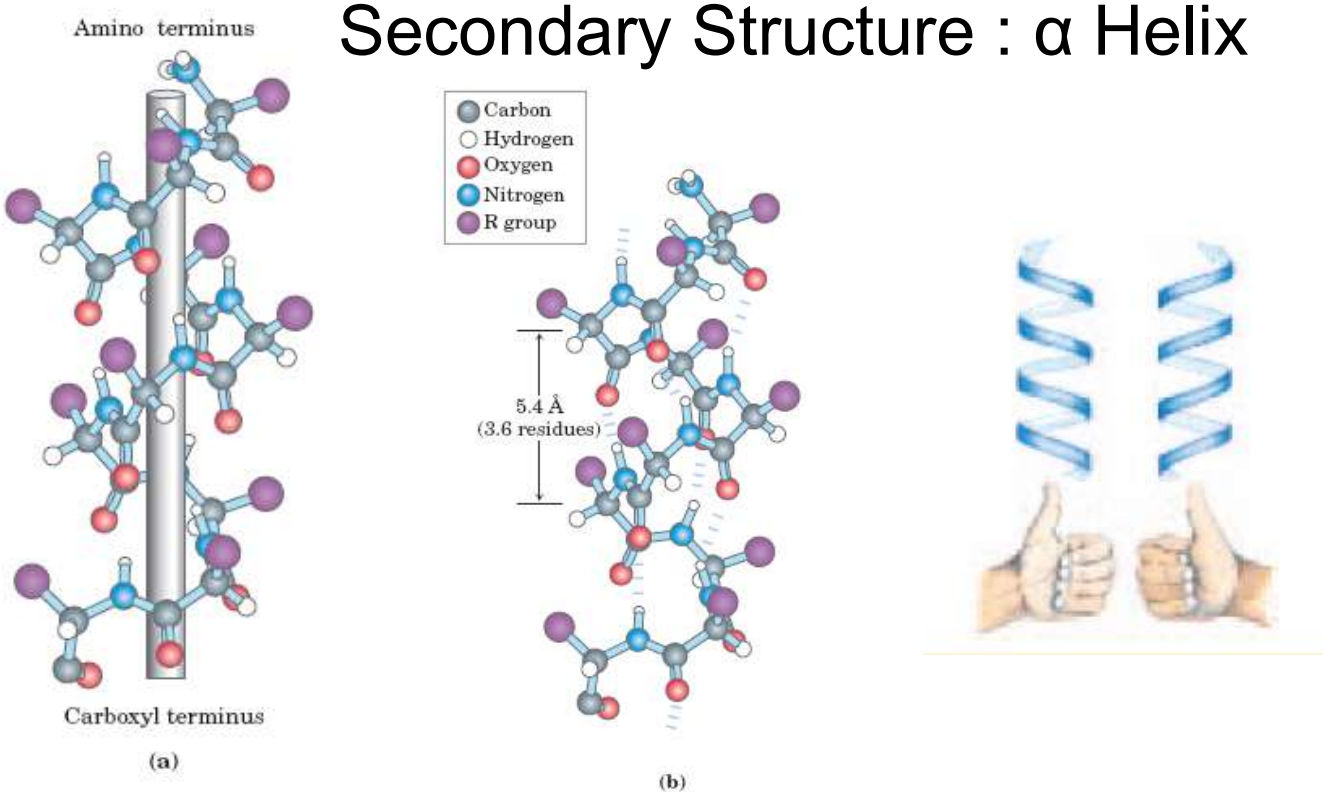


FIGURE 3–16 Levels of structure in proteins. The *primary structure* consists of a sequence of amino acids linked together by peptide bonds and includes any disulfide bonds. The resulting polypeptide can be coiled into units of *secondary structure*, such as an α helix. The helix is a part of the *tertiary structure* of the folded polypeptide, which is itself one of the subunits that make up the *quaternary structure* of the multisubunit protein, in this case hemoglobin.

Secondary Structure : α Helix



Secondary Structure : β sheet

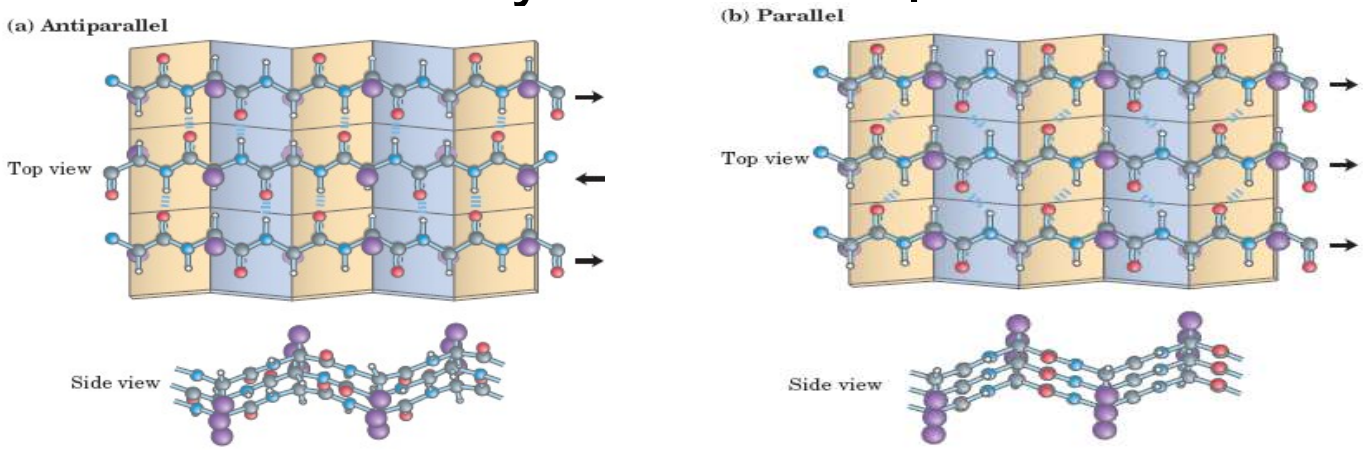
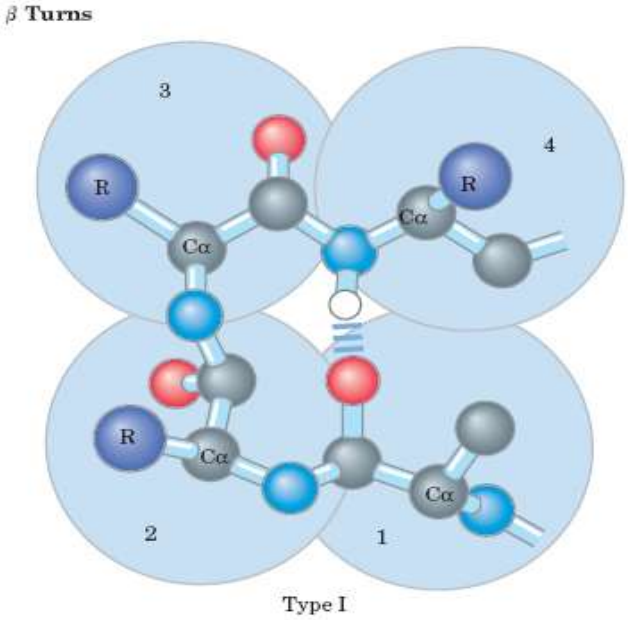


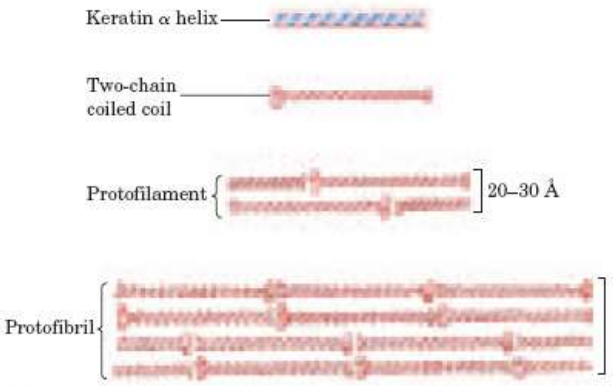
FIGURE 4–7 The β conformation of polypeptide chains. These top and side views reveal the R groups extending out from the β sheet and emphasize the pleated shape described by the planes of the peptide bonds. (An alternative name for this structure is β-pleated sheet.) Hydrogen-bond cross-links between adjacent chains are also shown. (a) Antiparallel β sheet, in which the amino-terminal to carboxyl-terminal orientation of adjacent chains (arrows) is inverse. (b) Parallel β sheet.

Secondary Structure : β turns

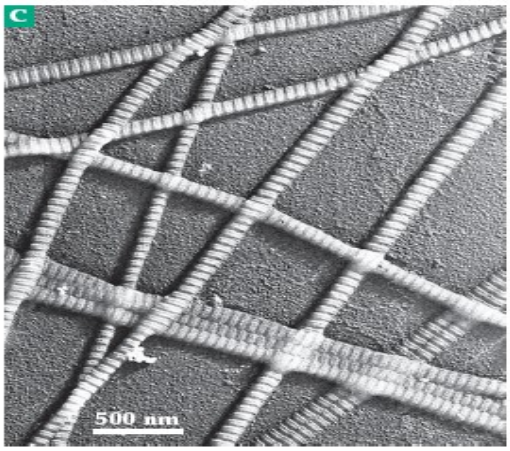


Glycine and proline

Tertiary structure



Kératine

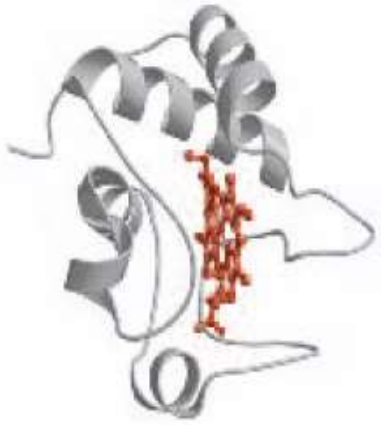


Collagène

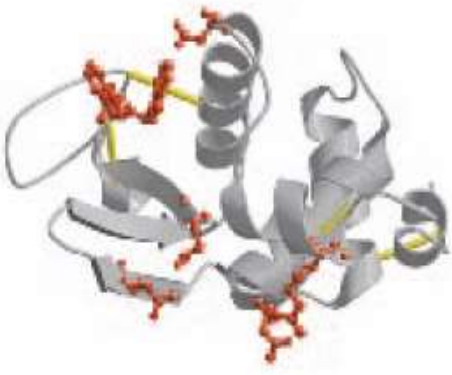
TABLE 4–1 Secondary Structures and Properties of Fibrous Proteins

Structure	*Characteristics*	*Examples of occurrence*
α Helix, cross-linked by disulfide bonds	Tough, insoluble protective structures of varying hardness and flexibility	α-Keratin of hair, feathers, and nails
β Conformation	Soft, flexible filaments	Silk fibroin
Collagen triple helix	High tensile strength, without stretch	Collagen of tendons, bone matrix

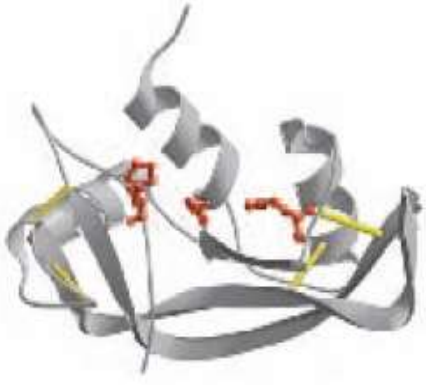
Globular proteins



Cytochrome *c*

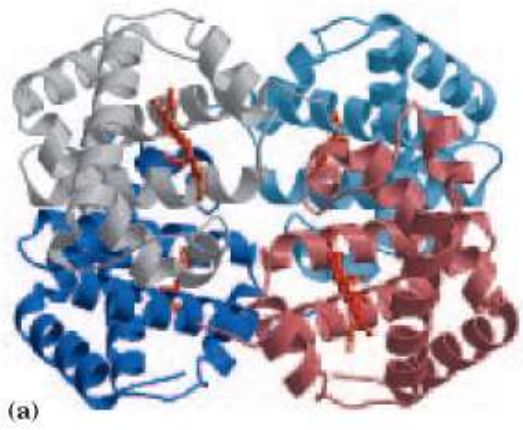


Lysozyme



Ribonuclease

Quaternary structure



Quaternary structure of deoxyhemoglobin.

II. Nucleic acids

Figure 5.8. Electron Micrograph of Part of the *E. coli* genome.

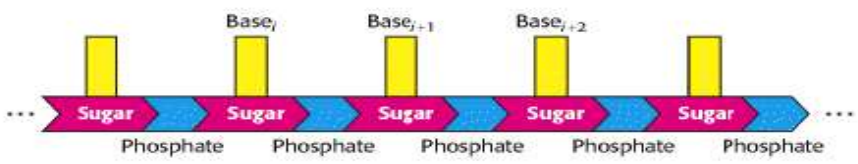
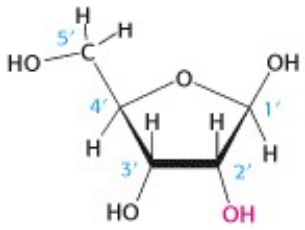


Figure 5.1. Polymeric Structure of Nucleic Acids.



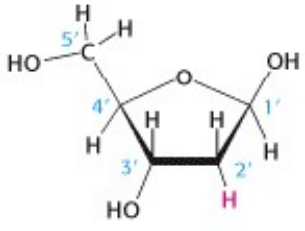


Figure 5.2. Ribose and Deoxyribose.

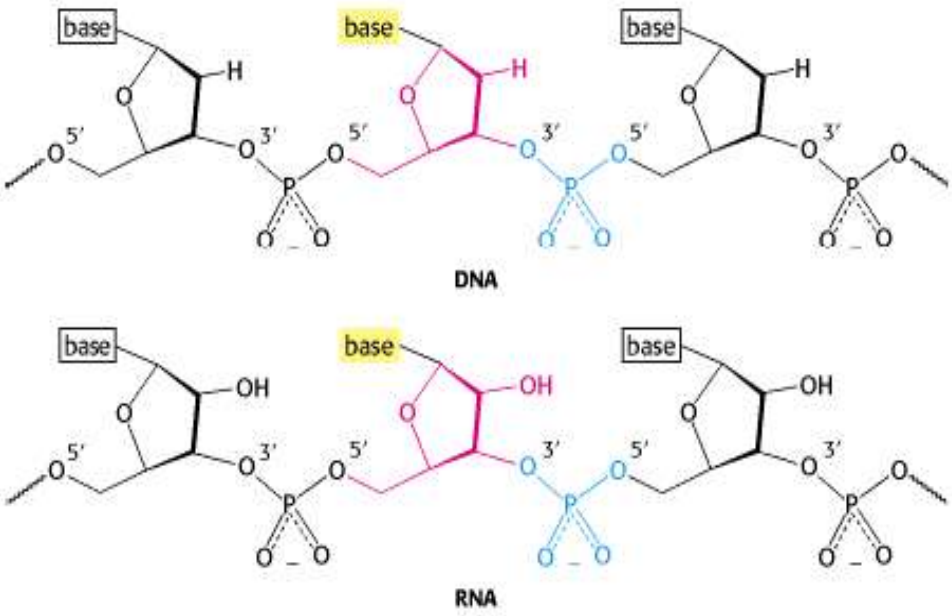
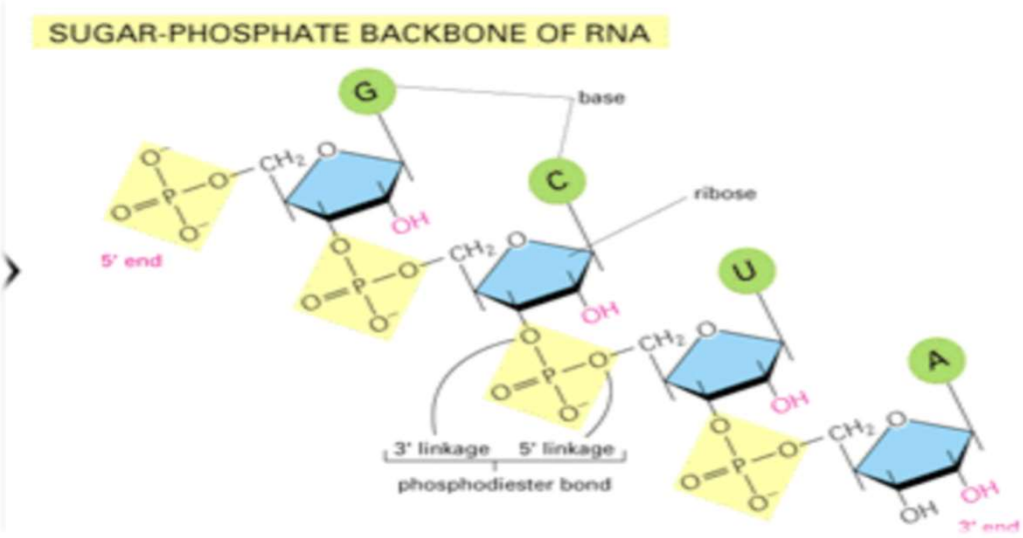


Figure 5.3. Backbones of DNA and RNA. The backbones of these nucleic acids are formed by 3′-to-5′ phosphodiester linkages. A sugar unit is highlighted in red and a phosphate group in blue.



5’ End : phosphate- group 5’P

3’ End : OH- group 3’OH

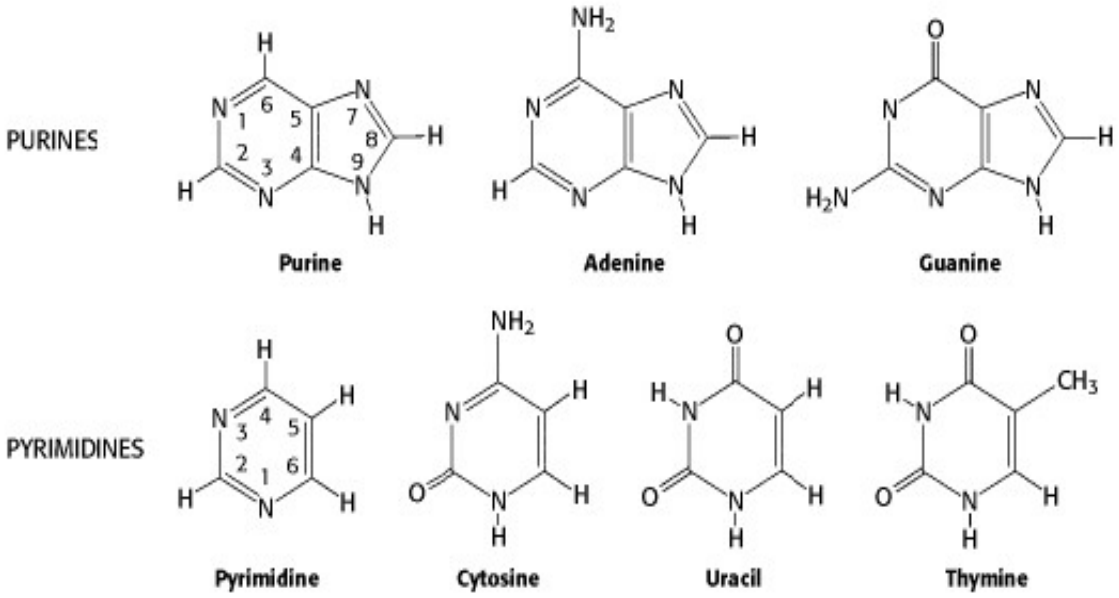
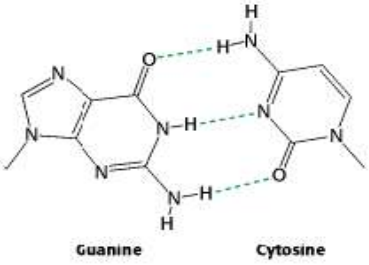
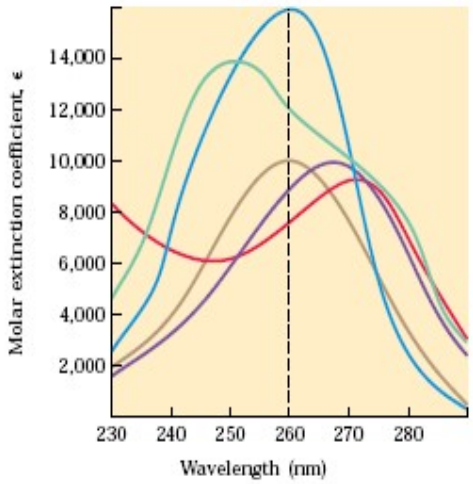


Figure 5.4. Purines and Pyrimidines. Atoms within bases are numbered without primes. Uracil instead of thymine is used in RNA.





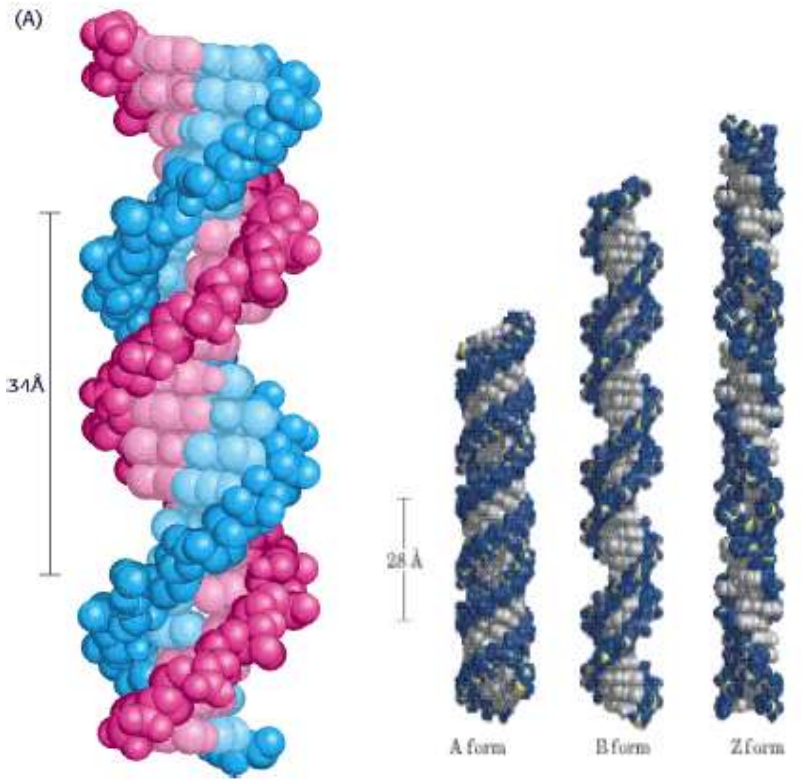
Molar extinction coefficient at 260 nm, ϵ_{260} ($M^{-1}cm^{-1}$)	
AMP	15,400
GMP	11,700
UMP	9,900
dTMP	9,200
CMP	7,500

FIGURE 8–10 Absorption spectra of the common nucleotides. The spectra are shown as the variation in molar extinction coefficient with wavelength. The molar extinction coefficients at 260 nm and pH 7.0 (ε_{260}) are listed in the table. The spectra of corresponding ribonucleotides and deoxyribonucleotides, as well as the nucleosides, are essentially identical. For mixtures of nucleotides, a wavelength of 260 nm (dashed vertical line) is used for absorption measurements.


James Watson


Francis Crick

Figure 5.12. Structures of the Base Pairs Proposed by Watson and Crick.



	A form	B form	Z form
Helical sense	Right handed	Right handed	Left handed
Diameter	~26 Å	~20 Å	~18 Å
Base pairs per helical turn	11	10.5	12
Helix rise per base pair	2.6 Å	3.4 Å	3.7 Å
Base tilt normal to the helix axis	20°	6°	7°
Sugar pucker conformation	C-3′ endo	C-2′ endo	C-2′ endo for pyrimidines; C-3′ endo for purines
Glycosyl bond conformation	Anti	Anti	Anti for pyrimidines; syn for purines

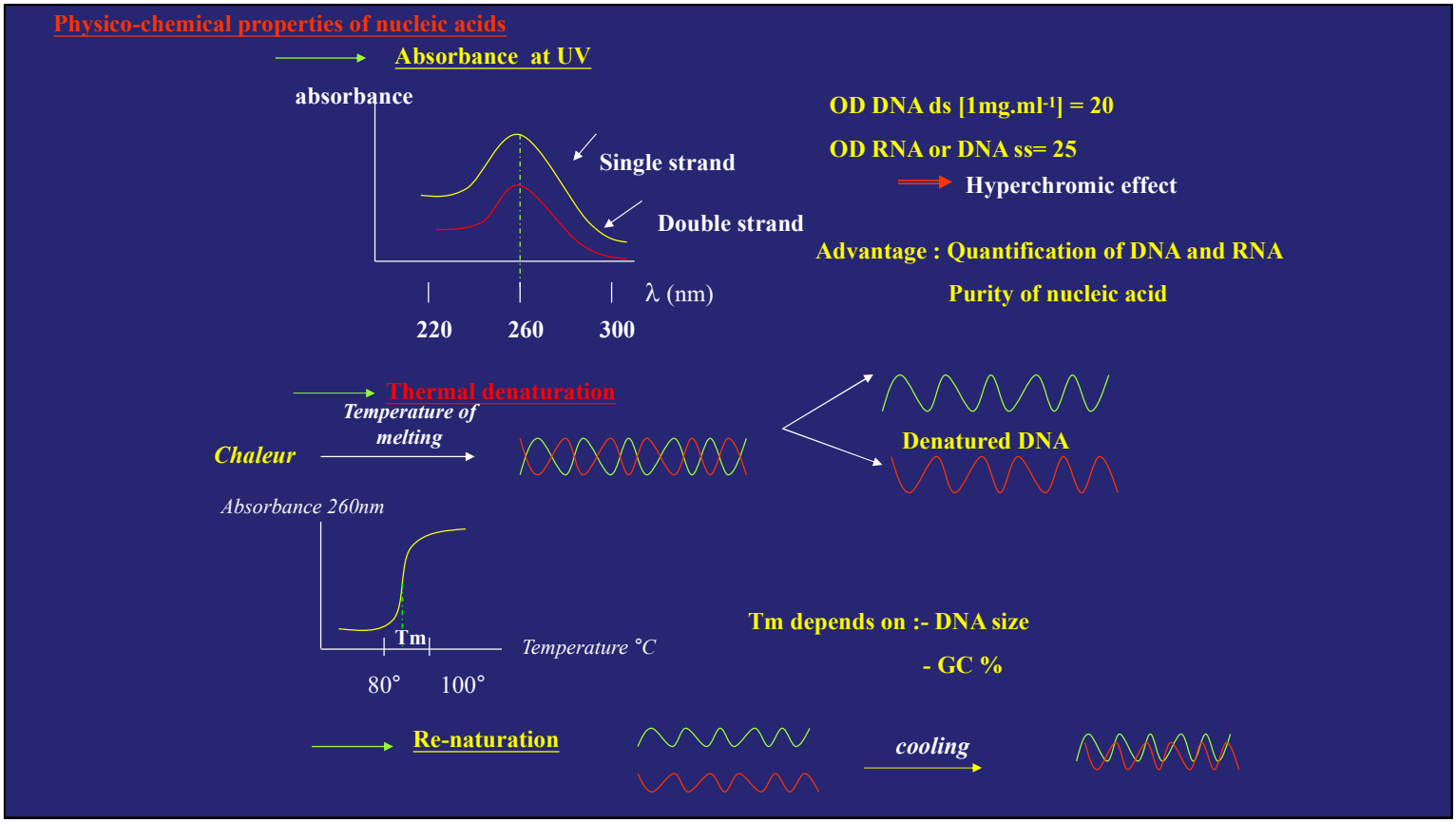
FIGURE 8–19 Comparison of A, B, and Z forms of DNA. Each structure shown here has 36 base pairs. The bases are shown in gray, the phosphate atoms in yellow, and the riboses and phosphate oxygens in blue. Blue is the color used to represent DNA strands in later chapters. The table summarizes some properties of the three forms of DNA.

Physicochemical properties of nucleic acids

- Important properties for the isolation and study of nucleic acids

- Stability : H bonds = specificity of base pairing : Hydrophobic and aromatic interactions
- Solubility : soluble in phenol, saline and alkaline solutions
  Precipitated by alcohol and strong acid solutions
  In aqueous solution, they are very acidic
- Viscosity : Long and thin DNA therefore very viscous solution
- Density : approximately 1,7 g/cm^3, possible isolation on ceasium gradient

- Denaturation : chemical by urea or formamide
  thermal : RNA progressive denaturation by heat
  DNA denaturation at a precise temperature (Tm) dependant on the content (property which allowed the creation of the PCR)



➢UV Absorption : The bases absorb in the UV with a maximum at 260 nm

Quantification : Pure nucleic acid solutions can be measured by measuring their UV absorbance at 260nm. An OD of 1 corresponds approximately to 50µg/ml of double-stranded DNA

DNA purity : we can also have information regarding the purity of the tested sample. In fact, DNA absorbs approximately 2 times less at 280nm than at 260nm (A_{260}/A_{280}=2).

The presence of proteins (aromatic amino acids absorb at 280nm) in the sample causes a decrease in this ratio.

Chapter I : Principal steps of biomacromolecules purification

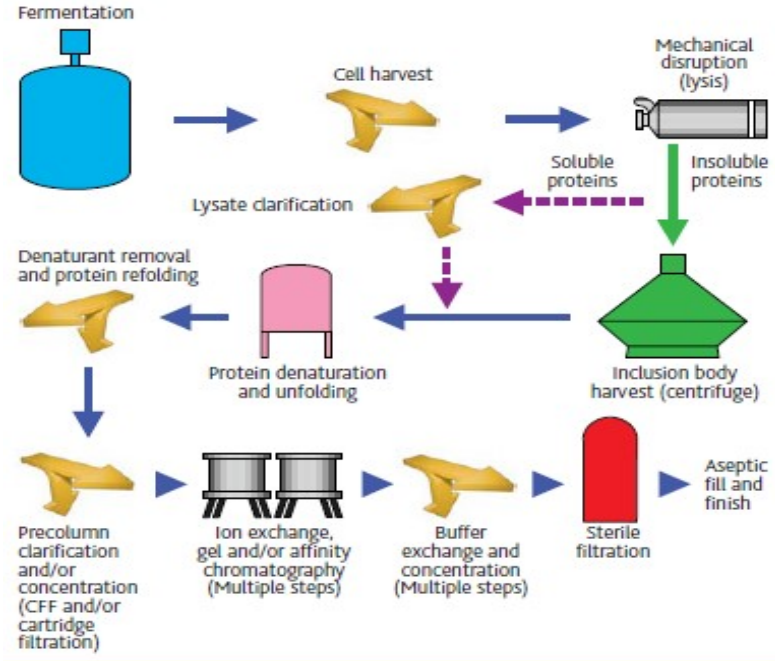


Fig 1. Purification by cross-flow membrane separation is integral to the manufacture of biological therapeutics. Membrane separation steps are depicted as bi-directional yellow arrows in this flowchart of a typical purification procedure for a recombinant protein.

I. Introduction

- The separation of biological molecules and macromolecules is a fundamental step in both analytical studies and biochemical assays. Various separation techniques are employed in biology, each based on the distinct **physicochemical properties and behavior of macromolecules in solution**.
- The primary objective of purification is to achieve a **high yield and maximum purity while minimizing costs**. Therefore, the selection of purification steps must be carefully optimized to reduce the number of processing stages without compromising efficiency.
- To accomplish this, it is essential to address the following key questions:

1. How much do I need ?

The required quantity of a purified protein depends on its intended application.

For instance, a comprehensive physicochemical characterization of a protein may necessitate several hundred milligrams of purified material, whereas a kinetic analysis of an enzyme-catalyzed reaction can often be conducted with only a few milligrams.

These varying quantity requirements influence both the choice of protein source and the purification strategy.

- ✓ For large-scale protein purification, initial steps typically employ techniques with high capacity but low resolution, such as salt or organic solvent precipitation.
- ✓ Intermediate purification steps often involve chromatographic methods with moderate capacity and resolution, including ion exchange chromatography and gel filtration.
- ✓ Finally, high-resolution but lower-capacity techniques, such as affinity chromatography and isoelectric focusing, are utilized in the final purification stages to achieve maximal purity.

Technique	Average amount required	Purity/activity of isolated protein
Crystallography	100 mg	Very high
Enzyme kinetics	10 mg	moderate
Antibody production	1 mg	Low/moderate
Mutagenesis studies	1-2 mg	Moderate
Multiprotein enzyme complex	1-10 µg	High

2. Do I want to retain biological activity ?

If the answer is yes, meaning that maintaining the biological activity of the protein is required, this constraint will limit the choice of purification techniques and the conditions under which they can be applied.

In general, most proteins retain their biological activity when handled in **neutral aqueous buffers at low temperatures**. However, specific purification methods vary in their compatibility with active proteins:

- ✓ Immunoaffinity chromatography is a highly selective method, but the harsh conditions required for elution can compromise protein activity.
- ✓ Reversed-phase chromatography relies on organic solvents for elution, making it unsuitable for active proteins.
- ✓ Ion Exchange Chromatography (IEC) is one of the most versatile techniques for protein purification while preserving activity.
- ✓ SDS-PAGE can be used regardless of whether protein activity is retained.

For the purification of labile molecules, it is essential to design a purification protocol with the fewest possible steps and minimal buffer changes to reduce the risk of denaturation or degradation. Additionally, various stabilizing agents are employed to enhance protein stability, inhibit microbial contamination, and lower the freezing point (see table below).

Common stabilizers for protein purification buffers

Stabilizer	concentration	Remarque
Metal-chelating agents (EDTA, AGTA)	0.1-1.0 mM	Chelation of heavy metals likely to inactivate proteins inactivation of certain proteases.
Bêta mercaptoethanol (BME), dithiothreitol (DTT)	5-20 mM (BME) -1.0 mM (DTT)	Avoid unwanted disulfide bond formation
Salts (KCl, NaCL….)	Minimum 50 mM	Maintain the buffer's ionic strength near the physiological ionic levels.
Glycerol, saccharose	5-20 %	Not all proteins are stabilized by glycerol, some are destabilized, Protein stability to freeze/thaw
Protease inhibitors (PMSF…)	Variable	Depend on the type of protease. Often, they will only be necessary during the initial steps of purification
Detergents (deoxycholate)	2-6 mM	Membrane proteins stabilization
Surfactants (triton X 100)	Variable	Membrane proteins stabilization
Sodium azide	0.01%	Antibacterial

3. Do I need a completely pure protein?

The concept of protein purity is complex and does not simply imply the absolute absence of contaminants. Ideally, a pure protein sample would contain only a single population of molecules with identical covalent structures and three-dimensional conformations, aside from water and intentionally added components such as buffer ions.

However, achieving absolute purity is both unattainable and unnecessary in most cases.

Instead, the essential criterion is that the protein preparation should be free from any contaminants that could interfere with the intended experiment. This is not merely a theoretical concern; as purification progresses, removing residual impurities becomes increasingly challenging, often necessitating additional purification steps that are time-consuming and result in reduced yields.

Therefore, an operational definition of purity must be established based on the specific requirements of each study. This definition will not only determine the purification strategy but may also influence the feasibility of the entire process. In cases involving labile proteins, achieving a highly purified sample may not be realistic; however, obtaining a preparation of sufficient purity for a particular experimental purpose may still be possible.

The key question remains: Does it matter whether the protein is 50%, 90%, or 99% pure? The answer depends on the intended application, emphasizing the need for a practical rather than an absolute approach to protein purification.

The required level of protein purity depends on the specific purpose of the purification.

For example:

- ✓A 50% pure protein may be sufficient for generating a monoclonal antibody, as long as the remaining contaminants do not interfere with the immune response. However, a 95% pure protein may be unsuitable for producing a monospecific polyclonal antibody, especially if the contaminants are highly immunogenic.
- ✓A relatively impure enzyme preparation may be acceptable for kinetic studies, provided it does not contain other enzymatic activities that could compete with or alter the reaction being analyzed.
- ✓A 95% pure protein is generally adequate for amino acid sequence analysis. In fact, an even lower purity may be acceptable, as long as precise quantification is performed to ensure that observed sequences do not originate from contaminants.

4. What source should I use?

The choice of protein source is often dictated by the specific research objective.

For instance, if the goal is to study the enzyme **ribulose-1,5-bisphosphate carboxylase (RuBisCO)**, it must necessarily be isolated from a **plant** source. However, the selection of the plant species can be optimized based on factors such as its availability, high enzyme content, ease of protein extraction, and low levels of interfering polyphenolic compounds.

In cases where the protein of interest is widely distributed across different organisms and species-specific differences are not relevant, plants are generally not the preferred source. Instead, microbial and fungal sources may be more advantageous, as they can be cultivated under controlled conditions, ensuring a consistent and reproducible supply of starting material. Additionally, in some cases, the expression levels of the target protein can be manipulated by adjusting growth media and environmental conditions.

However, microbial and fungal sources also present certain challenges. Their rigid cell walls make cell disruption more difficult, requiring specialized equipment for efficient large-scale extraction. As a result, unless a laboratory is equipped with appropriate mechanical or enzymatic disruption technologies, microorganisms may not always be the ideal choice for large-scale protein isolation.

Homogenizer

Benchtop Homogenizer

Dounce Homogenizer

In some cases, animal tissues such as the heart and liver serve as the most convenient protein sources.

For **large-scale protein purification**, these tissues are typically sourced from commercial abattoirs, whereas laboratory animals may be used for smaller-scale studies.

If the target protein is located in a soluble form within a subcellular organelle (e.g., mitochondria or chloroplasts), an additional decision must be made regarding tissue disruption. The choice depends on whether to:

- ✓ Completely disrupt the tissue under conditions that lead to organelle lysis, releasing their contents into the cytoplasmic extract.
- ✓ Preserve intact organelles through controlled homogenization, allowing their isolation via differential centrifugation or other fractionation techniques.

For animal tissues, the decision largely depends on the scale of purification:

Subcellular fractionation of a few hundred grams of tissue is a feasible approach and may simplify protein purification.

However, for large-scale work involving kilograms of tissue, the time and effort required for organelle isolation may become impractical, making purification from a total cellular extract the more efficient option.

In contrast, subcellular fractionation in plants is significantly more challenging due to cell wall rigidity and complex cellular structures, making it a less common approach in most purification workflows.

For membrane proteins, it is highly advantageous to first isolate a pure membrane fraction before initiating protein purification. The feasibility of achieving this depends on the organism and the specific membrane system being targeted. Efficient membrane isolation facilitates subsequent purification steps by reducing contamination from non-membrane-associated proteins.

For proteins present in very low abundance or sourced from organisms that are difficult to work with, an alternative approach is gene cloning and heterologous expression in a suitable host. This method allows for the production of recombinant proteins in larger quantities under controlled conditions. However, gene cloning and expression represent a major undertaking and are typically pursued only when conventional purification methods fail.

Once the recombinant protein has been expressed and extracted from the host cells, its purification follows the same principles and techniques as those applied to proteins derived from conventional sources.

5. Has it been done before?

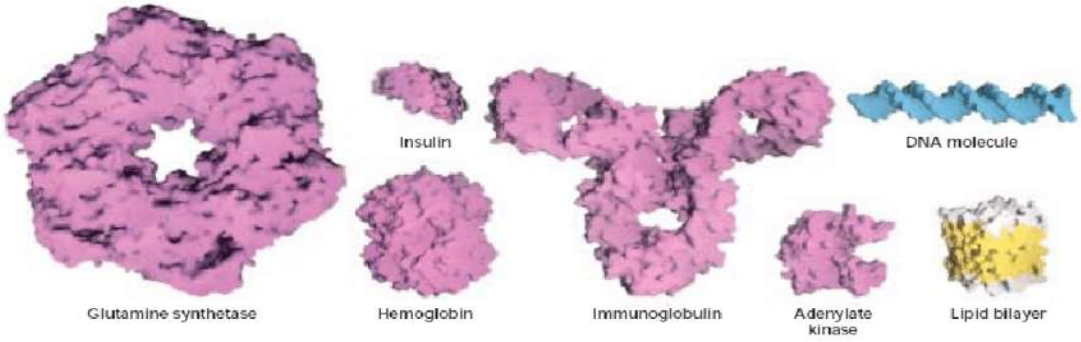
It is common to require the purification of a protein that has been previously isolated and characterized, either for use as an analytical tool or for conducting novel investigations. In such cases, the initial approach is typically to replicate the previously reported purification protocol. However, in practice, the procedure may not work exactly as described due to variations in starting material, experimental conditions, and laboratory techniques, all of which can significantly influence protein behavior during purification. These discrepancies are usually manageable, as minor adjustments can be made once experience with the specific protein's properties is gained.

If the exact protein of interest has not been previously purified, it is often beneficial to refer to purification protocols for closely related proteins. For example:

- If the same protein from a different organism has been isolated, particularly from a phylogenetically related species, its purification properties should be similar, requiring only minor modifications, such as adjustments to pH in an ion-exchange chromatography step.
- Even if the protein belongs to a more distantly related family, available literature may still provide valuable insights. For instance, if the target protein is a glycoprotein, this characteristic suggests specific purification strategies, such as lectin affinity chromatography.
- By leveraging existing literature and prior research, significant time and effort can be saved, preventing unnecessary trial-and-error approaches and streamlining the purification process.

II. Exploiting Differences

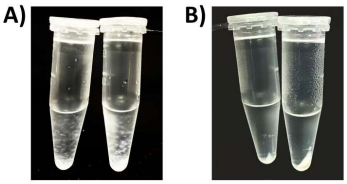
Protein purification involves the isolation of a specific protein from a complex mixture containing potentially thousands of other proteins with similar general properties. In many cases, the target protein may constitute less than 1% of the total protein content. Therefore, an effective purification strategy must fully exploit the distinguishing physicochemical properties of proteins to achieve efficient separation.



The following section outlines the key properties that differentiate proteins and describes the purification techniques based on these properties, along with practical considerations for their application.

A- Solubility

Proteins differ in their **surface composition of charged, polar, and hydrophobic amino acids**, which affects their **solubility** under specific conditions. This property allows for differential precipitation using neutral salts or organic solvents, providing a basic method for protein separation. However, this approach lacks precision, as precipitation occurs over overlapping concentration ranges, limiting its purification efficiency.

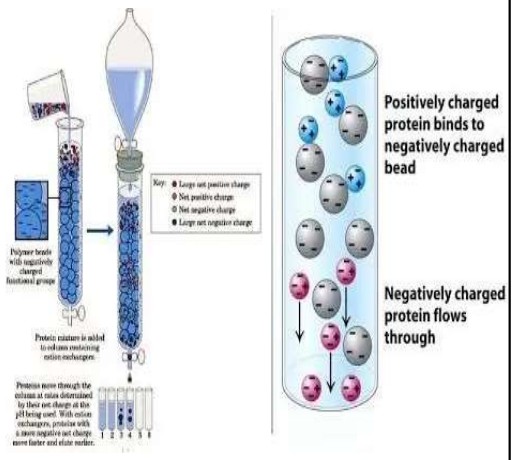


Despite its low resolution, fractional precipitation is widely used in early purification steps, particularly after extraction, due to its high yield, ease of large-scale application, and compatibility with centrifugation.

Additionally, it serves as a concentration step, reducing the water content of the protein solution, which is a significant practical advantage.

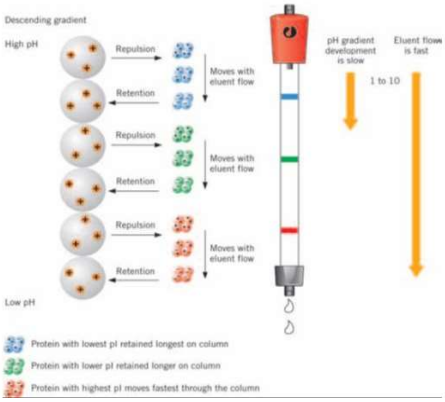
B- Charge

- Proteins vary in their content of charged amino acids (aspartic acid, glutamic acid, lysine, arginine, and histidine), leading to differences in their net charge at a given pH and their isoelectric point (pI)—the pH at which their net charge is zero.
- These charge differences are the basis of ion-exchange chromatography (IEC), one of the most powerful techniques in protein purification.
- In IEC, proteins with a net charge bind to a charged solid matrix of the opposite charge, with the strength of binding depending on the magnitude of the protein's charge.
- Proteins are then eluted by increasing the concentration of counter-ions, which compete for binding sites and displace the proteins from the matrix.

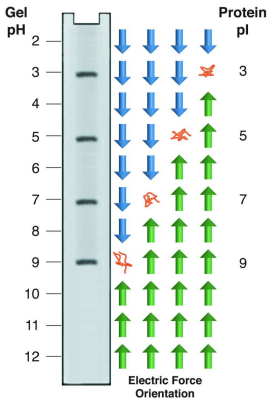


- Ion-exchange chromatography (IEC) offers moderate to high resolution, depending on the materials and conditions used.
- For large-scale purification (~100 g of protein), cellulose-based resins are preferred due to their high flow rates and suitability for large bed volumes, though they provide lower resolution. This makes them ideal for early purification steps.
- For higher resolution, Sepharose-based resins are commonly used, but due to their smaller scale, they are typically employed in later purification stages for final product refinement.
- It is important to note that proteins with the same charge at one pH may differ in charge at another pH. Therefore, purification protocols often include multiple IEC steps, either by adjusting pH with the same resin or using both anion- and cation-exchange resins to enhance separation efficiency.

Differences in isoelectric points (pI) between proteins can be exploited using two main techniques:



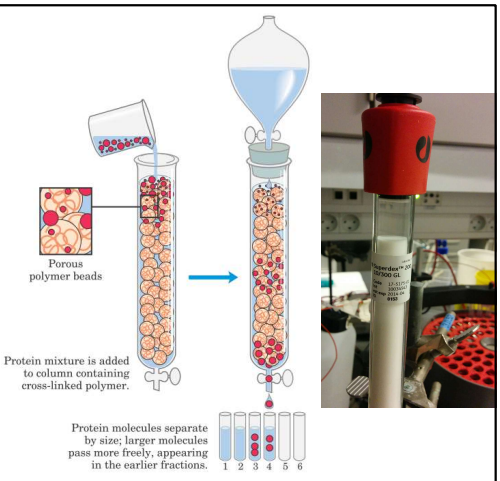
- **Chromatofocusing (CF)** is a variant of ion-exchange chromatography, where proteins bind to an **anion-exchange resin** and are eluted by gradually decreasing the buffer pH. Proteins elute in order of their isoelectric points, allowing for moderately high resolution and capacity. This technique is most effective for refining partially purified protein mixtures.



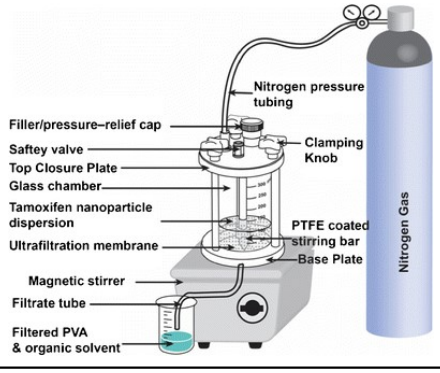
- **Isoelectric focusing (IEF)** involves applying an electric field within a stable **pH gradient**, causing proteins to migrate until they reach their isoelectric point (pI), where their net charge is zero and migration stops. If a protein diffuses away, it regains charge and moves back, leading to extremely high resolution. Despite its low capacity, IEF is valuable for separating proteins that are difficult to fractionate by other methods.

C-Size

Differences in molecular size can be exploited using size-exclusion chromatography (SEC-GF) and ultrafiltration (UF).

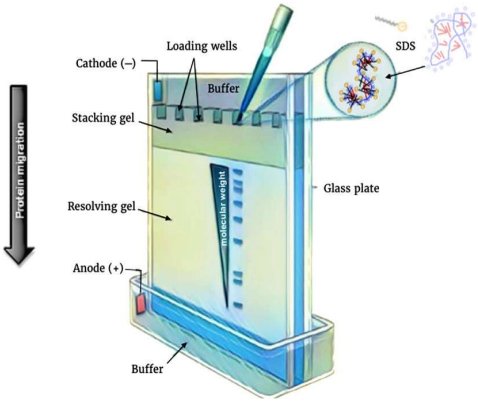


- Size-exclusion chromatography (SEC-GF) uses porous beads to separate proteins based on size: large proteins remain in the mobile phase and elute first, while smaller proteins enter the pores and elute later. Although it has limited resolution and low capacity (due to the need for small sample volumes), it is useful when the target protein is at an extreme size range.

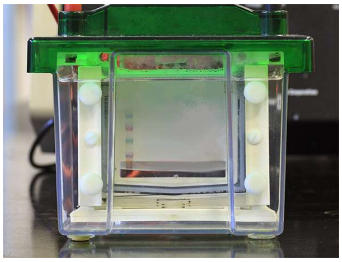


- Ultrafiltration (UF) involves passing a solution through a membrane with controlled pore sizes, allowing small molecules to pass while retaining larger proteins. This technique can be used for size-based separation and concentration of protein solutions. Unlike SEC-GF, UF is not limited by scale, making it suitable for large-volume applications.

A distinct approach to size-based protein separation is **SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)**.



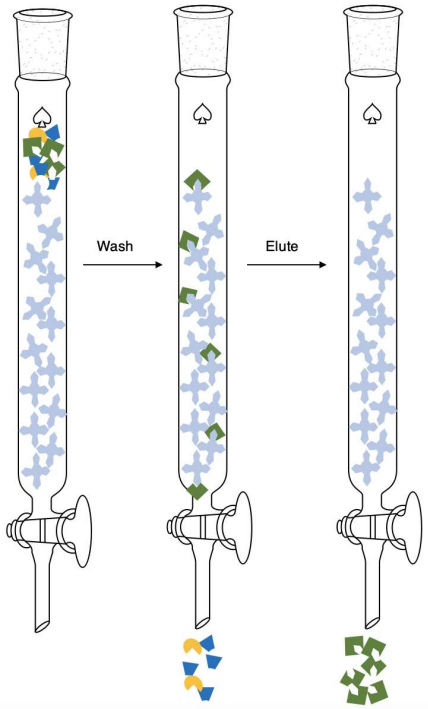
- In this technique, proteins are denatured and coated with SDS detergent, giving them a **uniform negative charge.**
- During gel electrophoresis, proteins migrate based solely **on size**, with smaller proteins moving faster due to the sieving effect of the gel.



- SDS-PAGE has exceptionally high resolution and is a fundamental tool in analytical protein chemistry.
- While primarily used for analysis, methods have been developed to extract proteins from gels, enabling small-scale purification from complex mixtures.

D- Specific Binding

Many proteins perform their biological functions by binding to specific molecules, such as substrates, receptors, or antigens. This property is exploited in affinity chromatography, where a ligand specific to the target protein is attached to a solid support and used as a chromatographic medium.



A protein extract or partially purified sample is passed through the column, where the target protein binds to the ligand due to its high specificity.

Elution is achieved by modifying solvent conditions or introducing a competitive solute that displaces the protein.

Among affinity-based methods, immunoaffinity chromatography is particularly powerful due to the high specificity of antibody-antigen interactions, enabling highly selective purification of target proteins.

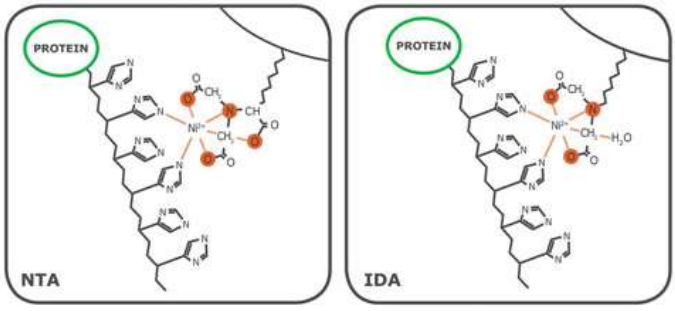
- *Lectin-affinity chromatography* utilizes the specific binding between plant lectins and carbohydrates, making it particularly useful for isolating glycoproteins or removing glycoprotein contaminants with high specificity.
- However, affinity methods using low-molecular-weight ligands (e.g., enzyme-substrate interactions) tend to be less specific, as the ligand may bind to multiple proteins.
- For example, **immobilized NAD** binds to various dehydrogenases, and benzamidine interacts with most serine proteases, enabling the isolation of protein families rather than individual species.

- In summary, affinity chromatography offers a range of selectivity levels, from moderate to very high, and in optimal cases, it enables single-step purification of a protein from a crude extract.
- However, affinity media often have limited capacity and can be cost-prohibitive, making large-scale applications impractical. As a result, affinity methods are typically reserved for the final stages of a purification protocol to achieve high purity.

E- Special properties

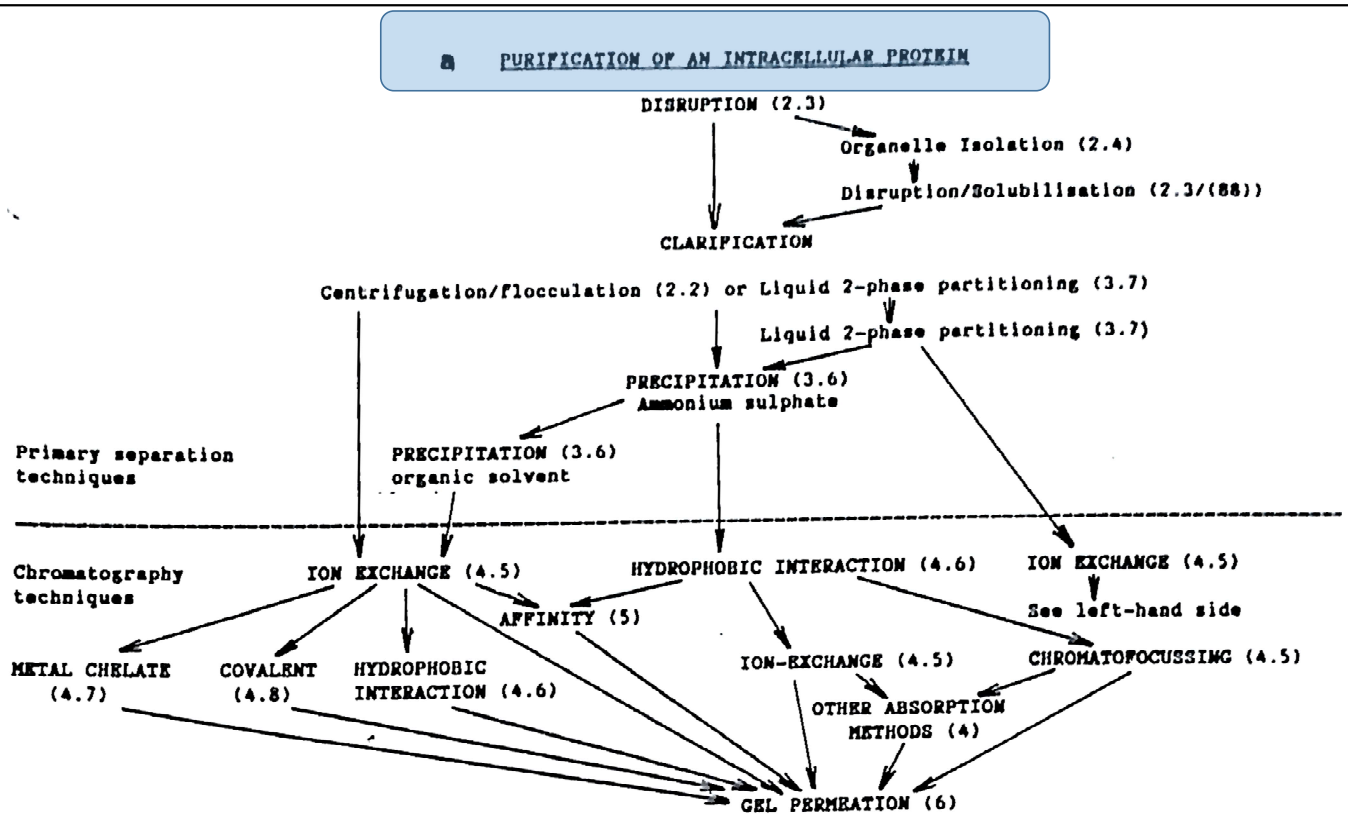
- Certain proteins exhibit exceptional stability under specific conditions, such as elevated temperatures or extreme pH values, which can be exploited for purification.
- For instance, heat-stable proteins can be selectively retained by heating a crude extract, causing denaturation and precipitation of contaminants while preserving the target protein.
- Similarly, proteins that remain stable at highly acidic or basic pH may be purified by selective precipitation of less stable impurities. However, this approach is ineffective for thermophilic organisms, where most proteins share thermostability. Preliminary stability tests are recommended to determine whether such properties can facilitate purification.

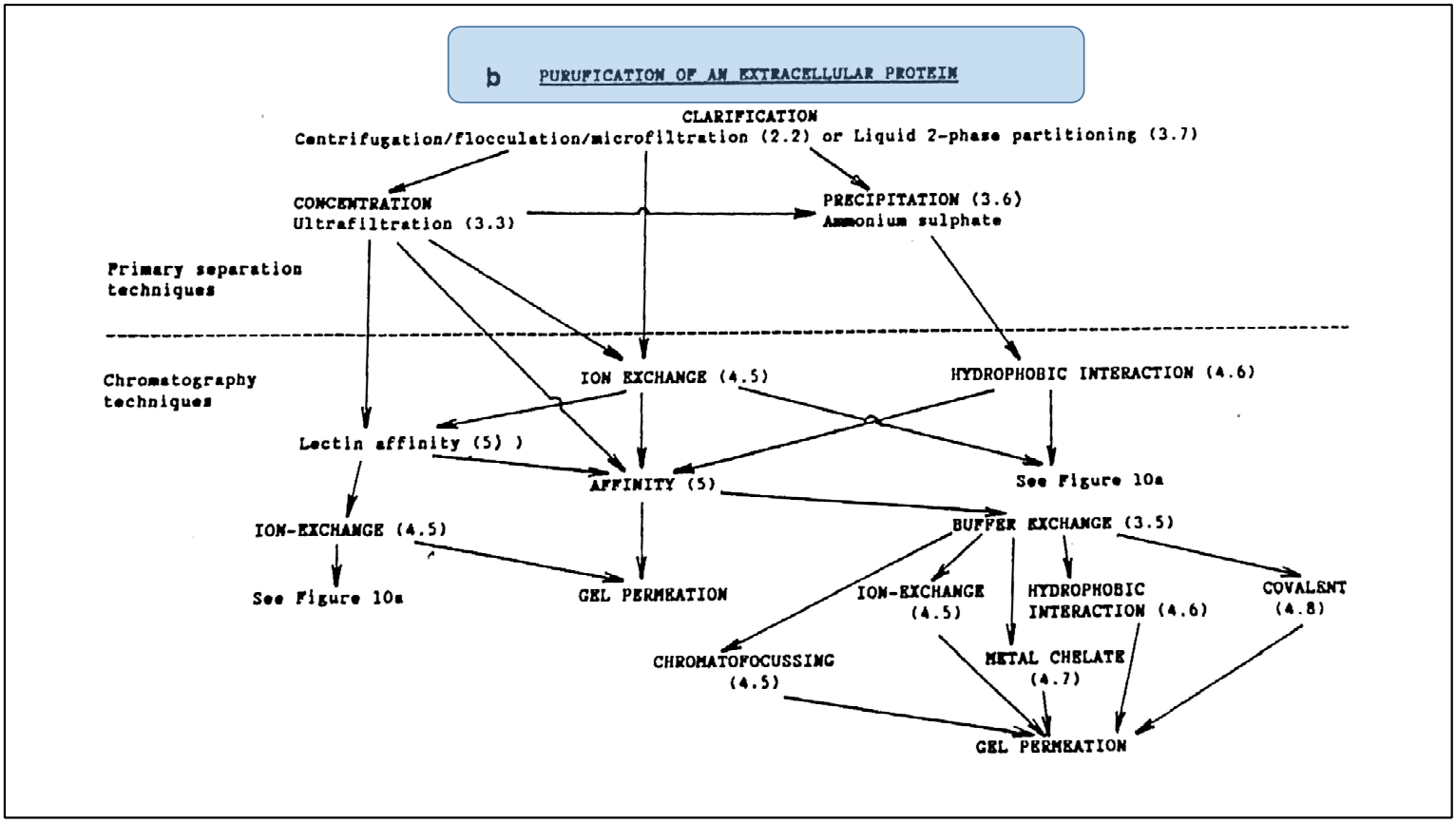
- In cases where conventional purification methods prove insufficient, genetic engineering can be employed to introduce specific properties that facilitate protein purification.
- For example, the addition of polyarginine or polylysine tails can enhance separation by ion-exchange chromatography, while polyhistidine tags enable purification via immobilized metal affinity chromatography (IMAC).
- However, these modifications are typically considered a last resort, unless recombinant DNA technology has already been chosen as the primary method for protein production and purification.

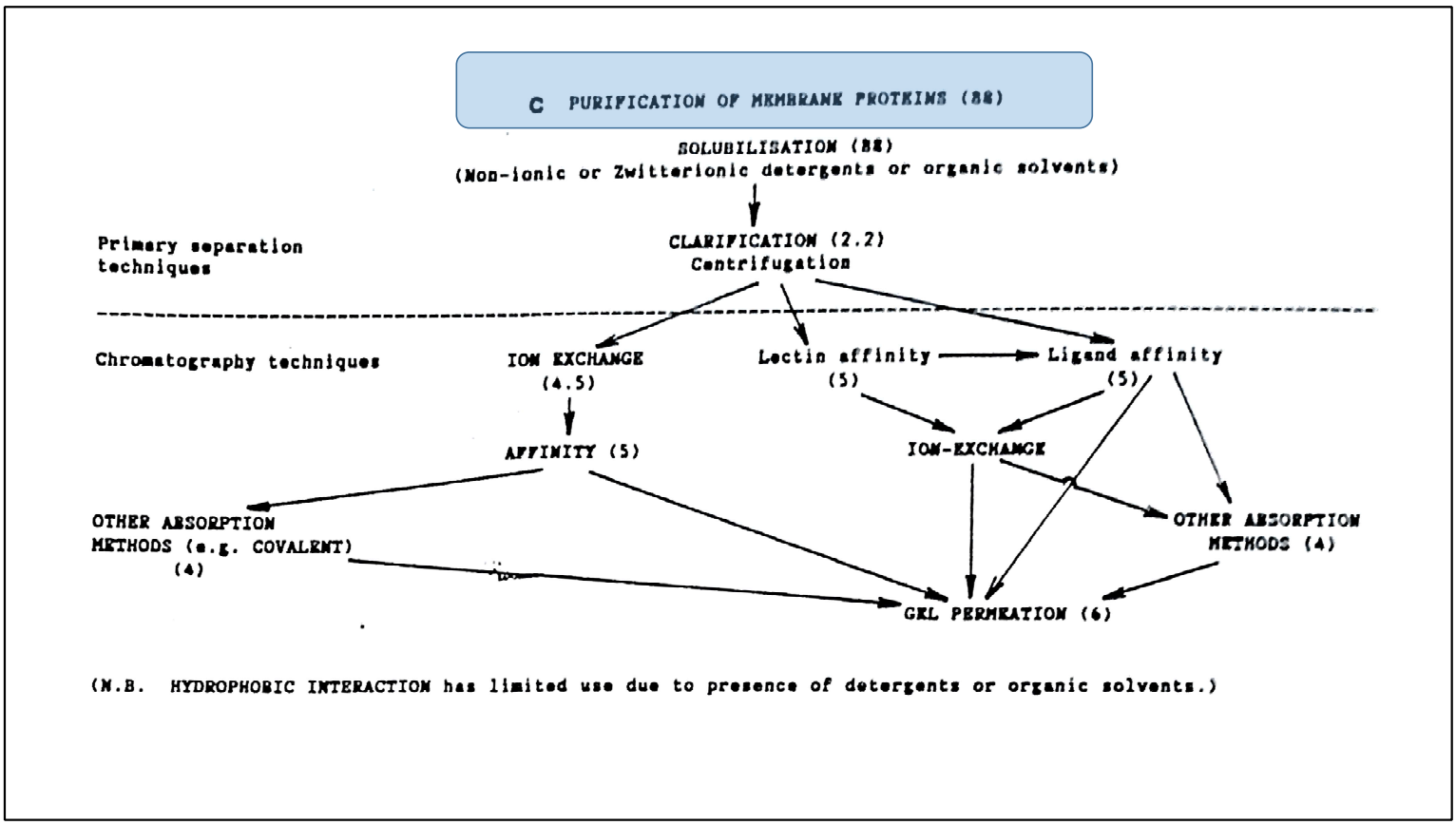


Ordering the Purification Steps

- Most purification strategies begin with homogenization, followed by precipitation, ion-exchange chromatography, affinity chromatography, and a final gel permeation step.
- This sequence is logical, as each technique exploits different protein properties.
- Precipitation handles large volumes, ion exchange removes contaminants before the expensive affinity step, and gel permeation provides final cleanup.
- The strategy should minimize steps and ensure compatibility between techniques.
- A universal approach is not possible, as purification depends on available materials and specific application requirements.




b PURUFICATION OF AN EXTRACELLULAR PROTEIN
CLARIFICATION
Centrifugation/flocculation/microfiltration (2.2) or Liquid 2-phase partitioning (3.7)
CONCENTRATION
Ultrafiltration (3.3)
PRECIPITATION (3.6)
Ammonium sulphate
Primary separation techniques
Chromatography techniques
ION EXCHANGE (4.5)
HYDROPHOBIC INTERACTION (4.6)
Lectin affinity (5))
AFFINITY (5)
See Figure 10a
ION-EXCHANGE (4.5)
BUFFER EXCHANGE (3.5)
See Figure 10a
GEL PERMEATION
ION-EXCHANGE (4.5)
HYDROPHOBIC INTERACTION (4.6)
COVALENT (4.8)
CHROMATOFOCUSSING (4.5)
METAL CHELATE (4.7)
GEL PERMEATION


C PURIFICATION OF MEMBRANE PROTEINS (8E)
SOLUBILISATION (8E)
(Non-ionic or Zwitterionic detergents or organic solvents)
Primary separation techniques
CLARIFICATION (2.2)
Centrifugation
Chromatography techniques
ION EXCHANGE (4.5)
Lectin affinity (5)
Ligand affinity (5)
AFFINITY (5)
ION-EXCHANGE
OTHER ABSORPTION METHODS (e.g. COVALENT) (4)
OTHER ABSORPTION METHODS (4)
GEL PERMEATION (6)
(N.B. HYDROPHOBIC INTERACTION has limited use due to presence of detergents or organic solvents.)

Documenting the Purification (Construction of a Purification Table)

- Maintaining an inventory during purification is essential to track volume, total protein content, and target protein yield.
- This prevents unexpected losses and ensures the protein remains active under purification conditions.
- Protein quantification at early stages can be done via 280 nm absorbance, while more accurate methods like Bradford assay are needed later.
- Enzymes can often be assayed spectrophotometrically, while other proteins may require bioassays, immunoassays, or gel quantification.
- Combining total protein assays with specific activity measurements helps evaluate purification efficiency, typically summarized in a purification table.

Table 3: Purification form used to record the various data collected purification of an enzyme

Purification Step	Vol. mL.	Protein mg/ml.	Enzyme activity U/ml.	Total Units	Specific Activity U/mg/ml	Fold Purification	Recovery
Homogenate	8500	40	1.8	15,300	0.045	1	100
45-70 % $(NH_4)_2SO_4$	530	194	23.3	12,350	0.12	2.7	81
CM-cellulose	420	19.5	25	10,500	1.28	28.4	69
AffinityChrom.	48	2.2	198	9500	88.4	1964	62
DEAE-Sepharose	12	2.3	633	7600	275	6110	50

$$\text{Specific Activity} = \frac{\text{Enzyme activity (Units/ml)}}{\text{Milligram protein/ ml.}}$$

$$\text{Fold Purification} = \frac{\text{Specific activity at any step}}{\text{Specific activity at initial step}}$$

$$\text{Yield purification} = \frac{\text{Total activity at any step (U)}}{\text{Total activity at initial step (U)}} \times 100$$