# PURIFICATION AND CHARACTERIZATIN OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM JAPANESE QUAIL ERYTHROCYTES

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**Master thesis**

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**2017**

**T.C.**
**BİNGÖL ÜNİVERSİTESİ**
**FEN BİLİMLERİ ENSTİTÜSÜ**

# GLUKOZ 6-FOSFAT DEHİDROGENAZ ENZİMİNİN JAPON BILDIRCIN (*Coturnix Coturnix Japonica*) ERİTROSİTLERİNDEN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

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**Ocak 2017**

REPUBLIC OF TURKEY
BINGOL UNIVERSITY
INSTITUTE OF SCIENCE

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**January 2017**

## ACKNOWLDGEMENT

This master study has been completed in the Bingol university Chemistry Department laboratories. All thanks and appreciatings are going to the Bingol University for providing necessary chemicals and equipments. Also I would like to thank Bingol University Research Project Department (BÜBAP) for sponsoring my study project number FEF.3.16.004.

First of all, all glory and thanks for Allah for helping in completing this master study. It is my pleasure to thank my valued supervisor Prof. Dr. Mehmet ÇIFTCI for his continuous encouragements and his guidance through all step of writing this thesis, without his recommendations, this thesis would not been made. My sincere gratitude's are for assistant Prof. Dr. Yusuf TEMEL for his insightful suggestion and clearing the way in front of me.

It is worth to mention the help and support from the director of the Institute of science Prof. Dr. Ibrahim ERDOGAN for managing the administration affairs of my study. My thanks are going to Dr. Adnan AYNA for making my thesis even better and clearer. Also I would like to thank Mr. Yusuf Karagözoğlu for helping me in my laboratory works.

I am deeply indebted to my family for them constant understanding and encouragement. I could never have completed my study without them.

My hearty thanks are going to my fellow lab mates (Sarkat and Hardi) who were with me through all good and hard times. Last but not least, I would like to deliver my deep gratitude to all of my friends who supported me even by a word.

**Ibrahim Hamdi Shafeeq**

**Bingol 2017**

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# ABBRIVIATIONS

| | |
|---|---|
| E-S | : Enzyme substrate complex |
| EC | : Enzyme Commission number |
| E-P | : Enzyme product complex |
| S | : Substrate |
| P | : Product |
| mmol | : Millimole |
| EU | : Enzyme unit |
| mg | : Milligram |
| g | : Gram |
| Da | : Dalton |
| kDa | : Kilo dalton |
| BSA | : Bovine serum albumin |
| CM | : Carboxymethyl |
| DNA | : Deoxyribonucleic acid |
| D-Isomers | : Dexo isomer |
| DEAE | : Diethylaminoethyl |
| $K_M$ | : Michaelis-Menten constant |
| $V_{max}$ | : Maximum velocity |
| rpm | : Round per minute |
| PPP | : Pentose phosphate pathway |
| G6P | : Glucose-6 phosphate |
| G6PD | : Glucose 6- phosphate dehydrogenase |
| $NAD^+$ | : Nicotinamide adenine dinucleotide oxidized form |
| $NADP^+$ | : Nicotinamide adenine dinucleotide phosphate oxidized form |
| NADPH | : Nicotinamide adenine dinucleotide phosphate reduced form |
| 6PGA | : 6-phosphogluconate |

| | |
|---|---|
| 6PGD | : 6-phosphogluconate dehydrogenase |
| PER | : Ammonium per sulfate |
| SDS | : Sodium dodecyl sulfate |
| PAGE | : Polyacrylamide gel electrophoresis |
| TEMED | : Tetramethylethylenediamine |
| TCA | : Trichloroacetic acid |
| GSH | : Glutathione (reduced) |
| RNA | : Ribonucleic acid |
| Ru-5-Phos | : Ribulose 5-phosphate |
| D.W | : Distilled water |
| Tris | : Tris (hydroxyl methyl) aminomethane |
| $R_f$ | : Retardation factor |
| M.W. | : Molecular weight |
| Log | : Logarithm |

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# GLUKOZ 6-FOSFAT DEHİDROGENAZ ENZİMİNİN JAPON BILDIRCIN (Coturnix Coturnix Japonica) ERİTROSİTLERİNDEN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

## ÖZET

Yüksek lisans tezi olarak sunulan bu çalışmada karbohidrat metabolizmasının önemli bir enzimi olan glukoz 6-fosfat dehidrogenaz (G6PD; EC 1.1.1.49) enzimi bıldırcın eritrosit dokularından 60,40 EU/mg spesifik aktiviteyle ve %77,17 verimle 2', 5'-ADP Sepharose-4B afinite kromatografisi kullanılarak saflaştırıldı. Bıldırcın eritrosit G6PD enzimi için saflaştırma katsayısı 4137 olarak bulundu. Enzim için yapılan karakterizasyon çalışmalarında; stabil pH Tris-HCl tamponu pH 7,5, optimum pH Tris-HCl tamponu pH 8,0, optimum sıcaklık 65 $^{o}$C, optimum iyonik şiddet 1 M Tris-HCl tamponu olarak bulundu. Enzimin saflığını kontrol etmek ve alt birim molekül kütlelerini tespit etmek amacıyla SDS-poliakrilamid jel elektroforezi yapıldı ve tek bant gözlendi. Bıldırcın eritrosit G6PD enziminin alt birim molekül kütlesi yaklaşık 78,8 kDa olarak hesaplandı. Son olarak enzimin $K_M$ ve Vmax değerleri sırasıyla $NADP^+$ koenzimi için 0,001 mM ve 0,124 EU/mL, G6P substratı için 0,012 mM ve 0,05 EU/mL olarak bulundu.

**Anahtar Kelimeler:** Bıldırcın, G6PD, eritrosit, saflaştırma, karakterizasyon.

# PURIFICATION AND CHARACTERIZATION OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM JAPANESE QUAIL *(Coturnix coturnix Japonica)* ERYTHROCYTES


## ABSTRACT

In this master's thesis study, glucose 6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) which is an importance enzyme for the carbohydrate metabolism, was purified from quail's erythrocyte and characterized. The purification was performed by preparation of hemolysate and 2', 5'-ADP Sepharose-4B affinity chromatography. G6PD from quail's erythrocyte was obtained with a yield of 77.17% having a specific activity of 60.40 EU/mg. protein. The overall purification fold was around 4137. The characterization studies were showed: the stable pH value of enzyme to be 7.5 in Tris-HCl buffer, optimum pH value to be 8.0 in Tris-HCl buffer. The optimum temperature was found at 65$^{o}$C and the optimum ionic strength at 1 M Tris-HCl. Molecular weight of G6PD enzyme from quail's erythrocyte was determined as 78.8 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Finally, the $K_M$ and $V_{max}$ values for substrate (G6P) and coenzyme ($NADP^+$) of the G6PD enzyme were calculated. $K_M$ and $V_{max}$ values for the $NADP^+$ coenzyme found as 0.001 mM and 0.124 EU/mL respectively, and the $K_M$ and $V_{max}$ values for G6P substrate found as 0.012 mM and 0.05 EU/mL respectively.



**Key words:** Quail, G6PD, erythrocytes, purification, characterization.

# 1. INTRODUCTION

## 1.1. Enzymes

Enzymes are biological catalysts that accelerate the biochemical reaction and are responsible for bringing all of the chemical reactions in living organisms. Without them, these reactions take place at a rate far too slow for the pace of metabolism. The majority of these biochemical reactions do not occur spontaneously. The phenomenon of catalysis makes biochemical reactions necessary for all life processes (Campbell and Farrell. 2012).

All biochemical reactions within human cells are depending on enzymes that needed for metabolic pathways in the body. The living cell is the site of huge biochemical activity called metabolism. This is the process of physical and chemical change which goes on continually in the living organism. The metabolism process includes energy production through nutrient, building of new tissue, tissue growth, replacement of old tissue, disposal of waste materials and reproduction. These building ups and tearing downs are take place in the face of an apparent paradox (Campbell and Farrell 2012).

### 1.1.1. Chemical Nature Of Enzymes

All enzymes are globular proteins except a small group of catalytic RNA molecules, they made up principally of chains of amino acids linked together by peptide bond (Figure 1.1). Enzymes could be precipitated and denatured with solvents, salts and other reagents. They have high molecular weights ranging from about 12000 to more than 1 million (Nelson and cox 2008).

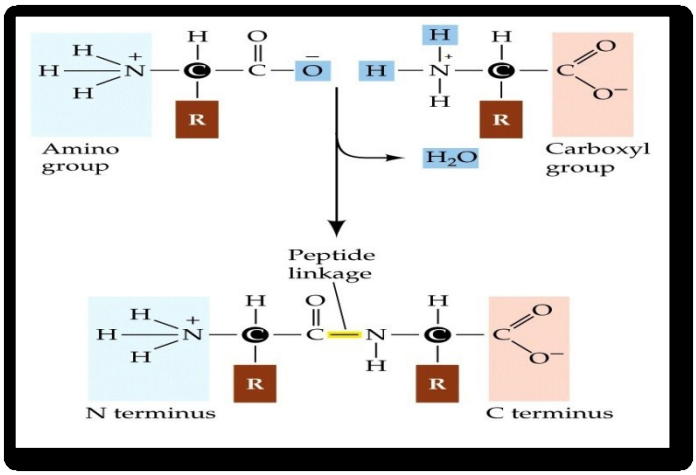



Figure 1.1. Typical protein structure – two amino acids joined by a peptide bond

Enzymes have a complex tertiary and quaternary structure in which polypeptides are folded around each other to form a roughly spherical or globular shape. Enzyme shape is maintained by hydrogen bonds and ionic forces. The three dimensional shape of an enzyme molecule is very important in which altered enzyme structures, cannot bind to their substrate and their catalytic activity would lost (Boisseau et al. 2010).

Many enzymes before their catalytic activity require the presence of other compounds called cofactors to do their functions. The whole activated complex is referred as the holoenzyme. The polypeptide portion of the enzyme is called an apoenzyme. apoenzyme (protein portion) plus the cofactor is called the holoenzyme (Nelson and cox 2008).

The cofactor may be:

- Coenzyme: nonprotein organic substance which is dialyzable, thermostable and loosely attached to the protein part.
- Prosthetic group: an organic substance which is dialyzable and thermostable which is firmly attached to the protein or apoenzyme portion.
- Metal ion: activator these include $K^{+}$, $Fe^{2+}$, $Fe^{3+}$, $Zn^{2+}$, $Mn^{2+}$, $Mg^{+2}$, $Cu^{2+}$ , $Co^{2+}$ , $Ca^{2+}$ and $Mo^{3+}$.

### 1.1.2. Naming And Classification

At the first times, the naming of enzymes was in a non-systematic way. For example, names like trypsin, pepsin contain no information on the enzyme function or the substrate on which the enzyme is catalysing. Later the naming of enzymes included adding the "ase" suffix to the substrate in which the enzyme acts on. For example the enzymes lactase acts upon the lactate and produces galactose and glucose. These method is known as "trivial naming" of enzymes. Presently enzymes are classified into six functional classes by the International Union of Biochemistry and Molecular Biology (IUBMB). As per the IUBMB system, each enzyme name starts with EC (enzyme commission number) followed by four digits. The first number represents the class, the second number strands for the subclass, the third number represents the sub-subclass or subgroup and the fourth number provides the particular enzyme (Fursule et al. 2009).

**The Six Classes Are Distinguished In The Following Manner:**

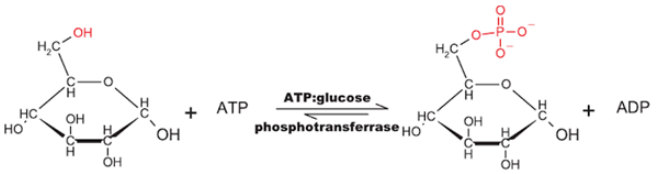
1. Oxidoreductases

This class encompasses all enzymes that catalyze redox reactions. The systematic name is formed according to donor: acceptor oxidoreductase.


ethanol ethanal (acrtaldehyde)

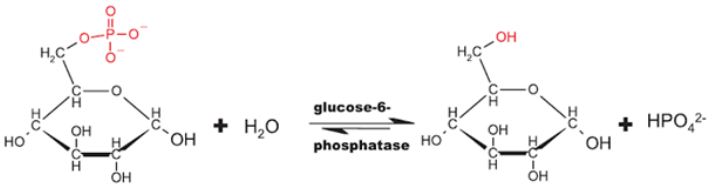
2. Transferases

Transferases catalyze the transfer of a chemical group from one molecule to another like (aldehyde, ketone, one carbon groups, acyl, alkyl, glycosyl, amino, sulphur or phosphorus containing groups). These chemical groups are not present in the free state during the transfer, e.g. Transaminases transferamino group, transmethylases transfer methyl group and transaldolases transfer aldehyde group.



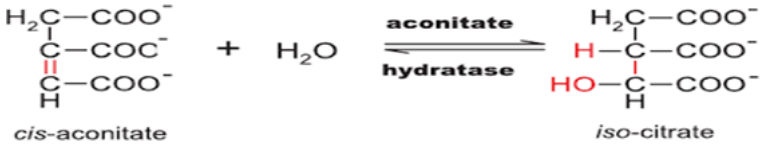

3. Hydrolases

This large group of enzymes brings about hydrolysis of various compounds. These reactions are enhanced in presence of water molecule, as one of the reactants. Since most of the hydrolytic reactions are reversible, they may also be called as synthetic or condensation enzymes, for example, peptidases, glucose -6phosphatase.



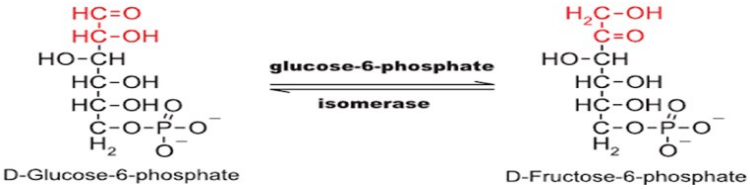

4. Lyases

Enzyme of this group recessively catalyze the removal of groups form their substrate by mechanisms other than hydrolysis (non- hydrolytical) and a double bond is introduced at the place of removal of the group. Lyasas act on C-C, C-O, C-N, C-S, and C-halide bonds. In most of the cases they require coenzyme. Examples are: fumarase, carboxylase, aconitate hydratase, ete.



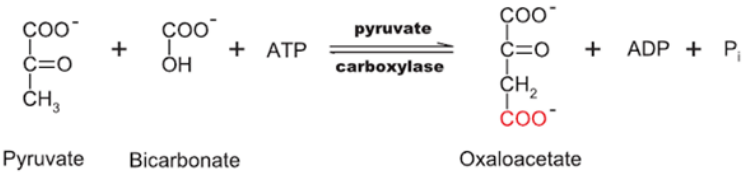

5. Isomerases

This class includes those enzyme which catalyze that reactions where intramolecular rearrangement take place in the substrate and optical, geometric or isomers are formed.




6. Ligases

Ligases catalyze the linkage of two molecules coupled with the cleavage of a pyrophosphate bond in Adenosine triphosphate (ATP) or similar compounds. Ligases may form C-O, C-S, C-N, or C-C bonds, e.g. Pyruvate carboxylase, glutamine synthetize, acetyl CoA carboxylase etc (Buxbaum 2015).




### 1.1.3. Specificity Of Enzymes

The enzyme characteristic that enables it to be used as a diagnostic tool for diseases or special reactions is the specificity of the enzyme to choose and select its substrate. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group (Khanna 2010). In general, there are four distinct types of specificity (Khanna 2010).

1. Absolute specificity: The enzyme will catalyze only one reaction.
2. Group specificity: The enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
3. Linkage specificity: The enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
4. Stereochemical specificity: The enzyme catalyse specific stereochemical groups in its reactions.

### 1.1.4. How Enzymes Work

Enzymes accelerate chemical reactions in the cell by reducing the activation energy of the reactions they catalyse (Figure 1.2). A special area on the enzymes structure, called the active site has a shape that fits with specific substrate molecules. An enzyme acts by binding to one or more specific molecules called substrates or reactants. The bindings occur at the active site. The enzyme and substrate form an enzyme-substrate complex. The interactions between enzymes and substrates, stresses or weakens some of the chemical bonds in the substrates. These stresses encourage connect between the two substrates leading to the formation of a different molecular. The result of the chemical interaction within the active site is a new product. While the product is released from the active site, the enzyme takes back its own original shape and would be free to work again. Although some of the enzyme reactions have specifically illustrated the formation of a single product from two substrate molecules, other enzymes catalyse the formation of two products from a single substrate (Campbell and Farrell 2012).

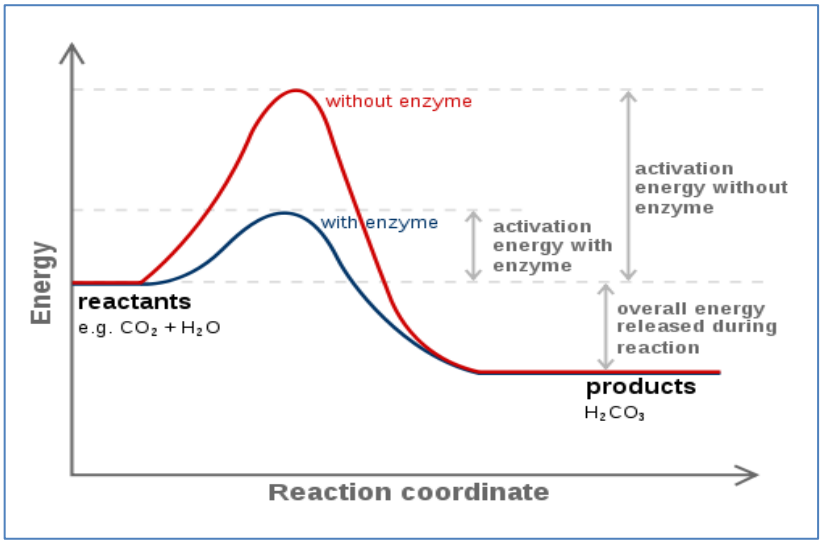



Figure 1.2. The lowering of the activation energy by an enzyme (Wikimedia 2008)

There are two main theories explaining how enzyme substrate complexes form: the lock-key theory and the induced-fit theory.

**The Lock- Key Theory**

The lock key hypothesis describes the catalytic functions of the enzymes that catalyse only one substrate. The method firstly postulated by Emil Fischer in 1894, he postulated the lock as the enzyme and the substrate as the key for the enzyme catalytic reaction and he referred his suggested method to the specificity and selectivity features of enzymes. In this analogy, the lock is the enzyme and the key is the substrate. Only the correct sized key (substrate) fit into the lock (enzyme) (Khanna 2010).

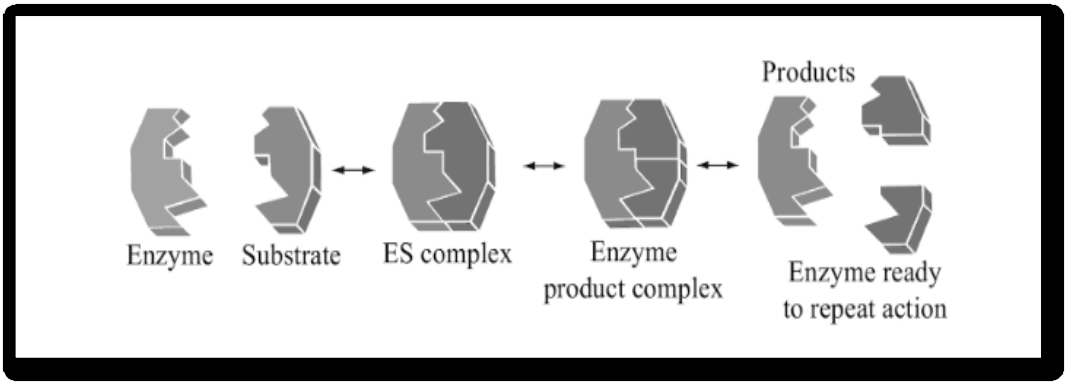



Figure 1.3. The lock-key theory (Khanna 2010)

**The Induced-Fit Theory**

In 1958 Daniel Koshland suggested a modification to the lock and key model. Enzymes have flexible structures; the active site of the enzyme can modify itself while interacting with substrates. The active sites amino acid side chains are melded into precise shapes to their substrates in which interact with the substrates and perform their enzyme catalytic activities. In some cases, the substrate molecule changes its shape slightly while it enters the active site of the enzyme (Khanna 2010).

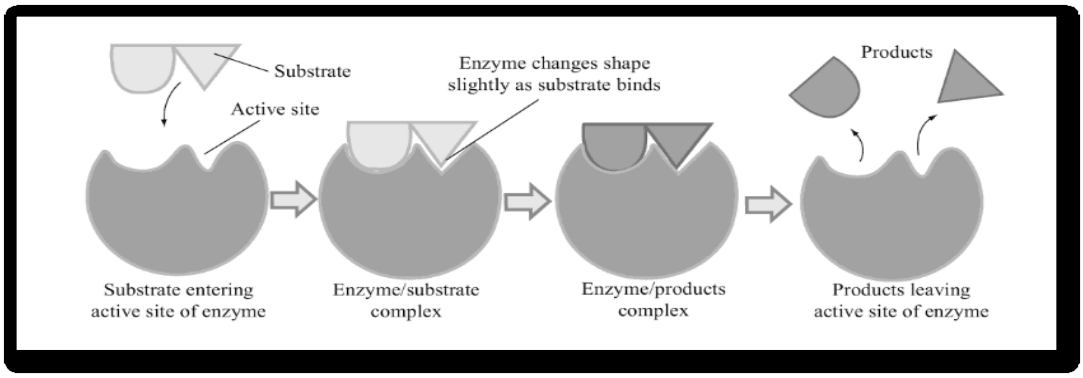



Figure 1.4. Induced-fit theory (Khanna 2010)

### 1.1.5. Chemical Equilibrium

Most of chemical reactions does not completely happen due to the reversibility of their reactions, this is also happens to the enzyme catalytic reactions. As the enzyme catalytic reactions mostly are reversible. The study of a large number of chemical reactions reveals that most do not go to true completion. This is also applies of enzymatically-catalysed reactions, because most enzyme reactions occur reversibly (Coursehero.com. 2016).

$$A + B \xrightarrow{k_1} C + D \qquad \text{forward reaction}$$

$$C + D \xrightarrow{k_2} A + B \qquad \text{reverse reaction}$$

Where $k_1$ is the forward reaction rate constant and $k_2$ is the rate constant for the reverse reaction.

Collecting the two reactions give:

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C + D$$

By applying this general equation to the enzymatic reactions, allows the enzyme reactions equilibrium.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} P + E$$

A steady state condition is reached when the forward reaction rates equals the backward rates. This is the basic equation upon which most of enzyme activity studies are based (Coursehero.com. 2016).

### 1.1.6. Factors Affecting Enzyme Activity

**Temperature**

Generally in chemical reactions an increase in temperature causes an increase in reaction rate. As the temperature of reactants increases, the particles move faster and are more likely to collide with sufficient energy to beat the activation energy of the substances reacting. Each enzyme has an optimum temperature in which at this point the maximum activity of the enzyme is achieved. Increasing the temperature above the optimum temperature leads to the break-down of hydrogen bonds that keep the shape of the active site. This minimizes the efficiency of the enzyme and the reaction rate falls. If the temperature decreased again the hydrogen bonds reform and the activity of the enzyme is restored. But if the temperature continues to rise the covalent bonds and disulfide bonds are break. This destroys the shape of the active site and the catalytic activity of the enzyme will lost permanently. Increasing in temperature permanently denatures the enzyme (Bettelheim et al. 2004).

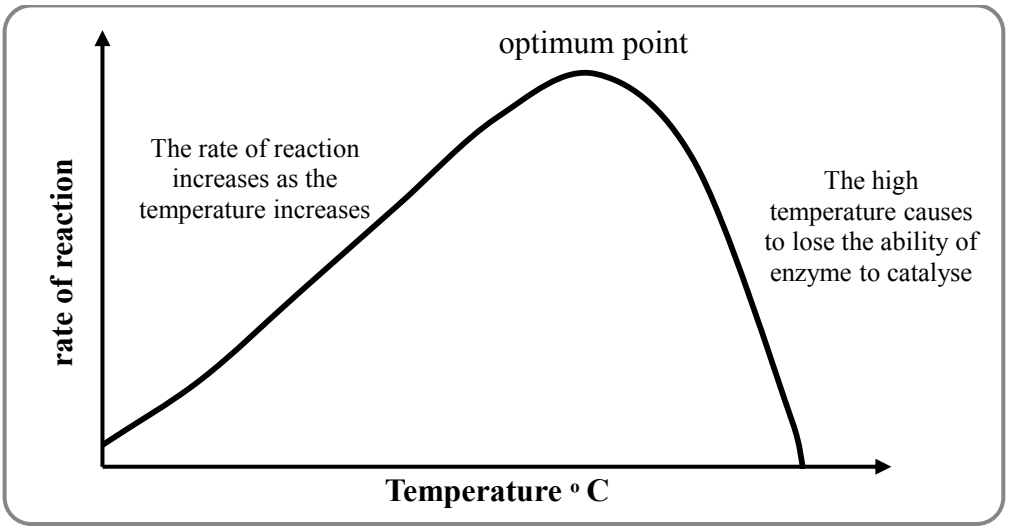



Figure 1.5. Effect of temperature on the rate of an enzyme controlled reaction

**pH**

The pH of the medium has a major effect on the rate of enzyme controlled reactions. The intermolecular bonds specially the hydrogen bonds that preserve the tertiary structure and the active site are very vulnerable to changes in hydrogen ion concentration. Every enzyme has an optimum pH at which it works at its optimum rate. A change in pH leads to change in the shape of the active site. It is not easy for the substrate to fit in and bind to the active site. The rate of reaction decreased due to the reducing of the activity of the enzyme. The substrate will not be able to bind to the active site at all if the change is too great and the enzyme can no longer catalyse the reaction. Generality the enzyme activity is restored as the pH returns to its optimum level (Satyanarayana and Chakrapani 2007).

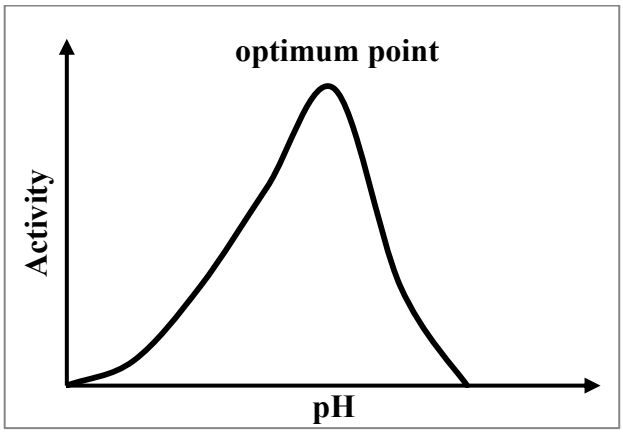



Figure 1.6. Effect of pH on the rate of an enzyme controlled reaction

**Effects of substrate concentration**

When the amount of enzyme is fixed at a constant level and substrate is slowly added, the reaction rate increases in proportion to the amount of substrate that is added this is due to the limited number of substrate molecules collide with enzyme molecules, and therefore the active sites of the enzymes are not working to full capacity. When the amount of substrate increased, the active sites gradually become fully utilised, until the point where all of them are working as fast as they can. The rate of reaction is at its maximum ($V_{max}$). After that, more addition of substrate will have no effect on the rate of reaction. In other words, when the substrate is in excess the rate of reaction levels off, a summary of the

effect of substrate concentration on the rate of enzyme action is given in figure 1.7 (Toole and Toole 2004).

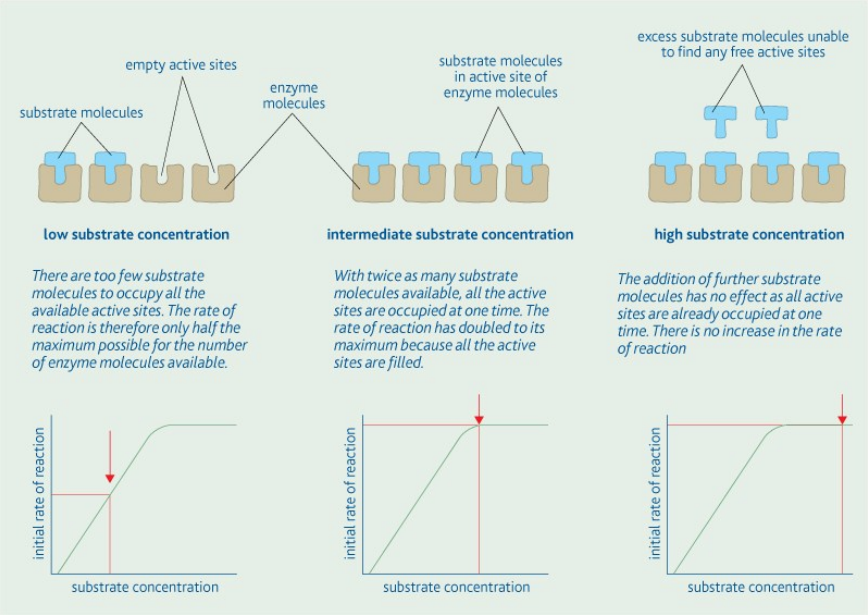



Figure 1.7. Effects of substrate concentration on the rate of enzyme action (Toole and Toole. 2004)

## Effect of enzyme concentration

If we increase the concentration of enzyme and keep the concentration of substrate constant, the rate increases linearly (Figure 1.8). This is the situation in practically all enzyme reactions, because the molar concentration of substrate is almost always much higher than that of enzyme (Bettelheim et al. 2004).

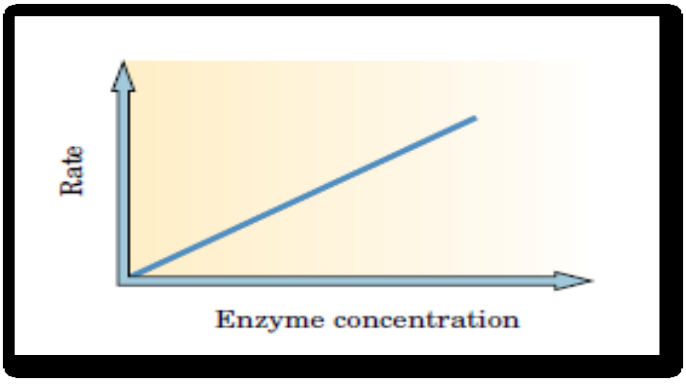



Figure 1.8. Effect of enzyme concentration on the rat of reaction

**Effect of inhibitors**

The formation of a normal enzyme-substrate complex may be inhibited by substances which prevent the catalytic enzymatic reaction, these substance known inhibitors. Some inhibitor to bind with the active site of enzyme competes with the substrate, these called competitive inhibition. Another types of the inhibitors, which bind to the enzyme other than the active sites and prevent the formation of enzyme substrate complex, these types of inhibitors known non-competitive inhibition (Segel 1968).

## 1.2. Pentose Phosphate Pathway

The pentose phosphate pathway (also known as phosphogluconate pathway or hexose monophosphate shunt, PPP) provides an alternative pathway for glucose oxidation. The pentose phosphate pathway was one of the first metabolic pathways which discovered after glycolysis (Embden–Meyerhof–Parnas pathway) and the tricarboxylic acid (Krebs) cycle (Stincone et al. 2015). In most tissues the oxidation of glucose about 80%–90% occur via glycolysis, and the remaining 10–20% occurs via the PPP (Wamelink et al. 2008). The pathway does not require oxygen, and does not generate ATP. The process occurs in the cytosol in most organisms, but in plants most of the process takes place in plastids. PPP has two major functions: one is production of NADPH, which is used as a reducing agent in many biosynthetic pathways (Synthesis of fatty acids, cholesterol, steroid hormones, sphingosine and some amino acid) and it is also important for protection against oxidative damage (Lagunas and Gancedo 1973; Bruinenberg et al. 1983; Thomas et al. 1991; Nogae and Johnson 1990); and the other function is synthesis of ribose 5- phosphate. Ribose and its metabolic product deoxyribose represent the sugar components of DNA and RNA, respectively, and therefore play key roles in dividing cells. Ribose is also a component of numerous other cellular intermediates including AMP, ADP, ATP, cAMP, coenzyme A, FAD, $NADP^+$ and NADPH (Cuperlovic-Culf 2013). PPP is the only way of ribose 5-P production in the body due to absence of ribokinase enzyme.

The pathway can be divided into two branches, the oxidative branch and the non-oxidative branch. The oxidative branch consists of three irreversible reactions. The

reactions in the non-oxidative branch are reversible. (See Figue 1.9 for an overview of the pathway, and Table 1.1 for its enzymes).

Table 1.1. Enzymes of the cytosolic pentose phosphate pathway. PPP enzymes

| **Enzyme** | **Abbreviation** | **EC number** |
|---|---|---|
| Glucose 6-phosphate dehydrogenase | G6PD | EC1.1.1.49 |
| 6-Phosphogluconolactonase | 6PGL | EC 3.1.1.31 |
| 6-Phosphogluconate dehydrogenase | 6PGDH | EC 1.1.1.44 |
| Ribose 5-phosphate isomerase | RPI | EC 5.3.1.6 |
| Ribulose 5-phosphate epimerase | RPE | EC 5.1.3.1 |
| Transketolase | TKL | EC 2.2.1.1 |
| Transaldolase | TAL | EC 2.2.1.2 |
| Sedoheptulokinase | SHPK | EC 2.7.1.14 |
| Sedoheptulose 1,7-bisphosphatase | SH17BPase | EC 3.1.3.37 |
| Sedoheptulose 7-phosphate isomerase | SHI | EC 5.3.1.28 |
| Glucose phosphate isomerase | GPI | EC 5.3.1.9 |
| Triosephosphate isomerase | TPI | EC 5.3.1.1 |
| Glyceraldehyde 3-phosphate dehydrogenase | GAPDH | EC 1.2.1.12 |

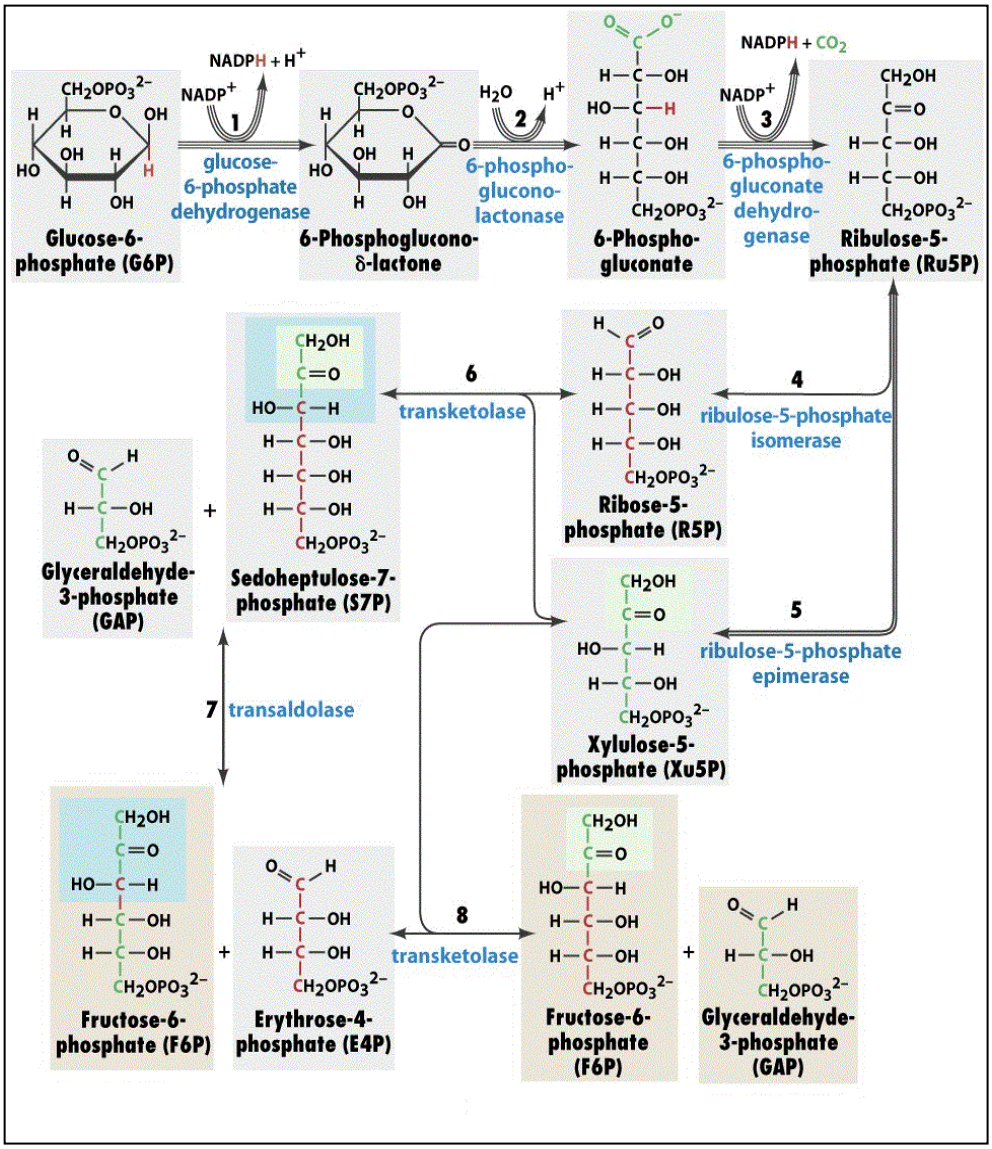



Figure 1.9. The Pentose phosphate pathway (BiochemDen 2015)

The oxidative phase of the pentose phosphate pathway starts with the dehydrogenation of glucose 6-phosphate at carbon 1, a reaction catalyzed by glucose 6-phosphate dehydrogenase with concomitant production of NADPH. The product 6-phosphoglucono-δ-lactone, which is an intramolecular ester between the C-1 carboxyl group and the C-5 hydroxyl group is rapidly and irreversibly hydrolysed by 6-phospho glucono lactonase to give 6-phosphogluconate. This six-carbon sugar is then oxidatively decarboxylated by 6 phosphogluconate dehydrogenase to produce ribulose 5-phosphate, $CO_2$ and NADPH (Wamelink et al. 2008).

The intermediates in the non-oxidative phase range from three-carbon to seven-carbon species. The ribulose 5-phosphate produced by the oxidative branch can be isomerized to ribose 5-phosphate by RPI, or epimerized to xylulose 5-phosphate by ribulose 5-phosphate epimerase. Ribose 5-phosphate and xylulose 5-phosphate are converted into GA 3-phosphate and sedoheptulose 7-phosphate by transketolase and further metabolized into erythrose 4-phosphate and fructose 6- phosphate by TAL. Finally, transketolase converts erythrose 4-phosphate and xylulose 5-phosphate into GA 3-phosphate and fructose 6-phosphate (Cohen. 2011).

**Regulation of PPP**.

The pentose phosphate pathway is controlled at the first step of the oxidative stage. Glucose-6-phosphate dehydrogenase is an allosteric enzyme. NADPH, $NADP^+$ and G6P regulate the oxidative phase of PPP by allosteric mechanism. When the cell has sufficient NADPH the pathway is inhibited, when the cell needs NADPH for biosynthesis it is stimulated. Regulation of the G6PD activity controls flux through the glycolytic pathway and pentose phosphate pathway (Voet and Voet 2011) (Figure 1.10).

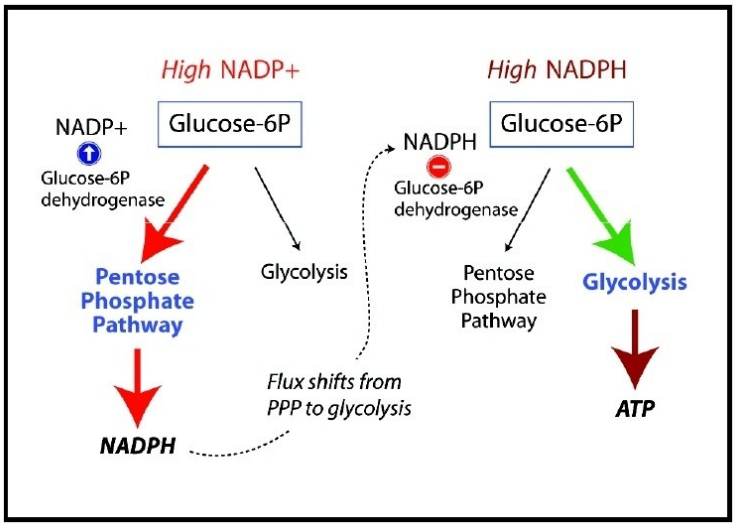



Figure 1.10. Regulation of PPP

## 1.3. Glucose 6-Phosphate Dehydrogenase

Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49, β-D-glucose 6-phosphate; NADP oxidoreductase) is the initial and the key regulatory enzyme in the pentose phosphate pathway in carbohydrate metabolism (Ulusu et al. 1999; Winiarska et al. 2003; Kaplan and Hammerman 2010; Del Campo and Patino 2011). G6PD is present in mammalian tissues, plant tissues and microorganisms (Gleason 1996; Kurutas and Tuncer 2000; Nemoto and Sasakuma 2000). The enzyme is located in cytosol and mitochondria in animal tissues, and in cytosol and chloroplasts in green plants (Schnarrenberger et al. 1973; Levy 1979; Ocheretina et al. 2000). It is a ubiquitous enzyme, so widely distributed among prokaryotes and eukaryotes that it is considered a housekeeping enzyme (Levy 1979). The first isolation of the enzyme was carried out from human erythrocytes by Yoshida and Huang (Scott and Tatum 1971). G6PD enzyme plays an important role in various cell functions; the principal role of this enzyme is to generate important reducing potential in the form of NADPH, which is required for the detoxification of reactive free oxygen radicals and hydroperoxides through two oxidative defence mechanisms involving catalase and glutathione peroxidase/reductase (Figure 1.11). NADPH is crucial for preserving and regenerating the reduced form of glutathione (GSH) (Scott et al. 1991), and it is also important in maintaining the catalytic activity of catalase (Kirkman et al. 1987; Gaetani et al. 1996).

Erythrocytes, which serve as oxygen carriers, are highly specialized. They are constantly under oxidative stress which can be produced from exposure to radiation, metals and redox-active drugs. However, in the metabolically restrictive erythrocytes, PPP is the only source of NADPH because the red blood cells lack some cellular organelles and other metabolic pathways to generate NADPH since G6PD catalyses the control step of PPP, the defence of the cells against oxidative damage depends ultimately and heavily on G6PD activity (Mason et al. 2007).

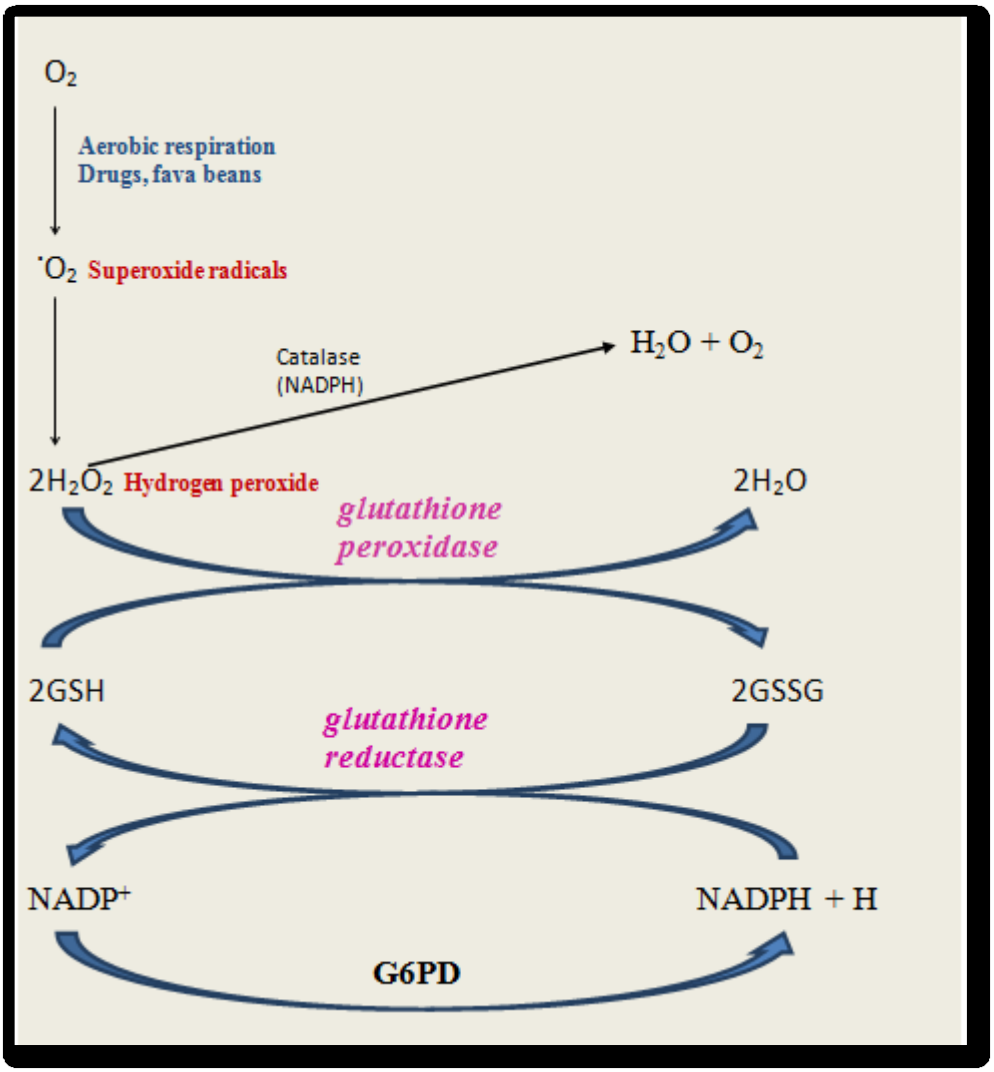



Figure 1.11. Two defence systems against oxidative stress

### 1.3.1. Catalytic Properties Of G6PD

Glucose 6-phosphate dehydrogenase is catalyses the oxidation of glucose 6-phosphate to 6-phosphogluconolactone with the concomitant reduction of $NADP^+$ to NADPH. Thermodynamically, this reaction is reversible (Horecker and Smyrniotis 1953; Beutler and Kuhl 1986), but in practice it is irreversible because the lactone ring hydrolyses quickly to 6-phosphogluconate. This reaction in vivo is further accelerated by the enzyme 6-phosphoglucono lactonase. G6PD can only utilize the β-anomer of glucose-6-phosphate rather than the open-chain form of the substrate (Levy 1979) since the β-anomer is thermodynamically stable. Alterations of the substrate at the anomeric carbon

atom lead to the loss of G6PD activity. Some G6PDs can utilize several phosphorylated and non-phosphorylated sugars at a lower rate (Levy 1979).

### 1.3.2. G6PD Deficiency

The most abundance enzymatic disorder of the red blood cells (RBC) s is the deficiency of G6PD enzyme (Glader 2008). In the G6PD deficiency, the oxidative stress in blood can denature the hemoglobin protein and causes the intravascular hemolysis. Some factors enhance oxidative stress such as drugs, infections, chemical agents, ingestion of fava beans, or ketoacidosis. The patients with deficient G6PD are unable to protect their RBCs due to the lack of the NADPH that produced during G6PD enzymatic catalytic reaction as the only source for the NADPH in RBCs is from the G6PD enzymatic catalytic reaction. The main cause to the G6PD enzyme deficiency is originated from a hereditary defect in the responsible gene for G6PD that is X-linked gene (Cappellini and Fiorelli 2008).

### 1.3.3. G6PD Structure

The subunit of the G6PD enzyme from human is consisted of 514 amino acid protein that encoded ta the Xq 28 allele (Camardella et al. 1995). The amino acid sequences of more than 100 G6PD enzymes from various sources were studied and their results showed big similarity in mostly all regions. Only three important regions in the enzyme structure were conserved regions (Kotaka et al. 2005).

The G6PD enzyme exists in various tissues from the organism in dimeric, tetrameric or hexameric form. However the active form of the enzyme is the dimeric structure enzyme. In the dimeric structure, each subunits of the enzyme has 59256 Da molecular weights (Levy 1979). The dimeric form or tetrameric form of the enzyme is affected by temperature, enzyme concentration and NADPH concentration. High pH, ionic strength, temperature, and high concentration of G6P favor the dimeric state, while low pH and ionic strength, and the presence of some divalent cations tend to favor tetramer formation. The physiological significance of the interconversion of these two oligomeric forms is still unclear (Cohen and Rosemeyer 1969b).

The difference between the human G6PD with the bacterial G6PD is the existence of a structural $NADP^+$ molecular in the humans G6PD that is absent in bacterial G6PD. This $NADP^+$ molecule in the structure of the human G6PD enzyme is very important for the stability of the enzyme. A second $NADP^+$ binding site is located at the expected position where it contacts the Gly41 and Asp42 residues in the dinucleotide binding fingerprint. The adenine of the structural NADP molecule lies between Tyr503 and Arg487 and the nicotinamide between Trp509 and Tyr401. The 2' phosphate makes hydrogen bonds to Arg487, Arg357, Lys238 and Lys366; the biphosphate interacts with Arg370 while Arg393 and Asp421 interact with the amide function of the nicotinamide. Asp421 is at the centre of the dimer interface (Wang et al. 2006) (Figure 1.12).

The G6PD enzyme structure had been investigated in (Leoconostoc mesenteroides) at a resolution of 2A°. The result of the study showed a non-equal dimeric structured enzyme with two domains for each subunit (Rowland et al. 1994).

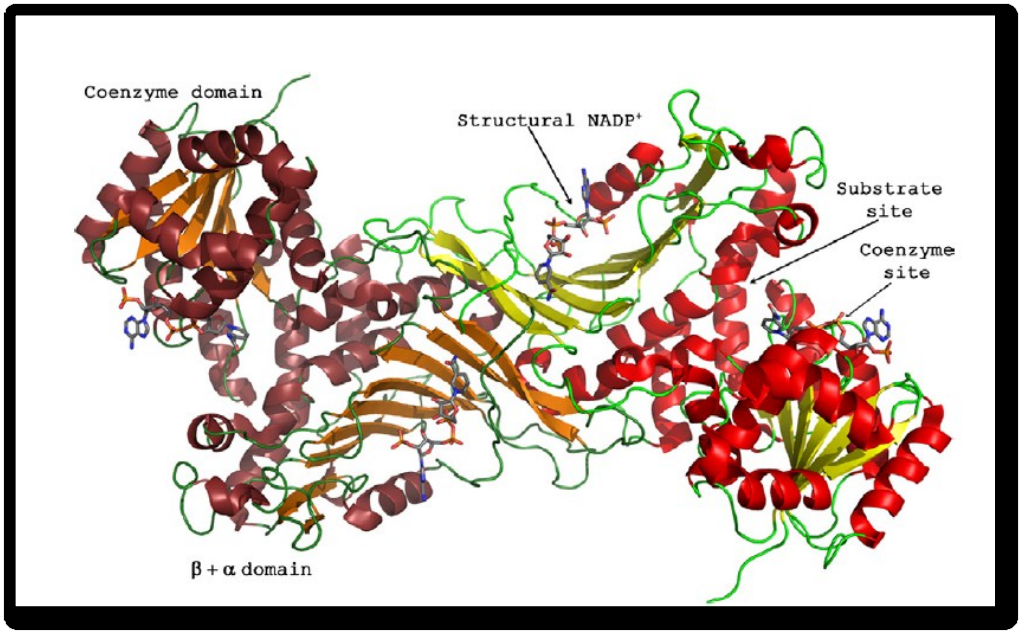



Figure 1.12. Dimer structure of the G6PD. One subunit is in red and green while the other is in shades of brown (Mason et al. 2007)

# 2. LITERATURE REVIEW

Glucose 6-phosphate dehydrogenase enzyme is the first and regulatory enzyme of pentose phosphate pathway it has been discovered at the RBC in 1931 by Warburg and Christian, at that time the enzyme was named “Zwischenferment” (Warburg 1931). The crystal form of the pure enzyme was obtained from Brewers' yeast for the first time, by Holtmann, Gubler and Kuby (Noltmann et al. 1961; Yue et al. 1969), later it was discovered in the red blood cells of other mammals (Hauschild et al. 2003).

The G6PD enzyme was purified and studied from widely different sources, including;

1. Human tissues, such as, human placenta (Kahler and Kirkman 1983, Aksoy 1992) from the human brain (Cho and Joshi 1990) and from human erythrocytes (Yoshida 1966; Ciftci et al. 2000; Buyukokuroglu et al. 2001; Ozmen et al. 2005).

2. Animal tissue, such as, such as, pig liver (Kanji et al. 1976), mouse liver (Velasco et al. 1994), rat kidney (Corpas 1995), pig small intestine (Tappia et al. 1998), bovine lens (Ulusu et al. 1999), rat brain ( Capellaci et al. 2001), rabbit small intestine (Ninfali et al. 2001), sheep Liver (Ciftci et al. 2002), dog liver (Ozer et al. 2002), sheep erythrocytes (Beydemir et al. 2002), turkey erythrocytes (Yilmaz et al. 2003), goose red blood cells (Beydemir et al. 2003), chicken liver (Erat 2005), sheep kidney cortex (Ulusu and Tandogan 2006), sheep brain cortex (Ulusu and Sengezer 2012), camel liver (Ibrahim et al. 2014) and rat lung (Adem and Ciftci. 2016).

3. Plants, such as, spinach (Schnarrenberg et al. 1973; Lendzian 1980), cotyledon (Gossling and Ross 1979), pea plant leaves (Fickenser and Schiebe 1986), maize leaves (Valenti 1984), wheat germ (Mirfakhra and Auleb 1989), soybean leaves

(Robinson 2000), potato (Wendt 2000), nicotiana tabacum (Knight 2001), barley leaves (Semenih 2001), parsley leaves (Coban et al. 2002), barley and barley roots (Esposito et al. 2003; Esposito et al. 2005), oilseed rape (Brassica napus L.) (Hutchings et al. 2005), and peach fruits (Wei-Fu et al. 2007).

4. Microorganism such as, enterobacteria (Bowman et al. 1967; Sanwal 1970), pseudomonas aeruginosa (Lessie and Neidhart 1967), pseudomonas multivorans (Wyk and Lessie 1974), drosophila melanogaster (Ganguly et al. 1985), acetobacter hansenii (Acetobacter xylinum) (Levy and Cook 1991), aspergillus niger and aspergillus nidulan (Wennekes et al. 1993), actinobacillus actinomycetemcomitans (Shah and Andrews 1994), arypanoma brucei (Heise and Opperdoes 1999), from aspergillus aculetaus (Ibraheem et al. 2005).

Various several methods was used for G6PD purification (Table 2.1), and some characterize on its properties were investigated (Table 2.2).

Table 2.1. Purification strategies of glucose-6-phosphate dehydrogenase from various sources

| Source | Purification method | Fold purification | % yield | References |
|---|---|---|---|---|
| Brewer's Yeast | Ammonium sulphate precipitation, calcium phosphate gel adsorption, ethyl alcohol fractionation, Alumina Cγ adsorption and chromatography on starch celite | 600-900 | 19% | Glaser and Brown (1955) |
| Human erythrocyte | DEAE Cellulose fractionation, Ammonium sulphate fractionation, Affinity and anion exchange chromatography | 47.2 | 35% | Chung and Langdon (1963) |
| *Neurospora crassa* | Ammonium sulphate precipitation, calcium phosphate gel adsorption, DEAE-Cellulose, hydroxylapatite and bio-gel A column chromatography | 2400 | 10% | Scott and Tatum (1971) |
| Pig Liver | Acid denaturation, Triton X-100 treatment, Sephadex G-200 and DEAE-cellulose ion exchange chromatography | 1130 | 40 | Kanji et al. (1976) |
| Dog liver | 2', 5'-ADP Sepharose-4B affinity gel chromatography followed by Sephadex G-100 SF gel filtration and rechromatography on 2', 5'-ADP Sepharose-4B | 2000 | 18% | Ozer et al.(2002) |
| *Bubalus bublis* (Buffalo) | 2', 5'-ADP Sepharose-4B affinity gel chromatography | 65 | 31% | Ciftci et al.(2003) |
| Goose erythrocyte | Ammonium sulphate precipitation and 2', 5'-ADP Sepharose-4B affinity gel chromatography | 3892 | 68.79% | Beydemir et al.(2003) |
| Rainbow trout | Ammonium sulphate precipitation and 2', 5'-ADP Sepharose-4B affinity gel chromatography | 1271.19 | 70.40% | Ciftci et al.(2004) |
| Goat Erythrocyte | DEAE-cellulose chromatography followed by CM-Sephadex column chromatography, gel filtration and ammonium sulphate precipitation | 45000 | 23% | Bayazit et al.(2005) |
| *Aspergillus aculeatus* | Ammonium sulphate fractionation, Rective Blue 2 Agarose affinity and Sephacryl S-200 Gel filtration chromatography | 229 | 9% | Ibraheem et al. (2005) |
| Lamb kidney cortex | 2', 5'-ADP Sepharose-4B affinity gel chromatography and DEAE Sepharose fast flow | 4202 | 26.4% | Ulusu et al.(2005); Ulusu and Tandogan (2006) |
| Taenia crassiceps | Salt fractionation, Ion exchange chromatography and affinity chromatography | 1417 | 32.3% | Rendon et al.(2008) |
| Polygonum cognatum | Ammonium sulphate fractionation and DEAE-Sephadex A50 ion exchange chromatography | 124.08 | 57.6% | Demir et al.(2009) |

Table 2.2. Some characteristic properties of G6PD from different sources

| Source | Molecular weight (kD) | Optimum temperature | Optimum pH | Stable pH | References |
|---|---|---|---|---|---|
| Pig liver | 67.5 | - | 8.5 | - | Kanji et al. (1976) |
| Bovine lens | 69.22±3.2 | 25 °C | 7.7 | - | Ulusu et al 1999 |
| Sheep Liver | 85 | 45-50 °C | 8.0 | - | Ciftci 2002 |
| Chicken erythrocytes | 73.177 | 60 °C | 9.0 in 1M Tris-HCl | 8.0 in Tris-HCl | Bakan et al.(2002) |
| Dog liver | 52.5 | | 7.8 | | Ozer et al 2002 |
| Turkey erythrocytes | 73 | 50 °C | 9.0 | 8.0 | Yilmaz et al 2003 |
| Buffalo (Bubalus bubalis) erythrocytes | 64.500 | 60 °C | 8.0 In Tris–HCl | 9.0 in 0.1M Tris–HCl | Beydemir et al 2003 |
| Goose Erythrocytes | 73.17 | 50 °C | 7.0 in Tris-HCl | 9.0 in Tris-HCl | Beydemir et al.(2003) |
| Rainbow trout erythrocytes | 64.26 | 45 °C | - | 8.9 | Ciftci et al.(2004) |
| Aspergillus aculeatus | 52.000 ± 1.100 | - | - | - | Ibraheem te al. 2005 |
| lamb kidney cortex | 67 | 45°C. | 7.7 in potassium phosphate buffer | - | Ulusu and Tandogan 2005 |
| sheep kidney cortex | - | 45 °C | 7.4 in potassium phosphate buffer | - | Ulusu and Tandogan 2006 |
| Camel Liver | 64 | - | 7.8 in Tris/HCI buffer | - | Ibrahim et al. 2014 |
| Pigeon Pea (*Cajanus cajan*) Seeds | 55 | 30 °C | 8.2 in Tris/HCI buffer | - | Singh1 and Srivastava 2014 |

# 3. MATERIAL AND METHODS

## 3.1. MATERIALS

### 3.1.1. Used Chemicals

Glucose 6 phosphate mono sodium salt (G6P), TEMED N,N,N,N tetra methylethyl diamine, trichloro acetic acid (TCA), bromothymol blue, glycin amino acid, potassium acetate, N,N,N,N methylene bis acrylamide, sodium dodecyl sulphate (SDS), serum albumin, ammonium persulphate, ethylene diamine tetra acetic acid (EDTA), tris trihydroxy methyl aminomethane, potassium chloride, potassium phosphate, isopropanol, beta mercapto ethanol, magnesium chloride, from sigma. Sodium bicarbonate, sodium hydroxide, ammonium sulphate, sodium chloride, sodium acetate, hydrochloric acid, phosphoric acid, glycerine, ethanol, methanol, acetic acid, from E-fluka company. 2', 5'-ADP Sepharose-4B, Commasie brilliant blue G-250 from Merck Company. β-nicotinamide adenine dinucleotide phosphate sodium salt from the Applichem.

### 3.1.2. Used Instruments

-Clinical centrifuge (Model Universal 320 R, Hettich) and (Allegra X-30R Beckman Coulter)
-pH meter: (Thermo Orion 3 stars)
-Vortex: (Lab Companion SK 300)
-Sensitive balance: (Denver instrument SI 234)
-Column chromatography: Sigma Aldrich
-Spectrophotometer: (SHIMADZU Model CC-10)
-Automatic pipette: (Transferpette, Eppendorf research)
-Electrophoresis tank: (Bio Rad Electrophoresis power supply Peristatic pump (Bio instruments ATTA SJ-1220) and (P Spectra J..P SELECTA, s.a.)

- Incubator
-Water bath
-Ice maker: (HOSHZAKI ICE MAKER FM-80EE)
-Ice bath

### 3.1.3. Preparation Of Solutions

#### 3.1.3.1. Hemolysate Preparation Solutions

1. 0.16 M KCl: 1.192 g KCl (0.016 mol) was dissolved in 50 mL of distilled water.
2. Ice-cold water.

#### 3.1.3.2. Activity Measurement Solutions:

1. 1 M Tris-HCl + 5 mM EDTA (pH= 8.0): 6.05 g (0.05 mol) Tris and 0.0605 g ($2.5x10^{-4}$ mol) EDTA was dissolved in 40 mL of distilled water. pH was adjusted to 8.0 with 0.1M HCl. Then the volume was completed to 50 mL with distilled water.
2. 0.1 M $MgCl_2$: 0.475 g (5 mmol) was dissolved in 50 mL of distilled water.
3. 6 mM G6P: 0.0091 g (0.03 mmol) G6P was dissolved in 5 mL of distilled water.
4. 2 mM $NADP^+$ solution: 0.00765 g (0.01 mmol) $NADP^+$ was dissolved in 5ml of distilled water.

#### 3.1.3.3. Affinity Column Chromatography Solutions

1. (0.1 M Potassium acetate / 0.1 M Potassium phosphate) pH = 6.0 (buffer that used for washing and equilibrating a column): 9.8 g of potassium acetate and 13.6 g of potassium phosphate was dissolved in 800 mL of distilled water. The pH was adjusted to 6.0. Then the volume was completed to 1L with distilled water.
2. 50 mM $KH_2PO_4$ + 1 mM EDTA + 1mM DTT, pH= 7.3 (buffer that used for washing affinity column after addition of the sample): 6.8 g $KH_2PO_4$, 0.292 g EDTA and 0.152 g DTT was dissolved in 950mL of distilled water. pH was adjusted to 7.3 with 0.1 M KOH. Then the volume was completed to 1L with distilled water.
3. 50 mM $KH_2PO_4$ + 80 mM KCI + 1 mM EDTA + 1mM DTT, pH= 7.3 ( buffer that used for washing the column after addition of the sample): 6.8 g $KH_2PO_4$, 0.292 g EDTA, 0.298 KCI and 0.152 g DTT was dissolved in 950 mL of distilled water. pH was

adjusted to 7.3 with 0.1 M KOH. Then the volume was completed to 1L with distilled water.

4. 80 mM $KH_2PO_4$ + 10 mM EDTA + 80 mM KCI + 0.5 mM $NADP^+$ (buffer that used for eluting glucose-6-phosphate dehydrogenase that attached to affinity gel): 0.544 g $KH_2PO_4$, 0.121g EDTA, 0.298 KCI and 0.01913 g $NADP^+$ was dissolved in 40 mL of distilled water. pH was adjusted to 7.8 with 0.1 M KOH. Then the volume was completed to 50 mL with distilled water.

5. 0.1 M Na-asetate + 0.5 M NaCl, pH= 4.5 (buffer that used for regeneration of the affinity column chromatography): 4.1 g Na-asetat (50 mmol) and 14.61 g NaCl (0.25 mol) was dissolved in 450 mL of distilled water. pH was adjusted to 4.5. Then the volume was completed to 500 mL with distilled water.

6. 0.1 M Tris + 0.5 M NaCl, pH= 8.5 (buffer that used for regeneration of the affinity column chromatography) 0.605 g Tris and 14.61 g NaCI was dissolved in 450 mL of distilled water. pH was adjusted to 8.5 with 0.1 M HCl. Then the volume was completed to 500 mL with distilled water.

#### 3.1.3.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Solution

1. 1 M Tris-HCl (pH= 8.8) separating gel buffer: 12.11 g Tris (0.1 mol) dissolved in 80 mL of distilled water, pH was adjusted to 8.8 with 0.1 M HCl. Then the volume was completed to 100 mL with distilled water.

2. 1 M Tris-HCl (pH= 6.8) Stacking gel buffer solution: 12.11 g Tris (0.1 mol) dissolved in 80 mL of distilled water, pH was adjusted to 6.8 with 0.1 M HCl. Then the volume was completed to 100 mL with distilled water.

3. Acrylamide/bis-acrylamide (30%/0.8% w/v) solution: 15 g acrylamide, 0.4 g bis-acrylamide was dissolved in 50 mL of distilled water.

4. 10% (w/v) ammonium persulfate solution: 1 g ammonium persulfate dissolved in 10 mL of distilled water.

5. 10% (w/v) SDS solution: 1 g SDS dissolved in 10 mL of distilled water.

6. Running buffer: (25 mM Tris; 0.2 M glycine; 10 % SDS) 1.51 g Tris base (12.5 mol) and 7.51 g glycine (0.1 mol) was dissolved in 450 mL $H_2O$. It was stirred until completely dissolved. Then 5 mL of 10% (w/v) SDS was added. Then pH was adjusted to 8.3 with 0.1 M HCl. Then the volume was completed to 500 mL with distilled water.

7. Sample buffer: 0.5 mL from 1M Tris-HCl(pH= 8), 1mL from 10% SDS, 1 mL from 100% glycerin and 1 mL from 0.1% bromothymol blue, are mixed then the volume was completed to 10 mL with DW, before using this solution every 950 µL of sample solution 50 µL of β-mercaptoethanol were added.
8. Fixation solution: 50% isopropanol, 10% TCA and 40% distilled water was mixed together.
9. Staining solution: 50 mL methanol, 10 mL acetic acid and 40 mL distilled water was mixed then 0.1 g Coomassie Brillant Blue R-250 was added to solution.
10. Destaining solution: 50 mL methanol, 10 mL acetic acid and 40 mL of distilled water were mixed.

#### 3.1.3.5. Solution Used In Bradford Method

1. Preparation of Coomassie reagent: 10.0 mg of CBB G-250 was dissolved in 5 mL of 95% ethanol and then 10.0 mL of 85% phosphoric acid was added. The mixture was adjusted to 100 mL with distilled pure water. The final solution was filtered through filter paper (Whatman No. 1) and was stored in a dark bottle at room temperature.
2. Preparation of Bovine serum albumen (BSA): 1 mg of BSA dissolved in 1 mL DW.

## 3.2. Methods

### 3.2.1. Determination Of Enzyme Activity

The G6PD enzyme activity assayed at room temperature as described in Beutler method (Beutler 1971). The assay mechanism depends on the reduction rates of $NADP^+$ to the NADPH by the G6PD catalytic activity in which the conversion rate interprets the amount of G6PD enzyme activity and the reduction rate is measured spectrophotometrically at 340 nm (Table 3.1).

$$\text{D-glucose 6-phosphate} + NADP^+ \xrightarrow{\text{G6PD}} \text{6-phospho gluconate} + NADPH + H^+$$

The enzyme assay was set as follows:

Table 3.1. Activity solution of G6PD enzyme

| Stock Solutions | Blank cuvette Volume (µL) | Sample cuvette Volume (µL) |
|---|---|---|
| 1 M Tris-HCl, pH 8.0 | 100 | 100 |
| 0.1 M $MgCl_2$ | 100 | 100 |
| 2 mM $NADP^+$ | 100 | 100 |
| $H_2O$ | 600 | 550 |
| Enzyme | - | 50 |
| Incubation for 5 min | | |
| 6 mM G6P | 100 | 100 |

The enzyme unit (EU) of G6PD is defined as the reduction of 1 µmol of $NADP^+$ into NADPH per one minute of time at 25°C. Since OD 340 of one µmol/mL of NADPH is 6.22 in a 1cm light path, the enzyme activity is calculated as:

$$\text{Activity (EU/mL)} = \frac{\text{(OD/min) x cuvette volume (µL)}}{\text{6.22 x enzyme volume (µL)}} \text{ x } f$$

When

OD: Optical density

*f*: dilution factor

### 3.2.2. Purification Of G6PD From Quail's Erythrocytes

#### 3.2.2.1. Preparation Of The Hemolysate

3 mL of quail's blood samples from Bingol's University Farm were collected in anticoagulant tubes then they centrifuged at (15 min, 4°C, 2,500 x g), after the plasma was removed, the packed red blood cells were washed with 0.16M cold KCl solution then centrifuged again at (15 min, 4°C, 2,500 x g) and the previous step was repeated for 3 times. The packed red blood cells were hemolyzed by adding ice-cold water as 1:5

volumes and then centrifuged at (4$^{o}$C, 10,000 x g, for 30 min) to remove the ghosts and intact cells (Ninfali et al. 1990).

### 3.2.2.2. 2', 5'-ADP Sepharose-4B Affinity Chromatography

Two grams of dried 2', 5'-ADP Sepharos-4B gel were used for 10 ml of column volume. The gel was washed with 400 mL of distilled water to remove foreign bodies and the air of swollen gel was eliminated. After removal of the air in the gel, it was suspended in the buffer containing (0.1M potassium acetate and 0.1 M potassium phosphate pH: 6.0) with a ratio of 25% buffer and 75% gel then it was packed into a column (1x10 cm). After precipitation of the gel, it was equilibrated with the same buffer. The gel was washed with equilibration buffer. The flow rates of washing and equilibration were adjusted with peristaltic pump to 50 mL/h. The hemolyzed enzyme solution obtained previously was loaded onto the column, and the flow rate was adjusted to 20 mL/h. Then, the column was sequentially washed with 25 mL of (50 mM $KH_2PO_4$ + 1 mM EDTA + 1mM DTT, pH=7.3) and 25 mL of (50 mM $KH_2PO_4$ + 80 mM KCI + 1 mM EDTA + 1mM DTT, pH=7.3). The washing with 80 mM KCl, 50 mM K-phosphate, (pH: 7.3) was continued until the final absorbance difference at 280 nm became 0.05. Finally, the enzyme was eluted with the solution of 80 mM K-phosphate 80 mM KCl 0.5 mM $NADP^{+}$ 10 mM EDTA (pH: 7.85). The solution that eluted from the column was collected in eppendorf tubes, and then the activity was measured for each tube. The tubes that containing activity were stored at -20°C. The protein was determined in the resultant solution. During all procedures, the temperature was kept at 4°C (Muto and Tan 1985; Ninfali et al. 1990).

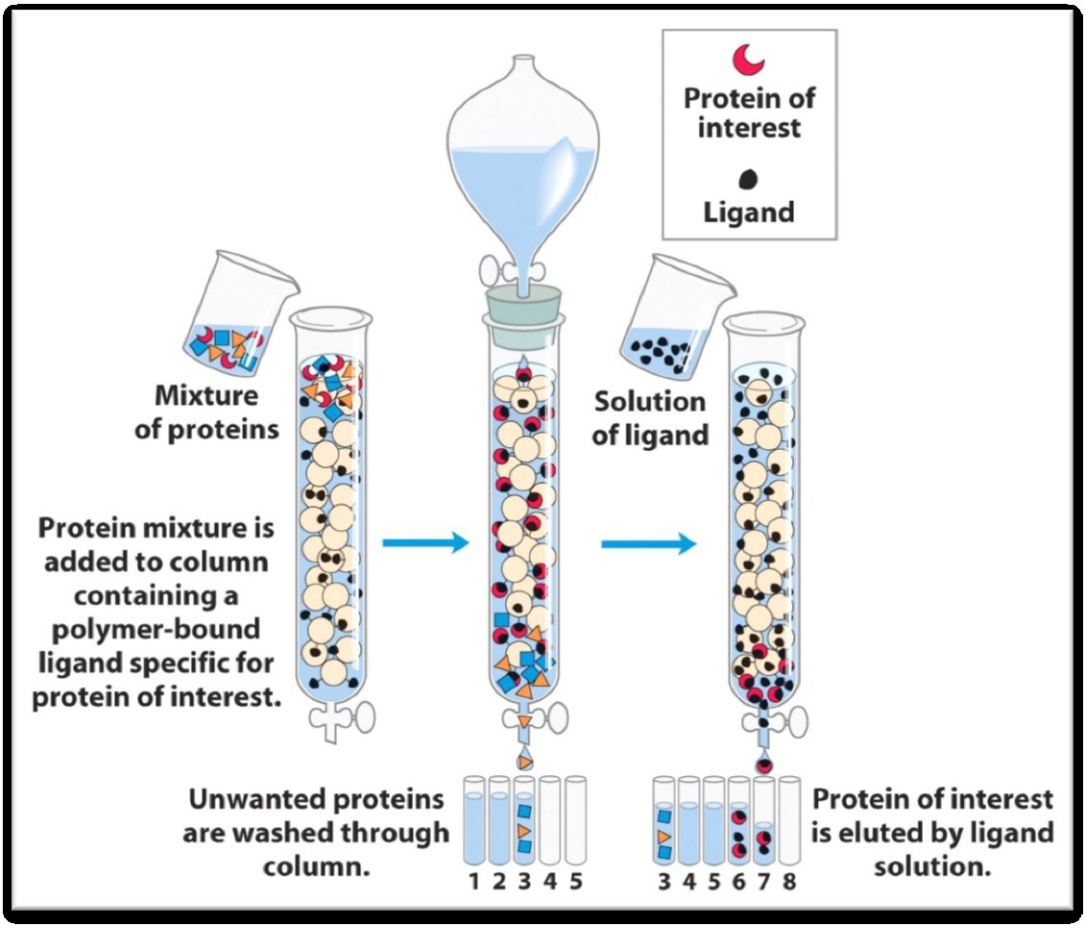



Figure 3.1. Protein purification steps by affinity chromatography (Lehninger, 2008)

### 3.2.3. Protein Determination

#### 3.2.3.1. Qualitative Protein Determination

The qualitative protein assay was carried out by the Warburg Method, this method based on the maximum absorbance of the tyrosine and tryptophan amino acids with the aromatic groups at 280 nm that present in the structure of the proteins.

#### 3.2.3.2. Quantitative Protein Determination

The Bradford total protein assay is the spectroscopic analytical method which is used to determine the total protein concentration of sample (Bradford 1976). In this method, Coomassie Brilliant Blue G-250 dye binds to proteins and changes their colour from green to blue. That colour change is monitored at 595nm in UV-visible

spectrophotometer. As the concentration of protein content is increase, the colour is getting darker and darker. Coomassie Brilliant Blue G-250 binds to arginine, lysine, and histidine residues in protein samples. Bradford assay was applied for both hemolysate and enzymes that eluted from 2', 5'-ADP Sepharos-4B affinity column, in order to calculate protein quantity before and after purification steps. A series of Bovine serum albumin (BSA) standards in different concentrations were prepared. According to this BSA standard, standard calibration curve was drawn with response to their absorbance values. Total protein content was calculated from standard calibration curve equation.

Table 3.2. Standard calibration curve for Bradford method

| Test tubes | BSA volume, (µL) | Water Volume, (µL) | Coomassie Reagent Volume (µL) |
|---|---|---|---|
| Blank | 0 | 0 | 5000 |
| Tube 1 | 10 | 90 | 4900 |
| Tube 2 | 20 | 80 | 4900 |
| Tube 3 | 30 | 70 | 4900 |
| Tube 4 | 40 | 60 | 4900 |
| Tube 5 | 50 | 50 | 4900 |
| Tube 7 | 60 | 40 | 4900 |
| Tube 8 | 70 | 30 | 4900 |
| Tube 9 | 80 | 20 | 4900 |
| Tube 10 | 90 | 10 | 4900 |

Table 3.3. Preparation of protein sample for Bradford method

| sample | sample Volume, (µL) | Coomassie Reagent Volume (µL) |
|---|---|---|
| Hemolysate | 100 | 4900 |
| Pure enzyme | 100 | 4900 |

Blank, BSA standards, and protein samples were prepared according to Table 3.2 and Table 3.3. After incubation for 10 mints, the absorbance for each tube was measured in

1cm disposable cuvettes at 595 nm in spectrophotometer. The absorbance values of sample should be in the dynamic range of BSA standard calibration curve. For over concentrations, sample dilution could be done.

### 3.2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Laemmli`s method was applied in the analytic gel electrophoresis of protein to estimate the molecular weight and purity of the purified enzyme (Laemmli 1970).

**Preparation of gel**

There are four different apparatus parts for casting gel (two glass plates: short plate and tall plate, also casting frame and casting stand). The glass plates were washed in warm detergent solution, rinsed subsequently in distilled water then dried. The short plate was placed in front of the tall plate and then putted it into the casting frame. After putting the two glass plates into the casting frame, it was checked for the bottom too see if it is flat. Casting frame was putted into the casting stand. After that the comb was putted between the glass plates and the marker was taken out approximately 1 cm beneath the comb, then the comb had been taken out. After that the separating gel was prepared as the following

Table 3.4. Separating gel solution

| Reagent | Volume (mL) |
|---|---|
| 1M Tris-HCl (pH=8,8) | 5 |
| Acrylamide/Bis-acrylamide (30%/0.8% w/v) | 4.4 |
| $H_2O$ | 3.13 |
| 10% SDS | 0.20 |
| TEMED | 0.100 |
| 10% APS | 0.08 |

APS and TEMED were added just prior to the pouring of the gel. The solution was mixed well and poured into the space between the two plates till the gel was at the same level as the mark. Isopropanol was carefully laid over the surface of the poured gel mixture to remove air bubbles. The gel mixture was allowed to polymerize, undisturbed at room

temperature for 30 minutes. After the separating gel was polymerize, the over laid isopropanol was removed carefully with filter paper and the stacking gel was prepared by the following.

Table 3.5. Stacking gel solution

| Reagent | Volume (mL) |
|---|---|
| 1M Tris-HCl (pH= 6.8) | 0.440 |
| Acrylamide/Bis-acrylamide (30%/0.8% w/v) | 0.80 |
| $H_2O$ | 2.45 |
| 10% SDS | 0.030 |
| TEMED | 0.030 |
| 10% APS | 0.040 |

After adding TEMED and APS to the stacking gel mixture, it was mixed well and poured immediately over the separating gel and a comb was inserted between the plates. The stacking gel was allowed to polymerize.

**Preparation of protein samples**

Enzyme samples were prepared by 200 µl of sample buffer, 150 µl of glycerol and 200 µl of enzyme that eluted from column. The samples were incubated for 5 min in a boiling water bath prior to loading

When the stacking gel was polymerized the completed gel cassette was removed from casting frame assembly and putted it into the electrode assembly, short plate facing inward. Sealing plate was used to close off the other side of electrode. The electrode assembly placed into the clamping frame then the clamping frame putted into the tank. Inner chamber were filled with running buffer and comb was removed between the two plates. The protein samples were loaded using a micropipette and the wells were completely and carefully filled with electrophoresis buffer.

The gel was ran at constant current (20 milli ampere 60 volts) for 30 minute at room temperature, then the current was a raised to (40 milli ampere 120 volts) for 2 hour. Electrophoretic mobility of the samples was determined by bromophenol blue front. At the end of the run the power pack was switched off. The gel and plates were laid flat on the table and a corner of the upper glass plate was lifted up and the gel was carefully removed.

**Staining of the gel**

After the completion of the electrophoresis, the gel was fixed with (10% trichloroacetic acid, 50% isopropanol and 40% distilled water) for 15 minutes and stained with Coomassie Brilliant Blue (CBB). The CBB staining solution was prepared using methanol, acetic acid and double distilled water in the ratio of 4:1:5 and 0.1g of CBB was added and the gel was stained overnight.

**Destaining of the gel**

The destaining of CBB stained gel was done by using methanol, acetic acid and double distilled water in the ratio of 5:1:4 till the appearance of clear bands on the gel.

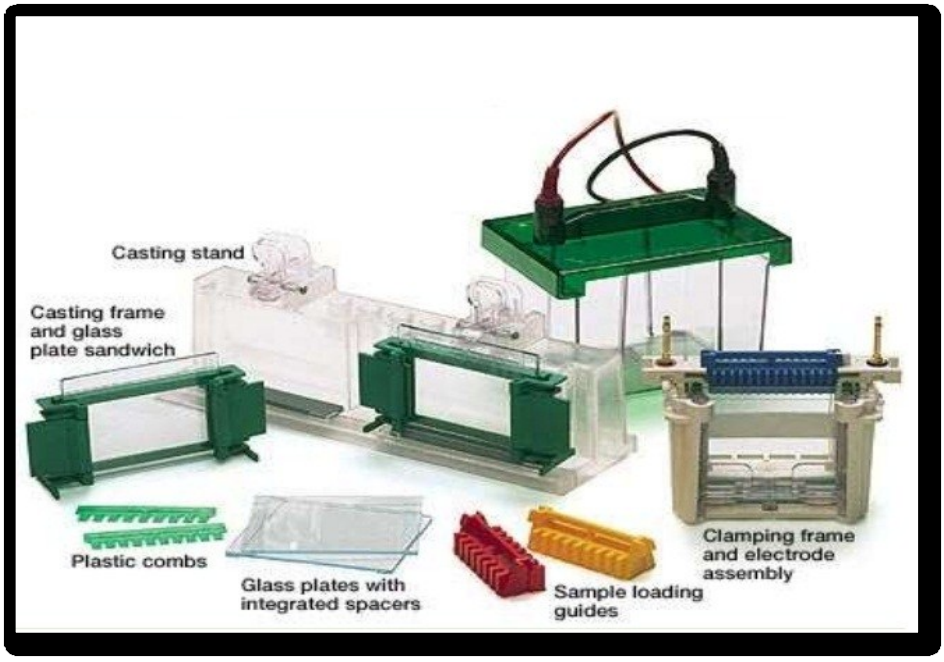



Figure 3.2. Apparatus of SDS –PAGE

### 3.2.4.1. Determination Of Molecular Weight Of G6PD By SDS PAGE

The molecular weight of the G6PD enzyme was determined by SDS-PAGE. From the resulted photograph of the gel, the relative migration distance ($R_f$) of the protein standards and the sample protein (G6PD) was determined. The migration distance (in centimeters) from the top of the gel to every major band in the gel measured by ruler and the relative migration distance ($R_f$) was determined using the following equation:

$$R_f = \frac{\text{Migration distance of the protien}}{\text{Migration distance of the dye}}$$

Based on the values obtained for the bands in the standard, the logarithm of the molecular weight of a standard and its relative migration distance ($R_f$) is plotted into a graph. Interpolating the value from this graph will then give us the molecular weight of the sample protein band.

### 3.2.5. Characterization Studies

### 3.2.5.1. Stable pH Determination

Two different buffers that have a various pH values were used. 1M Tris-HCl buffer and 1M K-phosphate buffer. The pH values of Tris-HCl buffer were (7.5, 8.0, 8.5, and 9.0) and the pH values of K-phosphate buffer were (5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). 400 mL of enzyme were mixed with 600 mL of each buffer, then kept at (+ 4°C). The activity was measured every 24 hour for 6 days for each mixture.

### 3.2.5.2. Optimum pH Determination

For the optimal pH determination, the enzyme activity was measured in 1 M Tris-HCl buffer and 1M potassium phosphate buffer within the pH values (7.5, 8.0, 8.5, 9.0) and (5.5, 6.0, 6.5, 7.0, 7.5, 8.0) respectively.

#### 3.2.5.3. Optimum Temperature Determination

The enzyme activity at optimum pH was measured between 0 °C to 90 °C, per 10°C for this purpose.

#### 3.2.5.4. Optimum İonic Strength Determination

For this purpose different concentration of Tris-HCl buffer and potassium phosphate buffer were prepared for measuring the activity of G6PD enzyme in quail's erythrocyte. The concentration were (0.2, 0.4, 0.6, 0.8 and 1M) respectively and the pH value of each buffer was fixed at 8.0.

### 3.2.6. Determination Of $K_M$ And $V_{max}$ Values Of The Substrate And Coenzyme (G6P And $NADP^+$) Of The G6PD Enzyme

The Lineweaver–Burk curves were used for determination of $K_M$ and $V_{max}$ (Lineweaver and Burk 1934) which were obtained by fixing the concentration of G6P at (0.6 mM), with five different concentrations of $NADP^+$ (0.0005, 0.001, 0.002, 0.005 and 0.01mM) and the same experiments were done for G6P in five different concentrations of G6P (0.012, 0.03, 0.06, 0.09, 0.12 and 0.18 mM) with fixing $NADP^+$ concentration at (0.2 mM). In this process the activity was measured at optimal pH (1MTris–HCl, pH 8.0).

# 4. RESULTS

## 4.1. Enzyme Purification

The prepared hemolysate was applied to 2', 5'-ADP Sepharose-4B affinity column. The process was described in section 3.2.2.2. After loading the elution buffer onto the column, the G6PD enzyme was eluted completely. The solution that was eluted from the column was collected in eppendorf tubes and the enzyme activity was measured for each tube. The maximum activity of the G6PD enzyme was found in the tube number 8 as shown in Table 4.1 and Figure 4.1. Additionally, specific activity of the enzymes, purification fold and perecentage of the purification was provided as well in Table 4.2.

Table 4.1. The activity of eluted fractions of the G6PD enzyme from quail's erythrocytes. Each of the fraction contains approximately 1.5 mL volume of the purified enzyme solution

| Number of tube | activity (EU/mL) |
|---|---|
| 5 | 0.002144 |
| 6 | 0.008574 |
| 7 | 0.063237 |
| 8 | 0.191854 |
| 9 | 0.12433 |
| 10 | 0.076099 |
| 11 | 0.043944 |
| 12 | 0.01179 |
| 13 | 0.004287 |

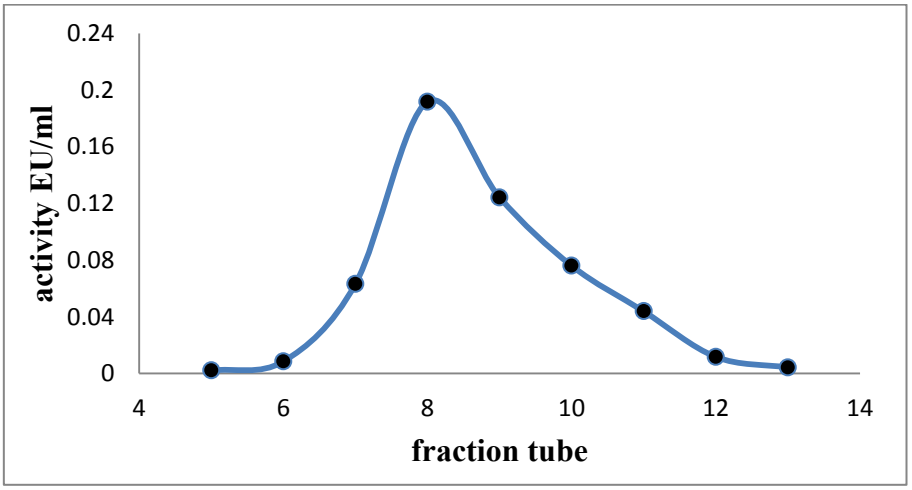



Figure 4.1. Elution graph of the G6PD enzyme in quail's erythrocytes purified by 2', 5'- ADP Sepharose-4B affinity chromatography

Table 4.2. purification table of G6PD enzyme

| Purification steps | Activity (EU/mL) | Total volume (mL) | Protein (mg/mL) | Total protein (mg) | Total activity (EU) | Specific activity (EU/mg) | Yield % | Purification fold |
|---|---|---|---|---|---|---|---|---|
| Hemolysate | 0.132 | 15 | 9.003 | 135.05 | 1.98 | 0.0146 | 100 | 1 |
| 2 ', 5'-ADP Sepharose-4B affinity chromatography | 0.191 | 8 | 0.00316 | 0.0253 | 1.528 | 60.40 | 77.171 | 4136.661 |

## 4.2. Proteins Determination

### 4.2.1. Qualitative Proteins Determination

Before loading G6PD elution buffer to column the absorbance for eluting solution during washing enzyme was measured at 280 nm, throughout the washing enzyme final absorbance difference was determined as 0.05 after eluting 25 mL of 50 mM $KH_2PO_4$ (pH 7.3) containing (1 mM EDTA and 1 mM DTT), and 23 mL of 50 mM $KH_2PO_4$ (pH 7.3) containing (1 mM EDTA , 80 mM KCI and 1 mM DTT). This difeerence of absorbace indicate that the column only contain proteins linked to the 2', 5'-ADP Sepharose-4B gel.

### 2.2.2. Quantitative Proteins Determination

The protein amount was determined quantitatively by the well-known Bradford's method (1976) in hemolysate and purified enzyme solution. Preparation of enzyme solution and drawing standard graph were described in section 3.2.3.2. By using the standard graph as shown in Figure 4.2 and absorbance values of sample and hemolysate Table 4.3 we determined the quantity (µg) of the G6PD enzyme.

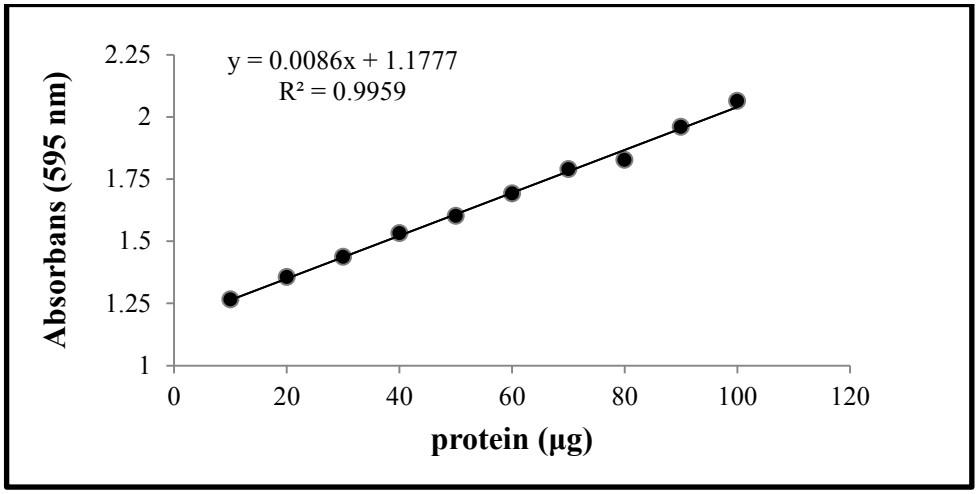



Figure 4.2. Standard graph used for quantitative determination of protein by Bradford method

Table 4.3. The Absorbance at 595 nm of enzyme sample and hemolysate for determination of amount of protein by Bradford's method

| No. | Sample | Absorbance at 595 nm |
|---|---|---|
| 1 | 1:100 Diluted hemolysate | 1.952 |
| 2 | Purified G6PD enzyme from quail erythrocytes | 1.205 |

Table 4.4. The amount of protein in heholysate and purified enzyme

| No. | Sample | Protein amount (µg /mL) |
|---|---|---|
| 1 | Hemolysate | 9003.4 |
| 2 | Purified G6PD | 3.174 |

## 4.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For checking the purity of the enzyme and determining the molecular weight of the purified enzyme from the quail's erythrocytes, SDS-PAGE was used. After running enzyme in the gel and staining it for the visualisation of the corresponding protein band, a photo of the gel was taken as shown in (Figure 4.3). Observation of a single band in the gel had proven that a pure G6PD was taken from quail's erythrocytes. Also from the gel photo the $R_f$ values were calculated for standard protein bands and the G6PD enzyme band (Table 4.5). Then $R_f$ - log MW graph was drawn (Figure 4.4). From this graph and by Laemmeli method the molecular weight of G6PD enzyme was calculated to be 78.88 kDa.

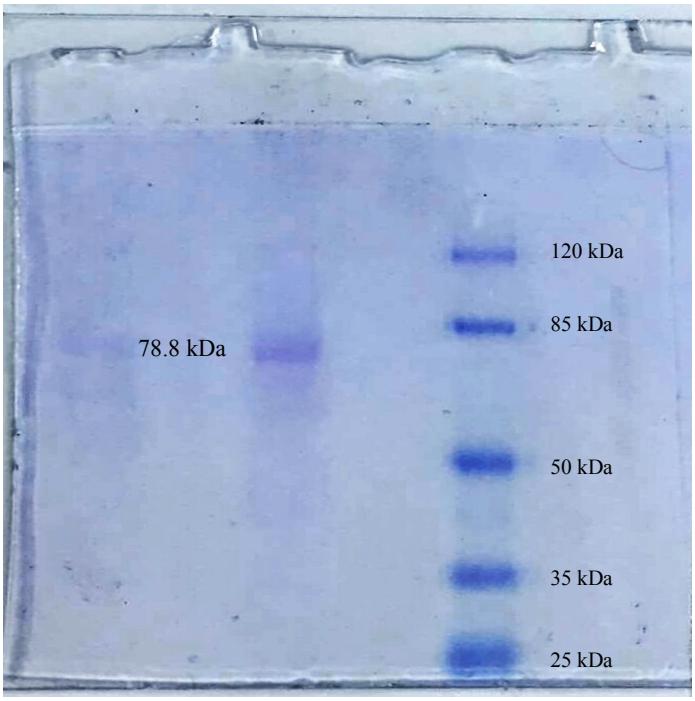



Figure 4.3. SDS-polyacrylamide gel electrophoresis of quail erythrocyte G6PD enzyme. Lane 1 : Standard proteins [ß -galactositase from E-coli: (180 kDa.), bovine serum albumin from bovine plasma: (85 kDa.), ovalbumin from chicken egg white: (50 kDa.), carbonic anhydrase from bovin erythrocyte: (35kDa.), ß-lactoglobulin from bovine milk: (25 kDa)]. Lane 2: G6PD from 2', 5'-ADP Sepharose-4B affinity chromatography : 78.88 kDa

Table 4.5. The $R_f$ values of the standard proteins and sample for the SDS-PAGE performed for determining the molecular weight of the G6DP enzyme in quail's erythrocytes

| Protein | Migration distance of the protein in centimeters | $R_f$ |
|---|---|---|
| ß -galactositase | 3.5 | 0.233 |
| Bovine serum albumin | 5.5 | 0.366 |
| Ovalbumin | 9.22 | 0.614 |
| Carbonic anhydrase | 12.23 | 0.815 |
| ß-lactoglobulin | 14.4 | 0.916 |
| Sample (G6PD) | 6.22 | 0.414 |

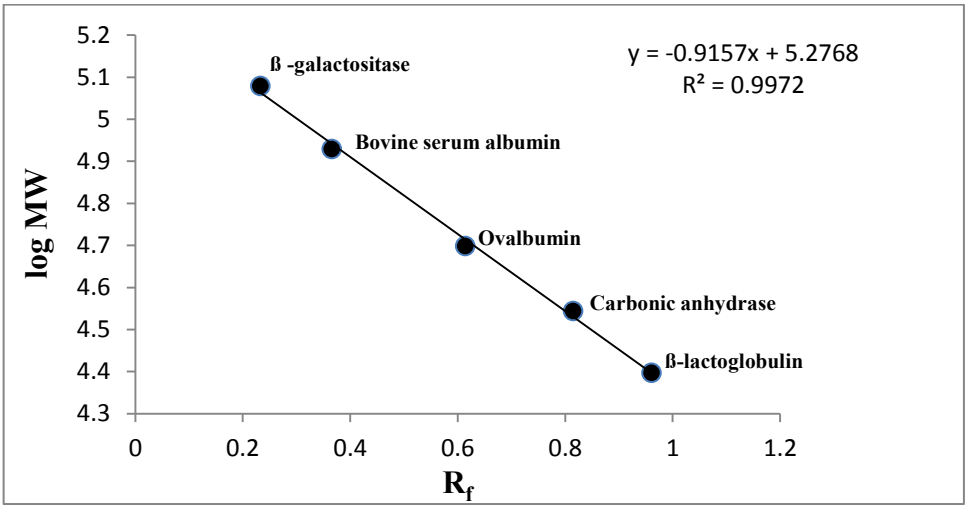



Figure 4.4. The calibration curve between Rf vs Log MW of standard protein for determining the molecular weight of the G6DD enzyme by laemmli method

## 4.4. Characterization

### 4.4.1. Stable pH

The activity of G6PD enzyme was mesuread to determine the stable pH as described in section 3.2.5.1. It was seen that the enzyme was stable in Tris-HCl buffer at pH 7.5 as shown in Table 4.6, 4.7 and Figures 4.5, 4.6.

Table 4.6. The activity of G6PD stored in different pH values of Tris-HCl buffer

| Time (days) | (pH=7.5) Activity (EU/mL) | (pH=8.0) Activity (EU/mL) | (pH=8.5) Activity(EU/mL) | (pH=9.0) Activity (EU/mL) |
|---|---|---|---|---|
| 1 | 0.0932 | 0.0991 | 0.1034 | 0.1039 |
| 2 | 0.0927 | 0.0969 | 0.0975 | 0.0964 |
| 3 | 0.0964 | 0.0969 | 0.0980 | 0.0986 |
| 4 | 0.0943 | 0.0959 | 0.1002 | 0.0975 |
| 5 | 0.0921 | 0.0911 | 0.0916 | 0.0932 |
| 6 | 0.0897 | 0.0897 | 0.08897 | 0.0901 |

Table 4.7. The activity of G6PD stored in different pH values of phosphate buffer

| Time (days) | (pH=5.5) Activity (EU/mL) | (pH=6.0) Activity (EU/mL) | (pH=6.5) Activity (EU/mL) | (pH=7.0) Activity (EU/mL) | (pH=7.5) Activity (EU/mL) | (pH=8.0) Activity (EU/mL) |
|---|---|---|---|---|---|---|
| 1 | 0.0557 | 0.0546 | 0.0573 | 0.0610 | 0.0627 | 0.0718 |
| 2 | 0.0471 | 0.0471 | 0.0514 | 0.0557 | 0.0519 | 0.0637 |
| 3 | 0.0493 | 0.0460 | 0.0535 | 0.0568 | 0.0562 | 0.0632 |
| 4 | 0.0460 | 0.0460 | 0.0466 | 0.0568 | 0.0503 | 0.066 |
| 5 | 0.0439 | 0.0450 | 0.0493 | 0.0444 | 0.0551 | 0.070 |
| 6 | 0.0418 | 0.0439 | 0.0479 | 0.0401 | 0.0547 | 0.0682 |

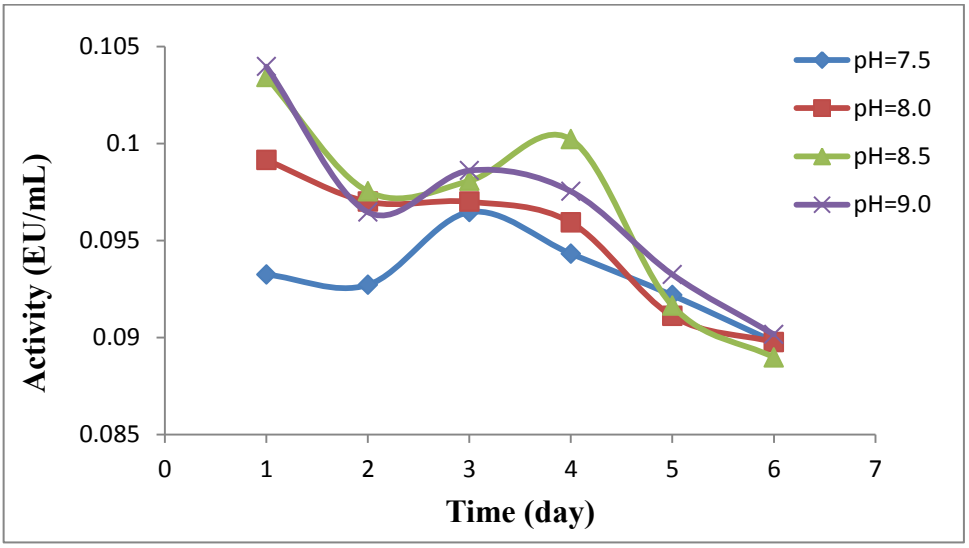



Figure 4.5. Stable pH graph of G6PD enzyme using Tris-HCl buffer

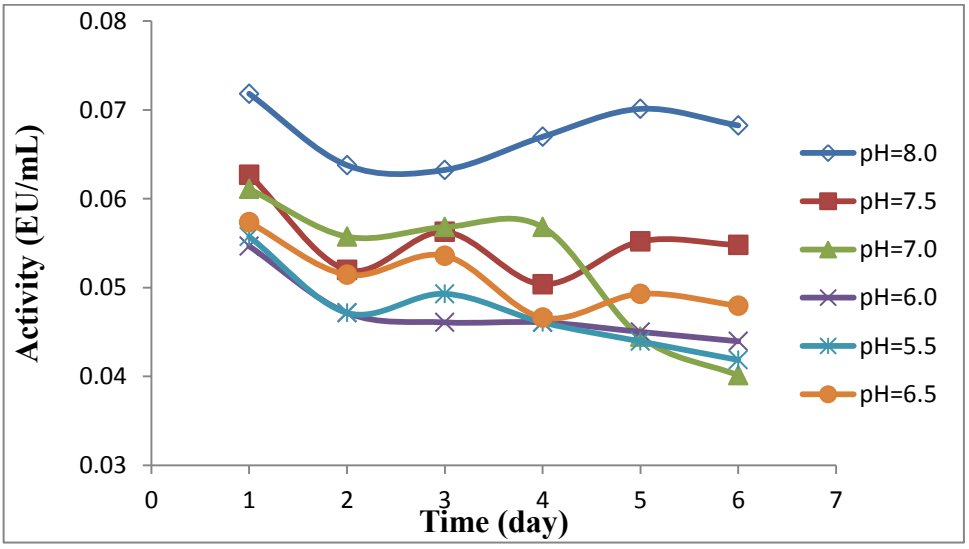



Figure 4.6. Stable pH graph of G6PD enzyme using phosphate buffer

### 4.4.2. Optimum pH

The activity of G6PD was measured in different buffers with varius pH values as described in section 3.2.5.2. It was found that the enzyme has an optimum pH at 8.0 in Tris-HCl buffer as shown as in the Tables 4.8, 4.9 and Figure 4.7.

Table 4.8. Activity of G6PD enzyme using potassium phosphate buffer for optimum pH

| pH | Activity (EU/mL) |
|---|---|
| 5.5 | 0.003751 |
| 6.0 | 0.014469 |
| 6.5 | 0.015541 |
| 7.0 | 0.030011 |
| 7.5 | 0.034298 |
| 8.0 | 0.040729 |

Table 4.9. Activity of G6PD enzyme using Tris-HCl buffer for optimum pH

| pH | Activity (EU/mL) |
|---|---|
| 7.5 | 0.056125 |
| 8 | 0.067215 |
| 8.5 | 0.062165 |
| 9 | 0.052657 |

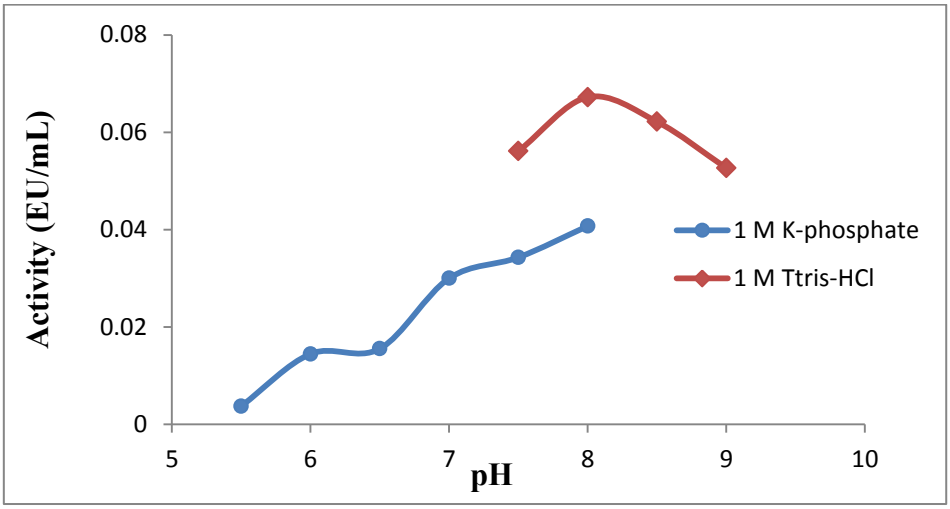



Figure 4.7. Optimum pH graph of G6PD enzyme

### 4.4.3. Optimum Temperature

For determination of the optimum temperature of G6PD enzyme from quail's erythrocytes, the activity of enzyme was assayed at different temperatures ranging from 0 to 90 ˚C. The enzyme has been shown to have the highest activity at 65 ˚C as shown in Table 4.10 and Figure 4.8.

Table 4.10. The effect of temperature on the activity of G6PD

| Temperatue (˚C) | Activity (EU/mL) |
|---|---|
| 0 | 0.020 |
| 10 | 0.025 |
| 20 | 0.054 |
| 30 | 0.062 |
| 40 | 0.097 |
| 50 | 0.153 |
| 60 | 0.178 |
| 65 | 0.194 |
| 70 | 0.157 |
| 80 | 0.015 |
| 90 | 0.004 |

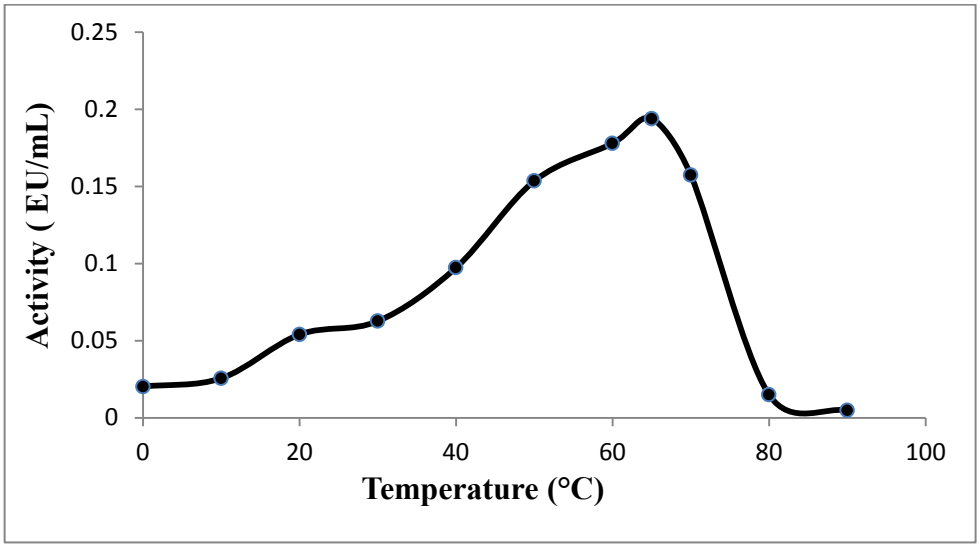



Figure 4.8. Optimum temperature graph of G6PD enzyme

### 4.4.4. Optimum Ionic Strength

The activity was mesuresd at different concentration for G6PD as described in section 3.2.5.4 G6PD enzyme has a high activity in Tris-HCl buffer at 1 M concentration as shown in Table 4.11, 4.12 and Figure 4.9.

Table 4.11. Activities of G6PD enzyme in quail's erythrocyte using different concentration of phosphate buffers

| Concentration (M) | Activity (EU/mL) |
|---|---|
| 0.2 | 0.06975 |
| 0.4 | 0.08602 |
| 0.6 | 0.07563 |
| 0.8 | 0.06899 |
| 1.0 | 0.06248 |

Table 4.12. Activities of G6PD enzyme in quail's erythrocyte using different concentration of Tris-HCl buffers

| Concentration (M) | Activity (EU/mL) |
|---|---|
| 0.2 | 0.058968 |
| 0.4 | 0.096999 |
| 0.6 | 0.085209 |
| 0.8 | 0.107717 |
| 1.0 | 0.115756 |
| 1.2 | 0.102210 |

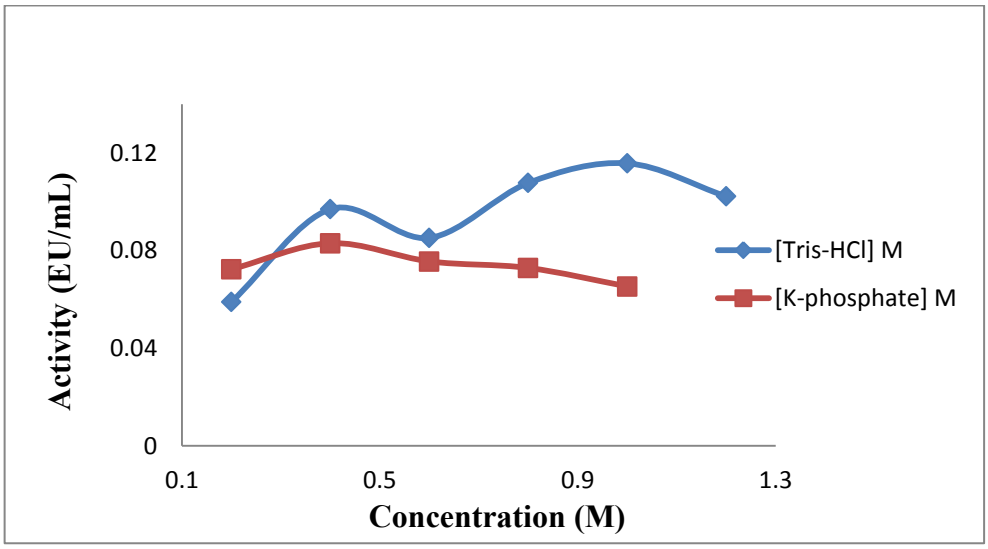



Figure 4.9. Optimum ionic strength graph of G6PD enzyme

## 4.5. Kinatic Studies ($K_M$ And $V_{max}$ Values For $NADP^+$ And G6P)

$K_M$ and $V_{max}$ values for $NADP^+$ coenzyme and G6P substrates of G6PD enzyme in quail's erythrocytes were determined by Lineweaver-Burk plot as shown in Figure 4.10 and Figure 4.11. $K_M$ and $V_{max}$ values for each substrate that calculated were shown in Table 4.13.

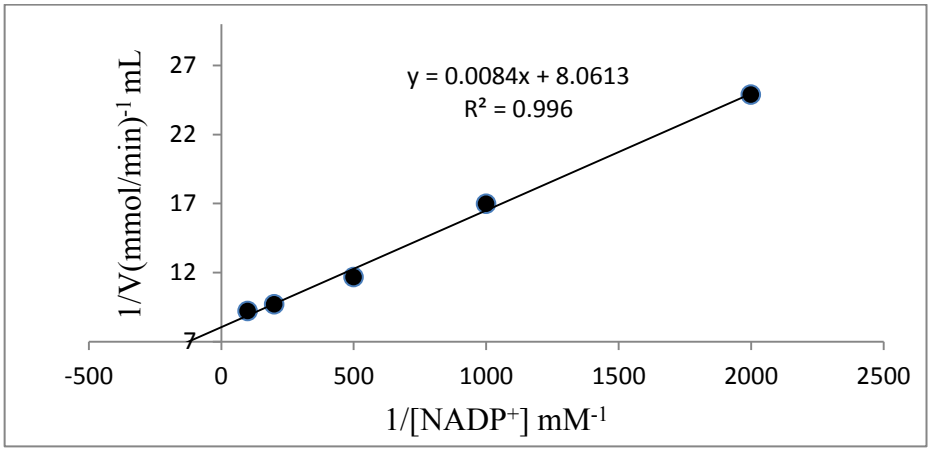



Figure 4.10. The Lineweaver-Burk plot for $NADP^+$ coenzyme using different concentration of $NADP^+$ with fixed concentration of G6P

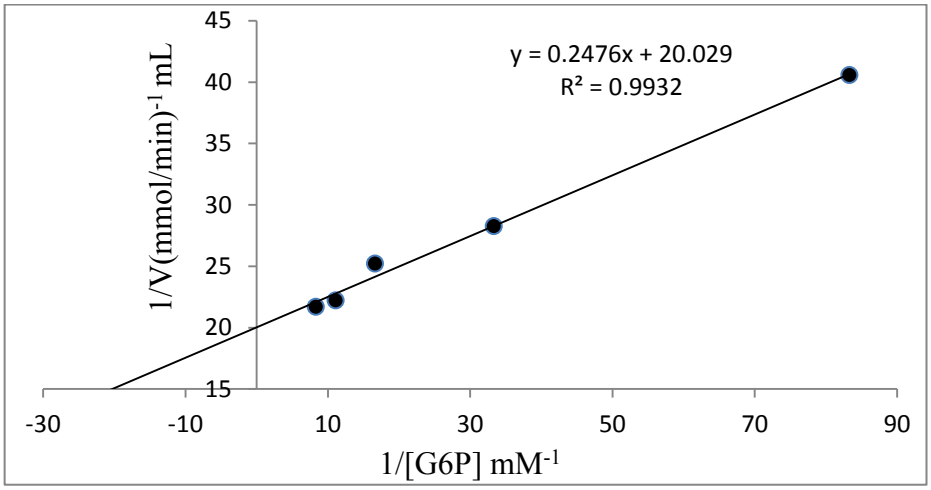



Figure 4.11. The Lineweaver-Burk plot for the G6P substrate using different concentration of G6P with fixed concentration of $NADP^+$

Table 4.13. $K_M$ and $V_{max}$ values for $NADP^+$ and G6P substrates of G6PD

| Substrate | $K_M$ (mM) | $V_{max}$ (EU/mL) |
|---|---|---|
| G6P | 0.012 | 0.05 |
| $NADP^+$ | 0.001 | 0.124 |

# 5. DISCUSSION

Glucose 6-phosphate dehydrogenase (G6PD, D-glucose-6-phosphate, NADPH oxidoreductase, E.C. 1.1.1.49) is the first enzyme of the pentose phosphate pathway. It generates ribose 5-phosphate (R5P) and NADPH from glucose 6-phosphate and $NADP^+$. R5P is required for the synthesis of nucleotides. NADPH has very important functions in the prevention of proteins, lipids, DNA, RNA and other molecules from oxidative damage in all cells (Luzzatto et al. 1978; Ying 2008; Mehta et al. 2000). NADPH also serves several vital functions in lipid synthesis, fatty acid chain elongation, synthesis of some amino acids and in cholesterol synthesis (Stanton 2012).

The pentose phosphate pathway is one of the key oxidative pathways for the oxidation of glucose, which is the main energy source of some cells, such as erythrocytes and brain cells (Mathews and Van Holde 1990; Mayes 1991; Thomas et al. 1983; Lukens 1993). The essential source for NADPH generation in all cells are the hexose monophosphate shunt. Considering one important enzyme in generating NAPH is G6PD, deficiency in the G6PD significantly reduces the amount of NADPH within the cells (Ciftci et al. 2002; Kufrevioglu et al. 2002). This decrease is in association with some diseases such as diabetic kidney disease and dialysis-associated anemia (Spencer and Stanton 2017). The most significant effect of the deficiency of G6PD is observed in erythrocytes as the only source for NADPH production in erythrocyte is PPP (Mason et al. 2007), whereas other cells in organisms have some other pathways to generate NADPH that can reduce the effect of oxidative stress.

G6PD is present in all mammalian tissues (Aksoy and Yasemin 1992), plants (Wendt et al. 2000) and microorganisms (Noltmann et al. 1961; Adewale et al. 2005). The structure of the enzyme is different from various sources. For example, the enzyme is homodimeric form in Leuconostoc mesenteroides (Ravera et al. 2010) whereas it is tetramer in human erythrocytes (Arese et al. 2012). It has been reported to G6PD enzyme

has been monomer, dimer, tetramer and hexamer, but only the dimeric or tetrameric forms have catalytic active (Cohen and Rosemeyer 1969; Babalola et al. 1976). Each monomer contains 515 amino acid residues with a molecular weight of 59 kDa (Takizawa et al. 1986). The gene of G6PD is located at the q28 position of the X chromosome in human beings (Pai et al. 1980; Szabo et al. 1984). The enzyme is often found in the cell as a mixture of dimer-tetramers (Luzzatto 1993; Beutler 1994).

Before starting the project, the results of the previous studies have been taken into account. As no research group was previously purified the G6PD enzyme in quail erythrocytes, the aim of project described in this paper was to characterize the G6PD enzyme of quail's erythrocytes. In order to do that, initially the protein was purified from the erythrocytes cells by preparation of hemolysate and 2', 5'-ADP Sepharose-4B affinity gel chromatography.

G6PD was purified by chromatographic methods from many different sources. Firstly it purified from human erythrocytes by Yoshida in 1965. In the previous years, CM-Sephadex, Sephadex G-200, DEAE-Sephadex, DEAE cellulose and hydroxylapatite columns frequently used in the enzyme purification (Scott and Tatum 1971; Kanji et al. 1976). Also Procion Red HE-3B-Sepharose. Procion Yellow H-3G Matrex gel Orange B. Cibacron blue F3G-Sephadex. Dye ligand affinity columns and nickel-chelate column as metal affinity chromatography are also used to purification the G6PD enzyme (Reuter et al. 1990; Johansson and Joelsson 1987; Hey and Dean 1983; Iyer et al. 2002). In recent days 2', 5'-ADP Sepharose-4B affinity chromatography are widely used as it purifies the enzyme in short time and effective way. This method was used for first time by De Flora and his co-workers (Levy 1979). The 2', 5'-ADP Sepharose-4B immobilized analog NADP structure, it bind and immobilize the enzyme that depend on the NADP. Therefore, this gel primarily used for purification of enzymes requiring NADP as a cofactors, such as 6-phosphogluconate dehydrogenase (Adem and Ciftci 2016; Adem and Ciftci 2012; Baqi 2016), thioredoxin reductase (Temel 2014).

For partial purification of the G6PD enzyme from the prepared hemolysate, ammonium sulphate precipitation was used. In order to achieve that, different percentages of the salt were added to the hemolysate to perform salting out experiments and the proteins were precipitated according to their size and separated by centrifugation. For this purpose 10-

60% of ammonium sulphate was added to the hemolysate. However the precipitation process did not come out with a desired outcome as non-sufficient precipitate was resulted from it to perform further experiments on it. For making this precipitation step possible, bulk amounts of the Japanese quail blood is needed. However, as we had small amount of blood we could not perform it. Therefore we neglect this step for purification process in our study; yet, this method is widely used for partially purifying the enzyme from other protein constituents.

Quail's erythrocyte G6PD has been purified to 4136.661 fold with a specific activity of 60.40 EU/mg. protein and 77.17% yield. These values vary for G6PD from different organisms. For example in the literature it was reported that the enzyme from human erythrocytes were purified to 13654 fold with a specific activity of 114.7 EU/mg. protein and 28% yield (Ciftci et al. 2000), chicken erythrocytes were purified 9150 fold with a specific activity of 20.862 EU/mg. protein and 54.68% yield (Yilmaz et al. 2007), bovine lens were purified 19.7 fold with a 13.7% yield (Ulusu et al. 1999), pork liver were purified 1000 fold with a specific activity of 1.24 EU/mg. protein and 40% yield (Kanji et al. 1976), penicillium were purified 2248 fold with a specific activity of 62.96 EU mg. protein and 28.33%yield (Malcolm et al. 1972), dog kidney were purified 2000 fold with a specific activity of 130 EU/mg. protein and 18% yield (Ozer et al. 2002), sheep kidney cortex were purified 13.84 fold with a 16.96% yield (Ulusu and Tandogan 2006), human erythrocyte were purified 91.50 fold with a 43% yield (Erat 2004).

After purification, the purity of enzyme was checked and molecular weight of the enzyme was determined by SDS-PAGE. The results revealed a single band suggesting the enzyme was obtained in a pure form. From the gel photograph Figure 4.3 the $R_f$ values of standard protein and G6PD enzyme were calculated Ttable 4.5 Then the calibration curve between $R_f$ and Log MW values of standard protein was plotted Figure 4.4. From this plot, the molecular weight of the G6PD in quail's erythrocyte was estimated as 78.88 kDa. The MW enzyme is similar to that of goose erythrocyte (Beydemir et al. 2003b), chicken erythrocytes (Yilmaz et al. 2002), grass carp (Hu et al. 2012), bovine lens (Ulusu et al. 1999), Gerze chicken erythrocyte (Mercan et al. 2011), buffalo erythrocyte (Ciftci et al. 2003). However, the molecular weight is different to that of rainbow trout liver (Cankaya et al. 2011) and dog liver (Ozer et al. 2002).

The amount of protein both in hemolysate and pure enzyme were determined quantitatively from Figure 4.2 by Bradford method (1976). This method is more advantageous than other protein determination methods. Because it takes less time, requires less amount reagent, Protein dye complex remains in solution for a long time and the sensitivity of this method is between 5-100 μg.

The stable pH of G6PD enzyme in quail's erythrocytes at different pH values was investigated in 1 M Tris-HCl buffer and 1 M phosphate buffer (Tables 4.6, 4.7 and Figures 4.5, 4.6). It was found that G6PD was more stable at pH 7.5 in Tris-HCl buffer than at other pH values. The stable pH of various G6PD were studied and reported in literature: The pH determined in that study was different as compared to rainbow trout erythrocytes (Ciftci et al. 2004), goose erythrocytes (Beydemir et al. 2003b), Lake Van fish erythrocytes (Ozdemir et al. 2007), buffalo erythrocytes (Ciftci et al. 2003), and chicken erythrocytes (Yilmaz et al. 2002).

It was found that the optimum pH of the G6PD enzyme in quail's erythrocytes was 8.0 in Tris-HCl buffer (Table 4.8, Table 4.9 and Figure 4.7). The optimum pH data of G6PD in literature suggested that this value has a similarity to G6PD of sheep liver (Turkoglu et al. 2002), pea leaves (Semenikhina et al. 1999), rainbow trout erythrocytes (Ciftci et al. 2004), human erythrocytes (Cohen and Rosemeyer 1969) and have a different optimum pH value from G6PD of Lake Van fish erythrocytes (Ozdemir et al. 2007), camel liver (Ibrahim et al. 2014), lamb kidney cortex (Ulusu et al. 2005) and Lake Van fish liver (Turkoglu et al. 2005).

Optimum temperature of enzyme measured between 0°C to 90°C and it was calculated to be 65$^{o}$C (Table 4.10 and Figure 4.8). This data was compared to literature it was similar to G6PD of chicken erythrocytes (Yilmaz et al. 2002) and buffalo erythrocytes (Ciftci et al. 2003) but it was different from rat liver and kidney (Corpas et al. 1995), sheep liver (Turkoglu et al. 2002), lamb kidney cortex (Ulusu et al. 2005) and sheep kidney cortex (Ulusu and Tandogan 2006).

Optimum ionic strength was determined at 1 M Tris-HCl buffer (Table 4.11, 4.12 and Figure 4.9). This data was different from rat lung, rat heart tissues (Adem and Ciftci, 2011) and human erythrocytes (Cohen and Rosemeyer 1969).

By using Figure 4.10, Figure 4.11, $K_M$ and $V_{max}$ values were calculated for G6P and $NADP^+$, which are substrates of the enzyme. The $V_{max}$ values of $NADP^+$ and G6P were found to be 0.134 EU/mL and 0.05 EU/mL respectively. Also The $K_M$ values for G6P and $NADP^+$ were found to be 0.0122 mM and 0.00133 mM respectively. It is seen that the $K_M$ value for $NADP^+$ is smaller when compared to the $K_M$ value for G6P. In this case, it can be said that the affinity of $NADP^+$ with enzyme is greater than G6P. Similar results were detected also by Ulusu et al. (1999), Corpas et al. (1995), Bilgi et al. (1995), Ozer et al. (2001), Ulusu and Tandogan (2006) and Hopa, (2010).

# CONCLUSION

Glucose 6-phosphate dehydrogenase (G6PD) is the first enzyme of the pentose phosphate pathway (PPP) and it is considered as a secondary anti-oxidant enzyme for its role in producing the necessary NADPH that is required for anti-oxidant effects of the free radicals in the cells.

The Glucose 6-phosphate dehydrogenase (G6PD) was purified from the erythrocytes of Japanese quail (*Coturnix coturnix japonica*) by the 2', 5'-ADP Sepharose-4B affinity gel chromatography and the purified G6PD enzyme was checked for its purity using the sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) method. The result was observing purely a single band on the gel which indicated that the enzyme had well-being purified and there was only one protein in the purified solution.

The purified G6PD enzyme was characterized for determining its behaviours like the stable pH value, optimum pH, optimum temperature value, optimum ionic strength, $K_M$ and $V_{max}$ values for the natural enzyme's substrate and its coenzyme as well as its molecular weight.

The information regarding the purification of the enzyme and the properties of the G6PD enzyme of quail's erythrocytes are summarized in the table 5.1 below:

Table 5.1. The summary of results for purification and characterization of the Japanese quail (*Coturnix coturnix japonica*) erythrocytes enzyme

| | |
|---|---|
| Specific activity of enzyme | 60.4 EU/mg |
| Purification yield % | 77.171% |
| Purification folds | 4136.661 folds |
| Molecular weight by SDS-PAGE method | ~ 78.88 kDa |
| Optimum pH value | pH 8.0, Tris-HCl buffer |
| Optimum Temperature | 65$^{o}$C |
| Optimum ionic strength | 1 M, Tris-HCl buffer |
| $V_{max}$ value for $NADP^{+}$ | 0.0.124 EU/mL |
| $V_{max}$ value for G6P | 0.05 EU/mL |
| $K_M$ value for $NADP^{+}$ | 0.001 mM |
| $K_M$ value for G6P | 0.012 mM |

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## CURRICULUM VITAE

I was born on June 27th 1990 in Zakho city of Kurdistan region in Iraq. I finished my primary, secondary and high school in Zakho. I started studying chemistry science at University of Zakho, college of science/ chemistry department in 2010 and graduated in 2014 holding a bachelor's degree in chemistry science. I started my graduated study in February 2015 at Bingol University/ Turkey and awarded the master's degree in biochemistry in Jan. 2017.