KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.

(For the candidates admitted from 2018 onwards)

DEPARTMENT OF BIOCHEMISTRY

SUBJECT	**:**	**PROTEINS PRACTICALS**		
SEMESTER	**:**	**II**		
SUBJECT CODE	**:**	**18BCU211**	**CLASS**	**: I B.Sc. Biochemistry**

Course Objective:
This practical course on proteins describes about the qualitative and quantitative analysis, as well as its purification and characterization.

Course outcome:
By the end of the course, students can be able to demonstrate the importance of the protein chemistry and their wide applications.

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.

SUGGESTED READING

Nelson, D.L. and Cox, M.M., (2013). Lehninger: Principles of Biochemistry 6th ed., W.H. Freeman and Company (New York), ISBN:13: 978-1-4641-0962-1 / ISBN:10:1-4292-3414-8.

Sheehan, D., (2009). Physical Biochemistry 2nd ed., Wiley-Blackwell (West Sussex), ISBN: 9780470856024 / ISBN: 9780470856031.

Cooper, T.G., (2011). The Tools of Biochemistry Wiley India Pvt. Ltd. (New Delhi), ISBN: 978-81-265-3016-8.

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University Established Under Section 3 of UGC Act 1956)
Pollachi Main Road, Eachanari Post, Coimbatore - 641 021. INDIA
Phone : 0422-6471113-5, 6453777 Fax No : 0422 -2980022-3
Email : info@karpagam.com Web : www.kahedu.edu.in

DEPARTMENT OF BIOCHEMISTRY

PRACTICAL PLAN

SUBJECT NAME: PROTEINS PRACTICAL

SUB.CODE: 18BCU211

SEMESTER: II **CLASS: I B.Sc., BIOCHEMISTRY**

S.No	Topics to be Covered	Supporting material with Page No.
1.	Estimation of proteins using UV absorbance and Biuret method	S1: 100-101
2.	Micro assay of proteins using Lowry/Bradford method.	S1: 102-103
3.	Isoelectric pH of casein	S1: 90-91
4.	Ammounium Sulphate fractionation of serum proteins.	S1: 122-223
5.	Separation of albumin from serum using an ion exchange chromatography.	S1: 112-114
6.	SDS-PAGE analysis of proteins	S1: 10-12

SUGGESTED READING

1. Nelson, D.L. and Cox, M.M., (2013). Lehninger: Principles of Biochemistry 6th ed., W.H. Freeman and Company (New York), ISBN:13: 978-1-4641-0962-1 / ISBN:10:1-4292-3414-8.

2. Sheehan, D., (2009). Physical Biochemistry 2nd ed., Wiley-Blackwell (West Sussex), ISBN: 9780470856024 / ISBN: 9780470856031.

3. Cooper, T.G., (2011). The Tools of Biochemistry Wiley India Pvt. Ltd. (New Delhi), ISBN: 978-81-265-3016-8.

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.

(For the candidates admitted from 2017 onwards)

DEPARTMENT OF BIOCHEMISTRY

COURSE MATERIAL

STAFF NAME : Mr. A. RAMAKRISHNAN
SUBJECT : PROTEINS PRACTICAL
SUBJECT CODE : 18BCU211
SEMESTER : II **CLASS : I B.Sc. Biochemistry**

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.

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 or casein. Take about 10ml of milk in a beaker slowly add 1N 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 no charge on the molecule is zero.

Remember that proteins remain in solution mainly because of the charges is neutralized, the proteins precipitate out. This is how and 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 Theabove 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:

Tris base	3.029 g
NaCl	4.383 g
EDTA	0.186 g
Na-deoxycholate	2.500 mg
SDS	500 mg
NP-40 [Nonidet-P-40(puriss)]	5.00 g

All these chemicals were dissolved in 400 ml double distilled water, pH was adjusted to 7.4 with 0.1 N HCl and then the volume was made up to 500 ml. The buffer was stored in refrigerator.

2) **Running gel buffer** (1.5 M Tris, pH 8.8): For 250 ml, 45.375 g Tris was dissolved in 200 ml double distilled water and pH was adjusted with 0.1 N HCl, the volume was made up to 250 ml and stored at room temperature.

3) **Stacking gel buffer** (0.5 M Tris, pH 6.8): For 250 ml solution, 15 g Tris was dissolved in double distilled water and pH was adjusted with 0.1N HCl before adjusting the volume up to 250 ml.

4) **SDS (10%):** 10 g SDS was dissolved in 100 ml double distilled water and stored at room temperature.

5) **Acrylamide (30%):** 29 g acrylamide (29%) and 1 g bis-acrylamide (1%) were dissolved in 100 ml double distilled water.

6) **Ammonium persulfate (10%):** 100 mg ammonium persulfate was dissolved in 1 ml double distilled water.

7) **10X electrophoresis buffer** (2.5 M Tris, 1.92 M Glycine and 1% SDS): For one litre of solution, 30 g Tris, 14.4 g glycine and 10 g SDS were dissolved in 800 ml double distilled water and volume made up to one litre and stored at room temperature.

8) **1X electrophoresis Buffer:** For 500 ml, 50 ml 10x SDS electrophoresis buffer + 450 ml double distilled water.

9) **10X Transfer buffer** (250 mM Tris and 1.92 M glycine): For one litre, 30.3 g Tris and 14.4 g glycine were dissolved in 800 ml double distilled water and the volume was adjusted up to one litre and stored at room temperature.

10) **1X Transfer Buffer with 20% methanol:** For 500 ml, 50 ml 10x transfer buffer + 350 ml double distilled water + 100 ml methanol were mixed and kept cold until used.

11) **2X Sample Buffer with reducing agent** (125 mM Tris-HCl (pH 6.8) 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.004% bromophenol blue): For 10 ml, 2.5 ml 0.5 M Tris-HCl (pH 6.8) and 4.0 ml of 10% SDS, 2.0 ml glycerol, 1.0 ml 2-mercaptoethanol, 0.4 mg bromophenol blue, 154 mg DTT were mixed and the volume was made up to 10 ml with double distilled water, a liquoted and stored at -20°C.

12) **10X Phosphate buffered saline** (0.1 M sodium phosphate): For 1 litre of solution, 13 g $NaH_2PO_4.4H_2O$ was dissolved in double distilled water, adjusted the pH to 7.2 with 0.1 N NaOH and the volume was made up to one litre. Stored at room temperature.

13) **Blocking buffer** (PBS with 10% glycerol, 5 % skimmed milk powder and 0.2% Tween-20): For 100 ml, 10 ml of 10X PBS, 10 ml of glycerol and 0.9 g of NaCl, 5 g BSA and 80 ml double distilled water were mixed and stirred well until dissolved and stored at 4ºC.

14) **Tris Buffered Saline (TBS)** (20 mM Tris, 500 mM NaCl, pH 7.5): For 500 ml, 1.21 g Tris and 14.62 g NaCl were dissolved in 400 ml double distilled water and pH was adjusted to 7.5 with HCl and made up the volume to 500 ml.

15) **T-TBS** (0.2% Tween-20 in TBS): For 100 ml, 200 µl Tween-20 was added to 100 ml of TBS. The solution was kept cold.

16) **Prestained SDS-PAGE standard:** Commercially available protein molecular weight marker was obtained from Fermantas Life Sciences, Molecular biology products (CA).

PREPARATION OF GEL

Running gel or separating gel (10%)

The following volume of solutions for two slab gel was prepared

Distilled water	4.0ml
Acrylamide (30%)	3.3 ml
Running gel buffer	2.5 ml
SDS (10%)	100 µl
APS (10%)	100 µl
TEMED	5 µl

Stacking gel (5%)

The following volume of solutions for two slab gel was prepared

Distilled water	3.40ml
Acrylamide (30%)	830 µl
Stacking gel buffer	630 µl
SDS (10%)	50 µl
APS (10%)	50 µl
TEMED	5 µl

Procedure

Protein extraction for immunoblotting was performed as follows: The rat liver tissue (100mg) was homogenized in lysis buffer [135 mMNaCl, 20 mMTris, 2 mMEDTA and 1 mM phenyl methyl sulfonyl fluoride (PMSF-pH 7.4)] and the volume was made up to 1 ml using the same buffer. The homogenates were centrifuged (15 min, 10,000 rpm at 4°C) and the protein content of the supernatant was determined by Lowry's method with BSA as standard. Aliquots of supernatant (50 µg total protein) were boiled for 5 min in sample buffer [0.2 M Tris–HCl buffer, 10% glycerol, 2% SDS, 0.02% β-mercaptoethanol]. Proteins were separated by Tris-Glycine-SDS discontinuous 12% polyacrylamide gel electrophoresis, and electro blotted onto PVDF membrane (Sigma chemicals, Mumbai) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, USA).

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.