PRACTICAL SYLLABUS

1. Estimation of proteins using UV absorbance and Biuret method.
2. Microassay of proteins using Lowry/Bradford method.
3. Isoelectric pH of casein.
4. Ammonium sulphate fractionation of serum proteins.
5. Separation of albumin from serum using anion-exchange chromatography.
6. SDS-PAGE analysis of proteins.

EXPERIMENT NO: 1

DATE:

ESTIMATION OF PROTEIN BY BIURET METHOD

Aim: To estimate the protein using Biuret method.

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a purple colour which can be measured at 540 nm.

Reagents Required:

1. Biuret Reagent: Dissolve 3 g of copper sulphate (CuSO4.5H2O) and 9 g of sodium potassium tartarate in 500 ml of 0.2 mol/liter sodium hydroxide; add 5 g of potassium iodide and make up to 1 liter with 0.2 mol/liter sodium hydroxide.

2. Protein Standard: 5 mg BSA/ml.

Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter, *etc*.,

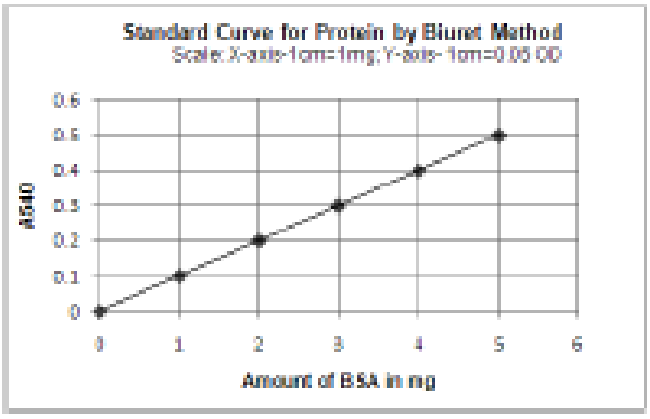
Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled test tubes.
2. Pipette out 1 ml of the given sample in another test tube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
4. Now add 3 ml of Biuret reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and warm at 37 ºC for 10 min.
6. Now cool the contents to room temperature and record the absorbance at 540 nm against blank.
7. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 540 nm along Y-axis.
8. Then from this standard curve calculate the concentration of protein in the given sample.

Result: The given unknown sample contains ----mg protein/ml.

Observations and Calculations

Volume of standard BSA (ml)	Volume of distilled water (ml)	Concentration of Protein (mg)	Volume of Biuret reagent (ml)	Incubate	A_{540}
0.0	1.0	00	3	At 37°C for 10 Min & Cool	0.00
0.2	0.8	1	3		
0.4	0.6	2	3		
0.6	0.4	3	3		
0.8	0.2	4	3		
1.0	0.0	5	3		
1.0 UK	0.0	To be estimated	3		



EXPERIMENT NO: 2

DATE:

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

AIM:

To estimate the amount of Protein present in given unknown solution.

PRINCIPLE:

Alkaline CuSo4 catalyses the oxidation of aromatic amino acids with subsequent reduction of sodium potassium molybdate tungstate of Folin's reagent giving a purple colour complex the intensity of the colour is directly proposition to the concentration of the aromatic amino acid in the given sample solution.

REAGENTS REQUIRED:

1. Stock Solution:

Bovine Serum albumin of 100mg is weighed accurately and dissolved in 100ml of distilled water in a standard flask (concentration 1 μg /ml).

2. Working Standard:

The Stock Solution of 10 ml is distilled to 100ml with distilled water in a standard flask (concentration 100 mg/ml).

3. Folin's Phenol Reagent:

Folin's Phenol Reagent is mixed with distilled water in the ratio 1:2.

4. Alkaline copper reagent:

Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide.

Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate.

Solution A, B, C is mixed in the proportion of 50:1:0.5.

Unknown Preparation:

The unknown protein is made upto 100 ml with distilled water.

PROCEDURE:

Working standard of 0.2 -1ml is pipette out into clean test tube and labeled as S1-S5. Test solution of 0.2ml is taken into test tube and labeled as T1. The volume is made upto 1ml of distilled water. Distill water of 1ml serve as blank.

To all the test tube 4.5ml of alkaline CUSO4 reagent is added and incubated at room temperature for 10 minutes. All the test tube 0.5ml of folin's phenol reagent is added. The contents are mixed well and the blue colour developed is read at 640 rpm after 15 minutes. From the standard graph the amount of protein in the given unknown solution is calculated.

RESULT:

The amount of protein present in the given unknown solution is mg (μg of protein).

EXPERIMENT NO: 3

DATE:

ISOELECTRIC PH OF CASEIN.

AIM: To estimate the Isoelectric pH of casein.

PROCEDURE:

Take about 10 ml of milk in a beaker. Slowly add 1*N* acetic acid in drops. At a particular stage a sudden flocculent precipitation takes place. Measure the pH. It will be found to be about 4.5. The major protein of milk is casein and its isoionic point is 4.5. At this pH, the net charge on the molecule is zero.

Remember that proteins remain in solution mainly because of the charges present on them which makes them hydrophilic. Once this charge is neutralized, the proteins precipitate out. This is how curd is prepared. The inoculum added contains lactobacilli which utilize the lactose of milk to produce lactic acid. When the pH reaches 4.5, casein is precipitated out.

EXPERIMENT NO: 4

DATE:

AMMONIUM SULPHATE FRACTIONATION OF SERUM PROTEINS.

AIM: To estimate the ammonium sulphate fractionation of serum proteins.

PRINCIPLE:

Ammonium sulfate precipitation is one of the most commonly used methods for protein purification from a solution. In solution, proteins form hydrogen bonds with water molecules through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium sulfate are added, these groups compete with the proteins to bind to the water molecules. This removes the water molecules from the protein and decreases its solubility, resulting in precipitation. Critical factors that affect the concentration at which a particular protein will precipitate include: the number and position of polar groups, molecular weight of the protein, pH of the solution, and temperature at which the precipitation is performed. The concentration at which antibodies precipitate varies among species; most rabbit antibodies precipitate with a 40% saturated solution, whereas mouse antibodies require 45-50% saturation.

PROCEDURE:

1. Allow serum or ascitic fluid to thaw, determine total volume, and centrifuge at 3000g for 30 minutes.
2. Transfer sample to beaker containing a stir bar and place on magnetic stirrer.
3. While sample is stirring, slowly add saturated ammonium sulfate to bring final concentration to 50% saturation.
    1. Volume of ammonium sulfate needed is equal to volume of sample.
    2. Adding the ammonium sulfate very slowly ensures that local concentration around the site of addition does not exceed the desired salt concentration.
4. Once total volume of ammonium sulfate is added, move beaker to 4°C for 6 hours or overnight.

5. Transfer to conical tube and centrifuge the precipitate at 3000g for 30 minutes.
6. Carefully remove and discard supernatant. Invert conical tube and drain well. For serum or ascites, resuspend pellet in 30%-50% of the starting volume in 1XPBS. For monoclonal antibody tissue culture supernatants, resuspend pellet in 10% of the starting volume in 1X PBS.
7. Transfer antibody solution to dialysis tubing and dialyze versus three changes of 1XPBS/0.08% Sodium Azide. Be sure to allow enough space for expansion of the antibody solution during dialysis. Normally twice the re-suspended volume is sufficient.
8. Remove antibody solution from the tubing and centrifuge to remove any remaining debris.
9. Determine the concentration and store at -80°C for long term storage

EXPERIMENT NO: 5

DATE:

SEPARATION OF ALBUMIN FROM SERUM USING ANION-EXCHANGE CHROMATOGRAPHY

AIM: To separate the albumin from serum using anion-exchange chromatography.

Methods: Two ion exchange resins were used in this study: Diethylaminoethyl cellulose (DEAE cellulose) resin; particle size of 60-130 μm; and Sodium Carboxymethyl cellulose (CM cellulose) resin; average molecular weight of ~90,000. All resins were prepared and recruited according to the standard preparation protocol provided by the manufacturer.

Buffer preparation: Since the pH of buffer should be 1 unit different from the product and also, the pH of serum human albumin is in the range of 4.8-5.6; so, the pH of DEAE cellulose resin should be above 6.6 and the pH of CM cellulose resin should be below 3.8. It has been demonstrated that the CM cellulose resin forms insoluble complexes with serum albumin with a maximum precipitation at pH 4-5 (9, 11). For preparation of solutions, the following steps were used: • For DEAE cellulose bis-tris buffer or bis-tris propane buffer with 20 molar concentration and Cl- as its counter-ion are considered the best buffers; however, we used tris buffer with 20 molar concentration in pH of 7.6 and Cl- as its counter-ion • For CM cellulose resin, the best buffer is lactic acid or formic acid buffer with 50 molar concentration in pH of 3.6 and Na+ as its counter-ion; we used this preparation with formic acid.

Determination of counterion concentration: For DEAE cellulose, the concentration of counterion is 0.05 to 0.25 molar. So, five different concentrations (0.05, 0.1, 0.15, 0.2 and 0.25 molar) of tris buffers were created. Then, 0.5 milliliter of each of these 5 buffers was added to 0.5 milliliter of DEAE cellulose. Afterwards, 0.5 milliliter of plasma is added and the resulting mixture was stirred by shaker to create a suspension. The resulting suspension was centrifuged for 5 minutes with 13000 rpm. Then, the extracted fluid was assessed for its protein content using SDS-page to find out the exact concentration of protein in which human serum albumin (HSA) was attached to the resin; i.e. the starting concentration of counterion; then, to find out the concentration in which HSA was detached from the resin; i.e. the concentration for counterion washout. For CM cellulose the concentration of counterion is 0.05 to 0.2 molar. The same

process of suspension production and washout which was described above for DEAE cellulose was done for CM cellulose by using 50mM formic acid for producing 0.05, 0.1, 0.15 and 0.2 molar concentrations. For DEAE cellulose, starting buffer concentration was 0.1 molar Chloride (Cl) solution in order to disappear HAS. However, for CM cellulose buffer, starting buffer concentration was 0.05 molar Sodium (Na+) solution in order to disappear HAS.

Technique of albumin purification: First, the resin was washed with 3 fold of the primary buffer volume. Each vial of resin contained 0.5ml of resin; so, at first, 0.5ml of tris buffer with 0.1M Cl-concentration was added to resin and mixed with 2500 rpm shaker to create a suspension; then, the yielding suspension was centrifuged for 5 minutes with 1300 rpm to achieve the resin through the final sediment. Again, the supernatant was removed and the above centrifugation process was repeated. After resin was prepared, the sample was added in the following process: • 0.5ml of the start buffer was added to the resin • 0.5ml of plasma was added to this mixture • The combination was mixed with shaker for 1 minutes with 2500 rpm • The final suspension was centrifuged for 5 minutes with 1300 rpm • The yielding supernatant was procured • 0.4ml of this solution was obtained and the remaining was wasted • 0.5 of the start buffer was added to the above 0.4 ml solution and mixed with shaker for 5 minutes with 2500 rpm • The latter suspension was centrifuged with 1300 rpm for 5 minutes and the supernatant was obtained • 0.4ml of this last solution was mixed with 0.5 ml of the start buffer inside vial number 1 and the remaining fluid was wasted • This latter solution was mixed with shaker for 1 minutes with 2500 rpm and then, centrifuged with 1300 rpm for 5 minutes • 0.4 ml of the solution in the latter stage was added to 0.5 ml of washing buffer and again mixed with shaker for 1 minutes with 2500 rpm; then, was centrifuged with 1300 rpm for 5 minutes • 0.4 ml of the above solution was spilled to vial number 2 • The above process of shaking and centrifugation was repeated twice Now it was the turn for separation of all resinattached proteins through the following order: • 0.5ml of 1M tris buffer was added to resin and was mixed with shaker for 1 minutes with 2500 rpm; then, centrifuged with 1300 rpm for 5 minutes • 0.4ml of the supernatant was spilled to vial number 3 and the remnant was wasted • The above process was repeated twice • the same steps were done for CM cellulose resin with its own buffers The results were finally analyzed with SDS PAGE technique, using 60 volts current for 3 hours

EXPERIMENT NO: 6

DATE:

SDS-PAGE analysis of proteins

AIM: To separate the proteins by SDS - PAGE.

METHODS:

An intact SDS PAGE electrophoresis system should include: a tank, lid with power cables, electrode assembly, cell buffer dam, casting stands, casting frames, combs(usually 10-well or 15-well), and glass plates (thickness 0.75mm or 1.0mm or 1.5mm). (Bio-rad brand one is recommended)

The SDS PAGE gel in a single electrophoresis run can be divided into stacking gel and separating gel. Stacking gel (acrylamide 5%) is poured on top of the separating gel (after solidification) and a gel comb is inserted in the stacking gel. The acrylamide percentage in SDS PAGE gel depends on the size of the target protein in the sample.

Acrylamide %	M.W. Range
7%	50 kDa - 500 kDa
10%	20 kDa - 300 kDa
12%	10 kDa - 200 kDa
15%	3 kDa - 100 kDa

Volumes of stacking gel and separating gel differ according to the thickness of gel casting:

Thickness of the gel	Vol. of stacking gel	Vol. of separating gel
0.75mm	2ml	4ml
1.0mm	3ml	6ml
1.5mm	4ml	8ml

- **For a 5 ml stacking gel:**

H_2O	2.975 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

- **For a 10ml separating gel:**

Acylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µ l	100µ l	100µ l	100µ l	100µ l
TEMED	10µ l	10µ l	10µ l	10µ l	10µ l

Note: AP and TEMED must be added right before each use.

- **5X Sample buffer (loading buffer):**

10% w/v	SDS
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

Make sure your target protein dissolved in the liquid phase, and no inappropriate ingredients present (e.g. guanidine hydrochloride can interact with SDS and cause precipitation) Generally, to treat your unprepared sample, you can use sonicator, lysis buffer or both to sufficiently make your target protein released, and centrifuge to make supernatant and pellet separated.

- **1x Running Buffer:**

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

(Approximately vol. of less than 1 liter is needed depending on the type of your electrophoresis system.)

SDS PAGE Protocol:

1. Make the **separating gel**:

Set the casting frames (clamp two glass plates in the casting frames) on the casting stands. Prepare the gel solution (as described above) in a separate small beaker. Swirl the solution gently but thoroughly. Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates. To make the top of the separating gel be horizontal, fill in water (either isopropanol) into the gap until a overflow. Wait for 20-30min to let it gelate. Make the **stacking gel**:

Discard the water and you can see separating gel left. Pipet in stacking gel untill a overflow. Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it gelate.

2. Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow untill the buffer surface reaches the required level in the outer chamber.

3. Prepare the samples: Mix your samples with sample buffer (loading buffer). Heat them in boiling water for 5-10 min.

4. Load prepared samples into wells and make sure not to overflow. Don't forget loading protein marker into the first lane. Then cover the top and connect the anodes.

5. Set an appropriate volt and run the electrophoresis when everything's done.

6. As for the total running time, stop SDS-PAGE running when the downmost sign of the protein marker (if no visible sign, inquire the manufacturer) almost reaches the foot line of the glass plate. Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel posessing higher percentage of acylamide, the time will be longer.

Note: Various factors affect the properties of the resulting gel. 1. Higher concentration of ammonium persulfate and TEMED will lead to a faster gelation, on the other hand, a lower stability and elasticity. 2. The optical temperature for gel gelation is 23°C-25°C. Low temperature will lead to turbid, porous and inelastic gels. 3. The pH is better to be neutral and the gelation time shoud be limited in 20-30 min.