19MBU202 BIOCHEMISTRY PRACTICAL II Semester – II

COURSE OBJECTIVE

- To familiarize the students with the some basic analytical techniques in Biochemistry.
- To make students to gain knowledge with these techniques used for purification and structural predication of bioorganic compounds.

COURSE OUTCOME

1. Students will get practical knowledge about various techniques used in Biochemistry.

EXPERIMENTS

1. Estimation of Protein by Lowry's method
2. Estimation of Cholesterol by Zak's method
3. Estimation of Phosphorus by Fiske Subbarow method
4. Determination of effect of pH, temperature and substrate concentration of Salivary Amylase
5. Separation of sugar by paper chromatography
6. Separation of amino acid by thin layer chromatography
7. Separation of plant pigments by thin layer / column chromatography

SUGGESTED READING

1. Biochemical Methods 1992, by S.Sadasivam and A. Manickam, Second Edition, New Age International Publishers, New Delhi
2. Laboratory Manual in Biochemistry, 1981. J.Jayaraman, New Age International publishers, NewDelhi

KARPAGAM ACADEMY OF HIGHER EDUCATION

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

DEPARTMENT OF BIOCHEMISTRTY

SUBJECT: **BIOCHEMISTRY PRACTICAL II**

SEMESTER: **II**

SUBJECT CODE: **19MBU213** **CLASS**: **I B. Sc. MB**

EXPERIMENTAL DETAILS
DEPARTMENT OF BIOCHEMISTRTY

S.No	Lecture Duration Hour	Topics to be Covered	Support Material/Page Nos
1	4	Estimation of protein by Lowry's method	Plummer D. T., (1998). An Introduction to Practical Biochemistry 3rd ed., Tata McGraw Hill Education Pvt. Ltd. (New Delhi), ISBN:13: 978-0-07-099487-4 / ISBN:10: 0-07-099487-0.
2	4	Estimation of cholesterol by Zak's method	
3	4	Estimation of phosphorus by Fiske Subbarow's method	
4	4	Determination of the effect of pH, temperature and substrate concentration of Salivary Amylase	
5	4	Separation of sugar by paper chromatography	
6	4	Separation of amino acid by thin layer chromatography	
7	4	Separation of plant pigments by thin layer/ column chromatography	
Total number of hours planned for practical= 28			

REFERENCES

Laboratory Manual in Biochemistry, J.Jayaraman, Madurai Kamaraj University, Madurai

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

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)

SUBJECT CODE:19MBU211

SUBJECT: BIOCHEMISTRY PRACTICAL II

CLASS : I BSC MICROBIOLOGY

Experiment No: 1

SEPARATION OF SUGAR BY PAPER CHROMATOGRAPHY

Paper Chromatography

Chromatography is the most powerful technique to separate chemically closely related substances into the individual components on the basis of their physicochemical properties. The compounds are separated on the basis of their partition coefficients between two immiscible phases. The static phase may be a solid or liquid while the mobile phase may be a solid, liquid or gas. Depending upon the static and mobile phases, a variety of chromatographic techniques are available. These include chromatography on paper, thin layer gel, ion-exchange resin etc. Although modern instrument facilities such as High Performance Liquid Chromatography (HPLC) are available for the separation of chemical substances, the classical techniques - paper chromatography and thin layer chromatography are still easy, can be set up even in an ordinary laboratory without much expenditure. It may be recalled that Calvin and his associates used paper chromatography to elucidate the pathway of carbon dioxide fixation in photosynthesis. The separation, identification and (semi) quantification of amino acids using paper chromatography is described below. The same methodology can be used to separate other smaller molecules such as sugars, organic acids etc. by changing the mobile phase and detection (spray) agents.

Principle

The separation of the solutes (amino acids) is based on the liquid-liquid partitioning of amino acids in paper chromatography. The partitioning takes place between the water molecule (static phase) adsorbed to the cellulosic matter of the paper and the organic (mobile) phase.

Materials

1. Whatman No.1 filter paper
2. Chromatography chamber
3. Hair-dryer or spot-lamp
4. Atomizer
5. Microsyringe or micropipette

Mobile Phase (Solvent System)

Mix n-butanol, glacial acetic acid and water in the ratio 4 : 1 : 5 in a separating funnel and stand to equilibrate for 30 min. Drain off the lower aqueous phase into a beaker and place it inside to saturate the chromatography chamber. Save the upper organic phase and use it for developing the chromatogram.

Dissolve different individual amino acids in distilled water at a concentration of 1mg/mL. Use very dilute (0.05N) HCl to dissolve the free amino acids tyrosine and phenylalanine. Dissolve tryptophan in very dilute (0.05N) NaOH.

Extraction of Sample

Grind a known quantity of the sample material (dry/wet) in a pestle and mortar with 10-fold volume of 70% ethanol. Shake the contents at 55°C for 30 min. Centrifuge the contents at 10,000rpm for 10 min. Collect the supernatant. Repeat the extraction of the pellet at 55°C at least twice. Pool the supernatants (for leaf extracts, treat with equal volume of petroleum ether 40-60°C) and shake vigorously. Discard the petroleum ether layer containing chlorophyll. Evaporate the alcohol fraction to dryness under vacuum using either a water-pump or rotary evaporator at 40-45°C. Dissolve the residue in a known volume of absolute ethanol or water for analysis.

Ninhydrin Reagent

Dissolve 100mg ninhydrin in 100mL acetone.

Elution Mixture

Prepare 1% copper sulphate solution. Mix ethanol and copper sulphate solution in the ratio 80:20 (v/v).

Procedure

1. Cut the chromatography sheet carefully to a convenient size (40 x 24cm). Draw a line with pencil across the sheet about 5cm away from one end. Mark a number of points at intervals of 3cm.

2. Apply a small volume (say, 25□L)of each amino acid as a separate small spot using a microsyringe. A stream of hot air from a hair-dryer facilitates fast drying of spot. The spot should be as small as possible for better resolution.
3. Similarly spot different known aliquots of sample extract.
4. After spotting, place the sheet in a stainless steel trough in the chromatography chamber, firmly hold it by placing a long steel rod over the sheet. The spot-end of the sheet should be in the trough (descending chromatography). Otherwise, the sheet may be rolled as a cylinder, tied together with fine thread and placed upright with the spots as the bottom in a large Petridish for upward movement of solvent (ascending chromatography).what mann no:1 filter paper was act into a circle of diameter 20 cm. pencil line was drawn 4 cm away from the centre. The circle was divided into four parts. The three amino acids and the unknown mixture was spotted along the pencil line at specific spots. As hole was made in the centre of the paper which was introduced into it. The paper was kept in the petridish containing 5 ml of solvent. The solvent was sucked by the wider which transfer solvent to the chromatographic chamber (Circular chromatoghraphy).
5. Add the organic (phase) solvent to the trough/petri dish and close the chamber airtight. Develop the chromatogram, preferably overnight or longer, until the solvent moves almost to the other end.
6. Note the solvent front and dry the chromatogram free of solvent in a fume chamber.
7. Spray the chromatogram with the ninhydrin reagent using an atomizer. Dry the paper for about 5 min at room temp followed by at 100°C in an oven for 2-3 min. Amino acids appear as purple spots; hydroxyproline and proline give yellow colored spots.
8. Mark all the spots and calculate their R_f values by the formula.

$$R_f = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

9. The amino acids present in the sample are then identified by comparing the *Rf* values with that of the authentic amino acids, co-chromatographed.
10. For quantitative estimation, cut each spot into several small bits and transfer to the bottom of the test tube.
11. Add 3mL of elution mixture. Shake the tubes vigorously for 15 min. Decant the liquid and elute the pieces with another 2mL of elution mixture. Repeat the elution with small aliquots until the bits are colorless. Combine and clear the eluate by centrifuging at 10,000rpm for 10

min. Read the intensity of purple color at 570nm in a colorimeter. Use the spot of leucine *(50□g)* run as standard for comparison.

Notes

1. Handle the chromatography sheet very carefully until developed as otherwise amino acids from fingers will contaminate. Hold the chromatographic paper between a fold of filter paper piece.
2. After developing the chromatogram with the first solvent system, it may be developed with second solvent system such as phenol: H_2O (80 : 20). This is then called bidimensional chromatography. In such case, the sample alone is applied at near one corner of the paper. Bidimensional chromatography is carried out for fine separation.
3. As the *Rf* value varies from run to run, due to solvent system, paper, room temperature, size of the chromatography chamber etc., it is advisable to co-chromatograph the standards every time.
4. Similarly, analysis of sugars, organic acid, phenolic compounds can also be done by paper chromatography (For solvent systems, spraying agents etc., see under thin layer chromatography).

Result: The amino acids are separated and visualized on Whatman paper

Experiment No: 2

SEPARATION OF AMINO ACIDS BY THIN LAYER CHROMATOGRAPHY

The separation and identification of organic compounds is a routine work in many service laboratories. Thin layer chromatography (tlc) is an easy technique to adopt for the said purpose. It is highly useful in research laboratories to separate, identify and characterize unknown compounds. A variety of small molecules like amino acids, sugars, organic acids, lipids etc. are separated by tic technique. The greater advantage of tic is the speed at which separation is achieved. When volatile solvents are used the time required to effect separation is only about 30 min and with nonvolatile solvents it is seldom longer than 90 min.

Principle

The general Principle involved in tlc is similar to that of column chromatography i.e. adsorption chromatography. In the adsorption process, the solute competes with the solvent for the surface sites of the adsorbent. Depending on the distribution coefficients, the compounds are distributed on the surface of the adsorbent. Of course, in tic the partition effect in the separation is also not ruled out. The adsorbent normally used contains a binding agent such as calcium sulphate which facilitates the holding of the adsorbent to the glass plate.

Materials

- Glass Plate (20 x 20 cm or 20 x 10 cm)
- Glass Tank with Lid
- Spreader
- Developing Solvents
- Adsorbent Silica GelG/Alumina
- Sample (should be extracted following the procedures indicated for each group of compounds. For e.g., extraction with 80% alcohol for amino acids and sugars)
- Standards Spraying Agent (This also differs as for the group of compounds of interest).

Procedure

Preparation of Plates

1. Place dry, clean glass plates (5 nos, 20 x 20cm) on the plastic base plate over a plane surface.
2. 1. Prepare a slurry of the adsorbent in water (sometimes buffer) in the ratio 1:2 (w/v).
3. Stir the slurry thoroughly for 1-2 min and pour into the applicator positioned on the head glass plate.
4. Coat the slurry over the glass plates at a thickness of 0.25mm for qualitative analysis by moving the applicator at a uniform speed from one end to the other. (One has to gain some experience by practicing to prepare uniformly coated plates.) Leave the plates to dry at room temperature for 15-30 min.
5. Heat the plates in an oven at 100-120°C for l-2h to remove the moisture and to activate the adsorbent on the plate. The dried plates in a rack can be stored in a desiccator over silica gel to prevent moisture absorption.

Sample Application

1. Leave 2.5cm from one end of the glass plate and at least an equal distance from the edges.

2. Apply the sample and standards by means of a micropipette or syringe as small spots. All spots should be placed at equal distance from one end of the plate. See that the adsorbent does not flake off at the sample application point. (Measured volumes are applied for quantitative analysis.)
3. Allow the sample to dry so that spotting can be done repeatedly for a more concentrated sample spot.

Developing Chromatogram

1. Pour the developing solvent into the tank to a depth of 1.5cm. Allow it to stand for at least an hour with a cover plate over the top of the tank to ensure that the atmosphere within the tank becomes saturated with solvent vapor. This is called equilibration.
2. After equilibration, remove the cover plate, and place the thin layer plate (sample applied) vertically in the and so that it stands in the solvent with the spotted end dipping in the solvent.
3. Replace the cover plate. The separation of the compounds occurs as the solvent moves upward. Develop the chromatogram at constant temperature in order to avoid anomalous solvent-running effects.
4. Once the solvent reaches the top of the plate, remove it from the tank, dry and proceed for the identification of the separated compounds.

Result: The amino acids are separated and visualized on TLC

Experiment No: 3

SEPARATION OF PLANT PIGMENTS BY COLUMN CHROMATOGRAPHY

. Chromatography is one of the techniques to separate biological molecules. There are many types of chromatography based on the physicochemical properties of the molecules.These different types of chromatography include gel filtration - ion-exchange - adsorption - affinity chromatography and so on, based on the molecular size and shape, ionic nature, molecular topography and biological specificity of the molecule.

Essentially, any chromatography consists of two phases: one is stationary phase which may be a solid, liquid or a solid/liquid mixture and is immobilized while the other, mobile phase, is a fluid which flows through the stationary phase. Chromatographic separations in practice may take any one of three modes-paper, thin layer or column chromatography.

In column chromatography, the stationary phase is packed in a cylindrical column made of plastic or glass.

The concept of gel filtration chromatography is the different molecules are separated based on the molecular size and shape where the stationary gel matrix serves as a sieve.

The principle underlying the ion-exchange chromatography is the attraction between the biological compounds and the stationary phase, each with opposite electrical charges, thus attracting each other. The ionic nature of chemical compounds is hence exploited.

Affinity chromatography is based on the attraction of a partially compound specifically to combine with the molecule of our interest. For instance, the inhibitor of an enzyme serves as affinity compound to separate that particular enzyme. However, the inhibitor has to be initially combined with an inert matrix to serve as stationary phase.

Majority of the chromatography is routinely carried out Using the column mode. The apparatus and general techniques used for gel exclusion, ion-exchange, adsorption and affinity chromatography have much in common. Gas-liquid chromatography and high performance liquid chromatography each have their own special apparatus, materials and protocols. The column chromatography nowadays been made sophisticated, easier and faster by combining together pumps, detectors, recorders etc. to the columns.

As a model, column chromatographic separation of proteins based on their molecular size by gel filtration is described below:

Principle

The basis of any form of chromatography is the partition or distribution coefficient (Kd) which describes the way in which a compound distributes itself between two immisible phases, such as solid/liquid or gas/liquid.

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for the solute to achieve complete equilibrium between the mobile and stationary phases. Each zone is called a theoretical plate and its length in the column is called the plate height. The more efficient the column is the greater the number of theoretical plates involved.

Materials

Ä Chromatographic column of suitable dimension made up of transparent plastic or glass: Generally, gel filtration is carried out in longer columns (up to 1 m) depending upon the type of gel filtration medium used and the size of the protein to be purified from the bulk

Ä Stationary phase: (ex) Sephadex G 100

There are different types and grades of gel filtration media available. It is to be chosen on the basis of the size of the protein under study

Ä Elution buffer

Ä Fraction collector

Ä Peristaltic pump

Ä Marker proteins (a set of highly pure proteins with known molecular weight (e.g.) cytochrome C, 12,400; carbonic anhydrase, bovine 1,50,000; b-amylase, potato 2,00,000; blue dextran 2,00,000 daltons.

Procedure

A. Packing the column

1. Suspend the gel (for instance, Sephadex G 100) in a large volume of water or preferably in elution buffer until the gel is fully swollen. The swelling can be done by overnight suspension or by heating in a water bath for 2-4 hr (Follow the manufacturer's instructions for this purpose, carefully).

2. Plug the bottom of column tube with glasswool or sintered filter and stand upright the column.

B. Packing the column

3. Make a good slurry of the gel (stationary phase) in a suitable buffer after proper swelling of the gel.

4. Pour a small volume of buffer into the column to avoid trapping of any air bubbles in the plug immediately followed by the slurry to the full of column. The top portion may be carefully, gently stirred prior to pouring additional slurry to the growing column, if necessary. Wait until the gel settle down to the desired height by gravitational force.

5. Place a suitable filter circle on top of the gel bed.

6. Equilibrate the column thoroughly by passing through the column buffer.

7. Apply the sample in column buffer onto the top of bed. The sample volume should preferably limited to 1-3% of the total bed volume. The sample can be applied to the top by careful pipetting or conveniently through the buffer pipeline.

8. Now connect the bufferline to the elution buffer to develop the chromatogram.

9. Protein molecules pass through the gel space while small molecules distribute between the solvent inside and outside the gel and then pass through the column at a slower rate.

10. The effluent emerging out of the column can be routed through a suitable spectrophotometer to monitor the absorbance at a particular wavelength (for proteins either 280, 230 or 210 nm) and the, data recorded. The effluent is then collected using a fraction collector. The effluent is manually collected in the absence of a collector in a fixed time-or volume intervals in tubes and measured subsequently.

11. The volume of mobile phase required to elute a particular solute is known as the elution volume while the corresponding time for elution of the solute at a given flow rate is known as the retention time.

12. The elution is continued (usually 2-3 times bed volume of buffer) until the absorbance monitored reached baseline value.

13. Thereafter the column is extensively reequilibrated with the column buffer for subsequent run.

14. The reference proteins are loaded onto the bed and the chromatography is carried out as above.

15. Plot the logarithms of molecular weight of marker proteins against their respective ratios of elution volume to column void volume (Ve/Vo), the column volume being the elution volume of a very large molecule such as blue dextran.

16. Compute the elution volume of protein of interest and deduce its molecular weight from the above linear graph.

Notes

1. The column chromatography is, nowadays, very much modernized/sophisticated and automated. A family of equipment such as pumps, cooling devices, detectors, collector and data processor put together make the technique much interesting and easier.

2. The column chromatography experiment is an art. Every step in the experiment needs to be carefully done for good results.

3. The experimenter should choose the right stationary and mobile phases, column etc. for optimal results. It is mostly trial and error when there is no details available on the molecule.

4. No single column chromatography procedure will result in the complete purification of a molecule except the affinity chromatography procedure. Therefore, chromatography exploiting two or more physicochemical traits of the molecule has to be employed for purification.

5. Column development using a single solvent is known as isocratic separation. However, in many cases by continuously changing the pH, ionic strength or polarity of the eluent, the resolving power of the eluent could be increased. This kind of elution is called as gradient elution.

Result: The plant pigments were separated and visualised

EXPERIMENT NO: 4 **DATE: _ /_ /_**

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

Objective

To estimate the amount of protein present in the given sample by Lowry's method.

Requirements

Spectrophotometers, test tubes with stand, pipettes, standard measuring flask, wash bottles and vortex mixer.

Description

The determination of protein concentration is an essential technique in all aspects of protein studies and proteomics. There are a wide variety of protein assays available none of the assays can be used without first considering their suitability for the application. Each method has its own advantages and limitations and often it is necessary to obtain more than one type of protein assay for research applications. Protein assays based on these methods are divided into two categories, dye binding protein assays and protein assays based on alkaline copper. The Lowry method is purely a protein assay based on alkaline copper. This method is sensitive to low concentrations of proteins. The method is sensitive down to about 0.01 mg of protein / ml and is best used on solutions with concentrations in the range 0.01-1.0 mg / ml of protein. The Lowry method is sensitive to pH changes and therefore the pH of the assay solution should be maintained at 10-

Sensitivity is moderately constant from protein to protein, and it has been so widely used that the Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances in which protein mixtures or crude extracts are involved.

Principle

Under alkaline conditions cupric ions (Cu 2+) chelate with the peptide bonds resulting in reduction of cupric ions (Cu2+) to cuprous ions (Cu3+). The peptide bonds of proteins react with copper under alkaline conditions to produce Cu+ , which reacts with the Folin reagent, and the Folin-Ciocalteau reaction, which is poorly understand, but in essence phosphomolybdic phosphotungstate is reduced to heteromolybdenum blue by the copper-catalyzed oxidation of

aromatic amino acids. The reactions result in a strong blue color which depends partly on the tyrosine and tryptophan content. The optical density is read at 660 nm. The amount of color produced is proportional to the amount of peptide bonds, i.e. size as well as the amount of protein / peptide. The unknown concentration of the given protein sample is calculated by comparing with the standard graph prepared by the concentrations of standard protein.

Reagent preparation Alkaline copper reagent:

Reagent A: Solution A (2% Na_2CO_3 in 0.1N NaOH, 0.2% Sodium potassium tartarate) and solution B (0.5% $CuSO_4$ in 1% sodium potassium tartarate) are mixed in 50:1 ratio before the experiment.

Reagent B: Folin-reagent is mixed with water in a ratio of 1:2

Standard solution: Stock standard is prepared by dissolving 100 mg bovine serum albumin (BSA) in 100 ml 0.9% saline (con: 1mg/1ml).

Working standard is prepared by making up of 40 ml stock standard to 100 ml with saline (con: 400 µg/ml).

Procedure

- The given protein sample was made up to 100 ml with distilled water.
- Aliquots of 0.2 ml and 0.4 ml were taken in different test tubes marked as T1 & T2 and made up to 2 ml with distilled water.
- Meanwhile, 0.1-0.5 ml of working standard was pipette out into different test tubes labeled as S1- S5 and made up to 2ml with distilled water. Simultaneously a blank with 2 ml distilled water was also taken and labeled as B
- To the entire test tubes 4.5 ml alkaline copper reagent was added and allowed to stand for10 minutes at room temperature.
- Then 0.5 ml Folin- Ciocalteau reagent was added to all the test tubes and mixed well at room temperature and kept in dark for 20 minutes. The blue color developed was read at 660 nm using a colorimeter after 20 minutes.
- A standard graph is prepared by plotting the standard protein concentrations in X-axis and against their OD values at Y-axis. From the standard, the concentration of protein in the

unknown sample is calculated.

Observation

Concentration of standard protein (BSA): 1mg/1ml

S.No.	PARTICULARS	B	S1	S2	S3	S4	S5	T1	T2
1	Volume of standard BSA (ml)	-	0.2	0.4	0.6	0.8	1.0	-	-
2	Concentration of standard(µg)	-	200	400	600	800	1000	-	-
3	Volume of test sample (ml)	-	-	-	-	-	-	0.5	0.7
4	Volume of distilled water (ml)	2.0	1.8	1.6	1.4	1.2	1.0	1.7	1.5
5	Volume of Alkaline copper reagent (ml)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
	Allowed to stand for 10 minutes								
6	Volume of Folin-Ciocalteau reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Mixed well and Allowed to stand for 30 minutes at dark room temperature								
	Optical density (OD) at 660 nm	0.0							

Results

The amount of protein present in 100 ml of the given unknown solution is found to be ……. µg or mg.

EXPERIMENT NO: 5 **DATE: _ / _ / _**

ESTIMATION OF CHOLESTEROL BY ZAK'S METHOD

Objective

To estimate the amount of cholesterol present in the given sample by ZAK's colorimetric method.

Requirements

Colorimeter, test tubes with stand, pipettes, standard measuring flask, wash bottles, spectrophotometer and vortex mixer. Ferric chloride, glacial acetic acid, standard cholesterol, concentrated sulphuric acid

Description

Lipids are a group of naturally occurring molecules that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others. Lipids may be regarded as organic substances relatively insoluble in water, soluble in organic solvents (alcohol, ether etc.) potentially related to fatty acids and utilized by living cell.

As its name suggests, cholesterol is a sterol. Sterols have similar chemical structures, consisting of multiple rings and a side chain, and their functions involve sending chemical messages in the body. Consuming too much or too little fat causes ill health, and in a society with an abundant food supply, people tend to consume more than enough fat and cholesterol. Zak's method the extracted cholesterol (5- cholestene-3-ol) from serum is first dehydrated to 3, 5- cholestadiene in the presence of sulfuric acid. Then oxidized and sulfonated by the catalytic action of Fe3+ ions to cholestapolyene sulfonate, a red coloured product which is read at 540nm (Green filter).

Reagent preparation

Stock ferric chloride solution: dissolved 840 mg dry ferric chloride in 100 ml of glacial acetate.

Ferric chloride precipitating reagent: 10ml stock is made up to 100 ml with glacial acetic acid.

Ferric chloride diluting reagent: 8.5 ml stock made up to 100ml with glacial acetic acid.

Conc. H_2SO_4

Standard cholesterol solution: stock standard is prepared by dissolving 100 mg of cholesterol in 100 ml of ferric chloride diluting reagent and the working standard cholesterol is prepared by diluting 10 ml of the stock to 100 ml in ferric chloride diluting reagent (con:0.1mg/1ml).

Procedure

- Aliquots of 2.5 ml and 5 ml of test sample supernatant are taken in different dry test tubes and 1.0 - 5.0 ml of standard cholesterol solution is also taken in a series of test tubes.
- The volume of all the test tubes including a blank is made up to 5 ml with ferric chloride diluting reagent.
- Then added 3 ml of conc. sulphuric acid to all the test tubes mixed well by swirling and allowed to stand for 20-30 minutes.
- The intensity of red colour formed is read at 540 nm.
- The concentration of cholesterol in the test sample is calculated from the standard graph plotted with the concentrations of standard cholesterol against their optical density.

OBSERVATION

Concentration of standard cholesterol: 100µg/1ml

S.No.	PARTICULARS	B	S1	S2	S3	S4	S5	T1	T2
1	Standard cholesterol (ml)	-	1.0	2.0	3.0	4.0	5.0	-	-
2	Concentration (µg)	-	100	150	200	250	300	-	-
3	Test sample (ml)	-	-	-	-	-	-	2.5	5.0
4	Ferric chloride reagent (ml)	5.0	4.0	3.0	2.0	1.0	0.0	2.5	0.0
5	Conc. sulphuric acid (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
6	Mixed well and allowed to stand for 20-30 minutes								
7	Read optical density at 540 nm								
8	Optical density (nm)	0.0							

Results

The amount of cholesterol present in the given sample is estimated as _____ µg/100ml

Experiment 7 : Estimation of salivary amylase activity by DNSA method

Aim:To determine the activity of amylase enzyme in saliva

Principle

Amylase is the hydrolytic enzyme which breaks down many polysaccharides like starch, amylose, dextrins and yields a disaccharide i.e., Maltose.

$(C_6H_{10}O_5)n + H2O \rightarrow n(C_{12}H_{22}O_{11})$

Reagents

1. Substrate (starch) : Mix 1 gm of soluble starch in 200ml of 0.1M Phosphate buffer (pH 6.8) boil for 3 minutes and cool to room temperature and filter it necessary
2. Enzyme : Saliva is the best and easily available source of amylase. Collect some saliva in a beaker and dilute it to 1:20 dilution with distilled water
3. 1% sodium chloride : It is necessary for enzyme activity
4. DNS (Dinitro Salicylic acid) : Dissolve 1.6 gm of NaOH in 20 ml of distilled water. Take 1gm of 3,5 DNS in NaOH solution. In other beaker take 30 gm of sodium potassium tartrate. Dissolve in 50 ml of distilled water. Mix this DNS solution and finally make the volume up to 100 ml with distilled water.
5. Standard solution of Maltose: It is prepared by dissolving 200mg Maltose in 100ml of water (2mg/1ml).

Procedure

Take 0.5 ml of substrate and 0.2 ml of 1% NaCl in a test tube and pre-incubated at 37°C for 10 minutes then add 0.3 ml of diluted saliva and incubate for 15 minutes at 37°C. Stop the reaction by the addition of 1 ml of DNS reagent mix well and keep the test tubes in boiling water bath for 10 minutes. Cool and dilute with 10 ml of distilled water. Read the color developed at 520 nm. Simultaneously setup the blank as per the test by adding DNS prior to the addition of enzyme.

Set up the standards of different test tubes and repeat the experiment as per the test and measure the color developed at 520 nm absorbance.

Preparation of Phosphate buffer

Dissolve 0.2M (2.7218 gms) of KH_2PO_4 in 100 ml of distilled water to this solution add

M (2.8053 gms) KOH drop by drop till the pH is set to 6.8. Then make it to 200 ml with distilled water. So the final concentration is 0.1 M of 200 ml phosphate buffer.

Table

S. no	Volume of standard (ml)	Volume of distilled water (ml)	Conc. of standard (ml)	Volume of DNSA	Heated in a boiling water bath for 10 minutes	Volume of distilled water (ml)	Optical Density at 520 nm
1	Blank	1.0	0.0	1.0		10	
2	0.2	0.8	0.4	1.0		10	
3	0.4	0.6	0.8	1.0		10	
4	0.6	0.4	1.2	1.0		10	
5	0.8	0.2	1.6	1.0		10	
6	1.0	0.0	2.0	1.0		10	

Calculation

- 1.5 mg of maltose formed/0.3 ml/15 minutes
- 1.5 x 4 mg of maltose formed/0.3 ml of enzyme/1 hour
- 1.5 x 4 x 3.3 mg of maltose formed/1ml of enzyme/1 hour
- 1.5 x 4 x 3.3 x 100 mg of maltose formed/100ml of enzyme/ 1 hour

Result

The amount of maltose in the given unknown sample is____grams of maltose formed per 100ml of enzyme per one hour.