Biochemistry

Unit 2 · Lesson 4 · Sorting and purifying proteins

The notes for Lesson 4 of Unit 2: chapters 2.7 to 2.8 of the unit book, with their figures, worked examples, exam traps and quizzes, in one file. The full unit book, with every chapter, the exam question bank and the glossary, is linked at the end.

Lesson 4 of 4 | 2 chapters | Chapters 2.7 to 2.8

About these notes

These are the notes for **Lesson 4 · Sorting and purifying proteins** of the University free n easy video lessons on Biochemistry Unit 2, Amino Acids and Proteins. They are chapters 2.7 to 2.8 of the unit book, unchanged, so a page here is the same page in the full book.

2.7 Classification and properties of proteins

There are many thousands of different proteins, so they are sorted in four ways: by shape, by chemical composition, by solubility and by biological function. Their properties, such as solubility, the isoelectric point, salting out and the colour reactions, all follow from the amino acids they contain and from their large size. These properties are used every day in labs to detect proteins, to tell one kind from another and to separate them.

You need: the isoelectric point (Chapter 2.2) and denaturation (Chapter 2.6).

Classification by shape, function and nutrition

Shape. Fibrous proteins are long and thread-like, insoluble in water and structural: keratin (hair, nails), collagen (tendon, skin), elastin, silk fibroin. **Globular proteins** are compact, roughly spherical, mostly water-soluble and do the cell's active work: enzymes, haemoglobin, myoglobin, albumin, antibodies, insulin.

FUNCTION	EXAMPLES
Catalytic (enzymes)	Amylase, trypsin, DNA polymerase
Transport	Haemoglobin (O_2), serum albumin (fatty acids, drugs), transferrin (iron)

FUNCTION	EXAMPLES
Storage	Ferritin (iron), casein (milk), ovalbumin (egg)
Structural	Collagen, keratin, elastin
Contractile and motile	Actin, myosin, tubulin
Defence	Antibodies (immunoglobulins), fibrinogen and thrombin (clotting)
Regulatory (hormones)	Insulin, glucagon, growth hormone
Receptors and signalling	Insulin receptor, rhodopsin

Nutritional value. A **complete** protein supplies all essential amino acids in good amounts (egg, milk). An **incomplete** protein lacks one or more: gelatin lacks tryptophan; zein of maize is poor in lysine and tryptophan.

Classification by composition and solubility

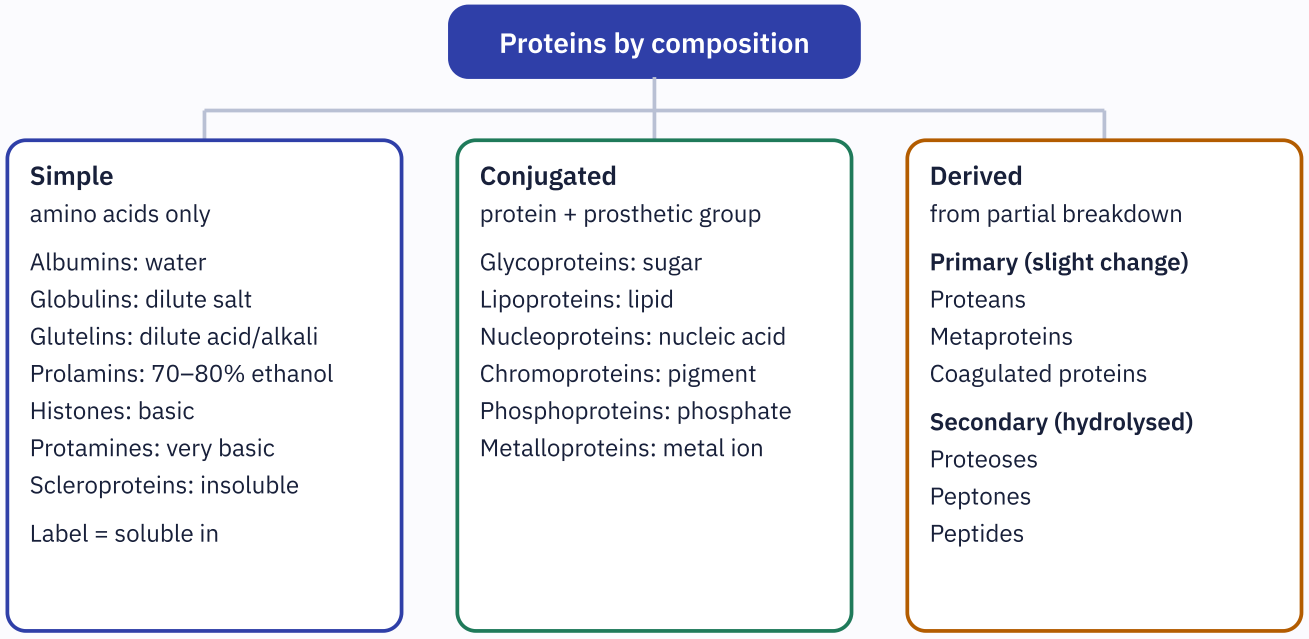


Figure 2.7.1 Classification of proteins by composition, the scheme used in most Indian biochemistry textbooks. Simple proteins are further sorted by the solvent that dissolves them; conjugated proteins are named after their non-protein part (prosthetic group); derived proteins are products of denaturation or partial hydrolysis.

Simple proteins give only amino acids on hydrolysis. Examples: **albumins** (serum albumin, ovalbumin; also coagulated by heat), **globulins** (serum globulins), **glutelins** (glutenin of wheat, oryzenin of rice), **prolamins** (gliadin of wheat, zein of maize), **histones** (bind DNA in chromatin),

protamines (in sperm) and **scleroproteins** or albuminoids (keratin, collagen, elastin). Gliadin and glutenin together make gluten, which gives chapati dough its stretch.

Conjugated proteins carry a non-protein **prosthetic group**. **Glycoproteins** carry sugars: mucins of saliva, immunoglobulins, many cell-surface proteins (sugar chemistry is in Unit 1). **Lipoproteins** carry lipids in blood: chylomicrons, VLDL, LDL and HDL (lipids are in Unit 3). **Nucleoproteins**: chromatin, ribosomes. **Chromoproteins** have a coloured group: haemoglobin (haem), flavoproteins (FAD), rhodopsin (retinal). **Phosphoproteins**: casein of milk, vitellin of egg yolk. **Metalloproteins**: ferritin (iron), ceruloplasmin (copper), carbonic anhydrase (zinc).

Derived proteins come from denaturation or partial hydrolysis of the above. Primary derived proteins are only slightly changed (metaproteins, coagulated proteins); secondary derived proteins are hydrolysed into smaller pieces (proteoses, peptones, peptides).

Physical properties

- **Size and colloidal nature.** Molar masses range from a few thousand to millions of g/mol. Proteins form colloids, do not pass through semipermeable membranes (the basis of dialysis) and scatter light.
- **Amphoteric; isoelectric point.** Their many ionisable side chains make proteins buffers. At the pI, net charge is zero, molecules stop repelling each other and solubility is lowest; they do not move in an electric field.
- **Salting in and salting out.** A little salt shields charges and increases solubility (salting in). A high concentration of a salt such as ammonium sulfate takes away the water the protein needs and precipitates it (salting out). Different proteins come out at different salt levels; this is the first step of many purifications (Chapter 2.8).
- **Optical and spectral.** Proteins are optically active and absorb UV light at 280 nm because of Trp and Tyr.
- **Denaturation and coagulation** by heat and other agents (Chapter 2.6).

Chemical properties: colour reactions and precipitation

TEST	REAGENT	POSITIVE RESULT	DETECTS
Biuret	Alkaline $CuSO_4$	Violet	Two or more peptide bonds (tripeptides and larger)
Ninhydrin	Ninhydrin, heat	Purple	Free α-amino groups
Xanthoproteic	Concentrated HNO_3, heat; then alkali	Yellow, turning orange	Aromatic rings (Tyr, Trp)
Millon's	Mercuric nitrate in HNO_3, heat	Red	Phenol group (Tyr)

TEST	REAGENT	POSITIVE RESULT	DETECTS
Hopkins–Cole	Glyoxylic acid, then concentrated H_2SO_4	Violet ring	Indole group (Trp)
Sakaguchi	α-Naphthol, alkaline hypobromite	Red	Guanidino group (Arg)
Lead acetate (sulfur)	Boil with NaOH, add lead acetate	Black precipitate (PbS)	–SH and S–S sulfur (Cys, cystine); not Met

Precipitation. Above their pI, proteins are negative and are precipitated by heavy metal cations (Hg^{2+}, Pb^{2+}, Ag^{+}); this is why raw egg white or milk is given as a first-aid antidote in heavy-metal poisoning. Below their pI, they are positive and are precipitated by acid reagents such as trichloroacetic acid (TCA), picric acid and tungstic acid, used to remove proteins from blood samples before analysis. Ethanol and acetone also precipitate proteins. **Hydrolysis** by acid, alkali or proteases breaks peptide bonds and gives free amino acids.

WORKED EXAMPLE

Question (6 marks). Three white protein samples are tested. **A** dissolves in water, coagulates on boiling, and gives positive biuret, Millon's and lead acetate tests. **B** is insoluble in water but dissolves in 70 percent ethanol; biuret positive. **C** dissolves in hot water and sets to a jelly on cooling; biuret positive, Hopkins–Cole negative. Identify the type of each and give an example.

1. All three are biuret positive, so all contain many peptide bonds: they are proteins, not free amino acids.
2. **A**: water-soluble and heat-coagulable is the definition of an albumin. Millon's positive shows Tyr; lead acetate positive shows Cys or cystine. Example: egg albumin (ovalbumin) or serum albumin.
3. **B**: soluble in 70–80 percent ethanol is the definition of a prolamin. Example: zein of maize or gliadin of wheat.
4. **C**: dissolves hot and gels on cooling, so it is gelatin, a derived protein made by boiling collagen. Hopkins–Cole negative fits: gelatin lacks tryptophan, which is also why it is a nutritionally incomplete protein.

EXAM TRAPS

- Biuret needs at least two peptide bonds; a dipeptide and free amino acids give a negative test.
- The lead acetate test detects Cys and cystine but not methionine, whose sulfur is held too tightly.
- Albumins dissolve in water; globulins need dilute salt. Swapping these is a common slip.
- Salting out is not denaturation: a salted-out enzyme redissolves with its activity intact.
- Heavy metals precipitate proteins above the pI (protein negative); acid reagents below the pI (protein positive).

CHECK YOURSELF

– REMEMBER **Classify conjugated proteins with one example each.** 4 marks

Glycoproteins (immunoglobulins), lipoproteins (LDL), nucleoproteins (chromatin), chromoproteins (haemoglobin), phosphoproteins (casein), metalloproteins (ferritin).

– REMEMBER **Which colour test is specific for tryptophan, and what is seen?** 2 marks

Hopkins–Cole test: a violet ring at the junction of the two layers.

– UNDERSTAND **Explain salting in and salting out.** 3 marks

At low salt, ions shield charged groups and reduce attraction between protein molecules, raising solubility (salting in). At high salt, the salt ions take up the water that hydrates the protein, so protein molecules associate and precipitate (salting out).

– UNDERSTAND **Why are proteins least soluble at their isoelectric point?** 2 marks

With zero net charge, molecules no longer repel each other electrically, so they come together and precipitate.

– APPLY **Why is raw egg white given in mercury poisoning?** 2 marks

Its proteins bind Hg^{2+} and precipitate it in the stomach, so less metal reaches the body's own proteins; the precipitate is then removed by vomiting or stomach wash.

– ANALYZE **A solution gives a positive ninhydrin test but a negative biuret test. What can you conclude?** 3 marks

Free amino groups are present, but there are fewer than two peptide bonds. So the solution contains free amino acids or dipeptides, not proteins.

BEYOND THE SYLLABUS

Classical colour tests are now mainly teaching tools. Labs identify proteins by mass spectrometry and classify them by sequence families and structural folds in databases such as UniProt, Pfam and CATH, and by function using Gene Ontology terms. Search "UniProt" and "protein families". This is not for the exam.

2.8 Protein purification and quantification

To study an enzyme or make a protein drug, you must separate one protein from the thousands of others in a cell and know how much you have. Purification uses differences in solubility, size, charge and binding to separate proteins step by step; quantification measures protein concentration by UV absorbance or a colour reaction. At other universities they usually appear in a bioanalytical or downstream processing course.

You need: isoelectric point and salting out (Chapter 2.7) and denaturation (Chapter 2.6).

Purification step by step

1. **Release.** Break cells (sonication, a homogeniser or lysozyme for bacteria) and spin off debris. The clear liquid is the **crude extract**. Work cold, with buffer, so the protein does not denature.
2. **Fractionation by solubility.** Add ammonium sulfate in steps; collect the precipitate that contains the target (salting out). Remove the salt by **dialysis** in a membrane bag that lets small ions through but keeps the protein.
3. **Ion-exchange chromatography** separates by charge. Above its pI a protein is negative and binds an anion exchanger (such as DEAE-cellulose); below its pI it binds a cation exchanger (such as CM-cellulose). Bound proteins are released by increasing salt or changing pH.
4. **Size-exclusion (gel filtration) chromatography** separates by size. Beads have pores; small proteins enter them and take a longer path, so **large proteins come out first**.
5. **Affinity chromatography** uses a ligand that binds only the target, such as a substrate analogue or, for recombinant proteins, nickel ions that bind a His-tag. Wash, then release with free ligand. It often gives the largest single step of purification.
6. **Check purity** by SDS-PAGE: SDS denatures and coats proteins with negative charge, so they move through a gel by size alone; a pure protein gives one band. Its position against

standards gives the subunit mass.

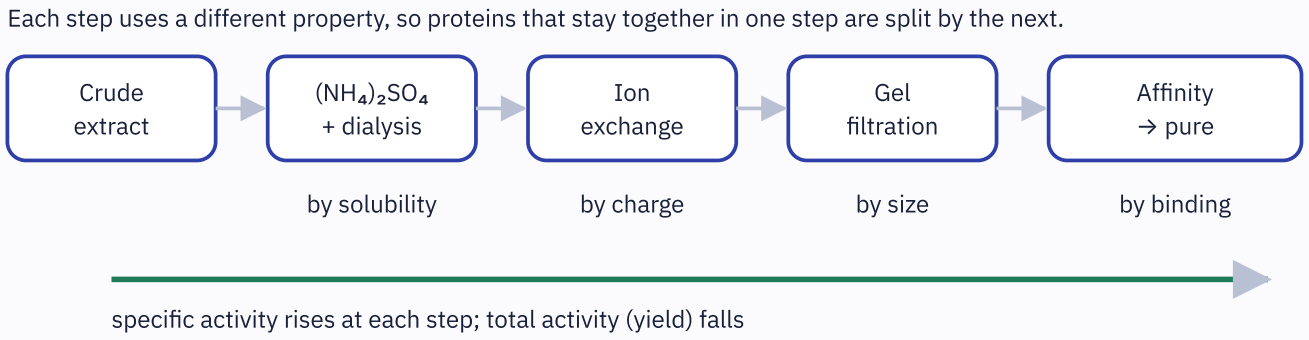


Figure 2.8.1 A typical purification. Notice that each method separates by a different property, and that the order usually goes from cheap, high-capacity steps (salting out) to selective, low-capacity ones (affinity).

Measuring progress and quantity

A purification is tracked with a **purification table**. **Specific activity** = total activity ÷ total protein (units per mg); it rises as other proteins are removed. **Yield** = activity at a step ÷ activity in the crude extract × 100. **Fold purification** = specific activity at a step ÷ specific activity of the crude extract.

Protein quantification methods:

- **UV absorbance at 280 nm**: fast and non-destructive, using the Beer–Lambert law $A = \varepsilon c l$ (A absorbance, ε molar absorption coefficient in $M^{-1}\,cm^{-1}$, c concentration in M, l path length in cm). Depends on Trp and Tyr content; nucleic acids interfere.
- **Biuret**: violet Cu^{2+}–peptide complex; simple but needs milligram amounts.
- **Lowry**: biuret reaction followed by the Folin–Ciocalteu reagent; about a hundred times more sensitive than biuret.
- **Bradford**: Coomassie Brilliant Blue G-250 binds protein and its absorption peak shifts to 595 nm; quick and sensitive.
- **BCA**: protein reduces Cu^{2+} to Cu^{+}, which forms a purple complex with bicinchoninic acid, read at 562 nm.

Colour methods need a **standard curve**, usually made with bovine serum albumin.

WORKED EXAMPLE

Question (6 marks). Complete the table and comment. Then find the concentration of the pure protein if A_{280} = 0.86 in a 1 cm cell, ε_{280} = 43,000 M^{-1} cm^{-1} and molar mass 66,000 g/mol.

STEP	PROTEIN (MG)	ACTIVITY (U)	SPECIFIC ACTIVITY (U/MG)	YIELD (%)	FOLD
Crude extract	10,000	200,000	20	100	1
$(NH_4)_2SO_4$	2,500	160,000	64	80	3.2
Ion exchange	400	120,000	300	60	15
Gel filtration	120	90,000	750	45	37.5
Affinity	20	70,000	3,500	35	175

1. Specific activity, for example ion exchange: 120,000 ÷ 400 = 300 U/mg. Yield: 120,000 ÷ 200,000 × 100 = 60 %. Fold: 300 ÷ 20 = 15.
2. Comment: the affinity step gives the largest jump (3,500 ÷ 750 ≈ 4.7-fold) with little loss. Overall the protein is purified 175-fold with 35 % yield.
3. Concentration: c = A ÷ (ε l) = 0.86 ÷ (43,000 × 1) = 2.0 × 10^{-5} M = 20 µM.
4. In mass units: 2.0 × 10^{-5} mol/L × 66,000 g/mol = 1.32 g/L = **1.32 mg/mL**.

EXAM TRAPS

- In gel filtration, large proteins come out first; in SDS-PAGE, small proteins move furthest. Do not mix these up.
- Specific activity, not total activity, shows purity.
- SDS-PAGE gives subunit mass, not the mass of the native multi-subunit protein.

CHECK YOURSELF

– REMEMBER **Define specific activity.** 1 mark

Enzyme activity per mg of total protein (units/mg).

– UNDERSTAND **Why do large proteins elute first in gel filtration?** 2 marks

They cannot enter the pores of the beads, so they travel only through the space between beads, a shorter path.

– APPLY **A protein has pI 5.0. Which ion exchanger binds it at pH 7.5?** 2 marks

At pH 7.5 (above pI) it is negative, so an anion exchanger such as DEAE-cellulose.

– APPLY **A step recovers 30,000 U in 5 mg from a crude extract of 150,000 U in 3,000 mg. Find yield and fold.** 3 marks

Yield = 30,000/150,000 × 100 = 20 %. Specific activities: 6,000 and 50 U/mg, so 120-fold.

– ANALYZE **Bradford gives 1.0 mg/mL but A_{280} suggests 3.0 mg/mL for the same sample. Suggest reasons.** 3 marks

The sample may contain nucleic acid, which also absorbs near 280 nm; or the ε used is wrong for this protein (unusually Trp-rich); or the Bradford standard (BSA) binds the dye differently from this protein.

BEYOND THE SYLLABUS

In industry these steps form downstream processing: monoclonal antibodies are captured on Protein A affinity columns and polished by ion exchange, often costing more than growing the cells. Search "downstream processing monoclonal antibodies". This is not for the exam.

Key terms of this lesson

TERM	MEANING OR FORMULA	CHAPTER
Biuret test	Violet with alkaline Cu^{2+}; needs two or more peptide bonds	2.7
Specific activity, fold, yield	Units/mg; SA(step)/SA(crude); activity(step)/activity(crude) × 100	2.8
Beer–Lambert law	$A = \varepsilon c l$	2.8

Glossary (terms used in this lesson)

Affinity chromatography Separation by specific binding of the target protein to a fixed ligand.

Amphoteric Able to act as both an acid and a base.

Conjugated protein	A protein with a non-protein prosthetic group, such as sugar, lipid or haem.
Denaturation	Loss of native shape and activity without breaking peptide bonds.
Dialysis	Removal of small molecules through a semipermeable membrane that keeps proteins.
Essential amino acid	One the body cannot make in enough amount; it must come from food.
Fibrous protein	A long, insoluble, structural protein such as keratin or collagen.
Globular protein	A compact, usually soluble protein such as an enzyme or haemoglobin.
Peptide bond	The amide bond between the α-carboxyl of one amino acid and the α-amino group of the next.
Prosthetic group	A non-protein part tightly bound to a protein and needed for its function.
Salting out	Precipitation of proteins by high salt concentration.
SDS-PAGE	Gel electrophoresis of SDS-coated proteins, separating them by subunit size.
Specific activity	Enzyme activity per mg of protein; a measure of purity.

The full unit book

Every chapter of Unit 2, the unit summary, the exam question bank with model answers and the full glossary: Biochemistry · Unit 2 · Amino Acids and Proteins (one file, works offline).

Sources and further reading

Textbooks

- David L. Nelson and Michael M. Cox, *Lehninger Principles of Biochemistry*, W. H. Freeman; chapters on amino acids, peptides and proteins, and on protein structure (from memory; check). Source of the pKa values used here.
- Jeremy M. Berg, John L. Tymoczko, Gregory J. Gatto Jr. and Lubert Stryer, *Biochemistry*, W. H. Freeman; chapters on protein composition and structure and on exploring proteins (from memory; check).
- Donald Voet and Judith G. Voet, *Biochemistry*, Wiley (from memory; check).
- U. Satyanarayana and U. Chakrapani, *Biochemistry*, Elsevier India; widely prescribed in Indian universities for the classification of proteins and colour reactions (from memory; check).

Open resources opened for this book

- G. N. Ramachandran, C. Ramakrishnan and V. Sasisekharan, "Stereochemistry of polypeptide chain configurations", *Journal of Molecular Biology* 7 (1963) 95–99, record at the Indian Academy of Sciences repository: repository.ias.ac.in/49564.
- C. B. Anfinsen, "Principles that govern the folding of protein chains", *Science* 181 (1973) 223–229 (the Nobel lecture): PDF copy. Source of the ribonuclease details in Chapter 2.6.
- Nobel Prize page for Anfinsen's lecture, 1972: nobelprize.org.

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