SEMESTER III

18BTU314B **BIO-ANALYTICAL TOOL PRACTICAL** **3H - 1C**

Total hours/week: L:0 T:0 P:3 **Marks: Internal: 40 External: 60 Total: 100**

Course Objectives: To understand the basic practical knowledge of bio-analytical techniques.

Course Outcomes: Students will able to select analytical techniques for problem solving.

Practical

1. Native gel electrophoresis of proteins
2. SDS-polyacrylamide slab gel electrophoresis of proteins under reducing conditions.
3. Preparation of the sub-cellular fractions of rat liver cells.
4. Preparation of protoplasts from leaves.
5. Separation of amino acids by paper chromatography.
6. To identify lipids in a given sample by TLC.
7. To verify the validity of Beer's law and determine the molar extinction coefficient of NADH.

References

1. Karp, G. (2010). *Cell and Molecular Biology: Concepts and Experiments* (6th ed.). John Wiley& Sons. Inc.
2. De Robertis, E.D.P. & De Robertis, E.M.F. (2006). *Cell and Molecular Biology* (8th ed.). Philadelphia: Lippincott Williams and Wilkins.
3. Cooper, G.M., & Hausman, R.E. (2009). *The Cell: A Molecular Approach* (5th ed.). Washington : ASM Press & Sunderland & MA: D.C. Sinauer Associates.
4. Becker, W.M., Kleinsmith, L.J., Hardin, J. & Bertoni, G. P. (2009). *The World of the Cell* (7th ed.). Pearson Benjamin Cummings Publishing, San Francisco.

Exp: 1 Native gel electrophoresis of proteins

Aim:

To learn the technique of Native PAGE.

Introduction:

Native" or "non-denaturing" polyacrylamide gel electrophoresis (PAGE) is done in the absence of SDS. While in SDS-PAGE the electrophoretic mobility of proteins depends primarily on their molecular mass, in native PAGE the mobility depends on both the protein's charge and its hydrodynamic size. Native electrophoresis is when the protein undergoes migration without denaturation. Under native PAGE conditions proteins migrate according to their charge, size and shape. In this situation polypeptides retain their higher-order structure and often retain enzymatic activity and interaction with other polypeptides.

Principle:

Electrophoresis of macromolecules is normally carried out by applying a thin layer of a sample to a solution stabilized by a porous matrix. Under the influence of an applied voltage, different species of molecules in the sample move through the matrix at different velocities. The electric charge driving the electrophoresis is governed by the intrinsic charge on the protein at the pH of the running buffer. This charge depends upon the amino acid composition of the protein. Since the protein retains its folded conformation, its hydrodynamic size and mobility on the gel will also vary with the nature of this conformation which means that higher mobility for more compact conformations and lower for larger structures like oligomers. Polyacrylamide Gel Electrophoresis (PAGE) is the most highly resolving electrophoresis method developed for separating protein molecules based on chargedensity. If native PAGE is carried out near neutral pH then it can be used to study conformation, self-association or aggregation, and the binding of other proteins or compounds. Thus native gels can be sensitive to any process that alters either the charge or the conformation of a protein.

The Native PAGE technique consists of the following three basic steps:

1. Preparation of acrylamide gel - Polyacrylamide is a synthetic gel which is thermo-stable, transparent, strong and relatively chemically inert and can be prepared with a wide range of average pore sizes. It can withstand high voltage gradients and is feasible to various staining and destaining procedures and can be digested to extract separated fractions or dried for autoradiography and permanent recording. A polymer gel is formed of acrylamide monomers

and the proteins are run through this gel by electrophoresis, hence this entire process is called Polyacrylamide Gel Electrophoresis (PAGE).

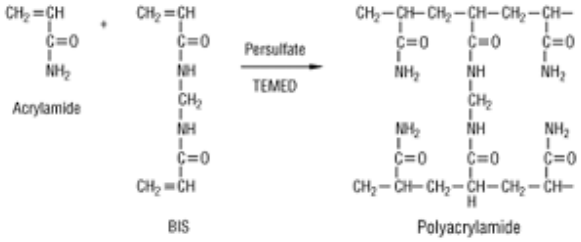


Fig1: Cross-linking of Polyacrylamide gel

2. Electrophoresis of protein – The porosity and the degree of crosslinking of a polyacrylamide gel depends upon the initial concentrations of Acrylamide and Bis-acrylamide which affects the resolution ofthe protein molecules to be separated. In a 12 – 15% Acrylamide gel large molecules are retarded during migration compared to the smaller molecules and in 4 – 8% Acrylamide gel molecules with higher molecular weight molecules have faster migration. The progress of gel electrophoresis is monitored by observing the migration of a visible tracking dye.
3. Visualization of protein fragments – After the completion of electrophoresis the polyacrylamide gel is stained with Coomassie® Brilliant Blue staining solution which is a methanol-based stain formulated for protein detection in polyacrylamide gel. Protein bands form an intense blue color when stained with this solution and can be easily distinguished on the gels after destaining.

Materials Required But Not Provided:

Glass wares: Conical flask, Measuring cylinder, Beaker

Reagents: Distilled water

Other requirements: Protein Electrophoresis apparatus, Micropipettes, Tips, Microwave/Burner/Hotplate

Procedure:

1. Assemble the electrophoresis unit such that the glass plates are clamped to the unit along with the spacers placed in-between them at two vertical edges.

2. Prepare 1% agarose (0.05g in 5ml of distilled water). Boil to dissolve the agarose and pour a thin horizontal layer at the lower edge of the plates to seal the assembly. Let it solidify by allowing it to cool down for 5-10 minutes

3. Preparation of 12% Separating Gel- To prepare separating gel, add the components as follows:

30% Acrylamide-bisacrylamide Solution - 6 ml

Distilled water* - 6.6 ml

1.5M Tris Buffer (pH 8.8) - 2.2 ml

10% APS Solution - 150 µl

TEMED - 18 µl

Pour the gel in-between the plates and allow it to solidify for an hour. Immediately after the gel is poured, add distilled water to level the gel. 4. After an hour pour off the water by inverting the casting assembly.

5. Preparation of 5% Stacking Gel- To prepare stacking gel, add the components as follows: 30% Acrylamide-bisacrylamide Solution - 1 ml

Distilled water* - 4.8 ml

1.5M Tris Buffer (pH 8.8) - 1.6 ml

10% APS Solution - 75 µl

TEMED - 10 µl

After addition of TEMED gently mix all the components by swirling the beaker. Pour the stacking gel on top of the separating gel and immediately place the comb avoiding air bubbles. Allow it to solidify for 30 minutes. Note: Acrylamide is a potential neurotoxin and should be treated with great care. Always wear an face mask and use gloves.

6. Pour 1X Tris-Glycine Gel Running Buffer in the unit such that the buffer connects the two electrodes, and hence completes the flow of current. Remove the comb from the Stacking Gel carefully.

7. Sample Preparation: Take 2 tubes for protein samples and 1 tube for Protein Marker. Label them respectively. Take 15 µl of each sample in the respective tube and add 15 µl of Native Gel Loading Dye to it.

8. Load 20 µl of the samples in the wells created by the comb in the Stacking Gel.

9. Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 130 volts and 90 mA until dye front reaches 0.5 cm above the sealing gel.

10. Carefully remove the gel from in-between the plates using spatula into the plastic tray containing distilled water. Wash the gel for 1 minute. Discard the water & proceed for staining destaining procedure.

Staining and Destaining of Gel:

1. After removing water, add 50 ml of Staining Solution to the tray containing gel, till the bands are visible. Sometimes the gel may have to be kept overnight in the staining solution for visualization of the bands.
2. Remove the gel from Staining Solution. The Staining Solution can be re-used 2-3 times.
3. Wash the gel by rinsing with distilled water till a considerable amount of stain leaches out from the gel. Keep changing the distilled water for 3-4 times.
4. Add 50 ml of Destaining Solution to the gel. Destaining should be carried out with constant moderate shaking.
5. Continue destaining till clear, distinct bands are observed.
6. Remove the gel from Destaining Solution. The Destaining Solution can be re-used 2-3 times.

Observation and Result:

Exp: 2 SDS-polyacrylamide slab gel electrophoresis of proteins under reducing conditions.

Aim:

To learn the technique of SDS-PAGE.

Introduction:

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their molecular weight. The electrophoretic mobility of proteins depends upon their size. The purpose of SDS-PAGE is to separate proteins according to their size. As proteins are amphoteric compounds, their net charge can therefore be determined by the pH of the medium in which they are suspended. Therefore, at a given pH and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and charge of molecules. As proteins are high molecular weight molecules, it needs porous gels to get separated. Polyacrylamide gels are those which provide a means of separating proteins by size as they are porous. This kit enables the students to learn the technique of SDS-PAGE.

Principle:

To separate different protein molecules of different shapes and sizes, they first have to be denatured so that the proteins no longer have any secondary, tertiary or quaternary structure. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone. SDS denatures all the proteins to their respective primary structure. SDS confers a negative charge to the polypeptide in proportion to its length.

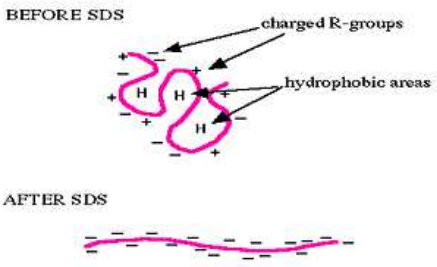


Fig1: Denaturation of protein by SDS

SDS treatment has two important features:

1. All proteins retain only their primary structure.
2. All proteins have a large amount of negative charge.

Polyacrylamide is the best gel recommended to provide such an environment. Polyacrylamide is a synthetic gel which is thermo-stable, transparent, strong and relatively chemically inert and can be prepared with a wide range of average pore sizes. It can withstand high voltage gradients and is feasible to various staining and destaining procedures and can be digested to extract separated fractions or dried for autoradiography and permanent recording. A polymer gel is formed of acrylamide monomers and the proteins are run through this gel by electrophoresis, hence this entire process is called Polyacrylamide Gel Electrophoresis (PAGE).

There are two layers of gel, namely Stacking or spacer gel, and Separating or resolving gel.

Stacking gel - The stacking gel contains large pores of polyacrylamide gel (generally 5%). This gel is prepared with Tris buffer of pH 6.8 which is of about 2 pH units lower than that of the electrophoresis buffer. This gel is formed over the separating gel.

Separating Gel - The separating gel contains small pores of polyacrylamide gel (5-30%). The Tris buffer used is of pH 8.8. In this gel, macro molecules separate according to their size.

The materials used in SDS-PAGE and their roles are as follows:

1. Tris: It is used as a buffer because it is an innocuous substance to most proteins. Its pKa is 8.3 at 20oC and reasonably a very satisfactory buffer in the pH range 7.0 – 9.0.

2. Acrylamide: This is a white crystalline powder and while dissolving in water, autopolymerisation takes place. It is a slow spontaneous process by which acrylamide molecules join together by head on tail fashion. But in presence of free radicals generating system, acrylamide monomers are activated into a free-radical state. These activated monomers polymerise quickly and form long chain of polymers. This kind of reaction is known as Vinyladdition polymerisation.

3. Bisacrylamide (N,N'-Methylenebisacrylamide): Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it has two acrylamide molecules coupled head to head fashion at their non-reactive ends.

4. Sodium Dodecyl Sulphate (SDS): SDS is the most common denaturing agent used to denature native proteins to individual polypeptides. When a protein mixture is heated to 100°C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus, polypeptides after treatment become a rod like structure possessing a uniform charge density, that is same net negative charge per unit length.

5. Ammonium Persulphate (APS): APS is an initiator for gel formation.

6. N, N, N', N'-tetramethylethylenediamine (TEMED): Chemical polymerisation of acrylamide gel is used for SDS-PAGE. It can be initiated by ammonium persulfate and the quaternary amine, N,N,N',N'- tetramethylethylenediamine (TEMED).

Materials Required

Glass wares: Conical flask, Measuring cylinder, Beaker

Reagents: Distilled water

Other requirements: Protein Electrophoresis apparatus, Micropipettes, Tips, Microwave/Burner/Hotplate

Procedure:

1. Assemble the electrophoresis unit such that the glass plates are clamped to the unit along with the spacers placed in-between them at two vertical edges.

2. Prepare 1% agarose (0.05g in 5ml of distilled water). Boil to dissolve the agarose and pour a thin horizontal layer at the lower edge of the plates to seal the assembly. Let it solidify by allowing it to cool down for 5-10 minutes

3. Preparation of 12% Separating Gel- To prepare separating gel, add the components as follows:

30% Acrylamide-bisacrylamide Solution - 6 ml

Distilled water* - 3 ml

2.5X Tris-SDS Buffer (pH 8.8)- 6 ml

10% APS Solution - 125 µl

TEMED - 18 µ

Pour the gel in-between the plates and allow it to solidify for an hour. Immediately after the gel is poured, add distilled water to level the gel.

4. After an hour pour off the water by inverting the casting assembly.

5. Preparation of 5% Stacking Gel- To prepare stacking gel, add the components as follows:

30% Acrylamide-bisacrylamide Solution - 1.3 ml

Distilled water* - 5.1 ml

5X Tris-SDS Buffer (pH 6.8) - 1.6 ml

10% APS Solution - 75 µl

TEMED - 10 µl

After addition of TEMED gently mix all the components by swirling the beaker. Pour the stacking gel on top of the separating gel and immediately place the comb avoiding air bubbles. Allow it to solidify for 30 minutes.

Note: Acrylamide is a potential neurotoxin and should be treated with great care. Always wear an face mask and use gloves.

6. Pour 1X Tris-Glycine-SDS Gel Running Buffer in the unit such that the buffer connects the two electrodes, and hence completes the flow of current. Remove the comb from the Stacking Gel carefully.

7. Sample Preparation: Take 2 tubes for protein samples. Label them respectively. Take 20 µl of each sample in the respective tube and add 5 µl of 5X Sample Loading Buffer to it. Boil the tubes containing Protein Samples at 100oC in a boiling water bath. Do not boil the tube containing PrestainedProtein Ladder.

8. Load 5 µl of Prestained Protein Ladder and 20 µl of the samples immediately after the heat treatment in the wells created by the comb in the Stacking Gel.

9. Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 100 volts and 10 mA until dye front reaches 0.5 cm above the sealing gel.

10. Carefully remove the gel from in-between the plates using spatula into the plastic tray containing distilled water. Wash the gel for 1 minute. Discard the water & proceed for staining destaining procedure.

Staining and Destaining of Gel:

1. After removing water, add 50 ml of Staining Solution in the tray containing gel, till the bands are visible. Sometimes the gel may have to be kept overnight in the staining solution for visualization of the bands.

2. Remove the gel from Staining Solution. The Staining Solution can be re-used 2-3 times.

3. Wash the gel by rinsing with distilled water till a considerable amount of stain leaches out from the gel. Keep changing the distilled water for 3-4 times.

4. Add 50 ml of Destaining Solution to the gel. Destaining should be carried out with constant moderate shaking.

5. Continue destaining till clear, distinct bands are observed.

6. Remove the gel from Destaining Solution. The Destaining Solution can be re-used 2-3 times. Protein

Observation and Result:

Exp: 3 Preparation of the sub-cellular fractions of rat liver cells.

Subcellular fractionation methods.

Separation of cellular compartments from one another is an important step for studying a specific intracellular structure or organelle or protein, or to assess possible associations between these macromolecular structures. Subcellular fractionation uses one or more of the properties of each compartment, such as buoyant density, surface charge density, size and shape, and is mainly based on differential centrifugation in media of high viscosity at 4°C. Media used for differential centrifugation are mainly sucrose, mannitol, glycerol, Ficoll 400 (a polymer of sucrose), Percoll (a colloidal silica) and iodixanol (OptiPrep). Sucrose is widely used because it is inexpensive. But they all have their advantages and limitations, which are discussed in detail by Harford and Bonifacino, 2011. Mainly these methods will be discussed here, with preference for the ones that are easily accessible to most labs and are less time-consuming, as speedy recovery is vital. Gel filtration, affinity chromatography, electrophoresis or selective density-shift perturbation can also be used. Variations in the conditions of the available protocols are dependent on the organelle, tissue or cell type and equipment used, and it is highly recommended to read the cited references for full details of each procedure. In the end, the purity and the yield of the fractionation should be assessed by detection of distinct markers in each collected fraction during the entire procedure.

Next, the most commonly used methods for isolation of organelles are presented. The use of rat liver has been discussed in detail and can be easily found in the literature; therefore the focus of this part of the review is on other model systems.

Isolation of cytoplasm, nucleoplasm, and chromatin

Cytoplasmic, nucleoplasmic and chromatin fractions can be easily prepared from a pellet of cultured cells. Cells are resuspended in a buffer containing 0.34 M sucrose, 10% glycerol and low concentration of a mild detergent (0.1% Triton X-100) as well as K+ and Mg+2 (which protect the nuclei from breaking) and the nuclei are pelleted by a low speed centrifugation while the supernatant is kept as the cytoplasmic fraction. Next, nuclei are lysed in a buffer containing chelating agents EDTA and EGTA and the insoluble chromatin fraction is pelleted by a low speed centrifugation while the supernatant is the nucleoplasmic fraction (Figure 1).

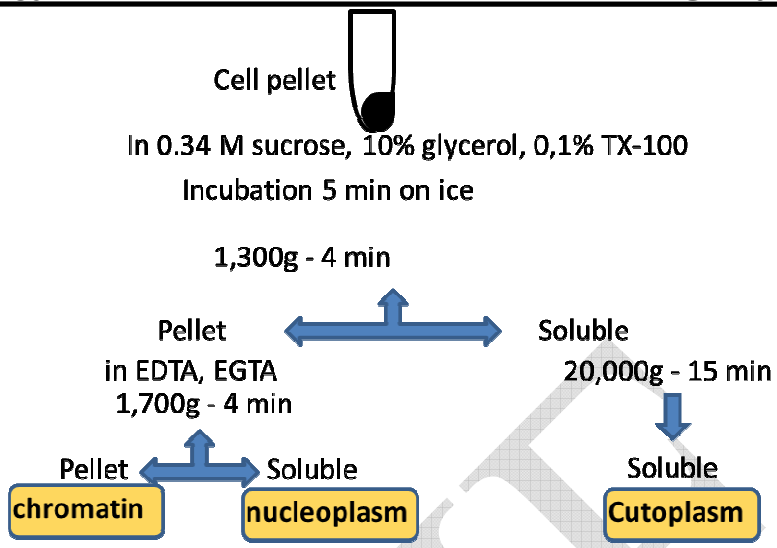


Figure 1. subcellular fractionation from cultured cells.

Some researchers use a 'quick and dirty' preparation of chromatin with its associated proteins from the cytoplasm/nucleoplasm. This is simply performed by lysing the cells in a lysis buffer containing 1% Triton X-100. In this buffer, chromatin and some cytoskeletal structures are insoluble and they can be recovered by centrifugation. The pellet can be resuspended in the buffer of choice e.g. Laemmli for SDS PAGE.

Exp: 4 Preparation of protoplasts from leaves.

Aim

To isolate protoplast by mechanical method

Introduction

- Protoplast is the living material of the cell where as an isolated protoplast is the cell from which the cell wall is removed.
- In plant breeding programme many desirable combination of characters could not be transmitted through the conventional method of genetic manipulation.
- Higher plants that could lead to the genetic process involving fusion between the subsequent developments of a product to a hybrid plant are known as somatic hybridization.
- Plant protoplasts can be isolated from cells by two methods that is mechanical and enzymatic method.

Principle

- Protoplast can be prepared from a variety of tissue but among them leaf mesophyll tissue from a wide range of plants have been proved to be the most ideal source of plant material for protoplast isolation.
- Leaves of Nicotiana tobacum is a highly standardized material for easy entry into the art of protoplast isolation and culture.
- Nowadays the mixed method i.e, single step method is very popular and this procedure is followed as routine work in most of the laboratories of the world.

Materials Required

- Plant leaves
- 2.5% sodium hypochloride
- 0.5% cellulase
- Pectinase
- 13% W/V mannitol
- Microscope

Procedure

i) Mature leaves were taken and rinsed briefly in tap water.

ii) The interior of the transfer chamber was wiped off with ethanol and all the subsequent procedures were conducted under aseptic conditions.

iii) The leaves were immersed in sodium hypochloride solution and washed well with distilled water.

iv) Then the leaves were kept in 13% mannitol solution for 1 hour to preplasmolyse it.

v) Then the leaves were held on a sterile tile, the point of the foreceps was inserted at the junction of the main vein or middle and the epidermal layer is stripped towards the edge of the lamina.

vi) The leaves were cut into small section and the 1 g of peeled leaf was transferred to a petri dish containing enzyme solution i.e., pectinase and cellulose. Then it was incubated at 25˚C for overnight.

vii) After incubation, the leaf strips were gently teased with foreceps to release the protoplast.

viii) First, the enzyme solution containing the protoplast was filtered through nylon mesh to remove the undigested tissue, the cell clumps and debris.

ix) Then the filtrate was transferred to a centrifuge tube and spin at 75 g for 5 min.

x) The debris in the supernatant is carefully removed and again centrifuged to get pure protoplasts.

Finaly the supernatant was discarded and the final pellet was observed under microscope.

Observation and Result:

Exp: 5 Separation of amino acids by paper chromatography.

Introduction

Chromatography is a technique for the separation of a mixture by passing it in solution or suspension through a medium in which the components move at different rates.

Paper Chromatography

This page is an introduction to paper chromatography - including two way chromatography.

Background

Chromatography is used to separate mixtures of substances into their components. All forms of chromatography work on the same principle.

They all have a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates. We'll look at the reasons for this further down the page.

In paper chromatography, the stationary phase is a very uniform absorbent paper. The mobile phase is a suitable liquid solvent or mixture of solvents.

Principle

Paper chromatography is a form of chromatography, where mixture of solutes separated in a filter paper. Two types of partitioning is involved in these methods,

1. Liquid - Liquid partition
2. Solid -Liquid partition

Liquid – Liquid partition:

Liquid – Liquid partition is between stationary phases. The water tightly bound to cellulose of the paper and the mobile organic liquid phase, which passes through the paper by capillary action during the separation process.

Solid – Liquid partition:

This is due to absorption of solutes to cellulose and desorption (elution) of the solutes by the same organic phase during separation process. This method can be used for separation of solutes, which are soluble both in water as well as in organic solvents.

Materials Required

Chromatography paper, Tyrosine, Arginine, Iso- Leucine, Amino acids mixture

Solvents (BAW), Ninhydrine Reagent.

Procedure

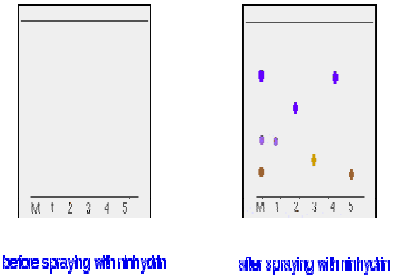
1. Draw a light pencil line about 2cm from the bottom of the paper across the narrow side.
2. Evenly space 4 marks across the pencil line. Label each mark so that can be easily identified latter.
3. Spot about 1µl of amino acid sample on to each mark.
4. Spots should be small; large spots lead to imprecise the results. Allow the sheet to air dry.
5. Add even solvent to the bottom of a beaker so that the entire bottom edge of the paper will be sitting in solvent (about 1 cm in the bottom).
6. Place a piece of filter paper along the side of the jar and wet with solvent. Prepare a piece of plastic wrap to cover the top.
7. Place the paper in the beaker, sample side down. Cover with the plastic wrap.
8. Allow the solvent to travel up the sheet until it has reached 85% of the way to the top.
9. Remove the paper carefully and immediately draw a thin pencil line at the "solvent front": the line, which defines how far, the solvent, has traveled.
10. Allow the chromatogram to dry completely.
12. Take the sheet to the hood, spray lightly with ninhydrin. Be careful to keep the spray in the hood: avoid inhaling it or getting it on hands.
11. Allow to dry and warm gently on a warm hot plate.
12. Amino acids present in the mixture should appear as Yellow/ purple/blue spots.
13. Make the center of the each spots

Result and Interpretation

Calculate Rf value for each amino acid according to this equation:

Rf = Distance traveled by the substances/Distance traveled by the solvent

Record Rf values for each amino acid as well as the mixture. Identify the substances present in the mixture. Compare the standard chart.



For example, if the substance traveled 0.7 cm from the base line while the solvent had traveled 5.0 cm, then the R_f value for the dye is:

Rf = 0.7/5.0=0.1

Rf Value Standard Chart Sheet

S.No	Amino acid	Solvent - I	Solvent - II
1	Histidine	0.07	0.69
2	Serine	0.10	0.36
3	Lysine	0.10	0.48
4	Arginine	0.11	0.59
5	Aspartic Acid	0.13	0.15
6	Glutamic Acid	0.16	0.25
7	Glycine	0.17	0.40
8	Alanine	0.22	0.54
9	Threonine	0.22	0.50
10	Proline	0.30	0.91
11	Tyrosine	0.32	0.64
12	Methionine	0.40	0.80
13	Valine	0.47	0.77
14	Tryptophan	0.47	0.83
15	Iso leucine	0.55	0.86
16	Phenylalanine	0.58	0.89
17	Leucine	0.60	0.86

Note:

Solvent-I n-butanol-acetic acid-water (4:1:5) v/v

Solvent-II Phenol-Water (4:1) v/v

Exp: 6 To identify lipids in a given sample by TLC.

Aim:

To separate a mixture of amino acids by Thin Layer Chromatography (TLC) and identify the test amino acids by measuring their Rf values.

Introduction:

Chromatography is the process through which biomolecules are separated and analyzed from a complex mixture. This separation process consists of two phases: a stationary phase and a mobile phase. The mobile phase consists of the mixture to be separated which percolates through the stationary phase. These two phases can be solid-liquid, liquid-liquid or gas-liquid. Thin Layer Chromatography (TLC) is a solid-liquid form of chromatography where the stationary phase is a polar absorbent and the mobile phase can be a single solvent or combination of solvents.

Principle:

Thin Layer Chromatography (TLC) is a type of chromatography which is based upon the distribution of biomolecules between two immiscible phases. TLC was originally developed to separate lipid molecules and can be used to identify components in a sample, and for preparative purposes. In TLC the stationary phase is a polar absorbent, like finely ground alumina (Al2O3) or silica (SiO2) particles which are coated on a glass slide or plastic sheet to create a thin layer of the particular stationary phase. Silica contains some free – OH groups which form hydrogen bonds or other Van-der-Waals interactions with the analyte components and as a result adsorption takes place. Sometimes a small amount of a binder such as plaster of Paris is mixed with the absorbent to facilitate the coating.

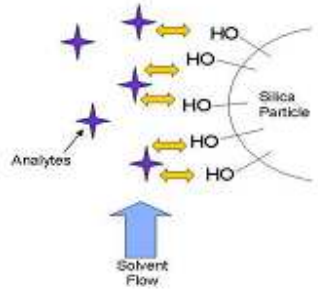


Fig 1: The free OH groups of silica interacts with the analytes (materials to be seprated)

The mixture to be separated is dissolved in a solvent and the solution is spotted at one end of the coated TLC plate next to the reference material. In order to determine whether an unknown substance is the same as a substance of known structure, it is necessary to run the two substances

side by side in the same chromatogram, preferably at the same concentration. The plate is placed with spotted end down in a covered jar containing a shallow layer of suitable solvent. The solvent (mobile phase) is allowed to move up the plate by capillary action through the adsorbent at its own rate and as a result differential partitioning occurs between the components of the mixture dissolved in the solvent and the stationary adsorbent phase. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it willspend in the mobile phase and the more slowly it will migrate up the plate. When the solvent front has moved to within about 1 cm of the top end of the adsorbent, the plate should be removed from the developing chamber. If the components of the sample are colored, they can be observed directly. If not, they can sometimes be visualized by shining ultraviolet light on the plate or by spraying the plate with a reagent (e.g. ninhydrin) that will react with one or more of the components of the sample. Sometimes the spots can be visualized by allowing the plate to stand for a few minutes in a closed container in which the atmosphere is saturated with iodine vapor.

The ninhydrin reaction is used to detect the presence of amino acids. Amino acids contain a free amino and carboxyl group which reacts together with ninhydrin to produce a characteristic blue colour (or occasionally pale yellow). In this reaction first an amino group is attached to the first or alpha carbon of the amino acid's carbon chain and then the nitrogen atom of the amino group reacts with ninhydrin to give a blue-purple product known as Ruhemann's purple as shown in figure 2. Some amino acids (e.g. proline, secondary amine) give yellow-orange colour.

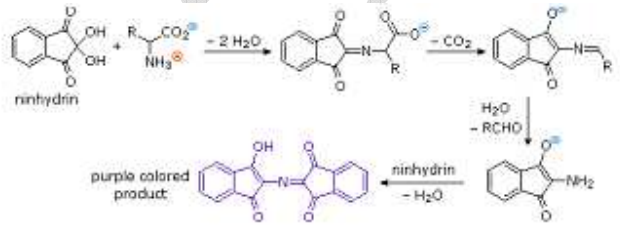


Fig 2: During ninhydrin reaction amino groups of proteins react with ninhydrin to form blue-violet compound called Rhumann's purple

In addition to qualitative results, TLC can also provide chromatographic separation of biomolecules because the distance traveled by a substance relative to the distance traveled by the solvent front depends upon the molecular structure of the substance. The relationship between the distance traveled by the solvent front and the substance is usually expressed as the Rf value which is also called 'retardation factor' and expressed as following:

Rf = distance traveled by substance / distance traveled by solvent front

The Rf values strongly depend upon the nature of the adsorbent and solvent. Therefore, for the separation and subsequent identification of amino acids in a given mixture, Rf values of individual amino acids have to be calculated by performing Thin Layer Chromatography.

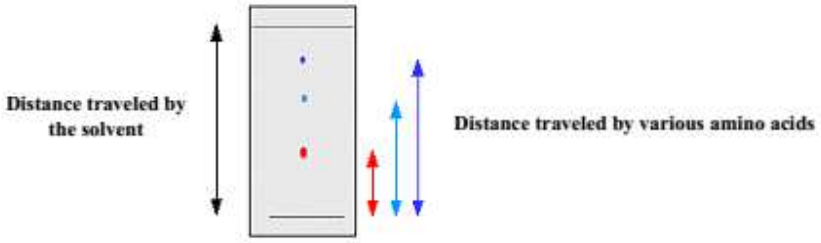
Materials Required:

Glass wares: TLC Chamber (with lid)

Other requirements: Micropipette, Tips, clean forceps, Hot air oven/Incubator

Procedure:

1. Take a TLC plate and with the help of pencil draw two straight lines on the white surface of the plate: one 2 cm from the bottom of the plate and another 1 cm from top of the plate. Never use a pen as dyes (used in ink) may interfere with the results by developing spots on the plate.
2. Mark 10 equidistant points on the bottom line for loading of amino acids samples and test sample. While marking the lines and points do not make a trough with the pencil.
3. Allow all the amino acid samples and test sample to come to room temperature. Then spot 1 µl of each amino acid and test sample along the bottom line on the TLC plate. While spotting use separate tips for each sample.
4. Allow the plates for air drying (~ 10 minutes). Further drying should be done by keeping the TLC plate at 700C in a hot air oven or incubator for 2 – 3 minutes.
5. Take 10 ml of solvent system in the TLC chamber (with lid) and wait for 10 minutes at room temperature.
6. Place the TLC plate inside the chamber with a clean forceps. While keeping the plate make sure that the spotted samples are near the solvent. Furthermore, the TLC plate should be in a straight position so that the solvent phase can move uniformly along the plate.
7. Allow the solvent front to reach the top line of the plate. After that take it out with the help of clean forceps and air dry the plate for 15 – 20 minutes. Keep the plates at 700C for 2 minutes for further drying.
8. Add 1 ml of the Developing Reagent on the plate and swirl the plate very carefully. Look for the development of the coloured spots of different amino acids and the test sample.
9. After the plate is air dried calculate the Rf values of each amino acid and different amino acids in the test sample as shown below:



The Rf value for each amino acid can be calculated by using the formula:

Rf = distance traveled by substance/distance traveled by solvent front

For example, if one amino acid (red) traveled 1.5 cm from the bottom line while the solvent had traveled 5.0

cm, then the Rf value for that amino acid is:

Rf = 1.5/5.0

= 0.3

Observation and Result:

Exp: 7 To verify the validity of Beer's law and determine the molar extinction coefficient of NADH.

Introduction

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are soluble dinucleotides that can be reversibly reduced by the addition of 2 hydrogen ions. While both molecules act as coenzymes in reversible reactions, they are involved in different types of reactions. NAD is generally used as an acceptor of reducing equivalents in catabolism, particularly glycolysis, the tricarboxylic acid cycle, and β-oxidation of fatty acids, while NADH, is reoxidized by complex I of the electron transport chain or by dehydrogenase enzymes during anaerobic metabolism. NADP is characteristically involved with reductive synthesis reactions, such as fatty acid and steroid synthesis. As demonstrated in Figure 1, NAD is a multiple ringed structure, which undergoes redox reactions within its nicotinamide ring . The closely related NADP molecule is phosphorylated on the 2' position of the adenosine ribose ring.

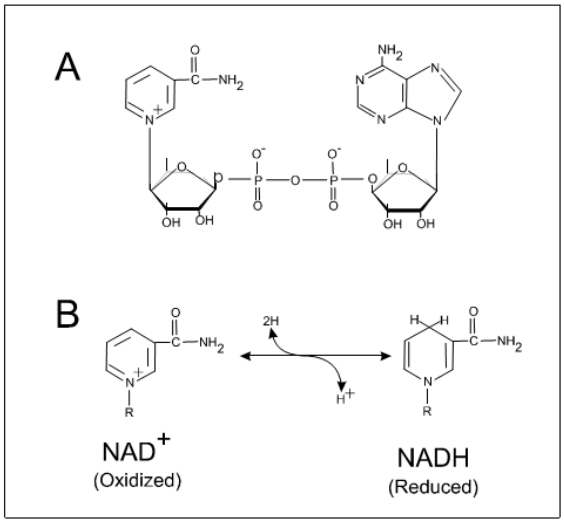


Figure 1. Structure and redox reaction of nicotinamide adenine dinucleotide (NAD+)

Principle

In terms of quantitation, enzymatic dehydrogenase reactions involving NAD or NADP take advantage of the property of the reduced forms, NADH or NADPH, to absorb light at a wavelength of 340 nm while the oxidized forms do not. Likewise, the reduced forms are capable of fluorescent emission at 445 nm when excited at 340 nm, while the oxidized forms are not. These two physical properties allow investigators to quantitate reactions that directly involve a change in the oxidative state of these coenzymes. An example of such a reaction is outlined in

Figure 2A, where the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase requires the conversion of equimolar amounts of NAD+ to NADH. Reactions of enzymes other than dehydrogenases, which are not directly linked with these coenzymes, can also be measured by coupling their product to a dehydrogenase, which uses that product as a substrate (Figure 2B). When the dehydrogenase is in excess, the rate of appearance or disappearance of NADH may then be used to quantify an enzyme that itself does not use NADH as a substrate.

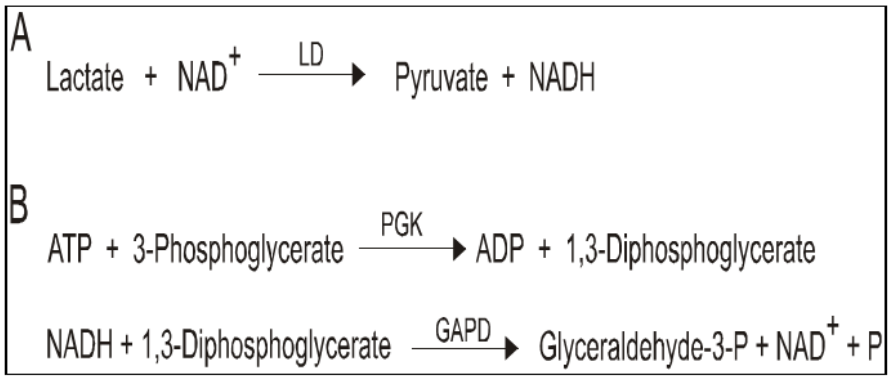


Figure 2. Outline of two reactions using NAD+ as a coenzyme.

Materials and Methods

Pre-weighed NADH and NAD+ vials were purchased from Sigma Chemical Company (St. Louis, MO). Regular and half area 96-well UV transparent microplates, catalogue number 3636 and 3679 respectively, and solid black fluorescence microplates, catalogue number 3915, were purchased from Corning. Stock solutions (1 mg/ml) of each compound were made using TE pH 8.0 (10 mM Tris, 1 mM EDTA) as the buffer. The concentrations of NADH solutions were checked by absorbance at 340 nm using a Shimadzu UV-1700 spectrophotometer. Further dilutions were then made using TE, pH 8.0 as the diluent and 200 µl aliquots of each dilution were dispensed into microplate wells in replicates of 4. Absorbance measurements were made using a Synergy™ 2 Multi-Detection Microplate Reader at 340 nm. Absorbance spectral scans were made using 100 aliquots in half-area UV-transparent microplates from 200 nm to 600 nm in 1-nm increments. The absorbance of TE buffer only was subtracted from the data prior to plotting. The fluorescence of all compounds was determined with the same microplate reader using a 340 nm, 30 nm bandwidth excitation filter and a 440 nm, 40 nm bandwidth emission filter in conjunction with a 400 nm cut off dichroic mirror. The data was collected from the top using the tungsten halogen light source.

Results