Department of Biotechnology

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Biochemistry Lab

(BT35L)

Lab Manual

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Experiment No 1:

PREPARATION OF SODIUM ACETATE BUFFER

Aim: Preparation of sodium acetate buffer of 0.1M and 4.7 pH (25ml)

Principle: Buffer is a solution used to maintain the pH of any biochemical reaction.

Procedure:

1. Weigh about 0.205g of sodium acetate. Dissolve it in 15ml distilled water.
2. Dip the electrode of the pre calibrated pH meter in the above solution.
3. Add acetic acid of 0.5M drop by drop till the pH reaches 4.7.
4. Make up the solution to 25ml with distilled water.
5. Store the buffer in a sealed container.

Experiment No 2:

ESTIMATION OF GLUCOSE BY DNS METHOD

Aim: To estimate the amount of glucose in the given ample by DNS method.

Principle: This method tests for the presence of free carbonyl group (C=O), of the reducing sugars. The reaction involves the oxidation of this of free carbonyl group (aldehyde functional group present in, glucose and the ketone functional group in fructose) and simultaneous reduction of , 3,5-dinitrosalicylic acid to 3-amino,5-nitrosalicylic acid (a coloured molecule) under alkaline conditions.

oxidation

aldehyde group ----------> carboxyl group

reduction

3,5-dinitrosalicylic acid ----------> 3-amino,5-nitrosalicylic acid

Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.

Reagents

- Dinitrosalicylic Acid Reagent Solution, 1%
  - Dinitrosalicylic acid: 10 g
  - Sodium sulfite: 0.5 g
  - Sodium hydroxide: 10 g
  - Add water to: 1 liter
- Potassium sodium tartrate solution, 40%

Procedures

1. Add 3 ml of DNS reagent to 3 ml of glucose sample in a lightly capped test tube. (To avoid the loss of liquid due to evaporation, cover the test tube with a piece of paraffin film if a plain test tube is used.)
2. Heat the mixture at 90º C for 5-15 minutes to develop the red-brown color.
3. Add 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution to stabilize the color.
4. After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 575 nm.

Experiment No 3:

ESTIMATION OF SAPONIFICATION VALUE OF FATS/OILS

Requirements

1) Fats and Oils [coconut oil, sunflower oil]
2) Conical Flask
3) 100ml beaker
4) Weigh Balance
5) Dropper
6) Reflux condenser
7) Boiling Water bath
8) Glass pipette (25ml)
9) Burette

Reagents:

Ethanolic KOH(95% ethanol, v/v)
Potassium hydroxide [0.5N]
Fat solvent
Hydrochloric acid[0.5N]
Phenolphthalein indicator

Procedure:

1. Weigh 1g of fat in a tared beaker and dissolve in about 3ml of the fat solvent [ethanol /ether mixture].
2. Quantitatively transfer the contents of the beaker three times with a further 7ml of the solvent.
3. Add 25ml of 0.5N alcoholic KOH and mix well, attach this to a reflux condenser.
   Set up another reflux condenser as the blank with all other reagents present except the fat.
4. Place both the flasks in a boiling water bath for 30 minutes. Cool the flasks to room temperature.
5. Now add phenolphthalein indicator to both the flasks and titrate with 0.5N HCl
6. Note down the endpoint of blank and test .
7. The difference between the blank and test reading gives the number of millilitres of 0.5N KOH required to saponify 1g of fat.
8. Saponification number of fat = mg of KOH consumed by 1g of fat.
9. Calculate the saponification value using the formula
   - Weight of KOH = Normality of KOH * Equivalent weight* volume of KOH in litres
   - Volume of KOH consumed by 1g fat = [Blank – test]ml

Experiment No 4:

ESTIMATION OF ACID VALUE OF FATS/OILS

Aim: To determine the acid value of fats/oils

Principle: The acid value is the number of milligrams of potassium hydroxide necessary to neutralise the acids in one gram of sample.

Reagents:

- Sodium hydroxide - 0.1 N aqueous solution, accurately standardised.
- Solvent - Ethanol. If the sample is not sufficiently soluble in this solvent, use a mixture of equal volumes of 96% v/v ethanol and diethyl ether.
- Phenolphthalein indicator - 1% solution in 96% v/v ethanol.

Procedure:

1. Weigh between 2 and 10 g of sample to an accuracy of 0.1 g, according to the acid value expected, into the flask.

2. Neutralise 50 ml of the solvent with 0.1 N sodium hydroxide solution using phenolphthalein indicator.

3. Add the neutralised solvent to the sample in the flask and dissolve.

4. Titrate, while swirling the flask, with 0.1 N sodium hydroxide solution to the phenolphthalein end point.

Calculation:

Weight (g) of sample = W 1

Volume (ml) of 0.1 N NaOH used = V Acid Value = 5.61 x V x N W 0.1

Where N is the exact normality of the sodium hydroxide solution used. Record the result to the nearest 0.1.

Experiment No 5:

PREPARATION OF PHOSPHOTIDYL CHOLINE FROM EGG YOLK

Aim: To extract and purify phosphotidyl choline from egg yolk.

Principle:

Procedure:

1. **Extraction**

1. Fresh eggs were taken, yolks were separated and blended with 1 L of cold acetone (4 ºC) with a mechanical homogenizer for5 min.
2. The homogenate was maintained for 1 h at 4 ºC to precipitate crude phospholipids and proteins.
3. The suspension was filtered (Büchner) and the precipitate was washed (5x, 400 mL acetone each).
4. The acetone filtrate, which contains neutral fats and pigments, was discarded and the residual solvent of precipitate was eliminated under vacuum.
5. The precipitate was transferred to a beaker, 2 L of cold (4 ºC) ethanol were added and the suspension was mechanically homogenized (5 min).
6. The mixture was filtered (Büchner) and the precipitate re-extracted with ethanol (1 L).
7. The ethanol extracts, which contain the phospholipids, were combined and the solvent removed in a rotatory evaporator (T< 40 ºC).
8. The resulting crude lecithin extract, VCE, was dissolved in petroleum ether (40 mL), acetone (2 L, 4 ºC) was added to precipitate the lecithin. After 2 h at 4 ºC, the supernatant cleared, and the solvent was separated from the precipitate by decantation.

9. This crude lecithin extract, was dissolved again in 40 mL of petroleum ether and the precipitation process was repeated. The extract was re-dissolved in chloroform and evaporated at reduced pressure.

2. Column chromatography

10. 2g crude lecithin, dissolved in 3.5 mL of chloroform, was applied on to the glass column (35 cm x 2 cm, Teflon tap, fritted glass bottom), packed with a suspension of 80 g of neutral Alumina (activated at 120 ºC for 12 h) in chloroform. Flow rate was 4 mL/min and 100 mL fractions were collected.
11. CE was eluted, successively, with: chloroform (1.0 L); chloroform: methanol (95:5 v/v, 0.1 L) and chloroform: methanol (9:1 v/v, 0.5 L). Each fraction was evaporated under reduced pressure and maintained in a desiccator under vacuum at -20 ºC.

3. TLC

12. Chloroform solutions of fractions of crude extract and standard phosphatyldilcholine (PC) were prepared and applied to silica gel TLC plates.
13. The lecithin containing fractions were pooled, dissolved in methanol, aliquoted in glass tubes and methanol was evaporated under a N2 flux.
14. The tubes were maintained for about 1 h in a desiccator under vacuum to eliminate the residual solvent. The tubes were sealed under vacuum and stored at -80 ºC.

Experiment No 6:

LOWRY METHOD FOR PROTEIN ESTIMATION
Aim:

Principle:

Reagents:

- **Lowry Reagent** (or) alkaline copper sulfate solution: Mix 50ml of solution A with 1 ml of solution B, just prior to use.
    - Solution A : 2% sodium carbonate in 0.1N NaOH.
    - Solution B : 0.5% copper sulfate solution in 1% sodium potassium tartarate solution (to be prepared fresh)
- **Folin-Ciocalteau reagent**: This is commercially available and has to be diluted with equal volume of water just before use.
- **Standard protein solution**: Dissolve 10mg of BSA in 100ml of distilled water in a volumetric flask. (for concentration-100 µg/mL)

Procedure:

1. Pipette out into clean glass tubes 0.2, 0.4, 0.6, 0.8, and 1.0 of the protein solution and make up the total volume to 2ml with addition of distilled water.
2. To each tube add 4ml of the alkaline-copper sulfate solution (Reagent C), mix well and allow to stand at room temperature for 10 minutes.
3. Pipette out 0.5ml of the FCR reagent and add into each tube, mix immediately after each addition. Allow the tubes to stand for 30 minutes.
4. Measure the Absorbance of the blue Purple color formed at 660nm. Prepare a blank tube with 2 ml of distilled water(instead of the standard protein) followed by all additions mentioned above and with 1ml of unknown solution (Sample) and perform the additions as in case of the standards.
5. Prepare a calibration curve with mg of protein on X-axis and O.D. (A660nm) on Y-axis and determine the amount of protein present in a given unknown sample.

Experiment No 7:

ESTIMATION OF AMINO ACID BY FORMAL TITRATION

Aim: Estimation of amino acid by formal titration

Principle: Amino acids are amphoteric in nature containing amino and carboxylic groups. They can be titrated against standard acid and alkali respectively. But it is difficult to titrate the carboxylic group of the amino acid in aqueous solution as amino group competes with added base for proton. To overcome this titration has to be carried out in presence of formaldehyde. Formaldehyde reacts with the amino group to form mono hydroxyl and di hydroxy methyl derivative.

Procedure:

1. Prepare of 0.1N Potassium bipthalate (mol. Wt. 204.22) by dissolving 2.04 g of Potassium bipthalate in 100ml dd H_2O.
2. Dissolve 400mg of NaOH in 100ml ddH_2O, Take this NaOH solution in burette and titrate against potassium biphthalate solution using phenolphthalein indicator and find the normality.
3. Prepare of 0.1N glycine by dissolving 750 mg of glycine in 100ml dd H_2O.
4. Take 10ml glycine solution in a conical flask, add 2Ml neutral formaldehyde solution and 2 drops of phenolphthalein indicator.
5. This mixture is titrated against standardized NaOH taken in the burette till a persistant pale pink color is observed.
6. The volume of NaOH consumed is noted.
7. The experiment is repeated for 3 trials to get concordant values.
8. The normality of glycine is calculated and weight of glycine present in 100ml is calculated.

Experiment No 8:
DETERMINATION OF ISOELECTRIC POINT OF GLYCINE (PI)

Aim: To determine the isoelectric point of Glycine.

Principle: Aminoacids exist as zwitter ions at a particular point (pH). This point is called as the isoelectric point of an amino acid. At this point the molecule is electrically neutral, since both positive and negative charges are present on the same molecule. The given amino acid is neutralized using an equimolar solution of NaOH and HCl to determine the P^I .

Procedure:

1. 10ml of 0.1N potassium bipthalate is titrated against NaOH solution using phenolphthalein as indicator till pale pink color is observed.
2. The titration is repeated for 3 trials to get concordant values. The average value of NaOH consumed is taken and the normality of NaOH is calculated.
3. Similarly 10ml of HCl solution is titrated against standard NaOH solution taken in a burette using phenolphthalein as indicator till pale pink color is observed. The titration is repeated for 3 trials to get concordant values.
4. 0.1N solution of glycine is prepared and 15ml of it is taken in a beaker and standardized HCl solution is added at regular intervals of concentration 1,1.5,2ml etc.
5. After addition of HCl solution, pH was recorded each time using pH meter. Same procedure is followed using NaOH solution.
6. A graph of pH vs volume of both HCl and NaOH is plotted. The point corresponding to the midpoint of the extrapolated line is the P^I ofGylcine

Experiment No 9:

SEPARATION OF AMINO ACIDS BY CIRCLULAR PAPER CHROMATOGRAPHY

Aim: Separation and identification of amino acids by paper chromatography

Principle: Separation of amino acids is based on partition co-efficient of amino acids in the given solvent system. Since they are colorless, ninhydrin is used for staining and Rf values.

Procedure:

1. A circular whattaman filter paper of cm diameter is taken. A small hole is made at the center and a circle of 1cm from the center is drawn.
2. The amino acids are applied as separate spots on the circle drawn. A small filter paper wick is inserted in the hole and is placed in a pre saturated chamber.containing solvent system (n butanol: acetic acid: water,4:1:5).
3. When the solvent reaches ¾ of the filter paper, it is removed from the chamber, dried and sprayed with ninhydrin reagent and dried.
4. The Rf value was calculated by measuring the distance of spots from center and the solvent front using the formula

   $$Rf = \text{distance travelled by aminoacid} / \text{distance travelled by solvent}$$

   .

Experiment No 10:

ESTIMATION OF IRON BY WONG'S METHOD

Aim: To estimate the amount of iron present in the given sample by wong's method.

Principle : Iron in the form of ferric ions gives red colour with potassium thiocyanate, the intensity of red colour is directly proportional to the iron present in the given sample. The intensity of red colour is measured at 540nm.

Reagents :

1. 30% sulfuric acid

2. 7% Potassium persulphate

3. 40% Potassium thiocyanate :

40 gms of thiocyanate is dissolved in 90 ml of distilled water, 4 ml of acetone is added and volume is made upto 100ml due to distilled water addition.

4. Stock Solution :

702.2 gm of ferrous ammonium sulphate is dissolved in 100ml of distilled water. Add 5ml of 1:1 Hcl then make up the volume to 1 litre by adding distilled water. The concentration of stock solution is 0.1 mg/ml.

5. Working Standard :

10 ml of stock solution is diluted to 100ml with distilled water. The concentration of working standard is 1mg/100ml (or) 10 micro gm/ml.

Procedure:

STANDARDS :

1. 1ml -5ml of standard solution ($FeCl3.6H_2O$) is pipetted out into a series of test tubes.

2. The volume is made utpo 5ml by adding distilled water.

3. Then 30% sulfuric acid is added to all the testtubes making the volume to 6ml.

4. Then 1ml of potassium persulfate solution was added.

5. Then 1.5ml of potassium thiocyanate solution is added to all the testtubes, and then incubated fr 20′ at room temperature.

6. The intensity of standards is observed at 540nm against blank.

BLANK :

1. Blank is prepared by adding 5ml of distilled water in a test tube.

2. To this testtube 1ml of 30% H_2SO_4, 1ml of potassium persulfate, and 1.5ml of potassium thiocyanate solutions are added.

3. Test Tubes are incubated for 20′ at room temperature.

4. This is used as a blank.

TEST :

1. Test is prepared by taking 1ml of test solution in a test tube.

2. To this 4ml of distilled water was added.

3. Then 1ml of 30% sulfuric acid, 1ml of potassium per sulphate and 1.5ml of potassium thiocyanate solutions are added.

4. After incubation at room temperature for 20′, the intensity of the solution is observed at 540nm against blank.