Kód předmětu: Bi8980

MASARYKOVA UNIVERZITA

Protein expression and purification

- VI. Cell disruption

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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

6.1. Raw material

6.1.1. Species used for protein purification

Vertebrates:

- Liver studies – rat
- Skeletal muscle studies – rabbit
- Organs (heart, brain, kidney, thymus) – cow or pig
- Blood or placenta – human

Invertebrates:

- Are very small
- Difficult to dissect organ of interest from each individual

Plants:

- Spinach (Spinacia oleracea) chloroplast
- Beta vulgaris

Microorganisms:

- Yeast
- Bacteria

6.1. Raw material

6.1.2. Freshness and storage

Yeast cakes remain viable:
- weeks (0℃)
- month (frozen)
- years (sealed under vacuum or in nitrogen)

Bacterial host cells: higher concentration of recombinant protein production
- protein insolubility
- biologically inactive proteins
- protein processing

6.1. Raw material

6.1.2. Freshness and storage

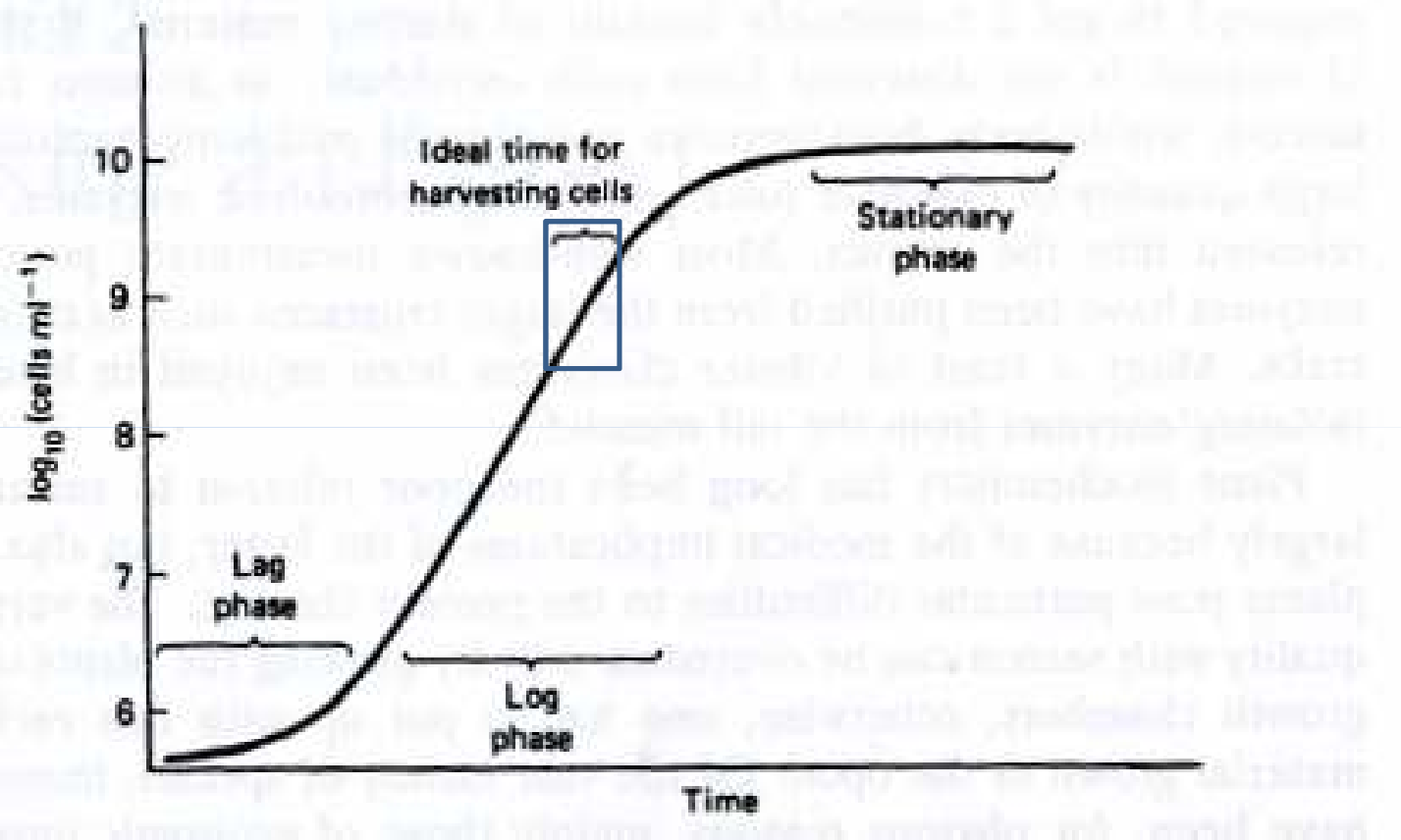


Figure 1. Growth of organisms in nutrient-rich medium. The ideal time for harvesting is toward the end of the log phase before growth rate slows, giving a high yield of cells. But specific enzymes may be maximum at an earlier or later stage, so some trials at different times are desirable.

6.1. Raw material

6.1.2. Freshness and storage

"Usually, the sooner the raw material is used, the better and more physiologically relevant the preparation will be."

- Natural degradative processes will have started.

Frozen storage:

- Free water freezes and ice crystals grow – very destructive for membrane layers and organelles, not for protein.
- Decreasing temperature causes the remaining liquid to become increasingly concentrated – solubility problem.
- pH changes drastically before complete solidification.
- Proteases liberated from lysosomes are activated.
- Freezing of extracts is sometimes preferable (proteolytic enzyme inhibitors).
- Thawing speed can be important – the faster, the better.

6.2. Cell disintegration and extraction

Extraction of protein from tissues and cells is perhaps the most critical step in any protein purification or proteomics strategy.

The principal aim:

- Achieve reproducibility.
- The highest degree of cell breakage.
- Minimal disruptive forces.
- Maintaining protein integrity (avoiding altering the native structure – biological activity).

Perturbation of native protein structure can be caused by exposure to:

- Extreme pHs,
- Extremes temperatures,
- Mechanical stress (shearing forces),
- Pressure, or
- Proteolytic degradation (association with host proteins).

6.2. Cell disintegration and extraction

Gentle

Technique	Example	Principle
Cell lysis	Erythrocytes	Osmotic disruption of cell membrane
Enzyme digestion	Lysozyme treatment of bacteria	Cell wall digested, leading to osmotic disruption of cell membrane
Chemical solubilization / autolysis	Toluene extraction of yeast	Cell wall (membrane) partially solubilized chemically; lytic enzymes released complete the process
Hand homogenizer	Liver tissue	Cells forced through narrow gap, rips of cell membrane
Mincing (grinding)	Muscle etc.	Cell disrupted during mincing process by shear force

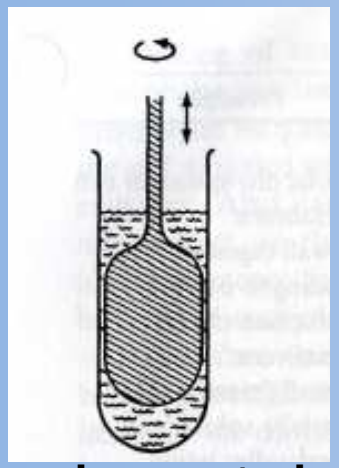
Moderate

Blade homogenizer (waring type)	Muscle tissue, most animal tissues, plant tissues	Chopping action breaks up large cells, shears apart smaller ones
Grinding with abrasive (e.g., sand, alumina)	Plant tissues, bacteria	Microroughness rips off cell walls

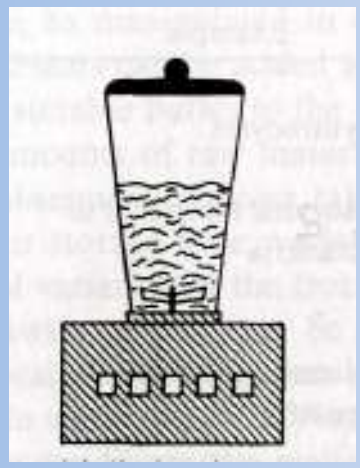
Vigorous

French press	Bacteria, plant cells	Cells forced through small orifice at very high pressure; shear forces disrupt cells
Ultrasonication	Cell suspensions	Microscale high-pressure sound waves cause disruption by shear forces and cavitation
Bead mill	Cell suspensions	Rapid vibration with glass beads rips cell walls off
Manton-Gaulin homogenizer	Cell suspensions	As for French press above, but on a larger scale
Nitrogen cavitation vessel	Cell suspensions	Explosive decompression (nitrogen cavitation)

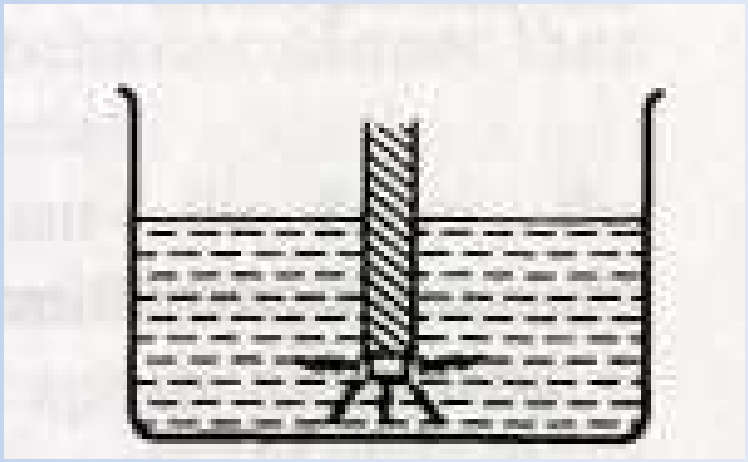
6.2. Cell disintegration and extraction



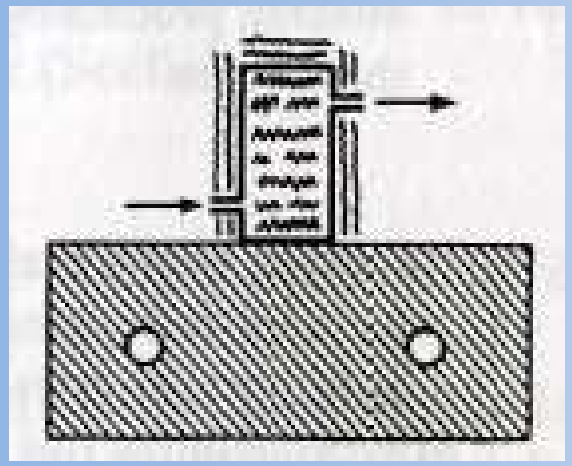
Hand-operated or motor driven



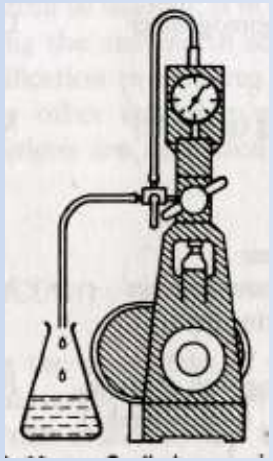
Waring blender



Ultrasound



Vibrating bead mill



Manton-Gaulin homogenizer

6.2. Cell disintegration and extraction

6.2.1. Mammalian tissues

- Frozen tissue stored below -50℃
- Dice tissues and cut away connective tissue and fat
- Extraction buffer: 2-3 vol/gram of tissue.
- Waring blender (30 s)
- Stir the homogenate (10-15 min)
- Check the pH
- Centrifuge (5,000-10,000 g/max 60 min)
- Decant extract through Miracloth
- Gentler conditions (Nonidet P-40), keep the sample cold, add protease inhibitors

6.2. Cell disintegration and extraction

6.2.1. Mammalian tissues

Protocol 1

Homogenization of Mammalian Tissue

To purify or characterize an intracellular protein, it is important to choose an efficient method for disrupting the cell or tissue that rapidly releases the protein from its intracellular compartment into a buffer that is not harmful to the biological activity of the protein of interest. One of the most widely used methods for disrupting soft tissues is homogenization. In this protocol, three processes for tissue homogenization using mechanical shear are discussed: chopping the tissue in a Potter-Elvehjem glass-Teflon homogenizer, a Dounce hand homogenizer, or a hand-held Waring Blendor. These methods are rapid and pose little risk to proteins other than the release of proteases from other cellular compartments. Proteolytic degradation can be minimized by the inclusion of protease inhibitors in the homogenization buffers.

6.2. Cell disintegration and extraction

6.2.1. Mammalian tissues

MATERIALS

CAUTION: See Appendix 5 for appropriate handling of materials marked with <!>.

Reagents

Appropriate animal tissue

Working with human tissue <!> presents a unique set of hazards.

Dithiothreitol (DTT) (0.5 M) <!>

Prepare a 0.5 M stock solution in cold H_2O and store frozen. Add the reagent to cold buffers at the indicated concentration just prior to use.

Homogenization buffer A

50 mM Tris-Cl (pH 7.5)
2 mM EDTA
150 mM NaCl
0.5 mM DTT <!>

Homogenization buffer B

50 mM Tris-Cl (pH 7.5)
10% (v/v) glycerol (or 0.25 M sucrose)
5 mM magnesium acetate
0.2 mM EDTA
0.5 mM DTT <!>
1.0 mM phenylmethylsulfonyl fluoride (PMSF) <!>

6.2. Cell disintegration and extraction

6.2.1. Mammalian tissues

The choice of homogenization buffer will depend on the nature of the extract required. Generally, use a buffer of moderate ionic strength at neutral pH (e.g., 0.05–1.0 M phosphate or Tris, pH 7.0–7.5). The appropriate buffer ionic strength should be chosen by trial and error to optimize the yield of the target protein. For example, the addition of 0.1 M NaCl or KCl <!> will increase the yield of those proteins that have a tendency to attach electrostatically to cell debris/membrane fragments. On the other hand, the association-dissociation behavior of some proteins is influenced markedly by ionic strength. If the purpose of the extraction is to isolate organelles, it is important to use low-ionic-strength buffers (e.g., 5–20 mM Tris, HEPES, or TES at pH 7.4) containing iso-osmotic sucrose or mannitol (0.25 M). Avoidance of proteolytic degradation of the target protein in a crude extract is a primary concern. In many cases, it may not be essential to add protease inhibitors to the homogenization buffer (due to the protective effect of bulk protein on a target protein), but some proteins are more susceptible to proteolysis than others, and some tissues (e.g., liver and pancreas) have much higher levels of proteases than others (e.g., heart). The use of protease inhibitor cocktails can be expensive if the extract volumes are large. Hence, carry out pilot experiments over a period of a few hours to ascertain whether there are measurable losses of the target protein activity. If proteolytic degradation is a problem, then include protease inhibitors in the homogenization buffers (for a list of protease inhibitors, see Table 2.2 and for the preparation of protease inhibitor cocktails, see Table 2.3). If the target protein is susceptible to oxidation or its activity is inhibited by heavy metals, then add DTT (1 mM) <!> (or 0.1 M β-mercaptoethanol <!>) and EDTA (0.1 M), respectively, to the extraction buffer.

6.2. Cell disintegration and extraction

6.2.1. Mammalian tissues

TABLE 2.2. Inhibitor cocktails used to control proteolysis during protein isolation

Tissue type	Protease inhibitors (working concentration)	Target protease type[a]	Stock solution[b]
Animal tissues	AEBSF (0.2 mM) <!> (*or* DCI [0.1 mM]or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; PMSF <!>: 200 mM in ethanol or isopropanol)
	benzamidine (1 mM)	serine	100 mM
	leupeptin (10 µg/ml) <!>	serine/cysteine	1 mg/ml
	pepstatin (10 µg/ml)	aspartic	5 mg/ml in methanol
	aprotinin (trasylol) (1 µg/ml) <!>	serine	0.1 mg/ml
	EDTA or EGTA[c] (1 mM)	metallo	100 mM
Plant tissues	AEBSF (0.2 mM) <!> (*or* DCI [0.1 mM] or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; PMSF <!>: 200 mM in ethanol or isopropanol)
	chymostatin (20 µg/ml) <!>	serine/cysteine	1 mg/ml in DMSO
	EDTA or EGTA[c] (1 mM) <!>	metallo	100 mM
Yeasts and fungi	AEBSF (0.2 mM) <!> (*or* DCI [0.1 mM]or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; PMSF <!>: 200 mM in ethanol or isopropanol)
	pepstatin (15 µg/ml)	aspartic	5 mg/ml in methanol
	1,10-phenanthroline (5 mM)	metallo	1 M in ethanol
Bacteria	AEBSF (0.2 mM) <!> (*or* DCI [0.1 mM] or PMSF [0.2 mM]) <!>	serine	20 mM in methanol (DCI: 10 mM in DMSO; PMSF <!>: 200 mM in ethanol or isopropanol)
	EDTA or EGTA[c] (1 mM)	metallo	100 mM

Adapted from North (1989).

Abbreviations: (AEBSF) 4-(2-aminoethyl)-benzenesulfonylfluoride; (DCI) 3,4-dichloroisocoumarin; (DMSO) dimethylsulfoxide; (EDTA) ethylenediamine tetraacetic acid; (EGTA) ethylene glycol bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; (PMSF) phenylmethylsulfonyl fluoride.

Mr values of inhibitors: AEBSF (240); PMSF (174); DCI (215); EDTA (disodium salt, dihydrate) (372); benzamidine (hydrochloride) (157); leupeptin (427); pepstatin (686); aprotinin (6500); chymostatin (605); 1,10-phenanthroline (198).

[a]For a review of proteolytic enzymes, see Neurath (1989) and Perlmann and Lorand (1970).

[b]Aqueous solution unless otherwise indicated.

[c]An efficient chelator of divalent metal cations other than Mg^{2+} (for which it has a 10^3-fold lower affinity) (Gegenheimer 1990).

6.2. Cell disintegration and extraction

6.2.1. Mammalian tissues

TABLE 2.3. Preparation of a general protease inhibitor cocktail

Inhibitor	Trial working concentration	Stock (100x) concentration	Recipe	Use[a]	Target protease type
AEBSF <!>	<1 mM	20 mM	239.5 mg/10 ml H_2O (100 mM)	4 ml	serine proteases
EDTA	1–10 mM	100 mM	19 mg/100 ml H_2O (0.5 M)	4 ml	metallo proteases
Leupeptin <!>	10–100 µM	2 mM	18.9 mg/2 ml H_2O (20 mM)	2 ml	cysteine/serine proteases
Pepstatin	1 µM	100 µM	6.8 mg/10 ml methanol (1 mM)	2 ml	aspartic proteases

Adapted from Calbiochem Technical Bulletin CB0578-0998.

[a]Mix the inhibitor solutions and bring to a final volume of 20 ml with H_2O or appropriate aqueous buffer. The resulting solution, 20 ml of a stock 100x protease inhibitor cocktail, can be aliquoted into microfuge tubes and stored at –20°C until required.

Waring Blendor

A mechanical shear homogenizer that uses rotating metal blades or teeth to disrupt the material. There are many variations of the traditional domestic food liquidizer in which the material is placed in a glass reservoir with the blades driven by a motor beneath it. Other models resemble the modern hand-held blenders in which the motor is overhead. The Waring Blendor is typically used to macerate large amounts (100–1000 ml) of hard animal tissue and plant tissue. For smaller volumes (~1–5 ml), the Ultra-Turrax (IKA Works) and its successor, the Polytron homogenizer, are widely used.

6.2.1. Mammalian tissues

Carry out all procedures at 0–4°C.

1. After the tissue is excised from the animal, trim and discard fat and connective tissue from the tissue. Place the tissue in cold Homogenization buffer A.
2. Dice the washed tissue into small pieces (i.e., 1-cm cubes) with a knife or, alternatively, pass the tissue through a meat grinder twice.

   Tissues such as liver, brain, kidney, and heart are readily homogenized in a Waring Blendor, but tissues such as skeletal muscle and lung are tougher, and it is advisable to grind them in a domestic meat grinder prior to homogenization. Very fibrous tissues such as mammary glands must be frozen prior to homogenization to facilitate disruption (the Ultra-Turrax homogenizer is widely used for this purpose). Cultured mammalian cells and small amounts (1–5 g) of soft tissue such as brain can be homogenized conveniently using a Dounce hand homogenizer (Dignam 1990). In all cases, prechill the homogenizers and glassware to 4°C, and work in a cold room while using the blender.

3. Add 3–4 volumes of Homogenization buffer B per volume of tissue, and transfer the mixture to the homogenizer.
4. Prepare the homogenate using one of the following methods:

   Potter-Elvehjem homogenizer: Homogenize the tissue with the apparatus set at 500–1500 rpm, allowing 5–10 seconds per stroke.

   Dounce hand homogenizer: Homogenize the tissue with 10–20 strokes of the pestle.

   Waring Blendor: Homogenize the tissue three to four times for 20–30 seconds each (no longer), pausing for 10–15 seconds between each homogenization.

6.2. Cell disintegration and extraction

6.2.1. Mammalian tissues

5. Pour the homogenate into a glass beaker, place the beaker on ice, and stir the homogenate gently for 30–60 minutes at 4°C to allow further extraction of proteins.

   Do not allow the homogenate to foam.

6. Remove cell debris and other particulate matter from the homogenate by centrifugation at 10,000*g* for 10–20 minutes at 4°C.

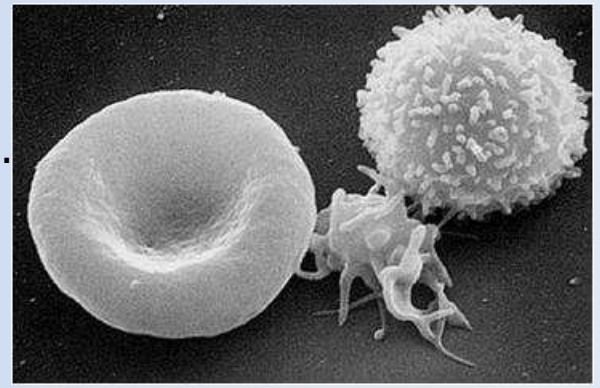
7. Filter the supernatant through two layers of cheesecloth (or a plug of glass wool) in a filter funnel to remove any fatty material that has floated to the surface. Carefully squeeze the cloth to obtain the maximum amount of filtrate (referred to as the "crude extract").

8. Proceed with the appropriate fractionation or analysis strategy as quickly as possible (see Chapter 1).

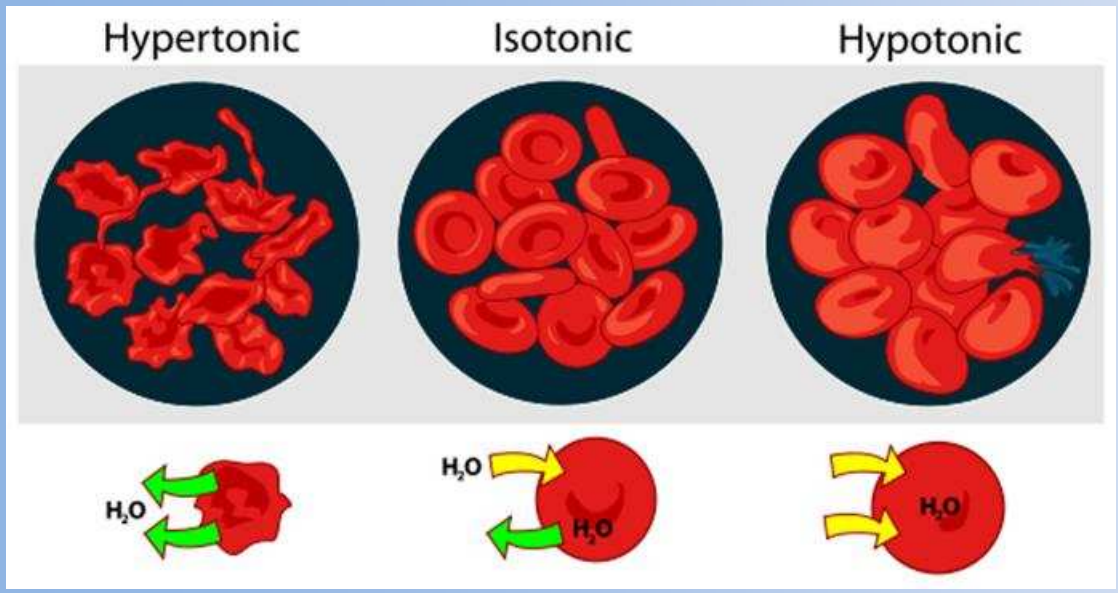
6.2. Cell disintegration and extraction

6.2.2. Erythrocytes

- Collect red blood cells by centrifugation.
- Rinse with isotonic NaCl (0.9%, 0.15 M).
- Osmotic lysis with water (2 vol/1 vol packed cells).
- Selectively remove hemoglobin (90%) – ethanol/chloroform.



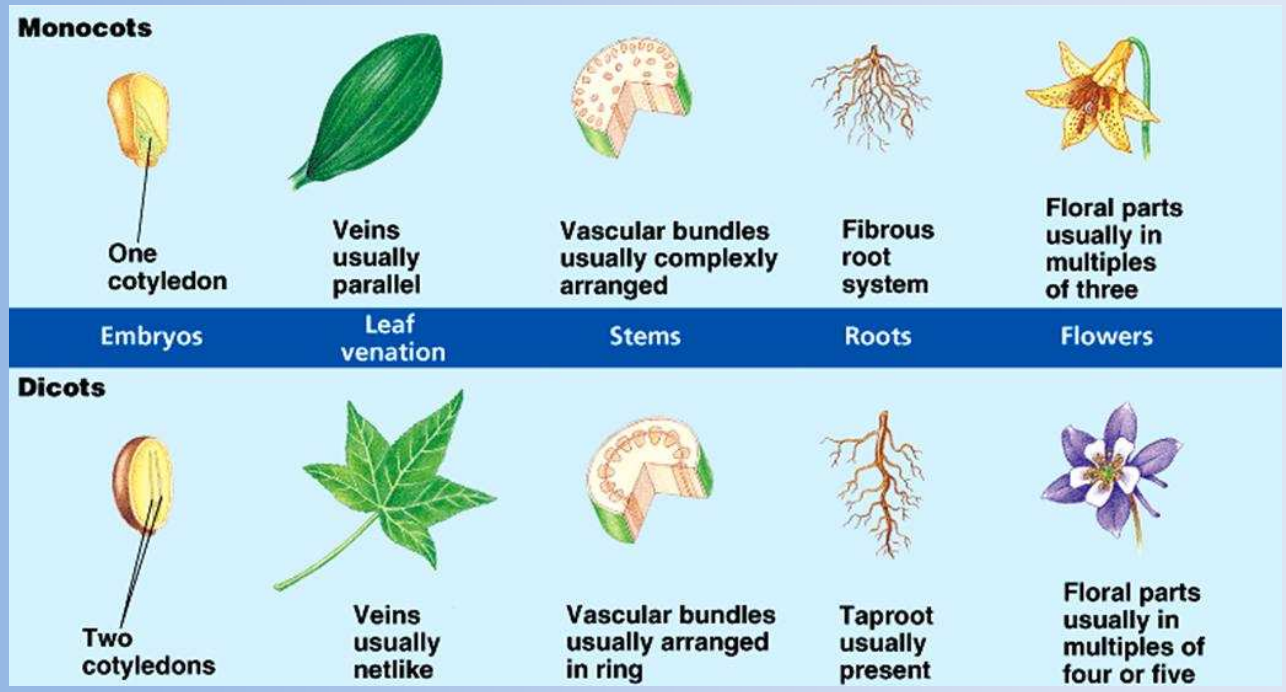
Scanning electron micrograph of blood cells. From left to right: human erythrocyte, thrombocyte (platelet), leukocyte.



6.2. Cell disintegration and extraction

6.2.3. Soft plant tissues

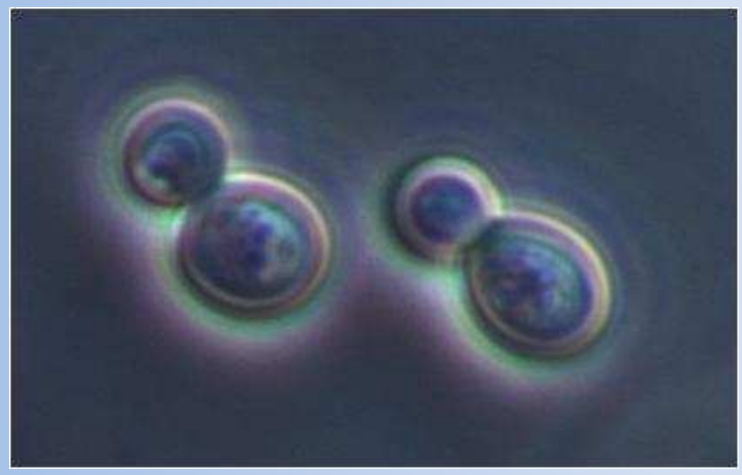
- 0.5-1 vol of cold extraction buffer
- 20-30 mM of β-mercaptoethanol
- Homogenize in blender (30 s)
- Centrifuge as soon as possible (2-3 x 10^5 g min)
  (minimize oxidative browning).
- Addition of powdered polyvinylpyrrolidone (for adsorbing phenols).



6.2. Cell disintegration and extraction

6.2.4. Yeasts

- Mechanical disruption (Manton-Gaulin homogenizer) 2-5 vol/gram wet weight.
- Toluene autolysate (35-40℃) after 20-30 min.
- Ammonia cytolysis (0.5 M NH_4OH) 2 vol/gram wet weight (16 -20 h). Bring pH down (1-2 vol of water plus acetic acid).
- Bead mill (1 g/3 ml of buffer) “Merckenschlager”.
- Manual beading (glass beads of diameter 0.5, suitable bottle, shaken manually for 5-10 min).
- Enzymatic lysis (mixture containing mannanases, glucanases and chitinases).



6.2. Cell disintegration and extraction

6.2.5. Bacteria

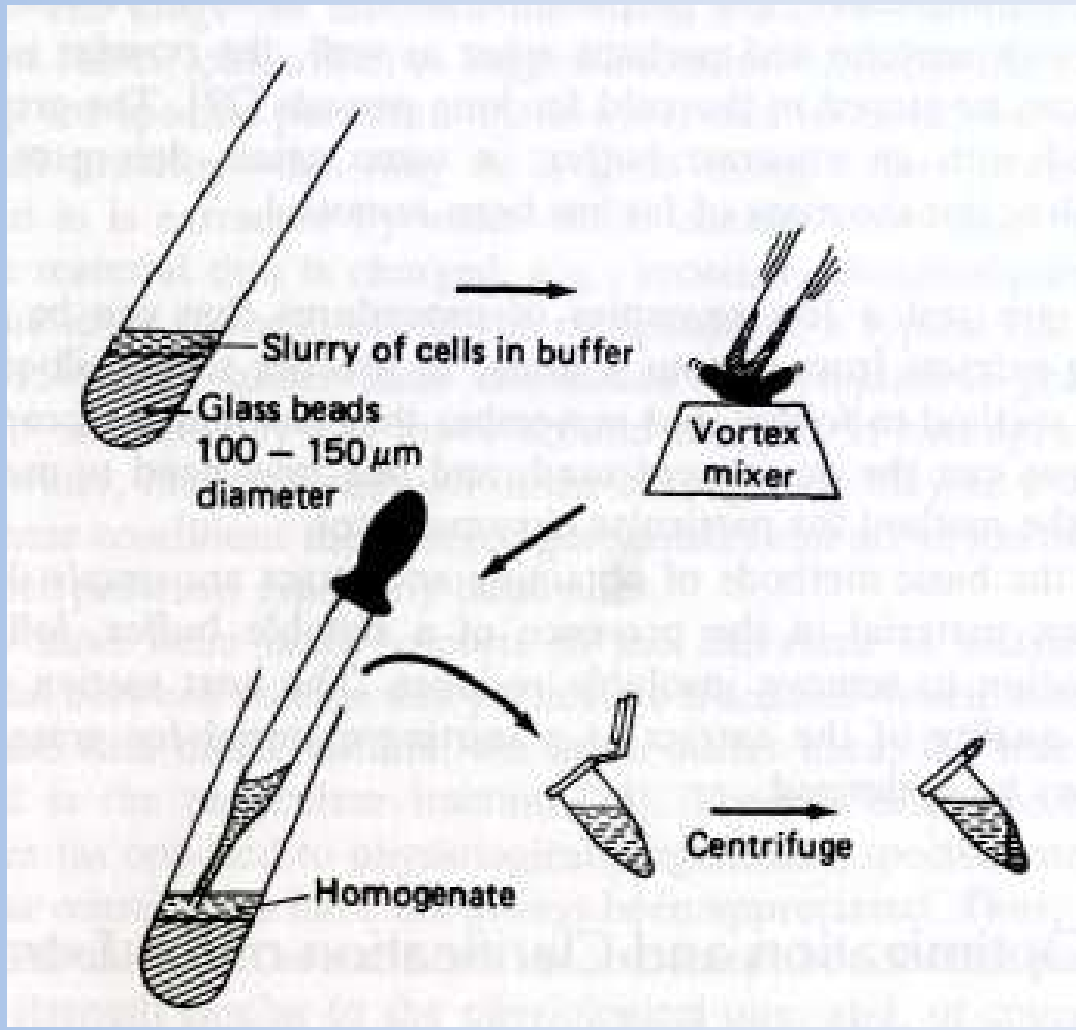
- Sonication
  - Dissolution of solids
  - Dispersion of particulates in liquids
  - Sonochemistry and chemical reaction acceleration
  - Degassing of solutions
- Bead-milling (vortexing with glass beads)
- French press (8,000–20,000 psi [55-140 MPa], 10-30 ml)
- Grinding with abrasive agents (alumina or sand)
- Lysozyme (enzyme digestion 0.2 mg/ml + DNAase 10 ug/ml)
- Nitrogen cavitation – 800 psi (5.5 MPa) 1 – 1000 ml

Sonication

6.2. Cell disintegration and extraction

6.2.5. Bacteria

Small-scale disintegration of bacteria. Glass beads are added to a small test tube (e.g. 15 mm diameter) to a depth of about 10 mm. A slurry of cells in buffer (e.g. 1 g wet weight in 5 ml of buffer) is added until up to 5 mm above the glass beads. The mixture is vortex-mixed for 2-5 min. The extract is sampled from the surface of the beads and centrifuged in a microcentrifuge tube.



6.2. Cell disintegration and extraction

6.2.5. Bacteria

A different problem can occur with *E. coli* expression system, where the expressed recombinant protein appears in the crude extract:

- Insoluble aggregates – “inclusion bodies” need to be solubilized in a strong denaturant (e.g. Guanidin hydrochloride or urea).
- Lysis conditions will depend on whether the lysate is to be used for:
  - Immunoprecipitation studies,
  - Western blotting,
  - 2D electrophoresis,
  - Native target protein isolation using conventional chromatographic purification procedures, or
  - Recombinant protein purification procedures that rely on the target protein expressed as fusion protein including a purification “handle” or “tag”.

6.2. Cell disintegration and extraction

6.2.5. Bacteria

Protocol 5

Small-scale Extraction of Recombinant Proteins from Bacteria

Bacteria are particularly convenient for producing recombinant proteins for purification purposes. To monitor induction as well as the levels of recombinant protein expression, it is important to have a rapid, simple method for estimating bacterial protein expression. This protocol describes the preparation of small-scale bacterial extracts using cell lysis with 0.5% Triton X-100.

1. Harvest the bacterial cells from 1 ml of culture by centrifugation in a microfuge for 1 minute at room temperature.
2. Pour off the supernatant and resuspend the bacterial cell pellet in 1 ml of chilled aqueous 0.5% (v/v) Triton X-100.
3. Sonicate the suspension for three cycles of 20 seconds each, cooling the cells on ice between treatments.
4. Centrifuge the suspension in a microfuge for 1 minute.
5. Add Laemmli buffer containing 100 mM DTT to the supernatant, boil the mixture for 2 minutes, and analyze for the target protein using analytical SDS-PAGE (see Chapter 3, Protocol 1).

6.2. Cell disintegration and extraction

6.2.5. Bacteria

Protocol 6

Large-scale Extraction of Recombinant Proteins from Bacteria

Bacteria are particularly convenient for producing recombinant proteins for purification purposes. Suitable extraction methods for bacterial cells include sonication, glass bead milling, grinding with alumina or sand, high-pressure shearing using the French pressure cell (French Press), and lysozyme treatment. These procedures are applicable for preparing extracts from a variety of Gram-negative bacteria such as *E. coli* and *Klebsiella pneumoniae* Gram-positive bacteria such as *Bacillus subtilis*. Disruption of bacterial cells by enzymatic means is commonly used because a relatively uniform treatment is obtained when cells are in suspension. A protocol for enzymatic disruption of *E. coli* follows.

6.2. Cell disintegration and extraction

6.2.5. Bacteria

1. Harvest the bacterial cells by centrifugation at 3000*g* for 15 minutes at 4°C.
2. Wash the cells with lysis buffer to remove the residual culture medium and harvest the washed cells by centrifugation as in Step 1.
3. Pour off the supernatant and weigh the wet pellet.
4. Resuspend the washed *E. coli* cells in ~3 ml of lysis buffer per gram of cell pellet and stir the suspension for 30 minutes at 4°C. If the pellet is not fully resuspended after 30 minutes, mix the suspension in a Waring Blendor at low speed for ~1 minute.
5. Add lysozyme to a concentration of 0.1% (w/v) and incubate for 35 minutes at 4°C, shaking gently.

   A faster rate of lysis may be obtained by increasing the lysozyme concentration to 1.0% (w/v) (10 mg/ml). Under these conditions, satisfactory lysis can be accomplished in as little as 5 minutes at temperatures as low as 4°C (Bollag et al. 1996).
6. Add in the following order:

   NP-40 to a final concentration of 0.5% (v/v)
   $MgCl_2$ to a final concentration of 5 mM
   DNase I to a final concentration of 40 µg/ml

   Stir the suspension for 30 minutes at 4°C to remove the viscous nucleic acid.

6.2. Cell disintegration and extraction

6.2.5. Bacteria

Stir the suspension for 30 minutes at 4°C to remove the viscous nucleic acid.

Bacterial extracts are 40–70% protein, 10–30% nucleic acid, 2–10% polysaccharide, and 10–15% lipid (Worrall 1996). The release of DNA upon cell lysis often results in a highly viscous extract that can cause serious problems in subsequent chromatographic purification steps. In addition to DNase I treatment, DNA can be removed from the cell extract (along with other nucleic acids, and in some cases, highly acidic proteins) by the addition of a neutralized solution of positively charged compounds such as protamine sulfate (up to 5 mg/g wet weight of cell pellet) (Scopes 1994) or polyethyleneimine (Burgess and Jendrisak 1975). Methods for DNA removal involving positively charged compounds should not be used with inclusion body preparations, since the precipitated DNA will cocentrifuge with the inclusion bodies.

7. Centrifuge the suspension at 23,000*g* for 30 minutes at 4°C.
8. Resuspend a small portion of the pellet in Laemmli buffer containing DTT.
9. Analyze aliquots of both the soluble protein fraction (supernatant) and pellet fraction for the presence of target protein using analytical SDS-PAGE (see Chapter 3, Protocol 1).

   If the bulk of the target protein is found in the insoluble pellet fraction, then inclusion bodies have likely formed, and the target protein will need to be solubilized and purified according to Protocol 8. If the target protein is found in the supernatant, this material should be stored at 4°C in readiness for the next purification protocol(s).

6.2. Cell disintegration and extraction

6.2.5. Bacteria

Protocol 7

Preparation of Clarified *E. coli* Extract Containing Histidine-tagged Proteins

Recombinant protein fused to a histidine peptide tag can be readily expressed in *E. coli* and subsequenctly purified on an immobilized metal ion affinity (IMAC) column (see Chapter 9). The goals of the extract preparation are to release the target protein from the cells and remove insoluble material that may foul the IMAC column filters and bed. The viscosity of the extract can be reduced by adding DNase (e.g., Benzonase) together with $MgCl_2$ to fragment the bacterial DNA. To avoid proteolysis of the target protein, PMSF or other protease inhibitors (but preferably not EDTA) can be included. Lysozyme, DNase, and $MgCl_2$ can often be omitted when using a high-pressure homogenizer. This protocol describes a typical lysis method. Other methods and conditions may be required for some proteins depending on the protein stability, solubility, and tendency to adsorb to cell debris.

6.2. Cell disintegration and extraction

6.2.5. Bacteria

Inclusion body formation can be prevented and the soluble fraction of the target protein increased by:

- Lowering temperature from 37℃ to 30℃, 22℃ or eve n lower,
- Varying the media composition and using different strains,
- Co-expression of molecular chaperones,
- Fusion of the target protein with a highly soluble protein, or
- Growing cells in the presence of sorbitol and glycyl betaine.

Two major advantages of inclusion bodies:

- By sequestering recombinant protein, these bodies permit the cell to express the protein at high levels.
- The inclusion bodies can be readily purified away from bacterial cytoplasmic proteins by centrifugation.

6.2. Cell disintegration and extraction

6.2.5. Bacteria

Protocol 8

Solubilization of *E. coli* Recombinant Proteins from Inclusion Bodies

Because molecular cloning techniques allow high levels of expression in bacteria, this is a particularly convenient system for producing recombinant proteins. Regrettably, these proteins are often difficult to purify due to their tendency to aggregate and precipitate within the bacteria to form insoluble inclusion bodies. The formation of inclusion bodies is especially common for nonbacterial proteins. Although no single method can be applied to every protein, a number of strategies are available to solubilize inclusion body proteins. One of these strategies is described in this protocol. A number of steps must be considered in solubilizing inclusion body proteins:

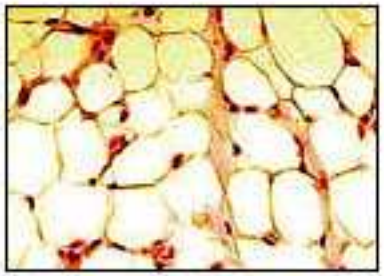
- cell lysis
- isolation of inclusion bodies
- washing of inclusion bodies
- solubilization of inclusion bodies
- renaturation (if required) of recombinant protein

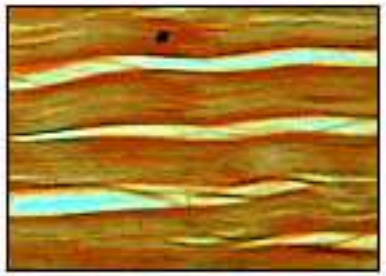
6.2. Cell disintegration and extraction

6.2.6. Fatty tissues

- Homogenization difficulties
- Large amounts of detergent
- Acetone powder


Areolar connective tissue


Adipose tissue


Fibrous connective tissue

6.3. Optimization and clarification of the extract

- Adjust the acidification (cause the aggregation).
- The best method is to make small-scale extract using a large volume (10ml/g) of extractant, and measure the amount of activity after different times of treatment.
- Isoosmotic buffer – for isolating organelles (sucrose, mannitol or sorbitol).
- EDTA (1–5 mM) destabilize complexes and inhibit proteases.
- β-mercaptoethanol (reducing condition).
- Typical buffers: 20-50 mM phosphate, pH 7–7.5; 0.1 M Tris-HCl, pH 7.5; 0.1 M KCl with a little buffer in it.
- Ionic strength inside (0.15–0.2 M).

6.3. Optimization and clarification of the extract

Provided that the protein wanted, acidification can be most beneficial if:

1. does not also isoelectrically precipitate at the pH used,
2. does not adsorb to the precipitate forming, and
3. remains stable at the subphysiological pH.

The extract should be:

- kept cold,
- controlled for pH after stirring 10-20 min,
- readjusted after pH measurement.

6.3. Optimization and clarification of the extract.

- Plant tissues - are more acidic.
- Microorganisms - contain large amount of nucleic acid.
  - The cell wall and extracellular material may either be finely dispersed (turbid extract) or partially solubilized (gum-like polysaccharide in solution).

A common procedure is to treat with substances that cause precipitation of the nucleic acids and associated compounds. These include:

- Streptomycin (precipitates ribonuclear proteins and clarifies the extract)
- Protamine (precipitates DNA and RNA complexes)
- Polyethyleneimine (precipitates nucleic acids and causes precipitation of aggregated nucleoproteins)

6.4. Extraction of membrane proteins

Many proteins and enzymes are not naturally present in an aqueous phase:

- cell membrane proteins in prokaryotes;
- in gram-negative organisms, periplasmic proteins immobilized between the outer and inner membranes;
- in multicellular eukaryotes, individual cell membranes and organelles (mitochondria, nuclei, endoplasmic reticulum, Golgi, vacuolar and lysosomal membranes).

Membrane proteins are categorized as:

- peripheral (loosely associated on the surface of the membrane) or
- integral.

6.4. Extraction of membrane proteins

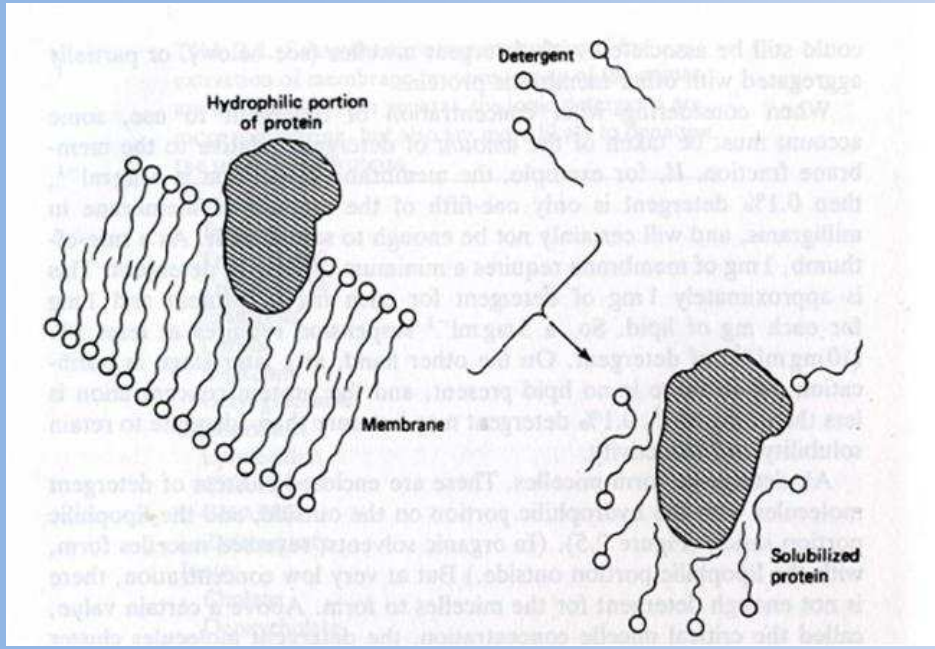
How to release membrane proteins as soluble:

1. Sonication
2. EDTA, EGTA at 1-10 mM
3. Mild alkaline conditions (pH 8-11) at low ionic strength
4. Dilute non-ionic detergent
5. Low concentrations of partially miscible organic solvents such as n-butanol
6. High ionic strength, e.g., 1 M NaCl
7. Phospholipase treatment

Some proteins have hydrophobic patches on the surface which will mutually attract, resulting in aggregation.

6.4. Extraction of membrane proteins

Some detergents commonly used for extraction of membrane proteins. In general, the ionic detergents are more solubilizing, but also are more likely to denaturate the solubilized proteins.



Nonionic:

- Tween 20
- Tween 80
- Triton X-100
- Triton X-114
- Emulgens
- Lubrol
- Digitonin
- Octyl glukoside

Zwitterionic:

- Lysoletcithin
- CHAPS
- CHAPSO
- Zwitergents

Ionic:

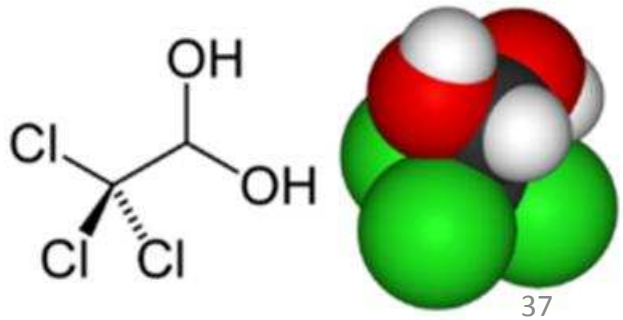
- Cholate
- Deoxycholate
- CTAB
- SDS – dodecyl sulfate

6.4. Extraction of membrane proteins

- 5 mg/ml protein suspension requires at least 1% of detergent.
- All detergents form micelles.
- Micelle clusters range from 30,000 to 100,000.
- Triton X-114 extracts membrane proteins at close to 0°C using 1-3% detergent.
- Choral hydrate in 100% aqueous w/v.

Chloral hydrate is produced from chlorine and ethanol in acidic solution. In basic conditions, the haloform reaction takes place and chloroform is produced.

$$4\ Cl_2 + C_2H_5OH + H_2O \rightarrow Cl_3CCH(OH)_2 + 5\ HCl$$



6.5. Differential detergent fractionation

Differential detergent extraction is an established method for cell fractionation, which partitions subcellular constituents into functionally and structurally distinct compartments.

Differential detergent fractionation (DDF) applicable for fractionation:

- cell grown in suspension
- monolayer culture
- whole tissues
- further subfractionation

DDF preserves the structural and functional integrity of cellular proteins and is useful in a variety of proteome research applications:

- Determine the subcellular localization of biological proteins.
- Semi-purify compartment-specific macromolecules.
- Enrich for low-abundance proteins.
- Investigate dynamic interactions between cytosolic and structural entities (membranes bind the cytoskeleton).
- Monitor treatment-induced compartmental redistributions of macromolecules.

6.5. Differential detergent fractionation

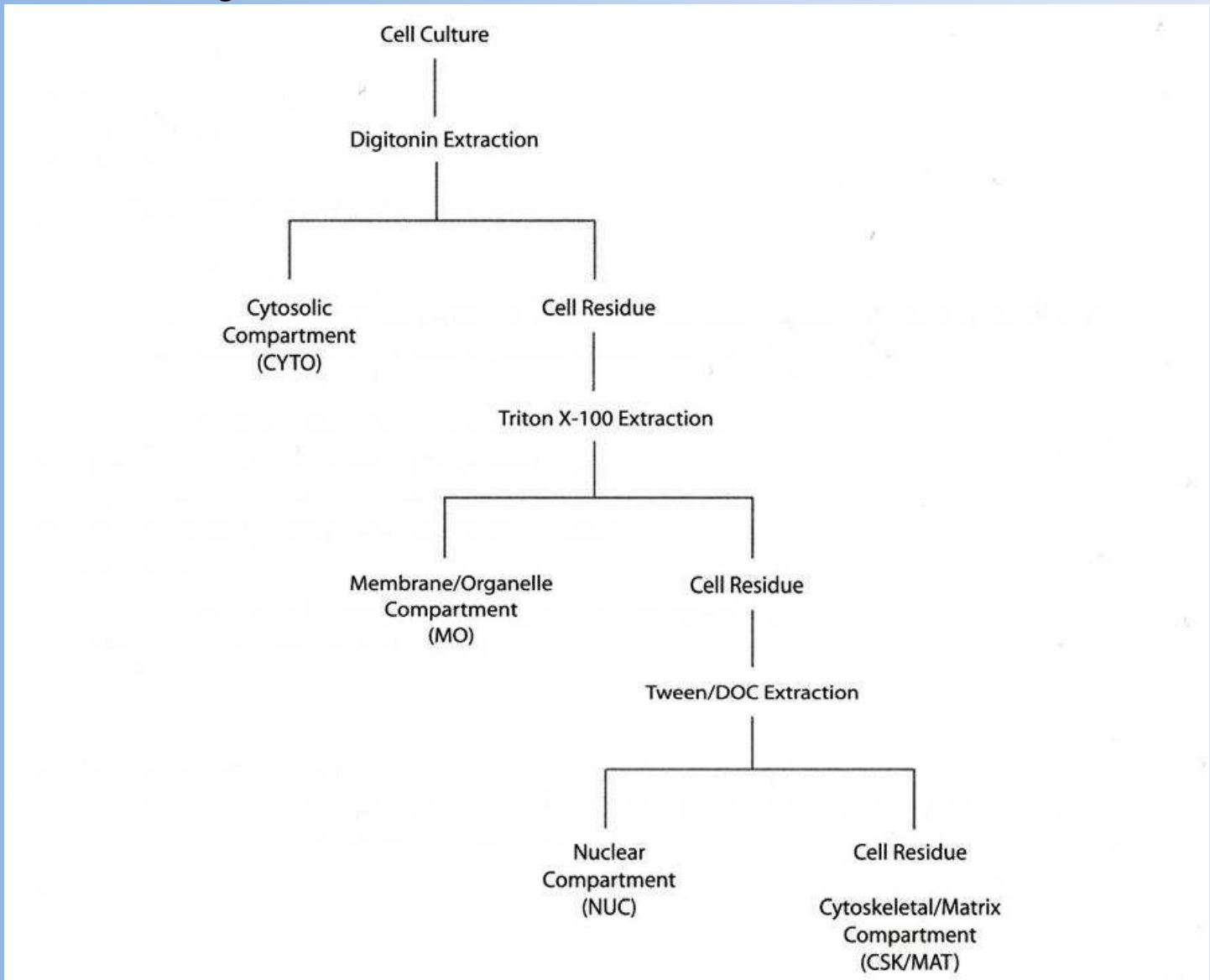


FIGURE 2.2. Schematic for differential detergent fractionation (DDF). (Courtesy of Melinda L. Ramsby and Gregory S. Makowski, University of Connecticut Health Center.)

6.5. Differential detergent fractionation

TABLE 2.6. Protein distribution in detergent fractions of hepatocytes

Cell fraction	Protein distribution in hepatocytes
Digitonin <!>/EDTA extracts (CYTO fraction)	• ~35% total hepatocytic cellular protein • enriched in cytosolic markers (90% LDH activity, 100% carbonic anhydrase <!> immunoreactivity) (Ramsby et al. 1994)
Triton extracts (MO fraction)	• constitutes the bulk of hepatocytic cellular proteins (~50% total protein) • enriched in markers for membrane and organelle proteins (Ramsby et al. 1994)
Tween/DOC (NUC fraction)	• ~5% of total hepatocytic cell protein • contains, exclusively, immunoreactivity for the nuclear protein p38 (Ramsby et al. 1994)
Detergent-resistant fraction (CSK/MAT fraction)	• ~ 7–10% of hepatocytic cellular protein enriched in intermediate filaments, actin, various cytoskeleton-associating proteins, and nuclear matrix • for monolayer cultures, this fraction also contains extracellular matrix (Ramsby et al. 1994)
Magnesium-precipitable fraction (tubulins and microtubule-associated proteins)	• 1–2% of total hepatocytic cellular protein • represents ~4–5% of the protein in digitonin <!>/EDTA extracts • includes tubulins, actin, and proteins presumed to interact with the cytoskeleton

6.6. Preparation of subcellular extracts

The aim of subcellular fractionation is to separate cellular compartments with minimal damage.

- Homogenization of tissues and cells followed by separation of cellular organelles:
  - achieves maximum cell breakage in a reproducible manner,
  - uses disruptive forces that minimize damage to the organelles of interest, and
  - retains the original structure and functional integrity of the organelles of interest.

The principal methods for disrupting cells (osmotic shock, ultrasonic vibration, mechanical grinding or shearing, and nitrogen cavitation):

- Centrifugal methods that separate organelles by size
- Immunoisolation methods that use antibodies
- Electrophoresis methods that separate proteins on the basis of surface charge distribution

6.7. Sample protocols

6.7.1. Lysis of cultured cells for immunoprecipitation

Protocol 2

Lysis of Cultured Cells for Immunoprecipitation

Cell lysis with mild detergent is commonly used with cultured animal cells. If low detergent concentrations are sufficient to cause cell lysis (e.g., 1% NP-40 or 1% Triton X-100), this method may be more gentle to the protein of interest than the homogenization methods discussed in Protocol 1. The choice of detergent must be tailored to the nature of the epitope recognized by the immunoprecipitating antibody. If the antibody recognizes a linear peptide epitope (e.g., a synthetic peptide), then use a harsh denaturing lysis buffer (e.g., RIPA buffer). On the other hand, if the antibody is directed toward a conformational epitope, use NP-40 lysis buffer (or 1% Triton X-100) (for lysis buffer details, see Table 2.4). This protocol was contributed by Hong Ji (Joint ProteomicS Laboratory of the Ludwig Institute for Cancer Research, and Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

6.7. Sample protocols

6.7.1. Lysis of cultured cells for immunoprecipitation

TABLE 2.4. Commonly used lysis buffers for lysing cultured cells

Buffer	Comments
NP-40 lysis 150 mM NaCl 1.0% NP-40 50 mM Tris-Cl (pH 7.4)	Probably the most widely used lysis buffer. It relies on the nonionic detergent NP-40 as the major solubilizing agent, which can be replaced by Triton X-100 <!> with similar results. Variations include lowering the detergent concentration or using alternate detergents such as digitonin <!>, saponin, or CHAPS.
RIPA lysis 150 mM NaCl 1% NP-40 0.5% sodium deoxycholate <!> 0.1% SDS <!> 50 mM Tris-Cl (pH 7.4)	A much harsher denaturing lysis buffer than NP-40, due to the inclusion of two ionic detergents (SDS <!> and sodium deoxycholate <!>). In addition to releasing most proteins from cultured cells, RIPA lysis buffer disrupts most weak noncovalent protein–protein interactions.

6.7. Sample protocols

6.7.1. Lysis of cultured cells for immunoprecipitation

Method 1: Lysing Cells Grown as Monolayer Cultures

1. Discard the culture medium, and wash the cells twice with ice-cold PBS.
2. Place the culture dishes on ice.
3. Add 1.0 ml of lysis buffer (chilled to 4°C) per 100-mm dish. For culture dishes of other sizes, adjust the volume of lysis buffer accordingly.
4. Incubate the cells for 10–30 minutes (dependent on cell lines being studied) on ice with occasional rocking of the dishes.
5. Tilt a dish on the bed of ice and allow the buffer to drain to one side; remove the lysate with a pipette and transfer it to a microfuge tube or other suitable centrifuge tube. Repeat with all of the remaining dishes.

   Although some researchers prefer to scrape the cells from the tissue-culture dish, this does cause some stress to the cells and is only required in unusual cases.
6. Centrifuge the lysate at 20,000*g* for 10 minutes at 4°C.
7. Carefully remove the supernatant to a fresh tube, making sure not to disturb the pellet. Store the lysate on ice until it is needed for the preclearing and immunoprecipitation (see Harlow and Lane 1999).

   The cell lysate can be snap-frozen using a dry ice/ethanol mixture and then stored at –70°C for long-term storage. However, for the analysis of protein complexes by immunoprecipitation, the use of a freshly prepared cell lysate is recommended.

6.7. Sample protocols

6.7.1. Lysis of cultured cells for immunoprecipitation

Method 2: Lysing Cells Grown in Suspension

1. Harvest the cells by centrifugation at 480*g* for 10 minutes. Pour off the supernatant and discard.
2. Carefully wash the cell pellet twice with ice-cold PBS, and then place the washed cell pellet on ice.
3. Resuspend the pellet in 1.0 ml of lysis buffer (chilled to 4°C) per 1×10^7 to 5×10^7 cells.
4. Incubate the cells for 15 minutes on ice with occasional vortexing of the tube.
5. Centrifuge the lysate at 20,000*g* for 10 minutes at 4°C.
6. Carefully remove the supernatant to a fresh tube, making sure not to disturb the pellet. Store the lysate on ice until it is needed for the preclearing and immunoprecipitation (see Harlow and Lane 1999).

   The cell lysate can be snap-frozen using a dry ice/ethanol mixture and then stored at –70°C for long-term storage. However, for the analysis of protein complexes by immunoprecipitation, the use of a freshly prepared cell lysate is recommended.

6.7. Sample protocols

6.7.2. Lysis of cultured cells for immunoblotting

Protocol 3

Lysis of Cultured Animal Cells, Yeast, and Bacteria for Immunoblotting

Cell lysis with detergents is commonly used with cultured animal cells. Typically, the ionic detergent SDS (e.g., 2% SDS) is sufficient for lysing cells for the purpose of immunoblotting studies. Both cultured animal cells and bacteria such as *E. coli* may be lysed in this manner. If the antigenic determinant recognized by the antibody being studied is dependent on the native spatial conformation and sensitive to reducing conditions, then dithiothreitol should be omitted from the lysis buffer and nonreducing/non-urea gels may need to be employed (Ji et al. 1997; Ji and Simpson 1999). This protocol was contributed by Hong Ji (Joint ProteomicS Laboratory of the Ludwig Institute for Cancer Research, and Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

6.7. Sample protocols

6.7.2. Lysis of cultured cells for immunoblotting

1. Add 1 ml of Laemmli sample buffer containing 100 mM DTT to 1×10^7 to 5×10^7 cells.
2. The cell lysate becomes highly viscous in Laemmli buffer due to the presence of released DNA. Two options are available for surmounting this viscosity problem.
   - Centrifuge the lysate at 100,000*g* for 20 minutes to remove DNA.
   - Lyse the cells by sonicating the mixture using four bursts of 15–30 seconds each. Transfer the samples to ice for 15 seconds between each sonication step.
3. Heat the collected supernatant or sonicated sample for 5 minutes at 95°C.
4. Centrifuge at 20,000*g* for 5 minutes.
5. Transfer the supernatant to a fresh tube.
6. Prepare the samples (supernatant) for electrophoresis (see Chapter 3, Protocol 1) and immunoblotting (see Appendix 4). When preparing cell culture extracts for immunoblotting, the protein sample must be
   - in a solution that is compatible with the gel electrophoresis system (e.g., the pH of the solution should be ~7.0 and the salt concentration ~200 mM) and
   - at a protein concentration that does not exceed the loading capacity for a particular gel system (for a discussion of gel electrophoresis variables, see Chapter 3). As a rule of thumb, for a conventional gel, do not load >150 µg of total protein per lane for a minigel.

6.7. Sample protocols

6.7.3. Disruption of cultured cells by nitrogen cavitation

Protocol 4

Disruption of Cultured Cells by Nitrogen Cavitation

Cell disruption by nitrogen decompression from a pressurized vessel is a rapid and effective way to homogenize cells and tissues, to release intact organelles, and to prepare cell membranes (Hunter and Commerford 1961). The principle of the method is simple: Cells are placed in a pressure vessel, and large quantities of oxygen-free nitrogen are first dissolved in the cells under high pressure (~5500 kPa, which is equivalent to 800 psi). When the gas pressure is suddenly released, the nitrogen comes out of solution as bubbles that expand and stretch the cell membrane, rupturing it, and releasing the contents of the cell. Nitrogen cavitation is well suited for mammalian and plant cells and fragile bacteria (i.e., bacteria treated to weaken the cell wall), but it is less effective at lysing yeast, other fungi, spores, or other cell types with tough cell walls. Features of the nitrogen cavitation method include the following:

6.7. Sample protocols

6.7.3. Disruption of cultured cells by nitrogen cavitation

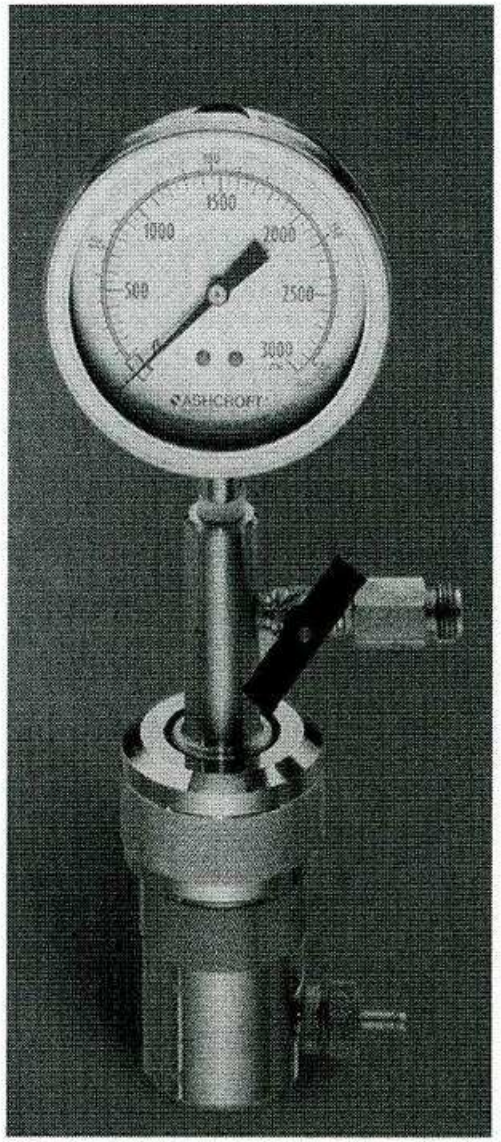


FIGURE 2.1. Nitrogen cavitation vessel. The image shown is the Parr Instrument Company Model 4639 Cell Disruption Vessel, designed specifically for small samples from 30 ml down to less than one milliter. (Parr Instrument Company 4636 and 4639 Cell Disruption Vessels.)

6.7. Sample protocols

6.7.3. Disruption of cultured cells by nitrogen cavitation

Cell type	Cell suspension	psi	No. cells used/ml	No. times through	No. cells remaining/ml	No. nuclei remaining/ml	% cells totally lysed
Cultured cells	KB	500	3.4×10^6	1	2.8×10^5	n.d.	93
	KB	500		2	2.0×10^3	n.d.	99.95
	KB	250	3.3×10^6	1	5.5×10^5	4.62×10^5	69.4
	KB	250		2	5.4×10^4	2.2×10^4	97.7
	KB	250		3	0	0	100
	KB	0	1.8×10^6	0	1.08×10^6	7.6×10^5	–
	KB	250		1	2.4×10^5	6.4×10^5	52.2
	KB	250		2	3.4×10^4	5.0×10^4	95.4
	KB	250		3	1.6×10^4	4.6×10^4	96.6
Tissue	rat liver	500	9.2×10^5	1	8.5×10^5	n.d.	7.8
		500		2	0		100
Blood	chicken red blood cells	1000	5.6×10^9	1	9.6×10^8	n.d.	83
Bacteria	*E. coli*	1500	1.3×10^{10}	1	1.2×10^{10}	n.d.	8
		1500		2	6.6×10^9	n.d.	50
	E. coli	1500	6.5×10^8	1	3.6×10^8	n.d.	45
		1500		2	3.3×10^8	n.d.	50

These values were determined with the Mini-Bomb cell disruption chamber. (KB cells) Human oral epidermoid carcinoma. n.d. indicates not determined. (Modified from Kimble/Kontes [http://208.72.236.210/html/pg-881455.html].)