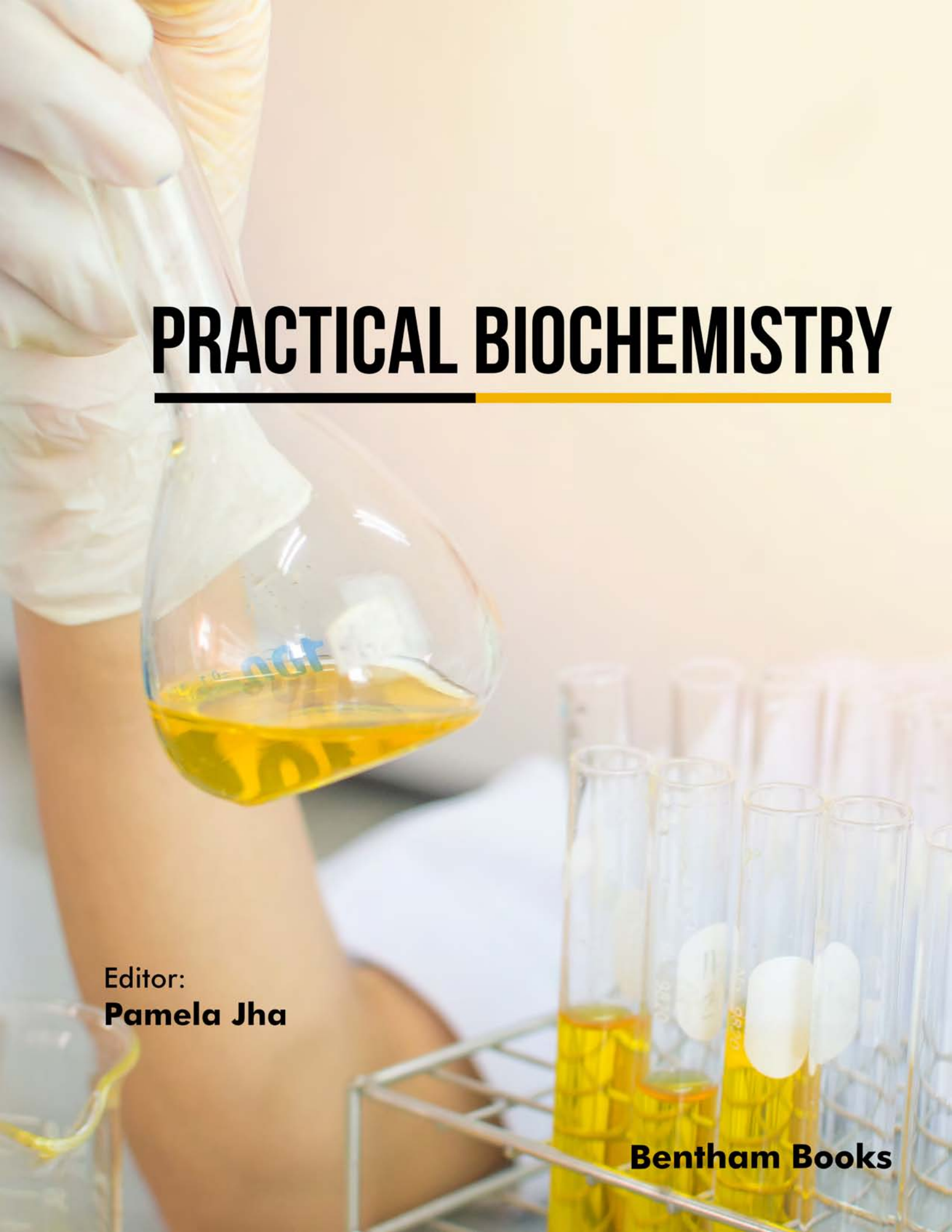

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CONTENTS

FOREWORD

Biochemical methods have contributed to diverse fields of science and technology over a century. They continue to be even more relevant today, half a century after molecular biology, genomics, and bioinformatics seem to hog the limelight. After all the genes are cloned and all the genomes are sequenced and analyzed bioinformatically, there is still no substitute for wet biochemistry to make progress in any area of modern biology or biotechnology. Apart from helping us in understanding the biological processes in any living organism, biochemical methods are critical in many applied branches such as medicine, agriculture, industrial microbiology, and biotechnology. Therefore, rigorous training in the protocols and practical skills of biochemistry greatly enhances the academic success and employability of students in every area of life sciences and biotechnology. This necessitates constant upgradation of books and manuals, and this book is the latest effort in that direction.

"Practical Biochemistry" edited by Dr. Pamela Jha is a comprehensive guide designed for students and early researchers in the whole gamut of life sciences. It encompasses various disciplines such as cell biology, molecular biology, and medical sciences, offering a holistic understanding of biochemistry through its underlying methods. The book rightly emphasizes both theoretical foundations and practical protocols, catering to individuals at the bachelor's and master's levels, as well as research scholars. Each experiment is accompanied by a quick recap of the relevant theory, including specific reactions and protocols, supplemented by 'viva voce questions' for interactive learning. Expected results with quantitative values are provided for many experiments, aiding in self-verification and comprehension. Dr. Pamela Jha's book is structured for quick and effective revision, making it a valuable resource for students and enthusiasts alike. Its user-friendly approach and comprehensive coverage ensure an enjoyable learning experience in the field of biochemistry.

I congratulate Dr. Jha and her team of authors and the publisher for this book.

N. Raghuram
University School of Biotechnology
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PREFACE

The first edition provides information that is important for understanding of some basic and advanced biochemical methods for qualitative and quantitative estimation of major macromolecules. This book is organized into 5 sections, *i.e.*, carbohydrates, proteins, nucleic acids, lipids, and other techniques and SDS. Each section comprises multiple chapters structured with abstract, keywords, introduction, aim, principle, requirements, procedure, probable dummy results, conclusion, precautions, and finally, references. In addition, each chapter is also supplemented with relevant interesting facts in the 'did you know' segment and 'viva voice' questions. I have attempted to write this book in a simple and lucid language to enable easy, focused learning.

I welcome constructive comments from students and readers. Finally, I express my gratitude to the contributors and publisher who have extended tremendous support in the production of this first edition. I wish everyone happy learning!

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CHAPTER 1

Introduction to Carbohydrates

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Abstract: Carbohydrates are one of the four major classes of biologically essential organic molecules in living organisms. They are the most abundant class of biomolecules in nature based on their mass. And they make up most of the organic bodies on earth due to their extensive role in every life form. Carbohydrates are polyhydroxy aldehydes or ketones or substances that result in such compounds upon hydrolysis. These macromolecules are composed of carbon (C), hydrogen (H), and oxygen (O). They are also referred to as saccharides *(sakcharon = sugar or sweetness)* since most of them have a sweet taste. Apart from that, carbohydrate serves as a primary energy source, a structural framework for nucleotides, and provides structural support to organisms. They also play a crucial role as mediators in cellular interaction. Carbohydrates are classified into monosaccharides, oligosaccharides, and polysaccharides. Monosaccharides are the simplest form of carbohydrates, and a few monosaccharides hydrolyse to form oligosaccharide, and many monosaccharides together form polysaccharides. They also act as a precursor for glycoproteins and glycolipids. In this section, we are discussing some basic concepts about carbohydrates.

Keywords: Carbohydrates, Cellular interaction, Energy, Glycoproteins, Glycolipids, Monosaccharide, Nucleotides, Oligosaccharide, Polysaccharides, Saccharides.

INTRODUCTION

Carbohydrates are the most abundant biomolecule on earth as they make up most of the organic matter. They are widely present in both plant and animal tissues, from serving as skeletal structures in plants, insects, and crustaceans to food reserves in the storage organs of plants and animals. A few carbohydrates (sugar and starch) are major chief dietary components in most parts of the world [1]. The

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metabolism of carbohydrates serves as a primary energy source for non-photosynthetic cells. They also serve as energy storage and metabolic intermediate. Deoxyribose and ribose sugar form a structural framework for DNA and RNA [2]. In plant cells and bacterial cells, polysaccharides are an essential element for cell wall formation. Cellulose, which is a key component of the plant cell wall, is the most abundant carbohydrate. In addition, carbohydrates are also linked with proteins and lipids, where they act as precursor molecules for the formation of glycoproteins and glycolipids.

Classification of Carbohydrates

Carbohydrates are mainly classified into 3 groups:

Monosaccharides (mono = one)

They are often called simple sugars and cannot be hydrolyzed into smaller units. They possess a free aldehyde (—CHO) or ketone (=CO) group and 2 or more hydroxyl (—OH) groups. They are represented by the general formula $C_n(H_2O)_n$ or $C_nH_{2n}O_n$ and can be subdivided into trioses, tetroses, pentoses, hexoses, heptoses *etc*., depending upon the number of carbon atoms they possess [3]. They are also classified as aldoses or ketoses, depending upon whether they contain aldehyde or ketone groups. D-glucose is the most abundant monosaccharide found in nature and is a 6- carbon sugar.

Oligosaccharides (oligo = few)

These are sugar compounds that can be hydrolysed to yield 2 to 10 molecules of the same or different monosaccharides. A disaccharide (general formula = $C_n(H_2O)_n - 1$) is an oligosaccharide that yields 2 molecules of monosaccharide on hydrolysis and one yielding 3 molecules of monosaccharide as a trisaccharide (general formula=$C_n(H_2O)_n - 2$) and so on. Examples of oligosaccharides are shown in Table **1.1**.

Table 1.1. Classification of oligosaccharides.

Oligosaccharides	No. of Sugar Molecules	Examples
Disaccharides	2	Sucrose, Lactose
Trisaccharides	3	Rhamnose, Gentianose
Tetrasaccharides	4	Stachyose, Scorodose
Pentasaccharides	5	Verbascose

Polysaccharides (poly = many)

These are sugar compounds that yield more than 10 molecules of monosaccharides on hydrolysis and have a general formula of $(C_6H_{10}O_5)_x$. They are further classified based on monosaccharide molecules produced after hydrolysis.

Homopolysaccharides

Polysaccharides yielding or made up of the same type of monosaccharides. *E.g*., Starch, Glycogen.

Heteropolysaccharides

Polysaccharides yielding or made up of different monosaccharides. *E.g*., Hyaluronic acid, Chondroitin.

GLYCOSIDIC BOND

A glycosidic bond is a covalent bond that joins the hemiacetal group of a saccharide or sugar molecule to one another and the hydroxyl group of another compound, like alcohol. This bond is not always a carbohydrate and is mainly formed as a result of a condensation reaction between alcohol or the hydroxyl (-OH) group of one group to the anomeric carbon of the sugar in which water molecule is released out and O-linkage is formed between the two molecules. Anomeric carbon is defined as the carbon atom to which the carbonyl (aldehyde or ketone) group is attached [4].

There are also types of glycosidic bonds and they are classified as:

***N*-glycosides**

In which linkage takes place *via* a nitrogen atom, also known as glycosylamines.

***C*-glycosides**

In which linkage takes place *via* a carbon; this type of linkage is said to be resistant to hydrolysis.

***S*-glycosides**

In which linkage takes place *via* a sulfur atom, also known as thioglycosides.

CHAPTER 2

Qualitative Tests for Carbohydrate Detection

Mrittika Sarkar[1], Sinchan Hait[2,*] and **Sai Joshi[3]**

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Abstract: Qualitative tests for the determination of carbohydrates in the given sample are a crucial part of before getting into major analytical procedures. Every mono-, oligo-, and poly-saccharide vary depending upon the number of carbohydrate molecule present in it as well as changes in the side chains. Thus, every carbohydrate molecule has distinct functions and properties. Depending upon these physio-chemical properties of the carbohydrates, they respond to certain specific chemical reactions under certain conditions. Only mono- and dis-saccharides respond to the solubility test as they are soluble in water at room temperature. The Molisch test is only for the determination of the presence of carbohydrate, not depending on the types of it. The iodine test gives a result for polysaccharides. Whereas Fehling, Benedict and Osazone tests distinguish between reducing and non-reducing sugars. The Bradford test differentiates between mono- and di-saccharide-reducing sugars. Seliwanoff test is only for sucrose which is a non-reducing sugar. Bial's test is to determine the presence of pentose sugars.

Keywords: Bradford test, Bial's test, Benedict test, Carbohydrate, Fehling test, Iodine test, Monosaccharide, Molisch test, Oligosaccharide, Osazone test, Polysaccharide, Qualitative, Solubility, Seliwanoff test.

INTRODUCTION

To identify the presence of carbohydrates, if present or not in an unknown sample, a certain standardised preliminary protocol is to be followed. There have been various ways to qualitatively determine of carbohydrate present in a given sample. These tests are based upon the physico-chemical properties of the carbohydrates: mono-, oligo-, and polysaccharide; and reducing and non-reducing sugars can be differentiated. Detection and characterizationof carbohydrates present in an unknown sample solution based on various qualitative chemical assays are discussed below (Fig. **2.1**).

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Solubility Test

Aim

To determine the type of carbohydrates present in each sample by solubility test.

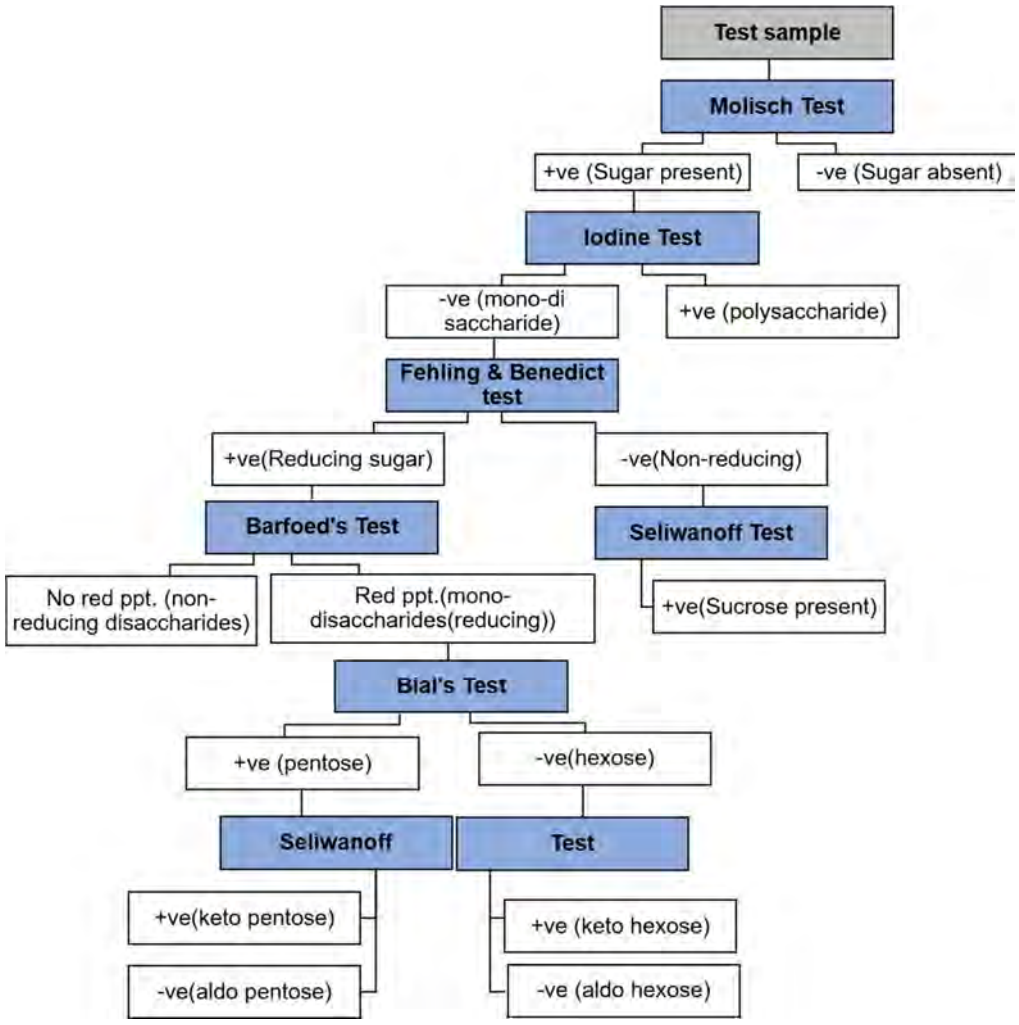

Fig. (2.1). Summary of all qualitative tests for carbohydrates.

Principle

Both monosaccharides (*e.g.*, glucose) and disaccharides (*e.g.*, sucrose) are crystalline solids at room temperature, but they are soluble in water because each

molecule has several -OH groups that readily engage in hydrogen bonding with water molecules. Whereas in the case of polysaccharides, they have a complex structure which has strong interaction among polysaccharide molecules via hydrogen bonds, so they are insoluble in water [1, 3].

Requirements

❖ ***Glasswares***

• Test tubes

❖ ***Other Requirements***

• Dropper

• Test tube holder

❖ ***Reagents***

• Sugar solution (5% glucose, 5% sucrose, 1% starch)

• Distilled water

Procedure

To 1ml of sugar solution, add a few drops of distilled water and mix well.

Applications

• This test can help to determine whether mono-disaccharides or polysaccharides are present in the solution and their ability to dissolve in water.

Conclusion

If it solubilizes, then it confirms the presence of monosaccharides or disaccharides (glucose, sucrose) or else the presence of polysaccharides (starch).

Molisch's Test

Aim

To determine whether carbohydrates are present in a given sample by the Molisch test.

CHAPTER 3

Quantification Tests for Carbohydrate Detection

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Abstract: Quantification of carbohydrates by the anthrone method is a colourimetric assay. In this method, first, the complex carbohydrates are hydrolysed in a highly acidic medium, resulting in monosaccharides. Thereafter, these monosaccharides are dehydrated to form 5-hydroxymethylfurfural (5-HMF) or 2-*furfural* (2-F), followed by reacting with anthrone reagent. This reaction produces a blue-green complex which is colourimetrically determined. In DNSA method 3,5-dinitrosalicylic acid (DNSA), a yellow colour reagent reacts with reducing sugar's carbonyl group and forms orange-red colour compound 3-Amino-5-nitrosalicylic acid (ANSA). The quantity of carbohydrate present in a sample is determined by Beer-Lambert's law. Apart from these techniques Folin-Wu, Hexokinase, Phenol-Sulphuric acid, Somogvi-Nelson, and GOPOD method are discussed in this chapter in detail.

Keywords: Anthrone, Carbohydrate, Colourimetry, Folin-wu, GOPOD, Hexokinase, Phenol-sulphuric acid, Somogvi-Nelson, 5-hydroxymethylfurfural (5-HMF), 2-*furfural* (2-F), 3,5-dinitrosalicylic acid (DNSA), 3-Amino-5-nitrosalicylic acid (ANSA).

INTRODUCTION

Quantification of carbohydrates is an important step to determine the amount of carbohydrate present in a given sample. Several methods have been developed for the same. In this chapter, we will be dealing with colourimetric assays for the quantitative determination of carbohydrates. The assays include the Anthrone

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method, DNSA method, Folin-Wu method, Hexokinase method, Phenol-Sulphuric acid method, Somogvi-Nelson Method, and GOPOD method. In these assays, the carbohydrate present in the sample reacts with certain chemical ingredients under certain specified conditions and forms a colour complex which is then detected *via* spectrophotometer.

The colour formation is directly linked with the concentration of the carbohydrate present in the sample. This is based on Beer-Lambert's law which states that the absorbance of a solution is dependent upon the concentration of material present in the given solution and path length.

$$A = \varepsilon c l \quad (1)$$

Where, A=absorbance, ϵ=molar absorption coefficient ($Mol^{-1}cm^{-1}$), c=conentraion of the solution (M), l=path length (cm)

ANTHRONE METHOD

Principle

The anthrone method for carbohydrate analysis is used to quantify total carbohydrates in an unknown sample. The carbohydrate content can be measured by hydrolyzing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides, which are the basic units of all carbohydrates. This method then uses a colourimetric approach which requires the aid of a chemical agent (anthranol) to determine the concentration of a chemical compound in a solution [1 - 4]. This method takes place in three steps:

Hydrolysis of Complex Carbohydrates

In this step, complex carbohydrate polymers react with water to break down into simple units of monomers. The disaccharide or long chain of polysaccharides breaks into two unionized components with each water molecule splitting. Here one part gains a hydrogen atom (H+) and the other gains a hydroxyl group (OH–) from a split water molecule. However, this process can be catalyzed by carrying out the reaction in an acidic condition (much like how the human digestive system uses various enzymes lactase, sucrase, and amylase, collectively called glycosidase or invertase in honey bees). Catalysis of the cleavage of *β 1-4 glycosidic bonds*occurs via a nucleophilic substitution reaction where an electron-rich nucleophile (electrophilic carbon atom of aldehydes and ketones of carbohydrate) attacks a positively charged electrophile (from acid) to replace a

leaving group [1 - 4]. The use of aqueous sulphuric acid (H_2SO_4) facilitates our purpose (Fig. **3.1**).

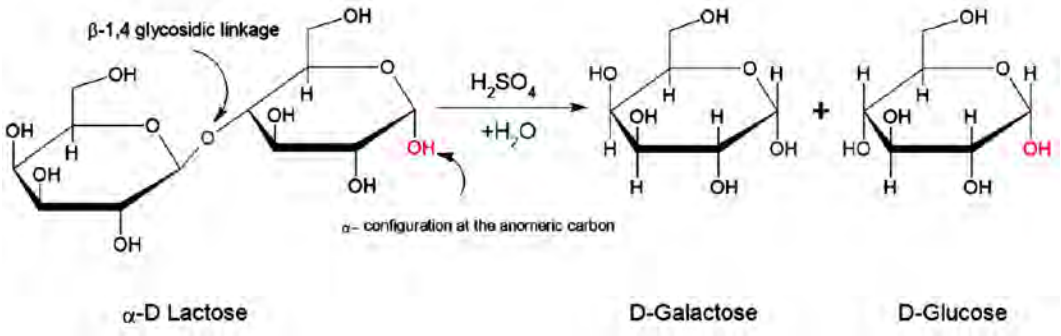

Fig. (3.1). Hydrolysis of carbohydrates.

Dehydration of Monosaccharide

Dehydration of 6-carbon monosaccharides or hexoses (*i.e.*, glucose, galactose, fructose) and 5-carbon monosaccharides or pentoses (*i.e.*, xylulose, arabinose, ribose) typically leads to the formation of 5-hydroxymethylfurfural (5-HMF) and 2-*furfural* (2-F) respectively [2, 3]. This step is observed in some qualitative tests for carbohydrates (Seliwanoff's test, Molisch's test) (Fig. **3.2**).

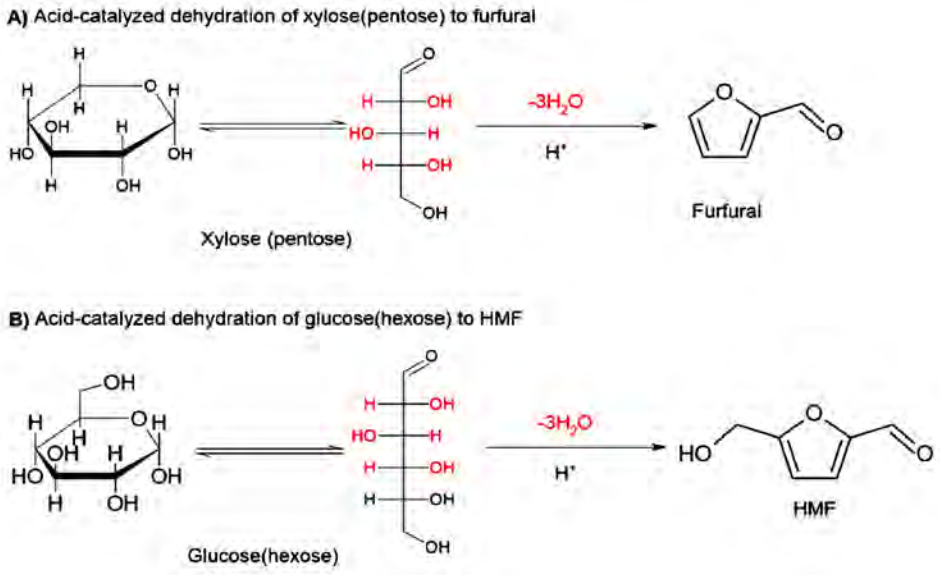

Fig. (3.2). Dehydration of monosaccharide.

CHAPTER 4

Introduction to Proteins

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Abstract: Proteins are the ambidexterity macromolecules which play a crucial role in biological processes. Through proteins, genetic information is expressed. They exhibit enormous functions which are crucial in various pathways. Some proteins act as an enzyme which is vital for catalyzing various metabolic reactions in living cells. All proteins are made up of amino acids which join together via a peptide bond to form a polypeptide chain which is a precursor for the further development of protein. A nascent protein undergoes several post-translational modifications to achieve its true function. Based on the amino acids present in the protein, its function determines its role as hormones, enzymes, antibodies, transporters, *etc*. In this chapter, a brief introduction to protein's structure and function is discussed.

Keywords: Amino acids, Antibodies, Enzyme, Gene expression, Hormone, Peptide bond, Protein, Polypeptide chain, Post-translation modification, Transporters.

INTRODUCTION

Proteins are one of the most important macromolecules, which are abundant in living organisms. The genetic information in nucleic acids is expressed *via* proteins. However, these macromolecules are constructed from 20 amino acids. In addition, depending upon these amino acid combinations, proteins can be enzymes, hormones, antibodies, transporters, muscle fibre, *etc*. In humans, the Recommended Dietary Allowance (RDA) for protein is 0.8 grams of protein per kilogram of body weight. Some of the major sources of proteins are milk, fish, meat, beans, *etc*. Proteins are composed of amino acids. A basic structure of amino acids contains an amino group ($-NH_2$) and a carboxylic acid group (-COOH) (Fig. **4.1**) [1, 2].

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These amino acids are joined by peptide bonds to form proteins (Fig. **4.2**). There are 20 amino acids present, these amino acids are mainly present in zwitterions (Fig. **4.3**) form, and they (except glycine) exhibit optical activity due to asymmetric α-carbon atoms (Fig. **4.4**).

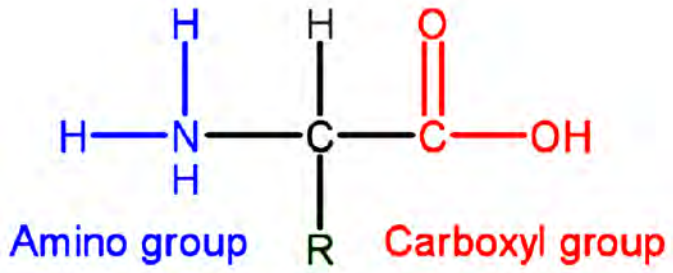

Fig. (4.1). Amino acid structure.

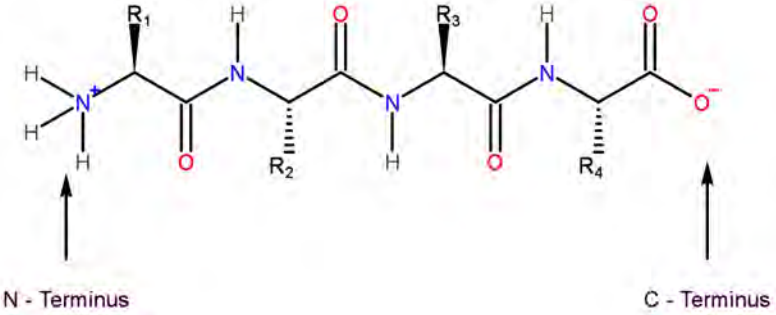

Fig. (4.2). Peptide chain.

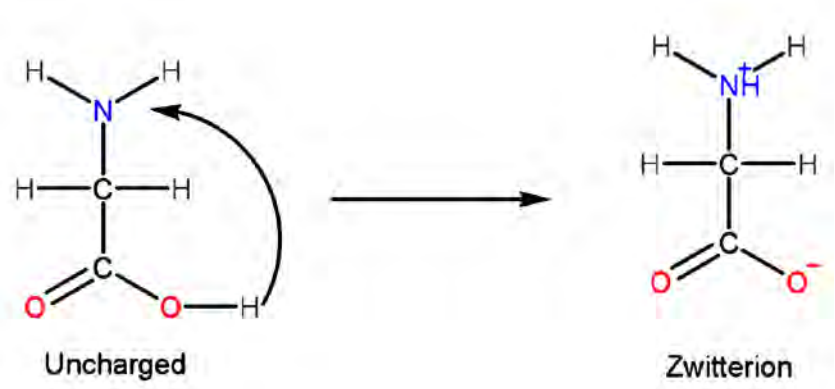

Fig. (4.3). Zwitterion.

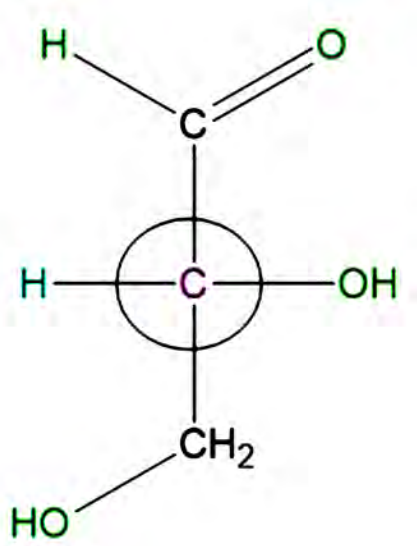

Fig. (4.4). Chiral carbon.

There are several methods available to determine amino acids, which help us to identify the protein.

PROTEIN STRUCTURE

The amino acid sequence of a protein determines its structural configuration and its function. A protein may contain 20 different amino acids (Table **4.1**) for Amino acid properties and convention, each containing an amine group and an acid group at the 2 terminals, with a distinct side chain.

Table 4.1. Amino acid properties and convention.

Amino Acid	Abbreviation	M_r	pKa Values			pI	Hydropathy Index	Occurrence in Protein (%)
			pK_1 (-COOH)	pK_2 (-NH_3^+)	pK_R (R group)			
Nonpolar, Aliphatic								
Glycine	Gly, G	75	2.34	9.60	-	5.97	-0.4	7.2
Alanine	Ala, A	89	2.34	9.69	-	6.01	1.8	7.8
Proline	Pro, P	115	1.99	10.96	-	6.48	1.6	5.2
Valine	Val, V	117	2.32	9.62	-	5.97	4.2	6.6
Leucine	Leu, L	131	2.36	9.60	-	5.98	3.8	9.1
Isoleucine	Ile, I	131	2.36	9.68	-	6.02	4.5	5.3

CHAPTER 5

Qualitative Tests for Protein Detection

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Abstract: Qualitative tests are the primary assays for detecting the presence of any protein in a given sample. Qualitative detection of proteins is based on the chemical interactions between amino acids and certain chemical reagents added to them. These chemical reactions yield colour formation, which corresponds to specific amino acid or a group of amino acids present in the sample. In this chapter, some of such most important assays for qualitative analysis are discussed in detail.

Keywords: Amino acid, Assay, Protein.

INTRODUCTION

Qualitative tests for protein are based on the detection of amino acids present in it. Each amino acid has unique chemical properties, and some have common properties too. Under various chemical conditions, these amino acids react with certain chemical agents and develop colour compounds in them. This formation of colour compounds can then be correlated with amino acid/s present in a given sample. In a few cases, the colour formation can be due to some contamination in the sample, so further analysis is required, such as qualitative tests, SDS-PAGE, western blotting, *etc*., which are explained in the next chapters.

AROMATIC AMINO ACIDS (TYROSINE, TRYPTOPHAN)

Xanthoproteic Test

Aim

To determine the presence of proteins in a given sample by Xanthoproteic test.

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Principle

The aromatic amino acids, such as phenylalanine, tyrosine and tryptophan, contain phenolic groups. Under alkaline aromatic amino acids, concentrated

HNO_3 nitrates the phenyl ring and produces dark yellow or orange colour compounds (nitro-derivatives) (Fig. **5.1**) [1, 3]. But, the benzene ring in phenylalanine is not activated (in the case of tyrosine and tryptophan the benzene ring is active), and so it is not nitrated by nitric acid.

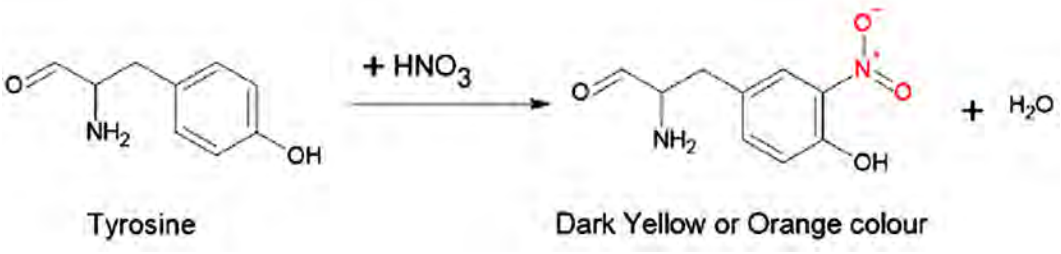

Fig. (5.1). Xanthoproteic test reaction.

Requirement

❖ ***Chemical***

• Conc. nitric acid

• 40% NaOH

❖ ***Glassware***

• Test tube

• 1 ml pipette

• Glass dropper

❖ ***Others***

• Test tube holder

• Bunsen burner

• Vortex

Procedure

1. Take 1 ml of the given sample in a test tube followed by a few drops of conc. HNO_3 and mix the contents.

2. Now boil the solution mix over a Bunsen burner for 2-3 minutes.

3. Allow the test tubes to cool down (Tip: if required use tap water to cool the content).

4. Add 0.1-0.3 ml of 40% NaOH solution and observe for colour change.

Results

❖ ***Positive Test***

The formation of yellow or orange colour means tyrosine and tryptophan are present.

❖ ***Negative Test***

No colour formation, which means the absence of tyrosine and tryptophan.

Precaution

1. Concentrated nitric acid is toxic and can cause serious injury to the skin. Prevent contact with the eye, clothing, and skin.

Application

• Xanthoproteic tests can be employed to identify aromatic amino acids (except phenylalanine).

Limitations

• Phenylalanine has a stable phenyl group; it does not react with HNO_3.

TYROSINE

Millon's Test

Aim

To determine the presence of protein in a given sample by Millon's test.

CHAPTER 6

Protein Determination by SDS-PAGE

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Abstract: SDS-PAGE is the abbreviated form of sodium-dodecyl sulphate-polyacrylamide gel electrophoresis. It is a commonly used gel electrophoresis for the qualitative estimation of proteins. It is based on the principle that treatment of protein with SDS, an anionic detergent, causes a constant negative charge throughout the protein surface. It also breaks the protein chain into smaller peptide chains. Hence, the separation of proteins is solely based on the size and shape of the fragments generated and is independent of the charge of the protein. This, combined with polyacrylamide gel electrophoresis, allows for visualization of these fragments where the smaller fragments retain at the bottom of the gel. This method has been discussed in detail in this chapter.

Keywords: Electrophoresis, SDS-PAGE, Size based separation, Protein.

INTRODUCTION

Electrophoresis through agarose or polyacrylamide gels is a standard method used to separate, identify and purify biopolymers, since both these gels are porous in nature. Polyacrylamide gel electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. The most commonly used form of polyacrylamide gel electrophoresis is the Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS- PAGE), used mostly for the separation of proteins which is an analytical method used to separate components of a protein based on size.

PRINCIPLE

SDS-PAGE stands for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis. Protein separation is generally achieved using a polyacrylamide

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gel. This gel is prepared by incorporating acrylamide with a polymerizing agent - ammonium persulfate (APS). TEMED (N,N,N',N'-tetramethylenediamine) is involved in catalyzing this polymerization reaction by enhancing the production of free radicals by APS. This converts acrylamide to polyacrylamide. The sievelike structure of polyacrylamide is manipulated to achieve protein separation [1 - 5]. The protein separation takes place based on the pore size present in the polyacrylamide gel, as shown in Fig. (**6.1**). The stacking gel allows all the protein samples to line up along the same length in the gel so that they enter the resolving gel at the same time. The resolving gel causes the separation of proteins based on molecular weight.

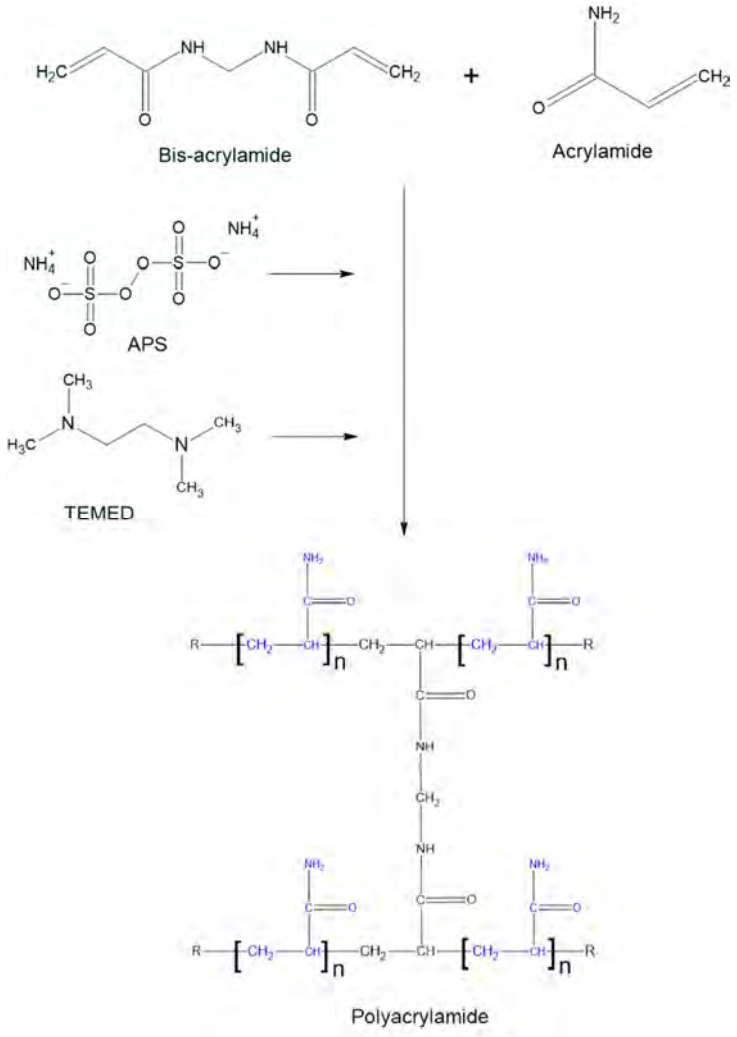

Fig. (6.1). Chemical reaction of gel formation.

In this technique, primarily the protein sample is treated with DTT (dithiothreitol) or 2-mercaptoethanol, as shown in Fig. (**6.2**). This breaks the disulphide bonds created by cysteine and methionine in the amino acid chain of the protein [1 - 3] [5]. Consequently, the binding of SDS to the protein sample denatures the protein and covers the protein sample with an overall negative charge. At pH 7, in the presence of 1% w/v SDS and 2-mercaptoethanol, proteins dissociate into their subunits and bind large quantities of detergent. Under these conditions, most proteins bind about 1.4g of SDS per gram of protein which completely masks the natural charge of proteins giving a constant charge mass to ratio. Hence, the larger the molecule, the greater will be the charge; this can be detected according to the electrophoretic mobility of the complex. Hence, the proteins get separated based on molecular weight see Fig. (**6.2**) [1 - 5].

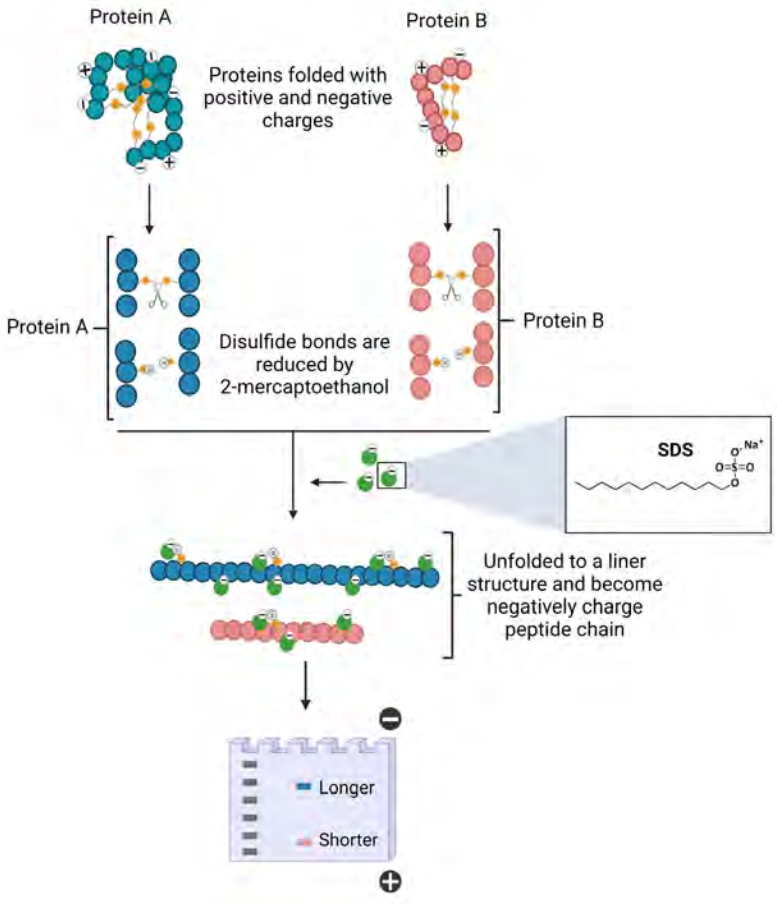

Fig. (6.2). Protein denaturation.

CHAPTER 7

Protein Determination by Western Blotting or Immunoblot

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Abstract: Western blotting, also known as immunoblot, is a chromatography-based technique in which quantitative detection of proteins can be performed based on their molecular weight. This is often performed after the SDS-PAGE technique. It is used to identify, analyze and quantify proteins from a protein mixture. The major steps include 1. Separation of proteins (*e.g.*, SDS-PAGE), 2. Transfer onto a solid support, 3. Utilizing primary and secondary antibodies for visualization. Western blotting can be used qualitatively and quantitatively. In this chapter, both of these techniques are explained in detail.

Keywords: Chromatography, Immunoblot, Protein, SDS-PAGE.

INTRODUCTION

Western blotting is a technique broadly used in research to separate and identify proteins. The separation of proteins based on size can be performed using SDS-PAGE; the size-based separated bands are then transferred onto a solid support such as a nitrocellulose membrane, which allows for visualization of the band for each protein. The incubation of the primary antibody with the membrane allows for the interaction between the protein of interest and the antibody specific to that protein. The bound antibody is detected through a secondary antibody which acts as a chromogen system (*e.g.*, HRP-conjugated protein) [1 - 5]. This technique can be used for both qualitative and quantitative analysis of protein samples. In this chapter, both techniques are discussed.

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QUALITATIVE WESTERN BLOTTING

Principle

The principle of western blotting (WB) or immunoblot is based on immuno-chromatography. The protein molecules are separated into polyacrylamide based on their molecular weight. Once proteins are separated on polyacrylamide, they are electro-transferred onto a nitrocellulose membrane or polyvinylidene difluoride (PVDF) [1 - 4]. Proteins attach to the membrane by hydrophobic interaction. Then these isolated proteins are detected by conjugation with a specific primary antibody and enzyme-labelled secondary antibody and substrate (Table **7.1**) by autoradiography or fluorescence or chemiluminescent methods.

Table 7.1. Colorimetric enzyme and substrate system for antigen detection of western blotting.

Enzyme	Substrate	Colour
Alkaline phosphatase	5-Bromo-4-chloro-3-indolyl phosphate (BCIP) with Nitro blue tetrazolium chloride (NBT)	Black or purple
Horseradish peroxidase	Naphthol AS-MX phosphate with fast red TR	Bright red
	4-Chloro-1-naphthol (CN)	Blue or purple
	3-Amino-6-ethyl carbazole (ACE)	Red or brown
	3,3'-Diaminobenzidine tetrahydrochloride (DAB) [carcinogenic]	Brown
Glucose oxidase	Phenazine methosulfate with NBT	Black or purple
β-Galactosidase	X-Gal	Blue

Did you know?

W. Neal Burnette discovered the western blotting technique taking inspiration from the northern blotting technique, but in 1979 his manuscript was rejected, and finally, in 1981, his manuscript was accepted and published in Analytical Biochemistry [4].

Requirement

❖ 1X transfer buffer

• 25 mM Tris base

• 192 mM glycine

• 0.1% SDS

• Adjust pH to 8.3

❖ Filter paper towel

❖ 5% skim milk

❖ 5% BSA

❖ Polyvinylidene fluoride (PVDF) membrane

❖ SDS-PAGE apparatus

Procedure

SDS-PAGE

See Chapter 6 for the SDS-PAGE procedure.

Electrotransfer

1. Cut one polyvinylidene fluoride (PVDF) membrane with the same dimensions as the gel.

2. Wet the sponge and filter paper in the transfer buffer and wet the PVDF membrane in methanol.

3. Preparation of the sandwich for electrotransfer is as follows: Sponge, 3 Filter Papers, Gel, PVDF, and 3 Filter Papers (Caution: ensure no air bubble present).

4. Keep the sandwich in the transfer apparatus, place it on ice, and the temperature during the process must be maintained at 4°C.

5. Pour the transfer buffer into the apparatus, and ensure that the sandwich is covered with the buffer.

6. Connect the apparatus to the electrical supply (caution: ensuring that the PVDF membrane is between the gel and a positive electrode).

7. Transfer for 90 minutes (Tip: running time depends upon gel thickness).

Blocking and Antibody Incubation

1. Take out the PVDF membrane and dip it in 5% skim milk in a buffer for 1 hour.

CHAPTER 8

Quantitative Tests for Protein Detection

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Abstract: Quantitative tests for protein are essential for understanding the amount of protein present in a sample. Various tests have been developed, such as BCA, biuret, Bradford, and Lowry tests. These are the most common and standardised procedures for the determination of protein. The mentioned techniques are colourimetric assays. However, over a while, several more advanced techniques are also being developed. In this chapter, quantitative methods for protein are discussed.

Keywords: BCA, Biuret, Bradford, Lowry, Protein.

INTRODUCTION

Quantitative tests help us to understand the concentration of protein in a given sample. For these, BCA, Biuret, Bradford, and Lowry tests are some standard assays which determine the same. These methods are colourimetric assays and the colour formation is equivalent to the concentration of protein resent in it.

BICINCHONINIC ACID (BCA) TEST

Aim

Quantification of protein using Bicinchoninic acid (BCA).

Principle

Bicinchoninic acid, sodium salt, is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ions (Cu^{+}) in an alkaline environment. This reagent forms the basis of an analytical method capable of monitoring cuprous ions produced in the reaction of protein with alkaline Cu^{2+}

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(biuret reaction). The colour produced from this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentrations [1].

At room temperature, cysteine, cystine, tryptophan, and tyrosine residues reduce Cu^{2+} to Cu^{1+}. At higher temperatures (37º C to 60º C), peptide bonds in protein also perform this reduction, which produces results that correlate more strongly to protein quantity than to the presence of specific residues. Each Cu^{1+} ion forms a complex with two BCA molecules, creating a purple-coloured product that absorbs light at 562 nm (Fig. **8.1**) [1]. Absorbance data from unknown samples can be plotted against a standard curve and the concentration of unknown samples can thus be calculated.

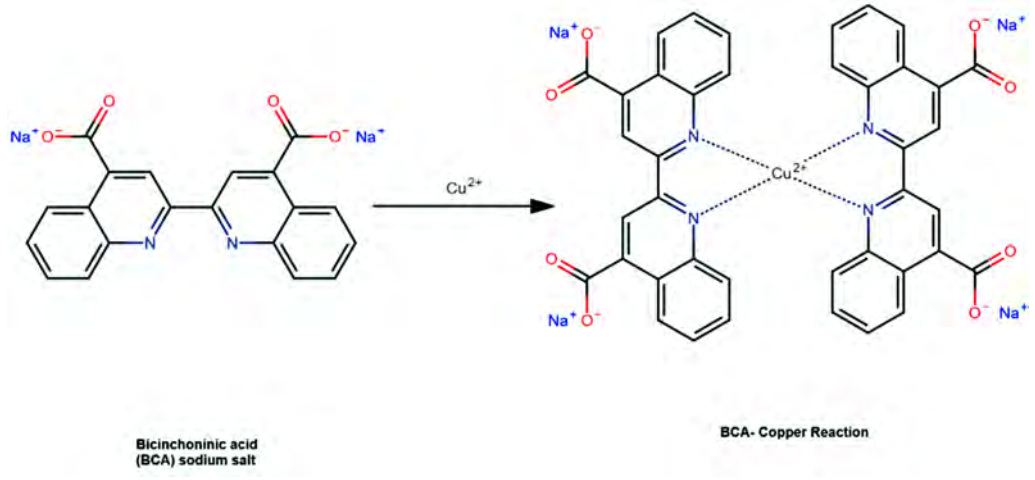

Fig. (8.1). Formation of the purple complex with BCA and cuprousion generated from the biuret reaction.

The bicinchoninic acid (BCA) or copper-based assay (range: 20-2000 ug/ml).

Requirements

• Reagents: Standard Bicinchoninic acid, sodium salt, Folin Ciocalteu reagent (2 N) diluted to 1N, Deionized water.

• Proteins: BSA (standard) and sample.

• Glasswares: Beakers, cuvette (specify), pipettes (10ml), measuring cylinder (100ml), test tubes, glass stirrer.

Procedure

Standard Bicinchoninic Acid Preparation

• Reagent A Components: An aqueous solution of 1% BCA-Na_2, 2% $Na_2CO_3.H_2O$, 0.16% Sodium-tartrate, 0.4% NaOH, and 0.95% $NaHCO_3$. If needed, appropriate addition of NaOH (50%) or solid $NaHCO_3$ is made to reagent A to adjust the pH to 11.25.

• Reagent B Components: 4% $CuSO_4.5H_2O$ in deionized water.

• Standard Working Reagent (S-WR) (prepared weekly or as needed): Mix 100 vol of Reagent A with 2 vol of Reagent B. S-WR is apple green in colour.

Standard Bicinchoninic Acid Preparation (For micro-assay)

• Micro-Reagent A (MA) components: An aqueous solution of 8% $Na_2CO_3.H_2O$, 1.6% NaOH, 1.6% Sodium tartrate, and sufficient $NaHCO_3$ to adjust the pH to 11.25.

• Micro-Reagent B (MB) components:

4% BCA-Na_2 in deionized water.

• Micro-Reagent C (MC) components: 4 vol of 4% (aq) $CuSO_4.5H_2O$ plus 100 vol of Micro-Reagent B.

• Micro-Working Reagent (M-WR) consists of 1 vol of MC plus 1 vol of MA. MA and MB are stable indefinitely at room temperature, but MC and M-WR should be prepared as needed.

MacroAssay

1. Pass all the prepared solutions through a 1-pm filter to remove insoluble debris associated with the salt.

2. Mix 1 vol of the sample (standard or unknown) with 20 vol of S-WR in a test tube.

3. Colour development proceeds immediately, even at room temperature, but it can be greatly accelerated by incubating the tubes in a constant temperature water bath. In this respect, the temperature chosen for the colour development is directly related to the desired sensitivity.

CHAPTER 9

Protein Purification

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Abstract: Protein purification is an essential step after protein isolation. Protein purification ensures the removal of all contaminants from the sample pool. Purification depends on the chemical and physical interactions of protein molecules and chemical moieties. These techniques are performed with tags and without tags. Tag purification involves ammonium sulfate precipitation and dialysis. Purification of proteins with tags utilizes affinity and size exclusion chromatography. There are various methods available for this, however, we will discuss some most common methods for protein purification.

Keywords: Ammonium sulfate precipitation, Affinity chromatography, Dialysis, Protein, Protein purification, Size exclusion chromatography.

INTRODUCTION

Protein purification techniques improve the protein quality in the sample by removing contaminants from it. Major techniques are performed with and without a tag for purification. Both techniques depend upon the physio-chemical interactions between the protein molecule and the chemical compound present in it. The techniques are discussed in detail below:

PURIFICATION OF DIFFERENT TYPES OF PROTEINS (WITHOUT TAG)

Ammonium Sulfate Precipitation

Aim

To use the ammonium sulfate precipitation method for protein purification.

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Principle

The ammonium sulfate precipitation method, also known as the salting out method, is a commonly used method for protein purification from a solution.

Most of the enzymes or proteins exist in cell fluids as soluble proteins; this solubility is attributed to the combined electrostatic effects of polar interactions of the protein with aqueous solvent and electrostatic forces between protein molecules and small aggregates of like charges [1, 3].

When the concentration of the ammonium sulfate increases, the small, highly charged ions of the salt compete with the proteins to bind to the water molecule. This removes the water molecule interactions or the 'hydration shell' around the protein and exposes the hydrophobic core of the protein to interact intermolecularly, thereby decreasing the protein solubility and resulting in precipitation [1, 3]. Hence precipitation of protein by the addition of salts like ammonium sulfate is known as 'salting out' (Fig. **9.1**).

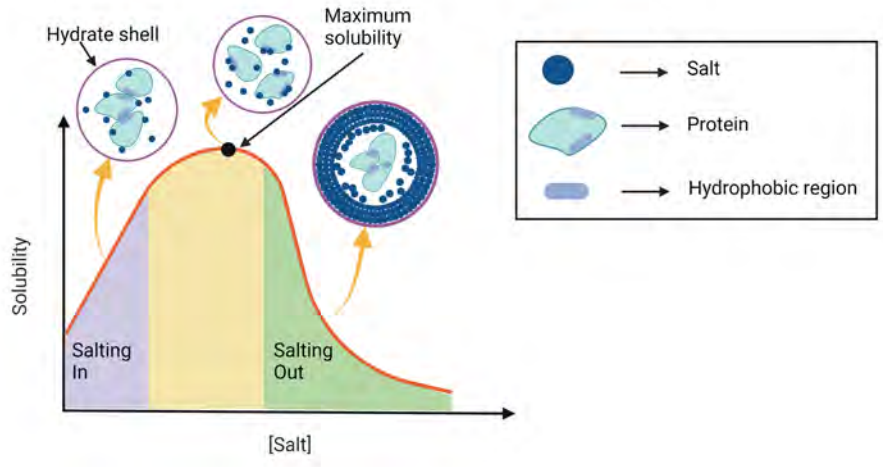

Fig. (9.1). Salting in and out curve.

Requirements

❖ *Chemicals and Sample*

• Protein sample

• Ammonium sulfate

• Phosphate buffer saline

❖ ***Instruments***

• Centrifuge

• Refrigerator

• Weighing balance

• Magnetic stirrer (optional)

❖ ***Miscellaneous***

• Conical flasks

• Stirrer

• Petri dish.

Procedure

1. The ammonium sulfate for precipitation needs to be in uniform, dry form; for that, place the salt in a dish and dry heat at 120 ºC in an oven.

2. Centrifuge the solution at 10000 g at 4 ºC for 10 mins to get rid of any visible precipitate.

3. Collect the precipitated protein by centrifugation for 30 minutes at ≥ 5,000 g at 4°C. Decant the supernatant. Dissolve the pellet (generally in 10 times lower volume than the initial volume) in the appropriate buffer.

4. Prepare ammonium sulfate of 45% saturation, *i.e.*, 277 g ammonium sulfate/litre solution. Stir gently for a minimum of 4 hr and a maximum of 16 hr at 4 ºC.

5. Add the prepared ammonium sulfate slowly while stirring in the prepared protein solution very slowly. This will ensure that the local concentration around the site of addition does not exceed the desired salt concentration.

6. Once the ammonium sulphate is added, move the container to the refrigerator at 4 ºC for 6 hours for sufficient precipitation to occur.

7. Transfer to a conical tube and centrifuge the precipitate at 3000g for 30 minutes.

8. Carefully remove and discard the supernatant. Invert the conical tube and drain well.

CHAPTER 10

Introduction to Nucleic Acids

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Abstract: Nucleic acids are biomolecules which carry all the genetic information in the form of DNA and RNA. The nucleic acids are composed of primarily a 5-carbon sugar, a phosphate and a nitrogenous basis. These nitrogenous bases can be adenine, guanine, thymine, cytosine and uracil. Nucleic acids can be measured qualitatively by UV absorbance, diphenylamine method/orcinol method, fluorescence dyes and capillary electrophoresis. It can be measured qualitatively by agarose gel electrophoresis.

Keywords: Capillary electrophoresis, Diphenylamine method, DNA, Fluorescence dyes, Nucelic acid, Orcinol method, RNA, UV absorbance.

INTRODUCTION

Nucleic acids are polymers of nucleotides, which are monomers made of three components: a 5-carbon sugar, a phosphate group, and a nitrogenous base (Fig. **10.1**) for nucleic acid composition. They are biomolecules which are also known as the energy currency of metabolism transactions due to the following functions [1 - 3]:

(a) They are an important chemical linkage between cellular response to hormones and other extracellular stimuli.

(b) They are the molecular repository of genetic information passed down from one generation to another, which is a fundamental property of life.

(c) The structural characteristics of proteins and, ultimately, every biomolecule is dependent on information programmed by the nucleic acid sequence of the cell. Nucleic acids comprise 3 components [1, 2]:

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5-carbon sugar, which is usually ribose, a phosphate group and a nitrogenous base.

Nitrogenous bases are of two kinds: (a) Pyrimidine: It is a heterocyclic aromatic ring containing two nitrogen atoms at the C1 and C3 positions of a 6-carbon ring.

(b) Purine: It is a pyrimidine ring with an imidazole ring.

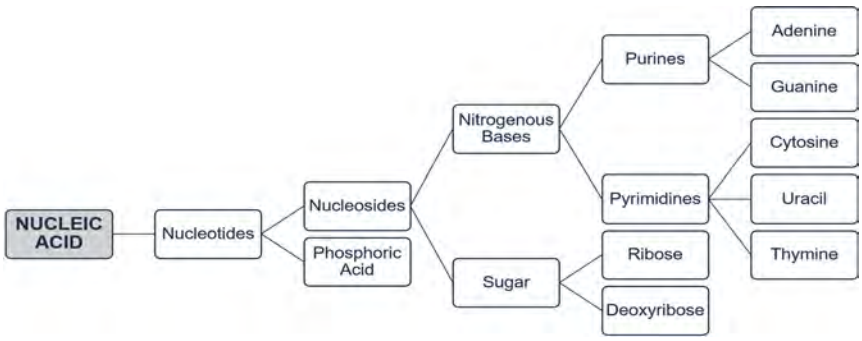

Fig. (10.1). Nucleic acid structure.

Nucleic acids can be further divided into [1 - 3]:

Deoxyribonucleic Acid (DNA)

The synthesis of functional biological products is largely dependent on cellular DNA, referred to as a **gene**. Cells possess thousands of genes and therefore their size tends to be very large.

Functions of DNA

Storage and transmission of genetic information in the form of genes.

Components of DNA

5 carbon sugar: Deoxyribose

Nitrogenous bases: Adenine (A), Guanine (G), Thymine (T), Cytosine (C)

Phosphate group

RNA: Ribonucleic Acid

RNA sequence is dependent on DNA sequence, and it has a broader range of functions than DNA. They are classified as:

Ribosomal RNA (rRNA)

They are a component of ribosomes, which play an important role in protein synthesis.

Messenger RNA (mRNA)

They carry genetic information from a gene which is to be translated to protein to the ribosome.

Transfer RNA (tRNA)

Translate data in mRNA into a specific amino acid sequence.

Components of RNA

5 carbon ring: Ribose

Nitrogenous bases: Adenine (A), Guanine (G), Uracil (U), Cytosine (C)

Phosphate group

Nucleic acids have two kinds of pentoses: DNA contains 2'- deoxy-D-ribose, whereas RNA contains D-ribose pentose sugar. Depending on this property of nucleic acids, the classification of DNA or RNA is established. Although DNA and RNA contain the major bases (A, G, C, T (DNA), U (RNA)), however, some minor bases have been identified. These are:

(a) In DNA, usually, the methylated forms of nucleic acid bases are found. *E.g.*: 5-methylcytidine occurs in the DNA of animals and higher plants.

(b) In viral DNA, hydroxymethylated or glycosylated forms of nucleic acid bases are found. *E.g.*: 5-hydroxymethylcytosine in the DNA of animals and bacteria infected by bacteriophages.

These altered forms of DNA play a role in the regulation or protection of the genetic sequence.

(c) Minor bases are also found in RNAs, especially tRNAs. *E.g.*: Inosine contains the base hypoxanthine.

The long chain nucleotides are formed by the phosphodiester linkage between the 5' - phosphate group of one nucleotide binds to the 3'- hydroxyl group.

CHAPTER 11

Qualitative Tests for Nucleic Acid Detection

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Abstract: Qualitative tests of nucleic acids are important to detect the presence or absence of nucleic acid in the given sample. It can be done by performing agarose gel electrophoresis. Nucleic acids are negatively charged and hence can be attracted towards a positive charge in an electric field. This phenomenon is used in agarose gel electrophoresis to detect nucleic acids. Based on the mass of nucleic acids, different bands are formed in the gel, which can be visualized using fluorescent dyes.

Keywords: Agarose gel, DNA, Nucleic acids, Negative charge, RNA.

INTRODUCTION

Detection of nucleic acid can be done efficiently using agarose gel electrophoresis. The pores of the agarose gel allow the sample to flow through it. The size of the pores determines the sieving property of the sample and, therefore the size of nucleic acid which can pass through the gel [1]. Nucleic acid with different sizes has different molecular masses, which are easily differentiated in the agarose gel.

AGAROSE GEL ELECTROPHORESIS

Aim

To test the presence of DNA/RNA in the sample by agarose gel electrophoresis.

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Principle

Having phosphate backbones, DNA (and RNA) molecules are negatively charged, which causes them to migrate toward positively charged anodes when they are exposed to an electric field. The mobility of NA molecules is influenced by the applied voltage. Sample migration is faster at higher voltages within a range. Large molecules move more slowly due to frictional drag and less efficiently pass through pore spaces in gels than smaller molecules [1 - 3].

The rate of migration (v) of DNA/RNA is dependent on electrophoretic mobility, given by the following equation:

$$v=(qE)/f$$

Where, v= velocity, q= charge on particle, E= electric field (volts/cm), f= frictional coefficient

The bands are visualized with fluorescent dyes, ethidium bromide or SYBR Green which intercalates between nucleic acid bases.

Range: Separates DNA fragments of size ranging from 100 bp to 25 kb.

Reagents and Materials

• **Equipment**

• Electrophoresis apparatus: agarose gel, an electrophoretic chamber with a cathode (negative terminal) at one end and an anode (positive terminal) at the other end.

• Casting tray

• Well combs

• Voltage source

• UV light source

• Microwave

• **Reagents**

DNA Electrophoresis

• TAE- 50X (Tris-base: 242 g Acetate (100% acetic acid): 57.1 ml EDTA: 100 ml 0.5M sodium EDTA. Add dH2O up to one litre. To make 1x TAE from this stock,

dilute 20ml of stock into 980 ml of dt water)

• Agarose (1%)

• Ethidium bromide (10 mg/mL)

RNA Electrophoresis

• **Gel loading buffer II** - Denaturing PAGE

• A 1-2X solution of 95% Formamide, 10 ml 10X MOPS running buffer, 18mM EDTA, and 0.025% SDS, Xylene Cyanol, and Bromophenol Blue.

• **Gel loading solution** - All-purpose, native agarose

• A 10X solution of 40% Sucrose, 0.17% Xylene Cyanol, and 0.17% Bromophenol Blue

Procedure

1. Prepare a standard 1% agarose gel.

2. Loading samples and running an agarose gel:

DNA

• Before the agarose gel solidifies, add the ethidium bromide (EtBr) at a concentration of approximately 0.2-0.5 g/mL (about 2-3 mL of stock solution per 100 mL of gel).

• Add a loading buffer to each of your DNA samples.

• Upon solidifying, place the gel into the electrophoresis unit.

• Fill the gel box with 1xTAE (or TBE) until the gel is covered.

• In the first gel lane, load the DNA ladder.

• Carefully load your samples into the additional wells of the gel.

• Run the gel at ~50–70 mA of current, until the dye line is approximately 75-80% of the way down the gel. In general, a gel run lasts between one and 1.5 hours, depending on the concentration and voltage of the gel.

• You must turn off the power, disconnect the electrodes, and carefully remove the gel from the gel box after the disconnection of the electrodes.

CHAPTER 12

Quantitative Tests for Nucleic Acid Detection

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Abstract: Nucleic acid quantification is essential in experiments associated with genetic engineering. Over periods, several techniques have been developed to quantify nucleic acids. UV-Vis spectrophotometer is the simplest technique where it is associated with absorbance, which is proportional to the concentration of the sample. There are some colourimetric methods, such as the diphenylamine (DPA) method, and the orcinol method, where nucleic acid reacts with diphenylamine and forms a bright blue colour complex, and orcinol reacts and forms a green colour complex. Apart from that, some advanced techniques, such as fluorescence techniques, are used for quantification. In this chapter, each of these methods is explained in detail.

Keywords: Diphenylamine (DPA) method, Fluorescence, Nucleic acid, Orcinol method, UV-Vis spectrophotometer.

INTRODUCTION

Besides the qualitative analysis of nucleic acids, quantification of the same is an important step. Over a while, several techniques have been developed, however, there are some standard assays available which are performed routinely. UV-Vis spectrophotometric analysis of quantification is the simplest one to perform [1] [3]. In the diphenylamine (DPA) method, the diphenylamine reacts with diphenylamine and forms a bright blue colour complex which is measured at 600nm [2]. Whereas, in the orcinol method, orcinol reacts with orcinol and forms a green colour complex, and the absorbance is measured at 660nm [1, 3]. In advanced techniques, fluorescence dyes are used, which bind to the nucleus acids and upon excitation, they emit some light which is then quantified to determine the amount of nucleic acid present in the sample [4 - 6].

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SPECTROPHOTOMETRY OF DNA AND RNA

Aim

To determine the concentration of DNA or RNA in a sample.

Principle

There are several ways to quantitate solutions of nucleic acids. If the solution is pure, one can use a spectrophotometer to measure the amount of ultraviolet radiation absorbed by the bases [1, 3].

Procedure

1. Use distilled water or 1X TE as a solvent to suspend the nucleic acids, and place each sample in a quartz cuvette.

2. Zero the spectrophotometer with a sample of solvent. For more accurate readings of the nucleic acid sample of interest, dilute the sample to give readings between 0.1 and 1.0.

For a 1 cm pathlength, the optical density at 260 nm (OD_{260}) equals 1.0 for the following solutions:

- a 50 μg/mL solution of dsDNA
- a 33 μg/mL solution of ssDNA
- a 20-30 μg/mL solution of oligonucleotide
- a 40 μg/mL solution of RNA

Troubleshooting

Contamination of nucleic acid solutions makes spectrophotometric quantitation inaccurate. Calculate the OD_{260}/OD_{280} ratio for an indication of nucleic acid purity. Pure DNA has an OD_{260}/OD_{280} ratio of ~1.8; pure RNA has an OD_{260}/OD_{280} ratio of ~2.0. Low ratios could be caused by protein or phenol contamination.

COLOURIMETRIC ASSAYS

Nucleic acids are isolated by homogenizing fresh samples, disrupting the cells, and precipitating the DNA and RNA with trichloroacetic acid and ethanol. To determine concentrations of nucleic acids a colourimetric assay is used.

Diphenylamine Reaction

Aim

To determine the concentration of DNA by the Diphenylamine (DPA) method.

Principle

Diphenylamine is specific for 2'-deoxypentose, including the deoxyribose characteristic of DNA. When a solution of diphenylamine and DNA is heated, the solution turns blue. The intensity of the blue colour, measured at 600 nm, is directly proportional to the concentration of the DNA. the lack of a hydroxyl substituent at the 2' position enables a ring-opening oxidation reaction, the aldehyde product of which further reacts with diphenylamine [$(Ph)_2NH$] to yield a bright blue complex (Fig. **12.1**) [1, 2].

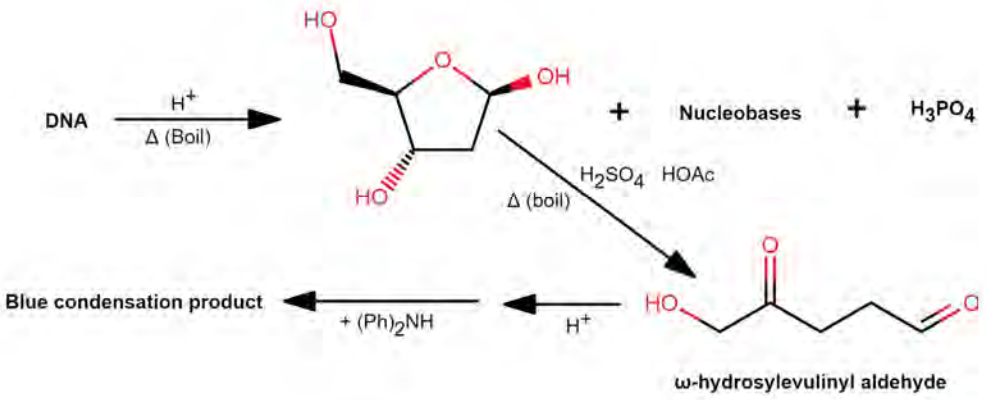

Fig. (12.1). In Dische's assay.

Procedure

Label six test tubes as shown in Table **12.1**, and add the appropriate volume of each solution. Tubes 1-4 are the standards, which contain known concentrations of DNA; tube 5 contains the extract prepared above with an unknown concentration of DNA. Make sure that you use the correct concentration of each stock solution. The problems, causes and probable solutions are mentioned in Table **12.2**.

Table 12.1. DPA method procedure.

Tube	The volume of DNA (µg/ml)	Stock Solution	Nucleic Acid Extract	5 % TCA
Blank	0	-	-	2.0 ml

CHAPTER 13

Purification of Nucleic Acids

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Abstract: Besides qualitative and quantification of nucleic acids, purification is an integral part of any genetic engineering experiments. In genetic engineering experiments, the purity of nucleic acid matters the most. Nucleic acid purification can be achieved by the phenol-chloroform method, alcohol-based purification, agarose gel electrophoresis, and chromatographic purification. These purifications are based on the interactions between bases of nucleic acids and chemical reagents. In this chapter, nucleic acid purification is explained in detail.

Keywords: Alcohol, Agarose gel electrophoresis, Chromatography, Genetic engineering, Nucleic acid, Phenol-chloroform.

INTRODUCTION

Five basic steps of DNA extraction are consistent across all the possible DNA purification chemistries [1]:

1. Disruption of the cellular structure to create a lysate.

2. Separation of the soluble DNA from cell debris and other insoluble material.

3. Binding the DNA of interest to a purification matrix.

4. Washing proteins and other contaminants away from the matrix.

5. Elution of the DNA.

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SEPARATION OF SOLUBLE DNA FROM CELL DEBRIS AND OTHER INSOLUBLE MATERIAL

Extraction of the Nucleic Acid Sample by Phenol-chloroform Method

Principle

DNA is insoluble in phenol because phenol is a nonpolar solution having a density of 1.07 g/cm^3, which is higher than the density of water (1.00 g/cm^3), thus forming two separate phases - an organic and aqueous phase when added to the cell sample. Chloroform protects genomic DNA and increases the efficiency of phenol to denature the protein. It allows proper separation of the organic phase and aqueous phase and keeps DNA protected in the aqueous phase. The mixing of phenol and chloroform forms a denser solution, thus enabling less cross-contamination from the organic phase in the aqueous phase, which is beneficial when the aqueous phase is removed from the solution to obtain a pure nucleic acid sample [2].

Binding to the Purification Matrix

The DNA of interest can be isolated using various methods and its purification can be performed by binding to the matrices using silica, cellulose and ion exchange depending on their characteristic binding capacity and efficiency.

Solution-Based Chemistry

It relies on alcohol precipitation rather than relying on a binding matrix. The cell debris and proteins are precipitated using a salt solution of high concentration which will remove proteins from the solution and centrifugation would separate the soluble nucleic acid from the cell debris and precipitated protein. The DNA is precipitated using isopropanol. Removal of remaining salt can be done by washing the pellet with ethanol and the DNA pellet is resuspended in an aqueous buffer like Tris-EDTA and is then ready for its use in downstream applications.

ALCOHOL-BASED PURIFICATION METHOD

Aim

To purify nucleic acid samples using alcohol.

Principle

The reaction between solute and solvent creates an insoluble aggregate called precipitate and the process is called precipitation.

DNA is a polar molecule with a net negative charge on the backbone (PO_3^-). In DNA precipitation, salt (sodium acetate) reacts with DNA and breaks up into Na^+ and CH_3COO^-. The positively charged sodium ion neutralizes negatively charged PO_3^- of the DNA. The hydrophilic nature of DNA helps it to dissolve in the water, but reacting with sodium acetate makes DNA less hydrophilic. According to Coulomb's law, "force of attraction between two opposite charges is inversely proportional to the dielectric constant." The low dielectric constant of ethanol protects the salt - DNA complex by shielding it against the water. Therefore, the chance of DNA getting dissolved in alcohol is less as compared to that of water and after adding alcohol, the force of interaction between PO_3^- and Na^+ increases. Alcohol forms hydrogen bonding with water, and thus, it protects the complex of PO_3^- and Na^+ by neutralizing the charge of water. Collectively, the DNA is pulled out from the bottom of the tube as a white cottony thread-like precipitate [3].

The most common salts utilized in DNA precipitation are sodium acetate, sodium chloride, ammonium acetate, and lithium chloride.

Lithium chloride is best used for RNA precipitation, whereas sodium acetate is employed for DNA precipitation.

Requirements

Glassware: test tubes, microcentrifuge tube, beaker.

Reagents: DNA sample (<1mg/ml), 3 M sodium acetate (pH 5.2), 1:1 phenol-chloroform, ice-cold 100% ethanol, 70% ethanol, TE buffer (pH 8.0).

Other Requirements: vortex, microcentrifuge, speed vac evaporator, pipettor.

Procedure

1. Take the DNA solution to be purified and add an equal volume of phenol and chloroform and place it in a 1.5ml microcentrifuge tube.

2. At room temperature, vortex vigorously for 10 seconds and microcentrifuge for 15 seconds at maximum speed so that the organic phase and aqueous phase are well distinct.

3. Carefully remove the aqueous phase containing DNA from the top using a 200µl pipettor and transfer it to a new tube. If a white precipitate is observed at the aqueous/organic interface, repeat steps 1 to 3.

4. Add 1⁄10 volume of 3 M sodium acetate of pH 5.2 to the solution of DNA. Mix by vortexing briefly or by flicking the tube several times with a finger.

CHAPTER 14

Introduction to Lipids

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Abstract: Lipids are water-insoluble hydrocarbon derivatives with many physiological functions. They are the structural components of membranes. The long chain fatty acid chains of lipids can be broken down to serve as energy fuels, as well as they play a role in intracellular signaling. The entire composition of lipid molecules in a cell constitutes its lipidome. In this chapter, we will discuss the different roles played by lipids by their chemical properties in detail.

Keywords: Hydrocarbon, Lipids, Lipidome.

INTRODUCTION

Lipids are a group of natural compounds such as fats, oils, chemicals, and certain parts of membranes that are assembled such that they don't interact with water.

The structure and properties of two delegate lipids are: stearic acid (an unsaturated fat) and phosphatidylcholine (a phospholipid) which are made up of chemical groups that form polar “heads” and nonpolar “tails” (Fig. **14.1**). The polar heads are hydrophilic, or soluble in water, though the nonpolar tails are hydrophobic, or insoluble in water. Lipid particles of this composition immediately form aggregate structures like micelles and lipid bilayers, with their hydrophilic ends arranged toward the watery medium and their hydrophobic ends shielded from the water.

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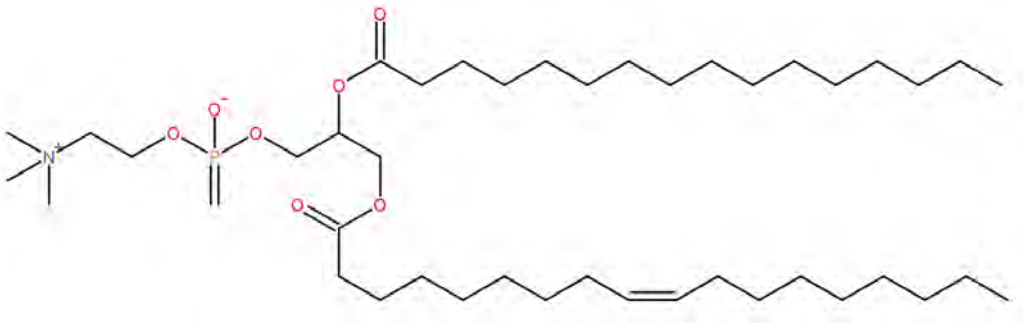

Fig. (14.1). Phospholipid (phosphatidylcholine).

Fats

Fats are any of a group of natural esters of glycerol and different fatty acids, which are solid at room temperature and are the primary constituents of animal and vegetable fat.

Glycerol

Glycerol is a basic polyol compound. Glycerol is found in lipids known as glycerides. Glycerol is an odourless, colourless, viscous liquid that is non-toxic and sweet-tasting.

Fatty acids

Fatty acids consist of a straight chain of a significant number of carbon molecules, with hydrogen atoms along the length of the chain and toward one side of the chain and carboxyl groups (—COOH) at the opposite end. It is the carboxyl group that makes it an acid (carboxylic acid) [1, 2].

Structure of Fatty Acids

Natural fatty acids, individuals from the class of compounds known as carboxylic acids, are made out of a hydrocarbon chain with one terminal carboxyl group (COOH) (Fig. **14.2**). The fragment of a carboxylic acid excluding the hydroxyl (-OH) group is called an acyl group. Under physiological conditions in water, this acidic group typically has lost a hydrogen ion (H^+) to form a negatively charged carboxylate group (-COO). Although the molecule overall is water-insoluble by its hydrophobic hydrocarbon chain, the negatively charged carboxylate is hydrophilic [1, 2]. This normal form for natural lipids, one that contains all-around isolated hydrophobic and hydrophilic parts, is called amphipathic.

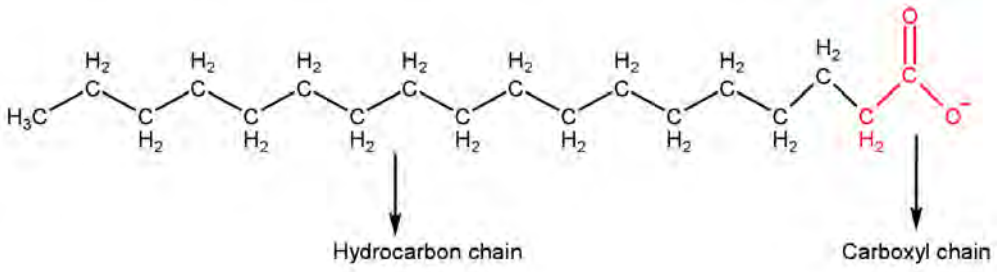

Fig. (14.2). Structural formula for stearic acid.

Classification on the Basis of Saturation

Saturated Fatty Acids

The most straightforward fatty acids are unbranched, direct or linear chains of CH_2 groups connected via a carbon-carbon single bond with one terminal carboxylic acid group [1, 2].

Unsaturated Fatty Acids

Unsaturated fats have at least one or more carbon-carbon double bonds. The term unsaturated shows that less than the most extreme conceivable number of hydrogen atoms are attached to every carbon in the atom. The quantity of double bonds is demonstrated by the conventional name - monounsaturated for atoms with one double bond or polyunsaturated for particles with at least two double bonds. Oleic acid (Fig. **14.3**) is an illustration of a monounsaturated fatty acid.

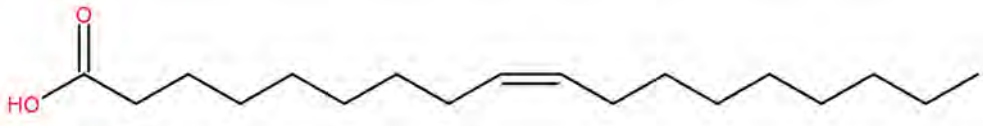

Fig. (14.3). Structural formula of oleic acid.

Fatty Acid Derivatives

Triglycerides

Triglycerides (synthetic name triacylglycerol), the principal method for storing fatty acids in natural frameworks, are a class of mixtures that comprise glycerol (three-carbon trihydroxy alcohol) with a fatty acid connected to every one of the three -OH groups by an **ester bond**. The structure of a typical triglyceride (tristearic acid) is shown in Fig. (**14.4**). Since this molecule contains just one kind of fatty acid, it is alluded to as a simple triglyceride [1, 2].

CHAPTER 15

Qualitative Tests for Lipid Detection

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Abstract: Qualitative tests are the primary assays for detecting the presence of any lipids in a given sample. Qualitative detection of lipids is based on the chemical interactions between components of lipids and certain chemical reagents added to them. These chemical reagents allow for the properties of lipids like hydrophobicity, translucency, or colourimetric analysis due to colour changes. These tests have been explained in detail in this chapter.

Keywords: Colourimetric, Hydrophobicity, Lipids, Translucency.

INTRODUCTION

Lipid detection through qualitative tests can confirm the presence of lipids in a mixture. Lipids have unique chemical properties. The solubility of lipid compounds in the mixture employs its detection through qualitative methods. The variation of translucency in mixtures allows for its detection. Under various chemical agents, a colourimetric determination can be correlated with the qualitative determination of lipids.

SOLUBILITY TEST

Aim

Qualitative test for analysis of lipids by solubility test.

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Principle

This test is used to know the solubility of lipids. Lipids are nonpolar in nature hence they are insoluble in the polar solvent while soluble in nonpolar solvents like chloroform, benzene and boiling alcohol. The general lipid structure of polar and nonpolar lipid molecules is shown below (Fig. **15.1 A-B**) [1 - 3].

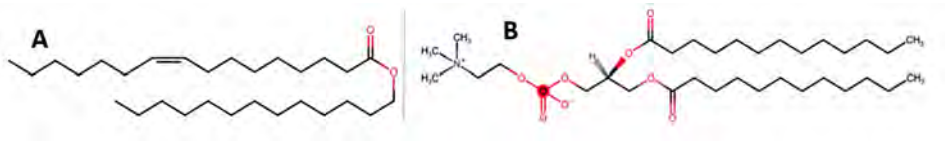

Fig. (15.1). Lipid molecules: **A.** Nonpolar unsaturated **B.** Polar saturated.

Requirements

❖ ***Glassware***:

• Test tubes

❖ ***Chemicals***:

• Olive oil

• Chloroform

• Distilled water

Procedure

1. In two clean, dry test tubes, add 1 ml of olive oil in both test tubes.

2. In the first test tube, mark it as A and add 1 ml of chloroform.

3. In the second tube, mark the test tube as B and add 1 ml of distilled water.

4. Shake both tubes vigorously for 2 minutes.

5. Allow the tubes to stand and note the formation of a homogenous solution with chloroform, indicating that the lipid is dissolved, and the formation of two layers with water indicates that the lipid is insoluble in water.

Observation

1. In test tube A, oil gets dissolved in chloroform.

2. In test tube B, oil drops float on the distilled water surface.

Inference

1. ***Positive result*** Lipids are soluble in a nonpolar solvent, *i.e.*, chloroform and partially soluble in ethanol which can solubilize upon heating.

2. ***Negative result*** Lipids are insoluble in a polar solvent, *i.e.*, water.

The sample contains fat, as it is not soluble in water (test tube B) but soluble only in organic solvents (test tube A).

TRANSLUCENT SPOT TEST

Aim

Qualitative test for analysis of lipids by Translucent Spot test.

Principle

A translucent spot test is also a preliminary test for lipids, which is characterized by a translucent and greasy spot. The lipid will not wet the filter paper, unlike water. The lipids will form a greasy or translucent spot due to their greasy texture, and penetrate the filter paper. Unlike lipids, the spot of water will disappear from the paper [1 - 3].

Requirements

❖ ***Miscellaneous***

• Filter paper

❖ ***Reagents***

• Water

• Olive oil

Procedure

1. Take filter paper.

2. Add 1 ml of water at one end and 1 ml of olive oil at the other end.

3. Observe the appearance of a translucent spot on the filter paper.

CHAPTER 16

Quantitative Tests for Lipid Detection

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Abstract: Quantification of lipids can be broadly classified into the following methods: gravimetric analysis, which employs the principle of phase separation, the acid value is based on the property of rancidity of fats, saponification value which relies on alkali required to saponify (hydrolyze) lipids containing mixture, iodine value depends on the interaction of fats with halogen iodine, and blood cholesterol estimation determines the cholesterol level by cholesterol esterase enzyme-based assay. These assays have been explained in detail in this chapter. Advanced techniques used for lipid quantification utilize the principles of chromatography (Thin-layer chromatography, gas chromatography, and HPLC) and spectroscopy (infrared spectroscopy, Raman spectroscopy, nuclear magnetic resistance spectroscopy, and fluorescence spectroscopy), and different colourimetric assays are discussed in detail in this chapter.

Keywords: Cholesterol, Fluorescent spectroscopy, Gravimetric, Gas chromatography, HPLC, Iodine value, Lipid, Raman spectroscopy, Thin-layer chromatography.

INTRODUCTION

Qualitative analysis of lipids allows for the detection of lipids in a mixture, however, quantification of the same is an important step. Over the period, several techniques have been developed; however, there are some standard assays available which are performed routinely. Manipulation of physical and chemical characteristics of lipids to estimate the number of lipids in a mixture allows us to understand the number of lipids in the mixture. The importance of lipid determination to maintain proper physiological functions is an important application of these quantitative techniques. Lipids like cholesterol and fats play an important role in the physiological functioning of the cells, and it ultimately

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impacts the entire composition of human functioning. Using quantitative techniques, a deeper understanding of lipid composition can be obtained. In this chapter, we have discussed different quantitative measures to estimate the number of lipids in a mixture.

GRAVIMETRIC ANALYSIS

Aim

Estimation of total lipids by the Bligh and Dyer method.

Principle

Methanol, chloroform and water are added to the sample in a two-step extraction and after phase separation, lipids are quantified in the chloroform phase. The mixtures with a known measure of water (80%) are broken down in this binary system and are then isolated by the transition from the monophasic system to the biphasic system induced by the addition of water: the final composition is situated inside the miscibility gap: the two samples with compositions given by the points at the end of the tie-line are coexist [1 - 5].

Requirements

❖ ***Reagents***

- Chloroform
- Methanol

❖ ***Other requirements***

- Homogenizer
- Whatman Filter Paper
- Plastic vials
- Measuring Cylinders
- Aluminium pans

Procedure

1. 5 g samples (preferably dry wet weight) are homogenized for 2 min in a homogenizer with 5 ml each of chloroform and methanol.

2. The mixture is homogenized for another 30 s after the addition of 5 ml of chloroform.

3. 5 ml of water is added to the mixture, and the sample is homogenized again for 30 s.

4. The mixture is then allowed to separate, initially, the lower solvent phase is removed and then passed through a Whatman #1 filter paper, and then the filtrate is saved in a labelled vial.

5. Another 5 ml of chloroform is added into the remaining pellet and aqueous phase and homogenized for another 2 min.

6. The resultant mixture is added to the previous filtrate by passing it through the Whatman #1 filter paper.

7. The filtrate is allowed to separate in the graduated cylinder, and the volume of the lower chloroform layer is recorded.

8. The lipids are then gravimetrically determined by placing 0.5 ml aliquots of the chloroform layer into pre-weighed aluminum pans (three pans per sample), allowing the samples to evaporate in a hood overnight, recording the weights, and then converting to per cent lipids.

Observation

Determination of Total Lipid Content

1. After weighing, a small volume of chloroform is added to each flask to detect the presence of non-lipid material that is insoluble.

2. If non-lipids are present, the chloroform is carefully decanted, and the flask is rinsed 3 times with the chloroform.

3. Then, the dry weight of the residue is determined and subtracted from the initial weight.

4. The lipid content of the sample is calculated as follows:

$$Total\ Lipid\ (g) = \frac{Weight\ of\ lipid\ in\ aliquot\ X\ Volume\ of\ chloroform\ layer}{Volume\ of\ aliquot}$$

Results

In general, lipid extraction from plants can be performed through two different

CHAPTER 17

Commonly Used Basic and Advanced Techniques

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Abstract: Different techniques are used in a biochemical laboratory to measure various parameters. For example, a pH meter is used to measure the pH of the sample and electrochemical cells are used to measure the redox potential. Zeta potential is another property which can be measured using zeta cells. Chromatography is a technique used to separate the components of a mixture. Two of the most common chromatography techniques are HPLC and ion exchange chromatography. HPLC is based on the interactions of the analytes in stationary as well as mobile phase. Whereas ion exchange chromatography is based on the interaction of ions and charged site of the stationary phase.

Keywords: Electrochemical cell, Electron transfer, HPLC, Ion exchange, pH, pH meter, Redox, UV detection, Zeta potential, Zeta cell.

INTRODUCTION

Measurement of certain properties of the sample is important for biochemical analysis and chemical interpretations. Redox potential is the tendency to lose and gain electrons. This can be measured by using an apparatus using electrochemical cells. Measuring pH is used to measure the acidity or basicity of a sample which can be achieved by using a pH meter. Similarly, zeta potential is an important property of colloidal solutions and is measured using zeta cells. Chromatography is a technique used to separate the components of a mixture. This involves a mobile phase and a stationary phase. The two-chromatography discussed is High-Performance Liquid Chromatography (HPLC) and ion exchange chromatography.

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MEASUREMENT OF REDOX POTENTIAL

Aim

To measure the redox potential of a sample using electrochemical cells.

Principle

The tendency of chemical species to acquire and lose electrons is called redox potential.

In an electrochemical cell, the redox reaction is not direct. The oxidation and reduction occur in different vessels, and the electrons are transferred from the reducing agent to the oxidizing agent through connecting wires. Hence in such cells, chemical energy is converted into electrical energy [1].

Half-cells are created by placing a piece of metal into a solution comprising a cation of the metal (*e.g.*, Cu metal in a solution of $CuSO_4$ or Cu^{2+}). A porous barrier or a salt bridge separates the two half-reactions. With a voltmeter, the (+) lead will make contact with one metal and the (–) lead with another (Fig. **17.1**). If a positive voltage is shown on the screen, the cell is correctly connected. The metal attached to the (+) lead is the cathode (reduction) and thus has a higher, more positive reduction potential. The metal attached to the (–) lead is the anode (oxidation) and has a lower, more negative, reduction potential.

Requirement

1. Millivolt
2. Redox electrodes
    a. Platinum and reference electrodes
    b. Combination electrodes
3. Thermometer (liquid in a glass or thermistor type)
4. Filter paper
5. Nitrate solutions of Cu, Zn (*i.e.* $Cu(NO_3)_2$, $Zn(NO_3)_2$)
6. 1 M $NaNO_3$ solution
7. Well plate
8. Alligator clips

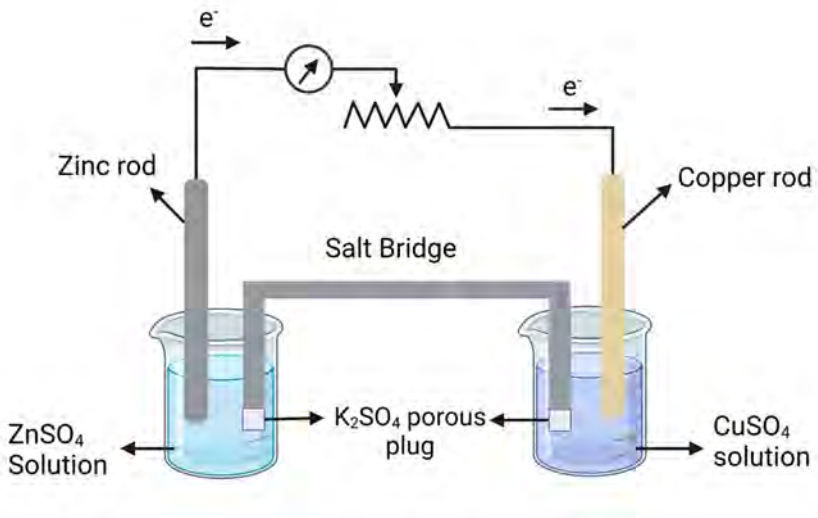

Fig. (17.1). Zn-$CuSO_4$ reaction in an electrochemical cell.

Procedure

1. Obtain a piece of filter paper and cut it into strips to use as salt bridges.
2. Add 1-2 mL of each metal nitrate $Cu(NO_3)_2$, and $Zn(NO_3)_2$ solutions to a different well in a well plate.
3. Add several drops of 1 M $NaNO_3$ to each filter paper strip. Place one end in the well containing the copper solution and the other end in the well with the zinc solution.
4. Clip metal copper into one alligator clip and zinc in the other. Place the metals in their respective solutions. If the voltage displayed in the meter is negative, then reverse the leads.
5. With a positive voltage displayed, wait about five seconds to take a voltage reading, and record the value. Be sure that you correctly label your data as you collect it.

Results

Theoretical values for these cells are:

[Zn(s) | Zn_2+(aq)] and [Cu_2+(aq) | Cu(s)] = 1.10 V

[Zn(s) | Zn_2+(aq)] and [Fe_2+(aq) | Fe(s)] = 0.32 V

[Fe(s) | Fe_2+(aq)] and [Cu_2+(aq) | Cu(s)] = 0.78 V

[Zn(s) | Zn_2+(aq)] and [Ag+(aq) | Ag(s)] = 1.56 V*

CHAPTER 18

Significant Experimental Hazard: Safety Data Sheet (SDS)

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Abstract: A safety data sheet or SDS is a datasheet prepared by the manufacturer to illuminate the user about any potential hazard and preserved by the owner. SDS also provides information about the handling and working of the product. SDS is also known as a material safety data sheet (MSDS) or product safety data sheet (PSDS). It must include information about the physical, environmental health, and health hazards; safety precautions, and protective measurements; transportation and storing. Guidelines for the preparation of such SDS are laid down by the occupational safety and health administration (OSHA). This chapter briefly explains the SDS of the significant experimental hazards of those chemicals used in the previous chapters.

Keywords: Hazard, MSDS, OSHA, PSDS, SDS, Safety.

INTRODUCTION

SDS is a document that provides guidelines and information for working with a certain chemical and about storage and transportation. In 2012, the United States made the safety data sheet in a consistent manner, which is globally accepted. The SDS cannot be presumed to be the same for an identical chemical name, as they have different formulations based on the country of origin. An SDS is written in English and contains various sections (s). The section(s) are discussed briefly below [1 - 3]:

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Section 1

Section 1 is about "identification" and provides information about the product identifier, including synonyms, recommended usage, and supplier information. Also, any special recommendations are provided by the supplier.

Section 2

Section 2 is about "Hazards identification". It identifies potential chemical hazards, warnings, and descriptions of any hazards that are not classified. In case of an unknown mixture of ingredients, acute toxicity must be mentioned.

Section 3

This section identifies "Composition or information on ingredients". Section 3 deals with ingredients details, including impurities and stabilizing additives. If a trade secret requires a specific chemical identity, and percentage can be withheld.

Section 4

Section 4 or "First-Aid measures" deals with primary care that needs to be taken for the individual who has been compromised to the chemical.

Section 5

Section 5 or "Fire-Fighting measures" gives recommendations in case of fire caused by the chemical, advice on hazards caused by chemicals during fire and precautions.

Section 6

Section 6 or "Accidental release measures" provides recommendations for any accidental release of the chemical. Also, advice about containment, cleanup, and exposure prevention to people, and the environment; from small spills to large.

Section 7

Section 7 or "Handling and storage" deals with safe handling and safe storage of the chemical.

Section 8

Section 8 or "Exposure control or personal protection" provides information on exposure limits, control, and personal protective measures.

Section 9

Section 9 or "Physical and chemical properties" inks different physical and chemical properties linked to the chemical or mixer.

Section 10

Section 10 or "Stability and reactivity" provides information on reactivity (description of specific test data on that chemical), chemical stability (on the stability of that chemical, description of stabilizer or any change in physical appearance), and others (indication of any possible hazardous reaction, conditions to be avoided, incompatible materials, and any anticipated hazard).

Section 11

Section 11 or "Toxicological information" inks toxicological data that may arise due to exposure, or routes of exposures, LD50, symptoms, *etc*.

Section 12

Section 12 or "Ecological information" informs about the environmental impacts of these chemicals if released into the environment. Data on aquatic and terrestrial organisms, biodegradation, bioaccumulation potential, groundwater contamination, and any other adverse effect.

Section 13

Section 13 or "Disposal consideration" guides on disposal mechanism, recycling of the chemical or its container and handling. This section also advises referring to section 8.

Section 14

Section 14 or "Transport information" guides on the safe transportation of the chemical.

Section 15

Section 15 or "REgulatory information" indicates any safety, health, and environmental regulations or guidelines to be followed which is not mentioned earlier in the SDS document.

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Pamela Jha

Dr. Pamela Jha, currently an Assistant Professor at SVKM's NMIMS Deemed to be University in Mumbai, brings extensive expertise in Biological Sciences, bolstered by post-doctoral fellowships from prestigious institutes. She has authored numerous publications, including research papers, review articles, and book chapters, and holds several granted patents. Her research focuses on biochemistry, industrial biotechnology, and toxicity, and has garnered significant recognition, reflected in a strong h-index, a substantial i10 index, and a high number of citations. Dr. Jha is an active member of multiple scientific societies and has presented her work at numerous national and international conferences. She is also deeply involved in academic governance, serving as a member of the Board of Studies and representing the Innovation Council.