B.Sc., BIOCHEMISTRY **2018-2019 batch**

Semester IV

17BCU414 PROTEIN PURIFICATION TECHNIQUES 3H-1C

PRACTICAL

Instruction hours per week: L:- T:- P:3 **Marks: Internal – 40; External - 60**

Exam: 3 hours

Course objectives

- To learn the sample preparation by ammonium sulphate precipitation.
- To fractionate proteins based on molecular weight, relative front, charge and mobility.

Course outcome

- The students were able to prepare protein sample from various sources.
- Learn to fractionate proteins based on their properties by applying the appropriate techniques like ion exchange chromatography, gel filtration chromatography, paper chromatography/TLC and electrophoresis

1. Preparation of the sample and ammonium sulfate precipitation of protein.
2. Ion-exchange chromatography.
3. Gel filtration chromatography.
4. Paper chromatography /TLC.
5. Electrophoresis.

REFERENCES

Sheehan, D., (2010). Physical Biochemistry: Principles and Applications 2nd ed., Wiley Blackwell (West Sussex), ISBN: 978-0-470-85602-4 / ISBN: 978-0-470-85603-1.

Freifelder, D., (1982). Physical Biochemistry: Applications to Biochemistry and Molecular Biology 2nd ed., W.H. Freeman and Company (New York), ISBN:0-7167-1315-2 / ISBN:0-7167-1444-2.

Plummer D. T., (1998). An Introduction to Practical Biochemistry 3rd ed., Tata McGraw Hill Education Pvt. Ltd. (New Delhi), ISBN:13: 978-0-07-099487-4 / ISBN:10: 0-07-099487-0

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)
(Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021
(For the candidates admitted from 2016 onwards)

DEPARTMENT OF BIOCHEMISTRTY

SUBJECT : PROTEIN PURIFICATION TECHNIQUES PRACTICAL -A

SEMESTER : IV

SUBJECT CODE: 17BCU414-B **CLASS : II B. Sc. BC**

EXPERIMENTAL DETAILS
DEPARTMENT OF BIOCHEMISTRTY

S.No	Lecture Duration Hour	Topics to be Covered	Support Material/Page Nos
1	3	Preparation of the sample and ammonium sulfate precipitation of protein.	Plummer D. T., (1998). An Introduction Practical Biochemistry 3rd ed., Tata McGraw H Education Pvt. Ltd. (New Delhi), ISBN:13: 978-07-099487-4 / ISBN:10: 0-07-099487-0
2	3	Ion-exchange chromatography.	
3	3	Gel filtration chromatography.	
4	3	Paper chromatography /TLC.	
5	3	Electrophoresis.	
Total No Of Hours Planned For Practical = 15			

REFERENCES

Sheehan, D., (2010). Physical Biochemistry: Principles and Applications 2nd ed., Wiley Blackwell (West Sussex), ISBN: 978-0-470-85602-4 / ISBN: 978-0-470-85603-1.

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Plummer D. T., (1998). An Introduction to Practical Biochemistry 3rd ed., Tata McGraw Hill Education Pvt. Ltd. (New Delhi), ISBN:13: 978-0-07-099487-4 / ISBN:10: 0-07-099487-0

Signature of the Staff

PRACTICAL SYLLABUS

1. Preparation of the sample and ammonium sulfate precipitation of protein.
2. Ion-exchange chromatography.
3. Gel filtration chromatography.
4. Paper chromatography /TLC.
5. Electrophoresis.

EXPERIMENT NO: 1 **DATE:**

PREPARATION OF THE SAMPLE AND AMMONIUM SULFATE PRECIPITATION OF PROTEIN

AIM: To prepare the protein sample and estimate the ammonium sulphate fractionation of 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

RESULT:

The concentration of the purified protein was found to be _____.

EXPERIMENT NO: 2 **DATE:**

ION-EXCHANGE CHROMATOGRAPHY

AIM: To separate the albumin from serum using ion-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.

Procedure:

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.

RESULT:

The purified protein by anion exchange chromatography was analyzed using SDS PAGE and was found to be _____ (kDa).

EXPERIMENT NO: 3 **DATE:**

GEL FILTRATION CHROMATOGRAPHY

AIM: To separate the proteins using Gel filtration chromatography.

PRINCIPLE:

The chromatographic medium for gel filtration is a hydrophilic gel made up from porous, fine-grain spheres of 10-300 µm diameter. This type of medium defines two solution compartments within the column: one is the freely moving mobile phase outside the gel particles, while the other is the restricted liquid compartment inside the porous particles.

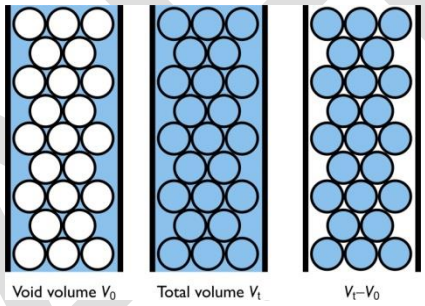

Figure. Liquid compartments inside a column packed with a porous gel. The individual liquid compartments are depicted as blue areas. V_0 = exclusion volume (void volume); V_t = total volume of the column; $V_t - V_0$ = combined volume of the liquid inside the gel particles and the material of the gel.

When a solution is moving through the gel filtration column, the movement of the solutes depends on two factors: the flow rate of the mobile phase and diffusion. Diffusion enables the molecules to explore the inside of the gel particles if their size so permits. The separation of a molecular mixture is based on the phenomenon that some molecules are excluded from the inside

of the gel particles due to their size. These molecules travel quickly in the mobile phase of the column, which is the only compartment available to them. Smaller molecules, on the other hand, spend various amounts of time inside the particles (stationary phase) and flow through the column slower.

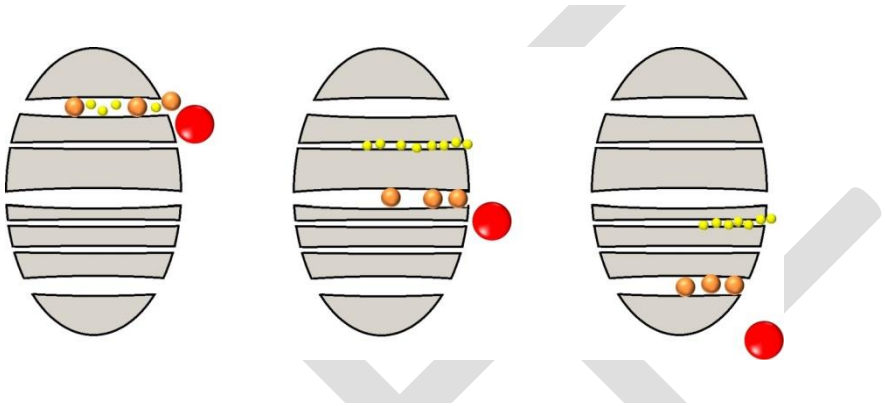

Figure - Travel of variably-sized molecules through a porous gel. During gel filtration (size exclusion) chromatography, molecules of different sizes will explore the available liquid spaces via diffusion. The largest molecules (red in the figure), due to their size, cannot enter the pores of the matrix at all. The movement of molecules with medium sizes (orange) is confined to the larger pores. The smallest molecules (yellow) can enter the gel particles through all pores. Therefore, the largest molecules will advance most rapidly through the column, whereas the smaller molecules will be retarded.

The result of a gel filtration experiment is usually depicted as an elution diagram. In this diagram, the concentration of the eluted compound is plotted against the volume of the eluent. The appearance of a given compound occurs at its elution volume (V_e). As in other distribution chromatographic methods, the elution of a compound is best characterised by its distribution coefficient (Kd):

$K_d = (V_e - V_o)/V_s$

where V_o equals the exclusion volume, i.e. the elution volume of a molecule that is larger than the largest pore size of the separating gel. Such a molecule therefore explores only the mobile phase, and is entirely excluded from the gel. V_s equals the volume of the stationary phase, i.e. the volume of the liquid inside the gel particles that is fully accessible only to molecules small enough to travel smoothly even through the smallest pores of the gel. V_s itself is difficult to determine. Therefore, in practice, it is replaced by the $V_t - V_o$ term, also accounting for the non-negligible volume of the gel itself. As a result, a constant pertinent to an apparent volume (K_{av}) is used instead of K_d (the latter would be valid only for real liquid volumes):

$K_{av} = (V_e - V_o)/(V_t - V_o)$

where K_{av} represents the portion of the gel volume that is accessible to a molecule of a given size. For a totally excluded macromolecule, $K_{av} = 0$; whereas, for small molecules diffusing freely in the entire volume of the gel, $K_{av} = 1$.

Planning a gel filtration experiment

(1) Choosing the gel type

Several different gel filtration media are available, which should be chosen according to the substance to be separated. These media differ in the chemical properties of the gel matrix, the pore size, the particle size, as well as the physical and chemical stability of the gel. The first developed and still widely used gel matrix is made of crosslinked dextran. Polymer beads made of dextran are known by the trade name Sephadex.

The pore size of the various Sephadex media is controlled by the number of crosslinks. The most popular ones are the entirely hydrophilic G-type gels. Numbers accompanying the G-type mark refer to the pore size and indicate the approximate molecular mass of excluded molecules in kDa. For example, Sephadex G-25 is used to separate relatively small molecules in the molecular mass range of 1000-5000 Da, including peptides. Alternatively, it can also be used to desalt larger proteins. To fractionate larger macromolecules up to 200-300 kDa, the G-150 or G-200 Sephadex gels are to be used. The mechanical properties of dextran gels having large pores are unfavourable due to the low density of crosslinks. These gels are easily compressible. Therefore, more rigid gels made of synthetic polymers are used to separate very large or elongated molecules.

If the size difference between the compounds to be separated is relatively large, e.g. during desalting of a macromolecule, it is practical to choose a gel in which the large-sized compound is eluted in the excluded volume (V_o; thus $K_{av} = 0$), while the small component elutes around V_t (thus $K_{av} = 1$). In this case, the fraction containing the macromolecules appears sharply, with minimal band broadening and dilution, in the shortest possible elution time.

In case of fractionating macromolecules and if the molecular weight of the compound of interest is known, the gel should be chosen so that the component of interest will elute approximately at the half of the entire fractionation range. For example, if a 100-kDa protein is to be isolated from a protein mixture, the use of a gel that spans the 10-250 kDa fractionation range is recommended.

(2) Choosing the particle size of the gel

Fine-sized beads fill the available space within the chromatographic column more efficiently. Therefore, the volume of the mobile phase will be reduced. This will result in a similar reduction in dilution and band broadening and, in turn, will yield a better resolution. On the other hand, the flow rate in a compact gel column is also reduced. Therefore, larger pressure should be applied when using super-fine beads. Indeed, special pumps are needed below a particle size of 10 µm. Naturally, only rigid, non-compressible gel types can be used in these cases.

For most purposes, the Fine and Medium type particle sizes (20-150 µm) are suitable. For preparative purposes and desalting, where high flow rates are required and the compounds of interest separate well even at a poor resolution, Coarse type gels can be used too.

(3) Choosing the size of the column

During gel filtration, the distance between two zones of separation increases proportionally to the square root of the column length. Long columns (> 100 cm) are used when a high resolution is required, while shorter (< 50 cm) columns are more practical when the aim is desalting or the separation of compounds that can be eluted at markedly different volumes.

Columns with diameters of around 1 cm are used for analytical purposes. By increasing the diameter, the amount of the applied sample, i.e. the capacity of the column, can be increased.

(4) Choosing the sample volume

A narrow start zone (relative to the column length) is sought if maximal resolution is to be achieved, e.g. for analytical purposes or in the case of compounds whose separation is difficult. Therefore, the sample volume in this case should be chosen to be 1-5 % of the column volume. The resolution cannot be further increased using smaller sample volumes, while the dilution will

be greater. The sample volume can be increased to as much as 15-20 % of the column volume if the compounds are readily separable, especially when working on a large scale.

(5) Choosing the eluent

The composition of the eluent does not directly influence the resolution of gel filtration. However, all components that have an effect on the molecules to be separated may influence the separation. The pH, ionic strength or the presence of detergents may influence the molecular state of the solutes. For instance, changes in molecular shape or the dissociation of multimeric proteins and enzyme-inhibitor complexes will change their chromatographic behaviour. In general, dilute (0.01-0.1 M) buffers are used that do not influence the structure of the compounds to be separated, but restrict the unwanted adsorption interactions between the gel matrix and the molecules of interest.

When the fractions containing the separated compounds are to be later concentrated, volatile buffers (e.g. ammonium bicarbonate) are practical to use that easily disappear during lyophilisation or film evaporation. The same considerations apply when the salt content of the gel filtration buffer should be subsequently eliminated.

(6) Choosing the flow rate of the eluent

During gel filtration, increasing the flow rate will deteriorate the resolution, because it prevents the formation of equilibrium between the mobile and the stationary phases. Generally, 5-10 mL/cm^2x hour is recommended as an optimal flow rate, but in most cases, a several times excess of this will not deteriorate the separation significantly. When doing preparative work, or if the

operation must be performed quickly for some reason, the advantage conferred by the higher flow rate may compensate for the deterioration of separation.

To achieve a higher flow rate, of course, a larger pressure must be applied. Therefore, in these cases, the mechanical stability of the gel matrix must be taken into consideration. Non-rigid gels may be compressed at pressures higher than allowed, which may lead to the complete clogging of the chromatographic column.

RESULT:

The purified protein by Gel filteration chromatography was found to be _____.

EXPERIMENT NO: 4 **DATE:**

PAPER CHROMATOGRAPHY /TLC

AIM: To separate and identify the amino acids in a mixture by thin layer chromatography.

PRINCIPLE: Thin layer chromatographic (TLC) technique readily provides qualitative information and with careful attention to details, it is possible to obtain quantitative data. Thin layer chromatography is a technique used to separate and identify compounds of interest. A TLC plate is made up of a thin layer of silica adhered to glass or aluminum for support. The silica gel acts as the stationary phase and the solvent mixture acts as the mobile phase. In the ideal solvent system the compounds of interest are soluble to different degrees. Separation results from the partition equilibrium of the components in the mixture.

In the simplest form of the technique, a narrow zone or spot of the sample mixture to be separated is applied near one end of the TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. This is called as the development of TLC plates. The separation depends on several factors; (a) solubility: the more soluble a compound is in a solvent, the faster it will move up the plate. (b) attractions between the compound and the silica, the more the compound interacts with silica, the lesser it moves, (c) size of the compound, the larger the compound the slower it moves up the plate.

The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value.

The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value.

$$Rf = \frac{distance\ moved\ by\ the\ substance\ from\ origin}{distance\ moved\ by\ solvent\ from\ origin}$$

Materials Required:

Reagents:

1. 2% solution of individual amino acids.
2. Solvent mixture of normal butanol, acetic acid and water in the ratio 12:3:5 by volume.
3. Ninhydrin reagent.

Requirements:

TLC plate.

1. TLC chamber.
2. Capillary tubes.
3. Reagent spray bottle.
4. Conical flasks.
5. Beakers.

Procedure:

1. Pour the solvent mixture in to the TLC chamber and close the chamber.
2. The chamber should not be disturbed for about 30 minutes so that the atmosphere in the jar becomes saturated with the solvent.
3. Cut the plate to the correct size and using a pencil (never ever use a pen) gently draw a straight line across the plate approximately 2 cm from the bottom.
4. Using a capillary tube, a minute drop of amino acid is spotted on the line.

5. Allow the spot to dry.
6. Spot the second amino acid on the plate [enough space should be provided between the spots].
7. Repeat the above step for spotting the unknown acid.
8. Place the plate in the TLC chamber as evenly as possible and lean it against the side(immerse the plate such that the line is above the solvent). Allow capillary action to draw the solvent up the plate until it is approximately 1 cm from the end.
9. Remove the plate and immediately draw a pencil line across the solvent top.
10. Under a hood dry the plate with the aid of a blow dryer.
11. Spray the dry plate with ninhydrin reagent.
12. Dry the plates in hot air oven at 105°C for 5 min. [Ninhydrin will react with the faded spots of amino acids and make them visible as purple coloured spots.]
13. After some time, mark the center of the spots, then measure the distance of the center of the spots from the origin and calculate the Rf values.

Rf value can be calculated using the formula:

$$Rf = \frac{distance\ moved\ by\ the\ substance\ from\ origin}{distance\ moved\ by\ solvent\ from\ origin}$$

The Rf values with butanol-acetic acid- water solvent are as follows: alanine 0.24, glutamic acid 0.25, glycine 0.2, leucine 0.58, valine 0.4, lysine 0.58, tyrosine 0.42.

RESULT:

The Rf values of amino acids were identified as _____

EXPERIMENT NO: 5 **DATE:**

ELECTROPHORESIS

AIM: To separate the proteins by Electrophoresis.

PRINCIPLE: SDS-PAGE was performed to accomplish the following: a) To observe the protein pattern of the enzyme mixture. b) To determine the homogeneity of the purified enzyme mixture. c) To determine the molecular weight of the purified enzyme.

REAGENTS REQUIRED:

1. Preparation of stock solution and buffers: 30% acrylamide a) Acrylamide: 29.2g b) N, N-methelyne–bis–acrylamide: 0.8g Added water, dissolved and made upto 100mL and filtered with Whatman no.1 filter paper.
2. Separating gel buffer: a) Tris-HCl: 1.5M, pH 8.8 18.171g of Tris was dissolved in 60mL of water and adjusted the pH to 8.8 with HCl and finally made upto 100mL with water.
3. Stacking gel buffer: a) Tris-HCl: 1M, pH 6.8 6.057g of Tris was dissolved in 60mL water and adjusted the pH to 6.8 with HCl and upto 100mL with water.
4. 10% SDS solution: 1g of SDS in 10mL of distilled water.
5. N,N,N'N'-Tetra methylene diammine(TEMED).
6. 10% Ammonium per sulphate (APS): 1g of APS in 10mL of distilled water.
7. Electrophoresis Buffer: a) Tris: 25mM, pH 8.3 b) glycine: 250mM,pH 8.3 c) SDS: 0.1%: Dissolved in minimum amount of water (500mL) and then added SDS. Allowed to settle and dissolved. This was finally made upto 2.5liters.
8. Sample buffer 4x: 5.0mL a) Tris (1M, pH 6.8): 2.1mL b) 2% SDS: 100mg c) Glycerol (100%): 1.0mL d) b-mercaptoethanol: 0.5mL e) Bromophenol blue: 2.5mg f) Distilled water: 0.4mL.

9. Staining solution (100mL): a) Alcohol: 40% b) Acetic acid: 10% c) Commassie Brilliant Blue (CBB): 259mg d) Distilled water: 50%.
10. Destaining solution (100mL) a) Alcohol: 50% b) Acetic acid: 10% c) Distilled water: 40%

PROCEDURE: Preparation of gel: The glass plates were washed in warm detergent solution, rinsed subsequently in tap water, deionised water and ethanol and dried. The unnotched outer plates were laid on the table and Vaseline (or grease) was coated. Spacer strips were arranged approximately at the sides and bottom of the plates. The notched inner plates were laid in position, resting on the spacer strips and the arrangement was mounted vertically. Sealing was done properly to avoid leakage. The volume of the gel solution required for making separating gel was calculated as follows (the reagents in the following table yield 20mL of solution after the addition of APS and TEMED)

Reagents	8%	10%	15%
H2O (ml)	9.3	7.9	4.6
30% acrylamide mix (ml)	5.3	6.7	10.0
1.5M Tris (pH 8.8) (ml)	5.0	5.0	5.0
10% SDS (ml)	0.2	0.2	0.2
10% APS (ml)	0.2	0.2	0.2
TEMED (ml)	0.012	0.008	0.008

APS and TEMED were added just prior to the pouring of gel. The solution was mixed well and poured into the space between the two plates leaving an inch of the upper space unfilled. Water was carefully laid over the surface of the poured gel mixture to avoid air contact, which reduces the polymerization reaction. The gel mixture was allowed to polymerize, undisturbed at room temperature for 60 minutes. In the mean time

gel mixture for stacking gel was prepared. (The reagents in the following table yield 10mL of solution after the addition of APS & TEMED)

After the separating gel was polymerized the over laid water was removed carefully with filter paper and an appropriate comb was inserted between the plates. 0.1mL of 10% APS and 10 l of TEMED were added to the stacking gel mixture. It was mixed well and poured immediately (to the brim) over the separating gel. The stacking gel was allowed to polymerize. Additional gel mixture was added when gel retracted significantly.

Preparation of protein samples:

The required volume of sample buffer was added to protein samples and they were loaded (the final concentration of sample buffer in the prepared sample should come to1x. If the protein was dried suspend it in 1x buffer). The samples were incubated for 2min in a boiling water bath prior to loading. When the polymerization was completed the comb was removed and the lower spacer strip was carefully removed. The Vaseline (or grease) from the bottom was removed with a piece of tissue paper. The gel was attached to the electrophoresis tank using appropriate clips/clamps. The lower reservoir was filled with 1x electrophoresis buffer, using a bent Pasteur pipette or syringe needle to remove any air bubble trapped beneath the bottom of the gel. The protein samples were loaded using a micropipette and the wells were completely and carefully filled with 1x electrophoresis buffer. The upper reservoir was also carefully filled with 1x electrophoresis buffer. The electrodes were connected to a power pack.

The gel was run at constant current (20 milli ampere 100 volts) for 4-6 hrs at room temperature. Electrophoretic mobility of the samples was determined by bromophenol

blue front. At the end of the run the power pack was switched off. The gel and plates were laid flat on the table and a corner of the upper glass plate was lifted up and the gel was carefully removed.

Staining of the gel:

After the completion of the electrophoresis, the gel was fixed with 10% trichloroacetic acid for 5minutes and stained with CBB. The CBB staining solution was prepared using methanol, acetic acid and double distilled water in the ratio of 4:1:5 and 0.25gm of CBB was added and the gel was stained over night.

Destaining of the gel:

The destaining of CBB stained gel was done by using methanol, acetic acid and double distilled water in the ratio of 5:1:4 till the appearance of clear bands on the gel.

RESULT:

The sample proteins are separated by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis. The proteins appeared as discrete bands in the gel. The relative molecular weights of the protein with respect to their bands were observed in Kilo Daltons.