UNITE

PREPARATIVE PROTEIN BIOCHEMISTRY

I.C. **LABORATORY OF BIOCHEMICAL METHODOLOGIES AND EXPERIMENTAL MODELS**

- MODULE: **BIOCHEMICAL METHODOLOGIES**

BIOS-07/A

5 ECTS (CFU)



Preparative protein biochemistry

BY THE END OF THE LESSON, THE STUDENT WILL BE ABLE TO:

- Distinguish preparative from analytical biochemistry, and justify the degree of purity that a given application actually requires.
- Describe the capture–intermediate–polishing strategy, and account for the trade-off between purity and yield that limits how many steps are worth taking.
- Choose a physical or enzymatic cell-disruption method suited to the cell wall, the scale of the preparation and the fragility of the target protein.
- Design an extraction buffer, selecting additives that protect against oxidation, proteolysis, dephosphorylation and metal or phenolic damage — and the detergents needed to release integral membrane proteins.
- Clarify a crude extract: remove cell debris and lipid, and manage the nucleic acids and capsular material that make lysates viscous.
- Compare the assays used to measure protein concentration (280 nm absorbance, lowry, BCA, bradford, kjeldahl), and state the interferences and assumptions that limit each of them.
- Select an appropriate affinity tag, and explain how the tag is subsequently removed by a protease or an intein.
- Compare the main expression hosts, justify secreting the product, and outline the route from plasmid to induced culture.

Preparative protein biochemistry

- **Preparative biochemistry** describes the **purification** of biological molecules such as: **proteins, viruses and nucleic acids** to a sufficiently high concentration so as to be useful for a range of processes:
  - Goal: obtain material
  - Works with large amounts
  - Product is recovered and used
  - Output: a purified sample

VS.

- **Analytical biochemistry** describes the application of small quantities of analyte to determine the composition or content of a biochemical sample:
  - Goal: obtain information
  - Works with minute amounts
  - Sample is often consumed
  - Output: a measurement

Preparative protein biochemistry

CHALLENGE: One protein out of thousands

10 000–20 000

different proteins in a typical cell or tissue homogenate

≈ 4

fractionation steps needed, on average, to purify a given protein

~100 %

purity typically demanded for pharmaceutical use

Why purify at all?

To provide material for structural or functional studies. The required final purity depends entirely on the purpose for which the protein will be used.

Preparative protein biochemistry

How pure is pure enough?

Monoclonal antibody production	< 50 % pure is acceptable — as long as the protein raises an immune response, antibodies against contaminants are usually ignored.
Enzyme kinetics	A relatively impure sample is fine, provided it contains no competing activities.
Amino-acid sequencing	~90 % pure, provided the sequence is analysed quantitatively so the deduced sequence is not that of a contaminant.
Monospecific polyclonal antibody	Highly purified antigen required — immunogenic contaminants would generate additional antibodies.
Therapeutic proteins	Extremely pure, to satisfy regulatory (safety) requirements.

The rule

- Each purification step necessarily loses some of the target protein.
- An extra step taking purity from 90 % to 98 % gives a purer sample — but may leave too little material for the planned experiments.
- Many protein studies tolerate 5–10 % (or more) contamination.
- Design the shortest sequence of steps that reaches the purity the application actually needs.

Preparative protein biochemistry

Three stages after extraction

1

Capture

Isolate, concentrate and stabilise the target protein from the crude extract. Speed and capacity matter more than resolution.

2

Intermediate

Remove the bulk of the remaining impurities. Resolution and capacity are balanced.

3

Polishing

Remove trace contaminants and closely related species to reach high final purity. Resolution is paramount.

Each stage is normally carried out by a different type of chromatography — the properties exploited must differ from step to step.

Preparative protein biochemistry

Cell disruption and extraction

The extraction buffer

Purification necessarily starts with disruption of the cells or tissue to release their protein content into an appropriate buffer — unless the protein is secreted. This initial extract is the starting point, so the composition of the buffer must be considered first.

0.1–0.2 M

ionic strength of a monovalent salt

pH 7–8

compatible with intracellular conditions

TRIS

or phosphate: the buffers most commonly used

4 °C

all steps, to slow unwanted proteolysis

Recombinant expression is now the norm — but poor folding, poor induction or missing post-translational modifications can still make it necessary to purify a native protein from an abundant natural source.

Preparative protein biochemistry

Cell disruption and extraction

Buffer Addictive: protein protection

- **Antioxidants / reducing agents**

Inside the cell proteins sit in a fairly reducing environment; once released they meet a more oxidising one. Free thiol groups from cysteine can then oxidise to inter- and intramolecular disulfide bridges. Dithiothreitol, β-mercaptoethanol, cysteine or reduced glutathione are added to prevent this.

- **Substrates and cofactors**

Low levels of substrate are often included when purifying an enzyme: binding at the active site stabilises the enzyme during the procedure. Cofactors that would otherwise be lost are also included, so that activity can still be detected when column fractions are screened.

- **Protease inhibitors**

Disruption destroys the organisational integrity of the cell: proteases packaged in lysosomes are released and may degrade the target. Each inhibitor is specific for one protease class.

Protease class	Inhibitors
Serine proteases	DFP, PMSF, TPCK
Thiol proteases	Iodoacetate, cystatin
Aspartic proteases	Pepstatin
Metalloproteases	EDTA, 1,10-phenanthroline
Exopeptidases	Amastatin, bestatin

Preparative protein biochemistry

Cell disruption and extraction

Buffer Addictive: chelators, phosphatase inhibitors and adsorbents

- **Phosphatase inhibitors** Sodium orthovanadate (Na_3VO_4) inhibits phosphotyrosyl phosphatases and is standard in protein-kinase assay buffers; being competitive, it is removed by dilution, inactivation with EDTA or dialysis. It is often combined with sodium fluoride (phosphoseryl/phosphothreonyl phosphatases). β-glycerol-phosphate acts as a pseudo-substrate, providing a ready source of organic phosphate.

- **EDTA** Chelates divalent metal cations, one per molecule, co-ordinated by four oxygen and two nitrogen atoms. It removes metals that react with protein thiols ($R–SH + Me^{2+} \rightarrow R–S–Me^{+} + H^{+}$) and inhibits metalloproteases. But sequestering free Mg^{2+} lowers the activity of ATP-dependent enzymes, interferes with nucleotide binding, and is incompatible with IMAC purification of His-tagged proteins.

- **Polyvinylpyrrolidone (PVP)** Added for plant tissue. Plants contain phenolics — monomeric (p-hydroxybenzoic acid) and polymeric (tannins) — that bind proteins through hydrophobic, ionic and hydrogen bonds and precipitate them. Phenolics are also oxidised by endogenous phenol oxidases to highly reactive quinones, which cross-link proteins. Insoluble PVP mimics the polypeptide backbone and adsorbs the phenolics, which are then removed by centrifugation; thiol reagents limit phenol oxidase activity.

- **Sodium azide** A bacteriostatic agent added at low concentration to buffers that will be stored for long periods.

Preparative protein biochemistry

Cell disruption and extraction

Special case: membrane proteins

Membrane-bound proteins are not released by simple cell disruption alone.

Extrinsic (peripheral)

- Bound only to the surface of the membrane, mainly by electrostatic and hydrogen bonds.
- Predominantly hydrophilic and relatively easy to extract.
- Released by raising ionic strength (e.g. 1 M NaCl) or by acidic (pH 3–5) or basic (pH 9–12) conditions.
- Once extracted, purified by conventional chromatography.

Intrinsic (integral)

- Embedded in the membrane, with large hydrophobic regions associated with lipids; poorly soluble in aqueous buffers.
- Extracted with buffer containing detergents — the choice is largely trial and error.
- Ionic: SDS, sodium deoxycholate, CTAB, CHAPS. Non-ionic: Triton X-100, Nonidet P40.
- Detergent must be kept in every subsequent buffer, at 10–100 times lower concentration than for extraction, to maintain solubility without disturbing the chromatography.

Preparative protein biochemistry

Cell disruption and extraction

Physical methods

Sonication

Ultrasound (< 20 kHz), 30–60 s

- Ideal for suspensions of cultured or microbial cells.
- Disruption by shear force and cavitation: gas bubbles under pressure decompress, releasing shock waves.
- Suitable for small volumes (50–100 cm^3); efficiency ~50–60 %.
- Generates considerable heat — keep on ice, e.g. in a rosette cell.
- Risk of damaging the target protein.

Repeated freeze–thaw

Gentle, requires no special equipment

- Cell suspension frozen (−20 °C, −80 °C or liquid N_2) and thawed in warm water; repeated three times.
- Shear forces from ice crystal formation disrupt the cells.
- A short sonication of the lysate afterwards breaks down genomic DNA, which makes the suspension stringy and viscous.

Blenders and bead mills

Shear force, for tissue and for cells with walls

- Ideal for mammalian or plant tissue: cut into small pieces and blend in buffer for about 1 min.
- Inappropriate for bacteria and yeast on its own.
- Adding small glass beads converts a blender into a bead mill: cells are trapped between colliding beads and physically disrupted.

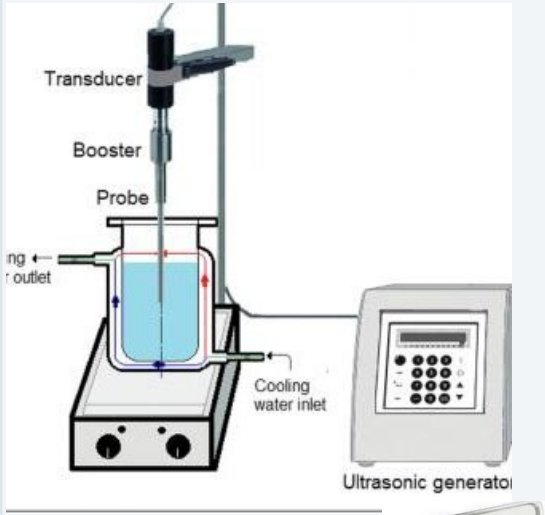
Preparative protein biochemistry

Cell disruption and extraction

Physical methods

Sonication

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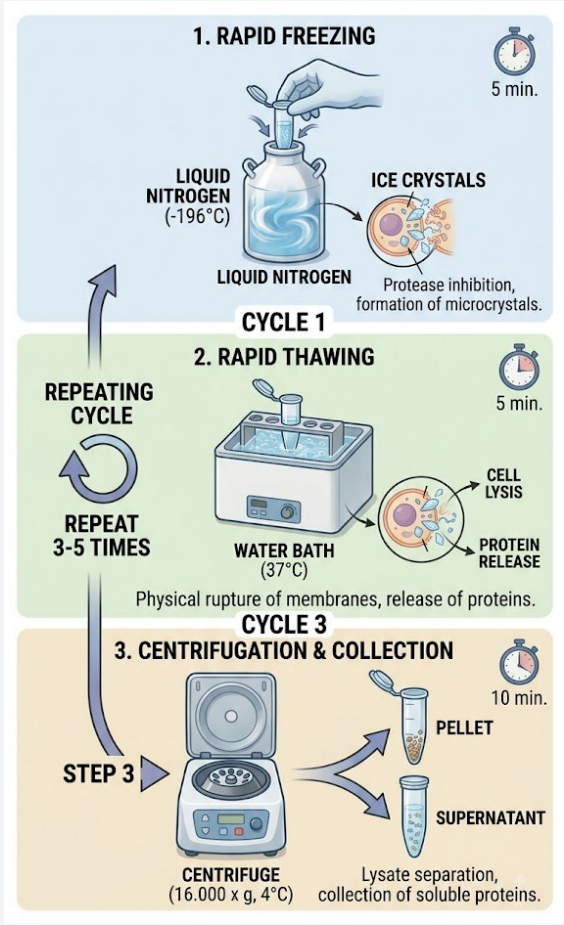
Probe Sonicators

Ultrasonic Bath Sonicators



Repeated freeze–thaw

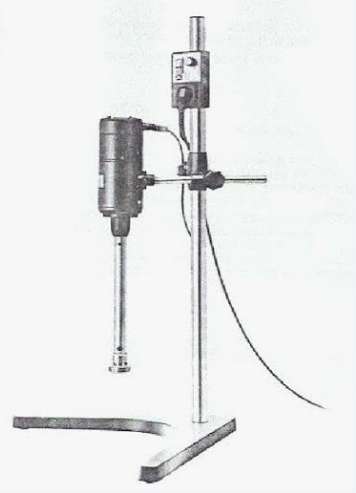
Gentle, requires no special equipment



Blenders and bead mills

Shear force, for tissue and for cells with walls

Ultra-Turrax (blender)





Precellys (bead mills)



Preparative protein biochemistry
Cell disruption and extraction
Physical methods (2)

Grinding with abrasives

- **Pestle and mortar** with sand or alumina and a little buffer: useful for bacterial or plant cells.
- Cell walls are physically ripped off by the abrasive.
- Only suitable for relatively small samples.
- The Dyno-mill is the large-scale version: a chamber of glass beads with rotating impeller discs; cells rupture between colliding beads.
- A 600 cm^3 laboratory model can process 5 kg of bacteria per hour.





Presses (homogenisers)

- French press, or the larger Manton-Gaulin press: excellent for microbial cells.
- A cell suspension (~50 cm^3) is forced by a piston pump at high pressure through a small orifice.
- Breakage results from shear as the cells pass the orifice, and from the rapid pressure drop on emerging, which lets compressed cells expand and burst.
- Multiple passes are usually needed; under carefully controlled conditions proteins can be selectively released from the periplasmic space.
- Hughes press: the cells are forced through as a frozen paste, often with an abrasive — ice crystals and abrasive together disrupt the wall.





Preparative protein biochemistry

Cell disruption and extraction
Enzymatic methods

- **Gram-positive bacteria (Lysozyme)** Lysozyme (from hen egg white) cleaves peptidoglycan. In a suitable buffer, the cell membrane then ruptures through the osmotic effect of the suspending buffer.
- **Gram-negative bacteria (Lysozyme + EDTA + non-ionic detergent)** EDTA removes divalent cations and destabilises the outer lipopolysaccharide layer; detergent solubilises the membrane. This permeabilises the outer membrane, giving lysozyme access to the peptidoglycan. In an isotonic medium the plasma membrane stays intact, allowing selective release of periplasmic proteins.
- **Yeast (Zymolyase or lyticase)** Both have β-1,3-glucanase activity together with a proteolytic activity specific for the yeast cell wall. Wall digestion is followed by osmotic shock or mild physical force; digestion alone releases periplasmic proteins selectively.
- **Filamentous fungi (Chitinase)** Degrades the chitin of the fungal wall.

Enzymatic methods are gentle and selective, but their cost limits them to laboratory-scale work.

Preparative protein biochemistry

Preliminary purification

Removal of cell debris

The initial extract — the homogenate or lysate — invariably contains insoluble matter: incompletely homogenised connective and vascular tissue and small tissue fragments.

1) Coarse removal

- Filter through a double layer of cheesecloth, or
- Low-speed centrifugation (5 000 ×g).

2) De-fatting

Floating fat is removed by coarse filtration through glass wool or cheesecloth.

3) Aggregation

- The solution is still cloudy with organelles and membrane fragments too small to filter out.
- Precipitate them with celite (diatomaceous earth, large trapping surface), cell debris remover (cellulose-based), or flocculants such as starch, gums, tannins or polyamines.

4) Final clarification

- Centrifuge the precipitate: typically 20 000 × g for 45 min, or 40 000 × g for 30 min in an ultracentrifuge.
- Filtration is an alternative.

Clarification: non-protein components

The cleared homogenate still contains DNA, RNA, carbohydrate, lipid and many small metabolites. Small molecules are removed later, during dialysis or size-based fractionation, and are of little concern — the problem is the macromolecules:

- Bacterial extracts are very viscous because of chromosomal DNA, and can be extremely difficult to centrifuge.
- **DNase I** in the extraction buffer reduces viscosity; the small fragments are removed later at dialysis or size-exclusion steps.
- **RNase** removes RNA in the same way.
- **Polyethyleneimine,** binds the same phosphate groups and precipitates DNA and RNA almost instantly.
- Capsular gum in bacterial extracts interferes with protein precipitation; it is digested by **lysozyme,** which works better in the presence of the detergent used for lysis.

Preparative protein biochemistry

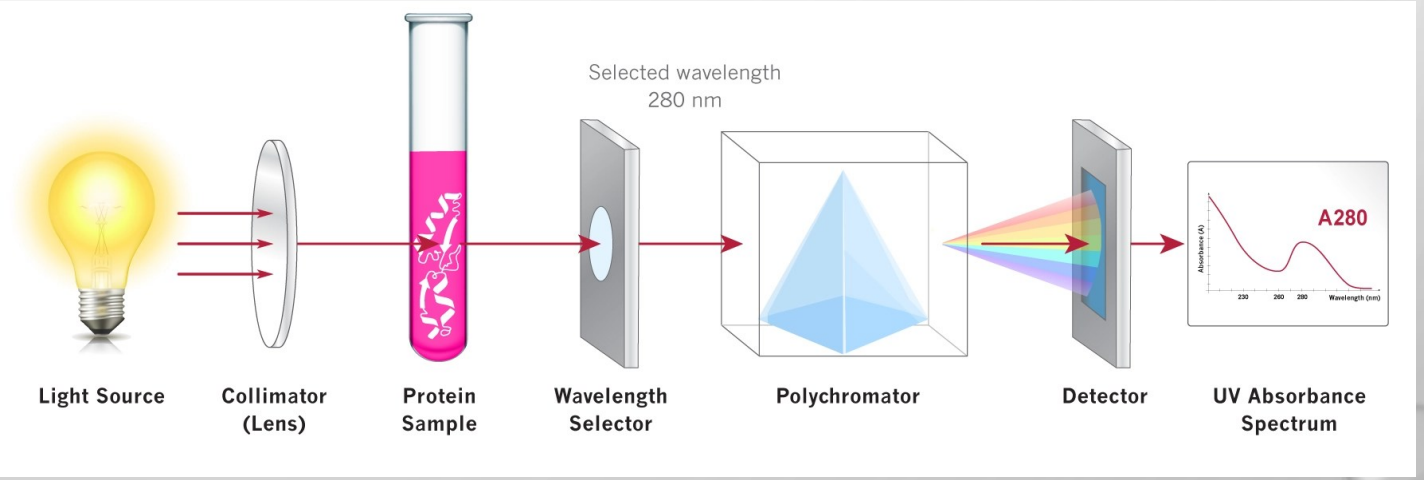
Determination of protein concentration

1) Ultraviolet absorption at 280 nm

- The **aromatic residues tyrosine and tryptophan** absorb maximally at 280 nm.
- Their proportions differ between proteins, so **extinction coefficients are characteristic of each protein** and can be estimated from its sequence.
- Sensitive: concentrations as low as ~10 μg cm^{-3} can be measured.
- **Non-destructive** — the sample can be recovered from the cuvette and used further. Invaluable when material is scarce.
- All modern chromatography systems have in-line UV detectors, typically at fixed wavelengths such as 230, 260 and 280 nm, to monitor elution continuously.

The nucleic acid problem:

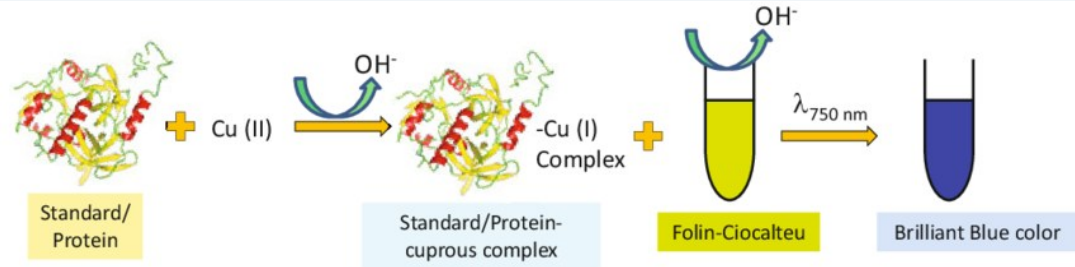
- Nucleic acids absorb at 280 nm as much as ten times more strongly than proteins, with their own maximum at ~260 nm.
- A few per cent of nucleic acid therefore distorts the reading badly.
- Measuring at both 260 and 280 nm allows a correction factor to be applied.



Preparative protein biochemistry
Determination of protein concentration
2) Lowry and bicinchoninic acid ($Cu^{2+} \rightarrow Cu^{+}$)

Lowry (Folin-Ciocalteau)

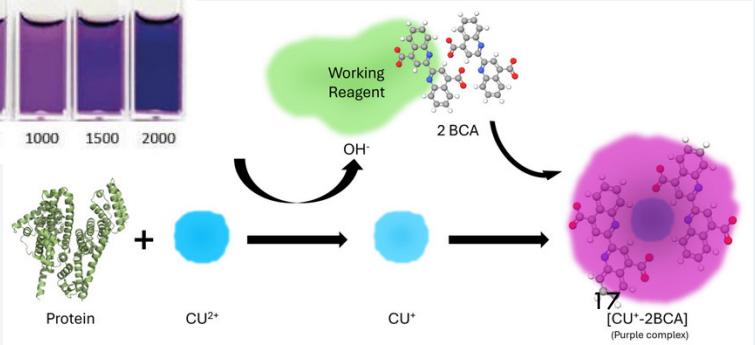
- The Folin-Ciocalteau reagent (sodium tungstate, molybdate and phosphate) with copper sulfate gives **a blue-purple colour**, read at **660 nm**.
- Detects down to 10 µg cm^{-3}; sensitivity is moderately constant between proteins.
- **Biuret reaction**: peptide bonds reduce Cu^{2+} to Cu^{+} under alkaline conditions.
- Folin-Ciocalteau reaction: phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids — so the colour depends partly on Tyr and Trp content.
- **Interference:** TRIS, the zwitterionic buffers PIPES and HEPES, and EDTA.



Bicinchoninic acid (BCA)

- Also depends on the reduction of Cu^{2+} to Cu^{+} under alkaline conditions.
- **Cu^{+} is then complexed by bicinchoninic acid**, giving an intense **purple colour** with a maximum at 562 nm.
- Twenty times more sensitive than Lowry: down to 0.5 µg cm^{-3}.
- More importantly, far more tolerant of the compounds that interfere with the Lowry assay — which is why it has largely replaced it.





Sara Standoli, a.a. 2026/2027

Preparative protein biochemistry
Determination of protein concentration
3) The Bradford method

Coomassie Brilliant Blue binding

- Relies on binding of the dye Coomassie Brilliant Blue to protein.
- At low pH the free dye absorbs maximally at 470 and 650 nm; on binding protein the maximum shifts to 595 nm.
- Practical advantages: the reagent is simple to prepare, and the colour develops rapidly and is stable.
- Sensitive down to 20 µg cm^{-3}.
- Only a relative method: dye binding varies with the content of the basic amino acids arginine and lysine, which makes choosing a standard difficult.
- Many proteins do not dissolve properly in the acidic reaction medium.

Absorption shift

Free dye



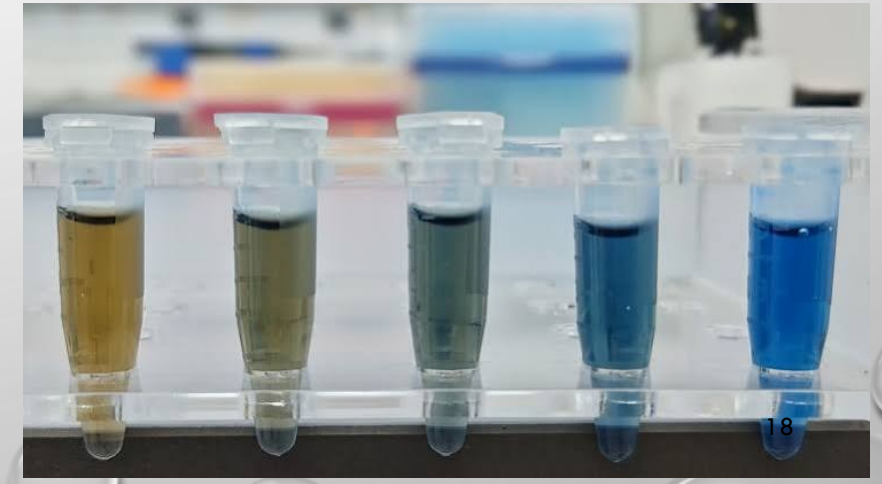
470 nm and 650 nm

Dye + protein



595 nm

The colour change on binding is what is measured; the bar lengths are illustrative only.

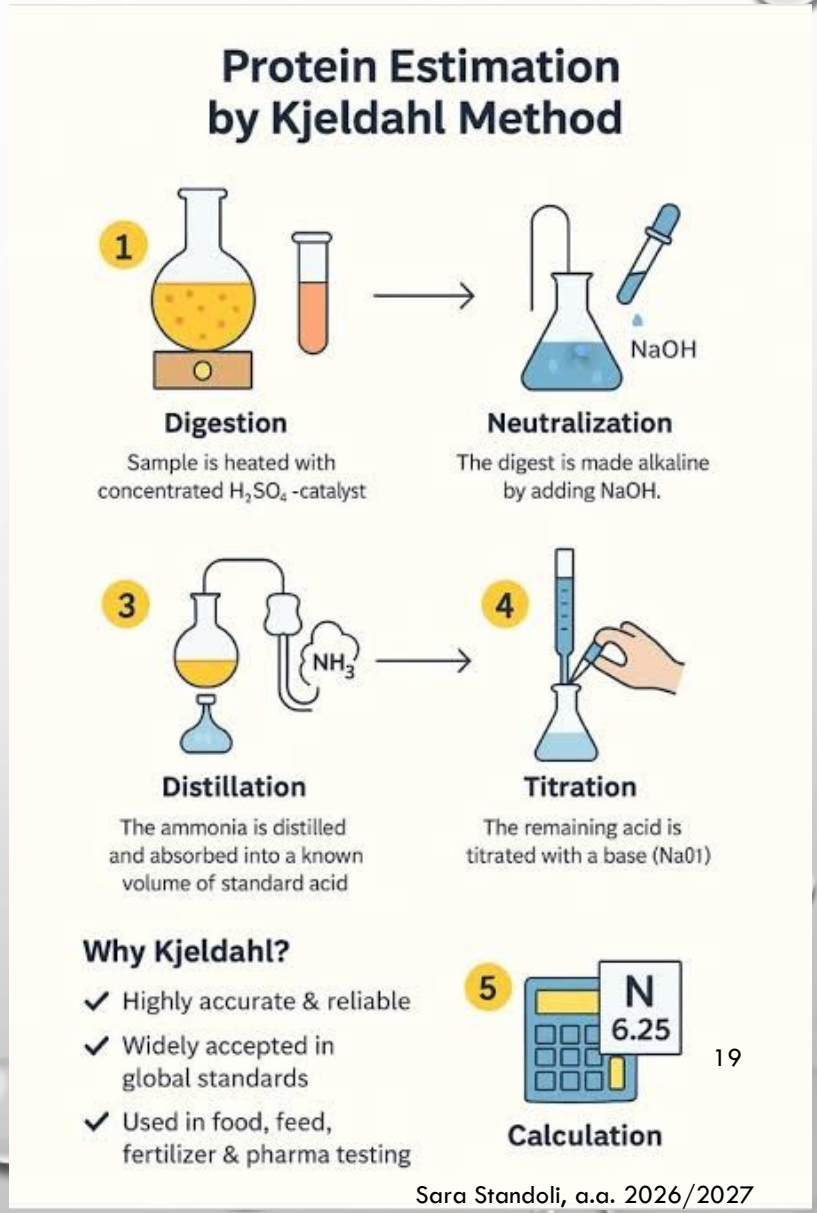


Preparative protein biochemistry
Determination of protein concentration
4) Kjeldahl analysis

Determining total nitrogen

- A general chemical method **for the nitrogen content** of any compound; used for complex solid and microbiological samples rather than purified proteins or column fractions.
- Digestion: boiling with concentrated sulfuric acid, sodium sulfate (to raise the boiling point) and a copper and/or selenium catalyst converts organic nitrogen to ammonia, trapped as ammonium sulfate. A clear solution signals completion.
- The ammonia is released with excess NaOH, steam-distilled in a Markham still, collected in boric acid and titrated with standard HCl using methyl-red-methylene-blue. An ammonium ion-selective electrode is an alternative.

⚠ Precise and reproducible for nitrogen — but the protein content deduced from it is complicated by the varying nitrogen content of individual proteins and by nitrogen in contaminants such as DNA. In practice, protein is assumed to be 16 % nitrogen by weight.



Preparative protein biochemistry

Determination of protein concentration

5) Comparing the assays

The only truly accurate method is acid hydrolysis followed by amino-acid analysis — far too slow for routine use. The practical alternatives are mostly colorimetric: a reagent produces a coloured product measured spectrophotometrically.

Method	λ	Sensitivity	Applications and notes
UV absorption	280 nm	10 μg cm^{-3}	General use; in-line monitoring in chromatography. Interference from anything absorbing strongly near 280 nm.
Lowry (Folin-Ciocalteau)	660 nm	10 μg cm^{-3}	General assay. Sensitive to TRIS, PIPES, HEPES and EDTA.
Bicinchoninic acid (BCA)	562 nm	0.5 μg cm^{-3}	General assay; tolerant of a broad range of compounds — hence its popularity.
Bradford (Coomassie Brilliant Blue)	595 nm	20 μg cm^{-3}	Simple and rapid, but binds only Arg and Lys.
Kjeldahl analysis	titration	1 mg cm^{-3}	Determines total N of any compound; used for complex samples. Any N-containing compound adds to the result (DNA, melamine).

None of the colorimetric methods is absolute: colour development depends partly on amino-acid composition and on prosthetic groups such as carbohydrate. BSA is the usual standard (cheap, pure, available), so results are only approximate.

Preparative protein biochemistry

Engineered Protein: Fusion constructs and affinity tags

TAG | **LINKER + PROTEASE SITE** | **TARGET PROTEIN**

The tag sits in frame at the N- or C-terminus and provides a handle for affinity chromatography; the engineered protease site lets it be cleaved off cleanly afterwards.

GST (*Glutathione-S-transferase*)

- Binds glutathione immobilised on agarose; eluted with excess reduced glutathione.
- In pGEX vectors thrombin cleaves the linker to remove it.
- Improves solubility, but less than MBP; four cysteines can oxidise and cause aggregation.

FLAG (*Immunoaffinity chromatography*)

- Short hydrophilic peptide (DYKDDDDK) captured by an immobilised monoclonal antibody.
- Binding is calcium-dependent; elution simply by chelating the calcium with EDTA.
- Enterokinase removes an N-terminal FLAG - or it is kept on to track the protein.

MBP (*Maltose-binding protein*)

- The solubility-enhancing tag of choice; well ordered, and assists folding when N-terminal.
- One-step purification on amylose resin.
- Add 2 mM glucose to the medium to repress the galactases that degrade amylose.

AviTag (*Acceptor peptide, 15 residues*)

- Biotinylated in vitro by BirA, or in transfected mammalian cells.
- Biotin is bound by streptavidin with a Kd of about 4×10^{-14} M.
- Yields natively folded, modified protein - ideal for capture, binding assays and detection.

His-tag (*Immobilised metal affinity chromatography*)

- Six histidines coordinate immobilised Ni or Co ions, Kd in the micromolar range.
- Eluted with 50-300 mM imidazole or at acidic pH; 10-20 mM in the wash raises purity.
- Two hexa-His tags in series allow harsher washing but need ~300 mM to elute.

Dual tags (*Combining the strengths of two*)

- His-tag + MBP in tandem: cheap, rapid capture plus a large gain in solubility.
- A TEV site between the His-MBP fusion and the target removes both at once.
- Combinations also allow the protein to be tracked, e.g. by Western blotting.

Preparative protein biochemistry
Tag removal and protein ligation

TEV protease

Patent-free and cheap to produce in the laboratory, in preference to commercial systems such as Factor Xa.

1. **Poor solubility**

   Expressed in bacteria as an MBP fusion carrying a TEV site: during expression the construct cleaves itself, releasing the active protease.

2. **Autolysis**

   TEV readily digests itself. The single change S219V removes this - and the mutant is slightly more active than the wild type.

3. **Clean-up after cleavage**

   TEV carries its own His-tag: after cleaving a His-tagged target, IMAC retains the tag, the protease and any uncleaved fusion. The pure target flows through.

Intein-mediated purification

Expressed protein ligation - no protease required

- Some post-translational modifications cannot be introduced by cloning, nor reliably by chemical or enzymatic means.
- Two peptides or proteins are joined covalently: one carrying a C-terminal thioester, the other an N-terminal cysteine.
- The method exploits the inducible self-cleavage of inteins - protein splicing elements - to separate the target from the affinity tag.
- Application: phosphorylation stoichiometry. For binding studies the exact degree of phosphorylation must be known, so the protein is ligated to a separately synthesised phosphopeptide of defined state.
- Ligated and unligated products are then separated, for example by size-exclusion chromatography.

An elegant piece of subtractive (negative) affinity chromatography: everything unwanted binds to the column, and the product does not.

Preparative protein biochemistry

Expression systems and secretion: Where to express it - and why secrete it

E. coli and yeast are explored first, then insect and mammalian systems; cultures run from dishes to shaker flasks and bioreactors.

Host	Key advantage	Limitation
Bacteria 1-4 µm, very rigid wall	Cheap; easy to manipulate, transform and induce	No glycosylation; folding problems and inclusion bodies
Yeast and fungi 5-10 µm, polysaccharide wall	Eukaryotic folding and secretion, still cheap	Wall must be digested; glycosylation differs from human
Mammalian cells ~10 µm, membrane only	Correct folding and post-translational modification	Expensive media; more complex chromatography
Plant cells ~100 µm, lignified wall	Large cells, easily broken by shear	Phenolics and polysaccharides complicate extraction

Why engineer the protein to be secreted?

1. **Easier purification:** Far fewer contaminating proteins in the medium than in a whole-cell lysate.
2. **Less degradation** Foreign proteins are attacked by intracellular proteases; export minimizes this.
3. **Lower toxicity:** Toxic products kill the cell; secretion prevents that and sustains production.
4. **Post-translational modification:** Most modifications, glycosylation above all, occur along the secretory pathway.

Signal sequences

- A short, usually N-terminal signal sets the pathway and the final destination, and is cleaved before secretion.
- The native signal sometimes works (human interferon in Pichia); yeast signals such as alpha-factor are also used.
- Prokaryotes do not glycosylate - hence eukaryotic hosts.

The bacterial envelope

- Gram-negative: thin peptidoglycan plus an outer membrane of lipopolysaccharide cross-linked by Mg^{2+} ions.
- Chelating that Mg^{2+} with EDTA destabilises the outer membrane - the basis of enzymatic lysis.
- Gram-positive: a thick 20-50 nm peptidoglycan shell, which takes up the Gram stain.

Preparative protein biochemistry

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Preparative protein biochemistry

From plasmid to culture: Transformation, selection and induction

1 Transform

The expression vector is introduced by heat shock or a short pulse of electricity; the brief stress momentarily makes the membrane permeable.

2 Recover

Conditions are kept gentle - enriched SOC medium, small volume - to improve cell viability.

3 Select

Cells are plated on antibiotic agar; the plasmid carries the matching resistance cassette, so only transformants grow.

4 Expand and induce

A single clone is expanded into a larger volume suitable for induction and substantial protein yield.

Induction: the lac operon and IPTG

Shortly after the lac operon was described, non-metabolisable analogues of lactose such as IPTG were used to relieve repression by the lac repressor and drive transcription and translation of the cloned gene. Gram-negative hosts can also export protein into the periplasmic space, giving a much cleaner starting extract than a whole-cell lysate.

A single colony is picked, not a scrape of the plate: the whole culture must derive from one plasmid molecule, or the population is heterogeneous.