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Practical Note
PHYSICAL BIOCHEMISTRY
(BCH 333)

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Experiment 1

1. Scanning spectrophotometry and spectrophotometric determination of concentration

1.1 Objectives:

1) Preparation of an absorption spectrum and determination of λ_{max}
2) Preparation a standard curve and determination of concentration of an unknown solution
3) Calculation of molar extinction coefficient

1.2 Introduction:

Measurements of absorbance of light by substances in solution are widely used in biochemistry because many compounds of interest to biochemists absorb light in ultraviolet (UV), visible or near infrared region.

The absorbance of light is characterized by two parameters, the wavelength of maximum absorption (λ_{max}) and the extinction coefficient (extent of absorption). The colour of substances and solutions is due to the substance or solution selectively absorbing light at other wavelengths and transmitting that which is visible.

Measurements of absorbance of light are made with a photometer, an instrument which directly measures light intensities. A photometer may be a filter photometer or a spectrophotometer. A spectrophotometer contains a monochromator which isolates a very narrow band of wavelength.

1.3 Principle:

The absorption of light by a solution is described by the Beer-Lambert law:

$$\text{Log}_{10}\ I_0/I = \varepsilon lc$$

I_0 = intensity of the incident light

I = intensity of the transmitted light

c = concentration of the absorbing substance

l = length of the light path through the solution

ε = extinction(absorption) coefficient

The quantity $Log_{10}(I_0/I)$ is the absorbance (optical density or O.D). Thus
The law can be rewritten as

$A = \varepsilon lc$

A is the absorbance.

Extinction coefficient has many units but they must relate to the units of concentration and length of light path. If the concentration if in molarity
E is the molar extinction coefficient(ε)

Thus,

$A = \varepsilon lc$

1.4 Materials:

1.4.1 Chemicals:

Trisodium citrate,dihydrate

Concentrated HCl

Ethanol,95%(v/v)

Bromophenol blue

1.4.2 Equipments:

Scanning spectrophotometer.

1.4.3 Glasswares:

Test tubes

Glass cuvettes

Preparation of solutions:

Citrate buffer,0.1M, pH 2.4

Dissolve 29.4 g of trisodium citrate, trihydrate, in 700 ml of water. Add conc. HCl to lower the pH to 2.4 and dilute to 1.0 liter with water.

Bromophenol blue, $1.5x10^{-3}$ M

Dissolve 0.1 g of bromophenol blue in 95% ethanol and make up to 100 ml with the ethanol.

Unknown bromophenol blue solution

Prepare the unknown by diluting the above solution.

1.5 Method:

1. Absorption Spectrum

   a) Take a test tube and add the following reagents:

Reagent	Volume (ml)
0.1 M citrate buffer, pH 2.4	9.0
$1.5x10^{-3}$ M bromophenol blue	0.2
95% ethanol	0.8

b) Mix and measure the absorbance of the solution from 340 to 620 nm at 20 nm intervals against a water blank. Remember to zero the instrument at each wavelength setting.

Use a scanning spectrophotometer if it is available.

2. Standard curve

   a) Set up 7 test tubes as follows:

Reagent	Tube number						
	1	2	3	4	5	6	7
0.1 M citrate buffer, pH 2.4 (ml)	9.0	9.0	9.0	9.0	9.0	9.0	9.0

$1.5x10^{-3}$M bromophenol blue (ml)	0.1	0.2	0.4	0.6	0.8	1.0	-
95% ethanol (ml)	0.9	0.8	0.6	0.4	0.2	-	-
Unknown (ml)	-	-	-	-	-	-	1.0
Molar concentration of bromophenol blue x 10^5	1.5	3.0	6.0	9.0	12.0	15.0	

b) Mix and measure the absorbance of all the tubes at 430 nm against a water blank.

1.6 Results:

1. Absorption Spectrum

If a scanning spectrometer is not available record the results as follows:

Wavelength (nm)	Absorbance
320	
340	
360	
380	
400	

etc. up to 620 nm.

Plot a graph of absorbance against wavelength (absorption spectrum)

From the graph or spectrum from a scanning spectrophotometer determine λ_{max} for bromophenol blue at pH 2.4

λ_{max} for bromophenol blue at pH 2.4 = nm

2. Standard curve and determination of concentration of unknown.

Record the absorbance in the table below:

Tube No	Molar concentration of bromophenol blue x 10^5	Absorbance at 430 nm
1	1.5	
2	3.0	
3	6.0	
4	9.0	
5	12.0	

6	15.0	
7		

Plot a standard curve of absorbance against Molar concentration of bromophenol blue x10^5

From the curve determine the molar concentration of the unknown (tube 7)

Molar concentration of bromophenol blue in unknown = x 10^{-5}

Calculate the concentration of the unknown also from the absorbance of one standard, tube 3.

i) $A_s = \varepsilon l c_s$ for tube 3

ii) $A_u = \varepsilon l c_u$ for tube 7

(s = standard, u = unknown)

$c_u = c_s A_u / A_s$

Calculated molar concentration of bromophenol blue in unknown=

3. Calculation of molar extinction coefficient.

Calculate the molar extinction coefficient by using absorbance and molarity values from tubes 3 and 4,

$A_3 = \varepsilon l c_3$

Molar extinction coefficient for tube 3 = A_3/lc_3

= $A_3/$ 1x 6x10^{-5}

= M^{-1} cm^{-1}

$A_4 = \varepsilon l c_4$

Molar extinction coefficient for tube 4 = A_4/lc_4

= $A_4/$ 1x 9 x 10^{-5}

= M^{-1} cm^{-1}

Average molar coefficient of bromophenol blue at 430 nm, pH 2.4

= M^{-1} cm^{-1}

1.7 Discussion and conclusion:

1.8 Questions:

1. What is Beer-Lambert's law?
2. What do you understand by λ_{max}?
3. What do you understand by molar extinction coefficient?

1.9 Reference:

Stenesh J.(1984). Experimental Biochemistry.Allyn and Bacon, Inc (Boston), pp 55.

Experiment 2

2. Spectrophotometric methods for determination of proteins

2.1 Introduction:

Quantitative determination of protein concentrations in solutions is often required in biochemistry. Several methods have been developed. However, most have limitations because either they are not sensitive enough or they are based on reactions with specific amino acids in the protein. Since the amino acid content varies from protein to protein no single method will be suitable for all proteins. Nonetheless, each method provides satisfactory results if proper experimental conditions are used and/or a suitable standard protein is chosen. Other important factors in method selection include the sensitivity and accuracy desired, the presence of interfering substances and the time available for the assay.

In this experiment three of these methods will be studied. Two are newer methods which are widely used and one is an older method. In the newer methods chemical reagents are added to the protein solutions to develop a colour whose intensity is measured in a spectrophotometer. The third method relies on direct spectrophotometric measurement.

2.2 Objectives:

Determination of proteins using three spectrophotometric methods.

a) Bicinchoninic acid (BCA, Smith) Method

This method is similar to the Lowry method but has an advantage of the colour being developed in a single step, because BCA can be included in the copper solution, instead of the two steps used in the Lowry method. The method has a high sensitivity, as low as 1 μg protein can be detected.

The reagents are available as kits.

Principle:

The blue colour resulting from this method is due to a) complex formation of the nitrogens of the peptide bonds with Cu^{2+} producing Cu^{+} under alkaline conditions b) Cu^{+} chelated by BCA to produce a copper-BCA complex with maximum absorption (λ_{max}) of 562 nm.

b) Bradford Method

This method is based on protein binding of a dye to arginine resdues. The method is very fast (15 min) and uses about the same amount of protein as the Lowry method. It is fairy accurate and samples that are out of range can be retested within minutes. The method is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. It has a high sensitivity and as low as 1 µg protein can be detected, about 1- 20 µg protein for micro assay or 20-200µg protein for macro assay.

Principle:

Binding of the dye Coomassie Brilliant Blue G-250 to protein in acidic solution causes a shift in wavelength of maximum absorption (λ_{max}) of the dye from 465nm to 595nm. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change. The absorbance at 595nm is directly proportional to the concentration of the protein. The colour is stable for one hour.

The method is useful since the extinction coefficient of the dye-albumin(protein) complex is constant over a 10-fold concentration range.

c) Warburg-Christian Method (A_{280}/ A_{260} Method)

This method is easy, sensitive and fast. It has a sensitivity of about 0.05- 2.0 mg protein/ml.

Principle:

This method is based on the relative absorbance of proteins and nucleic acids at 280nm and 260nm. Tyrosine and tryptophan residues in a protein absorb in the ultraviolet at 280nm. Since

the amounts of these residues vary greatly from protein to protein, the method is best used only for semiquantitative analysis of protein samples.

Nucleic acids which contaminate samples interfere with this method. This problem is overcome by the fact that nucleic acids absorb more strongly at 260nm than at 280nm, while the reverse is true for proteins.

A table has been compiled for A_{280}/ A_{260} ratio and the suitable correction factor(See table at the end)

2.3 Materials:

2.3.1 Chemicals:

Bovine serum albumin(BSA)

95% Ethanol

Coomassie Brilliant Blue G-250

Phosphoric acid

Kit for BCA method.

2.3.2 Equipment:

Spectrophotometer

2.3.3 Glasswares:

Quartz cuvettes

Polystyrene Cuvettes

Disposable cuvettes (Bradford method)

Test tubes

2.4 Method

Preparation of solutions:

a) BCA method:

Reagents A and B are available as parts of a kit. The chemical composition of each reagent is indicated.

Working standard solution (SWR): Mix 100 volumes of reagent A with 2 volumes of reagent B (work out the volumes depending on the number of students). The SWR should be green and is stable for 1 week.

Bradford reagent:

Commercial reagent is available but it can also be "Homemade".

Dissolve 100mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85%(w/v) phosphoric acid. After the dye has completely dissolved, filter through Whatman #1 paper just before use.

The reagent should be light brown. Filtration may have to be repeated to get rid of all blue components.

Albumin standard for BCA (2500µg/ml equivalent to 50µg in 20µl):

Dissolve 0.025g albumin in water and make up to 10ml.

Albumin standard for Bradford (200µg/ml):

Dissolve 0.02g albumin in water and make up to 100 ml.

A_{280}/ A_{260} Method

Prepare unknown consisting of a mixture of protein and nucleic acid.

Procedure:

a) **Bicinchoninic (Smith method)**

This method is available in kit form from Pierce Chemical Company (Rockford. Ill.).

1. Set up eight 1 ml test tubes as follows:

Tube	Water (µl)	Albumin standard (µl)	Unknown (µl)
A(blank)	20	-	-
B	16	4	-
C	12	8	-
D	8	12	-
E	4	16	-
F	-	20	-
G	-	-	20
H	-	-	20

2. Add 1 ml SWR to each tube and mix. Incubate for 30 min at 60°C.
3. Cool and read absorbance at 562 nm.

b) Bradford method:

1. Set up eight 18x150 mm test tubes as follows:

Tube	Water (ml)	Albumin standard (ml)	Unknown (ml)
A(blank)	1.0	-	-
B	0.8	0.2	-
C	0.6	0.4	-
D	0.4	0.6	-
E	0.2	0.8	-
F	-	1.0	-
G	-	-	1.0
H	-	-	1.0

2. Add 5 ml Bradford reagent to each tube, mix and incubate for 5 min at room temperature.
3. Measure the absorbance at 590 nm.

c) A_{280}/ A_{260} Method:

Measure the absorbance of the protein and nucleic acid mixture at both 280 nm and 260 nm against a blank (water).

Calculate the protein concentration in the unknown from the following equation:

A_{280} x correction factor = mg protein

2.5 Results:

Bicinchoninic (Smith method)

Tube	Protein concentration (µg/20(µl)	A_{562}
A	0	
B	10	
C	20	
D	30	
E	40	
F	50	
G		
H		

Plot a standard curve for absorbance at 562 nm against protein concentration (µg/ 20 µl/).

From the standard curve obtain the concentration of protein in the unknown.

Average protein concentration in the unknown = (µg/ml).

Bradford method:

Tube	Protein concentration (µg/ml)	A_{590}
A	0	
B	40	
C	80	

D	120	
E	160	
F	200	
G		
H		

Plot a standard curve of absorbance at 590 nm against protein concentration ((µg/ml).

From the standard curve obtain the concentration of protein in the unknown

Average protein concentration in the unknown = (µg/ml).

A_{280}/ A_{260} Method:

Unknown

A_{280} __________

A_{260} ___________

A_{280}/ A_{260} __________

Correction factor _______________

Unknown concentration __________________________ mg/ml

2.6 Discussion and conclusion:

2.7 Questions:

1. Why do proteins absorb at 280 nm?
2. Why do nucleic acids absorb at 280 nm?
3. What does A_{280}/ A_{260} signify?

2.8 References:

1. Boyer Rodney(2000). *Modern experimental Biochemistry*, 3rd Edition. Benjamin/Cummings (San Francisco),pp 41- 45
2. Bradford, M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of ligand-dye binding. *Anal*. *Biochem*.*72*, 248-254.
3. Smith, PK,Krohn, RI, Hermanaon GT et al.(1985). Measurement of protein using Bicinchoninic acid. *Anal*. *Biochem*.*150*, 76-85.
4. Stenesh J.(1984). *Experimental Biochemistry*, Allyn&Bacon,Inc(Boston) pp 69-79.
5. Stoscheck, CM.(1990) Quantitation of Protein.*Methods in Enzymology* *182*, 50-69.
6. http://www.ruf.rice.edu/~bioslabs/methods/protein/protein.html. Review methods for protein assay including absorbance and colorimetric (Lowry,Biuret,Bradford,BCA)

A_{280}/ A_{260} Method

A_{280}/ A_{260}	Correction factor	Nucleic acid (%)
1.75	1.12	0.00
1.63	1.08	0.25
1.52	1.05	0.50
1.40	1.02	0.75
1.36	0.99	1.00
1.30	0.97	1.25
1,25	0.94	1.50
1.16	0.90	2.00
1.09	0.85	2.50
1.03	0.81	3.00
0.98	0.78	3.50
0.94	0.74	4.00
0.87	0.68	5.00
0.85	0.66	5.50
0.82	0.63	6.00
0.80	0.61	6.50
0.78	0.59	7.00
0.77	0.57	7.50
0.75	0.55	8.00
0.73	0.51	9.00
0.71	0.48	10.00
0.67	0.42	12.00
0.64	0.38	14.00
0.62	0.32	17.00
0.60	0.29	20.00

Experiment 3

3 Homogenization, cell fractionation and marker enzyme assay in

3.1 cell fractions

This experiment consists of four parts which are as follows

a) Homogenization
b) Cell fractionation
c) Salting in and salting out
d) Dialysis

3.2 Objectives

- learn the techniques of tissue homogenization and different centrifugation
- perform enzyme assay on marker enzyme
- Salting in and salting out
- Dialysis

3.3 Introduction (Homogenization and cell fractionation)

Organelles are membrane-enclosed vesicles inside all eukaryotic cells that function in a variety of important cellular processes. In this lab, various subcellular fractions will be isolated from rat liver by a technique termed differential centrifugation. Then these fractions will be assayed for the presence of specific organelles using enzyme assays. In this experiment, first the technique of differential centrifugation will be carried out which will be followed by enzyme assay for acid phosphatase.

3.4 Cellular Fractionation

The arrangement of macromolecules within a cell is as important to cellular function as their catalytic activities. Cellular compartmentalization provides efficiency by bringing together related compounds that can interfere with each other (i.e. lysosomal hydrolytic enzymes). Cellular compartmentalization is accomplished in part by various subcellular organelles. In this lab module, several subcellular organelle fractions from liver cells will be isolated. The method

which will used to separate the various organelles utilizes **differential centrifugation** to isolate components of different densities. With this technique, the heaviest or most dense organelles, nuclei pellet in less time and with less force than is required to pellet lighter organelles such as mitochondria. First, a **cell homogenate** is made by rupturing the cell membranes in the tissue. The homogenate is then centrifuged for a short period of time to remove cell debris and nuclei. The supernatant is then transferred to another tube and centrifuged longer to pellet the lighter mitochondria.

For this type of fractionation, which tissue we use, and the method of homogenization are dictated by the biological system. Homogeneous cell populations from cell culture are well suited for cell fractionation. Some tissues, like those in the liver also have one cell type that predominates, so are also well suited. Most chlorophyll-free plant tissues are acceptable for preparing mitochondria, but recently-harvested plant tissues are usually required, making their use uncertain during winter months. Once a cell type is chosen (this experiment uses rat liver), it is important to obtain the organelles in a biochemically active, morphologically whole state. Homogenizers are used to break open the cells without damaging the organelles. Homogenizers have a precise clearance between the glass tube and pestle, which breaks the cell membrane leaving the smaller organelle membranes intact. The homogenization buffer is a solution which often includes sucrose to partially dehydrate the organelles, keeping them intact.

No technique used to isolate organelles is perfect. It is very difficult to get pure unbroken preparations of any organelle. Techniques providing optimal isolation of one organelle may completely rupture another organelle. Thus methods are often used to measure the contamination of one organelle fraction by another. This can be done by analyzing each organelle fraction for organelle-specific marker enzymes.

The purity of subcellular fractions is assessed by analyzing each fraction for various marker enzyme activities. It is well documented that some enzymes are located specifically within certain cell fractions. For example, succinate dehydrogenase is specific to mitochondria; glucose-6-phosphatase is specific to microsomes; acid phosphatase is specific to lysosomes; and lactate

dehydrogenase is specific to the cytoplasm. In this experiment acid phosphatase will be assayed in different fractions.

Subcellular Fraction	Relative Density	Marker Enzyme/Molecule
Nuclei	1	DNA, histones
Mitochondria	2	Succinate Dehydrogenase
Lysosomes	3	Acid Phosphatase
Microsomes	4	Glucose-6-Phosphatase
Cytosol	5	Lactate Dehydrogenase

3.5 Materials:

- Ice bucket
- Centrifuge tubes (50 ml and 15 ml disposable)
- Test tubes (12 x 75 mm, 13 x 100 mm)
- Cuvettes for Spec 20
- Single-edged razor blades
- Micropipettes & tips
- Rat liver

3.5.1 Chemicals

- Glycine
- Sucrose
- Triton WR 1339
- sodium dodecyl sulphate
- Tris

3.5.2 Equipments

- Tissue homogenizers
- Refrigerated centrifuge with J2-21 rotor

- Spectrophotometer
- Electronic balance
- Water bath

3.5.3 Glass wares

- Pipettes & Graduated cylinders
- Test tubes
- Beakers

3.6 Method:

Protocol for Rat Liver Fractionation

In the following procedures, keep the tissues and fractions **ice cold** whenever possible.

Tissue Homogenization**:**

A. Obtain a plastic weighing boat on ice containing pieces of rat liver.

B. Use a razor blade or scissors to dice the liver into small pieces.

C. Using an electronic balance, tare (zero) an empty weighing boat.

D. Use a spatula to add small pieces of liver to the boat until the mass is about one gm.

E. Place the boat with your one gm of liver on ice. Return the remainder of the liver to the ice bucket.

F. Mince the liver with a razor blade and transfer your one gm of liver tissue to a small glass (*Dounce*) homogenizer on ice.

G. Add ice-cold *Homogenization Buffer* (hypotonic solution of sucrose 0.25 mol/liter buffered with 20mmol/liter tris to pH 7) to the homogenizer.

H. Homogenize the tissue with up-and-down movement of the pestle until a homogeneous pink solution is obtained. Caution: Excessive grinding or heating can damage or inactivate subcellular fractions.

I. Pour the homogenate into a plastic 50 ml tube labeled "H" (for homogenate). Dilute the final concentration of the Homogenate to 1.0 gram tissue/10 ml of cold *Homogenization Buffer* and keep on ice.

J. Rinse the homogenizer with tap water, and return to the instructor.

Cell fractionation:

Fractionation of rat liver

Liver homogenate

↓

Centrifuge for 10 minutes at 600 g

↓

Supernatant →→ → → → Pellet is resuspended in homogenization buffer

↓ ↓

Centrifuge for 10 minutes at 600 g→→→→→ Pellet (Nuclei and cell debris)

↓

Supernatant

↓

Centrifuge for 10 minutes at 8000 g

↓

Supernatant →→→→→ Pellet (Mitochondria)

↓

Centrifuge for 10 minutes at 15 000 g

↓

Supernatant →→→→→ Pellet (Lysosomes)

↓

Centrifuge for 60 minutes at 100 000 g

↓

Supernatant (cytosol) →→→→→ Pellet (Microsomes)

Ideally each fraction should be Biosynthesis of non-essential amino acids amino acids from other non-essential amino acids resuspended in sucrose and the washings combined with the supernatants. This has the advantage of producing purer fractions, but the disadvantage of introducing an increasing dilution of cellular components.

Carefully resuspend the pellets in about 10 ml of sucrose and store on ice until required.

3.7 Assay for Acid Phosphatase

3.7.1 Objective:

To identify the lysosomal fraction after differential cell fractionation.

3.7.2 Introduction:

Acid phosphatase is present in lysosomes. The enzyme cleaves terminal phosphate groups and like other lysosomal enzymes operates maximally in acidic conditions. In this assay we will use a colorless compound, para-nitrophenol phosphate (pNPP) as the substrate for acid phosphatase. When the phosphate group of pNPP is cleaved, para-nitrophenol is generated. Para-nitrophenol is a yellow compound that is easily measured in a spectrophotometer.

3.7.3 Materials:

Different cell fractions obtained from the previous experiment.

Chemicals

- Glycine
- HCl
- Triton X-100
- Para-nitrophenol phosphate

3.7.4 Method:

Note: Store all reagents on ice during the exercise.

Buffer: 0.25 M of glycine-HCl buffer, pH 3.0 + 0.5% Triton X-100

Prepare 300 ml of 0.25 M of glycine-HCl, pH 3.0 and add 1.5 g of Triton X-100. Store at 4°C.

Substrate: 50 mM pNPP (mol. wt. 263 + 108 (6 H_2O) = 371)

Dissolve 1.85 g in 100 ml of water. Store at -20°C in freezer in 10-ml aliquots.

1. Label glass tubes (13 × 100 mm) and BLANK for crude homogenate, nuclei, mitochondria, lysosomes, mitochondria and cytosol etc. Pipette into each of these tubes:
0.1 ml Buffer [0.25 M glycine-HCl, pH 3.0, containing 0.5% Triton X-100]
50 µl Substrate [50 mM pNPP in water]
2. Make 1:10 dilution of A, Bs, Bp, Cs, Cp, Ds, and Dp [1:10 dilution prepared as follows: 0.1 ml of fractions + 0.9 ml of ice-cold Buffer A]. When ready, add to each of the (A, Bs, Bp, Cs, Cp, Ds, and Dp) 0.1 ml of the 1:10 dilution of the appropriate enzyme fraction. Add 0.1 ml Buffer A to the tube labelled BLANK. Note the starting time for each reaction.
3. Incubate all tubes at 37°C for 30 minutes.
4. Stop the reaction by adding 2.75 ml of ice-cold 0.2 M Na_3PO_4 (pH 12) to each tube.
5. Read Absorbance at 410 nm in a spectrophotometer adjusted to zero with the blank.
Determine the amount of para-nitrophenol liberated from the standard graph of para-nitrophenol.

3.8. Determination of protein content by Bradford protein assay

3.8.1 Objective:
To determine the protein content in various isolated subcellular fractions

3.8.2 Introduction:
The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis.
Assay materials including color reagent, and protein standard. The method described below is for the “Standard Procedure” with sensitivity to about 20 to 200 micrograms protein. Simply scale down the volume for the “microassay procedure, “which uses 1 ml cuvettes.
The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible

color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

3.8.3 Materials:

3.8.3.1 Equipments

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene (cheap) cuvettes may be used, however the color reagent stains both. Disposable cuvettes are recommended.

3.8.3.2 Chemicals

Coomassie Brilliant Blue
ethanol

phosphoric acid

Bovine serum albumin

Whatman paper #1

3.8.4 Method:

Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol; add 100 ml 855 (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through whatman #1 paper just before use.
The Bradford reagent should be a light brown in color. Filtration may have to be repeated to rid the reagent of blue components. “Homemade” reagent works quite well but it is usually not as sensitive as the commercial product.

Assay

Assays are performed with 1 ml sample plus 5 ml reagent

1. Prepare standards containing 0-200 micrograms protein/ml (bovine serum albumin or gamma globulin).
2. Prepare unknowns to estimated amounts of 20 to 200 micrograms protein per tube, same volume as the standard (1 ml).
3. (optional) Add 0.25 ml 1 M NaOH to each sample and vortex (This step is to be used only if the sample protein is not soluble in the dye-reagent).

4. Add 5 ml dye reagent and incubate 5 min.
5. Measure the absorbance at 590 nm.

Specific Activity

Specific Activity relates the enzyme activity to the amount of protein in the sample. We can measure the amount of protein, and we can measure the enzyme activity. Combining the two will give us the specific activity. Specific activity is defined in terms of enzyme activity per mg protein in the sample (ie, specific activity relates the enzyme units to the amount of protein in the sample).

3.8.5 Results:

Acid phosphatase activity in different subcellular fractions

<table>
<tr><th></th><th>Samples</th><th>Volume of Buffer</th><th>Volume of Substrate</th><th>Volume of the sample</th><th></th><th>Na_3PO_4</th><th>Absorbance at 410 nm</th></tr>
<tr><td>1</td><td>Crude homogenate</td><td>0.1 ml</td><td>50 µl</td><td>0.1ml</td><td rowspan="7">Incubate at 37^0 C for 30 minutes</td><td>2.75 ml</td><td></td></tr>
<tr><td>2</td><td>Nuclei</td><td>0.1 ml</td><td>50 µl</td><td>0.1ml</td><td>2.75 ml</td><td></td></tr>
<tr><td>3</td><td>Mitochondria</td><td>0.1 ml</td><td>50 µl</td><td>0.1ml</td><td>2.75 ml</td><td></td></tr>
<tr><td>4</td><td>Lysosomes</td><td>0.1 ml</td><td>50 µl</td><td>0.1ml</td><td>2.75 ml</td><td></td></tr>
<tr><td>5</td><td>Microsomes</td><td>0.1 ml</td><td>50 µl</td><td>0.1ml</td><td>2.75 ml</td><td></td></tr>
<tr><td>6</td><td>Cytosol</td><td>0.1 ml</td><td>50 µl</td><td>0.1ml</td><td>2.75 ml</td><td></td></tr>
<tr><td>7</td><td>Blank</td><td>0.1 ml</td><td>50 µl</td><td>0.1ml (H_2O)</td><td>2.5 ml</td><td></td></tr>
</table>

3.9 Standard graph of para-nitrophenol

Stock standard – 1 mg/ml paranitrophenol in triple distilled water (concentration 1000 µg/ml)

Working standard – 1 ml of Stock standard + 9 ml of water (concentration 100 µg/ml)

Serial number	Volume of working standard (ml)	Concentration (µg/ml)	Volume of water (ml)	Absorbance at 410 nm
1	0.1	10	2.9	
2	0.2	20	2.8	
3	0.4	40	2.6	
4	0.5	50	2.5	
5	1.0	100	2.0	
Blank	0.0		3.0	

3.10 Determination of protein in concentration in different subcellular fractions

Stock standard – 1 mg/ml paranitrophenol in triple distilled water (concentration 1000 µg/ml)

Working standard – 1 ml of Stock standard + 9 ml of water (concentration 100 µg/ml)

Serial number	Volume of working standard (ml)	Concentration (µg/ml)	Volume of water (ml)	Volume of the dye (ml)		Absorbance at 590 nm
Blank	0.0		3.0	5	Incubate for 5 minutes	
1	0.1	10	2.9	5		
2	0.2	20	2.8	5		
3	0.4	40	2.6	5		
4	0.5	50	2.5	5		
5	1.0	100	2.0			
	Volume (ml)					
Crude homogenate	0.1		2.9	5		
Nuclei	0.1		2.9	5		
Mitochondria	0.1		2.9	5		
Lysosomes	0.1		2.9	5		
Microsomes	0.1		2.9	5		
Cytosol	0.1		2.9	5		

Calculation of specific activity- Amount of paranitrophenol in 1 ml/ concentration of protein in 1 ml

3.11 Discussion and conclusion:

3.12 Questions:

1. Name the centrifugation technique used in this experiment to isolate the subcellular organelles?
2. What type of rotor was used in this experiment?
3. Which fraction had the maximum acid phosphatase activity?
4. Which fraction had the maximum protein content?
5. In which fraction was the maximum specific activity found?
6. What is the function of acid phosphatase in the body?

3.13 References

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. How cells are studied, Pages 135–198 (Chapter 4), *in* Molecular biology of the cell (Second edition). Garland Publishing, New York, 1218 pages.

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Experiment 4

4. Salting in and Salting out of proteins and Dialysis

4.1 Objective:

To learn the technique of isolation of proteins on the basis of their solubility. This experiment consists of two parts viz.,

Part I: salting in, salting out of proteins and dialysis of proteins.

Part II: Determinatioin of protein content by biuret assay

4.2 Introduction (Salting in and salting out of proteins)

Proteins show a variation in solubility depending on their solution ionic environments. These frequently complex effects may involve either specific interactions between charged side chains and solution ions, particularly at high salt concentration, reflect more comprehensive changes in the solvent properties.

Salting in

The effects of salts such as sodium chloride on increasing the solubility of proteins is often referred to as **salting in**. The **salting in** effect is related to the nonspecific effect the salt has on the ionic strength. When low concentrations of salt is added to a protein solution, the solubility increases. This could be explained by the following:

Salt molecules stabilize protein molecules by decreasing the electrostatic energy between the protein molecules which increase the solubility of proteins.

Salting out

When the ionic strength of a protein solution is increased by adding salt, the solubility decreases, and protein precipitates. This could be explained by the following:
The salt molecules compete with the protein molecules in binding with water.

Many globular proteins precipitate out at very low ionic strengths or in pure water. This happens because different proteins are able to interact favorably leading to formation of a complex. When this complex formation is extended between many protein molecules, it can lead to protein precipitation.

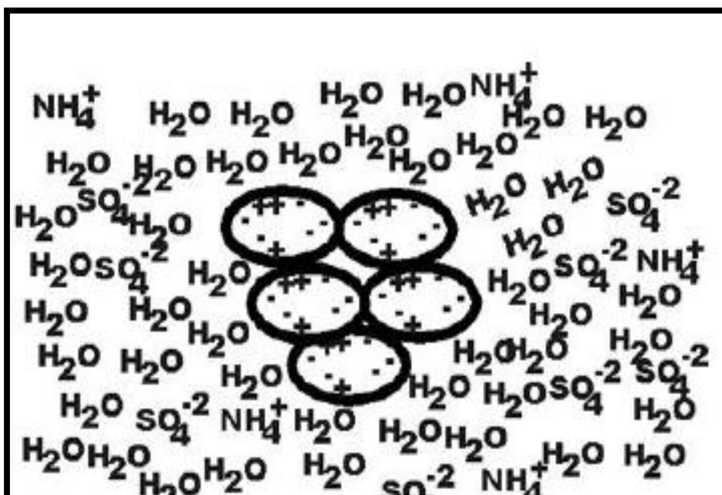
Some salts, such as high concentrations of ammonium sulfate, have general effects on solvent structure that lead to decreased protein solubility and **salting out**. In this case, the protein molecules tend to associate with each other because protein-protein interactions become energetically more favorable than protein-solvent interaction. Proteins have characteristic salting out points, and these are used in protein separations in crude extracts.

The most effective region of salting out is at the isoelectric point of the protein because all proteins exhibit minimum solubility in solutions of constant ionic strength at their isoelectric points.

The salt commonly used is **ammonium sulfate** because:

1. Its large solubility in water.
2. Its relative freedom from temperature effects.
3. It has no harmful effects on most of the proteins.

Note: the amount of salt needed to isolate a specific protein is determined from the salt's fractionation table.



Protein in Ammonium Sulfate

biocadmin.otago.ac.nz

http://biocadmin.otago.ac.nz/fmi/xsl/bioc2/learnbitslecture.xsl?-db=BIOC2web.fp7&-lay=Lectures&-recid=5450&-find

4.3 Introduction to Dialysis

Removal of salt molecules from the isolated protein solution through a semi permeable dialysis bag is called dialysis. The salt molecules move from the more concentrated solution (from inside the dialysis bag) to the less concentrated solution (e.g. distilled water).

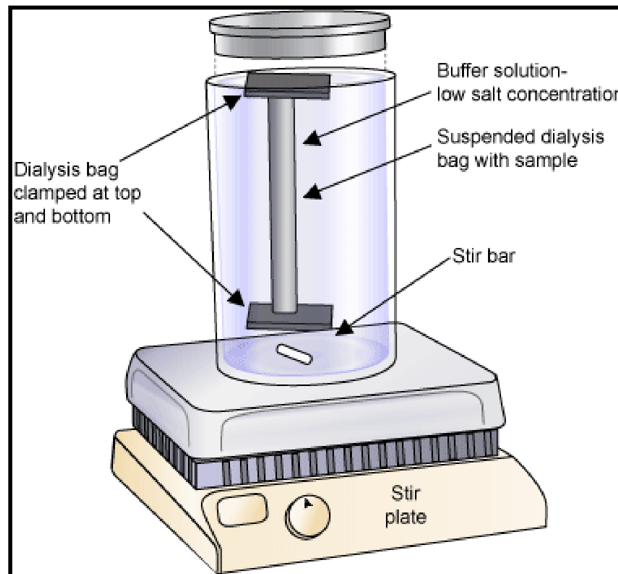


Figure1:……………………….

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Part I: Separation of Myoglobin

In this experiment myoglobin is isolated from skeletal muscle by salting out technique which discards up to 75% of the crude proteins in the protein purification process.

Part II: Biuret assay of protein

The biuret reagent is: alkaline copper sulphate. The biuret reagent reacts with peptides and proteins to give a purple colored cu^{2+}- peptide complex. This colored complex can be measured quantitatively by a spectrophotometer in the visible region. The color obtained is directly proportional to the number of peptide bonds present in the protein.

The assay is called Biuret because the reaction is positive with the biuret reagent. In this experiment the amount of isolated protein from the skeletal muscle is determined by the biuret assay and from the standard curve of bovine serum albumin (BSA).

4.4 Materials:

- Skeletal muscle
- Disposable cuvettes

4.4.1 Chemicals

- Solid ammonium sulphate
- Ammonium hydroxide solution (2 M)
- Bovine serum albumin

4.4.2 Equipments

- Waring blender
- Magnetic stirrer
- Spectrophotometer
- pH meter

4.4.3 Glassware

- Pipettes
- Graduated cylinders
- Test tubes
- Beakers
- Glass rods

4.4.4 Equipments

- Spectrophotometer
- Mettler
- Water bath

4.5 Method:

Part I:

Cut the skeletal muscle (100 g) into small pieces and homogenize for 10 seconds in 100 ml of distilled water at room temperature in a blender. Divide the homogenate into 5 equal parts (A group of three students can work with each part of the homogenate). Allow the homogenate to stand for 20 minutes and then centrifuge at 2000 rpm for 10 minutes t 4^0 C. Discard the residue and adjust the pH of the supernatant to 7.0 by the addition of NH_4OH (2M). Add solid ammonium sulphate to the supernatant to get a 0-35% saturation of the solution. The required amount of ammonium sulphate can be calculated from the table. The salt should be added slowly with constant stirring. Re-adjust the pH to 7.0 and allow the solution to stand at t 4^0 C for 40 minutes. Separate the resulting precipitate by centrifugation and discard. To the supernatant add ammonium sulphate at t 4^0 C to get 55% saturation. Centrifuge at 2500 rpm for 20 minutes and dissolve the resulting precipitate in 10 ml of distilled water. Take the red supernatant which contains myoglobin. This is a crude preparation of myoglobin.

Desalting

Remove the ammonium sulphate used in "the salting out" procedure by using dialysis tubing. Place the protein solution in a bag made by tying knots in the ends of cellulose dialysis tubing. Put the sealed bag in a large volume of cold water which is being moderately stirred. Continue the process until ammonium sulphate is completely removed and the protein solution is equilibrated with water (6-8 hours are required for complete dialysis). Determine the amount of protein in the dialysed sample by Biuret method.

Determination of protein by Biurette Method

Preparation of reagents

1. Protein standard (5 mg bovine serum albumin/ml). Prepare fresh.

2. Biuret reagent (Dissolve 3 g of copper sulphate ($CuSO_4.5\ H_2O$) and 9 g of sodium potassium tartrate in 500 ml of 0.2 mol/litre NaOH; add 5 g of potassium iodide and make up to 1 litre with 0.2 mol/litre NaOH).
3. Add 3 ml of biuret reagent to 2 ml of protein solution, mix and warm at 37^0 C for 10 minutes. Cool and read the extinction at 540 nm. Prepare a standard graph using BSA. Calculate the protein concentration in the sample from the standard graph.

4.6 Results:

Stock standard: 5 mg of BSA/ml

Working standard: Dilute to get a linear standard graph

	Volume of the standard (ml)	Concentration of the standard	Volume of H_2O (ml)	Volume of Biurette reagent (ml)	37^oC for 10 minutes	Absorbance at 540 nm
Blank			2	3		
1				3		
2				3		
3				3		
4				3		
5				3		
Sample				3		
Sample				3		

4.7 Discussion and conclusion:

4.8 Questions:

1. Explain why ammonium sulphate is used to precipitate proteins?
2. What is the principle of dialysis?
3. Which fraction had the highest protein concentration?

4.9 References:

Plummer DT. 1987. An Introductioin to Practical Biochemistry, Third Edition, Tata McGraw – Hill Publishing Company Limited, New Delhi, pp265- 270, 1987.

Experiment 5

5 Filtration using cheese cloth, filter paper, ultramembrane, nitrocellulose membrane

5.1 Objectives:

To learn different filtration techniques viz.,

a) Cheese cloth
b) Filter paper
c) Nitrocellulose membrane

5.2 Introduction:

Separation of insoluble substance from a liquid, by allowing it to pass through a porous material is called filtration. Filtration can remove suspended material from liquid as long as the pores in the filter paper are smaller than the particles of the suspended substance.

Filtration is done by passing a mixture through a filter. The **residue** is the substance that remains on the filter paper. The **filtrate** is the substance that flows through the filter paper.

5.3 Materials:

- Filter paper
- Cheese cloth
- Nitrocellulose membranes
- DNA

5.3.1 Glassware

- Beaker
- Glass rod
- Funnel

5.4 Method:

a) Filtration (removal of sediment) using Cheesecloth

1. Measure 500ml (2 cups) of distilled water into a large graduated cylinder
2. Add 30 ml (2 tbsp.) of finely crushed leaves into the water and stir gently.
3. Record the appearance of the mixture in the cylinder.
4. Place a clean piece of screen over a 1000-mL beaker, labeled Beaker A, and spread a piece of cheesecloth (or kitchen cloth) over the screen
5. Pour the mixture slowly through the filter (make sure it goes through both layers) into the beaker
6. Observe the liquid in the beaker and the solid on the filter and record.
7. Repeat steps 1-6 using Beaker B and a piece of cotton fabric placed over the screen. Make sure you rinse the screen in between.
8. Repeat steps 1-6 using Beaker C and a coffee filter placed over the screen. Make sure you rinse the screen in between.
9. Compare the results of Beaker A, B and C.

b) Filtration (removal of sediment) using Filter Paper

1. Fold the filter paper into four and fit it into the funnel.
2. When the filter paper does not fit well into the funnel, slightly adjust the folding in order to fit it perfectly.
3. In order to make the filter paper fit perfectly, cut the edge of the over lapping filter paper in contact with the funnel obliquely. Wet the paper with the solvent and press with fingers to fit the paper in.
4. Place a container in a manner that the leg of the funnel touches its inside wall.
5. Pour the unfiltered solution used in the earlier experiment in the funnel.

Filtration and Washing of a Precipitate

1. Put a glass rod on the mouth of the container and decant a sample slowly.
2. The filtration proceeds smoothly when the supernatant and then the mixture containing the precipitate are filtrated. Such operation is suitable when the filtrate is required.
3. When the precipitate is needed, quickly pour the swirling sample onto the filter paper. Next, add the filtrate to the remaining precipitate in the container, and then pour the mixture onto the filter paper again.
4. Wash the collected precipitate on the filter paper with a washing liquid a few times.

c) Nitrocellusose filter

Nitrocellulose filter is used to separate single stranded DNA and Proteins. Proteins and single stranded (ss) DNA adsorb to nitrocellulose filters. A solution containing ss DNA/protein is filtered through a nitrocellulose filter. The amount of ss DNA/protein adsorbed to the filter can be determined by measuring the absorbance of the solution before and after filtration at 260 and 280 nm respectively.

Adsorption of Protein

1. Prepare a solution of albumin and casein (5 g/litre)
2. Measure the absorbance at 280 nm
3. Filter the solution through a nitrocellulose filter and measure the absorbance of the filtrate at 280 nm.

Adsorption of Single Stranded DNA

1. Dissolve 10 mg of DNA (obtained from Sigma or any other commercial sample) in buffered saline and make up the volume to 100 ml.
2. Read the absorbance at 260nm.
3. Heat the solution by placing in a boiling water bath for 10 minutes.
4. Pass the solution quickly before it renatures through the nitrocellulose membrane.
5. Read the absorbance of the filtrate at 260 nm.

5.5 Results:

Filtration (removal of sediment) using Cheesecloth

	Volume of unfiltered solution (ml)	Volume of filtered solution (ml)
Beaker A		
Beaker B		
Beaker C		

Nitrocellusose filter

	Sample	Absorption at 280 nm	Absorption at 260 nm
1	Albumin		
2	Casein		
3	DNA		

5.6 Discussion and conclusion:

5.7 Questions:

1. At which wavelength does DNA have maximum absorption and why?
2. At which wavelength does protein have maximum absorption and why?
3. How would you separate a mixture of DNA from protein?

5.8 References:

Physical Biochemistry: Applications to Biochemistry and Molecular Biology
by David M. Freifelder.

Experiment 6

6. Paper and Thin Layer Chromatography (TLC)

6.1 Objectives:

Separation of amino acids using paper chromatography

Separation and identification of sugars in fruit juices using TLC.

6.2 Introduction:

Paper chromatography is one method for testing the purity of compounds and identifying substances. Paper chromatography is a useful technique because it is relatively quick and requires small quantities of material. In paper chromatography, substances are distributed between a stationary phase and a mobile phase. The stationary phase is usually a piece of high quality filter paper. The mobile phase is a developing solution that travels up the stationary phase, carrying the samples with it. Components of the sample will separate on the stationary phase according to how strongly they adsorb to the stationary phase versus how much they dissolve in the mobile phase.

Separation of compounds on a thin layer of silica gel is similar to paper chromatography. However, the method is rapid and separations can be completed in less than one hour. The substances used include silica gel, aluminium oxide, magnesium silicate etc.

In TLC the stationary phase is a layer (0.25 – 0.5 mm) of solvent spread uniformly over the surface of the glass pr plastic plate. The separation depends on

1. Partition of a solute between a moving solvent phase and a stationary aqueous phase. The solute therefore moves in the direction of a solvent flow at a rate determined by the solubility of the solute in the moving phase. Thus a compound with high mobility is more attracted to the moving organic phase than to the stationary aqueous phase bound to the support medium.
2. Ion exchange effect: Any ionized impurities in the support medium will tend to bind or attract oppositely charged ions and will therefore reduce the mobility of these solutes.
3. Temperature: since temperature can affect the solubility of a solute in a given solvent temperature is also an important factor and often a chromatography laboratory has a fixed temperature for optimum results.
4. The molecular weight of a solute also affects the solubility and hence chromatographic performance.
5. Adsorption of compound (solute) onto support medium: Although the support medium (silica gel) is inert, this is not always the case. If a solute tends to bind to the support medium this will slow down its mobility in the solvent system.
6. Composition of the solvent: Since some compounds are more soluble in one solvent than in the other the mixture of solvents used affect separation of the compounds.

Expression of the results:

The term "Rf" (relative flow) is used to express the performance of a solute in a given solvent system/support medium. The term Rf value may be defined as the ratio of the distance moved by a compound to that moved by the solvent. Rf value is constant for a particular compound, solvent system and insoluble matrix.

$$Rf = \frac{\text{Distance of migration of solute}}{\text{Distance moved by solvent}}$$

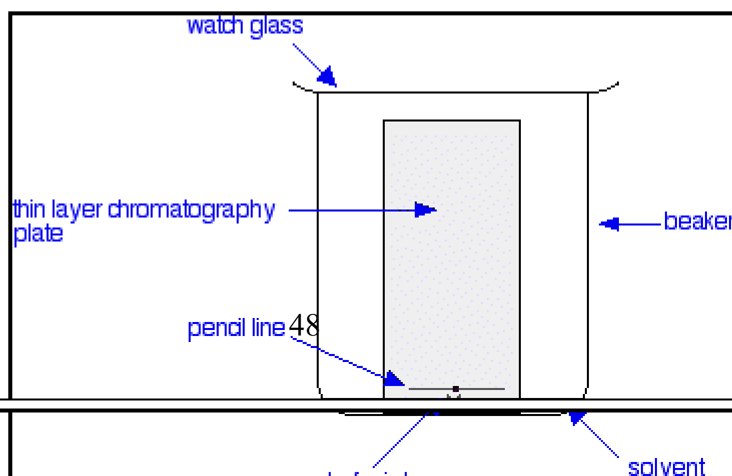


Figure 1: Thin layer chromatography

www.chemguide.co.uk/.../thinlayer.htmlwww.chemguide.co.uk/.../thinlayer.html

6.3 Materials:

Paper chromatography

1. Chromatography paper (30 cm X 30 cm)
2. Mixture of amino acids
3. Solvent 1- BuOH/HAc/H_2O 120/30/50 by volume
4. Solvent 2 – PhOh/H_2O/NH_3
5. 20 % ethanol
6. 0.2% ninhydrin in acetone
7. Microsyringe

Thin Layer Chromatography

1. Thin layer plates of silica gel: Prepare a slurry of silica gel G in 0.02 M sodium acetate and pour it onto the plate evenly. Dry the plate and activate it before use by heating at 150^0 C for 30 minutes.
2. Solvent: Ethyl acetate: isopropanol: water: pyridine (26:14:7:2).
3. Standard sugars (1% in 10% isopropanol) glucose, fructose, lactose, ribose, glucoronic acid, xylose, rhamanose, sucrose etc.
4. Mixture of fruit juices.

6.4 Method:

Paper chromatography

Place 150-200 ml of solvent 1 in a chromatography tank and allow the tank to equilibrate.

Prepare the chromatography paper by marking the origin 3 cm from one corner towards the center of the paper.

Spot by means of a micro-syringe, 10 µl of amino acid mixture allowing the solution to dry. Place the chromatogram in the frame and run in the solvent for about 16 hours.
Remove the chromatogram from the tank, mark the solvent front and allow the paper to dry in a current of cold air for about half an hour.

Thoroughly rinse the tank and prepare solvent 2. The requisite amount of ammonia is added to the phenol solution just before use.
Place 150-200 ml of solvent 2 in the tank and allow the tank to equilibrate.
Turn the chromatogram through 90^0 and place in the solvent 2.
Allow the chromatogram to run overnight.
Remove from the tank, mark the solvent and allow the paper to dry.
Place 0.2% ninhydrin in the dipping tray and dip the chromatogram.
Heat at 105^0 C for 10 minutes to develop the spots.
Outline the individual spots and compare the amino acid pattern with a standard amino acid map.

Thin Layer Chromatography
You will be provided with a silica gel plate. Place the plate on the bench with the silica gel side upward (handle the plate by the edges).

With a pencil draw a line about 2 cm from and parallel to the shorter edge of the plate. Draw the line gently so as not to break the surface of the gel. At equally spaced intervals mark the line at five different places using the tip of the pencil on the silica gel plate. Carefully spot the standard sugars solutions and the mixture of fruit juices with a 5 µl pipette without making a hole in the adsorbant. Each sample should be on different mark. The spot on the silica gel should be no more than 2 mm in diameter.

Into the chromatography chamber, pour enough chromatography solvent to a depth of about 1 cm. Place the gel coated plate in the chamber as shown in the figure. Cover the glass chamber tightly with aluminium foil. The solvent will rise up the silica gel by capillarity. When the solvent front is between 1 and 2 cm from the top of the gel, remove the chromatogram from the chamber and mark the position of the solvent front.Dry the plate in a stream of cold air in the

fume cupboard. Locate the sugars by spraying the plate with aniline-diphenylamine and heating the plate briefly at 100^0C in an oven. Note the colour of each sugar and measure the R_f values.

6.5 Results:

Calculate the Rf value for each spot and identify the sugars by comparing them with spots of standard sugars.

Sample	Rf	Identification
Glucose		
Fructose		
Sucrose		
Ribose		
Glucoronic acid		
Sugars in Fruit Juices		
1		
2		
3		
4		
5		
Amino acids		

6.6 Discussion and Conclusions:

6.7 Questions:

1. Why is TLC compared with paper chromatography?
2. Why is aniline-diphenylamine used to develop colour?
3. Why is ninhydrin used to identify the amino acids?

6.8 References:

1. An introduction to Practical Biochemistry by Plummer D.T (1971). McGraw Hill book Company, UK.
2. Physical Biochemistry by David Friefelder (1976) W. H. Freeman and Company, USA.

Experiment 7

7. Separation of proteins by gel filtration and determination of molecular by gel filtration

7.1 Objectives:

1. The objective of this experiment is for students to learn the principles of gel filtration chromatography by separating the proteins according to the molecular weights.
2. A practical experience on gel filtration chromatography in the laboratory
3. Importance of gel filtration chromatography and procedures in purification.

7.2 Introduction:

Gel filtration chromatography is an important method in the repertoire of the protein purifier (Prenata, 1989; Stellwagen, 1990). Other names for this method include size exclusion, gel exclusion, molecular-sieve and gel permeation chromatography. This separation method is unique in fractionating without requiring protein binding, thus significantly reducing the risk of protein loss. Gel filtration is well suited for biomolecules that very sensitive to the changes in pH, concentration of metal ions and cofactors and harsh environmental factors.

If the protein is relatively large (over 100 KDa), gel filtration could be used as an initial step in the purification.

7.3 Principle:

Gel filtration chromatography separates proteins according to their size. The gel filtration matrix contains pores which permit the buffer and smaller proteins and protein complexes. Therefore, excluded larger proteins migrate around the matrix particles and elute from the column before the smaller proteins (figure 1). Medium-sized proteins can enter the larger size pores in the matrix, and so they reach the end of the column later. Small proteins are able to enter all the pores, and they have the largest volume to pass through before emerging from the column last.

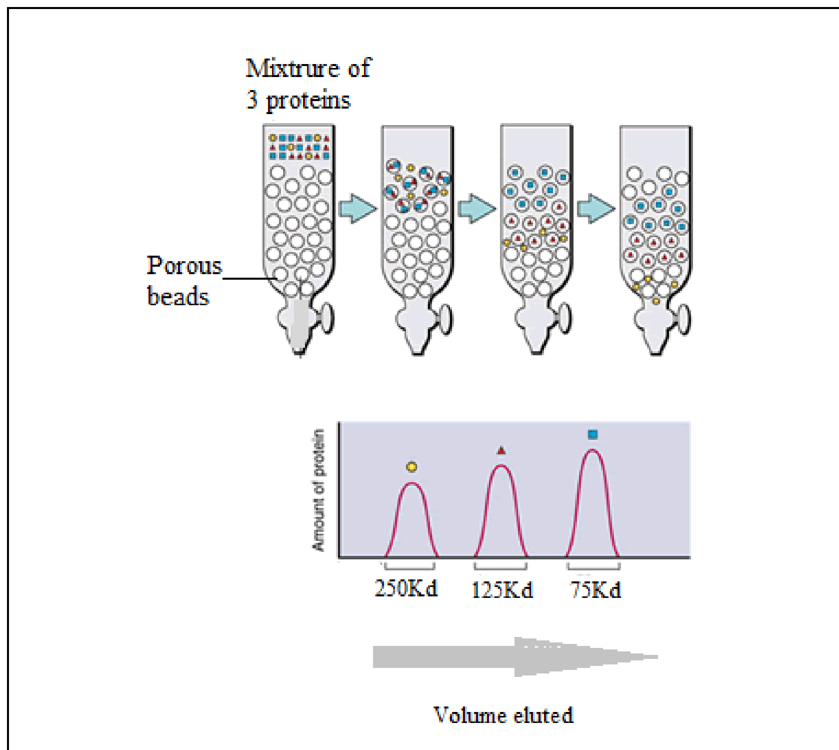


Figure 1: The principle of gel filtration chromatography (Figure has been taken from-(http://mortada8.maktoobblog.com/category/instrumental-analysis-studies/chromatography-studies/)

The gels used as molecular sieves consist of cross-liked polymers that are generally inert, do not bind or react with the material being analyzed, and are uncharged. The space within the gel (between the bead particles) is filled with liquid and this liquid occupies most of the gel volume and act as a mobile phase. The gels currently in use are of three types: dextarn, agarose, and polyacrylamide.

Dextran is a polysaccharide composed of glucose residues and prepared with various degrees of cross-linking to control pro size, and is supplied in the form of dry beads that swell when water is added. It is commercially available under the trade name Sephadex. It is mainly used for separation of small peptides and globular proteins with small to average molecular mass.
Polyacrylamide gels are prepared by cross-linking acrylamide with N,N'methylenebisacrylamide. Also, the pore size is determined by the degree of cross-linking. The separation properties of polyacrylamide gels are mainly the same as those of dextrans. They are marketed as Bio-gel P, and are available in wide range of pore sizes.
Agarose is a linear polymer of D-galactose and 3,6-anhydro-1-galactose and forms a gel that is held together without cross-links by hydrogen bonds. It is dissolved in boiling water and forms a gel when cooled. The concentrated of the material in the gel determines the size of the pores – which are much larger than those of sephadex and Bio-gel P. This makes it useful for the analysis or separation of large globular proteins or long, linear molecules such as DNA. Agarose is supplied as wet beads called Sepharose and Bio-gel A.

The pore size determines the range of molecular weight in which fractionation occurs.
The gel beads come in various sizes: coarse (large), medium, fine, and superfine. The rule is the larger the beads, the more rapid the flow rate and the poorer the resolution. This is because as the flow rate increases, the time available for the molecules to equilibrate between the mobile phase and the pore space in the stationary phase decreases. The larger beads are for very large preparation in which resolution is less important than time. While, super fine is used if maximum resolution is required – for example for analytical work – but it is very slow.

7.4 Advantages of gel filtration:

It is the best method for separation of molecules differing in molecular weight because:

1. It doesn't depend on temperature, pH, ionic strength and buffer composition, so, separation can be carried out under any conditions.
2. There is very little adsorption.
3. There is less zonal spreading than in other techniques.
4. The elution volume is related to the molecular weight.

To estimate the molecular weight for a protein, several proteins with known molecular weights are run on the column and their elution volumes determined. If the elution volumes are then plotted against the log molecular weight of the corresponding proteins, a straight line is obtained for the separation range of the gel being used. If the elution volume of a protein of unknown molecular weight is then found, it can be compared to the calibration curve and the molecular weight determined.

It is important that the gel should be homogenous, free from bubbles, free from cracks, and free from spaces between the walls. And it should be covered by the liquid "mobile phase" all the time.

7.5 Terms:

"Total bed volume": the total volume of material in the column (both solid and liquid).

"Void volume": the volume of the mobile phase in the column.

"Loading the column": small volume of sample is placed on the stationary phase and allowed to enter the column.

"Eluting the column": as different substances move through the column, they separate and appear in the effluent when particular volumes of liquid have passed through the column.

"Elution volume": the amount of liquid that must be added to produce a peak of a particular solute in the effluent.

The gel filtration material that will be used in the experiment is called Sephadex G-100 and it will separate molecules with molecular weights from 4,000 to 150,000. Those molecules which are with molecular weight larger than 150,000 will be excluded from the beads. We will separate a mixture of blue dextran m.wt.= 2,000,000 and bromophenol blue m.wt.= 669.99 .

7.6 Materials:

1- Sepadex G-100 .

2- Sample solution (mixture of bromophenol blue and blue dextran).

3- 0.1 M sodium chloride solution.

4- Chromatography column.

5- Graduated centrifuge tubes (at least 25).

6- Pasteur pipette.

7- Plastic cuvettes.

8- Spectrophotometer.

7.7 Method:

1- You have been provided with a column (2x30 cm) packed with sephadex G-100 in 0.1 M sodium chloride solution (NaCl)

2- Try to ensure that the column is not allowed to run dry.

3- Clean and dry at least 25 test tubes.

4- Carefully remove the layer of NaCl solution from above the resin (gel matrix) using a pasture pipette, and let only a very thin layer of salt.

5- Again using the pasture pipette, very slowly layer the sample mixture solution on the top of the resin, by adding the tip of pipette on the wall of column. Care should be taken not to disturb the gel beads.

6- Open the screw clip, and start to collect the fractions of about 3 ml each.

7- After the sample mixture penetrates the gel so that you can see the gel beads, carefully fill up the column with NaCl solution, and complete the collection of fractions.

8- Collect at least 25 fractions.

9- Read the absorbance of each fraction at 560 nm with the help of spectrophotometer using 0.1 M NaCl as a blank.

10- Record your results in the table.

11- Plot a graph of O.D (absorbance) at 560 nm against fraction number.

The graph should show two well separated peaks.

7.8 Results:

1- From the curve, indicate which peak is for promophenol blue and which is for blue dextran and give your explanation.

2- How can you know the number of substances in your sample?

3- Find the elution volume to each substance in your sample.

7.9 Discussion and conclusion:

7.10 Questions:

1- Can get filtration chromatography be used to separate small molecules from proteins?
2- What is the difference between gel filtration and gel electrophoresis?

7.11 References:

Prenata, A.Z. (1989), Separation on the basis of size: gel permeation chromatography. pp.298-302 in protein purification methods: A practical approach. Harris and S.Angal, eds.IRL Press, new York.

Stellwagen, E. (1990), Meth. Enzymol. 182;317-328. Gel filtration.

Daniel M. bollag, Michael d. Rozycki and Stuart J. Edelstein, (1996). Protein Methods. Second edition

Experiment 8

8. Separation of proteins by ion exchange chromatography

8.1 Objectives:

1- The objective of this experiment is for students to learn the principles of ion exchange chromatography by separating the charged molecules using a salt gradient.
2- Identification of the factors influencing ion exchange chromatography.
3- A practical experience on ion exchange chromatography in the laboratory
4- Importance of ion exchange chromatography and procedures in purification.

8.2 Introduction:

Ion exchange chromatography (IEC) is the most commonly used chromatographic method for protein purification. Its popularity stems from the possibility of high resolution protein separation, the relative ease of use, reproducibility and availability. The ion exchange principle

permits the protein to bind even when a large buffer volume is applied, making this method especially useful for an initial purification step from a crude extract. Ion-exchange chromatography separates molecules based on their charged groups, which cause the molecules to interact electrostatically with opposite charges on the stationary-phase matrix. The stationary phase carries ionizable functional groups coupled to an inert matrix material. Because of the principles of electroneutrality, these immobilized charges are electrostatically associated with exchangeable counterions from the solution. Charged molecules to be purified compete with these counterions for binding to the charged groups on the stationary phase and are thereby retarded on the basis of their charge. Different types of molecules will bind to the matrix with affinities that depend on both the conditions used and the types and number of individual charged groups. These differences lead to resolution of various molecule types by ion- exchange chromatography.

8.3 Principle:

Separation and purification of proteins using ion exchange chromatography is based primarily on differences in the ionic properties of surface amino acids. Exposed arginine, histidine, and lysine residues are generally positively charged and aspartic acid and glutamic acid residues possess a negative charge at neutral pH. Thus at a given ph, a protein will possess an overall net charge. At a lower pH, the net charge will be more positive and at a higher ph, the net charge will be more negative. The pH at which the positive charges equal the negative charges (in other words, the net charge of the protein is zero) defines that protein's isoelectric point (PI). For IEC, a good rule to follow when separating a protein whose isoelectric point is known is to select a working pH which is 1 unit away from the PI of the protein. At this pH, the protein will possess a high enough net charge to bind well to the ion exchange column.

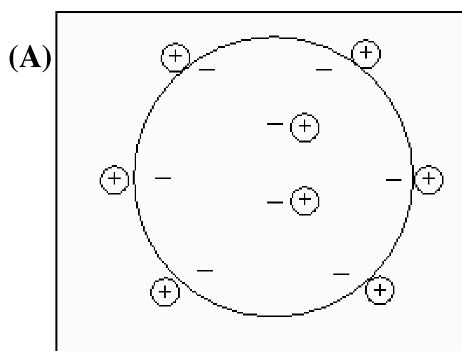
How does an ion exchanger bind protein?

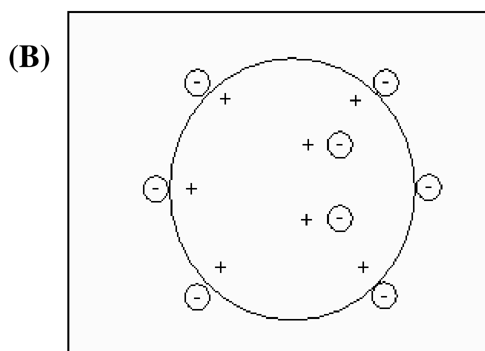
Assuming that the protein possesses a net charge, an ion exchanger needs an opposite charge in order to bind that protein. Ion exchangers are typically composed of a charged (ion exchange)

group attached to an insoluble matrix. A positively charged group, such as DEAE (diethylamino ethyl), defines the matrix as an anion exchange matrix whereas a negatively charged group, such as CM (carboxymethyl) makes a cation exchange matrix.

DEAE: $-CH_2-CH_2-N^+H(CH_2CH_3)_2$

CM: $-CH_2-COO^-$

(A) 

(B) 

A Cation exchange resin   B Anion exchange resin

Thus, if a protein has a negative net charge at a given pH, an anion exchange matrix should be used for its purification.

8.4 Materials:

Chromatography

Dowex-50 resin

Hydrochloric acid (4M)

Hydrochloric acid (0. 1M)

Glass wool

Amino acid mixture final concentration 2mg/ml in 0.1M HCl

Tris-HCl buffer (0. 2M, pH 8.5)

Sodium hydroxide (0.1M)

Separatory funnel (500ml)

Acetate buffer (4M, pH 5.5)

Ninhydrin reagent

Methyl cellosolve

Ethanol (50% v /v)

Ninhydrin (2g/1 in acetone)

8.5 Method:

Step-1: It is necessary to make sure that the resin is in proper state before it is used for chromatography, for example, if a cationic exchanger (Dowex-50) is used, it must be in the acid form or must contain H. This is achieved by suspending the resin in 4N HCl for 15 minutes. This assures that the resin is saturated with H ions. Now filter the suspension through a Buchner funnel and repeatedly wash with distilled or deionized water, till the filtrate is of neutral pH.

Step-2: Setting the column: The resins are available in several mesh sizes. Generally for amino acids 200-400 mesh is used. Suspend 10g of the resin (Dowex-50) in water, stir and allow to settle. The supernatant containing any light particles is decanted. Now add HCl O.1M enough to cover the resin, stir well and let it stand for about 1 hour. This is to equilibrate the resin to pH about 1-2. Use the suspension to set up a column (about 30xO.9cm). Run through the column, about two column volumes of the HCl solution. Adjust the flow rate to about 1ml/3min.

Step-3: Loading: The amino acid mixture to be separated is dissolved in HCl O.1M, at this pH; all amino acids will have positive charges. Place O.2ml of the sample on the top of the column without disturbing the top layer. Now the tap is opened and the sample allowed to percolate through. When the level just reaches the top layer, stop the flow.

Step-4: Elution: The amino acids are now bound to the column and these can be released and eluted by increasing the pH of the eluant buffer. As the pH increases, the protonated form of the amino acids will start ionizing, and when the pI value is reached, they will have no net charge and therefore will not be bound to the resin any more, they will be eluted out. Since the pI of each amino acid is different, they will be eluted at different pH values and thus the separation is achieved.

Now apply 2ml of 0.1M HCl to the top of the resin and connect the column to a reservoir containing 500ml of 0.1M HCl and collect at total of forty 3ml fractions. Test five of the tubes at a time for the presence of amino acids by spotting a sample from each tube onto a filter paper; dip this in the acetone solution of ninhydrin and heat in an oven at 105°C. If amino acids are

present, they will show up as blue spots on the filter paper. When the first amino acid has been eluted, remove the reservoir of 0.1M HCl and allow the level of acid to fall to just above the resin. Run 2ml of 0.2M Tris-HCl buffer (pH 8.5) onto the column, then connect to a reservoir of this buffer and continue with the elution until the final amino acid is removed from the column.

Detection of amino acids:

Adjust the pH of each tube to 5 by the addition of a few drops of acid or alkali. Add 2ml of the buffered ninhydrin reagent and heat in a boiling water bath for 15 min. Cool the tubes to room temperature, add 3ml of 50% (v/v) ethanol and read the extinction at 570 after allowing the tubes to stand for 10 min. Set up the appropriate blanks and plot the absorbance for each tube against the number of fractions.

8.6 Results:

1. How many amino acids do you have in your sample?
2. In what order are they eluted and why?
3. Explain why aspartate comes off the column before glutamate?

Discussion and conclusion:

8.7 Questions

1- Why does ion-exchange chromatography generally use a salt gradient or a pH gradient?

2- Is ion-exchange chromatography reverse phase chromatography?

3- What should we be careful of when loading a column chromatography?

8.8 References:

Cooper, G.T., (1977) The Tools of Biochemistry, John Wiley & Son, New York.

Dryer, R.L. and Lata, G.F., (1989). Experimental Biochemistry Oxford University Press, New York.

Jayaraman, J. (1981), Wiley Estern Limited, New Delhi.

Minch, M.J. (1989) Experiments in Biochemistry, Prentice Hall, Engle wood Cliffs, New Jersey.

Daniel M. bollag, Michael d. Rozycki and Stuart J. Edelstein, (1996). Protein Methods. Second edition

Experiment 9

9. Affinity chromatography of Concanavalin A lectin protein from jack bean

9.1 Objectives:

1. In this experiment, students will learn the principle of affinity chromatography. They will isolate a carbohydrate-binding lectin protein from an extract of jack bean meal.
2. Identify the factors affecting affinity chromatography.
3. Get hands-on experience on affinity chromatography in the laboratory
4. Study the significance of affinity chromatography and approaches in purification.

9.2 Introduction:

Affinity chromatography is a method by which selective purification of a molecule or group of molecules from complex mixtures is carried out. This is based on a highly specific biological interaction between the two molecules. The interaction is characteristically reversible and

purification is accomplished through a biphasic interaction with one of the molecules (the ligand) immobilized to a surface while its partner (the target) is in a mobile phase as part of a complex mixture. The capture step is generally followed by washing and elution, and this results in the recovery of a highly purified protein. Highly selective interactions allow for a rapid, often single step, process, with potential for purification in the order of several hundred to thousand-fold.

9.3 General features of the support material:

The matrix should be macroporous, have a consistent particle and pore size, with good flow properties. Pore size of a matrix is inversely correlated to its surface area, and the surface area, in turn, directly influences the amount of immobilized ligand and thus the capacity. The pore size correlates to the exclusion limit, which is the size (molecular weight) or the size range of proteins that are not able to enter the pore. The particle size distribution should be as consistent as possible so that smaller particles do not fill the void volume and restrict the flow.

9.4 Selectivity:

One important feature of an affinity matrix is its selectivity. It should be specific for the protein of interest as determined by the specific ligand coupled to the matrix and inactive to all other compounds that are present in the complex sample. Given that most applications are performed in aqueous solutions, often having a low ionic strength, the support should be hydrophilic and contain a limited charge that may lead to unwanted ionic interaction. Nonspecificity can come from the support itself, such as hydrophobicity related to the polystyrene beads and negative charge on the surface of silica. It can also be introduced when adjusting a matrix to accept a particular ligand.

9.5 Stability:

The affinity matrix must also be chemically and physically stable during the process, so that the support material as well as the attached ligand does not react with the solvents used in the process, nor should they be degraded or damaged by enzymes and microbes that might be present in the sample.

9.6 Magnetic affinity beads:

Magnetic separation can considerably shorten the purification process by rapid recovery of affinity beads at each step (e.g., binding, wash, and elution), and reduce sample dilution that is typically associated with traditional column-based elution. The capability of miniaturization and parallel screening of multiple conditions, such as growth conditions for optimal protein expression and buffer conditions for purification, makes magnetic separation amenable to high-throughput analysis which can appreciably shorten the purification process.

9.7 Selection of Ligands:

Selection of the appropriate ligand needs a certain degree of knowledge and understanding of the nature of interactions between the ligand and the target molecule; where the ligand must exclusively bind the target molecule and should be stable in different binding and elution conditions.

9.8 General considerations for ligand design and selection:

Affinity between ligand and target molecule is one of the most important factors when developing a new affinity purification material. Low affinity may reduce the binding efficiency thus resulting in poor yield while high affinity may cause inefficient elution or inactivation of the target protein by harsh elution conditions also resulting in low yield. The density of ligand on the surface should also be optimized. Very high density of the ligand can have an undesirable effect and lead to loss of binding capacity either because of close proximity of binding sites causing steric hindrance or strong binding that may prevent effective elution. Other factors that can influence selection of the best ligand are ability to be sterilized, stability of the ligand, proper storage conditions and cost.

9.9 Lectins:

Lectins are a varied group of proteins which bind carbohydrates with high degree of specificity where each lectin has its own specificity profile. They are frequently used in affinity purification or enrichment of carbohydrate moieties of complex glycoconjugates, such as polysaccharides, glycolipids, and glycoproteins. They are also used for a specific isolation of different

glycolforms of a specific protein depending on the nature of glycosylation. Lectin from Canavalia ensiformis, known as Conacanavalin A (ConA) has an affinity for a-D-mannose, a-D-glucose, and N-acetylglucosamine and is the most commonly used lectin.

9.10 Purification Method:

Purification by affinity begins with proper handling of the sample and the matrix, followed by discriminatory binding of the target, washing to remove nonspecific background, and, finally, elution of the bound target. Successful affinity purification depends on a number of significant factors including, the amount and accessibility of the ligand on the resin, the strength of the interaction, and the integrity of protein to be immobilized.

9.11 Sample preparation:

When preparing the sample for purification, conditions should be selected to retain the proper fold and functionality of the target of interest. Insoluble materials should also be removed and viscosity reduced because both of these factors could block the column, decrease the flow rate, and enhance back pressure.

9.12 Binding and wash:

Efficiency of binding is associated with the strength and the kinetics of protein–ligand interaction which can be influenced by the nature of the interaction, the concentration of applied target, the amount of immobilized ligand, and the flow rate used for binding. Affinity immobilization onto solid support can be accomplished if the sample is passed through a column that is packed with the affinity matrix, usually under ambient pressure and a slow flow rate. Generally, the higher the flow rate the less will be the binding efficiency, particularly , when the interaction between the ligand and protein is weak or the mass-transfer rate in the column is slow. After binding, protein bound by nonspecific interactions can be removed by washing.

9.13 Elution:

Elution of bound target from the resin is basically the reverse process of binding, where conditions are optimized to reduce the Ka that is weakening the interaction between target and ligand. The elution condition should not result in the denaturation of the target protein, unless such conditions are compatible with downstream applications.

9.14 Method:

Affinity chromatography of Concanavalin A lectin protein from jack bean

In this experiment, students will learn the principle of affinity chromatography. They will isolate a carbohydrate-binding lectin protein from an extract of jack bean meal. The saccharide binding Concanavalin A (Con A) lectin protein will be isolated from jack bean meal that binds to the glucose-based saccharide (dextran) of Sephadex. Thus no ligand needs to be chemically coupled to Sephadex as the glucose molecules making up the dextran polymer of Sephadex will act as the ligands. Con A is a mannose-binding protein present in jack bean meal. The structures of Mannose and glucose are similar and Con A binds to glucose but not as strongly as it does to mannose or mannose containing oligosaccharides. A one molar (1M) solution of dextrose (glucose) is used to extract the Con A from the Sephadex column.

The biological activity exhibited by Con A in this experiment is its ability to bind to horseradish peroxidase, a mannose containing glycoprotein with enzymatic activity. Horseradish peroxidase has an oligosaccharide core that contains mannose. Con A binds to the mannose of the enzyme without affecting the peroxidase activity. Con A eluted infractions from jack bean meal are adsorbed to a nylon membrane. The ligand binding activity is maintained by Con A while bound to the nylon membrane. The binding is determined by incubating the adsorbed membranes in a solution of horseradish peroxidase. The enzyme will bind to the membrane bound Con A and the bound horseradish peroxidase will convert the substrate to a colored product.

9.15 Materials:

Affinity Gel

Jack bean meal

NaCl

1 M NaCl/1M Dextrose

Con A Control

Horseradish peroxidase

ABTS Substrate

Standard dilution buffer

Membrane

Columns (syringe) and column tips

Cheesecloth for column

50 ml conical tubes

Transfer pipettes

Petri dishes

9.16 Equipments and glassware:

Shaking platform or vortex

Rotating or rocking platform

Clinical centrifuge

Ring stands with clamps for columns

Micro test tubes or small glass test tubes for collecting fractions

Test tubes (15 ml) to collect eluting

50 ml, 100 ml beakers or flasks

Beaker or flask

Graduated cylinders: 10 ml, 100 ml, and 250 ml

10 ml pipettes

Forceps

Distilled water

Filter paper

9.17 Method:

1. In the 50 ml capped tube, suspend the 1.5 gram of jack bean meal in 12 ml of 1M NaCl.
2. Extract the Con A from the Jack Bean meal at room temperature for thirty minutes with frequent, vigorous mixing (jack bean meal should be kept in suspension) or by placing on a shaking platform or vortex with the tube placed horizontally.

Column preparation

• Remove the plunger from the syringe.

• Fold the cheesecloth in half twice and then again to fit into the barrel of the syringe.
• Use the plunger or a pencil to push the cheesecloth to the bottom of the syringe (remove the plunger).

Note: The jack bean meal should be swirled vigorously in the extraction fluid to ensure even dispersal.

During the extraction, proceed to steps 3 through 6.

3. Re-suspend slurry of affinity gel by inverting tube several times.
4. Pour the 10 ml of slurry into affinity column and allow the gel to settle.
5. Start the elution of the affinity column and let liquid flow from column.

If the gel leaks from the column it should be saved in a beaker. Remove and repack the cheesecloth at the bottom of the column and repack the column.

Note: Collect this wash in a 50ml beaker. If a considerable amount of affinity gel comes out of the column, then add one more column volume of wash solution and collect all of the wash in the beaker. Remove the packing at the bottom of the column and repack the column. Pass all of the wash through the column. The gel should be retained in the column. If the buffer is leaking from the capped column, dab a small amount of petroleum jelly or stopcock grease on the column end piece. Be careful not to plug the column opening with the jelly or grease.

6. When affinity gel surface in the column is moist and no liquid is visible, gently fill the rest of the column with a solution of 1 M NaCl. Let the wash flow through; when the last of the wash enters the column, stop the flow. The surface of the gel should be moist, not dry, and a very small amount of sodium chloride solution should remain on the surface of the gel.
7. Pour the extract into a clean conical centrifuge tube and centrifuge at 2000 rpm or at high speed for 15 minutes. Transfer the supernatant to a clean tube and spin again if there are pieces of un-dissolved jack bean meal remaining. Discard the pellets.
8. Save 0.5 ml of the volume of the extract and label it as “Jack bean meal extract - Sample #2”. There should be no precipitate.
9. Charge the column by gently pouring the remaining extract into the affinity column.

10. Start the column and collect the flow through (effluent) in bulk in a clean 50 ml beaker. As the last of the extract enters the column, stop the flow. The surface of the gel should be moist, not dry and no extract should be present on the surface of the gel.
11. Wash the affinity column four times by filling the column with 1M NaCl. The column is then eluted and the effluent discarded into a 50 ml beaker. These steps are repeated three more times. When the last (fourth) elution wash enters the column, collect a few drops and stop flow. Label this tube “Effluent - Sample #3”. The surface of the gel should be moist, not dry and no wash should be present on the surface of the gel.
12. Pour 5 ml of elutant, 1M NaCl/1M Dextrose, into the column.

Note: Most of the NaCl solution is absorbed by the dry meal the final volume of extract should be about 7-9 ml.

13. Allow the column to flow and collect the first 0.5 ml fraction. This is “Eluate fraction 1-Sample #4”.
14. Stop the column flow and let the column set for 10 minutes.
15. Allow flow to continue and collect 0.5 ml fractions. Collect fractionsuntil all the column is eluted. Label the initial fraction collected as “Eluate fraction 5 -
Sample #5”
16. Elute the final fraction as “Last eluate fraction collected - Sample #6”.

Note: The total number of fractions collected from the column elution may
vary, but each group should get 7 – 9 fractions.

17. Assay the following fractions for enzyme binding activity:

1: Con A Control
2: Jack bean meal extract (Step 8)
3: Effluent (Step 11)
4: Eluate fraction 1 (Step 13)
5: Eluate fraction 5 (Step 15)

6: Last eluate fraction collected (Step 16)

Application of samples to membrane:

In all steps, use gloves and forceps to handle the membrane.

Sample Application:

1. Place a piece of membrane on a paper towel.
2. Using a micropipette, apply 10 micro liters of each sample to the membrane. Each membrane should have the pattern as shown.

Sample Numbers:

1. Con A Control, 1 mg/ml
2. Jack bean meal extract
3. Effluent
4. Eluate fraction 1
5. Eluate fraction 5
6. Last eluate fraction collected

Note: Apply the sample slowly. The size of the 10 µl spot should be no larger than 10-11 mm in diameter

Allow the membrane to dry completely for 15 minutes at room temperature or 10 minutes in a 37°C incubation oven.

Optional Stopping Point:

The experiment can be stopped after step 19 and resumed during the next lab period.

Detection of Con A protein

1. Obtain three 60 mm diameter Petri dishes and a 50 ml beaker which will be used for steps 2 through 9.

2. Label a different Petri dish for each of the following:

• Standard dilution buffer (SDB)

• Horseradish peroxidase (HRP)

• Substrate (SUB).

3. Pour 40-45 ml distilled water into a beaker. Label this beaker as “Wash”.
4. Pipette 10 ml of Standard Dilution Buffer into Petri dish labeled "SDB".
5. Pipette 5 ml of diluted horseradish peroxidase into Petri dish labeled "HRP".
6. Dip the dried membrane in the Petri dish with the Standard Dilution Buffer (SDB) and transfer the wet membrane to "HRP" dish. Incubate the membrane in "HRP" dish at room temperature for 10 minutes with frequent mixing or place on a shaking or rotating platform.
7. Lift the membrane from the enzyme (HRP dish). Drain excess liquid from the membrane. Dip the membrane in the Standard Dilution Buffer (SDB dish) and drain excess SDB from the membrane.
8. Place the membrane in the dish labeled "SUB". Carefully pipit 2 ml of the ABTS substrate overtop of the center of the membrane (the substrate should spread evenly over the entire membrane). Avoid disturbing the dish and causing the substrate to spill off of the membrane. Allow the membrane to soak undisturbed for 30-90 seconds or until the color is sufficiently developed. Do not allow the membrane to become too dark.
9. Lift the membrane from the substrate solution and immediately immerse the membrane in the wash beaker of distilled water. Wash the membrane in the water by successive dipping for one minute.
10. Lift the membrane from the water and drain excess water from the membrane.
11. Place the membrane on a piece of filter paper and allow it to dry at room temperature for 30 min. Alternatively, place the membrane in a 37°C incubation oven for
10 min. After the membrane is dry, record the precipitated substrate in Samples 1 - 6. Initially, precipitin dots will appear very dark but will become lighter after the membrane dries.
12. Store the membrane in a plastic bag.

9.18 Results:

Wavelength (nm)	Absorbance

9.19 Discussion and conclusion:

9.20 Questions:

1. What pattern of enzyme (HRP*) binding activity would you expect if you assayed the 0.5 ml effluent fractions?

2. Dextrose is used to elute the bound Con A from the affinity gel column, yet the bound dextrose was not removed from the Con A containing eluate fractions before adsorbing the fractions to the membrane. Why does Con A bind to HRP if the dextrose is still present in the Con A binding site?

3. Con A adsorbs strongly to the membrane, yet the HRP* protein binds only slightly. If HRP bound as strongly to the membrane as Con A, then the assay may not be possible unless an intermediate step was done. What would that intermediate step be?

9.21 References:

Urh Marjeta, Simpson Dan, Zhao Kate. 2009. Affinity Chromatography. Methods in Enzymology. 463: 417-438.

Affinity Chromatography of Glucose Binding Protein. 2005. EDVO-Kit #277. The Biotechnology Education Company EDVOTEK, Bethesda, MD, USA.

Experiment 10

10. SDS-Polyacrylamide Gel Electrophoresis

10.1 Objectives:

1. To detect the purity of the protein
2. Determine of protein molecular weight
3. To be familiar with SDS-PAGE protocols (Linear slab gel)

10.2 Introduction:

Sodium Dodecyl Sulfate-PolyacrylAmide gel Electrophoresis (SDS-PAGE) is a low-cost, reproducible and rapid method for qualifying, comparing and characterizing proteins. This method separates proteins based primarily on their molecular weights (laemmli, 1970).

In general, fractionation by gel electrophoresis is based on differences in size, shape and net charge of macromolecules. Systems where you separate proteins under native conditions cannot Distinguish between these effects and therefore proteins of different sizes may have the same mobility in native gels. In SDS-PAGE this problem is overcome by the introduction of an anionic detergent SDS which binds strongly to most proteins. When hot SDS is added to a protein all non-covalent bonds are disrupted and the proteins acquire a negative net charge. A Concurrent treatment with a disulfide reducing agent such as β-mercaptoethanol or DTT (dithiothreitol) further breaks down the macromolecules into their subunits. The electrophoretic

Mobility of the molecules is now considered to be a function of their sizes i.e. the migration of the SDS-treated proteins towards the anode is aversely proportional to the logarithms of their molecular weights, or more simply expressed: Small proteins migrate faster through the gel (figure 10. Compare this with the situation in gel filtration.

The polyacrylamide gel is formed by co-polymerization of acrylamide and a cross-linking monomer N,N'-methylene. To polymerize the gel a system consisting of ammonium persilfate (initiator) and tetramethylene ethylene diamin (TEMED) is added.

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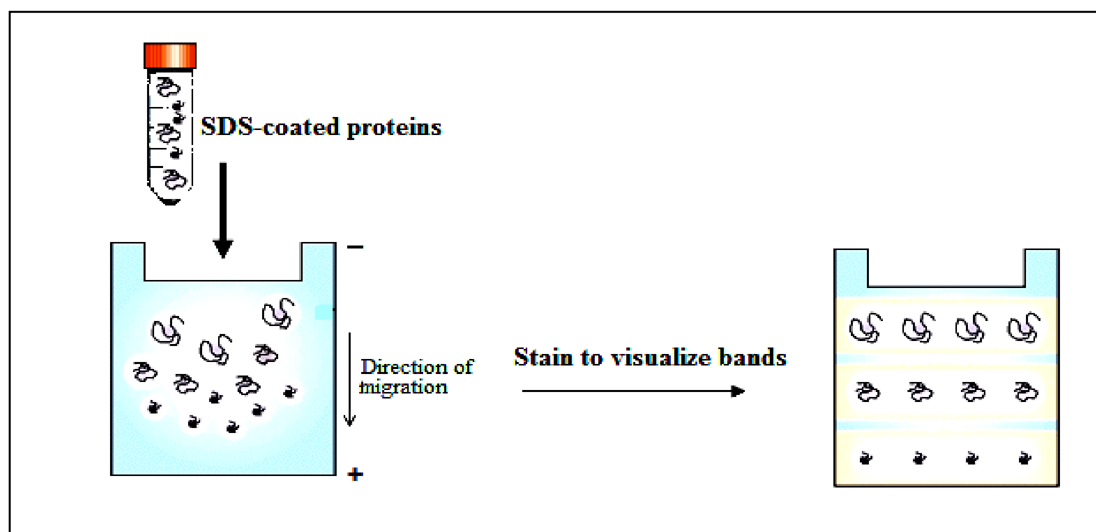

http://tainano.com/chin/Molecular%20Biology%20Glossary.htm)

10.3 Principle:

The mobility (R_f) of a molecule in gel electrophoresis is determined by its free solution mobility, Y0 (= mobility in a gel of zero %) and the sieving action of the gel matrix. In denaturing protein electrophoresis, the addition of SDS to the electrophoresis buffer uniformly coats the proteins with negative charges, equalizing the charge to mass ratio for all proteins, thus making Y0 the same for all species. In this case, relative mobilities are determined solely by the sieving action of the gel. This sieving action is proportional to the molecular weight (MW) of the particular protein. Theoretical treatments suggest that log(R_f) should vary with MW, but most users use an empirical plot of log(MW) vs. R_f for several standards of known MW to determine the MWs of unknowns.

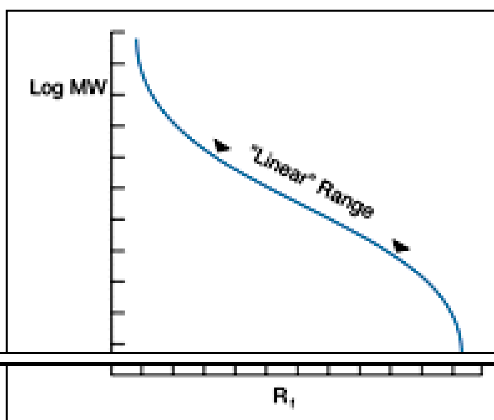

Figure 2: graph of log MW vs. R_f is sigmoidal, it is nearly linear for a range of molecular weights depending on the percentage monomer of the gel. (Figure has been taken from http://www.nationaldiagnostics.com/article_info.php/articles_id/55)

In practice the proportionality of log(MW) vs. R_f holds true for most proteins, provided they are fully denatured, and provided the gel percentage has been chosen to match the molecular weight range of the sample. In fact, the actual plot of log(MW) vs. R_f is sigmoidal (Figure 2), because at high MW, the sieving affect of the matrix is so large that molecules are unable to penetrate the gel, while at low MW, the sieving effect is negligible, and proteins migrate almost at their free mobility, which in SDS is independent of MW.

10.4 Materials:

Assemble minigel using glass front plate, white back plate, and spacers (1.5mm or 1.0mm). Make sure plates have been well washed and scrubbed with ethanol. Place in Pouring Apparatus, being certain that spacers are flush with the bottom of the glass and white plates. Tighten against rubber on bottom for a tight seal.

Buffers and solutions

SDS-PAGE Running buffer (5x) pH 8.4

Tris	15 g
Glycine	72 g
SDS	5 g

Made up to 1L with distilled water.

SDS-PAGE disruption buffer

10% (w/v) SDS	2.0 ml
1M Tris/HCl, pH 6.8	0.5 ml
Glycerol	0.6 ml
β-Mercaptoethanol	0.5 ml

Bromophenol blue 0.01 g

Made up to 10 ml with distilled water.

SDS-PAGE Stain

Glacial acetic acid 70 ml

Methanol 400 ml

Coomassie brilliant blue R 0.25%

Made up to 1L with distilled water.

SDS-PAGE destain

Glacial acetic acid 70 ml

Methanol 400 ml

Made up to 1L with distilled water.

10.5 Method:

Polyacrylamide gel electrophoresis will carry out according to the method of Laemmlli (1970). The composition of the gel mixes is shown in Table 1.1A and 1.1B. The samples (30μl) will denature by adding 10μl of 1.6x disruption buffer and then place for 5 minutes in boiling water. The gel will run at a constant current of 35mA until the blue dye reache the bottom of the gel. The M_r of the protein was determine using protein standard markers (SDS-7 (Sigma); Table 1.2). Then the gel is stain with 0.2% Coomassie brilliant blue R-250 in 20% methanol and 0.5% acetic acid for 20 min. Then destain the gel using 30% methanol until the protein bands become visible (Hellman *et al*., 1995).

Table 1.1A Separation gel contents

Stock solutions	Volume of stock solution required to make 12% polyacrylamide gel
1.5 M Tris/HCl, pH 8.8	2.0 ml
Acrylamide stock	3.2 ml
Water	2.8 ml
10% SDS	80 μl
10% Ammonium persulphate (fresh)	100 μl

TEMED	20 µl

Mix first 4 reagents, then just before you are ready to pour, add APS, stir to dissolve, and add TEMED. Pour gel using Pasteur pipette, pour running gel solution along side of gel to avoid bubbles. Pour until running gel is about 2.5 cm from top of white back plate.
Carefully layer about 1 cm of ethanol on top of running gel. This will aid polymerization by keeping O_2 away from gel surface. Let polymerize for 10-15 minutes. Pour out H_2O prior to pouring stacking gel, and let it drain for a minute.

Table 1.1B Stacking gel contents.

Stock solutions	**Volume of stock solution required to make 12% polyacrylamide gel**
0.5M Tris/HCl, pH6.8	1.0 ml
Acrylamide stock	1.0 ml
Water	3.0 ml
10% SDS	80 µl
10% Ammonium persulphate (fresh)	100 µl
TEMED	20 µl

As before, mix first four solutions, then add APS and TEMED. Pour stacking gel using Pasteur pipette, and place comb into gel. If necessary, remove any bubbles at bottom of teeth by replacing comb back into gel. Allow stacking gel to polymerize (about 30 minutes).
After stacking gel has polymerized, place gel into electrophoresis chamber. Add electrophoresis buffer to inner and outer reservoir, making sure that both top and bottom of gel are immersed in buffer. Be sure to mark the location of the wells using a Sharpie on the glass plate. Add Running Buffer to bottom, then add buffer to the area behind the gel, adding enough buffer so buffer enters back of gel. remove comb carefully, make sure not to tear the well ears. Flush wells with Pasteur Pipette before loading gel in order to remove unpolymerized acrylamide and contaminants (figure 3).

Table 1.2 Relative molecular mass markers (Sigma).

Protein standards	**M_r**
Bovine serum albumin	66000
Ovalbumin	45000
Glyceraldehyde 3-phosphate dehydrogenase	36000
Carbonic anhydrase	29000
Trypsinogen	24000
Soybean trypsin inhibitor	20100
α-Lactalbumin	14200

Preparing and loading samples:

1- Combine protein sample and 5x sample buffer (i.e. 20µl +5 µl) in an Eppendorf tube.

2- Heat at 100oC for 2-10minutes.

3- Spin down protein solution for 1 second in microfuge.

4- Introduce sample solution into well using Hamilton syringe. Be careful to avoid introducing air bubbles as this may allow some of sample to be carried to adjacent well.

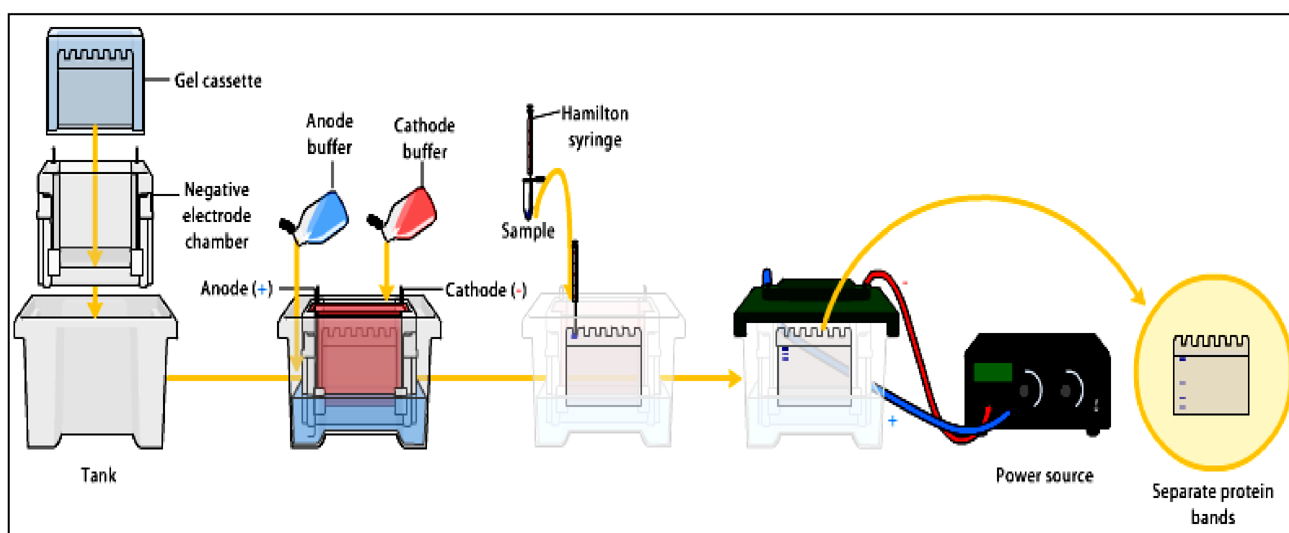

Figure 3: steps of SDS-PAGE electrophoresis(Figure has been taken from - http://protocolsonline.com/category/proteomics/western-blotting/)

10.6 Results:

The Mw of unknown protein

The purity of the sample

10.7 Discussion and conclusion:

10.8 Questions:

1- What is the role of the following;
SDS, TEMED, β-Mercaptoethanol

2-What is the differences between Coomassie blue and silver staining regards the sensitivity?

10.9 References:

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Laemmli U. K. (1970) *Nature* 227, 2304-2313. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4.

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