BIOCHEMICAL METHODS

A Comprehensive Guide

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A COMPREHENSIVE GUIDE

May 2024

VYD PUBLISHERS

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ISBN: 978-81-967529-5-8

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ISBN: 978-81-967529-5-8

Cover Page and Graphic Design By Miss. Urvi Dhumal

Published By: VYD Publishers, Satara

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PREFACE

Entering the realm of **"Biochemical Methods: A Comprehensive Guide,"** meticulously crafted to meet the needs of students, researchers, and anyone intrigued by the fascinating world of biomolecules and their analysis. Jointly written by Dr. Ms. Ashiya Momin and Dr. Mrs. Savita Nalawade, this comprehensive guide delves into the essential methods and procedures in the field of biochemistry, offering practical insights for students, researchers, and anyone fascinated by the world of biomolecules.

Biochemistry lies at the heart of understanding the intricacies of life, delving into the molecular foundation of living organisms. It reveals the secrets of cellular processes, metabolism, and the building blocks of life. To unravel these mysteries, one must possess a solid grasp of the techniques fundamental to the discipline.

In this book, we explore a range of laboratory procedures and methods integral to biochemistry. The content covers a broad spectrum of techniques, from protein estimation using Biuret and Lowry methods to the isolation of essential biomolecules like cholesterol and lecithin. We delve into the determination of amino acid concentrations, glucose levels, and pH optimization, providing a well-rounded foundation for anyone venturing into the captivating world of biochemistry. Throughout the chapters, you will find step-by-step instructions, explanations, and practical tips to ensure a clear understanding of each technique. Furthermore, the methods described in this book are not confined to the laboratory; they can also be applied to solve real-world biological challenges and contribute to advancing our knowledge of living organisms.

Whether you are a student embarking on your academic journey or a seasoned researcher looking to refine your laboratory skills, "**Biochemical Methods: A Comprehensive Guide**," authored by Dr. Ms. Ashiya Momin and Dr. Mrs. Savita Nalawade, aims to be your reliable companion. By

following the guidance within these pages, you can confidently conduct experiments, analyse results, and contribute to the ever-evolving field of biochemistry.

As the authors of this book, we have strived to share our knowledge and experience in biochemistry with the hope that it will inspire a deeper appreciation for the subject and empower individuals to explore the frontiers of scientific discovery. We encourage you to embrace the hands-on learning experience that this book offers and to use the techniques presented here as tools to uncover the marvels of life at the molecular level.

Dr. Ms. Ashiya Momin

Dr. Mrs. Savita Nalawade

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PROTEIN ESTIMATION TECHNIQUES

Estimation of Proteins by Lowry's Method

Aim: Quantitative estimation of proteins in the given sample using Lowry's method.

Principle:

Proteins are unbranched heteropolymers of amino acids joined by peptide bonds. The main principle of this method is that the aromatic amino acids (phenylalanine, tyrosine, tryptophan) present in the protein molecule reduce phosphomolybdate, producing a blue colour. Additionally, CuSO4 reacts with peptide bonds, producing a violet colour. The intensity and colour are directly proportional to the concentration of the protein, which can be measured quantitatively using a colorimeter.

Chemicals:

- Standard Bovine Serum Albumin (BSA): 100 µg/ml.
- NaOH.
- 2% Sodium citrate or Na-K tartarate.
- 1% $CuSO4 \cdot 5H_2O$.
- Folin phenol reagent.
- Sodium Carbonate.
- Colorimeter.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of standard BSA solution into clean and dry test tubes.
2. Adjust the volume to 1 ml in each tube with distilled water.
3. Add 3 ml Lowry's reagent to each test tube and let it stand for 15 minutes at room temperature.
4. Add 0.5 ml Folin phenol reagent to each tube and wait for 30 minutes.
5. Measure the optical density of the solution at 660 nm.
6. Plot the graph of optical density at 660 nm per mg of BSA.
7. Pipette out 0.5 ml of the unknown sample and repeat the above procedure.
8. Find the optical density of the unknown sample at 660 nm and determine the unknown concentration using the standard graph.

Preparation of Reagents:

Lowry A solution: Dissolve 2% sodium carbonate in 0.1 N NaOH.

Lowry B1 solution: 2% sodium citrate/Na-K Tartarate.

Lowry B2 solution: Dissolve 1 gm $CuSO4 \cdot 5H_2O$ in 100 ml distilled water.

Lowry C solution: Mix 100 ml Lowry A solution with 1 ml each of Lowry B1 and Lowry B2 solutions.

Folin phenol reagent: Combine 100 gm sodium tungstate + 25 gm sodium molybdate in 700 ml distilled water. Add 50 ml 85% phosphoric acid, followed by 100 ml concentrated HCl. Reflux for 50 hours, add 150 gm lithium sulfate and 50 ml distilled water, and a few drops of Br2 water. Boil the mixture for 15 minutes, cool, and dilute to 1 litre. Stir at 4°C in a brown bottle.

Observation Table:

Standard BSA Concentration: 100 µg/ml

Sr. No.	Standard BSA Solution (ml)	Distilled Water (ml)	Lowry 'C' (ml)		Folin Phenol (ml)		µg of protein	O.D at 660 nm
1.	0.0	1.0	3	Wait for 15 Min	0.5	Wait for 30 min	00	
2.	0.2	0.8	3		0.5		20	
3.	0.4	0.6	3		0.5		40	
4.	0.6	0.4	3		0.5		60	
5.	0.8	0.2	3		0.5		80	
6.	1.0	0.0	3		0.5		100	

Sr. No.	Standard BSA Solution (ml)	Distilled Water (ml)	Lowry 'C' (ml)		Folin Phenol (ml)		µg of protein	O.D at 660 nm
1.	0.0	1.0	3	Wait for 15 Min	0.5	Wait for 30 min	00	
2.	0.2	0.8	3		0.5		20	
3.	0.4	0.6	3		0.5		40	
4.	0.6	0.4	3		0.5		60	

5.	0.8	0.2	3		0.5		80	
6.	1.0	0.0	3		0.5		100	
7.	Unknown A 0.4	0.6	3		0.5		?	
8.	Unknown B 0.4	0.6	3		0.5		?	

From Graph:

Unknown A Solution Containing: ------- µg of protein in 0.4 ml.

Therefore, Concentration of Unknown A: ---------- µg/ml.

Unknown 'B' solution containing: -------- µg of protein in 0.4 ml.

Therefore, Concentration of Unknown B: -------- µg/ml.

Results:

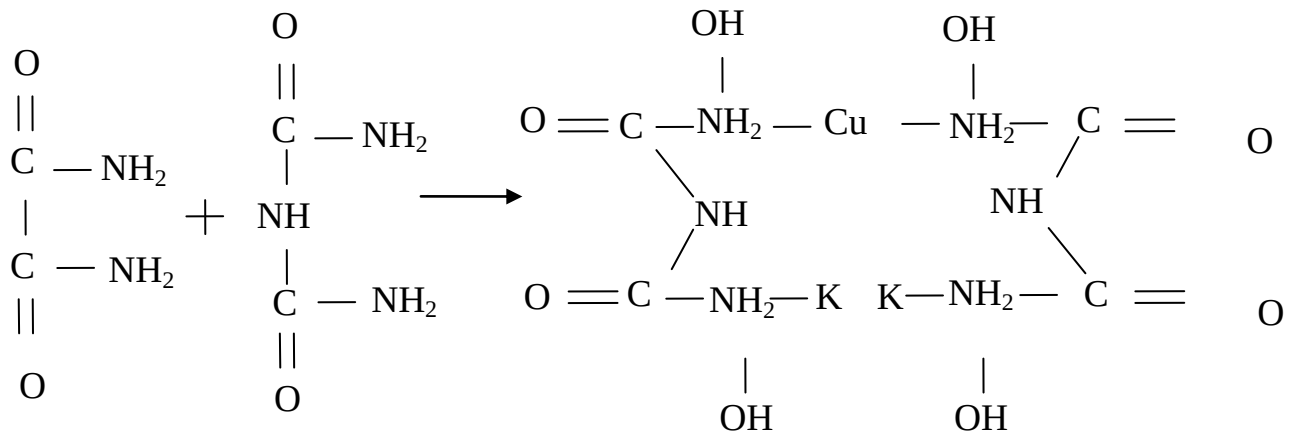
1. The concentration of protein in the given unknown 'A' sample: --------- µg/ml.
2. The concentration of protein in the given unknown 'B' sample: --------- µg/ml.

Estimation of Proteins by Biuret Method

Aim: Quantitative estimation of proteins in the given sample using Biuret method.

Principle:

Proteins are unbranched heteropolymers of amino acids joined by peptide bonds. The Biuret reaction, used in this method, occurs with substances whose molecules contain two carbonyl groups (CO-NH2). Proteins give a positive test since there are pairs of CO-NH groups in the molecule. The Biuret reagent, containing alkaline CuSO4, reacts with peptide bonds in an alkaline medium to form a violet-coloured complex. The intensity of colour is directly proportional to the number of peptide bonds, i.e., the concentration of protein, and can be quantitatively measured using a colorimeter.



Oxamide Biuret

Cupri- potassium biuret complex.

Materials/Chemicals:

- Casein
- Egg albumin
- NaOH
- KI
- Na-K Tartarate
- Colorimeter

Procedure:

1. Pipette out 0.5, 1.0, 1.5, 2.0 and 2.5 ml of standard casein solution into clean and dry test

9. tubes.
2. Adjust the volume of each test tube to 2.5 ml with distilled water.
3. Add 0.5 ml Biuret reagent to each test tube.
4. Incubate the tubes at room temperature for 30 minutes.
5. Measure the optical density of the solution at 530 nm.
6. Plot the graph of optical density at 530 nm vs. mg of casein in solution.
7. Pipette out 1 ml of the unknown sample and repeat the procedure.
8. Measure the optical density of the unknown sample at 530 nm and determine the unknown protein concentration using the standard graph.

Preparation of Reagent:

1. Standard casein solution (5 mg/ml): Dissolve 1.50 mg of casein in 250 ml of 0.2 N NaOH (keep at 4°C).

2. Biuret reagent: Dissolve 9 gm of Na-K tartarate in approximately 400 ml of 0.2 N NaOH. Dissolve separately 3 gm of CuSO4.5H20 in a minimum amount of water. Add the CuSO4 solution to the alkaline tartarate solution with constant stirring, then add 5 gm of KI and dilute to 1 litre with 0.2 N NaOH.

Observation:

Standard Casein Concentration: 5 mg/ml

Sr. No	Std. Protein (ml)	D/W(ml)	Biuret reagent (ml)		mg of Protein	O.D At 530 nm.
1)	0.0	2.5	5	Wait for 50 min. at room temp.	0.0	-
2)	0.5	2.0	5		2.5	
3)	1.0	1.5	5		5.0	
4)	1.5	1.0	5		7.5	
5)	2.0	0.5	5		10.0	
6)	2.5	0.0	5		12.5	

Standard Casein Concentration: 5 mg/ml

Sr. No	Std. Protein (ml)	D/W (ml)	Biuret reagent (ml)		mg of Protein	O.D At 530 nm.
1)	0.0	2.5	5	Wait for 50 min. at room temp.	0.0	
2)	0.5	2.0	5		2.5	
3)	1.0	1.5	5		5.0	
4)	1.5	1.0	5		7.5	
5)	2.0	0.5	5		10.0	
6)	2.5	0.0	5		12.5	
Unknown A	2.0	0.5	5		-	
Unknown B	2.0	0.5	5		-	

From Graph:

Concentration of unknown A Solution : …… mg/2ml
: …… mg/ml
Concentration of unknown A Solution : ……mg/2ml
:…… mg/ml

Calculations:

For egg albumin:

Weight of egg = ….. gm

Protein in ml = …… ml

=……. ml dilute 200 by diluted water

Sr. No	Std. Protein (ml)	D/W (ml)	Biuret reagent (ml)		O.D at 530 nm
Unknown	0.5	2.0	5	Wait for 30 min	

From Graph:

0.5 ml of the unknown sample contains: … mg of protein

1 ml of the sample contains: … mg of protein

Total egg protein solution contains: … mg

Result:

1. The concentration of protein (Casein) in the given Unknown A sample:
    i. Unknown A sample = … mg/ml
    ii. Unknown A sample = … mg/ml
2. The concentration of egg albumin in the given unknown sample = … mg/ml

Amino Acid Analysis and Separation Techniques

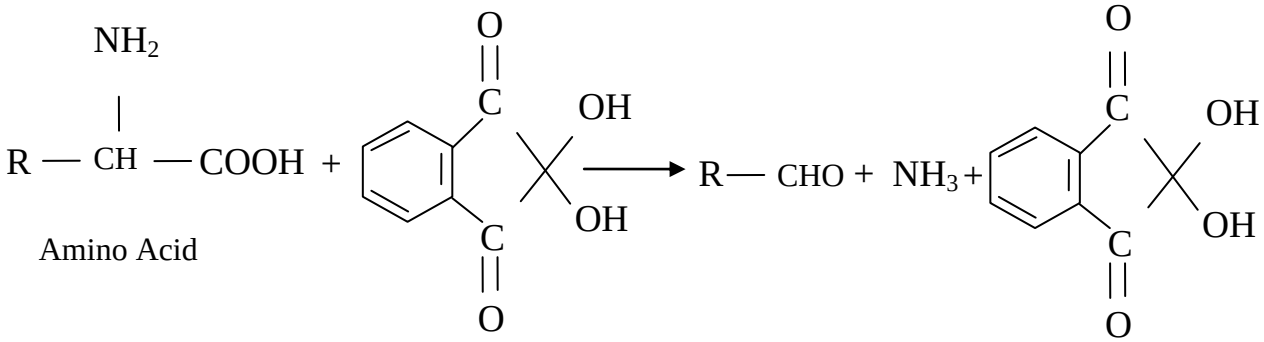
Determination of Amino Acid by Ninhydrin Method

Aim: Quantitative estimation of the amino acid (Leucine) in the given sample using the Ninhydrin method.

Principle:

Amino acids are the building blocks of proteins, and they are primary amines (except proline). In the Ninhydrin method, amino acids react with Ninhydrin reagent, forming a purple-colored complex called "Ruhemans complex." The intensity of the purple colour complex is directly proportional to the concentration of the amino acid in the solution, which can be measured quantitatively using a colorimeter.

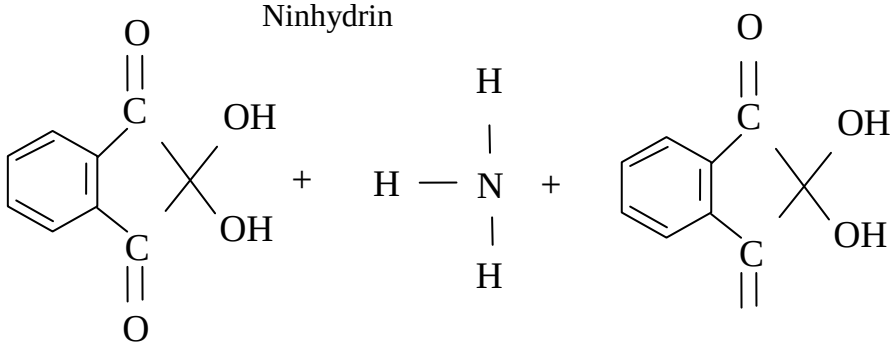
Reaction:



Amino Acid

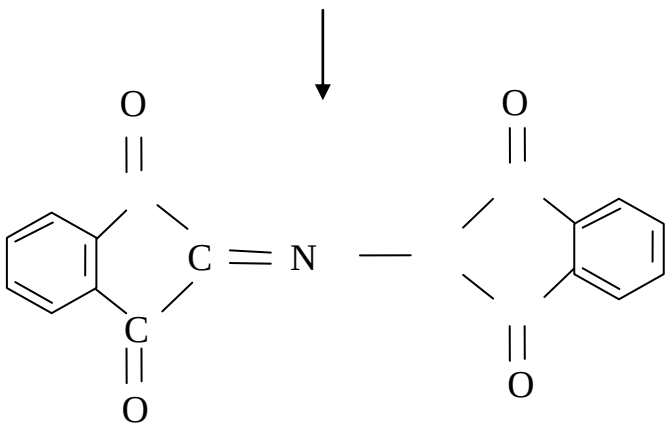
Oxidised form of Ninhydrin

Reduced Ninhydrin



Ninhydrin (Oxidised)

Ninhydrin Reduced



Purple Complex

Chemicals:

- Standard Leucine Solution
- Acetate buffer (4M) pH 5.4
- Ethylene glycol / Methyl cellulose
- Ninhydrin
- 50% ethanol
- Sodium bisulphite

Preparation of Reagents:

- **1% Sodium Bisulphite**: Dissolve 1.04 gm of sodium bisulphite in 100 ml of distilled water.
- **Acetate Buffer (4M, pH: 5.4)**: Dissolve 65.9 gm of sodium acetate in 200 ml of distilled water and 2.8 ml of glacial acetic acid in 50 ml of distilled water, mix the solution.
- **Ninhydrin Reagent:** Dissolve 3 gm of Ninhydrin in 100 ml of Ethylene glycol solution or methyl cellulose.
- **0.1% Ninhydrin:** 100 ml Acetone, 2 drops glacial acetic acid (4:1:7), 65.9 mg/ml

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of standard Leucine solution into clean and dry test tubes.
2. Adjust the volume of each test tube to 1 ml with distilled water.
3. Add 0.5 ml of freshly prepared acetate buffer to each tube and then add 0.5 ml of Ninhydrin reagent.
4. Keep all the tubes in a boiling water bath for 20 minutes and then cool.
5. Add 2 ml of 50% ethanol to each test tube.
6. Measure the optical density of the solution at 530 nm.
7. Plot the standard graph of 0.5 ml at 530 nm vs. µg Leucine.
8. Pipette out 0.5 ml of the unknown sample and repeat the above procedure.
9. Find the O.D. at 530 nm of the unknown sample and use this value to determine the unknown Leucine concentration using the standard graph.

Observation Table:

Standard Leucine Concentration: …….µg/ml

From Graph:

Unknown Solution Contains: ……..µg of leucine / 0.5 ml

Concentration of unknown solution: …… µg/ml

Sr. No.	Standard Leucine 'ml'	Distilled Water 'ml'	Acetate Buffer 'ml'	Ninhydrin 'ml'		30% Ethanol	µg of Leucine	O.D at 530 nm
1.	00	1.0	0.5	0.5	Keeep in Boiling Water bath for 20 min and	2	00	
2.	0.2	0.8	0.5	0.5		2	13	
3.	0.4	0.6	0.5	0.5		2	26	
4.	0.6	0.4	0.5	0.5		2	39	
5.	0.8	0.2	0.5	0.5		2	52	
6.	1.0	00	0.5	0.5		2	65	
7.	Unknown A. 0.5	0.5	0.5	0.5		2	-	

Standard Leucine Concentration: 65 µg/ml

- 100 ml
- 6500 µg/100ml
- 0.065 mg/100ml

Sr. No.	Standard Leucine 'ml'	Distilled Water 'ml'	Acetate Buffer 'ml'	Ninhydrin reagent 'ml'		50% Ethanol 'ml'	µg of Leucine	O.D. at 530 nm
1	00	1.0	0.5	0.5	Keep in Boiling Water bath for 20 min and Cool	2.0	00	-
2	0.2	0.8	0.5	0.5		2.0	13	
3	0.4	0.6	0.5	0.5		2.0	26	
4	0.6	0.4	0.5	0.5		2.0	39	
5	0.8	0.2	0.5	0.5		2.0	52	
6	1.0	00	0.5	0.5		2.0	65	

Result:

The concentration of amino acid in the given unknown sample: ……..µg/lit.

Thin-Layer Chromatography for Amino Acid Separation

Aim: Separation of Amino Acids by Thin-Layer Chromatography

Principle:

TLC is based on both absorption and partition chromatography. It is conducted on open layers of absorbent materials like silica gel and alumina supported on a glass plate. The separation of samples relies on the partition coefficient and adsorption of the sample onto the thin TLC layer. Adsorption is less prominent than partition, with two phases involved: a mobile phase and a stationary phase. The organic solvent system comprises various solvent combinations, while water held in silica gel acts as the stationary phase. The movement of the sample is based on its solubility in the aqueous and organic phases. The difference between these phases creates partition, and the solute's movement is proportional to its partition coefficient. Each solute has a specific RF value, which can be determined by calculating the value. The RF value is crucial for identifying unknown samples.

Reagents:

- Standard Amino acids (Ala, Ile, Pro)
- Amino acids mixture
- Silica gel
- $CuSO_4$
- Solvent system: Butanol: CH_3COOH: H_2O in a 4:7:1 ratio
- Ninhydrin

Procedure:

Preparation of TLC Plate:

1. Dissolve a pinch of $CuSO_4$ in 20ml distilled water.
2. Add approximately 10g of silica gel to form a thin, uniform slurry.
3. Spread this slurry on a glass plate.
4. Activate the plate by keeping it in an oven at 110°C for 12 hours.
5. Cool the plate and mark a line at 2 cm from one side.

Application of Sample:

1. Make spots at equal distances on the marked line.
2. Apply the given sample to the respective spots using capillaries, repeating the procedure twice.
3. Place the plate in the organic solvent system.
4. Allow the solute to run for 3-4 hours.
5. Remove the plate and mark the solvent front.
6. Dry the plate and spray ninhydrin to visualize the separating mixture.
7. Measure the distance traveled by the solute and calculate the RF value.

Calculation:

$$Rf = \frac{\text{Distance Travelled by Solvent}}{\text{Distance Travelled by Solute}}$$

Total distance travelled by solvent: 17cm

For Standard:

- RF Value of Alanine: 1.5/17 = 0.088
- RF Value of Isoleucine: 8.5/17 = 0.50
- RF Value of Proline: 4.0/17 = 0.235

For Mixture:

- RF Value of 1st spot: 1.5/17 = 0.088
- RF Value of 2nd spot: 8.4/17 = 0.494
- RF Value of 3rd spot: 3.8/17= 0.223

Result:

The mixture contains Alanine, Isoleucine, and Proline.

Paper Chromatography for Amino Acid Separation

Aim: Separation of Amino Acids by Paper Chromatography Method.

Principle:

Chromatography involves the separation of compounds in a sample through the existence of two phases: a stationary phase (solid or liquid) that remains fixed, and a mobile phase (liquid or gas) that moves. Paper chromatography, a type of partition chromatography, utilizes a paper strip as the absorbent column. The separation is based on the relative solubilities of components in two liquid phases—the fiber cellulosic filter paper serves as the stationary phase, and the solvent serves as the moving phase.

Chemicals:

- Amino Acid Mixture
- Standard Amino Acids
- Solvent system: Butanol: Acetic acid: Water (12:3:5)
- Ninhydrin

Procedure:

1. Take a strip of Whatman paper No. 1 and draw a line 2 cm from one end.
2. Place a drop of the solution on the paper, dry it, and repeat the procedure 2-3 times.
3. Place the strip in a chamber containing the solvent system at the spotted point without dipping it in the solvent.
4. Allow the solvent to run for 2/3 hours.
5. Remove the strip and dry it.
6. Apply the ninhydrin solution and dry it.
7. Calculate the Rf value for developed spots.

Calculation:

$$Rf = \frac{\text{Distance Travelled by Solute}}{\text{Distance Travelled by Solvent}}$$

Distance Travelled By Solvent: 13cm

For Standard Amino Acids:

- Rf Value of Proline: 4.8/13 = 0.3692
- Rf Value of Leucine: 8.6/13 = 0.6692
- Rf Value of Arginine: 2/13 = 0.1615

For Mixture:

- Rf Value of 1st spot: 4.5/13 = 0.3538
- Rf Value of 2nd spot: 8.9/13 = 0.6923
- Rf Value of 3rd spot: 1.2/13 = 0.1538

Result:

Standard Amino Acids:

- Rf value of Proline: 0.3692
- Rf value of Leucine: 0.6692
- Rf value of Arginine: 0.1615

Mixture:

- Rf value of 1st Spot: 0.3538
- Rf value of 2nd Spot: 0.6923
- Rf value of 3rd Spot: 0.1538

The mixture contains Proline, Leucine, and Arginine as amino acids.

Bio-molecule Isolation Techniques

Isolation of Cholesterol and Lecithin from Egg Yolk

Aim: Isolation of Cholesterol and Lecithin from egg yolk.

Principle:

Lecithin, a phosphatidylcholine, and cholesterol, a steroid, exhibit selective solubility in basic solutions, facilitating their separation. Cholesterol readily dissolves in organic solvents like chloroform, acetone, and buffer, while lecithin is soluble in $CHCl_3$ and ether but insoluble in acetone. This property is exploited to isolate lecithin and cholesterol from egg yolk. Ether is employed to isolate cholesterol and lecithin, and acetone is used to separate them from each other. Cholesterol is estimated using an iron reagent, and lecithin is weighed and tested by Reincke's salts, which produces a pink color with the choline group of lecithin. Cholesterol, being a steroid and an unsaponifiable lipid, maintains the fluidity of eukaryotic membranes and serves as a precursor for steroid hormones.

Requirements:

- Acetone
- Ether
- Chloroform
- Iron reagent
- Concentrated H_2SO_4
- Reineke's Salt

Preparation of Reagents:

1. Iron Reagent:

- Mix 2.5 gm $FeCl_3 \cdot 6H_2O$ with 100 ml H_3PO_4.
- Add 4 ml of Concentrated H_2SO_4.
- Adjust the volume to 100 ml.

2. Standard Cholesterol Solution:

- Dissolve 100 ml Cholesterol solution in 100 ml alcohol.
- Take 1 ml from the stock and dilute it up to 10 ml with alcohol.

Procedure:

1. Weigh the egg yolk (20 gm).
2. Add 20-30 ml ether and stir well.
3. Allow it to settle, then decant the supernatant.
4. Wash it with ether until the layer is colorless.
5. Evaporate the colorless supernatant to a volume of 50 ml in a hot water bath.
6. Slowly add about 100 ml acetone to the supernatant with constant stirring.
7. Filter the precipitate formed (lecithin).
8. Dry and recrystallize the precipitate by adding acetone and ether in a 2:1 ratio.
9. Weigh and test the precipitate with Reincke's Salt for lecithin confirmation.
10. Further evaporate the filtrate to 4-5 ml, which contains cholesterol.
11. Estimate the cholesterol concentration by diluting the filtrate and using the iron reagent.
12. Calculate the weight of cholesterol and lecithin per egg.

Observations:

Weight of Egg Yolk: 20 gm

Weight of Lecithin: 5 mg/egg

Confirmatory tools for Lecithin:

Test	Observation	Inference
Take a small amount of lecithin on filter paper and immersed in Reinekin salt for 5 min then wash with water	Pink Colour	Lecithin is Present and confirmed

Standard Concentration of Cholesterol: 100 µg/ml

Sr. No.	Standard Cholesterol	Alcohol (ml)	Cholesterol Reagents		OD at 530 nm	µg of Cholesterol
1.	0.0	2.0	4	Wait for ½ hours in dark		
2.	0.4	1.6	4			
3.	0.8	1.2	4			
4.	1.2	0.8	4			
5.	1.6	0.4	4			
6.	2.0	0.0	4			
Sample	0.1	1.9	4			

Calculations:

The Sample is from graph, 2:10 diluted.

From Graph,

Unknown concentration of cholesterol sample = **…….. µg / ml sample.**

Unknown concentration of cholesterol sample = **……..µg/ml of sample.**

Results:

Concentration of Cholesterol :------- **mg extract**

:------- **mg/egg**

Weight of Lecithin in Egg Yolk : **------ gm/egg**

Isolation of DNA from *Pseudomonas putida*

Aim: Isolation of DNA from *Pseudomonas putida*

Principle:

DNA, the genetic material of cells, is double-stranded with an α-helical structure, containing the bases A, T, G, and C. In this method, DNA is isolated from the organism using 70% ethanol. DNA precipitates while salts are removed. Lysozyme is employed to open the cell and extract DNA. Neutral phenol chloroform solution is used to separate DNA from proteins present in the cell.

Reagents:

- EDTA
- SDS
- Lysozyme
- 70% ethanol
- Sucrose
- Neutral phenol chloroform solution
- Sodium Acetate
- Isopropanol
- Sterile Distilled Water

Functions of Reagents:

- Saline EDTA: For osmotic shock treatment with high pH, inhibits deoxyribonucleic activity.
- SDS: An anionic detergent that lyses most non-metabolizing cells, facilitating removal and solubilisation of membrane proteins.
- Lysozyme: Used to lyse the cells.
- 70% ethanol: Used in a cold state to precipitate nucleic acid and remove salt.
- Sucrose: Used for sedimentation in an alkaline sucrose gradient to separate DNA based on size and shape.

- Neutral phenol chloroform solution: Phenol removes protein from DNA-containing solutions, and chloroform eliminates any traces of phenol in nucleic acid preparation.
- Sodium acetate: Maintains pH of the solution.
- Isopropanol: Reduces foaming, resists subsequent phase separation, and acts as a precipitant against DNA.

Procedure:

1. Grow the organism for 24 hours in 100 ml sterile medium.
2. Collect the mass by centrifugation at 8000 rpm for 20 min.
3. Suspend 20 mg cells in 500 µl lysozyme solution.
4. Add 250 µl 2% SDS and vortex mixture for 1 min.
5. Add 250 µl neutral phenol chloroform solution and vortex for 30 seconds.
6. Centrifuge at 13,000 rpm for 20 minutes.
7. Transfer the aqueous phase to a fresh micro centrifuge tube.
8. Add 250 µl neutral phenol chloroform solution and vortex for 15 minutes. Centrifuge at 13,000 rpm for 10 min.
9. Transfer the aqueous phase to a new micro centrifuge tube. Add 0.1 volume of 3M sodium acetate and wait for 10 minutes.
10. Centrifuge at 13,000 rpm for 5 minutes.
11. Decant the supernatant and wash the DNA pellet with 70% ethanol (500 µl).
12. Rehydrate the DNA in 50 µl of sterile distilled water for 2 hours at 65°C.

Result:

The DNA of *Pseudomonas putida* was successfully isolated.

Isolation of Amylase Producing Microorganism from Soil

Aim: To isolate amylase-producing organisms from Soil

Theory:

Soil is a dynamic environment containing various organisms. Amylase producers are isolated from soil due to the presence of starch in the form of plant waste material. Isolation is carried out on starch agar, where amylase, an extracellular enzyme, acts on α 1-4 and α-1-6 linkages of starch, breaking it down into glucose. Amylase producers exhibit a zone of clearance on starch agar, and amylase activity is confirmed by iodine staining. The culture is then maintained on a replica plate.

Principle:

Amylase-producing organisms secrete the enzyme amylase, which hydrolyzes starch into simpler sugars. The isolation method utilizes starch agar as a substrate. Amylase activity results in a clear zone around colonies, detected by iodine staining. Characteristics like size, shape, colour, and motility aid in identifying and isolating amylase producers.

The principle is based on amylase's ability to hydrolyse starch, forming a zone of clearance on starch agar. This zone, confirmed by iodine staining, helps isolate and identify amylase-producing organisms. Collected colony characteristics provide additional information for identification and further study.

The isolation of amylase-producing organisms from soil relies on their ability to break down starch, forming a visible zone of clearance on starch agar. This principle is confirmed by iodine staining. The isolated colonies exhibit specific characteristics, facilitating identification and further analysis. The overall aim is to successfully isolate organisms capable of producing amylase enzymes from the soil environment.

Requirements:

- Sterile pipettes
- Sterile dilution blanks
- Soil sample
- Sterile starch agar plates

Starch Agar Plate:

- Starch: 20g
- Peptone: 5g, Beef extract: 3g
- Distilled water: 1000ml
- pH: 7
- Iodine

Procedure:

1. Prepare soil dilutions ranging from 10^-1 to 10^-10.
2. Apply 0.1 ml of a higher dilution onto a starch agar plate and spread it elliptically.
3. Incubate the plates at 37°C for 24 hours.
4. Perform a replica plate on the second day, with the master plate stored in the freezer and the replica plate incubated at room temperature for 24 hours.
5. On the next day, pour iodine on the master plate and observe a zone of clearance around amylase-producing colonies. Note down colony characteristics, perform gram staining, and check motility.

Colony Characteristics:

Media: Starch agar plate.

Incubation Temperature: 27°C

Incubation Period: 24 hrs.

Size	2mm
Shape	Circular
Colour	White
Elevation	Low convex
Margin	Entire
Surface	Smooth
Opacity	Opaque
Consistency	Moist
Gram staining	Gram positive large rods
Motility	Non motile

Observation:

- Colonies on the starch agar plate exhibited a zone of clearance around amylase-producing colonies, while other plates remained blue.
- After gram staining and motility testing, gram-positive and non-motile organisms were observed.

Result:

Amylase-producing colonies were successfully isolated from the soil.

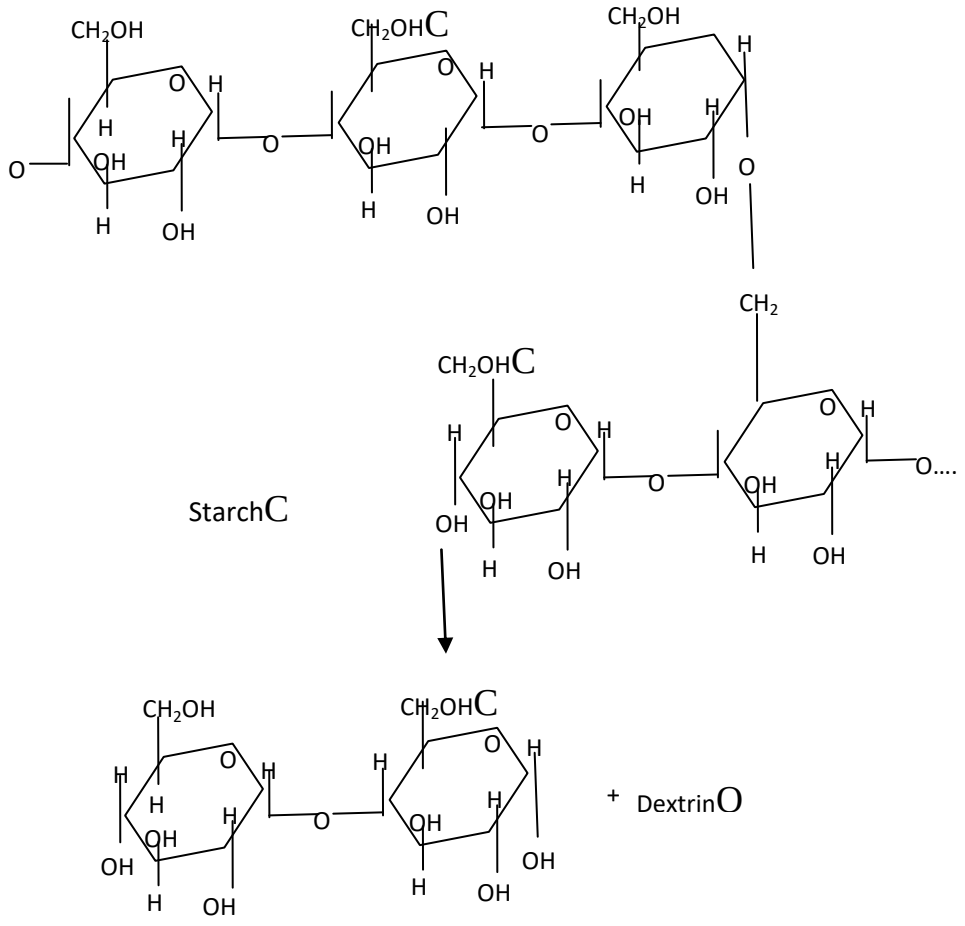
Isolation of β-Amylase from Sweet Potato

Aim: To determine the enzyme activity of β-amylase from sweet potato and analyze its performance under the influence of inhibitors.

Introduction:

β-Amylase, an exoenzyme, specifically hydrolyzes α-1→4 linkages in starch, producing maltose. This study aims to quantitatively measure β-amylase activity by estimating maltose concentration using the DNSA method.

Reaction:



Principle:

Enzyme activity is defined as the product formed in µg or mg/ml of enzyme per unit time. The activity of β-amylase is quantitatively measured by estimating the concentration of maltose released from the substrate using the DNSA method. In this method, 3,5-dinitrosalicylic acid in an alkaline solution is reduced to 3-amino-5-nitrosalicylic acid. This reaction is carried out by the aldehyde group of the reducing sugar maltose. The intensity of

the colour is directly proportional to the number of CHO groups, representing the concentration of reducing sugar.

Chemicals:

- Standard maltose solution
- β-amylase enzyme
- Acetate buffer
- β-mercaptoethanol
- CuSO4 / HgCl2

Procedure:

1. Take clean and dry test tubes and add 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of maltose to each.
2. Adjust the volume of each tube to 1 ml with distilled water and add 2.5 ml of DNSA solution to each tube.
3. Place the tubes in a boiling water bath for 10 minutes.
4. Cool the tubes and record the optical density (O.D.) at 530 nm.
5. Plot the standard graph of O.D. at 530 nm versus the concentration of maltose.

Isolation of β-amylase from sweet potato:

1. Clean the sweet potato, peel it, and weigh it to about 100 gm.
2. Cut it into small pieces and homogenize it in 100 ml of sodium acetate buffer (pH 4.8) by adding 1-2 drops of β-mercaptoethanol.
3. After homogenization, measure the crude extract, and take some of it as the enzyme source.
4. Determine the enzyme activity by measuring the conversion of starch into maltose through the action of α-amylase.

Addition without inhibitor:

	Substrate	**Buffer**	**Enzyme**
Test	1ml	1ml	0.5 ml
C1	-	2.0ml	0.5ml
C2	1ml	1.5ml	-

1. Incubate this reaction mixture for 3 minutes from the time of enzyme addition at room temperature (R.T.).
2. Then add 0.5 ml NaOH to each test tube to inactivate the enzyme.
3. Take 0.5 ml of the reduction mixture from each tube and transfer it to another 3 tubes.
4. Adjust the volume up to 1.0 ml with distilled water, then add 2.5 ml DNSA in each test tube.
5. Keep the tubes in a boiling water bath for 10 minutes. Cool them, and record the optical density (O.D.) at 530 nm.
6. Calculate the maltose concentration using the standard graph and determine enzyme activity.

Enzyme activity in presence of inhibitors:

Procedure is similar as above but in reaction mixture C1, C2 and Test inhibitor is added.

Observation Table:

Standard maltose concentration: **1000 µg/ml.**

Sr. No.	**Standard maltose solution (ml)**	**Distilled water (ml)**	**DNSA (ml)**		**µg of Maltose**	**O.D. at 530 nm.**
1.	0.0	1.0	2.5	Keep in Boiling water bath for 10 minutes and then cool down	00	
2.	0.2	0.8	2.5		200	
3.	0.4	0.6	2.5		400	
4.	0.6	0.4	2.5		600	
5.	0.8	0.2	2.5		800	
6.	1.0	0.0	2.5		1000	

Determination of enzyme activity of β – amylase without inhibitors

	Substrate	Buffer	Enzyme
Test	1.0	1.0	0.5 ml
C1	-	1.5	0.5ml
C2	1.0	1.5	-

Incubate the reaction mixture for 3 minutes and stop the reaction by adding 0.5 ml 0.5 M NaOH.

Reaction Mixture	Distilled water (ml)	DNSA (ml)		O.D. at 530 nm
Test (0.5)	0.5	2.5	Keep in Boiling water bath for 10 minutes	
C1 (0.5)	0.5	2.5		
C2 (0.5)	0.5	2.5		

μg of reducing sugar : 0.86 - (0.62 + 0.03)
: 0.86 - 0.65
A : 0.21

Calculation:
From Graph:
Enzyme activity:…… μg of reduction sugar / 3 min/0.5 ml R.M

:……μg reduction sugar /min/ml R.M.

But the 1ml crude extract is diluted to 50 ml

Activity of β – amylase: …….. μg of maltose /min /ml R.M.

Determination of activity of β – amylase with inhibitors:

	Substrate	Buffer	Inhibitor	Enzyme
Test	1ml	0.5	0.5	0.5
C1	-	1.5	0.5	0.5
C2	1ml	1.0	0.5	-
Sample	1ml	1.0	-	0.5

Incubated the reaction mixture for 3 min.

Then add 0.5 ml NaOH to stop reaction

Reaction Mixture (ml)	Distilled water (ml)	DNSA (ml)		O.D. at 530 nm
Test (0.5)	0.5	2.5	Keep in Boiling water bath for 10 minutes	
C1 (0.5)	0.5	2.5		
C2 (0.5)	0.5	2.5		
Sample (0.5)	0.5	2.5		

Calculation:

Enzyme activity in absence of inhibitor: 0.60 – 0.50
: 0.10
Enzyme activity in presence of inhibitor: 0.60 – 0.55
: 0.05

From Graph:

Reducing sugar generated in absence of inhibitors: ……µg/3min/0.5 R.M

Reducing sugar generated in presence of inhibitors:…… µg /3min/ 0.5 R.M

Total volume of reducing mixture: 3ml

Reducing sugar in absence of inhibitor: ……µg/3min/3ml R.M

Reducing sugar in presence of inhibitor:…….. µg/3min/3ml R.M

Enzyme activity in absence of inhibitors:

: …… µg/3min/ml enzyme

: ……µg/min/ml enzyme

Enzyme activity in presence of inhibitors:

: ……. µg/3min/ml enzyme

: ……. µg/min/ml enzyme

% Inhibition by $HgCl_2$: $\frac{120 \times 100}{240}$

: ……..%

Result:

Activity of β – amylase without inhibitor: …… µg/min/ml of enzyme.

Activity of β – amylase with inhibitor:……. µg/min/ml of enzyme.

Result:

1. Activity of β-amylase without inhibitor: ……. µg/min/ml of enzyme.
2. Activity of β-amylase with HgCl2 inhibitor: ……. µg/min/ml of enzyme.
3. % Inhibition by HgCl2: ……..%

Conclusion:

The study successfully determined the enzyme activity of β-amylase from sweet potato and evaluated its inhibition by HgCl2, providing valuable insights into enzyme kinetics and potential applications.

Isolation of Casein from Milk and its Characterisation

Aim: Isolation of casein from milk and its characterisation.

Principle:

Casein is the principal protein in milk, a conjugated protein containing phosphoric acid as a prosthetic group. Different forms of casein (α, β, κ) exist, with kappa (κ) being present in milk. Casein can be readily isolated as it is insoluble at its isoelectric pH (PI), which is 4.5. The isoelectric pH can be adjusted by adding acetic acid. At the PI value, casein precipitates from milk and can be isolated. Milk typically contains about 3.5% casein.

Chemicals:

- 50 ml milk sample
- Glacial acetic acid
- pH paper

Procedure:

1. Take 50 ml of milk in a beaker and add 50 ml of distilled water.
2. Add glacial acetic acid drop wise with constant stirring into the milk sample.
3. Adjust the pH to 4.5 by drop wise addition of acetic acid, using pH paper.
4. Allow the precipitate to settle down and decant the upper liquid portion.
5. Filter with cheesecloth, dry the residue, and measure the yield.
6. Perform characteristic tests for casein.

Observations:

Sr. No.	Test	Observation	Inference
1.	Casein + cold distilled H_2O	Insoluble	Casein is insoluble in cold water
2.	Casein + distilled water Heat	Insoluble	Casein is insoluble in hot water
3.	Test for protein: casein + NaOH + $CuSo_4$	Violet colour	Protein is present
4.	Test for phosphorus: Casein + H_2O + ANSA solution+ AMM. Molybdate Wait for few minutes	Blue colour	Phosphorus is present

Result:

The amount of casein in the given milk sample (50 ml) under wet conditions is …….. grams.

Isolation and Characterization of Starch from Potato

Aim: Isolation of starch from potato and its characterization.

Principle:

Starch is a major carbohydrate found in plants, stored in the form of fine granules within cells. Potato tuber cells contain starch, which is released by mechanically breaking the cells. Since starch is insoluble in cold water, water-insoluble starch settles at the bottom and can be isolated.

Materials:

- Knife
- Potato
- Potato peeler
- Cold distilled water

Procedure:

1. Wash the potato thoroughly with water.
2. Weigh 100 gm of the potato and peel it with a potato peeler.
3. Cut the potato into small pieces.
4. Suspend the smaller pieces in a small amount of water for 10 minutes for soaking.
5. After 10 minutes, homogenize the mixture in a beaker and allow it to settle down.
6. Starch settles at the bottom; filter it using cheesecloth to remove the potato pulp.
7. Allow the filtrate to settle for 15 minutes; starch settles at the bottom.
8. Filter the solution again using filter paper, separate out the starch, and dry it.
9. Weigh the dried starch and perform characteristic tests.

Characteristic Tests for Starch:

Sr. No	Test	Observation	Inference
1.	Small amount of starch + cold water	In soluble	Starch is insoluble in cold water
2.	Small amount of starch + water heat	Soluble	Starch is soluble in hot water
3.	Starch + distilled water + I_2 solution	Blue colour	Starch is present
4.	Starch + distilled water + saliva (Keep it for 5 min) + I_2 solution	No blue colour	Starch is hydrolysed into glucose by amylase
5.	Starch + distilled water + saliva (Heat for few minutes and Keep it for 10 min) + I_2 solution	Blue colour	Starch is not hydrolysed into glucose

Result:

Amount of starch isolated from 100 gm of potato under wet conditions: ……. grams.

Enzyme Activity and Kinetics

Determination of Amyloglucosidase Activity

Aim: To determine the activity of the amyloglucosidase enzyme and its Km value.

Principle:

Amyloglucosidase hydrolyzes α 1-4 and β 1-4 linkages between glucose molecules in amylose and amylopectin, splitting glucose units from the non-reducing ends. This extracellular enzyme, often used in the starch industry for glucose production, is secreted by microorganisms like *Aspergillus rhizopus*. Activity is measured by allowing the enzyme to react with starch, and the formed glucose is quantified using the DNSA method. Km is calculated by plotting 1/[s] vs 1/vo.

Chemicals:

- Acetate buffer solution, pH: 4.2
- DNSA Solution
- 1% Starch
- Standard Glucose
- 0.5 N NaOH

Procedure:
For standard glucose graph:

1. Take a clean test tube and add 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of standard glucose.
2. Adjust the total volume to 1.0 ml with distilled water.
3. Add 2.5 ml DNSA solution.
4. Boil in a water bath for 10 min.
5. Cool and record O.D at 530 nm.
6. Plot the graph of Concentration of glucose vs Optical Density at 530 nm.

Enzyme activity and Km determination:

1. Prepare a series of reaction mixtures with varying substrate concentrations.
2. Incubate for a specific time.
3. Stop the reaction and quantify glucose using DNSA.
4. Plot the graph of 1/[s] vs 1/vo to determine Km.

For the Determination of Enzymatic Activity:

Test tubes	Substrate	Buffer	Enzyme
C1	-	2.3	0.2
C2	1	1.5	-
Test	1	1.3	0.2

1. Take three clean test tubes designated as C1, C2, and Test.
2. In tube C1, add 2.3 ml of buffer.
3. In tube C2, add 1 ml of substrate and 1.5 ml of buffer.
4. In tube Test, add 1.3 ml of substrate, 0.2 ml of enzyme, and 2 ml of buffer.
5. Incubate all tubes for 10 minutes at room temperature.
6. Stop the reaction by placing the tubes in a heat inactivation unit for 10 minutes.
7. Take 1 ml of the reaction mixture from each tube and proceed with the DNSA method.

For the Determination of Km:

1. Take a clean test tube and sequentially add 0.0, 0.1, ..., 1.0 ml of 1% starch solution.
2. Adjust the volume to 2.5 ml with buffer and add 0.2 ml of enzyme.
3. Incubate for 10 minutes at room temperature.
4. Stop the reaction exactly after 10 minutes by heat inactivation for 2 minutes.
5. Proceed with the DNSA method and measure the optical density (O.D.) at 530 nm.
6. Calculate Km by plotting the Lineweaver-Burk (L:B) plot.

Observation Table:

Concentration of Standard Glucose: 500 µg/ml

Sr. No.	Standard Glucose (ml)	Distilled Water (ml)	DNSA (ml)		µg of Glucose	O. D. at 530 nm
1.	0.0	1.0	2.5	Keep in boiling water bath for 10 min,	0.0	
2.	0.2	0.8	2.5		100	
3.	0.4	0.6	2.5		200	
4.	0.6	0.4	2.5		300	
5.	0.8	0.2	2.5		400	
6.	1.0	0.0	2.5		500	

Preparation of Reaction Mixture:

	Substrate	Buffer	Enzyme	
Test	1	1.3	0.2	Incubate for 10 min at R.T. and stop reaction by heat inactivation
C1	-	2.3	0.2	
C2	1	1.5	-	

	Reaction Mixture	Distilled Water	DNSA		O.D at 530 nm
Test	1.0	1.5	2.5	Incubate for 10 min at R.T. and stop reaction by heat inactivation	
C1	1.0	1.5	2.5		
C2	1.0	1.5	2.5		

Determination of Km of Amyloglucosidase:

Sr. No.	Starch	Buffer	Enzyme
1.	0.0	2.5	0.2
2.	0.1	2.2	0.2
3.	0.2	2.1	0.2
4.	0.3	2.0	0.2
5.	0.4	1.9	0.2
6.	0.5	1.8	0.2
7.	0.6	1.7	0.2
8.	0.8	1.5	0.2
9.	1.0	1.3	0.2

Sr. No.	Reaction mixture	Distilled Water	DNSA	O.D. at 530 nm
1	0.5	2	2.5	
2	0.5	2	2.5	
3	0.5	2	2.5	
4	0.5	2	2.5	
5	0.5	2	2.5	
6	0.5	2	2.5	
7	0.5	2	2.5	
8	0.5	2	2.5	
9	0.5	2	2.5	

Results:

Calculations for Enzyme Activity:

A: Test - (C1 + C2)

From the Graph:

Activity of enzyme: …….. µg / ml enzyme / 10 min / ml R.M.

Enzyme activity: …….. µg / ml Enzyme / Minute

Results:

Activity of Amyloglucosidase: …….. µg / ml Enzyme / Minute

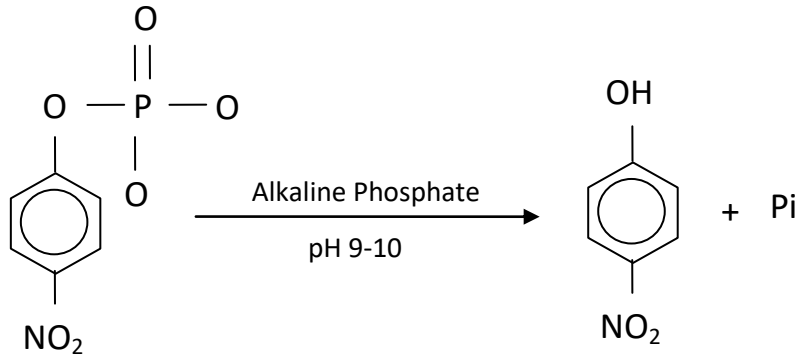
Km of Amyloglucosidase: …….. Moles / liter

Determination of Alkaline Phosphatase Activity

Aim: To determine the enzyme activity of alkaline phosphatase and its specific activity.

Principle: Enzyme activity is quantified as the amount of product formed per milliliter of enzyme per unit time. Alkaline phosphatase catalyzes the removal of O-phosphate groups from p-nitrophenol phosphate, resulting in the hydrolysis of the substrate to P-nitrophenol. The yellow colour of P-nitrophenol at alkaline pH is measured calorimetrically at 420nm.

Reaction:



FNPP PNP

Reagents:

- Standard PNP: 20 µg/ml
- Carbonate-bicarbonate buffer of pH 10

Substrate: PNPP (p-Nitrophenyl Phosphate)

- 10% TCA (Trichloroacetic acid)
- Standard BSA: 100 µg/ml (prepared in distilled water)
- Lowery C: 100 ml Lowry A + 1 ml Lowry B1 + 1 ml Lowry B2

Preparation of Reagents:

Standard BSA: Dissolve 1g of BSA in 100 ml distilled water. Pipette out 1 ml and dilute to 10 ml.

Standard PNP: Dissolve 20 µg of PNP in 10 ml carbonate-bicarbonate buffer. Pipette out 1 ml and dilute to 100 ml buffer.

P-nitrophenyl phosphate (PNPP): Dissolve 75 mg in 50 ml distilled water.

Lowry C: Mix 100 ml Lowry A with 1 ml Lowry B1 and 1 ml Lowry B2.

Procedure:

Determination of Specific Activity by Lowry's Method:

1. Take 6 test tubes and add standard BSA solutions (0.0, 0.2, 0.4, 0.6, 0.8, 1.0 ml).
2. Adjust the volume to 1 ml with distilled water.
3. Add 3 ml of Lowery C to each tube and wait for 15 minutes.
4. Add 0.5 ml of fresh Folin-phenol reagent to each tube.
5. Wait for 30 minutes and measure O.D. at 660 nm.
6. Plot a standard graph of O.D. vs. concentration of BSA.

Determination of Enzyme Activity:

Preparation of Reaction Mixture:

	Substrate	**Buffer**	**Enzyme**
C1	-	2ml	0.5ml
C2	1ml	1.5ml	-
Test	1ml	1.0 ml	0.5ml

Then, follow the Fiske-Subbarao procedure by taking 1 ml of the reaction mixture from each tube.

Determination of Specific Activity:

Specific activity is defined as enzyme activity per milligram of protein.

$$\text{Specific Activity} = \frac{\text{Enzyme Activity}}{\text{mg of Protein}}$$

Calculation:

Protein Content:

In crude extract

From the graph:

Concentration of Protein : 43 µg of protein in 0.5 ml enzyme

: 86 µg of protein/ml enzyme

Dilution : 1:10

Concentration of Protein : 860 µg of Protein/ml enzyme

In purified enzyme:

Concentration of Protein : 27 µg of Protein in 0.5 ml enzyme.

: 54 µg of protein/ml enzyme

Dilution : 1:10

Concentration of Protein : 540 µg of Protein/ml Enzyme

Specific Activity:

Crude Extract : $\frac{\text{Activity of Enzyme}}{\text{Protein Content}} = \frac{22.4}{860}$

: 0.0260 of PNP/ µg of protein / min

Purified Enzyme : $\frac{45.2}{540}$

: 0.083 of PNP /µg of protein /min

Result:

- Activity of Enzyme: ……. µg of PNP/ml enzyme/minute
- Specific Activity of Crude Extract: ……... of PNP/µg of protein/minute
- Specific Activity of Purified Enzyme:……... of PNP/µg of Protein/minute

Determination of Acid Phosphatase Activity from Rat Kidney

Aim: To determine the acid phosphate activity from Rat Kidney.

Introduction:

Acid phosphate is a cytosolic membrane-bound enzyme found in organisms. It acts on monoesters of orthophosphoric acids. Its optimum pH is 5, and it consists of subunits. Acid phosphate serves as a marker enzyme for lysosomes and is abundantly found in organs such as the kidney, liver, and muscles.

Principle:

Enzyme activity is quantified based on the amount of product formed in micrograms or milligrams per milliliter of enzyme per unit time. Acid phosphate catalyzes the removal of phosphate from β-glycophosphate, which can be measured using the Fiske-Subbarao method. In this method, released inorganic phosphate reacts with ammonium molybdate in an acidic solution to form phosphomolybdate acid. A reducing agent, ANSA, is then added, which reduces molybdenum to give a blue colour without affecting uncombined molybdic acid.

Reagents:

- Standard M/S KH_2PO_4
- Ammonium molybdate
- ANSA
- Standard β-glycophosphate
- Acetate buffer pH 5-6
- Saline 0.14 M

Procedure:

I. Isolation of Enzyme:

1. Retrieve the kidney from a rat and immerse it in 10 ml of saline in a beaker.

2. Remove the saline and cut the kidney into small pieces while maintaining cold conditions.
3. Homogenize the kidney pieces in 30 ml of acetate buffer with pH 5.6.
4. Centrifuge the homogenate and collect the supernatant.
5. Use the supernatant as the enzyme source after dilution.

II. Preparation of Standard Graph:

1. Take a test tube and add 0.4, 0.8, 1.2, 1.6, and 2.0 ml of standard phosphate.
2. Adjust the volume to 3.6 ml with distilled water.
3. Add 1 ml of ammonium molybdate to each test tube.
4. Add 0.4 ml of ANSA to each test tube.
5. Measure the OD at 660 nm after 10 minutes and plot the graph of OD vs Concentration of Phosphate.

III. Determination of Enzyme Activity:

Prepare the reaction mixture as follows:

	Substrate	**Buffer**	**Enzyme**
C1	-	2ml	0.5ml
C2	1 ml	1.5ml	-
Test	1 ml	1ml	0.5 ml

Incubate the test tubes for 10 minutes at room temperature and then stop the enzymatic activity by adding 0.5 ml of NaOH.

Procedure:

1. Then take another three test tubes and make following addition in them.
2. Add 1 ml reduced mixture from each test tubes 0.1 to 3.6 ml distilled water.
3. Then add 1 ml amm. Molybdate and then 0.4 ml ANSA.

Observation Table:

Standard concentration of phosphate: 20 μg/ml

Sr. No	Standard PO4 ml	Distilled Water	Amm. Molybdate	ANSA	µg of – PO4	O.D. at 660nm
1.	0.0	3.6	1	0.4	0	
2.	0.4	3.2	1	0.4	8	
3.	0.8	2.8	1	0.4	16	
4.	1.2	2.4	1	0.4	24	
5.	1.6	2.0	1	0.4	32	
6.	2.0	1.6	1	0.4	40	

Determination of Enzyme Activity:

	Substrate	Buffer	Enzyme	
C1	-	2.0	0.5	Incubate for 10 min at R.T. and Inactivate by adding 0.5 m NaOH (0.5 ml)
C2	1.0	1.5	0.0	
Test	1.0	1.0	0.5	

	Reaction Mixture (ml)	Distilled Water (ml)	Amm. Molybdate	ANSA ml	O.D. at 660nm
C1	1.0	2.0	1.0	0.4	
C2	1.0	2.0	1.0	0.4	
Test	1.0	2.0	1.0	0.4	

A : Test – Control
: 0.80 – (0.04 + 0.19)
: 0.80 - 0.23
A : 0.57

Calculation:

1ml of reaction mixture : 23 µg of inorganic phosphate

3 ml of total reaction mixture : x µg

X : 23 × 3

X 69 g of product /total reaction mixture /10min

3 ml reaction contains 0.5 ml enzymes

Enzyme Activity:

69 µg of PNP /0.5 ml enzyme /10 min

: 138 µg of PNP/ ml enzyme /min

: 13.8 µg of PNP /ml of enzyme /min

Dilution factor : 1:50

Enzyme activity : 13.8 × 50

Enzyme activity : 690 µg of PNP/ ml of Enzyme /ml

Result:

Activity of acid phosphatase from Rat Kidney: ----------µg of PNP / ml of enzyme / minute

Determination of Invertase Activity

Aim: To determine the activity of invertase.

Principle:

Enzyme activity is defined as the amount of product formed (in µg or mg) per ml of enzyme per unit time. In this experiment, the activity of invertase is measured by quantifying the concentration of glucose released from the substrate using the DNSA (3,5-dinitrosalicylic acid) method. DNSA reacts with reducing sugars, such as glucose, in an alkaline medium to form a colored product. The intensity of the color is directly proportional to the concentration of reducing sugars.

Chemicals:

- Standard glucose solution
- Invertase enzyme
- Acetate buffer
- DNSA Reagent

Procedure:

For Standard Graph:

1. Prepare standard glucose solutions in six test tubes ranging from 0.0 to 1.0 ml.
2. Adjust the volume of each tube to 1.0 ml with distilled water.
3. Add 0.5 ml of DNSA reagent to each tube.
4. Place the tubes in a boiling water bath for 10 minutes.
5. Cool the tubes and measure the absorbance at 530 nm.
6. Plot a standard graph of optical density versus the concentration of glucose.

For Enzyme Activity Determination:

1. Prepare reaction mixtures in three test tubes as follows:

   Test: 1 ml substrate + 1 ml buffer + 0.5 ml enzyme

   C1: 1 ml substrate + 1.5 ml buffer

   C2: 2 ml buffer + 0.5 ml enzyme

2. Incubate the test tubes for 10 minutes and stop the reaction by adding 0.5 ml of 0.5 N NaOH.
3. Transfer 0.1 ml of each reaction mixture to separate test tubes.
4. Adjust the volume of each tube to 2.5 ml with distilled water and add 2.5 ml DNSA reagent.
5. Place the tubes in a boiling water bath for 10 minutes, cool, and measure the absorbance at 530 nm.

Observation Table:

Standard glucose concentration: 500 µg/ml.

Sr. No.	Standard Glucose	Distilled water (ml)	DNSA ml		µg of Sucrose	O.D. at 530 nm
1.	0.0	1.0	2.5	Keep in boiling water bath for 10 min	-	
2.	0.2	0.8	2.5		200	
3.	0.4	0.6	2.5		400	
4.	0.6	0.4	2.5		600	
5.	0.8	0.2	2.5		800	
6.	1.0	0.0	2.5		1000	

Determination of activity of invertase
Substrate: Sucrose: 20 mg/ml

	Substrate	Buffer	Enzyme
Test	1ml	1ml	0.5 ml
C1	1ml	1.5 ml	-
C2	-	2ml	0.5 ml

Incubate for 10 minute, stop reaction by adding of 0.5 ml 0.5 N NaOH

Sr. No.	Reaction mixture	Distilled Water	DNSA		O.D. at 530 nm
C1	0.1	0.9	2.5	Keep in boiling water bath for 10 min	
C2	0.1	0.9	2.5		
Test	0.1	0.9	2.5		

Calculations:

A: Test – Control

A: 0.38 -0.03

A: 0.35

From graph:

Activity of invertase : ……µg of reduced sugar per 10 min/0.1 ml reaction mixture.

: ……µg of reduced sugar / min per ml reaction mixture.

Result:

The activity of invertase : **…….µg of reduced sugar / min / ml reaction mixture.**

Determination of Km Value of Invertase

Aim: To determine the Km value of invertase.

Introduction:

Michaelis constant (Km) is defined as the substrate concentration at which the reaction rate is half of its maximum velocity. Km is determined with the help of the Michaelis-Menten equation:

$$\text{Vo} = \frac{(\text{S})\text{Vmax}}{Km + (S)}$$

A graph of Vo versus (S) gives a hyperbolic curve. Vmax is the maximum reaction rate. When there is little change in the reaction rate as substrate concentration increases, Km can be determined approximately from this curve. The Km, which is formulated by the Lineweaver-Burk Plot, is also called the double reciprocal plot. The equation is the reciprocal of the Michaelis-Menten equation:

$$\frac{1}{Vo} = \frac{\text{Km}}{Vmax} - \frac{T}{(S)} + \frac{1}{Vmax}$$

In the case of the LB plot of 1/Vo versus 1/(S), a straight line is obtained, having a slope of km/Vmax. The intercept of 1/Vmax on the 1/Vo Y-axis and the intercept of -1/Km on the X-axis are obtained.

Principle:

Invertase is a glycosidase enzyme found in yeast that catalyzes the hydrolysis of sucrose to form glucose and fructose. Enzyme activity of invertase is defined as the formation of product as reducing sugar in µg/ml of enzyme per minute. DNSA is used to determine the concentration of the product, resulting in a colour change proportional to the sugar concentration, which is measured by a calorimeter at 530 nm and determines the activity of an enzyme.

Procedure:

For Standard Graph of Glucose:

1. Take clean and dry test tubes.

2. Add 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of glucose solution to each tube.
3. Adjust the volume of each test tube up to 1 ml with distilled water.
4. Then add 2.5 ml of DNSA solution to each tube.
5. Place the tubes in a boiling water bath for 10 min.
6. Cool the test tubes and record the absorbance at 530 nm.
7. Plot the standard graph of absorbance at 530 nm versus the concentration of glucose.

For Determination of Km of Invertase:

1. Take clean and dry test tubes and add sucrose solution as substrate in varying volumes: 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml respectively.
2. Adjust the volume of each test tube to 1.0 ml with buffer.
3. Then add 0.5 ml of invertase enzyme to each test tube.
4. Incubate the tubes at 55°C in a boiling water bath for 10 minutes.
5. After exactly 10 minutes, add 0.5 ml of 0.5 N NaOH to each tube to stop the reaction.
6. Take 0.5 ml of solution from each tube for DNSA test and measure the absorbance at 530 nm.

Observation table:

Standard glucose concentration: 500 µg/ml

Sr. No.	Standard Glucose (ml)	Distilled Water (ml)	DNSA (ml)		µg of glucose	O.D. at 530 nm
1.	0.0	1.0	2.5	Incubate the test tube at 55°C for 10 minutes	-	
2.	0.2	0.8	2.5		100	
3.	0.4	0.6	2.5		200	
4.	0.6	0.4	2.5		300	
5.	0.8	0.2	2.5		400	
6.	1.0	0.0	2.5		500	

Determination of sub. Concentration (S):

Sr. No.	Sucrose (ml)	Buffer (ml)	Enzyme (ml)		0.5 N NaOH
1.	0.0	2.0	0.5	Incubate the test tube at 55°C in water bath for 10 minutes	0.5
2.	0.1	1.9	0.5		0.5
3.	0.2	1.8	0.5		0.5
4.	0.3	1.7	0.5		0.5
5.	0.4	1.6	0.5		0.5
6.	0.6	1.4	0.5		0.5
7.	0.8	1.2	0.5		0.5
8.	1.0	1.0	0.5		0.5
9.	1.2	0.8	0.5		0.5

For V:

Sr.No.	V µg/ml/min	V Moles /liter	1/Vo
1.	70	0.388×10^{-3}	257.73
2.	100	0.555×10^{-3}	180.180
3.	160	0.888×10^{-3}	112.61
4.	190	0.055×10^{-3}	94.78
5.	230	0.277×10^{-3}	78.80
6.	380	0.388×10^{-3}	47.37
7.	400	0.388×10^{-3}	45.00
8.	420	0.388×10^{-3}	42.91

Sr.No.	1/(S)	1/(Vo)
1.	85.54	257.73
2.	42.92	180.180
3.	28.97	112.61
4.	21.41	94.78
5.	14.26	78.80
6.	10.69	47.37
7.	8.55	45.00
8.	7.12	42.91

From Graph:

$\frac{-1}{Km}$: 8.5

Km : $\frac{1}{8.5}$

Km : ……..moles/liter

Result:

The km of invertase is **……..moles/liter**

Determination of α-Amylase Activity

Aim: Identification and quantification of enzyme activity of salivary α-amylase**.**

Introduction:

Salivary α-amylase, present in saliva, hydrolyzes α-1,4-D-glucose units. Its action can be conveniently demonstrated using starch as a substrate. Under appropriate conditions, α-amylase catalyzes the hydrolysis of starch and higher dextrins, producing maltose and other products. The presence of reducing sugars resulting from this hydrolysis can be detected using DNSA method, where 3,5-dinitrosalicylic acid in an alkaline solution is reduced to 3-amino-5-nitrosalicylic acid, resulting in a color change that is directly proportional to the concentration of sugar.

Procedure:

For Standard Graph of Glucose:

1. Take clean and dry test tubes and add 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of glucose solution to each.
2. Adjust the volume of each test tube to 1.0 ml with distilled water.
3. Add 2.5 ml of DNSA solution to each test tube.
4. Boil the tubes in a water bath for 10 minutes, then cool and measure the OD at 530 nm.
5. Plot the standard graph of OD at 530 nm vs. concentration of glucose.

Preparation of Reaction Mixture:

1. Sample: 1 ml substrate + 1 ml buffer + 0.5 ml enzyme (saliva diluted to 1:30).
2. Substrate blank: 1 ml substrate + 1.5 ml buffer. (Incubate these test tubes for 30 min at room temperature. Inactivate the enzyme after exactly 3 min by boiling in a water bath for 5 min.)

Preparation of Enzyme Activity Sample:

1. Take 0.5 ml of enzyme reaction mixture from each test tube to another 3 test tubes.
2. Adjust the volume to 1.0 ml with distilled water.

3. Add 2.5 ml of DNSA solution to each tube.
4. Boil in a water bath for 10 minutes, then cool.
5. Measure the OD at 530 nm.
6. Calculate the concentration of glucose using the standard graph and determine enzyme activity.

Reagents:

- Salivary α-amylase
- 1% Starch Solution
- Standard glucose: 500 µg/ml
- DNSA reagent
- Phosphate buffer (0.02M)

Observation Table:

Standard glucose concentration: 500 µg/ml.

Sr. No.	Standard Glucose	Distilled Water	DNSA		µg of glucose	O.D. at 530 nm
1.	0.0	1.0	2.5	Keep in Boiling water bath for 10 minutes	00	
2.	0.2	0.8	2.5		100	
3.	0.4	0.6	2.5		200	
4.	0.6	0.4	2.5		300	
5.	0.8	0.2	2.5		400	
6.	1.0	0.0	2.5		500	

Preparation of Reaction mixture:

	Substrate	Buffer	Enzyme
Test	1ml	1ml	0.5 ml
C1	1ml	1.5ml	-
C2	-	2 ml	0.5 ml

Activity of α-amaylase

Reaction mixture (ml)	Distilled water (ml)	DNSA (ml)		O.D. at 530 nm
0.5 ml from test	0.5	2.5	Keep in Boiling water bath for 10 minutes	
0.5 ml from C1	0.5	2.5		
0.5 ml from C2	0.5	2.5		

Calculations:

A : Test – constant (C1 + C2)

A : ……..

From Graph:

Enzyme Activity : ……..µg of reduced sugar /minute per 0.5 ml / reaction mixture.

: …….. µg of reduced sugar / 2.5 ml reaction mixture /minute.

:…….µg of reduced sugar / 2.5 ml reaction mixture /minute.

Result:

Enzyme Activity:

: …….. µg of reduced sugar /minute/ml of reaction mixture.

: ……. µg of reduced sugar /minute / ml of saliva.

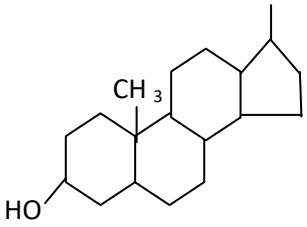
Estimation of Compounds

Estimation of Cholesterol

Aim: Quantitative estimation of cholesterol in the given sample.

Introduction:

Cholesterol is a steroid derivative of a saturated tetracyclic hydrocarbon. It is insoluble in water but readily soluble in organic solvents such as alcohol and chloroform. Cholesterol is a constituent of cell membranes in higher plants and animals, maintaining the fluidity and providing mechanical stability to the plasma membrane. In higher organisms, cholesterol, upon exposure to UV light, is converted into ergosterol, a precursor of vitamin D.



Principle:

In the presence of a strong dehydrating agent like concentrated H2SO4, cholesterol undergoes intramolecular rearrangement. This rearranged compound reacts with FeCl3.6H2O in the presence of H3PO4 to form an orange-colored complex. The intensity of the color is directly proportional to the concentration of cholesterol, which can be measured calorimetrically at 530 nm.

Chemicals:

- Standard cholesterol solution
- Alcohol
- Concentrated H2SO4
- H3PO4
- Calorimeter

Procedure:

1. Pipette out 0.0, 0.4, 0.8, 1.2, 1.6, and 2 ml of standard cholesterol solution into clean and dry test tubes.

2. Adjust the volume of each tube to 2 ml by adding alcohol.
3. Add 4 ml of cholesterol reagent to each tube.
4. Incubate the tubes in the dark for 10 min.
5. Measure the OD of the solution at 530 nm.
6. Plot the graph of OD at 530 nm vs. µg of cholesterol.
7. Pipette out 1 ml of the unknown sample and repeat the above procedure.
8. Determine the OD at 530 nm of the unknown sample and use this value to determine the unknown concentration of the sample using the standard graph.

Preparation of Reagents:

Standard cholesterol solution: 100 µg/ml

- Dissolve 100 mg of cholesterol in 100 ml alcohol to create a stock solution.
- Dilute 1 ml from the stock solution to 10 ml with alcohol.

Iron Reagent:

- Dissolve 2.5 g $FeCl_3$ in 100 ml H_3PO_4 solution.

Cholesterol Reagent:

- Mix 40 ml iron reagent with 460 ml concentrated H_2SO_4 solution.

Observation Table:

Standard Cholesterol concentration: 100 µg/ml

Sr. No.	Standard Cholesterol (ml)	Alcohol (ml)	Cholesterol reagent (ml)		µg of cholesterol	O.D. at 530 nm
1.	0.0	2.0	4	Wait for 30 minute and keep in dark	0.0	
2.	0.4	1.6	4		40	
3.	0.8	1.2	4		80	
4.	1.2	0.8	4		120	
5.	1.6	0.4	4		160	
6.	2.0	0.0	4		200	
7.	1.0	1.0	4		-	
8.	1.0	1.0	4		-	

From Graph:

Concentration of unknown A: ……. µg/ml

Concentration of unknown B:…….. µg/ml

Result:

- The Concentration of cholesterol in given unknown 'A' sample: ……. µg/ml
- The Concentration of cholesterol in given unknown 'B' sample:…….. µg/ml

Estimation of Glucose by Anthrone Method

Aim: Quantitative estimation of glucose in the given sample by Anthrone Method.

Introduction:

Glucose is an aldohexose and serves as a unit of many biologically important polysaccharides such as cellulose, glycogen, and starch. It is produced by green plants through photosynthesis, where CO2 and light are utilized as sources of energy. Glucose acts as a significant energy source for cells, generating as much as 38 ATPs, which are utilized for various biological functions. Glucose typically exists in the pyranose form, where the carbonyl carbon atom reacts with the fifth carbon atom to form a closed-ring structure.

Principle:

The Anthrone reaction provides a rapid and convenient method for the detection of hexoses, aldopentoses, and hexauronic acids, whether free or present in polysaccharides. The resulting blue-green solution exhibits absorption maxima at 620 nm, although some carbohydrates may produce other colours. However, the reaction is not suitable for glycoproteins containing large amounts of tryptophan, which give a red colour.

Chemicals:

- Standard glucose solution
- Anthrone reagent

Preparation of Reagent:

Anthrone reagent: Dissolve 200 mg of Anthrone in 100 ml of ice-cold 95% H2SO4 solution.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8, and 1 ml of standard glucose solution into clean and dry test tubes.
2. Adjust the volume of each tube to 1 ml with distilled water.
3. Add 5 ml of cold Anthrone reagent to each test tube.
4. Place these tubes in a boiling water bath for 8 minutes and then cool them.
5. Measure the optical density of the solution at 620 nm.

6. Plot the standard graph of optical density at 620 nm vs. µg of glucose.
7. Pipette out 0.5 ml of the unknown sample and repeat the above procedure.
8. Determine the OD of the unknown sample at 620 nm, and using this value, determine
9. the unknown glucose concentration using the standard graph.

Observation table:

Sr. No.	Standard glucose (ml)	Distilled water (ml)	Anthrone (ml)		µg of glucose	O.D. at 620 nm
1)	0.0	1.0	5	Keep in boiling water bath for 8 min and cool it	0.0	
2)	0.2	0.8	5		20	
3)	0.4	0.6	5		40	
4)	0.6	0.4	5		60	
5)	0.8	0.2	5		80	
6)	1.0	0.0	5		100	
Unknown A	0.5	0.5	5		-	
Unknown B	0.5	0.5	5		-	

From Graph:

Concentration of unknown A solution: …. µg/0.5 ml

Concentration of unknown A solution: ….µg/ml

Concentration of unknown B solution: ….µg/0.5 ml

Concentration of unknown B solution: ….µg/ml

Result:

The Concentration of glucose in given unknown sample A: ……. µg/ml

The concentration of glucose in given unknown sample B: ……..µg/ml

Estimation of Glucose by Phenol-H2SO4 Method

Aim: Quantitative estimation of glucose in the given sample by the Phenol - H2SO4 Method.

Introduction:

Glucose is a polyhydroxy aldehyde, an aldohexose, and a fundamental unit of many biologically important polysaccharides such as cellulose, glycogen, and starch. It is produced by green plants through photosynthesis using CO2 and light energy as sources. Glucose serves as a primary energy source for cells, producing up to 38 ATP molecules utilized for various biological functions. Glucose exists in the pyranose form, where the carbonyl carbon atom reacts with the fifth carbon atom to form a closed-ring structure.

Principle:

Glucose, upon reduction with concentrated H2SO4, undergoes dehydration to form a furfural derivative. Furfural and its derivatives react with phenol to produce a brightly colored complex. The intensity of the color is directly proportional to the concentration of glucose in the solution, which can be determined calorimetrically at 470 nm.

Chemicals:

- Standard Glucose solution
- 7.5% Phenol
- Concentrated H2SO4
- Colorimeter

Reagent Preparation:

7.5% Phenol: Dissolve 15 mg of phenol in 200 ml of distilled water.

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of standard glucose solution into clean and dry test tubes.
2. Adjust the volume of each tube to 1.0 ml with distilled water.
3. Add 0.5 ml of 7.5% phenol to each tube.
4. Then add 5 ml of concentrated H2SO4 to each tube.

5. Incubate the test tubes for 30 minutes.
6. Measure the optical density of the solution at 470 nm.
7. Plot the standard graph of optical density at 470 nm Vs. µg of glucose.
8. Pipette out 0.5 ml of the unknown sample and repeat the above procedure.
9. Determine the optical density at 470 nm of the unknown sample and use this value to determine the unknown glucose concentration using the standard graph.

Observation Table:

Standard glucose concentration: 100 µg/ml

Sr. No.	Standard glucose (ml)	Distilled water (ml)	7.5 % phenol (ml)	Concentrated H_2SO_4 (ml)		µg of glucose	O. D. at 470 nm
1)	0.0	1.0	0.5	5	Keep in boiling water bath for 30 min	00	
2)	0.2	0.8	0.5	5		20	
3)	0.4	0.6	0.5	5		40	
4)	0.6	0.4	0.5	5		60	
5)	0.8	0.2	0.5	5		80	
6)	1.0	0.0	0.5	5		10	
A	0.5	0.5	0.5	5		-	
B	0.5	0.5	0.5	5		-	

From Graph:

- The Concentration of glucose in given sample A: …… µg/0.5 ml
- The Concentration of glucose in given sample A: …… µg/ml
- The Concentration of glucose in given sample B: …… µg/0.5 ml
- The Concentration of glucose in given sample B:…… µg/ml

Result:

The Concentration of glucose in given sample A: …… µg/ml

The Concentration of glucose in given sample B:……. µg/ml

Estimation of Glucose by Glucose Oxidase Method

Aim: To Estimate glucose by the glucose oxidase method.

Principle:

Glucose oxidase is a dimeric protein with a molecular weight of 16 kDa containing bound FAD per monomer as a cofactor. Glucose oxidase and β-D-glucose O2 + oxidoreductase catalyze the oxidation of β-D-glucose to D-glucolactone and H2O2 using molecular oxygen. The initial product of glucose oxidase is D-glucolactone 1,5-lactone, which hydrolyzes spontaneously to gluconic acid.

The peroxidase breaks down H2O2 to water and oxygen using dye as an electron donor. At some time, the reduced dye is oxidized, from which the color comes. Since the amount of H2O2 produced indicates how much reaction takes place, the intensity of color is measured at 530 nm, which is directly proportional to glucose concentration.

Reaction:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} H_2O_2 + \text{Gluconic acid}$$

$$H_2O_2 + \text{Phenol} + \text{Reduction Dye} + H^+ \xrightarrow{\text{Peroxidase}} H_2O + \text{Oxidased}$$

Chemicals / Material:

- Standard glucose solution: 100 µg/ml
- Phenol reagent
- Glucose oxidase: 100 mg
- Peroxidase: 2-4 mg
- 35 mg 4-amino phenazone
- Sodium azide
- Colorimeter

Preparation of reagents:

Phenol reagent: Dissolve 200 mg of phenol in 100 ml of distilled water.

Colour reagent: Dissolve 1 gm Na2HPO4 2 H2O + 100 mg glucose oxidase + 2-4 mg peroxidase + 35 mg 4-amino phenazone + 100 mg sodium azide in 100 ml of distilled water. Adjust the pH to 6.8 to 7.0.

Procedure:

1. As per the observation table, take 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of standard glucose solution in test tubes.
2. Add 0.5 ml of phenol reagent to each test tube.
3. Also, add 3 ml of coloured reagent to each test tube.
4. Incubate all the test tubes for 10 minutes at 37°C.
5. Measure the OD at 530 nm.
6. Plot the standard graph of OD at 530 nm versus glucose concentration.
7. Repeat the same procedure for the unknown sample and calculate the sample concentration from the standard graph.

Observation Table:

Standard glucose concentration: 100 µg/ml

Sr. No.	Standard glucose (ml)	Distilled water (ml)	Phenol reagent (ml)	Coloured reagent (ml)		O.D. at 530 nm	Concentration of glucose µg/ml
1.	0.0	1.0	0.5	3	Wait for 10 minutes at 37°C	0.0	
2.	0.2	0.8	0.5	3		0.04	
3.	0.4	0.6	0.5	3		0.08	
4.	0.6	0.4	0.5	3		0.10	
5.	0.8	0.2	0.5	3		0.18	
6.	1.0	0.0	0.5	3		0.22	
7.	Unknown 0.5	0.5	0.5	3		0.08	

Calculations:

From the graph:

- The concentration of the unknown sample: ……. µg/0.5 ml
- The concentration of the unknown sample: ……. µg/ml

Result:

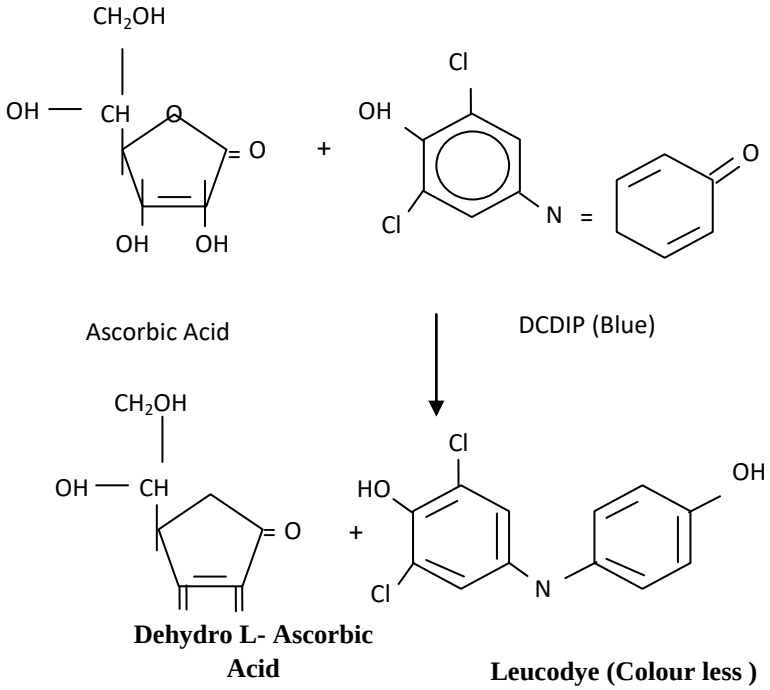
The unknown concentration of the sample is ……. µg/ml.

Estimation of Vitamin C by DCPIP Method

Aim: Estimation of ascorbic acid (Vitamin C) in the given biological sample (lemon) by the DCPIP method.

Principle:

Chemical methods for the determination of vitamin C are mostly based on its reducing properties. To increase the specificity of oxidation-reduction methods, 2,3-dichlorophenol indophenol dye is used. This method depends on the stoichiometric reduction of the dye to 2,6-dichlorophenol indophenol, a colorless compound, by ascorbic acid. In the absence of interfering substances, the extent of reduction is proportional to the amount of ascorbic acid present. The titration is carried out in the presence of acetic acid to prevent air oxidation of vitamin C.



Chemicals:

- Standard L-ascorbic acid solution
- 2,6-dichlorophenol indophenol dye
- Lemon Juice

- 3% metaphosphoric acid
- Acetic acid
- Sodium Bicarbonate

Preparation of Reagent:

Dichlorophenol indophenol dye (DCPIP): Dissolve 40 mg sodium bicarbonate in 40 ml hot water, then add 50 mg of DCPIP dye, heat it slightly, cool it, and dilute it to 200 ml with distilled water.

Procedure:

Part I:

1. Fill the burette with DCPIP solution.
2. Pipette out 10 ml of standard L-ascorbic acid into a conical flask.
3. Add 1 ml of glacial acetic acid.
4. Titrate this solution with DCPIP dye until a slightly pink color is obtained as an endpoint.
5. Calculate the burette reading as 'X' ml.

Part II:

1. Cut 5-6 lemons and squeeze the juice into a beaker, filtering if necessary.
2. Take 40 ml of juice in a measuring cylinder and dilute it up to 300 ml with 3% metaphosphoric acid.
3. Pipette out 10 ml of this biological sample into a conical flask.
4. Add 1 ml of glacial acetic acid to the conical flask and titrate this with DCPIP dye until the endpoint is reached.
5. Calculate the burette reading as 'Y' ml.

Observations:

Part I:

Solution in Burette : 2,6-dichlorophenol indophenol dye
Solution in Pipette : Standard ascorbic acid + 1 ml acetic acid
Solution in Indicator : Dye itself
Change in color : Colorless to slight pink
Burette Reading (C.B.R.) 'X' : 20.2 ml

Burette Level	Burette reading in ml			C.B.R. 'x'ml
	I	II	III	
Final	20.2	20.3	20.2	X=20.2 ml
Initial	0.0	0.0	0.0	
Difference	20.2	20.3	20.2	

Part II:

Solution in Burette : 2,6-dichlorophenol indophenol dye

Solution in Pipette : Lemon juice (diluted) + 1 ml acetic acid

Solution in Indicator : Dye itself

End Point : Colorless to slight pink

Burette Reading (C.B.R.) 'Y' : 3.2 ml

Burette Level	Burette reading in ml			C.B.R. 'Y'ml
	I	II	III	
Final	3.2	3.1	3.2	Y=3.2 ml
Initial	0.0	0.0	0.0	
Difference	3.2	3.1	3.2	

Calculations:

For 2 mg of Ascorbic acid, 20.2 ml of dye is required.

For 'Z' mg of Vitamin C in lemon, 3.2 ml of dye is required.

'Z' : $\frac{3.2 \times 2}{20.2}$ mg of Vitamin C / 10 ml

: $\frac{3.2 \times 2 \times 40}{20.2}$ mg of Vitamin C / 40 ml of lemon juice

: 12.67 mg of Vitamin C / 40 ml of lemon juice

'Z' : 12.67 mg

Amount of Vitamin C in 40 ml sample: 12.67 mg

Result:

Amount of Vitamin C in the given Lemon juice: 12.67 mg/40 ml

Lipid Analysis

Qualitative Test for Lipids

Sr. No.	TEST	OBSERVATION	INFERENCE
1.	**Emulsification test:** 2 ml. O. S. + 3 ml of water shake well	Droplets are observed	Oil confirmed
2.	**Sudan III test:** 3ml O. S. + 2 drops of Sudan III stain. Take out one to two drops on a clean and dry slide. Observe under microscope	Brick red coloured droplets	Oil confirmed
3.	**Saponification test** 1 ml O. S. + 3 ml of alc. 10% NaOH boil, cool & excess of Na2 SO4	Soap is formed & it rises to the surface	Oil confirmed

Estimation of Lipids by Sulpho-phosphovanillin Reagent

Aim: To estimate total Lipids using Sulpho-phosphovanillin reagent from tissue.

Principle:

Lipids react with vanillin in the presence of sulphuric and phosphoric acids to form a pink-coloured complex.

Chemicals :

- Total Lipid Standard – 300mg cholesterol in 50ml Glacial acetic acid
- Vanillin reagent
- Chloroform
- Methanol
- sulphuric acid
- Sodium sulphite
- Homogenate : 100 mg chilled tissue was homogenized in 10 ml Folch mixture (chloroform: methanol = 2: 1). A pinch of sodium sulphite was added, and the solution was filtered.

Procedure:

1. Take 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard into labelled test tubes.
2. Unknown sample – Take 1 ml of homogenized sample.
3. Boil the standard tubes and unknown sample for 2 hours and dry them.
4. Add 1 ml of concentrated H_2SO_4 to all tubes. Prepare a blank test tube containing 1 ml of conc. H_2SO_4 and boil for 5 minutes, then cool it.
5. In the cooled solution, add 2 ml of Vanillin reagent. Pink color develops.
6. Measure the optical density at 530 nm.

Observation Table:

Volume of standard Cholesterol (ml)	Conc. of Cholesterol (µg)	Boil for two hours and dry it	Volume of Conc. H_2SO_4 (ml)	Boil for 5 min. and cool it	Volume of Vanillin reagent (ml)	Absorbance at 530 nm (Optical Density)
0.2	1200		1		2	
0.4	2400		1		2	
0.6	3600		1		2	
0.8	4800		1		2	
1.0	6000		1		2	
Unknown 1.0	?		1		2	
Blank -	00		1		2	

(Graph- Plot a graph of Optical density Vs Concentration of standards. Coincide the OD of the unknown sample with the Conc. of the standard. This is the Conc. of the unknown sample.)

Result:

The given unknown sample contains ……. µg of lipid.

Estimation of Lipids by Gravimetric Method

Aim: To estimate the total lipid content in a given sample using the gravimetric method.

Principle:

The gravimetric method for lipid estimation involves extracting lipids from the sample using a suitable solvent, followed by evaporation of the solvent and subsequent weighing of the lipid residue. Lipids are insoluble in water but soluble in organic solvents such as chloroform, methanol, or a mixture of chloroform and methanol. By extracting lipids with these solvents and evaporating the solvent, the lipid content can be determined gravimetrically.

Chemicals:

- Chloroform
- Methanol
- Petroleum ether
- Anhydrous sodium sulphate (Na2SO4)
- Standard lipid solution (for calibration)

Procedure:

1. Weigh an empty, clean, and dry flat-bottomed glass vial accurately and record its weight as W1 grams.
2. Take a known volume (e.g., 10 ml) of the sample in a separating funnel.
3. Add an equal volume of a suitable solvent mixture (e.g., chloroform: methanol, 2:1) to the separating funnel.
4. Shake the separating funnel vigorously for several minutes to ensure thorough mixing of the sample with the solvent.
5. Allow the mixture to stand until the layers separate.
6. Collect the lower layer (containing lipids dissolved in the solvent) in a weighed glass vial.
7. Evaporate the solvent in a fume hood or under reduced pressure using a rotary evaporator until a constant weight is achieved.
8. Weigh the glass vial containing the lipid residue and record its weight as W2 grams.

9. Repeat the procedure with a known standard lipid solution to prepare a calibration curve.

Observation Table:

Sample	Initial weight of vial (g)	Final weight of vial with lipid residue (g)	Weight of lipids (g)
Unknown 1	W_{1a}	$W_{2\,a}$	$W_{2\,a} - W_{1a}$
Unknown 2	W_{1b}	W_{2b}	$W_{2b} - W_{1b}$
...	...	...	...
Unknown n	W_{1n}	W_{2n}	$W_{2n} - W_{1n}$

Calculations:

1. Calculate the weight of lipids for each sample using the formula:

   Weight of lipids = Final weight of vial with lipid residue - Initial weight of vial.

2. Plot a calibration curve using the known concentrations of the standard lipid solution and the corresponding weights of lipids obtained.

3. Determine the concentration of lipids in the unknown samples using the calibration curve.

Result:

The lipid content in the given unknown samples is determined as per the calibration curve obtained from the standard lipid solution.

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VYD Publishers
B-3,Balaji Nagar Apartment
406, Mangalwar Peth, Satara.
415-002.
Reg No : 2331400317359270
Email Id : vydpublishers@gmail.com
www.teamvyd.com

VYD PUBLISHERS

ISBN 978-81-967529-5-8
9 788196 752958