

Introduction to Protein Techniques

542B-Spring 2008

Lecture 1

Sample preparation in 5 steps

1. Cell disruption using physical or chemical methods in an appropriate buffer
2. Clarification by centrifugation
 - low speed (3000 to 4000 x *g*) to remove debris
 - high speed (10,000 to 12,000 x *g*) to clarify extract
 - ultra-high speed (60,000 x *g*) to pellet membranes
3. Protein precipitation
4. Removal of salts by dialysis, membrane filtration or size exclusion chromatography
5. Column chromatography for purification

Sample disruption methods

1. Physical methods

- Bead beater-use glass beads 0.1 -0.2 mm
- Homogenizer
- Grinder (freeze sample –liquid nitrogen)
- Mortar and pestle-use liquid nitrogen

2. Chemical methods

- chloroform or toluene-to solubilize the membrane

3. Enzymatic methods

- Lysozyme with or without EDTA to digest the peptidoglycan layer

Centrifugation

1. Low speed- to remove debris such as unbroken cells, glass beads, usually from 3,000 to 5,000 x g
2. High speed- to clarify extract remove particulate matter, usually from 10,000 to 12,000 x g
3. Ultra high speed at 60,000 x g to isolate membranes

NOTE: Always use 'g' and not 'rpm'; g takes into account the radius from tip of tube to center of rotor, whereas rpm does not; consequently, if 'rpm' is used and the rotor size is not known, one cannot repeat centrifugation parameters.

The formula for Relative Centrifugal Force (RCF) is
$$= 1.12 r(\text{rpm}/1000)^2$$

r= radius, rpm=revolutions per min

Ammonium sulfate precipitation

1. Disrupts water structure around the protein molecule
2. Allows hydrophobic regions to interact
3. Protein precipitates out of solution
4. In general, higher MW proteins precipitate out at lower concentrations of ammonium sulfate than lower MW proteins
5. Therefore, differential ammonium sulfate precipitation provides a pre-purification step for proteins and is one of the classical methods used for protein purification

Salt removal/exchange

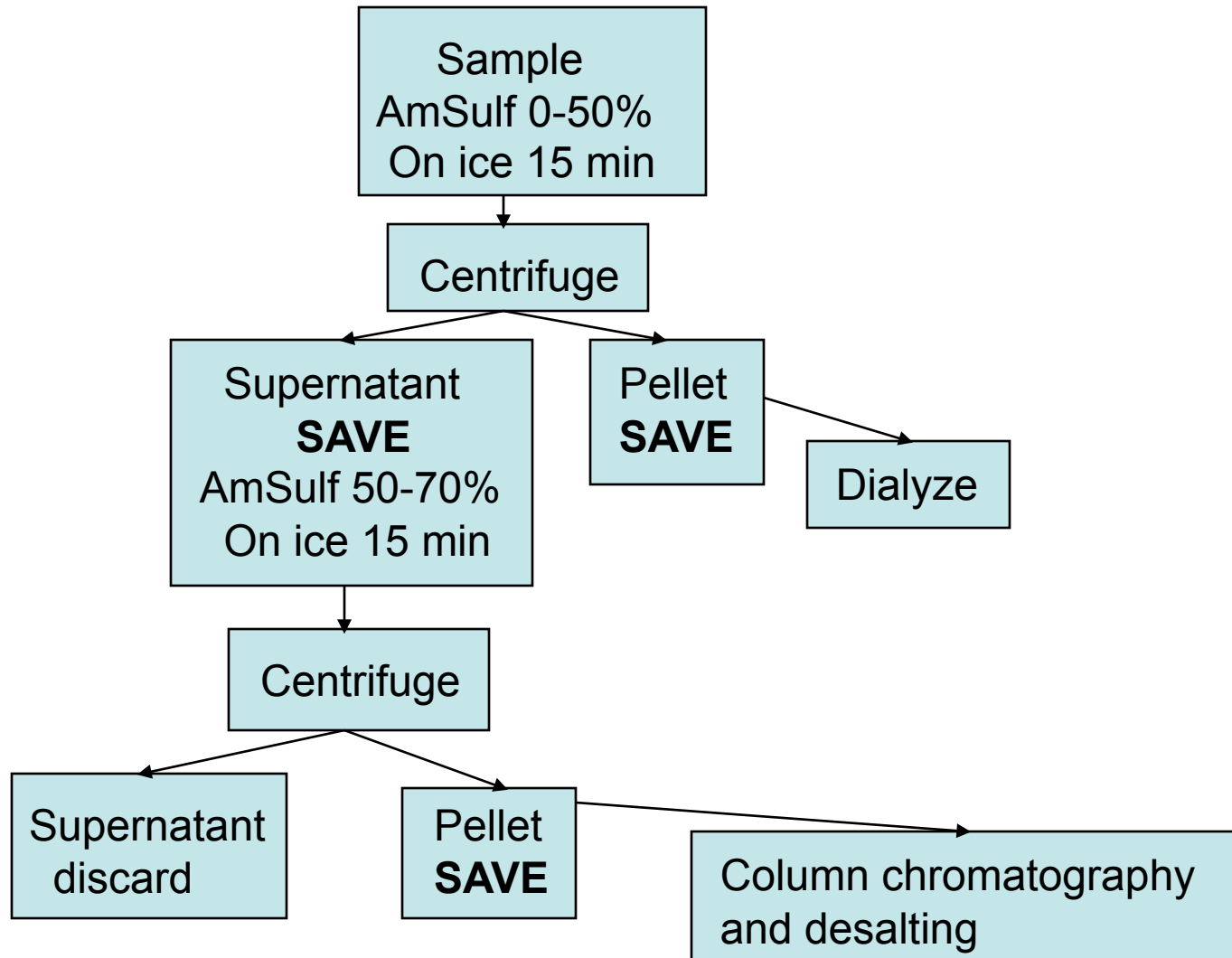
1. Dialysis-a procedure to remove salts from protein extracts. The porous cellulose tubing containing the protein extract is clamped off on both ends and suspended in a buffer or low salt solution.
 - The membrane has a molecular weight cut-off of around 10,000-12,000. Proteins remain in the bag, where as the ions and other low molecular weight components diffuse out of the bag.
 - Exceptions for diffusion are highly charged ions, such as ATP (retarded), SDS (will not diffuse out).

Note: as salts move out, water moves in (Donnan equilibrium). Leave empty tubing above liquid.

2. Membrane filtration-removes water and salts
3. Size exclusion chromatography

Concentration

1. Freeze-drying -removes water->high salt and protein concentration.
2. Membrane filtration -removes water and salt; many molecular cut-off membranes available
3. Ammonium sulfate precipitation-precipitates the protein, roughly according to the molecular size. High MW proteins at lower concentrations; lower MW proteins at higher concentrations
 - Is the preferred method because it is gentle to proteins and enzymes (use metal-free ammonium sulfate)
 - Partially purifies proteins in that polysaccharides, oligonucleotides, lipids are not precipitated
 - But lose peptides that may be lower than 10,000 in molecular mass-they will still be soluble at >70% ammonium sulfate
4. Differential ammonium sulfate precipitation –advantage is that this constitutes a partial purification



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Lecture 2

Chromatography

1. Chromatography derives from the word chroma= color (Latin) and graphy = chart
2. Originally from experiments using filter paper to separate dye components
3. Now it refers to separation of molecules on a column filled with a matrix that affects the mobility of the molecule (analyte)

Types of chromatographic techniques

1. Gel filtration (permeation)-separation by molecular weight
2. Size exclusion-separation by molecular weight, used for desalting or buffer exchange of proteins
3. Ion exchange-separation based on charge
4. Hydrophobic interaction-separation based on hydrophobic interaction
5. Affinity chromatography-separation based on ligand-ligand interaction
 - antibody/antigen
 - CibraconBlue-nucleotide containing proteins/enzymes
 - metal ion interaction- Ni-for his tag proteins, iron-binding proteins
 - substrate analog or inhibitor-enzyme purification

Chromatography

6. High performance liquid chromatography (HPLC)-separation of analytes on a silica gel or polymer-based packing functionalized for different applications, reverse phase, normal phase, ion exchange, gel permeation
 - high pressure, stainless steel components
7. Fast protein liquid chromatography (FPLC)
 - separation of proteins under lower pressure than HPLC, glass, Teflon components
 - functionalized resin for ion exchange, affinity purifications

HPLC

1. Advantages

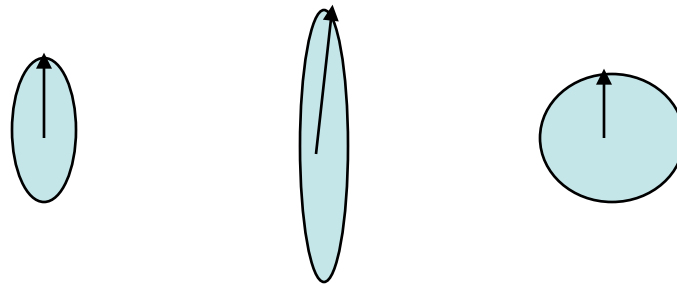
- Fast- 1hr for gel permeation experiments as compared to up to 3 days using open column methods (low pressure)
- low molecular weight components such as peptides, up to 1 hour
- silica gel is available derivatized with alkanes: e.g., C-4 to C-18, and can be modified with any desired functional group, depending on the application

2. Disadvantages

- for silica gel-based packing the usable pH ranges from pH 2-7
- for polymer packing the usable pH ranges from pH 1-14, however eventually polymer components may be leached off the column

Desalting/size exclusion

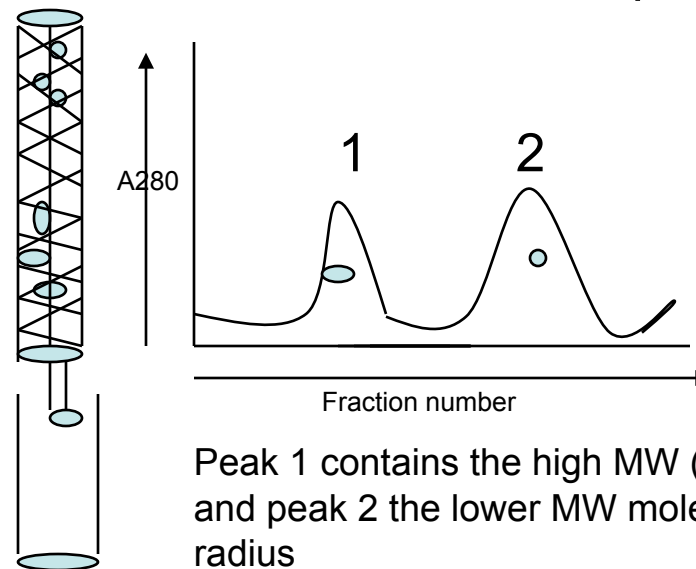
1. In size exclusion chromatography (SEC, organic solvent system) and gel filtration chromatography (GFC, aqueous solvent system) molecules are separated based on their molecular size. The elution properties depend on the Stoke's radius of the molecule:



The arrow indicates the radius, r

2. Given the molecules are the same molecular weight, the molecules with the largest Stoke's radius will elute ahead of the molecules with the smallest Stoke's radius.

In general, the larger MW proteins always elute first, followed by the smaller MW proteins, because the larger molecules cannot enter the pores of the matrix, whereas the smaller ones may partially enter the pores and will be slowed down. Salts however, are small, enter the pores completely and are last to elute or be displaced by the water molecules.



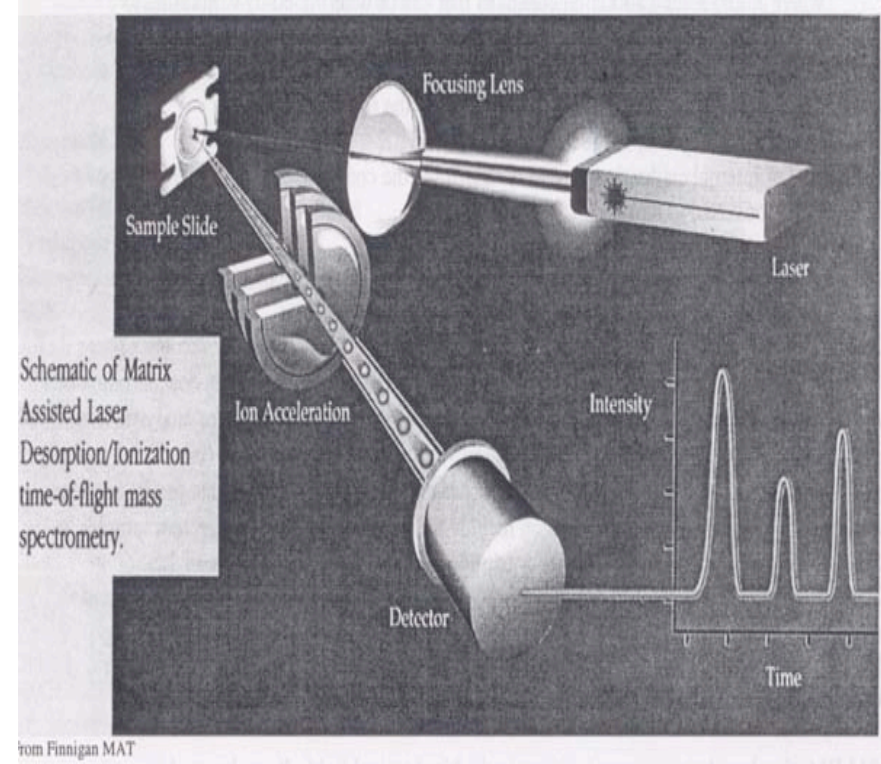
Peak 1 contains the high MW (or larger Stokes's radius) and peak 2 the lower MW molecules (or smaller Stoke's radius)

Reversed-phase HPLC

1. In reversed phase chromatography the solute (molecule to be separated) has a high affinity for the stationary phase. It will be eluted with an organic solvent of increasing concentration and eventually will be displaced by the solvent.
2. The more non-polar (hydrophobic) the solute is, the tighter it binds to the column. A higher concentration of organic solvent is necessary to elute the solute and the longer it takes to elute the solute from the column.
3. Thus in reversed-phase chromatography elution of the solutes depends on their degree of hydrophobicity.
4. Advantage is taken of this property of the column for desalting peptides for mass spectrometry using “Ziptips”, or C-18 cartridges to clean up samples.
5. Proteins contain many hydrophobic or non-polar regions. Usually they are located on the inside folds of the protein. Proteins may be purified on silica gel derived with C-4 to C-8 alkanes, as we will do in class with your protein samples.

MALDI-TOF mass spectrometry

1. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry
2. The mass spectrometer for the biochemist, as one mass spec inventor (Dr. George Karas) from the University of Münster, Germany, called this instrument.
3. With the introduction of this instrument, molecular weights of proteins could be determined in a matter of seconds, rather than days using SDS-PAGE.
4. Very small amounts are necessary, in the order of picomoles compared to μ moles for SDS-PAGE (1-10 ng compared to 1 μ g for a protein of 50,000 Dalton).
5. Causes soft ionization; single, double and triply charged ions
6. Mass range up to 150,000 m/z
7. Not tolerant of salts and detergents



Matrix-analyte

1. Deposit 0.5 μ l of sample containing about 1-10 pmol of protein or peptide or a concentration of about 1 mg/mL onto the target plate..
2. Add 0.5 μ l of saturated matrix (in 30% acetonitrile/0.1% trifluoroacetic acid) to the sample, mix and let dry.
3. For proteins use sinapinic acid.
4. For peptides use α -cyano-4-hydroxycinnamic acid.
5. Fire the laser according to the voltage setting and laser power indicated by the Protein Facility personnel and acquire spectra.

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Lecture 3

Historical background

1. MW of a protein is one of the parameters that describes the protein; other parameters include isoelectric pH (pI), primary sequence, secondary, tertiary and quaternary structure.
2. Earlier methods for measuring molecular weight included, gel filtration, analytical ultracentrifugation, light scattering.
3. These were time consuming methods, and except for gel filtration, require expensive instrumentation and specific skills.
4. SDS-PAGE changed this almost overnight starting in the late 60's. The seminal paper published in 1969 was that by Weber and Osburn.
5. An improvement over SDS-PAGE for measuring MW is MALDI-TOF

Primary sequence information and amino acid composition from blots

1. The second most important discovery was that information could be obtained from proteins electro-transferred (blotted) to polyvinylidene difluoride (PVDF) first described by Matsudaira in 1987 (Matsudaira, P., “Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes”, J. Biol. Chem. 262 (21):10035-10038, 1987).
2. Both sequence and amino acid composition could now be obtained from small amounts of protein.

SDS-PAGE and blotting for amino acid analysis and protein/peptide sequencing

1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent (e.g. 2-mercaptoethanol, dithiothreitol) is a technique for the separation of polypeptide subunits according to their molecular weight.
2. Protein is denatured with the detergent SDS and protein disulfides (from cysteine-cysteine bonds) are reduced with 2-mercaptoethanol.

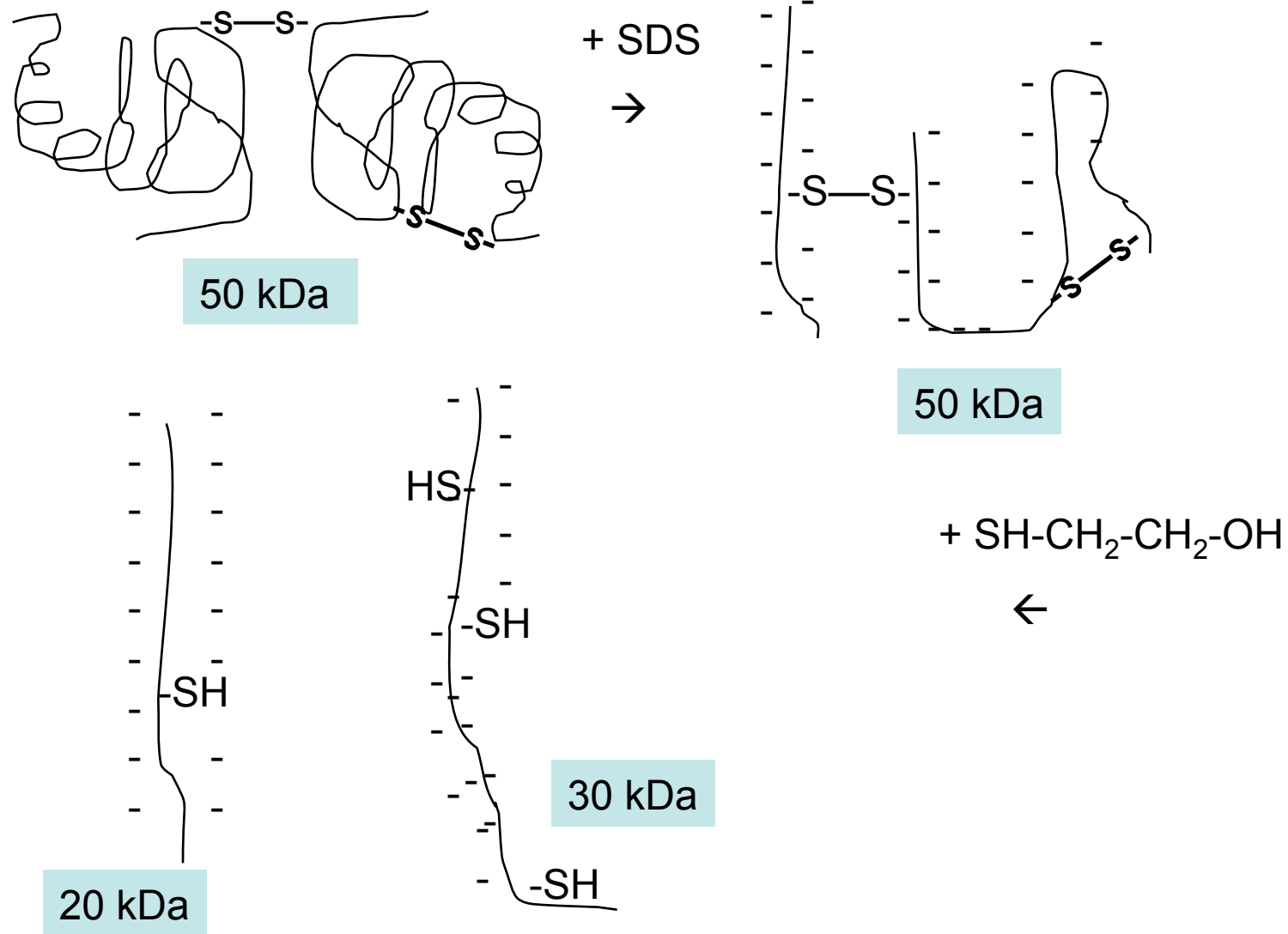
3. Binding of SDS to the protein confers a net negative charge-



by associating with the nonpolar residues of the protein therefore interfering with the hydrophobic interactions responsible for the protein's structure.

4. Binding of SDS will linearize the protein molecule, and as a result the molecular weight of the protein will be proportional to its size, provided all disulfide bonds, both intra- and intermolecular bonds, have been reduced from P-S-S-P to P-SH and HS-P.

Denaturation and reduction



Electrophoresis-Separation of proteins in an electric field

1. The negatively-charged proteins are separated under the influence of an applied electric field through a gel of known percent acrylamide or pore size, towards the positive electrode (anode-marked red).
2. At a given percent acrylamide, the higher MW proteins move slower compared to the lower MW proteins.
3. When the solvent front (Bromphenyl Blue indicator dye) reaches the bottom of the gel, electrophoresis is stopped.

4. The gel is removed and stained with one of several procedures (Coomassie Brilliant Blue R-250, silver, fluorescent dye).
5. After destaining, the bands are measured in comparison to standard molecular weight markers.
6. The subunit molecular weights of the sample proteins are determined from a graph of \log_{10} MW versus distance of migration in mm.

Gel matrix

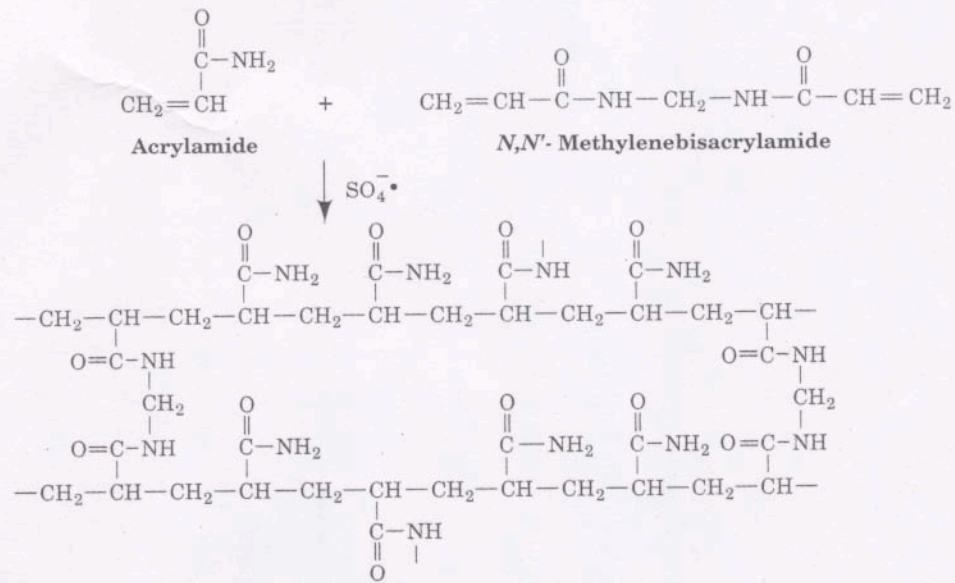


Figure 5-21

The polymerization of acrylamide and *N,N'*-methylenebisacrylamide to form a cross-linked polyacrylamide gel. The polymerization is induced by free radicals resulting from the chemical decomposition of **ammonium persulfate** ($S_2O_8^{2-} \rightarrow 2SO_4^{\cdot -}$) or the photodecomposition of riboflavin in the presence of traces of O_2 . In either case, ***N,N,N',N'*-tetramethylethylenediamine**

(**TEMED**), a free radical stabilizer, is usually added to the gel mixture. The physical properties of the gel and its pore size are controlled by the proportion of polyacrylamide in the gel and its degree of cross-linking. The most commonly used polyacrylamide concentrations are in the range 3 to 15% with the amount of *N,N'*-methylenebisacrylamide usually fixed at 5% of the total acrylamide present.

Taken from Voet and Voet, *Biochemistry*, John Wiley & Sons, Inc. (1990)

Plot of \log_{10} MW versus distance

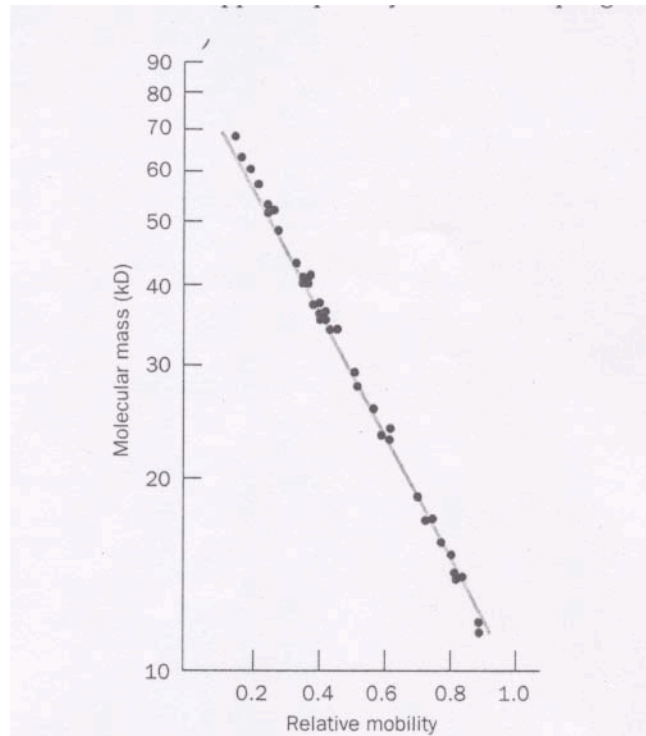


Figure 5-27
A logarithmic plot of the molecular masses of 37 different polypeptide chains ranging from 11 to 70 kD versus their relative electrophoretic mobilities on an SDS-polyacrylamide gel. [After Weber, K. and Osborn, M., *J. Biol. Chem.* **244**, 4406 (1969).]

Taken from Voet and Voet, *Biochemistry*, John Wiley & Sons, Inc. (1990), p. 99

1. Prepare the gel as shown in your manual, you will work in pairs, two students/gel, two gels/apparatus.

- Assemble the cassette and test for leakage

- Put the pipet tip into the solution when adding TEMED and APS

- Invert gel solution several times to ensure complete mixing

- Running gel, 12% acrylamide, 30 min

- Stacking gel, 4%, 30 min (needed when not running a gradient gel; allows pre-sieving of proteins and also forms the wells).

2. Denature protein sample

3. Load protein and standards underneath the buffer layer in the gel

- standard lane

- protein lane-----

- protein lane

- blank lane

- blank lane

- standard lane

- protein lane

- protein lane

Gel is cut in half after running the electrophoresis,
1/2 gel for staining, 1/2 gel for electroblotting

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Lecture 4

Blotting to PVDF

1. Blotting is a technique for the electrophoretic transfer of DNA, RNA or protein to a suitable membrane.
2. The method of Towbin et al., 1979 (Proc. Natl Acad Sci. USA, 76:4350-4354, 1979) is the most commonly used for the transfer of macromolecules to nitrocellulose.
3. For protein sequencing and amino acid analysis, the proteins are transferred to polyvinylidene difluoride (PVDF) membrane (Matsudaira, J. Biol. Chem. 262 (21):10035-10038, 1987).
4. Bands visualized on the blot with Coomassie Brilliant Blue R-250 are excised, cut into smaller pieces, washed extensively with deionized water and used directly for N-terminal sequencing or amino acid analysis

5. The amount of protein to be loaded on the gel for transfer should be ~ 1 μg ; expect about a 50% loss during the transfer.
6. The lowest amount that will give a limited sequence is from 5-10 pmol, although 1 pmol can be detected; 1 pmol = 30 ng of a protein with a mass of 30,000 dalton.
7. When no N-terminal sequence is obtained, even though the required amount of protein was transferred to the blot, means that the N-terminal was blocked.

8. Reasons for a blocked N-terminal

a. post translational modification

- methylated or acetylated N-terminal

- pyroglutamine, cyclized glutamic acid as the N-terminal amino acid

b. alkylation due to impurities

- unpolymerized acrylamide in the gel (cure gel overnight before use)

- impure reagents for staining and destaining (e.g. aldehydes in acetic acid, methanol); use sequencing grade reagents whenever possible

9. Blank cycle

- a. due to cysteine; it is converted to cysteic acid during the sequencing cycle and elutes at the solvent front.
- b. to detect cysteine, the protein or peptide must be chemically modified with vinyl pyridine to form the pyridylethyl adduct of cysteine

10. No protein blotted to PVDF

- a. cassette inverted, membrane must face anode
- b. proteins have pI that is greater than the pH of the blotting buffer; titrate buffer to higher pH, use a different buffer of higher pH
- c. stain the gel after blotting

11. Difficulty blotting proteins

- a. Add 0.05% SDS final concentration to the blotting buffer

12. Results show 20 N-terminal residues

a. Protein is partially degraded, keep frozen, add protease inhibitors (e.g., phenylmethylsulfonyl fluoride, EDTA, leupeptin, pepstatin, etc).

-degradation not always visible by SDS-PAGE

b. For structural studies, keep protein frozen, never store a protein longer than three days at 5°C.

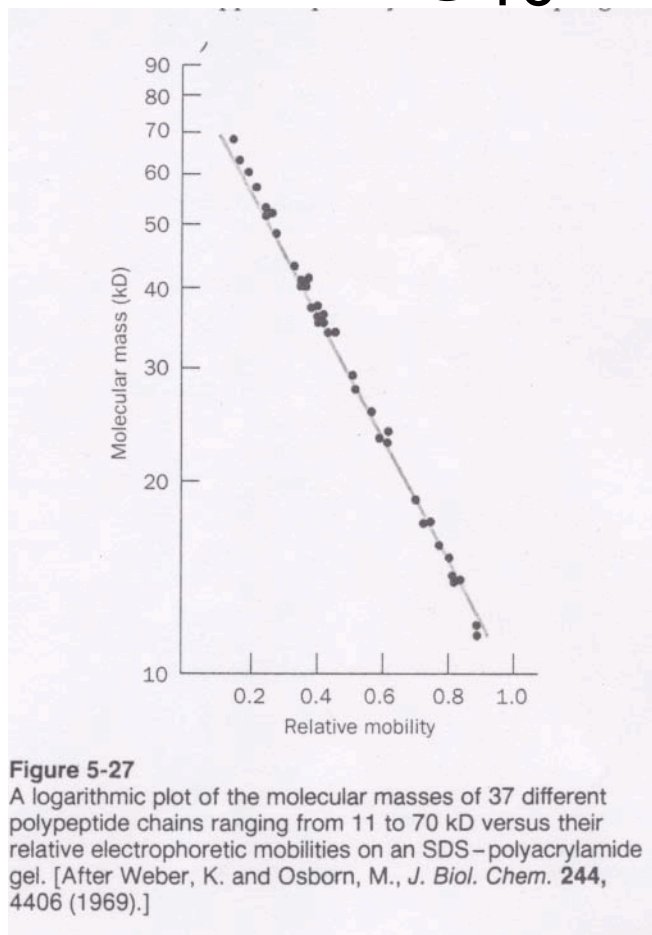
Caution

1. Always wear gloves
-N-terminal sequences of finger prints, dandruff, saliva, have been seen in samples
2. Always keep protein samples frozen; proteolytic enzymes from *Pseudomonas* spp. and molds in buffer solutions degrade proteins

Procedure

1. Soak gel in transfer buffer without methanol for 20 min.
 - Excess SDS must be removed, it interferes with binding to the PVDF; but enough SDS is required to allow the protein to be transferred out of the gel.
2. Soak Whatman #1 filter paper in transfer buffer + 10% methanol
3. Dip PVDF in 100% methanol to “activate” the membrane, allow to drain, soak in transfer buffer + 10% methanol (not appropriate for nitrocellulose)
4. Assemble gel cassette as shown in the manual
 - All gels will be placed in one blotting apparatus
 - Place PVDF onto the gel by carefully allowing it to roll onto the gel, and by excluding air bubbles

Plot of \log_{10} MW versus distance



- Use a ruler to determine the mobility in mm of the MW marker proteins
- Plot mobility versus the MW on 2-cycle semi-log paper
- Find the mobility in mm of the unknown proteins
- From the mobility value of the sample, use the standard curve to determine the MW

MW values and instructions on page 9 of Part I

Taken from Voet and Voet, *Biochemistry*, John Wiley & Sons, Inc. (1990), p. 99

Protein Assay

1. Bradford dye-binding protein assay (BioRad) works well with purified protein extracts
2. For crude extracts which may contain nucleic acid, lipids, polysaccharides, etc., the Folin-Lowry protein assay is more appropriate (see Manual).

Protein Assay

1. Always prepare a “blank”; a “blank” contains everything your samples contain, except for the analyte (protein).
2. Make dilutions of the standard protein, normally, bovine serum albumin (BSA), to construct a standard curve. To get accurate results one should do the standards in triplicate. In class we will do a single tube/standard concentration.
3. Make several dilutions of the sample to be tested. In class we will do a single dilution.
4. Add the dye reagent, vortex, incubate for 10 min at room temperature, and read absorbance at 595 nm.
5. Construct a standard curve, estimate concentration of the unknown protein from the graph.
6. Alternatively, input data in to a linear regression program, determine, correlation coefficient, slope and intercept; calculate protein concentration of the unknown from the equation of a straight line:
 $y = mx + b$, where y =absorbance, m = slope, b = intercept
 $x = (y - b) / m$

Demo

During the 1-hour blotting procedure go to the Protein Facility
-Demo on protein sequencing