Tissue Homogenization

BCH 332 lecture 1

Homogenization is a technique used in tissue preparation

- Tissue preparation is needed in several application areas, including research, pharmaceuticals, molecular biology, food science, forensics and toxicology.

Homogenization

- Means to prepare uniform dilution of a known quantity of tissue suspended within a known quantity of a suitable diluents and uniformly macerate or crush the tissue in such a manner as to disperse minute fragments of tissue evenly throughout the mixture.
- Tissue homogenization is a process used to prepare tissue samples for certain types of studies.
- It involves lysis or breaking cells to release their contents.

Homogenization

Tissue Sample

Blender

Homogenate

Potter-Elvehjem

French Press

Sonication

Detergent or
Organic Solvents

- Homogenization methods used for the proteomics purposes can be divided into five major categories:

- 1. Mechanical;
- 2. Ultrasonic;
- 3. Freeze–thaw;
- 4. Osmotic and detergent lysis

Types of Tissue Preparation Techniques

- Tissue preparation techniques can be categorized into mechanical, digestion or extraction instruments.

Mechanical Techniques

- Homogenization or grinding remains the most popular and, generally, the most practical means of preparing tissues for a range of qualitative or quantitative applications.
- Homogenizers consists of a small stainless steel probe-style blender with a generator and a set of blades causes vigorous mixing and turbulence as well as physically shearing the sample into small pieces.
- A weighed amount of sample, which can be 10 mg–1 g in size, in a vial with a known volume of buffer solution.
- The pH of the buffer can be tailored to the desired extraction conditions. The resulting product, or homogenate, is semisolid in nature and called the homogenate.
- Lengthening the homogenization step or centrifuging the homogenate and decanting the supernatant will minimize large particles in the homogenate.

- 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.
- There are three processes for homogenization of animal tissues using mechanical shear: a Potter-Elvehjem glass-Teflon homogenizer, a Dounce hand homogenizer, or a handheld Waring Blendor

Different types of homogenizers

Hand homogenizer

Waring Blendor

A Potter-Elvehjem glass-Teflon homogenizer

- 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).

- Avoiding proteolytic degradation of the target protein in a crude extract is a primary concern. In many cases, it might not be essential to add protease inhibitors to the homogenization buffer (because of 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).

- If proteolytic degradation is a problem, then protease inhibitors are included in the homogenization buffers.
- If the target protein is susceptible to oxidation or if its activity is inhibited by heavy metals, 1 mM dithiothreitol (or 0.1 M -mercaptoethanol) and 0.1 M EDTA, respectively, are added to the extraction buffer.

Sonication

- Sonication is one alternative to homogenization.
- In the case of sonication for cell lysis, ultrasound (high-frequency) energy is applied to samples to agitate and disrupt the cell membranes
- Ultrasonic devices are mainly used to homogenize small pieces of soft tissues (brain, blood, liver).
- Tough and dense tissues are not recommended to homogenize using this equipment.

Bead Beater

- The bead beater represents a more hands-off approach to tissue sample preparation.
- The bead beater is a unique but simply designed apparatus that uses small beads and a high-speed rotor to rupture cells.
- A solid polytetrafluoroethylene impeller rotating at high speeds forces thousands of minute beads to collide in a specially shaped vessel. Cells are disrupted quickly, efficiently and safely.
- Samples are placed in tubes with a defined amount of beads and buffer solution and then agitate them for 15–20 min.
- During longer agitation times, the unit is refrigerated to prevent sample heating from the beads' movement.
- Various bead sizes are available, and bead types include glass, stainless steel and ceramic.

Bead Beater


Benchmark
Cycle
Speed
Time
10
4350
30
START
STOP
SET
BEADBUG

Stabilization of Samples

BCH 332 lecture 2

- Before embarking on a detailed analysis of a biomolecule using biophysical techniques it is often useful to know something about the stability of the sample to chemical variables, especially pH, temperature and solvent polarity.
- This knowledge can help us to design a suitable solvent or set of chemical conditions which will maximize the stability of the analyte for the duration of the experiment and may also help us to explain unexpected results.
- For example, we sometimes find loss of enzyme activity during column chromatography which may be partly explained by the chemical conditions experienced by the protein during the experiment.

- A good indication of the most stabilizing conditions may often be obtained from knowledge of the biological origin of the biomolecule.
- It is also wise to assess the structural and functional stability of the analyte over the range of experimental conditions encountered in the experiment during its likely time-span.

- We can distinguish two main types of effects as a result of variation in the chemical conditions to which biomolecules are exposed.
- *Structural effects reflect often irreversible* structural change in the molecule (e.g. protein/nucleic acid denaturation; hydrolysis of covalent bonds between building blocks of which biopolymers are composed).
- *Functional effects are frequently more subtle and may be reversible* (e.g. deprotonation of chemical groups in the biomolecule resulting in ionization; partial unfolding of proteins).

pH Effects

- Most biomacromolecules are *labile to* alkaline or acid-catalyzed hydrolysis at extremes of the pH scale but are generally *stable in the range 3–10.*
- *It is usual* to analyse such biopolymers at pH values where they are structurally stable and this may differ slightly for individual biopolymers.
- For example, proteins normally expressed in lysosomes (pH 4) are quite acid-stable while those from cytosol (pH 7) may be unstable near pH 3.
- Aqueous solutions in which sample molecules are dissolved usually comprise a *buffer to prevent changes in pH during the experiment.*

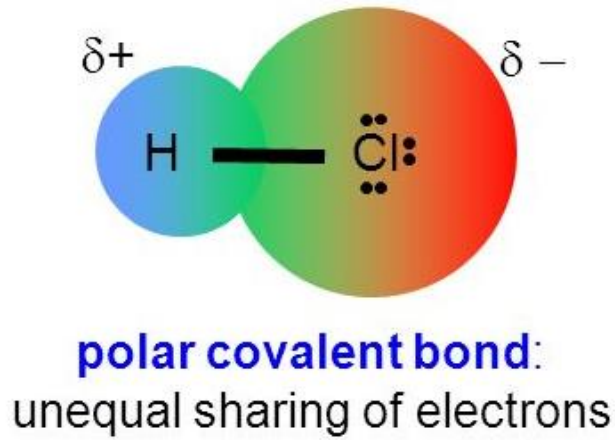
Temperature Effects

- Three main effects of temperature on biomolecules are important for the biophysical techniques.
- These are effects on structure, chemical reactivity and solubility.
- Heat can disrupt noncovalent bonds such as hydrogen bonds which are especially important in the structure of biomacromolecules. This can lead to denaturation of proteins and DNA or to disruption of multimolecular complexes in which they may be involved.

- Extensive heating can result in disintegration of the covalent structure of biomacromolecules.
- Thus proteins can break down into component peptides or nucleic acids into smaller polynucleotide fragments as a result of exposure to heat.
- The rate of chemical reactions are effected by temperature.

- Thirdly, temperature usually increases the solubility of molecules in a solvent as well as the rate of diffusion through the solvent.
- Temperature is therefore usually tightly controlled during biophysical experiments.

Effects of Solvent Polarity



polar covalent bond:
unequal sharing of electrons

- Polarity arises from unequal affinity of atoms bonded together for shared electrons called electronegativity.
- In extracting, analyzing and purifying biomolecules these intricate structural interactions are often lost which can result in aggregation, precipitation or loss of structure and, hence, of biological activity. If it is desired to retain biological activity we use aqueous solutions to handle largely hydrophilic biomolecules, nonpolar solvents to dissolve mainly hydrophobic samples and detergent solutions for molecules which possess both classes of groups.

Some Common Buffers

- Some of these buffer components are of biological origin (e.g. glycine; histidine; acetate).
- *Good's buffers were developed by N.E. Good to facilitate buffering* in the pH range 6–10.5.

Good's rules to select for ideal buffers:

- 1. **pKa between 6-8**
- Most biological reactions take place at a neutral pH, between 6-8. Ideal buffers should have pKa values in this range to provide maximum buffering capacity at a neutral pH.
- 2. **Solubility in water**
- Because biological systems are aqueous systems, good solubility in water is required. Low solubility in nonpolar solvents (such as fats, oils and organic solvents) is considered beneficial. This prevents the buffer from accumulating in nonpolar compartments in biological systems such as cell membranes.
- 3. **Membrane impermeability**
- A buffer should not readily pass through cell membranes. Zwitterionic buffers contain both positive and negative charge so do not pass through biological membranes. MOPS and HEPES are zwitterionic buffers, while Tris and phosphate buffers do not isomerize into zwitterions.
- 4. **Minimal Salt effects**
- The ideal buffer should have minimal salt effects upon the biological system. If salt is required, suitable ions can be added, but highly ionic buffers can cause problems in biological systems.

Table 1.3. Some common buffers used in biochemistry

Buffer	pK_a
Phosphate[a]	0.85, 1.82, 6.68
Histidine[a]	1.82, 5.98, 9.17
Phosphoric acid[a]	1.96, 6.7, 12.3
Formic acid	3.75
Barbituric acid	3.98
Acetic acid	4.8
Pyridine	5.23
Bis TRIS [Bis-(2-hydroxy-ethyl)imino-tris-(hydroxy-methyl)-methane]	6.46
PIPES [1,4-piperazinebis-(ethanesulphonic acid)]	6.8
Imidazole	7.0
BES [*N*, *N*-*B*is(2-hydroxy-ethyl)-2-amino-ethane-sulphonic acid]	7.15
MOPS [2-(*N*-morpholino)propane-sulphonic acid]	7.2
HEPES [*N*-2-hydroxyethyl-piperazine-*N*'-2-ethane-sulphonic acid]	7.55
TRIS [hydroxymethyl)amino-methane]	8.1
TAPS [W-tris(hydroxymethyl)methyl-2-aminopropane sulphonic acid]	8.4
Boric acid	9.39
Ethanolamine	9.44
CAPS [3-(cyclohexylamino)-1-propane-sulphonic acid]	10.4
Methylamine	10.64
Dimelhylamine	10.75
Diethylamine	10.98

[a] There are polyprotic with several pK_a values.

Additional Components Often Used in Buffers

- In addition to buffer components such as weak acids/bases and their salts, buffers frequently contain a range of other components of which a selection is shown in Table 1.4.
- These may be necessary to maintain stability of the biomolecule, to control levels of metal ions, to ensure reducing/ oxidizing conditions or to keep the biomolecule dissolved and/or denatured.

Table 1.4. Additional reagents sometimes added to buffers

Chemical	Function
2-Mercaptoethanol	Reducing agent
Dithiothreitol	Reducing agent
Sodium borohydride	Reducing agent
Divalent metals plus O_2	Oxidising agents
Performic acid	Oxidising agent
Leupeptin	Protease inhibitor
Phenylmethyl sulphonyl fluoride (PMSF)	Serine protease inhibitor
Ethylene diamine *NNN′N′* tetra-acetic acid (EDTA)	Metal chelator/ metalloprotease inhibitor
Ethylene glycol-bis(*β*-aminoethyl ether) *NNN′N′* tetraacetic acid (EGTA)	Calcium chelator
Urea	Denaturing agent
Guanidinium hydrochloride	Denaturing agent
Sodium dodecyl sulphate (SDS)	Anionic detergent
Cetyltrimethyl ammonium chloride	Cationic detergent
3-(3- cholamidopropyl)dimethy lammonio)-1-propane sulphonate (CHAPS)	Zwitterionic detergent
Triton X-100	Nonionic detergent
Digitonin	Nonionic detergent
Protamine K	Binds DNA

- The tissues should be homogenized in buffered, balanced salt solution, media rich in carbon source.
- Sucrose is used to provide sufficient osmotic potential to prevent organelles swelling and bursting.
- It is important to maintain the pH and critical levels of certain inorganic ions.
- For example Mg2+ ions help to maintain nuclear and ribosome integrity.

- On the other hand chelating agents such EDTA may be added to remove divalent cations such as Mg^{2+} and Ca^{2+} when it is important to inactivate membrane proteases.
- Inhibitors such as Diisopropyl fluorophosphate (for serine residues) may be included to inhibit protease activity.
- Alternatively artificial protease substrates such as bovine serum albumin may be added to ameliorate protease activity.

- Many enzymes need to be maintained with their sulphydryl groups in a reduced state which requires adding reducing agents to extraction medium such as 2- mercaptoethanol, dithiothreitol, reduced glutathione or cysteine at concentration in the mM range.

- An organic based aqueous medium is sometimes preferred to salt solutions in tissue extractions. Citrate has been used in the isolation of nuclei because of its ability to inactivate neutral deoxyribonucleases. Glycerol, ethyleneglycol and ethyleneglycol polymers have been used for preparation of plastids.
- A nonaqueous medium may be used in the isolation of organelles. The suspending fluid is usually a blend of a light and heavy organic solvents such as ether-chloroform. The density of the medium can be varied so that the required organelles either float or sediment from the remainder of the homogenate in subsequent centrifugation.