PROTEIN EXTRACTION AND PURIFICATION

I: Materials

Animal tissue/cell, Microorganisms and Plant tissue/cell. However, plant tissue/cell is unique in that it contains:

1) lignin
2) pigments, terpenes
3) polyphenols: known as flavonoids and tannins; can react with proteins by hydrogen bonding with peptide bond oxygens, or by covalent modification of amino acid residues such as -OH, thiols, and primary amines.

II: Techniques of cell disintegration:

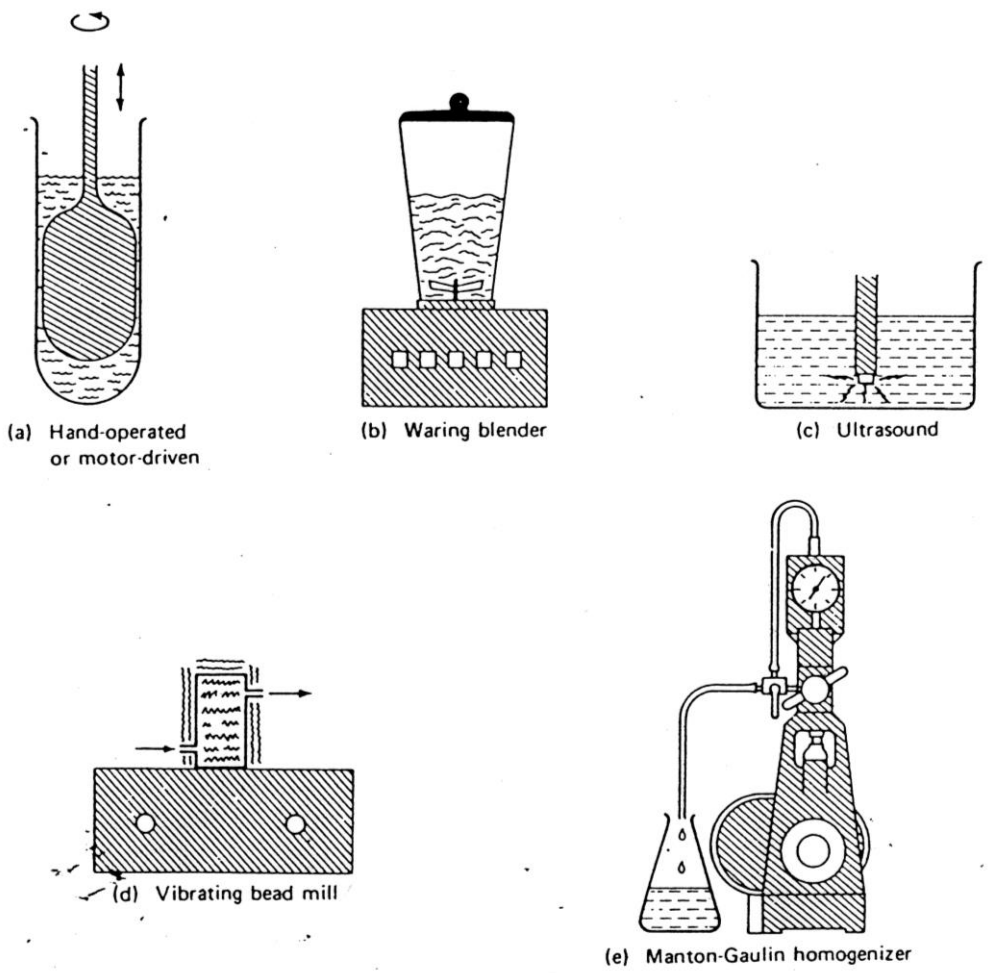


Figure 1.2. Equipment used for breaking up cells to obtain an extract: (a) hand-operated or motor-driven glass homogenizer, (b) Waring blade-blender (food processor), (c) ultrasonic probe, (d) vibrating glass bead mill, (e) Manton–Gaulin cell disintegrator.

Techniques of cell disintegration

Table 1.1. Cell Disintegration Techniques

Technique	Example	Principle
Gentle		
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 off cell membrane
Mincing (grinding)	Muscle etc.	Cells 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	Micro-scale 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

III. Removal of interferring substances

1. **Salts:** In most cases, salts do not interfere by a strong binding to the protein, but rather by, disturbing the electrophoresis process.

***methods to remove salts:**

1) **dialysis,** a general method to remove salts from halophilic organisms and in some biological fluids (urine, sweat, and spinal fluid).

Dialysis:

*Proteins can be separated from small molecules by dialysis through a semi-permeable membrane.

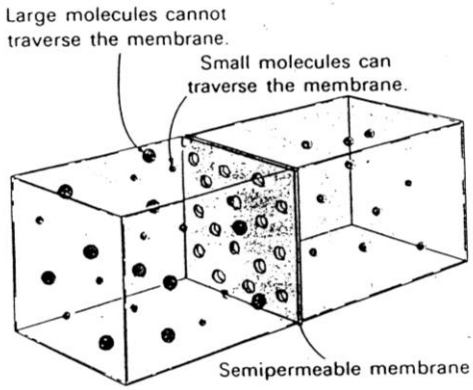


Figure 2-21
Separation of molecules on the basis of size by dialysis.

PREPARATION OF DIALYSIS TUBING

1. Cut the tubing into pieces of convenient length (10–20 cm).
2. Boil for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH 8.0).
3. Rinse the tubing thoroughly in distilled water.
4. Boil for 10 minutes in 1 mM EDTA (pH 8.0).
5. Allow the tubing to cool, and then store it at 4°C. Be sure that the tubing is always submerged. From this point onward, always handle the tubing with gloves.
6. Before use, wash the tubing inside and out with distilled water.

Note

Instead of boiling for 10 minutes in 1 mM EDTA (pH 8.0) (step 4), the tubing can be autoclaved at 20 lb/sq. in. for 10 minutes on liquid cycle in a loosely capped jar filled with water.

2) **precipitation** with 10% TCA in water and resolubilization in a medium convenient for electrophoresis.

3) **chelators** to remove calcium from calcium-binding proteins.

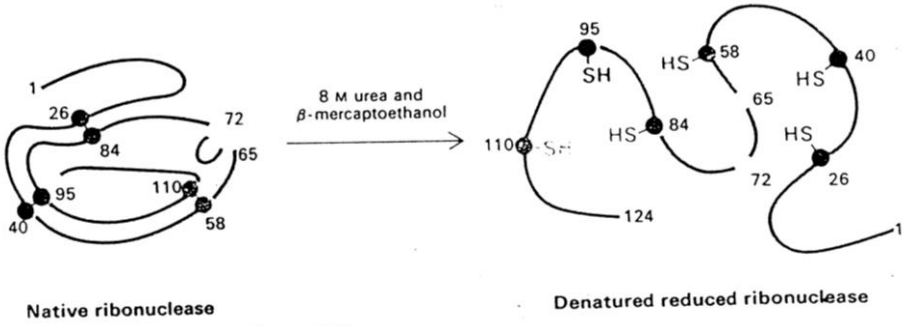


Figure 2-45
Reduction and denaturation of ribonuclease.

2. Lipids: Lipids give problems because of their supramolecular structure and not easily solubilize in aqueous solution.

***methods to remove lipids:**

1) As a general rule, the presence of **detergents** in the extraction buffer is an efficient way to disrupt the membranes and solubilize the lipids.
2) For high lipid levels, delipidation is achieved with **organic solvents**. For examples, **ethanol** or **acetone**, often provides a partial but useful delipidation.

*A severe loss of proteins may be experienced, either because some proteins are soluble in organic solvents, or perhaps because the precipitated proteins do not resolubilize.

***Special attention must be paid to the final removal of the organic solvents prior to solubilization.** If the solvent is not efficiently removed, emulsion problems or precipitation by the remaining solvent may arise. If the precipitated protein pellet is dried too extensively in order to remove the solvent completely, a tight and dry pellet appears with extremely severe losses even in media of high denaturing and solubilizing power.

3. Nucleic acids: Several types of problems are encountered because of nucleic acids.

1) Nucleic acids behave as polyanions and are therefore able to bind many proteins through electrostatic interactions resulting in completely artifactual results with severe streaking.
2) When separation with isoelectric focusing is to be performed, nucleic acids bind carrier **ampholytes** to give complexes.
3) Nucleic acids are very long molecules that are able to increase the viscosity of the solution considerably and also to clog the small pores of the acrylamide gels used to separate proteins.

***methods to remove nucleic acids:**

1) Nuclease digestion is initially used by a mixture of RNAses and DNAses, followed by TCA precipitation.
2) Extraction pH can be increased (with the addition of a basic polyamine, e.g. spermine) so that all the proteins will behave as anions and will be repelled from the anionic nucleic acids.
3) Phenol/ammonium acetate precipitation followed by resolubilization of the protein pellet.

4. Polysaccharides: Polysaccharides (starch, glycogen, etc.) pose problems only because they are huge molecules, which could clog polyacrylamide gels.

***methods to remove polysaccharides:**

1) Ultracentrifugation. The same methods used for preventing protein-nucleic acids interactions can also be used (solubilization in SDS or presence of organic polycations such as spermine or high pH).
2) Selective precipitation of the proteins with TCA, ammonium sulfate, phenol/ ammonium acetate followed by resolubilization of the protein pellet may be solutions.

5. Other Compounds: Apart from these major classes of interfering compounds, other interferring substances can be found, mainly in extracts from plants. These include lignins, polyphenols, tannins, alkaloids, pigments, terpenes, and organic acids.

***methods to remove these compounds**

1) precipitation of proteins with organic solvents
2) with the addition of **polyvinylpyrrolidone** (**PVP**) that can trap polyphenols.

III. Components in the Buffer Solution

1. Buffers:

*The physiological pH in most cells is 7.0-7.5 at 37℃. Most biological happenings occur in the pH range, 6-8, yet many commonly used buffers suffer from serious drawbacks in this range.

*Certain buffers have their limitations:

Tris: 1) is a poor buffer below pH 7.0
2) possesses a potentially reactive primary amine
3) is affected by buffer concentration and temperature.

Phosphate: 1) is a feeble buffer in the pH range 8-11
2) precipitates or binds many polyvalent cations such as Ca^{++}, Mg^{++}
3) inhibits a large variety of enzymes, including kinases, DH, phosphatases ....etc.

***Design an appropriate buffer according to the following criteria:**

1) pKa between 6-8: prepare a buffer very near the working pH.
2) high solubility in aqueous systems
3) minimal salt effects
4) minimal effects on dissociation due to concentration, temperature and ionic composition. Ideally, the dissolution constant of the buffer system should not shift as **concentration** changes. If the shift in the dissociation of a buffer material is small, a **stock solution** can be convinently prepared and diluted as required without readjustment of the pH. e.g. when diluted to 0.05 M, the pH of a 0.5 M

phosphate buffer at pH 6.6 rises to 6.9. Another 10-fold dilution increases the pH to 7.1.

*Tris is particularly notorious for its large dissociation constant shift with **concentration** and **temperature**. Tris decreases approximately 0.1 pH unit per 10-fold dilution. So does temperature. If you happen to use pH 7.0 Tris, made up in the cold room at 4℃, when you incubate at 37℃, the pH will be dropped 1.05 units to 5.95.

5) chemical stability: The buffer should be stable under working conditions to oxidation and light and should be unaffected by the biochemical system.

6) insignificant light adsorption between A_{240} and $A_{700.}$ Buffer components which will not adsorb UV or visible light are convenient in photometric experiments.

7) easily available in pure form

2. chelators.

*chelators: the most commonly used chelating agents is EDTA (ethylenediamine tetraacetic acid). The concentration of EDTA ranges from 0.1-5 mM.

3. reducing agents

1) **DTT** (dithiothreitol) may be used at 0.5-1.0 mM, and oxidation results in the formation of a stable intramolecular disulfide which does not endanger protein sulfhydryls.

2) **β-mercaptoethanol** must be used at a concentration of 5-20 mM. Within 24 h of its introduction into the buffer, β-mercaptoethanol becomes oxidized, after which it may accelerate protein inactivation.

4. detergents

1) **ionic detergents:** It contains + or – charged head groups. **SDS** or LiDS (sodium or lithium dodecyl sulfate); SDS is a strong denaturant.

2) **nonionic detergents:** It contains uncharged hydrophilic head groups. **Triton X-100** (polyoxyethylene [9-10} *p-t*-octyl phenol), **Nonidet P-40** (polyoxyethylene [9] *p-t*-octyl phenol), **Tween 20** (PEG [20] sorbitan monolaurate).

*NP-40 exhibits weaker absorbance than TritonX-100 at A_{280}. Therefore, it is a better detergent used in protein isolation.

*Ionic and nonionic detergents are good solubilizing agents that increase the solubility of hydrophobic proteins but are not efficient to breaking protein-protein interactions. They are often used in combination with **urea**.

3) **zwitterionic detergents:** It contains both head groups that possess + and – charges. **CHAPS** (3-[(Cholamidopropyl)dimethyl-ammonio]- 1-propanesulfonate). It has the

same properties as Triton X-100 but is much more effective in breaking protein-protein interactions.

Table 1. Detergent properties.

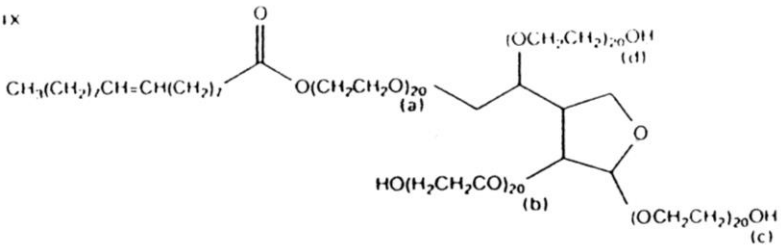
Detergent	*Charge* character	*Mol. wt (monomer)*	*CMC (% w/v)*	*Mol. wt micelle*	*Readily dialysable*	*Comments*	*Source*[a]
Digitonin	Non-ionic	1229		70 000	–	Solubility increased by warming	SCW
α-D-glucopyranoside, octyl	Non-ionic	292	0.29		+	Pure preparations (e.g. Wako) exhibit best solubility and solubilizing properties	
β-D-Glucopyranoside	Non-ionic					Hexyl, heptyl and nonyl glucosides also available	
e.g. *n*-Decyl		320	0.07		–		SC
n-Dodecyl		348	0.0066		–		SC
n-Octyl		292	0.73	8000	+		ACFS
Octyl-β-D-thioglucopyranoside	Non-ionic	308	0.28		+	May be superior to glucosides in some respects	SC
β-D-Maltoside, dodecyl	Non-ionic	511	0.08	50 000	–		SC
N-Methyl glucamides							
Heptanoyl (MEGA-7)		307			+	Mild, non-denaturing.	CS
Octanoyl (MEGA-8)		321			+	other derivatives can be readily produced	CS
Nonanoyl (MEGA-9)		335	0.80		+		CS
Decanoyl (MEGA-10)		349	0.80		+		CS
Polyoxyethylene alcohols	Non-ionic						
e.g. $C_{11}E_8$		542	0.005	65 000	–	The Brij series also fall into this category and have	SC
LuBrol PX		582	0.006	64 000	–	broadly similar characteristics. So too do the emulphogens which are based on the isoalcohols	SC
Polyoxyethylene octyl phenols	Non-ionic						
e.g. Nonidet P-40		603	0.023		–	Polyoxyethylenes prone to oxidation, especially if	
Triton X-100		625	0.015	90 000	–	heavy metal ions are present. Protect with 0.2 mole butylated hydroxytoluene. Absorb strongly at 280 nm, but non-absorbing reduced versions are available from Aldrich	ACFS
Polyoxyethylene sorbitol esters	Non-ionic						
e.g. Tween 80		1310	0.0015	76 000	–	Tweens 20, 40 and 60 have CMCs of 0.006, 0.003 and 0.003 (% w/v) respectively	AFS
CHAPS	Zwitterionic	615	0.49	6150	+	Stored desiccated at RT or 4°C since hygroscopic	ACFS
CHAPSO	Zwitterionic	631	0.50	6310	+		
Deoxy BIGCHAP	Zwitterionic	878	0.25	8800	+	Reduced interference in ion-exchange chromatography	CS
C16 Lyso PC	Zwitterionic	495	0.0004	92 000	–		CFS
Zwittergent 3-14	Zwitterionic	364	0.011	30 110	–	Range of compounds 3-6 to 3-16 of increasing hydrophobic character. Part of class known as sulphobetaines	C
Cholate, Na	Anionic	430	0.60	4300	+	Also available as the glyco- and tauro-cholates	ACFS
1-Decanesulphonate, Na	Anionic	244	0.80		+	Also pentane and octane sulphonates	S
Deoxycholate, Na	Anionic	414	0.21	4200	+	Also available as the glyco- and tauro-cholates	ACFS
Dodecyl sulphate, Na (SDS).	Anionic	288	0.23	18 000	partly	K salt insoluble. Strong denaturant but non-denaturing ether sulphates can be synthesized (42)	ACFS
Cetylpyridinium Cl	Cationic	358	0.25		+		S
Trimethylammonium bromides	Cationic					Form insoluble complexes with SDS	
e.g. Cetyl (CTAB)	Cationic	365	0.04	62 000	–	Not readily soluble at 5% w/v in H_2O	AFS
Dodecyl (DTAB)	Cationic	308	0.43		+	at room temperature. Strong denaturant	SE
Myristyl (MTAB)	Cationic	336	0.15	19 000	partly		S

[a]A, Aldrich; C, Calbiochem; E, Eastman Kodak; F, Fluka; S, Sigma; W, Wako. Catalogues often contain useful lists of references.

Triton X-100
PEG (9 – 10) p-t-octyl phenol
Nonidet P40

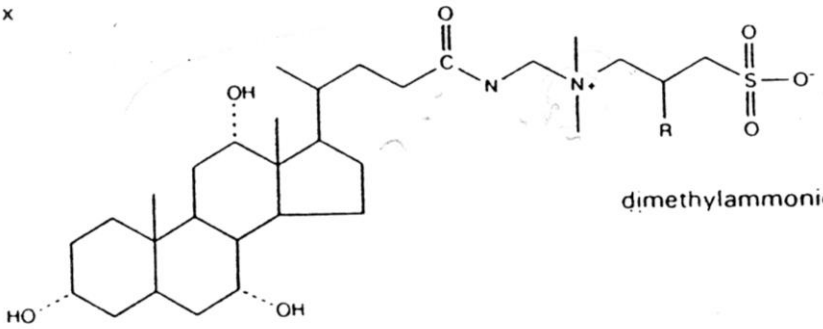
Triton N series
(polyoxyethylene nonyl phenols)

IX



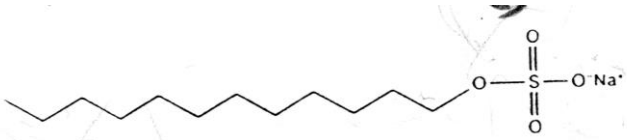
Tween series
a + b + c + d = number in series, here shown as 80

X



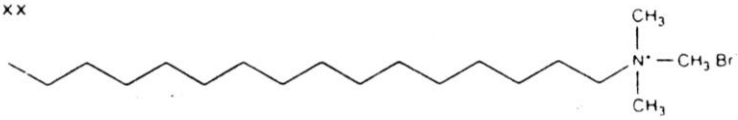
CHAPS (R = H)
3[(3-Cholamidopropyl) dimethylammonio]-1-propane sulphonate

XVII



SDS
Sodium dodecyl sulphate
Sodium lauroyl sulphate

XX



$C_{16}TAB$ Hexadecyl trimethylammonium bromide Cetrimide

5. protease inhibitors: protease inhibitors include

1) irreversible inhibitors:

   PMSF, The most popular one. It inhibits efficiently the **wide range** of serine proteases generally present in a cell or a tissue.

   TLCK, TPCK. These inhibitors are very potent but also **very specific**, so that they do not inhibit efficiently the wide range of serine proteases.

2) reversible inhibitors:

   many of these inhibitors are short peptides (**pepstatin, antipain, leupeptin**) or small molecules (b**enzamidine**).

   *More frequently, two or more inhibitors are used instead of one.

3) For plant samples which are very rich in proteases, solubilization of the sample in **boiling SDS** or homogenization in TCA/acetone has been proposed.

Proteases and protease inhibitors

A. Endopeptidases

1. Serine proteases

They bring about hydrolysis of a peptide bond by attack upon the **carbonyl carbon** by a **nucleophilic serine residue** in the active site of the enzyme. The active site **serine** and **histidine** residues are both effective targets for inhibitors (Table 8).

acetylcholinesterase

• Phenylmethanesulphonyl fluoride

Synonyms	PMSF
Mol. wt	174
• Effective concentration	0.1 – 1 mM
Stock/solvent	20 – 50 mM in dry solvents (propan-2-ol, MeOH, EtOH)
Stability of stock solution	At least 9 months at 4°C
Notes	Not as effective or toxic as DipF. Inhibits cysteine proteases (reversible by reduced thiols). Active towards all serine proteases

L-1-Chloro-3-[4-tosylamido]-7-amino-2-heptanone · HCl

Synonyms	TLCK, Tosyl lysyl chloromethyl ketone, Tos Lys CH_2Cl
Mol. wt	369.4 (hydrochloride)
Effective concentration	10 – 100 μM
Stock/solvent	10 mM in aqueous solution (pH 3.0 – 6.0)
Stability of stock solution	Prepare fresh as needed
Notes	Active towards some trypsin-like serine proteases

L-1-Chloro-3-[4-tosylamido]-4-phenyl-2-butanone

Synonyms	TPCK, Tosyl phenylalanyl chloromethyl ketone, Tos Phe CH_2Cl
Mol. wt	351.9
Effective concentration	10 – 100 μM
Stock/solvent	10 mM in methanol or ethanol
Stability of stock solution	Several months at 4°C
Notes	Active towards some chymotrypsin-like serine proteases

Leupeptin

Synonyms	*N*-Acetyl-Leu-Leu-Arg-al
Mol. wt	542.7 (hemisulphate, monohydrate)
Effective concentration	1 – 100 μM
Stock/solvent	10 mM in water or buffer
Stability of stock solution	1 week at 4°C, 1 month at −20°C
Notes	Leupeptin is an amino acid aldehyde the aldehyde being contributed by an arginine residue It inhibits trypsin-like serine proteases and most cysteine proteases

Antipain

Synonyms	[(S)-1-carboxy-2-phenylethyl]-carbamoyl-Arg Val-Arg al
Mol. wt	604.7
Effective concentration	1 – 100 μM
Stock/solvent	10 mM in water or buffer
Stability of stock solution	1 week at 4°C, 1 month at −20°C
Notes	Antipain is an amino acid aldehyde, the aldehyde being contributed by an arginine residue It has a similar specificity to leupeptin

Chymostatin

Synonyms	Phe-(Cap)-Leu-Phe-al
Mol. wt	582.7
Effective concentration	10 – 100 μM
Stock/solvent	10 mM in DMSO
Stability of stock solution	Stable for months at −20°C
Notes	Chymostatin is an amino acid aldehyde the aldehyde being contributed by a phenylalanine residue It inhibits chymotrypsin like serine proteases and most cysteine proteases

2. Cysteine proteases

Their activities depend upon the **nucleophilic cysteine residue** that is a target for inhibitors (Table 9). E-64 is an **epoxide** that seems to be highly specific for the **nucleophilic cysteine residue**. Others such as **iodoacetic acid** and **iodoacetamide** are less specific and can inactivate many enzymes that involve a cysteine in the active site.

Table 9. Inhibitors of cysteine proteases.

Iodoacetate

Synonyms	IAA
Mol. wt	208.0 (Sodium salt)
Effective concentration	10 – 50 μM
Stock/solvent	10 – 100 mM in water
Stability of stock solution	Decomposes slowly; prepare fresh
Notes	IAA is not specific for the active site cysteine residue of serine proteases and can inhibit many other proteins and enzymes

3. Aspartic proteases

They employ **a pair of aspartic residues** to labilize and hydrolyse the scissile peptide bond. The most effective inhibitor of aspartic endopeptidase is the tight binding transition state analogue **pepstatin** (Table 10A).

Table 10. Inhibitors of aspartic and metallo-proteases

A. *Aspartic proteinases* (acid protease)

Pepstatin

Synonyms	Pepstatin A
Mol. wt	685.9
Effective concentration	1 μM
Stock/solvent	1 mM in methanol
Stability of stock solution	Stable for months at −20°C
Notes	Pepstatin is a transition state analogue that is a potent inhibitor of cathepsin D, pepsin, renin and many microbial aspartic proteases

4. Metallo-proteases

Their activities are dependent on the **electron-withdrawing ability of a metal ion** (to date, exclusively **zinc**) to labilize the peptide bond in the substrate. Metallo-protease inhibitors direct an **electronegative moiety** to the vicinity of the **metal ion** and are tight binding but are **reversible inhibitors** (Table 10 B). Simple cheators, such as **EDTA or 1, 10-phenanthroline** are also effective inhibitors, but might be unfavorable as components of purification buffer.

B. *Metallo-proteases*

EDTA

Synonyms	None
Mol. wt	372.24 (disodium salt, dihydrate)
Effective concentration	1 mM
Stock/solvent	0.5 M in water, pH 8.5
Stability of stock solution	Stable for months at 4°C
Notes	EDTA acts as a chelator of the active site zinc ion in metallo-proteases but can also inhibit other metal ion-dependent proteases such as the calcium-dependent cysteine proteases. EDTA may interfere with other metal-dependent biological processes

IV. Extraction principles

1. Should obtain the **best yield** and the **highest quality**. However, maintaining quality is always superior to obtaining yield.
2. Many ways have been developed for avoiding protein degradation (quality maintenance)
   1) keeping work at **low temperature**.
   2) add **protease inhibitors** in the extraction buffer:PMSF, leupeptin, chymoststine, TPCK (N-tosyl-L-phenylalanine chloromethyl ketone), TLCK (N-tosyl-L-lysin chloromethyl ketone)
   3) add **chelating reagents** in the extraction buffer: EDTA

4) add **reducing agents** in the extraction buffer: DTT, β-mercaptoethanol
5) add **denaturants** in the extraction buffer: SDS, Triton X-100, NP-40, urea
6) others: **antioxidants** such as ascorbic acid, **PVP** (polyvinylpyrrolidone)

V. Extraction Methods

1. **Tris-buffer extraction (nondenaturing)**
   The extraction buffer contains 30 mM Tris-HCI, pH 8.7, 1 mM DTT, 1 mM EDTA, 1 mM ascorbic acid, 5 mM $MgCl_2$, 10 mg PVP
2. **SDS extraction**
   The extraction buffer contains 4% SDS, 5% β-mercaptoethanol, 5% sucrose, and 10 mg insoluble PVP.
3. **TCA-acetone extraction**
   The extraction buffer contains 10% TCA, 0.07% β-mercaptoethanol in cold acetone.
4. **Phenol extraction**
   This method allows the removal of polysaccharides, phenolic compounds, and nucleic acids.

VI. Protein precipitation and concentration

1. precipation by pH alteration

a. pH > pI the protein surface is predominantly negatively charged
pH < pI the overall charge on protein surface is positive

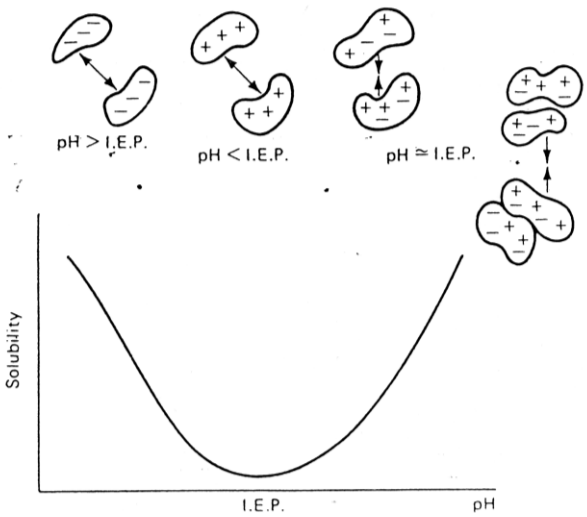


Figure 3.3. Solubility of a globulin-type protein close to its isoelectric point (IEP).

b.
Isoelectric precipitation is often used to precipitate unwanted protein, rather than to precipitate the protein of interest.

2. precipitation by increasing the ionic strength (salting out)

1) When high concentrations of salt are present, proteins tend to aggregate and precipitate out of solution. This technique is referred to as "salting out". Factors such as pH, temperature and protein purity play important roles in determining the salting out point of a particular protein.
2) Salting out is dependent on the hydrophobic nature of the surface of the protein. The hydrophobic patches consist of the side chains of Phe, Tyr, Trp, Met, Leu, Ile, Val. Protein with large or more hydrophobic patches will aggregate and precipitate before those with smaller and fewer patches, thus resulting in **fractionation**.
3) Salting out is usually performed at 4℃ to decrease the risk of inactivation.
4) The effectiveness of the salt for salting-out is mainly determined by the nature of anion, multi-charged anions being the most effective; the order of effectiveness is phosphate> sulfate>acetate>chloride: monovalent cations are the most effective with NH_4^+>K^+>Na^+.
5) Ammonium sulfate is the salt of choice because it combines many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price.
6) Few proteins precipitate below 24% ammonium sulfate while most do by 80% of ammonium sulfate. Frequently, ammonium sulfate precipitation results in removal of RNA and DNA.
7) Ammonium sulfate precipitation is often a good way of stabilizing proteins for storage.

TABLE I
FINAL CONCENTRATION OF AMMONIUM SULFATE: PERCENTAGE SATURATION AT 0°[a]

Initial concentration of ammonium sulfate (percentage saturation at 0°)	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

[a] Adapted from "Data for Biochemical Research" (R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones, eds.), 2nd Ed. Oxford Univ. Press, London, 1969. Similar tables prepared for 25° and 20° are found in Ref. 2 (p. 76) and 10 (p. 303), respectively. Saturated solutions of ammonium sulfate are 3.90, 4.04, and 4.10 *M* at 0°, 20°, and 25°, respectively. Appendix A of Ref. 10 also presents a table for the liquid addition of miscible organic solvents or of a saturated solution of ammonium sulfate to raise an initial concentration (%, v/v) to a given higher concentration.

3. precipitation with organic solvents

1) organic solvents cause precipitation of proteins largely by changing the solvation of the protein with water.
2) common used organic solvents include acetone, trichloroacetic acid (TCA), ethanol, and ammonium acetate. Protein precipitation can be best performed in 80% rather than 100% acetone since phospholipids are soluble only in aqueous acetone. Similarly, phenolic compounds are also best removed using aqueous acetone
3) keep sample concentration at a range of 5-30 mg/ml and salt concentration at 0.05-0.2 M. If the salt concentration is too high, electrostatic aggregration will be impaired, high levels of organic solvents are required, and denaturation is more likely. On the other hand, at a very low salt concentration, a very fine precipitate may be formed that can be difficult to sediment.
4) most proteins larger than 15 kDa precipitate with 50% organic solvent.

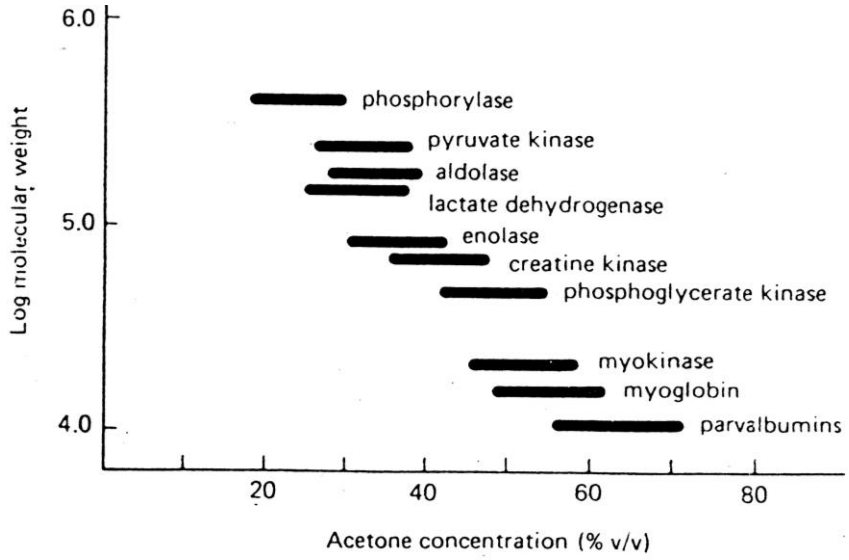


Figure 3.10. Approximate precipitation ranges in acetone at 0°C, pH 6.5, $I = 0.1$, of some proteins found in muscle tissue extracts. [From Scopes (29).]

4. Precipitation with PEG

1) PEG is a nonionic water-soluble polymer, cause little protein denaturation, while inducing precipitation at a discrete PEG concentration for a given protein. This property, along with its low heat of solution and short equlibration time for precipitation, makes it a useful reagent for protein fractionation.
2) Maximum protein precipitation is generally achieved with a final PEG concentration of 30%.

6. Ultrafiltration

1) Concentration of protein by ultrafiltration. proceeds by forcing the liquid in a protein solution through a membrane which retains the protein of interest. The Centricon Microconcentrator has a starting capacity of 2 ml. A 2-ml sample may be concentrated to less than 50 µ1 in a 30 min centrifugation.
2) At present, two models are available, Centricon-10 and Centricon-30, with 10 kDa and 30 kDa average molecular weight cutoffs. The protein of interest should be 30-50 larger than the stated cutoff to ensure that it will be retained. The recovery of proteins is typically greater than 90%.

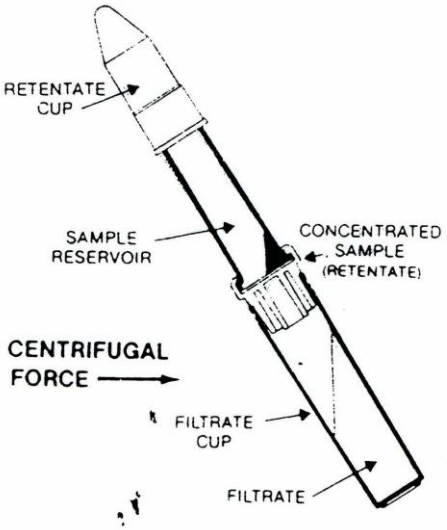


Figure 4.1. Concentration of a protein solution using the Amicon Centricon system.

VII. Determination of Protein Concentration

* **Bradford method** is the most widely used method.

Table 1. Reagents Compatible with the Bio-Rad Protein Assay When Using the Standard Procedure.*

Acetate, 0.6M	KCl, 1.0M
Acetone	Malic Acid, 0.2M
Adenosine, 1 mM	$MgCl_2$, 1.0M
Amino Acids	Mercaptoethanol, 1.0M
Ammonium Sulfate, 1.0M	MES, 0.7M
Ampholytes, 0.5%	Methanol
Acid pH	MOPS, 0.2M
ATP, 1 mM	NaCl, 5M
Barbital	NAD, 1 mM
BES, 2.5M	NaSCN, 3M
Boric Acid	Peptones
Cacodylate-Tris, 0.1M	Phenol, 5%
CDTA, 0.05M	Phosphate, 1.0M
Citrate, 0.05M	PIPES, 0.5M
Deoxycholate, 0.25%	Polyadenylic Acid, 1 mM
Dithiothreitol, 1M	Polypeptides (MW<3000)
DNA, 1 mg/ml	Pyrophosphate, 0.2M
EDTA, 0.1M	rRNA, 0.25 mg/ml
EGTA, 0.05M	tRNA, 0.4 mg/ml
Ethanol	total RNA, 0.30 mg/ml
Eagle's MEM	SDS, 0.1%
Earle's Salt Solution	Sodium Phosphate
Formic Acid, 1.0M	Streptomycin Sulfate, 20%
Fructose	Triton X-100, 0.1%
Glucose	Tricine
Glutathione	Tyrosine, 1 mM
Glycerol, 99%	Thymidine, 1 mM
Glycine, 0.1M	Tris, 2.0M
Guanidine-HCl	Urea, 6M
Hanks' Salt Solution	Vitamins
HEPES Buffer, 0.1M	

*Interference may be caused by chemical-protein and/or chemical-dye interactions. Table 1 lists those chemical reagents not directly affecting the development of dye color. Since every protein-chemical reagent combination has not been assayed, it is possible that some of the listed reagents produce interference in combination with certain proteins. However, with respect to proteins such as bovine albumin and globulin, the above listed reagents show little or no interference.

The concentrations of reagents shown above are for the standard assay procedure (see page 8). Equivalent concentrations of reagents compatible with the Microassay procedure (page 9) are 1/40 of those listed above. For example, 5M NaCl in a sample assayed by the Standard Assay is equivalent to 0.125M NaCl in a sample assayed by the Microassay. This is due to the differences of sample-to-dye ratios between the Standard and Microassay procedures.

Table 2. Comparison of the Bio-Rad Protein Assay with Four Commonly Used Methods.

Method and Sensitivity	Chemical Interference	Protein/Protein Variation	Technique: Speed Complexity
Bio-Rad (Bradford) 1 μg	Slight	Significant	Rapid Simple (one reagent)
Lowry 1 μg	Great	Significant	Moderate Moderate
Biuret 100 μg	Moderate	Low	Moderate Simple
Kjeldahl 1 μg	Moderate	Low	Slow Complex
Absorbance[a] 10 μg	Moderate	Significant	Rapid Simple

a. At 280 nm.

Comparison of Bio-Rad, Lowry and Biuret Protein Assays

In addition to the high specificity for protein, the assay may display significant protein-to-protein variation. This variation is also observed in the Lowry and biuret procedures. For comparison purposes, Bio-Rad, Lowry and biuret assays were performed on 23 different commercially obtained proteins. The Bio-Rad Protein Standard I (bovine gamma globulin) was used for the Lowry and Bio-Rad assays, with bovine serum albumin being used for the biuret method. The results of this study are recorded in Table 3. All three assays displayed considerable variation in response to different proteins, but the averages were comparable. The data from this comparison study show that the substitution of the Bio-Rad Protein Assay for the biuret or Lowry assays will yield comparable results in most cases.

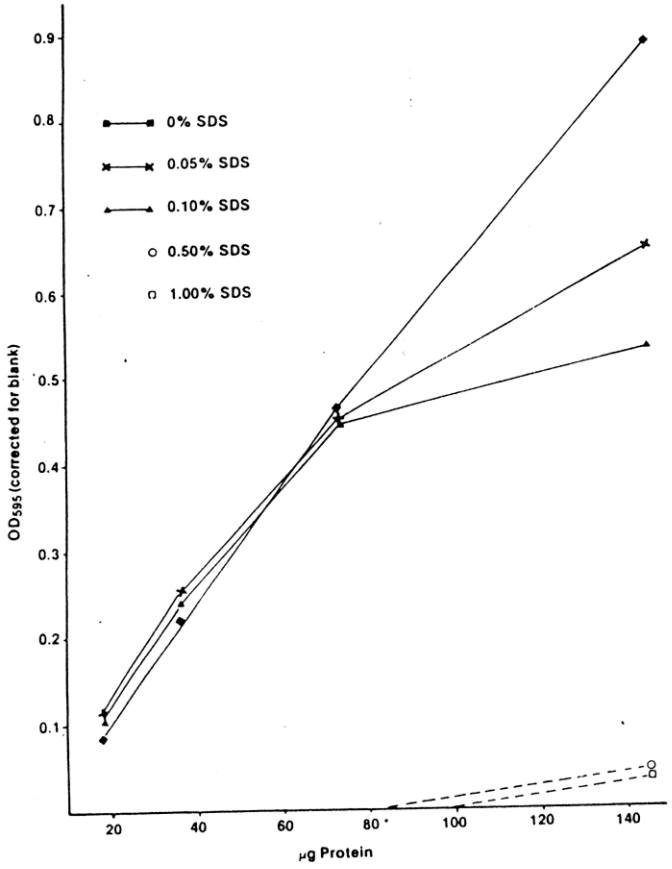


Fig. 3. The effect of various levels of SDS on the Bio-Rad Protein Assay. Each standard curve was obtained by using Protein Standard I (bovine gamma globulin) in the Standard Procedure (20-140 μg).

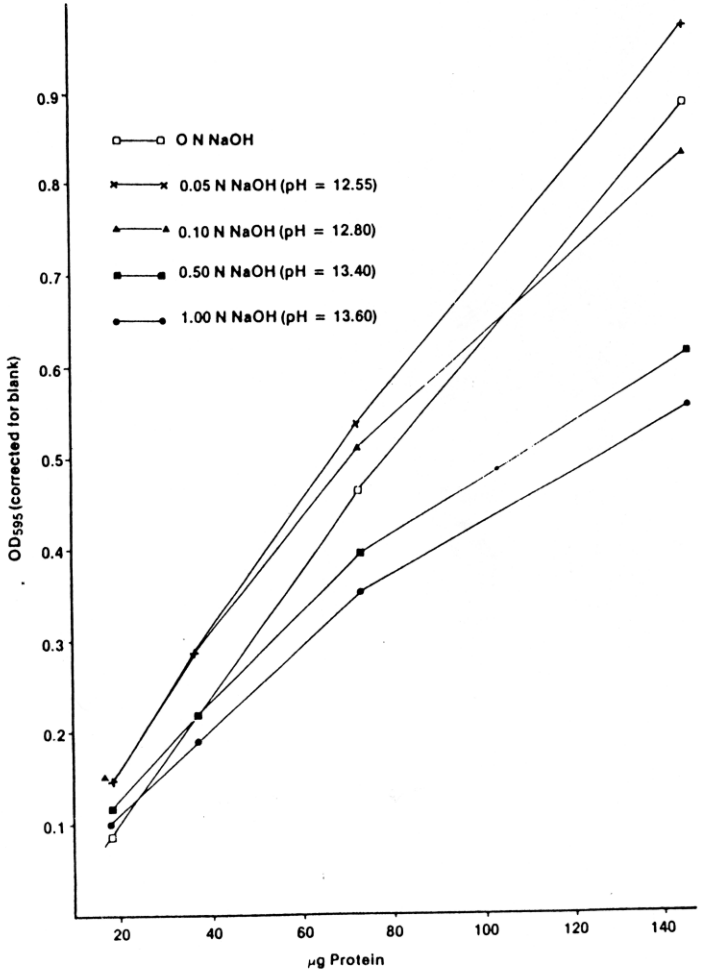


Fig. 4. The effect of NaOH on the Bio-Rad Protein Assay. Each standard curve was obtained by using Protein Standard I (bovine gamma globulin) in the Standard Procedure (20-140 μg).

Standard Assay Procedure

(20–140 μg Protein; 200–1400 μg/ml)

Prepare several dilutions of protein standard containing from 0.2 to about 1.4 mg/ml. Prepare a standard curve each time the assay is performed.

1. Place 0.1 ml of standards and appropriately diluted samples in clean, dry test tubes. Place 0.1 ml sample buffer in "blank" test tube.
2. Add 5.0 ml diluted dye reagent. 5x conc → dil to 1x → (filter)
3. Vortex (avoid excess foaming); or mix several times by gentle inversion of test tube.
4. After a period of from 5 minutes to one hour, measure OD_{595} versus reagent blank.
5. Plot OD_{595} versus concentration of standards (see Figure 1). Read unknowns from the standard curve.

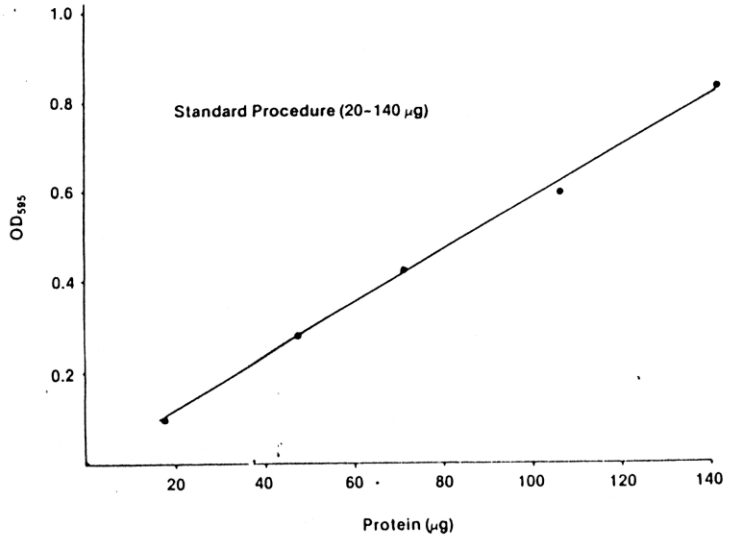


Fig. 1. Typical standard curve for the Bio-Rad Protein Assay (from 20-140 μg) using the Bio-Rad Protein Standard I. OD_{595} corrected for blank.

Microassay Procedure

(1–20 μg Protein; ≤25 μg/ml)

Prepare several dilutions of protein standard containing from 1 to 25 μg/ml. Prepare a standard curve each time the assay is performed.

1. Place 0.8 ml of standards and appropriately diluted samples in clean, dry test tubes. Place 0.8 ml sample buffer in "blank" test tube.
2. Add 0.2 ml Dye Reagent Concentrate.
3. Vortex (avoid excess foaming); or mix several times by gentle inversion of the test tube.
4. After a period of from 5 minutes to one hour, measure OD_{595} versus reagent blank.
5. Plot OD_{595} versus concentration of standards (see Figure 2). Read unknowns from the standard curve.

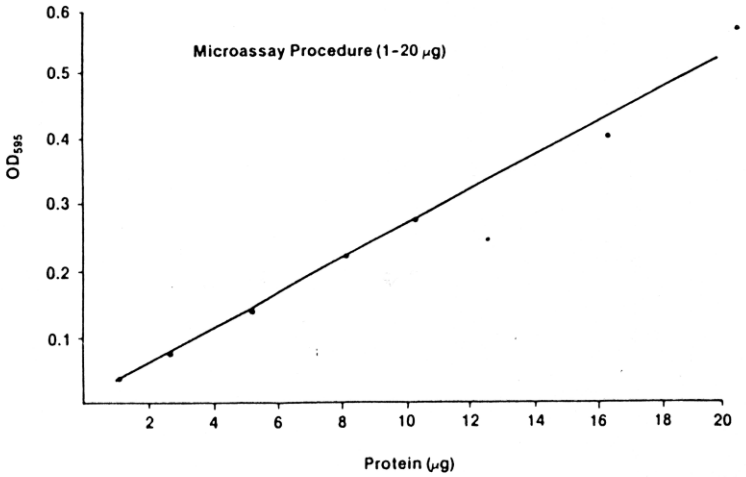


Fig. 2. Typical standard curve for the Bio-Rad Protein Assay (1-20 μg) using the Bio-Rad Protein Standard I. OD_{595} corrected for blank.