PURIFICATION AND CHARACTERIZATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM JAPANESE QUAIL *(Coturnix coturnix japonica)* ERYTHROCYTES

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Master thesis

Chemistry Department

Supervisor: Prof. Dr. Mehmet ÇIFTCI

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T.C.
BİNGÖL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

6-FOSFOGLUKONAT DEHİDROGENAZ ENZİMİNİN JAPON BILDIRCIN (*Coturnix coturnix japonica*) ERİTROSİTLERİNDEN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

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LIST OF ABBREVATIONS

E-S	: Enzyme substrate complex
EC	: Enzyme Commission number
Da	: Daltons
kDa	: Kilo daltons
K cat	: Turnover number
mmol	: Millimoles
EU	: Enzyme units
mg	: Milligrams
g	: Grams
BSA	: Bovine serum albumin
CM	: Carboxymethyl
DNA	: Deoxyribonucleic acid
D-Isomers	: Dextrorotatory isomer
DEAE	: Diethylaminoethyl
L-Isomer	: Levorotatory isomer
K_M	: Michaelis-Menten constant
Vmax	: Maximum velocity
S	: Substrate
P	: Product
rpm	: Round per minute
E-P	: Enzyme product complex
Q_{10}	: Temperature coefficient
PPP	: Pentose phosphate pathway
NAD^+	: Nicotinamide adenine dinucleotide oxidized form
$NADP^+$	: Nicotinamide adenine dinucleotide phosphate oxidized form
PER	: Ammonium per sulfate
SDS	: Sodium dodecyl sulfate

RNA	: Ribonucleic acid
G6P	: Glucose-6 phosphate
G6PD	: Glucose 6- phosphate dehydrogenase
NADPH	: Nicotinamide adenine dinucleotide phosphate reduced form
6PGA	: 6-phosphogluconate
6PGD	: 6-phosphogluconate dehydrogenase
TCA	: Trichloroacetic acid
GSH	: Glutathione (reduced)
LKB1-AMPK	: Liver kinase B1- Activated protein kinase pathway
ROS	: Reactive oxygen species
Ru-5-Phos	: Ribulose 5-phosphate
NMN	: Nicotinamide mononucleotide
Met13	: Methionine 13
D.W	: Distilled water
DDW	: Double distilled water
Tris	: Tris (hydroxyl methyl) aminomethane
R_f	: Retardation factor
TEMED	: Tetramethylethylenediamine
M.W.	: Molecular weight
PAGE	: Polyacrylamide gel electrophoresis

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6-FOSFOGLUKONAT DEHİDROGENAZ ENZİMİNİN JAPON BILDIRCIN *(Coturnix coturnix japonica)* ERİTROSİTLERİNDEN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Yüksek lisans tezi olarak sunulan bu çalışmada Japon bıldırcın eritrosit dokularından 6-Fosfoglukonat dehidrogenaz enzimi (6PGD) 52,84 EU/mg spesifik aktiviteyle ve %69 verimle 2', 5'-ADP Sepharose 4B afinite kromatografisi kullanılarak saflaştırıldı. Japon bıldırcın eritrositlerinden saflaştırılan 6PGD enzimi için saflaştırma katsayısı 4984 olarak bulundu. Saflığının kontrolü için SDS-poliakrilamid jel elektroforezi (SDS-PAGE) yapıldı ve tek bant gözlendi. SDS-PAGE yöntemi kullanılarak enzimin alt birimlerinin molekül kutlesi 81 kDa olarak hesaplandı. Optimum iyonik şiddet 0,5 M Tris-HCl, optimum pH ve stabil pH 0,5M Tris-HCl tamponu pH 8,0 olarak bulundu. Optimum sıcaklık 60 ºC olarak bulundu. Ayrica Japon bıldırcın 6PGD enziminin K_M ve V_{max} değerleri Lineweaver-Burk grafiklerinden sırasıyla 6PGA substratı için K_M değeri 0,120 mM, V_{max} değeri 0,191 EU/mL, $NADP^+$ substratı için K_M değeri 0,017 mM, V_{max} değerise 0,228 EU/mL olarak hesaplandı.

Anahtar Kelimeler: Japon bıldırcın, 6PGD, eritrosit, saflaştırma, karakterizasyon.

PURIFICATION AND CHARACTERIZATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM JAPANESE QUAIL *(Coturnix coturnix japonica)* ERYTHROCYTES

ABSTRACT

In this master's thesis study, the 6PGD enzyme from Japanese quail erythrocytes was purified with specific activity of 52.84 EU/mg and 69% yield of purification by 2', 5'-ADP Sepharose 4B affinity gel in a single chromatographic method. The purification folds of the enzyme were 4984 folds. The purified enzyme was checked using SDS polyacrylamide gel electrophoresis (SDS-PAGE) method; the result of gel showed a single band. The subunit molecular weight of the enzyme was calculated as 81 kDa by the SDS-PAGE method. The characterization studies of the 6PGD enzyme from erythrocytes of Japanese quail showed: the optimum ionic strength to be at 0.5 M Tris-HCl, optimum and stable pH values to be at 0.5 M Tris-HCl buffers pH 8.0. The optimal temperature for the enzyme activity was found at 60°C. Finally, the K_M and V_{max} values for the 6PGD enzyme from Japanese quail's erythrocytes were calculated respectively for the 6PGA the K_M value found as 0.120 mM, Vmax value as 0.191 EU/mL and for $NADP^+$ the K_M value as 0.017 mM and Vmax value as 0.228 EU/mL.

Key words: Japanese quail, 6PGD, erythrocytes, purification, characterization.

1. INTRODUCTION

1.1. Enzymes

Enzymes are considered as biocatalysts of life, catalyst is a substance that causes the reaction rate to be increased in a certain amount of time without actually being part of its reactants neither product. These biocatalysts are all synthesized by living cells. (Satyanarayana and Chakrapani 2007).

Metabolism process in any biological system is mapped by enzymes that catalyze all reactions in the biological system (Berg et al. 2002). These polymers of amino acids are the main reason why all chemical reactions happen very smoothly in our bodies as they catalyze all metabolic reactions in living cells. From here the importance of enzymes come into the view as well as their clinical significances; any imbalances in the enzyme's catalytic activity or concentrations could lead to very critical health conditions (Murray et al. 2012). The catalytic power of enzyme is about a factor of one million times of increasing the velocity of the reaction, without the actions of enzymes the life we know could be impossible (Berg et al. 2002).

The position of some enzymes is fixed in some organelles of the cells. This localization of enzymes causes the reacting substrates or products to be away from other competing reactions and consequently, only the most favorable reaction happens in that precise cell or organelle which makes an enormous amounts of the enzymes to be very specific and purposeful to target their substrates. The nature of a cell is defined by the occurrence of the enzymes that located and synthesized in it and also determines which type of metabolism reaction happens in that specific cell (Harvey and Ferrier 2011).

All enzymes are made up by amino acid polymers except a tiny group of RNA catalyst enzymes which known as ribozymes. The activity of an enzyme as a catalyst is dependent on its protein structure. If a protein denatured, the enzyme loses its activity directly.

The size of enzymes as other proteins lay between 12000 to over 1 million Daltons (Nelson and Cox 2005). The specificity and catalyzing power of enzymes are two of the most important features of enzymes. The catalyzing process of an enzyme happens due to special sites on the enzyme's structure called active sites (Berg et al. 2002). The active site is an efficient part of the enzyme that provides different mechanisms for the happening of the reaction and increases the rate of reaction. Enzymes are very selective in choosing their substrates. They only choose one substrate or group of substrates that all lay under one category (Harvey and Ferrier 2011). The active site of enzymes occupies only a small part of the whole enzymes volume. The enzyme's activity happens in the active sites. The amino acids and cofactors are arranged precisely in a way that complement with the enzyme's substrate structure forming the E-S complex (Copeland 2000).

According to the cellular requirements, the catalytic activity of enzymes are regulated (increased or decreased) and consequently, the rate of the desired product is achieved (Harvey and Ferrier 2011).

The efficiency of an enzyme is measured by the number of substrate molecules those changed to product by one molecule of the enzyme in one second of time. This number is called as turnover number or K_{cat} that is normally about 10^2-10^4 per sec. for a variety of enzymes (Harvey and Ferrier 2011). Some small molecular weight chemical compounds or ions cause a decrease in the *in vitro* and *in vivo* enzyme activities. These compounds are called inhibitors and the process of inhibition of the enzymatic activity in biological systems is the main regulation mechanism for the enzyme activities. Likewise to inhibitors, another group of small molecular weight compounds and ions activate the enzymes and cause an increase over the enzymatic reaction rates and they are called activators. Inhibitions and activations have great importance in enzyme studies. Studying of the inhibitor and activator effects on enzymes is very useful for health issues and drug synthesis, because a lot of drugs and toxic compounds are designed upon the basis of enzymatic inhibitions or activations (Keha and Küfrevioğlu 2004; Gözükara 1997; Taranci 2011).

Enzyme unit is the amount of enzyme that converts 1μmol of substrate into the product per one minute in definite conditions. However the specific activity of the enzyme is the

amount of EU in one mg of the protein. It's considered as a measurement for the purity of the process of enzyme's purification, at maximum values of specific activity the enzyme purification reaches to homogeneity (Keha and Küfrevioğlu 2004).

1.1.1. Historical perspective

The studies of the enzymes began at 1810 by Joseph Gay-Lussacs while he was doing studies on fermentation by determining that the sugar is decomposed to ethanol and CO_2. In 1835, while Jacob Berzelius was studying chemical catalysis he had stated that an extract from malt that was known as diastase which is now called as alpha amylase has an ability to catalyze the starch hydrolysis process more actively than the H_2SO_4 does. In 1894, the lock and key hypothesis was proposed by Emil Fischer. The discovery he came up was his assumption from those glycolytic enzymes which could able to differentiate the stereo isomeric sugars. He postulated that specificity of the enzymes (the lock) for their substrates (the key) arises from their complementary three-dimensional geometrical shapes. In mid 1930s, John Northrop and Moses Kuintz had proven a direct relationship between the activity of pepsin, trypsin and chymotrypsin with the protein amounts that exist through the reaction medium. Since then the enzymes were said to be proteins. In 1963 bovine pancreatic (ribonuclease A) enzyme amino acid sequences reported. In 1965, first X-Ray structure of an enzyme (hen egg white) lysozyme had been found, after these times techniques for the enzyme purifications had improved to date, and thousands of enzymes had been purified and characterized since then by using a variety of purification techniques and the process is to be continued (Voet and Voet 2011).

1.2. Enzyme nomenclature

Generally, each enzyme has two names, one easy short name for using on a daily basis to recognize it from other enzymes. This naming referred as recommended name, and one more revealing systematic name that elucidate the whole information regarding that specific enzyme (Harvey and Ferrier 2011).

In the beginning of enzymology, naming of enzymes was in an uncontrollable fashion, for example, names as pepsin, trypsin or chymotrypsin carries no prove relevant to

neither the action of the enzyme nor the substrate. In addition sometimes “-ase” suffix added to the substrates that enzyme acts on like lactase (enzyme that acts on lactose), this nomenclature is considered as non-systematic naming (Satyanarayana and Chakrapani 2007).

Enzymes could be classified under two major broad categories, which are intracellular enzymes those which act only in cells that were synthesized in and extracellular enzymes those in which act within the cells and *in vitro* too (Satyanarayana and Chakrapani 2007).

For the sake of clarity, the enzymes are classified into six major groups according to the nature of work they do in their reactions. These major groups are subdivided into smaller groups that give the whole information regarding the enzymes nature and function. Each enzyme is specifically assigned to a special enzyme commission number (E.C) that defines exactly what that specific enzyme is (Harvey and Ferrier 2011).

According to this system, each enzyme belongs to a broader group of enzymes that all have common features in their functioning. Each group of the six major groups is subdivided into subgroups that represent reactions which enzymes catalyze (Nelson and Cox 2005).

Table 1.1. International classifications of enzymes

No.	**Class**	**Type of catalyzed reaction**
1	Oxidoreductases	This group transfer electrons (hydrogen or hydride ions)
2	Transferases	This group catalyze transfer reactions
3	Hydrolases	This group catalyze hydrolases reactions
4	Lyases	This group catalyzes the reaction of addition groups to double bonds or removing groups to form double bonds.
5	Isomerases	This group transfer groups in the same molecules to form isomers.
6	Ligases	This group catalyze reactions to form C-C,C-N,C-O and C-S bonds through condensation reactions.

1.3. Enzymes and cofactors

The ability of an enzyme to catalyze a reaction is sometimes based upon the existence of special molecules known as the cofactors. The enzyme without its cofactor is called apoenzyme, the catalytically active enzyme is referred as haloenzyme, which is apoenzyme with the cofactor. The cofactors can be divided into two major groups including metal cofactors and small organic molecules. The small organic molecule cofactors are often called coenzymes, which are generally derived from vitamins. These coenzymes are capable of attaching to the enzymes strongly or weakly. Those which strongly bound to the enzymes are referred as prosthetic groups. While the weakly bound coenzymes are often referred as co-substrates as they attach to the enzyme only like substrates and products do. These coenzymes could be used by a group of enzymes, and from here comes the differentiation of coenzymes from substrates. However a group of enzymes that all use one coenzyme are all share the same mechanisms for their activities (Berg et al. 2002).

1.4. Enzyme substrate specificity

Enzymes are bind with the substrate molecules in a similar way to that proteins bind for themselves. These bindings involve Van der Waals, electrostatic, hydrogen bonding and hydrophobic interactions. Usually, the surface of the substrate contains a space that is exactly compatible to the specific clefts of the enzymes. The active sites amino acid residues on the surface of the cleft are designed in a way that allows them to interact selectively with the substrate molecules. A molecule with different geometrics to the enzyme would not bind with it and the enzyme substrate complex would not be formed (Voet and Voet 2011; Segel 1968).

1.4.1. Stereo specificity

Enzymes are very specific for binding and catalyzing the reactions of chiral substrates. The stereo specificity of enzymes comes from the reality that proteins are naturally L-isomers, L-amino acids. For example, L-polypeptides are hydrolyzed by trypsin without those of D- polypeptides. On the other hand, the metabolic enzymes of glucose are all

selective to D-glucose residues. So enzymes are stereospecific in reactions they participate in as catalysts (Voet and Voet 2011).

1.4.2. Geometric specificity

Enzymes are very specific in identifying their substrate chemical groups. This is recalled as geometric specificity, which is more favorable than the stereo specificity. The geometric specificity of enzyme varies in a big way from one enzyme to another. There are enzymes designed for catalyzing exactly one compound. However, the other majority of enzymes are able to catalyze reactions of small similar groups of compounds (Voet and Voet 2011).

1.5. Henri-Michaelis-Menten model for enzyme kinetics

Enzymes are bind to their substrates and form an unstable temporary E-S complex that is later forms product. Henri-Michaelis-Mentens equation describes the kinetic features of enzymes, it shows the velocity of an enzymatic reaction in given substrate concentration [S] in a comparison with the maximum velocity of that reaction (Segel 1968).

The K_M constant could be defined as the concentration of the substrate that produces half maximum rate of a reaction (1/2 V_{max}) in an enzymatic catalysis reaction. It means the attached enzyme molecules to the substrate molecules are of 50% rate and it equals to the K_M value (Satyanarayana and Chakrapani 2007; Keha and Küfrevioğlu 2004 ; Segel 1968). K_M is a constant value and is considered as an enzymatic characteristic or feature. It shows how tough the E-S bound is. Low K_M value assembles strong affinity for binding between the substrates and enzymes; high K_M value represents a weak E-S bound affinity. Most of the enzymes have K_M values between 10^{-5}- 10^{-2} even though K_M is not dependent on [E] (Satyanarayana and Chakrapani 2007).

V_{max} on the other hand is the maximum rate of the enzymatic reaction and it happens while all the enzyme molecules are interacted with the substrate molecules in the E-S form (Segel 1968).

Rates of reactions are determined by K_1, K_2 and K_3 that represent the reaction rates.

$$K_M = K_2 \frac{K_3}{K_1} \quad 1.1.$$

By performing some mathematical calculations, the 1.1 equation, the Michaelis-Menten equation derived as follows:

$$V = \frac{V_{max} \cdot [S]}{K_M + [S]} \quad 1.2.$$

$$V = V_{max} \frac{[S]}{K_M} + [S] \quad 1.3.$$

V: measured velocity

V_{max}: maximum velocity

S: substrate concentration

K_M: Michaelis – Menten constants

1.6. Factors affecting enzyme activity

The relationship of the E-S complex is determinant for the enzyme activity. There are some factors that are affecting the enzyme’s activity. The enzyme concentration during the reaction determines the rate of enzymatic reactions, since the rate of enzymatic reaction is proportional to the enzyme concentration (Copeland 2000). This enzyme characteristic is used for finding the enzyme activities in serum samples for diagnosing diseases, in which by using a determined volume of the sampled serum with keeping constant rates of the other reaction components like the substrate, pH value and temperature, the enzyme’s activity could be assayed spectrophotometrically (Satyanarayana and Chakrapani 2007; Segel 1968).

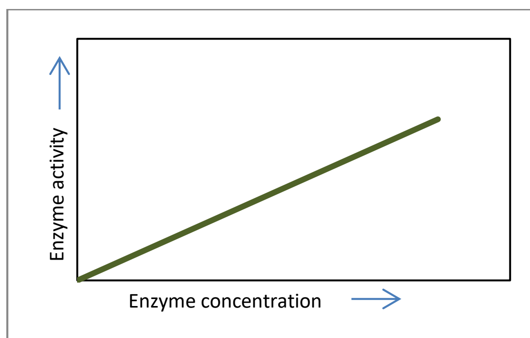

Figure 1.1. The effect of enzyme concentration on the activity of enzymes: the enzyme activity increases as the concentration of the enzyme elevates in a reaction medium

In addition, substrate concentrations also affect the rate of the reactions. In first-order reactions in which ([S] is smaller than K_M), the velocity of the reaction will be proportional to the substrate's concentration at that point of reaction. In a zero ordered reaction, which is when ([S] is more than K_M value); the reaction rate will not be dependent on the substrate's concentration. Instead, the velocity of the reaction is depends upon the chemical conversion rates of the E-S complex in to E-P and product formation that releases back the free enzyme (Copeland 2000; Satyanarayana and Chakrapani 2007; Segel 1968).

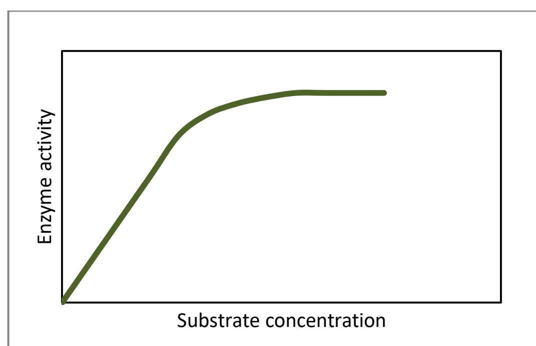

Figure 1.2. The effect of substrate concentration on the activity of enzymes: in the first order reactions the enzyme activity depends on the amount of substrate concentrations in the reaction medium, while in zero ordered reactions the enzyme's activity will no longer depend on the substrate concentrations

Velocity of the enzymatic reaction is highly dependent on the temperature. By increasing the temperature, reaction velocity increases that eventually produce a bell-shaped curve. The increase in the enzyme's activity that produced when a temperature of a reaction medium elevates by 10°C is called temperature coefficient (Q_{10}). The way elevating

temperature affects the enzyme's activity is by causing an increase in the enzyme substrate collision numbers, interactions, and also producing much higher activation energy for the reacted molecules to drive the reaction faster (Satyanarayana and Chakrapani 2007; Segel 1968).

The optimum temperature in the majority of enzymes lies between 40°C to 45°C. Some enzymes make an exception for these numbers since they are able to keep their activities for the catalyzing reactions to up to 100°C. These enzymes like (Venom phosphokinase, muscle adenylate kinase). Plant enzymes, because of their stable structures and conformations have the optimum temperatures of about 60°C. However, exposing most of the enzymes to elevated temperatures above 50°C make them to denature, that is the decomposition of the native tertiary structure of the polypeptide and the active sites on the enzymes. Most of the enzymes lose their activities at temperatures of about 70°C and higher (Satyanarayana and Chakrapani 2007).

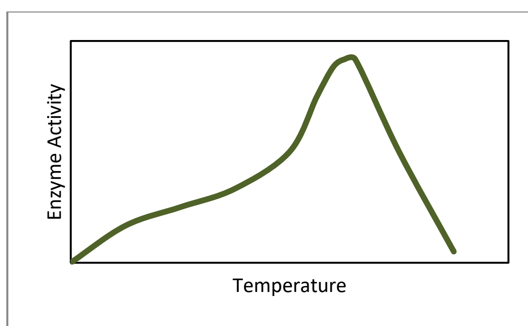

Figure 1.3. The effect of temperature on the activity of enzymes: elevating temperatures increase the enzymes activity to a specific extend when higher temperatures cause denaturation to the protein structure of the enzymes

The pH value as well effectively causes the activity of the enzymes to change. Each enzyme has a pH value in which its maximum velocity of the reaction achieved, this value referred as an optimal pH value in which any deviations from this optimal point cause a decrease in the enzyme's activity. Also, exposing enzymes to extremely acidic or basic mediums completely deactivates the enzymes (Satyanarayana and Chakrapani 2007).

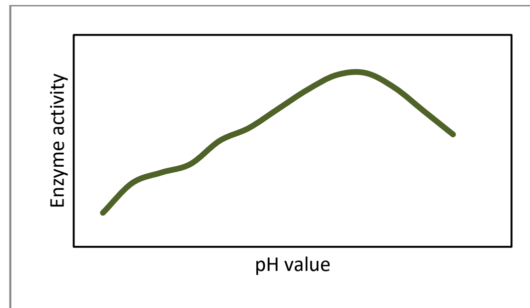

Figure 1.4.The effect of pH on the activity of enzymes: changing the pH range of the reaction medium alters the enzyme's activity

A lot of higher organism enzymes exhibit their maximum activities at pH values of about 6-8, neutral pH values. The way pH value works in reaction mediums is by making a change in charges of the ionic states of amino acids, especially the active sites, substrates and E-S complex.

Moreover the product concentration indicates how much enzyme activity should be exhibit. Generally if there were too many products, then the enzyme's activity would be decrease. This works in different ways for different enzymes. A group of enzyme active sites interacts with the product in ways that inhibit the enzyme-substrate interactions and cause the production to be decreased or stopped. Another way of enzyme inhibition is caused by a feedback which is referred as allosteric regulation of enzymes (Satyanarayana and Chakrapani 2007; Segel 1968).

Some of the enzymes need metallic cations for achieving their maximum activities like Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Na^{+} and K^{+}; and in some cases anions role for activating the enzymes like Cl^{-} for the amylose enzyme. These metallic activators work through various mechanisms from binding with the substrates, forming enzyme substrate metal complex, direct participations through the reaction and making conformational changes in the enzymes. The enzymes which require metals for their activity are separated into two categories: metal activated enzymes (enzymes that are activated by metals) which are not tightly bound to the metals and a metal could be exchanged readily by other ions. The example within this category is ATPase (Mg^{2+}, Ca^{2+}) and enolase Mg^{2+}. However, the second category is the metalloenzymes in which the bound between the metal and the enzyme is much stronger that makes them stable and non-exchangeable

easily. Examples of this type are alcohol dehydrogenase, alkaline phosphatase, carboxy peptidase and aldolase that contain zinc, phenol oxidase copper, pyruvate oxidase (manganese), xanthine oxidase (molybdenum), cytochrome oxidase (iron and copper), (Satyanarayana and Chakrapani 2007).

When the ideal circumstances are available for enzymatic reactions, the amount of time required for the reaction to occur is much less than the absence of the ideal circumstances. If pH value or temp changed for a reaction, then the time required for that reaction changes as well, most of the times increases.

The enzymes are inactivated if they expose to ultraviolet, beta, gamma and X-rays. This deactivation happens because of peroxidase formation, an example of the enzyme deactivation is the inhibition of salivary amylase activity by exposing it to the UV rays (Satyanarayana and Chakrapani 2007).

1.7. Pentose Phosphate Pathway

Hexose monophosphate shunt is a more complicated pathway than glycolysis in which the reaction of three molecules of glucose 6-phosphate produce three molecules of CO_2 with three molecules of five carbon sugars. By the end of the reaction processes two molecules of glucose 6-phosphates are reformed alongside with a glycolytic intermediate molecule, which is glyceraldehyde 3-phosphate. This pathway is considered as a way for completion of glucose oxidation; because, two molecules of the glyceraldehyde 3-phosphates are reform in a glucose 6-phosphate molecule (Murray et al. 2003).

The PPP had been first introduced in 1930s by the German biochemist Otto Warburg, who announced the NAD^+s function as an electron carrier. This finding led to the identification of $NADP^+$ coenzyme that take role in the conversion of the glucose to the G6-P by the G6PD. The studies through three decades had been conducted by Arthur Kornberg, Frank Dickens, Terry Wood, Fritz Lipmann, Hans Klenow, Severo Ochoa and so many others resulted in the presentation of the basic version of processes in the pathway in 1955 (Stincone et al. 2015).

Pentose phosphate pathway takes place in the cytoplasm of the cells since the enzymes in which catalyze the reactions of the PPP process are cytosolic enzymes. Here in PPP $NADP^+$ is used for dehydrogenation as the hydrogen atom acceptor. The reactions in this pathway are divided into two phases (irreversible oxidative phase, reversible non oxidative phase). First phase is the phase of dehydrogenation and decarboxylation of glucose 6-phosphate that yields a pentose sugar (ribulose 5-phosphate). Second phase is converting of ribulose 5-phosphate to glucose 6-phosphate by a series of reactions catalyzed by (trans ketolase and trans aldolase) enzymes (Murray et al. 2003).

The disturbances in the PPP were linked to human disorders containing Alzheimer's disease, parasite infections, cancer developing and metabolic syndromes (Stincone et al. 2015).

PPP considered as one of the basic metabolic processes within the cell, it balances the carbon amounts within the cells, which participate in biosynthesizing the amino acids and nucleotides in order to prevent the oxidation effects of the cells and the anabolism to be continued (Stincone et al. 2015).

1.7.1. The oxidative phase (generating of NADPH)

The reaction of glucose 6-phosphate to 6-phosphogluconate passes through a transition state which is contains the formation of 6-phosphogluconolactone, this reaction is catalyzed by an $NADP^+$ dependent enzyme which is glucose 6-phosphate dehydrogenase (G6PD). This 6-phosphogluconolactone is hydrolyzed by gluconolactone hydrolase enzyme. 6-phosphogluconate dehydrogenase enzyme is responsible for the second oxidation stage in the pentose phosphate pathway which is another $NADP^+$ dependent enzyme that donates its H^+ to the $NADP^+$. The process of decarboxylation continues until the formation of the ketopentose ribulose 5-phosphate is accomplished. A glucose 6-phosphate dehydrogenase enzyme in an endoplasmic reticulum hexose 6-phosphate dehydrogenase provides the required amount of NADPH that is needed for hydroxylation reactions.

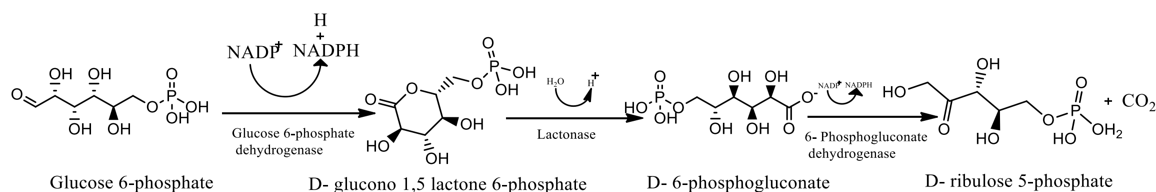

Figure 1.5. The oxidative phase of pentose phosphate pathway

1.7.2. The non-oxidative phase (generating ribose precursors)

Two enzymes use ribulose 5-phosphate as a substrate for their reactions, which are ribulose 5-phosphate -3-epimerase that changes the third carbon configuration and makes the xylulose 5-phosphate. And ribose 5-phosphate ketoisomerase that in turn catalyzes the reaction of converting ribulose 5-phosphate to its aldehyde form aldo-pentose (ribose 5-phosphate). This ribose 5-phosphate is used in synthesizing of nucleotides and nucleic acids. Transketolase converts the first and the second carbons from a ketose sugar to the aldose sugar. As a result, two carbons of the aldose sugar would be decreased. The Mg^{2+} and thiamin diphosphate (Vitamin B1) are functioning as coenzymes in the reaction.

Transformation of the two carbon units of xylulose 5-phosphate to ribose 5-phosphate is catalyzed by trans ketolase. Later this reaction produces a sedoheptulose 7-phosphate which is a seven carbon ketose, with a three carbon glyceraldehyde 3-phosphate. These two products undergo transaldolation (trans aldolase). The transformation of three carbons of dihydroxyl acetone from the ketose sedoheptulose 7-phosphate to aldose glyceraldehyde 3-phosphate, forming a fructose 6-phosphate (ketose) and a four carbon aldose erythrose 4-phosphate, are catalyzed by trans aldolase. Later through a reaction catalyzed by transketolase the xylulose 5-phosphate will give two carbon atom compound to the erythrose 4-phosphate and fructose 6-phosphate with glyceraldehyde 3-phosphates are produced. For guaranteeing the complete oxidation of the glucose to CO_2 through the PPP, the fructose 1,6-biphosphatase enzyme which is responsible for conversion of glyceraldehyde 3-phosphate to glucose 6-phosphate should be available. This process is the reverse to glycolysis and is called gluconeogenesis (Murray et al. 2003).

1.8. The 6PGD enzyme

6-phospho-D-gluconate-nicotinamide adenine dinucleotide phosphate-oxidoreductase decarboxylation (6PGD) enzyme commission number E.C 1.1.1.44 is the third enzyme of the pentose phosphate pathway. It is responsible for the conversion of 6-phosphogluconic acid 6PGA to D-ribulose 5-phosphate and CO_2 with existing of $NADP^+$ coenzyme (Beydemir et al. 2004).

The enzyme is located in the cytoplasm of all living cells, including microorganisms, plants and animal cells (Toews et al. 1976).

The 6PGD enzyme has a dimeric structure and from all of its species is depend on the $NADP^+$ as a coenzyme for its activity (Pearse and Rosemeyer 1974). Each one of the enzyme's subunits has three domains for attaching to the coenzyme $NADP^+$, the α domain from β-α-β domains has the most of the interface of the enzymes dimer (Adams et al. 1991), a monomer from the dimeric structure of the sheep liver 6PGD enzyme has a molecular weight of 51 kDa that independently acts on its substrate (Villet and Dalziel 1972; Silverberg and Dalziel 1973).

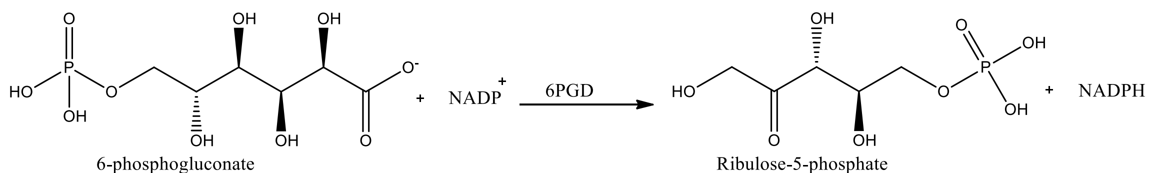

Figure 1.6. The reaction catalyzed by 6PGD enzyme in the oxidative phase of the PPP

In the enzyme's reaction, NADPH is also produced. It has the function of protecting cells against oxidizing agents by producing the reduced glutathione GSH (Bianchi et al. 2001; Nelson and Cox 2005). Besides that, NADPH plays a role in producing of many biomolecules as fatty acids, steroids and some amino acids (Human et al. 1983; Srivastava and Beutler 1970). Due to its functions 6PGD could be called as an anti-oxidant enzyme. In cases of totally absence or partially deficient 6PGD with maintaining the normal range of G6PD enzyme, also in the absence of G6PD too, the production levels of NADPH would be less than normal. In some cases no NADPH productions would be observed at all. This condition causes an increase of the erythrocyte's

sensitivity towards the activity of the hemolytic oxidative agents and leads to damaging of the cells.

The 6PGD enzyme activity is linked to disorders like cancer and Alzheimer (He et al. 2007).

The enzyme's kinetic properties, its role in the pentose phosphate pathway, regulations of the enzyme, genetics and the enzyme's role for the production of fatty acids had been studied for long time (Zera et al. 2014).

The 6PGD enzyme from the prokaryotic and eukaryotic microorganisms sequenced, the result showed it had 468 amino acids. The arrangement of the protein amino acids is almost similar in the prokaryotic and eukaryotic microorganisms, reversibly the ovine 6PGD that comprises of 466 amino acids shows the central region on the protein to be alike of those of bacterial enzyme but different at the protein C- and N- terminals (Reizer et al. 1991).

6PGD can be targeted in order to decrease the cell proliferations of cancer cells while lowering the enzyme's activity by using inhibitors like liver kinase B1- Activated protein kinase pathway (LKB1-AMPK) that showed non toxicity effects and did not caused a decrease to the NADPH levels in the blood. At the same time the (LKB1-AMPK) caused the tumor growth to be attenuated (Lin et al. 2015).

The sequences of the amino acids for 6PGD enzymes from about 40 various sources had been reported containing pig (Harbitz et al. 1990), *Bacillus subtilis* (Fujita and Fujita 1987), sheep (Carnes and Walker 1983), human (Kwok et al. 1996) and *Escherichia coli* (Nasoff et al. 1984). The three-dimensional enzyme structure for sheep's liver enzyme is the only one that was determined at a resolution of 2.5 A° (Adams et al. 1983).

The sequencing results for the 6PGD enzyme from 37 various sources showed that the highest important regions of the enzyme's structure like the active site, in which the enzymes functions of binding to the substrates and coenzymes are happen, is located in conserved regions in the enzyme's structure. In addition some other regions of the enzymes structures from all different species showed a big similarity in their locations (Goulielmos et al. 2004).

2. LITERATURE REVIEW

6-phosphogluconate dehydrogenase (6PGD) enzyme is the third enzyme of the pentose phosphate pathway. It has been intensively investigated by biochemists for 70 years. Such attention came from the enzyme's critical role in pentose phosphate pathway process. The enzyme was purified from different sources. This began in 1951 and continued until the recent study. The enzyme was isolated from *Saccaromyces cerevisia* (Horecker and Smyriniotis 1951), *Candida wills* (Pontremoli et al. 1961), *Drosphila melanogaster* (Kazazian et al. 1965), *Bacillus stearothermophilus* (Pearse and Harris 1973) *Neurospora* (Scott and Abramsky 1973). The first purification of the enzyme from a mammalian tissue was from sheep's liver tissue (Villet and Dalziel 1969), later from human erythrocytes (Pearse and Rosemeyer 1974), rabbit's mammary gland (Betts and Mayer 1975), human brain (Weisz et al.1985), *Dicentrarchus labrax L.* liver (Medina-puerta and Garrido-pertierra 1986), from the maize's cytoplasm (Bailey-Serres and Nguyen 1992), rat's erythrocytes (Beydemir et al. 2004), Van's lake fish liver (Guler 2010), rainbow trout *(Oncorhynchus mykiss)* (Taranci 2011), chicken's liver (Erat and Sakiroglu 2007), rat's kidney (Adem and Çiftci 2012) and yellow catfish (Zhuo et al. 2015). Here is a review of what information obtained from these all researches regarding this specific enzyme by time from 1950s to nowadays with a summary of purification techniques and methods that used for the enzyme.

The M.Ws of the 6PGD enzymes purified from various sources were different in their sizes. Sheep's liver 6PGD enzyme had M.W of 129 kDa (Villet and Dalziel 1969); *Dicentrarchus labrax L.* liver had 100 kDa (Medina-puerta and Garrido-pertierra 1986); rat's erythrocytes 6PGD had 59.5 kDa (Beydemir et al. 2004); rat kidney's enzyme had 56 kDa (Adem and Çiftci 2012); yellow catfish 6PGD enzyme had 50.1 kDa (Zhuo et al. 2015); *Bacillus stearothermophilus* had a 100 kDa 6PGD enzyme (Pearse and Harris 1973); maize's cytoplasm 6PGD enzyme had 55 kDa

(Bailey-Serres and Nguyen 1992); the enzyme from *Neurospora* had the molecular weight of 110-112 kDa (Scott and Abramsky 1973), Van's Lake Fish liver enzyme had 57 kDa (Guler 2010); rainbow trout *(Oncorhynchus mykiss)* erythrocyte's enzyme had a molecular weight of 58.5 kDa (Taranci 2011); rat lung's enzyme had 107 kDa (Adem and Çiftci 2016); and from the rat erythrocytes, the enzyme had 111 kDa (Beydemir et al. 2004).

The methods used for the enzyme's purification from different sources were different. The enzyme of sheep's liver had been purified using ultracentrifugation, DEAE Cellulose and CM- Cellulose ion exchange chromatography methods (Villet and Dalziel 1969); the 6PGD enzyme of *Bacillus stearo thermophillus* purified by hydroxyl apatite, DEAE-Sephadex and CM Sephadex ion exchange chromatography (Pearse and Rosemeyer 1974). The ammonium sulfate precipitations and DEAE cellulose ion exchange chromatography methods had been used for purification of 6PGD enzyme from *Neurospora's* (Scott and Abramsky 1973). The 6PGD from human erythrocytes had been purified by DEAE Sephadex and CM Sephadex ion exchange chromatography methods (Pearse and Rosemeyer 1974); 6PGD enzyme of *Dicentrarchus labrax L.* liver's had been purified by the gel filtration chromatography, Sephacryl S-200 ion exchange chromatograph and 2', 5'-ADP Sepharose 4B affinity chromatography to near homogeneity (Medina-puerta and Garrido-pertierra 1986). Ammonium sulfate precipitation, DEAE cellulose ion exchange chromatography and 2', 5'-ADP agarose affinity chromatography methods had been used for the purification of the enzyme from the cytoplasm of maize's root cells (Bailey-Serres and Nguyen 1992). For the erythrocytes enzyme of rat, ultracentrifugation, ammonium sulfate precipitation and 2', 5' ADP Sepharose 4B affinity gel chromatography methods had been used (Beydemir et al. 2004); also 2', 5'-ADP Sepharose 4B affinity gel chromatography method used for rat's kidneys, yellow catfish's, human erythrocytes, Van's lake fish liver, rainbow trout's *(Oncorhynchus mykiss)* erythrocytes enzyme and the chicken's liver 6PGD enzyme purifications (Adem and Çiftci 2012; Zhuo et al. 2015; Aykuz et al. 2004; Guler 2010; Taranci 2011; Erat and Sakiroglu 2007).

Tests had been made throughout the literature for 6PGD enzyme in order to determine the best pH conditions for the enzyme to do its catalytic functions. The results of some of them were shown as following: the optimum pH value for the Van's cat erythrocytes was

at pH 8.0 using 0.1 M Tris-HCl (Kiliç 2007); rat's erythrocyte 6PGD enzyme had its optimal pH value using Tris-HCl pH 7.0 (Beydemir et al. 2004). The liver and kidneys enzymes of rats had their maximum enzyme activities at pH 8.0 (Corpas et al. 1995); Van's lake fish liver enzyme had its optimum activity at pH 8.0-9.0 (Guler 2010). The optimum pH value for rainbow trout's *(Oncorhynchus mykiss)* 6PGD was at 7.5, and the most stable pH value for the 6PGD enzyme was at pH 7.0 (Taranci 2011) and the 6PGD enzyme from yellow catfish's (*Pelteobagrus fulvidraco*) liver had the optimum pH value at 7.85 (Zhuo et al. 2015).

Experiments carried out for the determination of the optimum temperature for 6PGD enzyme activity from different sources showed a range of temperatures starting from 45℃ to 60℃. The optimum temperature of rat's erythrocytes 6PGD enzyme was at 45℃ (Beydemir et al. 2004). Yellow catfish (*Pelteobagrus fulvidraco*) hepatic 6PGD enzyme's optimum temperature was 60℃ (Zhuo et al. 2015). The parsley 6PGD obtained highest activity at 50℃ (Demir et al. 2003). Chicken's liver 6PGD best activity was at 60℃ (Erat 2005); Van's lake fish liver optimum temperature was between 35 to 40℃ (Guler 2010); the rainbow trout *(Oncorhynchus mykiss)* erythrocyte's enzyme optimum temperature was at 40℃ (Taranci 2011); and Van's cat erythrocytes 6PGD enzymes optimum temperature was 50℃ (Kiliç 2007).

The affinity of 6PGD enzymes towards each of the 6PGA and $NADP^+$ had been calculated from different studies and various sources of the enzyme. The Van's cat erythrocytes had K_M and V_{max} values for $NADP^+$ as 0.048 M and 0.158 EU/mg respectively, and for 6PGA as 0.351M and 0.162 EU/mg (Kiliç 2007). The K_M and V_{max} values for $NADP^+$ and 6PGA were found for erythrocyte 6PGD enzyme from rainbow trout *(Oncorhynchus mykiss)* as 0.00279 mM, 0.0417 EU/mg, 0.0207 mM and 0.257 EU/mg respectively (Taranci 2011). The yellow catfish liver's 6PGD enzyme K_M values were founded as 169.3 µM and 91.1 µM for 6PGA and $NADP^+$ respectively and V_{max} values as 51.109 EU/mg and 6.6776 EU/mg respectively for 6PGA and $NADP^+$ (Zhuo et al. 2015), the K_M value for *E. coli* bacteria's 6PGD enzyme was calculated as 11µM and 10 µM for both 6PGA and $NADP^+$ respectively (Wang and Zhang 2009), rat's liver 6PGD enzyme had its own K_M values for $NADP^+$ 0.258 M and for 6PGA 0.157 M; for rat's kidneys the K_M value for $NADP^+$ founded as 0.056 M and for 6PGA as 0.049 M (Corpas et al. 1995) and for the rat's erythrocytes enzymes the K_M and V_{max} values had

been calculated as 0.059M and 0.063 EU/mg for $NADP^+$ and 0.194 M and 0.054 EU/mg for 6PGA (Beydemir et al. 2004).

An enzyme activity unit of 6PGD can be defined as the amount of the reduction rate of $NADP^+$ to NADPH in µmol/min and a specific activity of an enzyme could be defined as EU/mg of protein (Villet and Dalziel 1969).

The response of the 6PGD enzyme from different source towards some antibiotics, drugs, heavy metals and some organic compounds had been taken under investigations by researchers so as to show if they exhibit inhibition effects on the activity of the enzyme. Some of them showed inhibitory effects on the enzyme's activity such as antibiotics netilmicin sulfate, cefipime, amikacin, isepamycin, chloramphenicol, ceftazidim, teicoplanin, ampicillin, oflaxacin, levofloxacin, cefotaxime, penicillin, gentamicin sulfate, ciprofloxacin, that showed an inhibitory effect of the human erythrocytes enzyme activity *in vitro* (Aykuz et al. 2004). The larnoxicam, metronidazole, imipenem, ornidazole, vanequycin, clindamycin and amoxicillin drugs caused a decrease in the enzyme activity *in vitro* from human erythrocytes (Adem and Ciftci 2007). The rat lungs enzyme activity had been inhibited by the levofloxacin, furosemide, ceftazidime, cefuroxime and gentamicin *in vitro* and levofloxacin *in vivo* (Adem and Ciftci 2016). The erythrocytes 6PGD enzyme from rat had been inhibited by lead acetate, while lead acetate with naringenin and naringenin did not affect the enzyme activity *in vivo* (Demirdag et al. 2015). The enzyme from chicken liver had been inhibited *in vitro* by aflatoxin non-competitively and by cefepime competitively. However, cefazolin and ampicillin did not caused any change on the enzyme's activity (Erat and Sakiroglu 2007). ATP competitively inhibited the 6PGD enzyme from rat's liver while NADPH and NADH non competitively inhibited the activity of the rat's erythrocytes 6PGD enzyme (Beydemir et al. 2004), and nicotine caused an activation of muscle, lungs and testicular 6PGD enzyme of rats with no effect of heart and liver's enzyme *in vivo* while nicotine with vitamin E activated the muscle, testicles and liver enzymes with no observed effect on heart and lungs enzymes of rat *in vivo*.

A study suggested that the effect of vitamin E on the 6PGD enzyme is increasing the enzyme's activity in liver tissues of nicotine administrated rats (Ciftci et al. 2008). Even though phenolic compounds (polyphenols) are not harmful compounds, yet they

exhibited some inhibitory characteristics against the activity of 6PGD human erythrocytes enzyme *in vitro*. The effect of 12 phenolic compounds was checked on the 6PGD enzyme activity. The activity of the 6PGD enzyme had been inhibited by narengenin, caffeic acid, ellagic acid, ferullic acid and sinapic acid. However, chlorogenic acid, coumaric acid and syringic acids hadn't showed any inhibitory effects (Adem et al. 2014). The heavy metals such as cadmium, copper, mercury, iron, aluminum, zinc, silver and nickel exhibited some inhibitions on the 6PGD enzyme activity of rainbow trout's *(Oncorhynchus mykiss)* erythrocytes enzyme (Taranci 2011).

The ionic strength effect on the 6PGD enzyme activity from different sources was investigated. The optimum ionic strength for 6PGD from Van's lake fish liver was at 0.02 M ammonium sulfate salt (Guler 2010). The rainbow trout *(Oncorhynchus mykiss)* erythrocyte's enzyme had its optimum ionic strength at 0.8 mM salt concentration (Taranci 2011).

The enzymes in thermophilic microorganisms have a very important role in molecular biology and industrial applications. The gene which is responsible for coding of the thermostable 6PGD from the hyperthermophilic bacteria (*Thermotogamaritima)* had been cloned and overexpressed in an *E-coli* bacterium successfully. The expressed 6PGD enzyme in *E.coli* kept its primary biochemical characteristics and had the ability to produce NADPH as in its origin bacterium (Wang and Zhang 2009).

A useful, easy, fast and sensitive fluorimetric micro-technique method was used for measuring the activity of 6PGD enzyme in human erythrocytes. The technique based on the natural fluorescent emissions that emitted by NADPH during the enzymatic catalytic reaction that convert $NADP^+$ to NADPH by the catalytic activity of 6PGD. The method provides a better precision and more sensitivity in comparing with spectrophotometric analysis. It needs small quantities of samples, substrates and coenzymes in the experiment (Tan and Whitehead 1969).

The effect of 6PGD enzyme in development of cancer cells had been taken under investigations, and a study conducted on the role of the 6PGD enzyme in cancer development showed that by suppressing the 6PGD enzyme in cancer cells the process of RNA biosynthesis, lipogenesis are decreased, and ROS levels are increased. This change

decreased the proliferation and growth of tumor cells. The 6PGD could be considered a possible anti-cancer target (Lin et al. 2015). The ribulose 5-phosphate production by catalytic effect of 6PGD causes the activation of the AMPK inhibition process. This inhibition happens because of changing the active complex of LKB1. This in turn leads to acetyl coA carboxylase 1 and lipogenesis process to be activated. A new link was founded in the study in between the oxidative pentose phosphate pathway and lipogenesis by inhibition effects of LKB1-AMPK signaling on Ru5-P. Also new powerful inhibitors to the 6PGD enzyme activity were suggested that restricted the enzyme activity without any clear toxic effects to the nude mice xenografts in which used for the study. These effective inhibitors were physicion with S3 derivatives (Lin et al. 2015).

The oxidative pentose phosphate pathway has a significant role in the tumor growth process. The 6PGD enzyme is mostly activated due to acetylation of lysine in epidermal growth factor stimulated cells and in human cancer cells. Acetylation process of K76 and K294 enhances the formation of an active dimer of 6PGD enzyme and the affinity of 6PGD enzyme to bind to $NADP^+$. Cancer cell proliferation is significantly reduced by decreasing the amount of acetyl mutants of 6PGD. The decreasing of tumor growth and cell proliferations are caused by the ribulose 5-phosphate that produced from the 6PGD catalyzed reaction and from the lacking of the NADPH formation from the whole oxidative phase pentose phosphate pathway. The non-sufficient NADPH caused the RNA and lipid biosynthesis to be decreased with marked elevations of ROS. The activity of 6PGD enzyme could be controlled in primary leukemia by elevating the lysine acetylation process. That could be used as a potential path for 6PGD control in cancer cells (Shan et al. 2014).

The causes of 6PGD enzymatic deficiencies in human had been investigated and searched for. The deficiencies of 6PGD enzyme in some patient red blood cells (RBC) are related to a genetic disorder which is silence PGD° allele. The factors causing this disorder include the lack of specific protein synthesis or synthesis of unstable proteins and the deletions in the responsible gene for protein-coding enzyme as the strongest factor. Finding such deletion would be a difficult task unless some major chromosomal deviations happened. By using some chromosomal analysis methods these deletions could be notable (McKusick and Ruddle 1977). Even small deletions could be found by searching for the availability of markers that are closely linked to the targeted genes in

certain cases by using chromosome maps. The gene codes for the 6PGD enzyme in human is located on the first chromosome on the P arm and there are some related and closely linked markers to it that make the search for even small deletions in the PGD° allele possible (Ajmar et al. 1979).

The structure of the 6PGD enzyme had been investigated. A study conducted within the 6PGD enzyme crystal structure showed that the enzyme has significant differences in nicotinamide mononucleotide (NMN) part of the $NADP^+$ and NADPH. In the reaction of the complexed oxidized coenzyme with the methionine (Met_{13}) side chain, the reaction happened from the Si face of the $NADP^+$. This interaction affects the reaction type between $NADP^+$'s nicotinamide rings and methionine side chain. This change causes a variable effect in the catalytic activities of the 6PGD enzyme. In order to see this effect, the Met_{13} residues were converted into different other residues like V.I.C.F and Q, in a site direction mutagenesis process. The result of this mutation caused a decrease in the activity of enzyme by decreasing the affinity of the enzyme towards the $NADP^+$. However, its affinity towards NADPH remained unchanged. Among other factors, Met_{13} is an important factor of the catalytic reactions direction mechanism of the 6PGD with other factors like the geometrical, shape, and space to the third carbon of 6-phosphogluconate ring (Cervellati et al. 2005).

3. MATERIAL AND METHOD

3.1. Material

3.1.1. Chemicals

The chemicals used for conducting all of the experiments throughout this study were all analytical graded from Sigma, Merck, Fishcer Scientific, ChemSolute bio and Applichem and they are listed as follows:

From Sigma: ammonium sulfate, magnesium chloride, sodium chloride, sodium bicarbonate, sodium hydroxide, potassium phosphate, potassium chloride, EDTA ethylene diamine tetra acetic acid, tris (trihydroxy methyl amino methane), isopropanol, β-mercaptoethanol, acrylamide, TEMED (N, N, N, N tetramethylethylenediamine), silver nitrate, hydrochloric acid, phosphoric acid, glycerine, ethanol, methanol, acetic acid, bovine serum albumin, 6PGA (6-phosphogluconate monosodium salt), 2', 5'-ADP Sepharose 4B. From Merck: potassium acetate, bromothymol blue, sodium acetate, potassium hydroxide, glycine amino acid and trichloroacetic acid. From Fishcer Scientific: SDS (sodium dodecyl sulfate), Commasie Brilliant Blue G-250, Brilliant Blue R-250 electrophoresis grade. From the Applichem: $NADP^+$ and from the ChemSolute Bio: ammonium persulfate.

3.1.2. Instruments

The instruments used for conducting all experiments throughout the whole study are listed as follows:

Clinical centrifuge	: (Model Universal 320 R, Hettich) and (Allegra X-30R Beckman Coulter)
Spectrophotometer	: (Shimadzu Model CC-10) UV 180

pH meter : (Thermo Orion 3 stars)
Electrophoresis tank : (Bio Rad)
Electrophoresis power supply : (Bio Rad)
Peristatic pump : (Bio instruments ATTA SJ-1220) and (P Spectra J.P. Selecta, s.a.)
Vortex : (Lab Companion SK 300)
Sensitive balance : (Denver instrument SI 234)
Column chromatography : Sigma Aldrich
Automatic pipette : (Transferpette, Eppendorf research)
Ice maker : (Hoshzaki ice maker FM-80EE)
Refrigerator, Hot plate stirrer, Water bath and Ice bath

3.1.3. Preparation of used solutions

The solutions used during conducting experiments throughout this study and their purposes of using them are listed as follows:

3.1.3.1. Hemolysate preparation solution

1. Preparation of the red blood cells (RBC) washing solution with 0.16 M KCl solution; 11.92 g (0.16 mol) of KCl was dissolved in a small amount of distilled water (D.W) then the volume was completed to 1L with D.W.

2. Preparation of ice water solution; 100 mL of D.W placed in the refrigerator until it partially froze then used for the hemolysate preparation by adding 4 volumes of the ice water to one volume of the packed RBCs.

3.1.3.2. Enzyme activity measurement solutions

1. 500 mM Tris-HCl/ 5 mM EDTA (pH 8.0) enzyme activity measurement buffer solution; 3.028 g of Tris (50 mmol) and 0.0292 g (0.1 mmol) EDTA were dissolved in 80 mL of D.W, then the pH adjusted to 8.0 with diluted HCl and the volume was completed to 100 mL with D.W.

2. Preparation of 6 mM of the 6PGA substrate solution used for measuring enzyme activity; 0.091 g (0.3 mmol) of 6PGA were dissolved in 40 mL of D.W then the volume was completed to 50 mL with D.W.

3. Preparation of 2 mM $NADP^+$ solution; 0.0795 g (0.1 mmol) $NADP^+$ was dissolved in 40 mL D.W then the volume was completed to 50 mL with D.W.

4. Preparation of 50 mM KH_2PO_4 pH 8.0; 0.68 g (5 mmol) KH_2PO_4 was dissolved in 80 mL of D.W, then the pH adjusted to 8.0 with diluted HCl and KOH later the volume was completed to 100 mL with D.W.

3.1.3.3. Preparation of affinity gel chromatography solutions

1. Preparation of 0.1 M potassium acetate/ 0.1 M potassium phosphate pH 6.0 buffer used for balancing and washing the affinity column; 9.8 g potassium acetate (0.1 mol) with 13.6 g potassium phosphate (0.1 mol) were dissolved in 800 mL of D.W then the pH adjusted to 7.3 and the volume was completed to 1L by D.W.

2. Preparation of 0.1 M potassium phosphate/0.1 M KCl pH 7.3 buffer used for loading samples to the affinity column and washing the affinity column; 13.6 g potassium phosphate (0.1 mol) with 7.45 g (0.1 mol) KCl were dissolved in 800 mL of D.W then the pH adjusted to 7.3 by a diluted HCl and KOH solution, then the volume was completed to 1L by D.W.

3. Preparation of washing buffer solution for column chromatography; 0.1M potassium phosphate/ 0.1 M potassium acetate pH 7.3, 9.8 g of potassium acetate (0.1 mol) and 13.6 g /potassium phosphate (0.1 mol) were dissolved in 800 mL of D.W, the pH adjusted to 7.3 by using diluted HCl and KOH solution then the volume was completed to 1L by D.W.

4. Preparation of elution buffer for 6PGD enzyme from the affinity gel column; 80 mM potassium phosphate, 80 mM KCl, 0.5 mM $NADP^+$ and 10 mM EDTA pH 7.3, 5.44 g potassium phosphate (0.04 mol) 2.98 g KCl (0.04 mol) 0.1913 g $NADP^+$ (0.025 mol) and 1.21 g EDTA (0.003 mol) were mixed and dissolved in

400 mL D.W then the pH adjusted to 7.3 and the volume was completed to 500 mL.

5. Preparation of the first regeneration buffer for washing the column chromatography; 6.05 g tris (0.05 mol) and 14.61 g NaCl (0.25 mol) were dissolved in 400 mL of D.W, then the pH adjusted to 8.5 and the volume was completed to 500 mL by D.W.

6. Preparation of the second regeneration buffer for washing the column chromatography; 0.1 M sodium acetate/ 0.5 M NaCl pH 4.5, 14.61 g NaCl (0.25 mol) and 4.1 g sodium acetate (0.05 mol) were dissolved in 400 mL of D.W, then the pH adjusted to 4.5 and the volume was completed to 500 mL by D.W.

3.1.3.4. Preparation of protein determination solutions

3.1.3.4.1. Bradford's reagent

1. 0.1 g of Coomassie Brilliant Blue G-250 was dissolved in 50 mL of 50% ethanol then the solution diluted to 100 mL by concentrated 85% H_2SO_4 solution, and volume completed to 250 mL by double distilled water (DDW). The solution labeled as stock solution. This stock solution should be prepared four weeks before using, and remains stable for a long time if kept in 2-8°C. Working reagent (W.R) was prepared from the stock solution by filtering and diluting it with 1:4 volumes of DDW.

2. Standard protein solution is prepared by dissolving 5.0 mg/mL of BSA in 0.1 w/v% SDS solution.

3.1.3.5. Solutions used for SDS-PAGE

1- Preparation of 1 M Tris-HCl pH 8.8 solution; 12.11 g of tris was dissolved in 80 mL of D.W. The pH adjusted to 8.8 and the volume was completed to 100 mL with D.W.

2- Preparation of 1 M Tris-HCl pH 6.8 solution; 12.11 g of tris was dissolved in 80 mL of D.W. The pH adjusted to 6.8 and the volume was completed to 100 mL with D.W.

3- Preparation of 30% acrylamide - 0.8% bisacrylamide solution; 15 g of Acrylamide, 0.4 g bisacrylamide and 34.6 g of D.W mixed to form 50 mL mixture solution.

4- Preparation of 10% ammonium persulfate solution; 1 g of ammonium persulfate is dissolved in 10 mL of D.W.

5- Preparation of 10% sodium dodecyl sulfate (SDS) solution; 1 g of SDS is mixed with 9 g of D.W to form 10 mL 10% SDS solution.

6- Preparation of running buffer for SDS-PAGE; 1.51 g of tris, 7.51 g of glycine was mixed and dissolved in 450 mL of D.W and then 5 mL of 10% SDS was added to the mixture, the pH adjusted to 8.3 and the volume was completed to 500 mL with D.W.

7- Preparation of the sample buffer; 5 mL from 1 M Tris-HCl solution, 1 mL from 10% SDS solution, 1 mL from 100% glycerin solution, 1 mL from bromothymol blue solution were added into a tube and the volume was completed to 10 mL by D.W, before using this buffer for each 950 µl of the buffer 50 µl of β-mercaptoethanol should be added.

8- Preparation of 0.04% bromothymol blue solution; 0.1 g of an indicator was dissolved in 16 mL of 1 M NaOH solution, and the volume was completed to 25 mL with D.W.

3.1.3.5.1. Staining with Coomassie Brilliant Blue R-250

1- Preparation of stabilization solution (a solution used for stabilizing the proteins in the gel); 50% isopropanol, 10% TCA and 40% of D.W mixed to form a stabilizing solution.

2- Preparation of the gel staining solution; 0.1 g Coomassie Brilliant Blue R-250 was dissolved in 50 mL of methanol, 10 mL of acetic acid and 40 mL of D.W to form 100 mL of the gel staining solution.

3- Preparation of the gel washing solution; 50 mL of ethanol, 10 mL of acetic acid were mixed with 40 mL of D.W to form 100 mL of the washing solution.

3.1.3.5.2. Staining the gel with silver nitrate

1- Preparation of the gel fixation solution to; 60 mL of D.W, 30 mL of 96% ethanol and 10 mL of acetic acid were mixed to form 100 mL of fixation solution.

2- Preparation of the gel reduction solution; 4.8 g sodium acetate, 45 mL of ethanol and 80 mL of D.W were mixed, and the mixture's pH adjusted with acetic acid to pH 6.0 then 150 mg of sodium thiosulfate and 3 mL of glutaraldehyde added to the mixture and finally the volume of the solution was completed to 150 mL with D.W.

3- Preparation of the dye solution; 150 mg of silver nitrate was mixed with 150 mL of D.W and 30 µL of formaldehyde to form the gel dye solution.

4- Preparation of the first washing solution; 3.75 g of sodium carbonate was dissolved in 270 mL of D.W and then 120 µL of formaldehyde added to the solution to form first washing solution used in the gel.

5- Preparation of the second washing solution; 1 mL of glycerin added to 250 mL of D.W containing 5% of acetic acid to form the second washing solution used in the gel.

3.1.3.5.3. Preparation of separation gel

5 mL of 1 M Tris-HCl pH 8.8, 4.4 mL of 30% acrylamide, 0.8% bisacrylamide, 0.61 mL 1% SDS, 0.4 mL of 5% TEMED and 11.94 mL of D.W all mixed up together and finally 0.8 mL of 1.5 % ammonium persulfate (PER) added. This gel should be freshly prepared for each electrophoresis process.

3.1.3.5.4. Preparation of stacking gel

2.24 mL of Tris-HCl pH 6.8, 1 mL of 30% acrylamide and 8% bisacrylamide, 0.1 mL of 10% SDS, 0.1 mL of 5% TEMED, 7.36 mL of D.W were all mixed into a container and lastly 0.2 mL of 1.5% PER is added to the gel.

3.2. Methods

The methods used for the experiments in this study are described below:

3.2.1. Protein determinations

The protein tests for the hemolysate and eluted purified 6PGD Japanese quail's erythrocytes were performed both qualitatively and quantitatively as described below:

3.2.1.1. Qualitative determination of proteins

The eluted fractions from the affinity column were tested for existence of proteins qualitatively. The absorbances of fractions were measured against the elution buffer as blank at 280 nm which is the maximum wavelength (λ_{max}) for tryptophan, phenyl alanine and tyrosin (Segel 1968).

3.2.1.2. Quantitative determination of proteins by Bradford's method

The Bradford assay protocol is considered as the least time-consuming methods among other quantitative methods for determination of proteins. The prepared solutions from section 3.1.3.4 were pipetted into previously labeled test tubes as in table (3.1). The range of measurements should be between 10 to 100 μg of proteins. After pipetting of all solutions and 10-minute incubation time at room temperature, the absorbance was measured at 595 nm against the blank, the blank solution mostly has a high absorbance but had no influence upon the measurement. After the reaction happens, the color remains stable for a long time. The standard curve was prepared by using various BSA concentrations against absorbance (Bradford 1976). Bovine serum albumin (BSA) concentration is 1 mg/mL, D.W is distilled water and W.R is working reagent.

Table 3.1. Preparation of the calibration curve for quantitative determinations of proteins using Bradford's method: incubate the test tubes for 10 minutes at room temperature and measure the absorbance at 595 nm against blank solution that has no standard proteins in it

	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**	**Tube 8**	**Tube 9**	**Tube10**
BSA	10 µl	20 µl	30 µl	40 µl	50 µl	60 µl	70 µl	80 µl	90 µl	100µl
D.W	90 µl	80 µl	70 µl	60 µl	50 µl	40 µl	30 µl	20 µl	10 µl	0 µl
W.R	4.9mL	4.9mL	4.9mL	4.9mL	4.9mL	4.9mL	4.9mL	4.9mL	4.9mL	4.9mL

3.2.2. Sample collection and preparing the hemolysate

Blood samples of the Japanese quail birds were obtained using decapitation method from the agriculture faculty's research farm of the Bingol University. Blood samples were kept at 4°C and transferred to the laboratory within EDTA containing tubes. In order to distinguish the erythrocyte from the plasma, blood samples were filtered to remove any impurities and equally distributed into eppendorf tubes then centrifuged at 2500 rpm at 4°C for 15 minutes. The plasma and leukocytes were removed using a dropper then the precipitated erythrocytes washed for three times by 0.16 M KCl solution and each time the solution centrifuged for 15 minutes at 2500 rpm at 4°C. Later in order to obtain the hemolysate, the RBCs were hemolyzed by using 1:5 volume of ice water, the hemolysate solution was mixed well to ensure the cell membrane decomposition. Then, for the removal of the cell debris in the hemolysate, the blood was centrifuged for 30 minutes at 4°C using a speed of 10000 g. After the centrifugation completed, the supernatant was isolated from the precipitate and used for further enzyme purification processes that include the quantitative protein determinations and loading the hemolysate to the 2', 5'-ADP Sepharose 4B affinity gel chromatography column (Ninfali et al. 1990).

3.2.3. 2', 5'-ADP Sepharose 4B affinity gel chromatography

For preparing the affinity gel, 2 g of solid 2', 5'-ADP Sepharose 4B were poured into a 10 mL (1×10 cm) volume tube, the substance washed repeatedly using 400 mL of distilled water (D.W) for removing any foreign materials in the gel if existed; during the washing process, the gel swelled up. The air bubbles removed inside the swelled gel by water trompe using a balancing homogenizing buffer (0.1 M potassium acetate/ 0.1 M

potassium phosphate pH 6.0). By the end of this process, the suspension of the gel was accomplished in 1×10 cm closed column system where the gel packed.

In the next step, the packed gel was washed using a balancing buffer and the flow rate controlled by a peristatic pump. The washing process done by using a setting of 20 mL/hour of the peristatic pump (Ninfali et al. 1990). For checking the gel in the column for homogenization with the balancing buffer, samples were taken from the top of the column and from the eluate as well and checked for their pH values. After the column was homogenized with the (pH 7.3) balancing buffer, the column would be ready for using. The prepared hemolysate from previous step loaded into the affinity column then washed repeatedly and respectively with 25 mL volume portions of 0.1 M potassium acetate/ 0.1 M potassium phosphate (pH 7.3). Then 25 mL of 0.1 M KCl / 0.1 M potassium phosphate pH 7.85 the flow rate set up fixed at 20 mL/hour by using the peristatic pump and temperature kept at 4℃. Through this method the majority of desired 6PGD enzyme attached to the 2', 5'-ADP Sepharose 4B affinity gel. The unwanted enzymes as long as other proteins removed from the gel and column while eluted readily from the column with the washing buffers. After removing all of the unwanted debris and proteins, an elution buffer containing 80 mM potassium phosphate/ 80 mM KCl + 5 mM $NADP^+$ and 10 mM EDTA pH 7.3, used to elute out the attached 6PGD enzyme from the gel in the column. The eluate should be collected in fractions using labeled eppendorf tubes, and each fraction should be tested for the enzyme's activity. The fraction containing highest enzyme activity indicates the existence of the highest amount of the 6PGD enzyme, and should be checked by SDS-PAGE for determining the purity of the purification process. The temperature controlled at 4℃ as well as flow rate at 20 mL/ hour during the entire purification process using the peristatic pump (Ninfali et al. 1990; Morelli et al. 1978).

The purified fraction of the Japanese quail erythrocyte's 6PGD enzyme used for the characterization and kinetic studies of the enzyme.

3.2.4. SDS-PAGE

The eluted 6PGD enzyme from the affinity column checked for determining of its purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis. 0.1% SDS containing

3% and 10% of acrylamide and bisacrylamide respectively were used in stacking gel and separation gels (Laemmli 1970).

At the beginning of the process, the instruments used in experiment were washed with distilled water and the glass plates washed with alcohol. Then the glass plates sat up correctly. Later, the separation gel containing 0.1% SDS poured through the glass and left over for 20 minutes for forming a stable gel layer. After the separation gel formed, the upper layer of the gel (stacking gel) containing 0.1% SDS also poured in to the glass plate, the comb was putted inside the stacking gel in order to make wells for the standard proteins and samples, also it left over for 20 minutes to change into the stable gel.

The standard proteins those loaded into the specified wells of the gel were comprised of the 120 kDa protein β-galactosidase from E. coli, 85 kDa protein bovine serum albumin from bovine plasma, 50 kDa protein the egg-white ovalbumin, the 35 kDa protein carbonic anhydrase (CA) protein from human erythrocytes and 29 kDa standard protein was CA from cattle's erythrocytes. After formation of the gel was completed, the glass plates containing the gel inside of them placed into the electrophoresis tank.

The electrophoresis tank was filled with running buffer until the glass plates were covered. The protein samples previously prepared for electrophoresis by using 20 µl proteins in 100 µl of sample buffer and boiled for denaturation. Then samples were loaded into the wells of the gel inside the glass plates using a suitable micropipette. Later, the tank covered by the electrophoresis cover and both the anode and cathode charges plugged in. Finally, the cables connected with the power supply and the electrophoresis ran at 80 V for 30 minutes then at 120 V for two hours.

After completion of electrophoresis and separation of the proteins on the gel, the gel removed from the glass plates and was transferred into a stabilizing buffer solution containing (50% isopropanol + 10% TCA + 40% DW) and stayed for 15 minutes. Following the stabilizing solution the gel transferred into a coloring solution containing (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid and 40% DW) for two hours and washing solution containing (50 mL of methanol, 10 mL acetic acid and 40 mL of D.W) until the clearing of the gel color completes after about 15 minutes, and the gel color checked for protein bands. The washing buffer could be reused for other

gel electrophoresis processes by removing its color using active charcoal as well as most of the other used buffers and solutions in the process.

A second method for staining the SDS-PAGE was by using silver nitrate as a staining solution, which included using of a fixation, reduction, dying and two washing solutions. The preparation of the solutions described in section 3.1.3.5 above. After the gel removed from the electrophoresis tank it was placed on a fixation solution, containing ethanol and acetic acid for 30 minutes. Then, it was transferred into a reduction solution that composed of sodium acetate, ethanol, sodium thiosulfate and glutaraldehyde for another 30 minutes. After the reduction solution, the gel washed with D.W for three five minute times, afterwards the gel placed into the dye solution that contained silver nitrate and formaldehyde for 20 minutes. The dye solution should be prepared shortly before using it and should be stored in a dark place. The gel washed by two washing solutions. Firstly, by using first washing solution, that contained sodium carbonate and formaldehyde. Then, the gel washed by the second washing solution, containing glycerin and acetic acid. The bands should be appeared during washing the gel; all steps need to be performed with continuous shaking to the gel and solutions (Laemmli 1970).

The last step in the gel electrophoresis is taking a photograph of the gel and checking, whether a purification process completed successfully or not, single band proteins on the sample lanes indicate a protein solution having only protein type and it means a good purification as well. Whereas multiple bands on the gel indicate that the enzyme is poorly purified.

3.2.5. Determination of the enzyme molecular weight

The molecular weight of the 6PGD enzyme purified from Japanese quail's erythrocytes has determined by SDS-PAGE. From the resulted photograph of the gel the retardation factor (R_f) values of the standard proteins and samples used for plotting a standard curve versus the standard protein molecular weights. The R_f value = X_e / X_{dye} where X_e is the distance that the protein traveled inside the gel, and X_{color} is the distance traveled by the dye.

By calculations from the standard curve of R_f vs log M.W. The unknown molecular weight of 6PGD enzyme from the quail's erythrocytes could be found.

3.2.6. Enzyme activity determination

The enzyme activity of the 6PGD Japanese quail's erythrocyte enzyme measured spectrophotometrically at 25°C using Beutler's method. In this method the absorbance of reduction of $NADP^+$ to NADPH was measured depending on time at 340 nm.

In the presence of 6PGD the following reaction happens.

The enzyme assay composed of two cuvettes one of them is blank and another one for the assayed enzyme, each cuvette contains chemical components as seen in the table (3.2).

After adding the whole components into the cuvettes, the mixture allowed for 5 minutes of incubation at room temperature. Then, the optical density differences were measured against time for 3 minutes. And the enzyme activity determined following the equation 3.1 (Beutler 1971).

Table 3. 2. The assay protocol for 6PGD from Japanese quail's erythrocytes

No.	Materials	Blank	Test
1	0.5 M Tris- HCl pH 8.0	200 µl	200 µl
2	6PGA	100 µl	100 µl
3	$NADP^+$	100 µl	100 µl
4	D.W	600 µl	570 µl
5	Sample	0.00 µl	30 µl
6	Total volume	1000 µl	

$$\text{Enzyme activity EU/mL} = \frac{\frac{\Delta O}{3\ \text{min.}}}{6.22} \times \frac{V_T}{V_E} \times f \qquad 3.1.$$

EU/mL is an enzyme unit in one milliliter, ΔO is a change in the optical density, 6.22 is (extinction coefficient) the amount of reduction of 1 mM $NADP^+$ to NADPH, and it's a constant value; V_T is the total volume of the reaction components and V_E is the volume of the enzyme assayed in the reaction. If the enzyme assay contained any dilutions of the enzyme solutions or substrates, the dilution factor (*f*) should be considered in the calculations.

3.2.7. Kinetic properties and characterization of the enzyme

Some kinetic features and properties of the 6PGD Japanese quail erythrocyte's enzyme had been investigated using methods described below:

3.2.7.1. Determination of the optimum pH

For determination of the optimum pH value for 6PGD enzyme activity of Japanese quail erythrocytes two different buffers with different ranges of pH values used. First is Tris-HCl buffer, 0.5 M pH ranges of 7.0 - 7.5 - 8.0 - 8.5 and 9.0. Second buffer is potassium phosphate 1 M, pH ranges from 5.5 - 6.0 - 6.5 - 7.0 - 7.5 and 8.0. The enzyme assayed as in table 3.2, for each measurement one of the buffers with different pH values used, the method is described by Beutler (Beutler 1971). The pH value which the enzyme has its maximum activity at is assigned as the optimum pH value for the 6PGD enzyme activity.

3.2.7.2. Determination of the stable pH

For finding the most stable pH value for the 6PGD enzyme, two buffers 0.5 M Tris-HCl and 1 M potassium phosphate used; the Tris-HCl buffer pH ranged from 7.0 – 9.0 and pH range of potassium phosphate buffer was from 5.5 to 8.0. The purified 6PGD enzyme mixed with each buffer solution at 1:2 volume ratios then stored at 4℃ over 7 days with two measurements of the enzyme's activity per day. The results from the activity of enzyme-buffer mixture that gave the lowest difference in between the first, and the last measurements, were assigned as the most suitable solutions and stable pH values for the enzyme storage in which the enzyme maintained its activity the most.

3.2.7.3. Determination of the optimum ionic strength

For determining the effect of ionic strength on the 6PGD from Japanese quail erythrocytes, two different series concentrations of Tris-HCl buffer pH 8.0 and potassium phosphate buffer pH 8.0 with concentrations of (10 mM, 20 mM, 50 mM, 100 mM, 200 mM, 400 mM, 500 mM, 600 mM, 800 mM and 1000 mM) used in order to determine the maximum activity of the 6PGD enzyme. The concentration of the buffer that gave highest enzyme activity assigned as the best ionic strength for the 6PGD enzyme from the Japanese quail erythrocytes.

3.2.7.4. Determining the optimum temperature

In order to determine the optimum temperature for the maximum enzyme's activity, the 6PGD enzyme of quail erythrocytes assayed using 0.5 M, Tris-HCl buffer pH 8.0 at different ranges of temperatures (0 to 80°C). The temperature at which the enzyme gave its maximum activity assigned as the optimum temperature for the 6PGD Japanese quail erythrocyte's enzyme.

3.2.7.5. Kinetic studies

For determining the K_M and V_{max} values of the substrate and coenzyme (6PGA and $NADP^+$) of the 6PGD enzyme, a method contained of using stable concentrations of $NADP^+$ with 8 different concentrations of 6PGA (10 mM, 25 mM, 50 mM, 100 mM, 200 mM, 300 mM, 400 mM and 500 mM) for finding the 6PGAs (K_M and V_{max}), and likewise, for $NADP^+$ coenzyme, stable concentrations of 6PGA used in the enzyme's assay with 8 different concentration of the $NADP^+$ (10 mM, 25 mM, 50 mM, 100 mM, 200 mM, 300 mM, 400 mM and 500 mM) coenzyme solution for finding the $NADP^+$s (K_M and V_{max}) values. The data collected from these measurements used for plotting of the Lineweaver-Burk graphs (Figure 4.10 and 4.11) for both $NADP^+$ and 6PGA in which the values for K_M and V_{max} were found by calculations in order to indicate the affinity of the 6PGD enzyme from Japanese quail erythrocytes towards the two substrate and coenzyme.

4. RESULTS

The purification and characterization results of the whole experiments carried out through this study on 6PGD enzyme of the Japanese quail erythrocytes are collected in this chapter; all of the experiments performed using the procedures, materials and methods described in chapter 3.

4.1. Protein determinations

The experiments conducted for the qualitative and quantitative determinations of proteins, for checking the abundance and to determine the amount of proteins in the hemolysate and purified enzyme samples.

4.1.1. Qualitative determinations of proteins

The absorbance of the eluate (hemolysate) before eluting out the Japanese quail 6PGD erythrocyte enzyme from the 2', 5'-ADP Sepharose 4B affinity gel column, was measured at 280 nm and indicated the absence of the proteins in the eluate, confirming that all of the 6PGD enzyme were attached to the particles of the affinity gel in the column before eluting them out with the elution buffer. So, while the 6PGD eluted using its special elution buffer, the purity of the eluted enzyme was guaranteed (section 3.2.1.1).

4.1.2. Quantitative determination of protein

The quantitative determination of protein was done as described in (section 3.2.1. 2), the resulted absorbance used to plot a calibration curve for determination of the amount of proteins in both the hemolysate and the purified samples, Table 4.1.; 4.2.; Figure 4.1.

Table 4.1. Determination of the amount of protein by Bradford's method

No.	Sample	Absorbance
1	Hemolysate	1.949
2	6PGD Japanese quail erythrocytes purified enzyme	1.177

Table 4.2. The 6PGD enzyme activity and absorbance of the samples assayed for Bradford's method for quantitative determination of protein

No.	Sample	Activity EU/mL	Protein mg/mL	Volume mL
1	Hemolysate	0.086	8.097	25
2	6PGD purified enzyme	0.297	0.005625	5

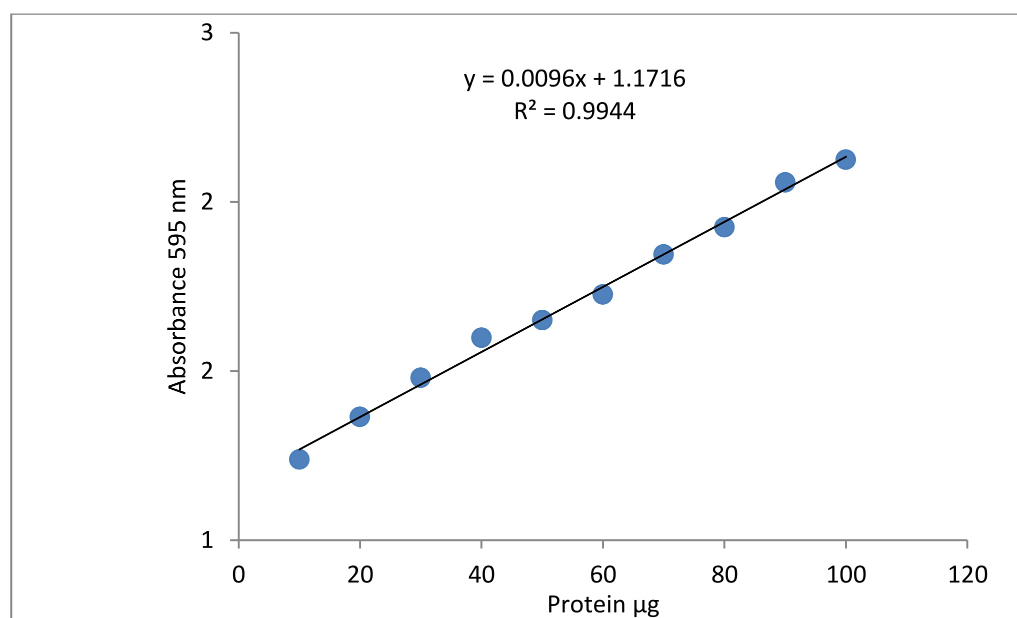

Figure 4.1. The standard calibration curve for quanitative determination of protein by Bradford's method: for determining the amount of proteins in the hemolysate and the purified 6PGD Japanese quail erythrocyte enzyme

4.2. Purification of the 6PGD enzyme

The purification of the 6PGD enzyme of the Japanese quail erythrocytes was performed using the affinity gel chromatography. After preparation of the hemolysate and the affinity column, the mixture of enzymes and proteins loaded on the affinity gel (section 3.2.3), then by washing the column with washing buffers and later eluting the 6PGD enzyme, the enzyme has been purified.

4.2.1. 6PGD enzyme elution from 2', 5'-ADP Sepharose 4B affinity column

The prepared hemolysate from the Japanese quail's erythrocytes was loaded into the affinity column. After washing process of the hemolysate was accomplished with the washing buffers, the 6PGD enzyme was eluted using a specific elution buffer (3.2.3). Then, the eluate fractions from the affinity column were collected in the eppendorf tubes labeled from 1 to 15. Following that they were checked for their 6PGD enzyme activity and they had their maximum activity in the fifth eppendorf with an activity of 0.2979 EU/mL (Table 4.3; Figure 4.2). The purification folds, specific activity of enzyme and percentage of the purification in each step is also provided in Table 4.4.

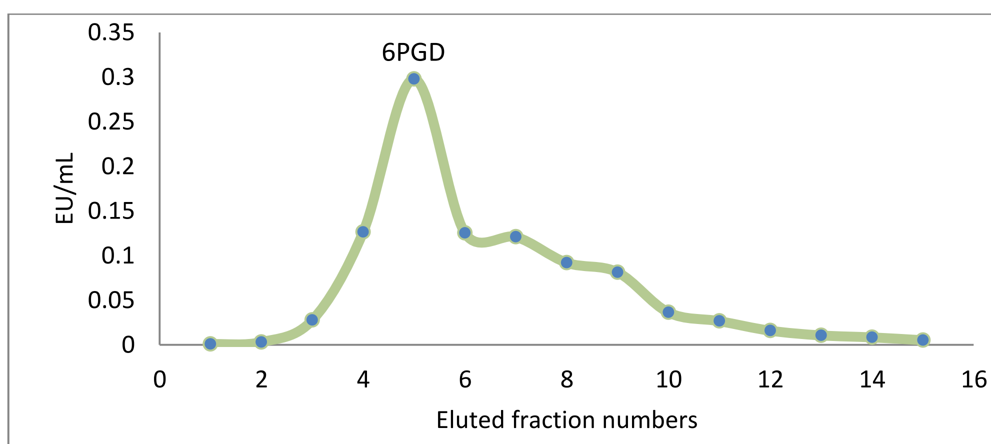

Figure 4.2. The enzyme's activity EU/mL of the eluted fractions of 6PGD Japanese quail erythrocytes from the 2', 5'-ADP Sepharose 4B affinity column chromatography: the column used for this purification was a 1×10 cm column, containing 2 g of 2', 5'-ADP Sepharose 4B affinity gel, the elution runned by setting the flow rate to 20 mL/hour through peristatic pump, each collected fraction of the enzyme had a 1.5 mL volume

Table 4.3. The Japanese quail erythrocyte 6PGD enzyme activity of the eluted fractions

Fractions Eppendorf tube no.	**Activity (EU/mL)**
1	0.0009
2	0.0032
3	0.0278
4	0.1264
5	0.2979
6	0.1254
7	0.1211
8	0.0921
9	0.0814
10	0.0364
11	0.0267
12	0.016
13	0.0107
14	0.0085
15	0.0053

4.2.2. SDS-PAGE

The SDS-PAGE experiment conducted for checking of the purity and the determining of molecular weights of the purified enzyme that eluted from the affinity column (section 3.2.4), the results of the test showed a single band protein on the gel for two of the eluates that had the maximum activity of the 6PGD in them. This proved the existence of only one type of protein in the eluate fraction which is the 6PGD enzyme from the Japanese quail erythrocytes (Fig. 4.3).

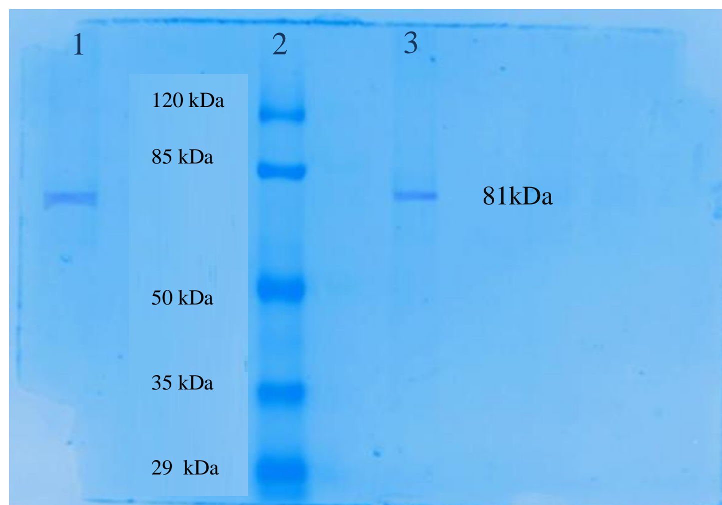

Figure 4.3. The SDS-PAGE photograph for the 6PGD Japanese quail's erythrocytes enzyme: the 120 kDa protein is β-galactosidase from *E. coli*, 85 kDa protein is bovine serum albumin from bovine plasma, 50 kDa protein is the egg white ovalbomin, the 35 kDa protein is carbonic anhydrase protein from human erythrocytes and 29 kDa standard protein is CA from cattle's erythrocytes. The 81 kDa protein in lane one and three is the 6-phosphogluconate dehydrogenase enzyme protein from Japanese quail's erythrocytes

4.3. Purification yield, folds of purified enzyme and specific activity

The quality and efficiency of the purification method was calculated using the calibration curve of the Bradford's method (1976) for quantitative protein determinations (Figure 4.1). The amount of proteins, volumes and activities in the hemolysate and purified enzyme samples (Table 4.1 and 4.2) used for calculation of the purification and the resulted purified enzyme solution. The purification result shows 69% yields of purification, 4984 fold purification of the proteins, and the specific activity of 52.84 EU/mg for the purified 6PGD enzyme (Table 4.4).

The total activity = Activity EU × V mL

The total proteins = Proteins mg × V mL

The specific activity = Total activity / Total proteins

The purification folds = Specific activity of purified enzyme / specific activity of the hemolysate

The yield% of purification = Total activity of purified enzyme / total activity of the hemolysate ×100.

Table 4.4. The information about purification of the 6PGD Japanese quail's erythrocytes enzyme using 2', 5'-ADP Sepharose 4B affinity gel chromatography

No	Sample	Volume mL	Protein mg/mL	Total protein mg/mL	Activity EU/mL	Total activity EU/mL	Specific activity EU/mg	%Yield	Purification folds
1	Hemolysate	25	8.097	202.425	0.086	2.15	0.0106	100	1
2	2', 5'-ADP ADP Sepharose 4B affinity column	5	0.00562	0.02812	0.297	1.485	52.84	69	4984

4.4. Characterization of 6PGD enzyme

The characterization experiments of the 6PGD enzyme of quail erythrocyte were conducted for the determinations of the enzyme's molecular weight (4.4.1), optimum conditions of the enzymes catalyzing reactions like optimum pH value (4.4.2), optimum ionic strength (4.4.3) optimum temperature (4.4.4), the best pH for storing the enzyme in which the enzyme keeps its activity for the longest time (4.4.5) and K_M and V_{max} values (4.4.6).

4.4.1. Molecular weight

The enzyme's molecular weight was determined by using the method described in section 3.2.5. The retardation factor (R_f) (distance traveled by each one of the standard proteins and the purified 6PGD enzyme), were measured and plotted against the logarithm of the standard proteins molecular weights, then from the linear curve the molecular weight of the unknown 6PGD Japanese quail's erythrocyte enzyme determined to be ~81 kDa. (Table 4.5; Figure 4.4).

Table 4.5. The R_f values of the standard proteins and purified ezyme samples for the SDS-PAGE

No.	Sample	R_f	Distance travelled by proteins in centimeters
1	120 kDa	0.2	1.2 cm
2	85 kDa	0.3	1.8 cm
3	50 kDa	0.55	3.3 cm
4	35 kDa	0.75	4.5 cm
5	29 kDa	0.9	5.4 cm
6	6PGD enzyme from quail erythrocytes	0.35	2.1 cm

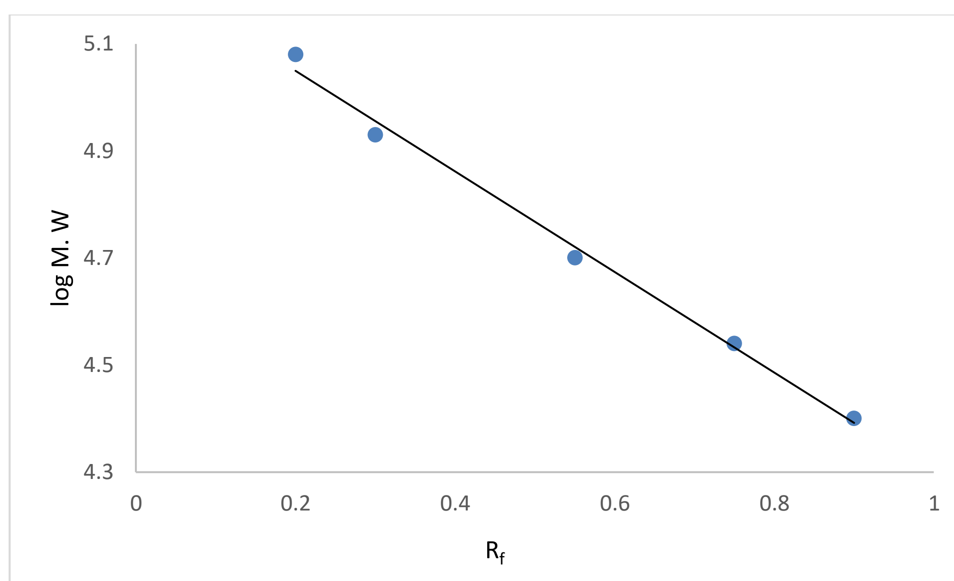

Figure 4.4. The calibration curve between R_f vs Log M. W. for determination of the 6PGD enzyme molecular weight by Lammelie’s method (1970)

4.4.2. Optimum pH

The optimal pH value test for the quail’s 6PGD enzyme activity was performed using two different buffer solutions 0.5 M Tris-HCl buffer and 1 M potassium phosphate buffer as described in section 3.2.7.1. The results showed that the optimum pH value for the enzyme’s activity was at pH 8.0, in Tris-HCl buffer (Table 4.6; 4.7; Figure 4.5).

Table 4.6. The 6PGD enzyme activities using different pH value potassium phosphate buffer solution in the enzyme assay

pH	Activity (EU/mL)
5.5	0.016
6.0	0.025
6.5	0.021
7.0	0.030
7.5	0.034
8.0	0.039

Table 4.7. The 6PGD enzyme activities using different pH value in Tris-HCl buffer solution in the enzyme assay

pH	Activity (EU/mL)
7.5	0.038
8.0	0.071
8.5	0.066
9.0	0.064

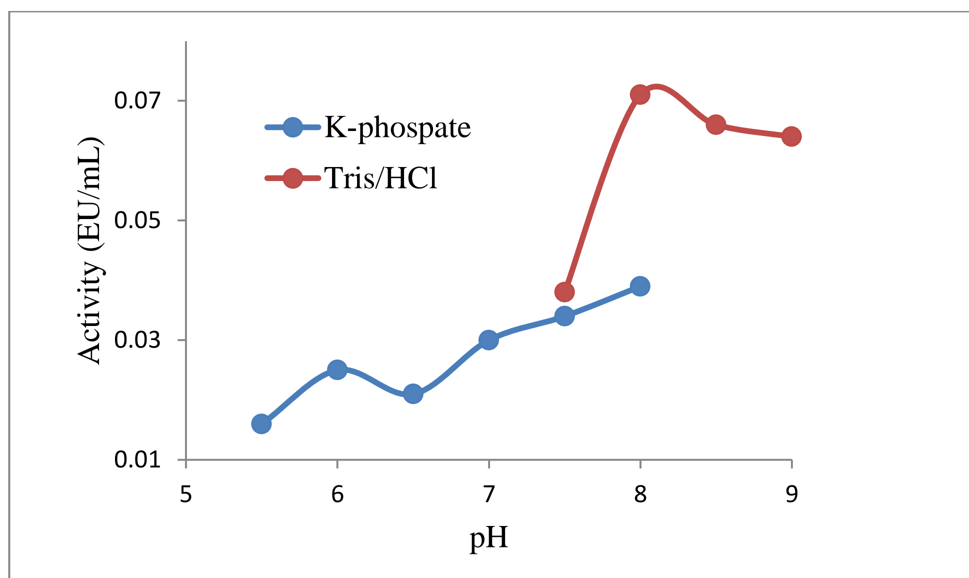

Figure 4.5. The optimum pH value test of the 6PGD enzyme's activity from quail erythrocytes

4.4.3. Optimum ionic strength

The effect of ionic strength on the enzyme activity was tested as described in section 3.2.7.3. The optimum ionic strength was determined at pH 8.0, in the 500 mM Tris-HCl, buffer (Table 4.8; Figure 4.6).

Table 4.8. The best effect of ionic strength on the 6PGD enzyme from quail erythrocytes

[Tris-HCl] (mM)	10	20	30	40	50	100	200	400	500	600	800	1000
Activity (EU/mL)	0.04	0.03	0.038	0.040	0.011	0.011	0.008	0.036	0.067	0.036	0.040	0.049

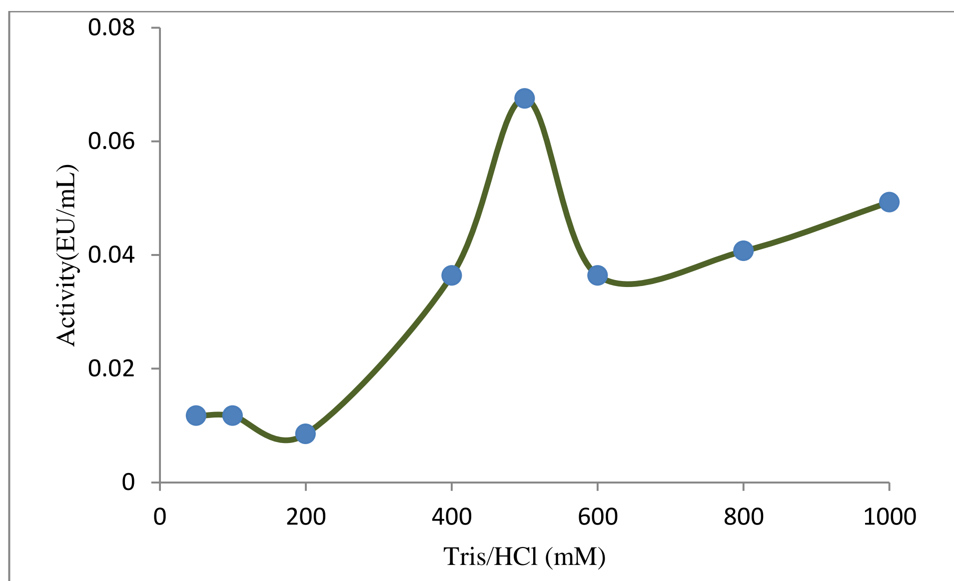

Figure 4.6. The optimum ionic strength effect on 6PGD Japanese quail's erythrocyte enzyme

4.4.4. Optimum temperature

The optimal temperature test for the enzyme's activity conducted as described in section 3.2.7.4, an increase in the activity of the enzyme was observed due to the elevating temperatures. The activity increased until 60℃ where the enzyme had its maximum activity. However, the enzyme lost its activity when the temperature elevated more than 60℃ due to the denaturation of the enzyme's protein structure by the effect of heat (Table 4.9; Figure 4.7).

Table 4.9. The effect of temperature on the 6PGD Japanese quail erythrocyte's enzyme activity

Temp. (˚C)	0	10	20	30	40	50	60	70	80
Activity (EU/mL)	0.0178	0.0482	0.0768	0.1375	0.1786	0.2500	0.3447	0.0107	0.000

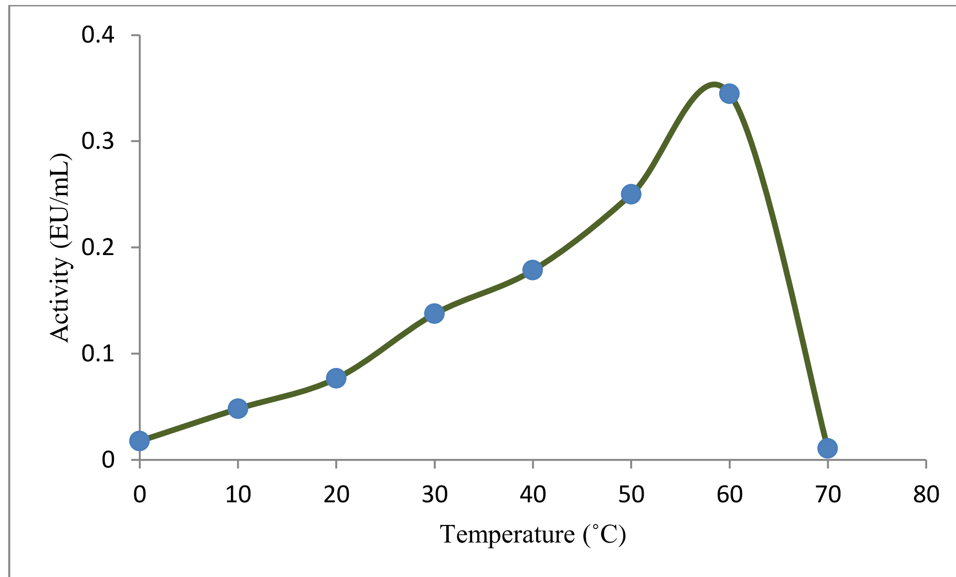

Figure 4.7. The effect of elevating temperatures on the 6PGD Japanese quail's enzyme activity

4.4.5. Stable pH

The enzyme's stability tested against time and storage conditions in acidic/basic mediums as described in section 3.2.7.2. The samples were assayed twice a day. The most suitable buffer solution that mostly kept the activity of the 6PGD enzyme of Japanese quail was 0.5 M Tris-HCl pH 8.0 buffer. The enzyme maintained its activity for highest levels along seven days of measurements in the buffer. However, the most suitable pH value of the stored 6PGD enzyme in the potassium phosphate buffer was at pH 7.5 (Table 4.10, 4.11; Figure 4.8, 4.9).

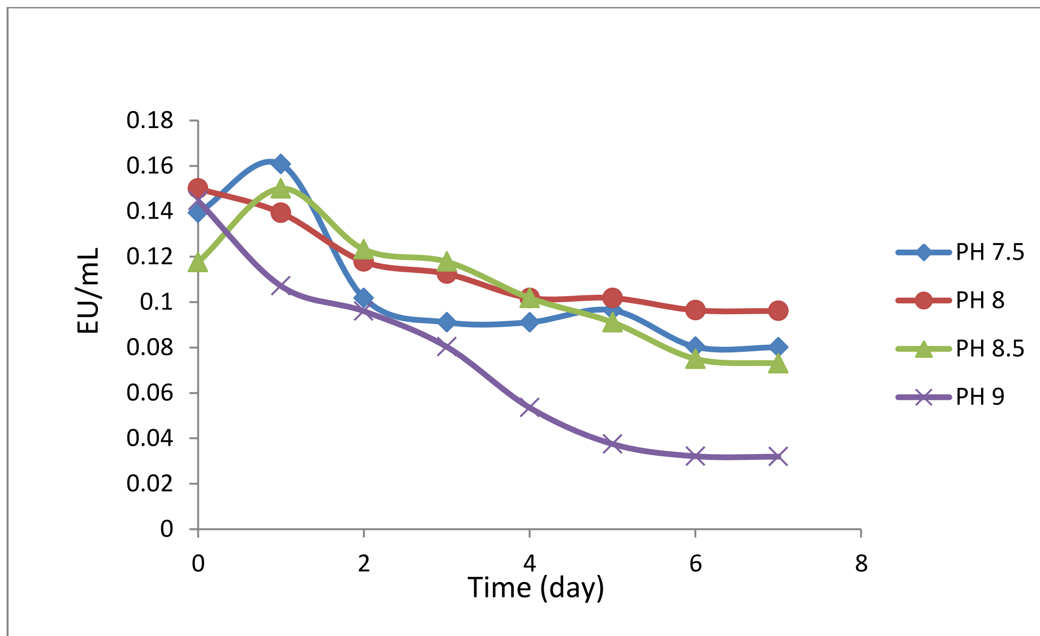

Figure 4.8. The stability of the 6PGD Japanese quail's erythrocyte enzyme in Tris-HCl buffer over seven days time

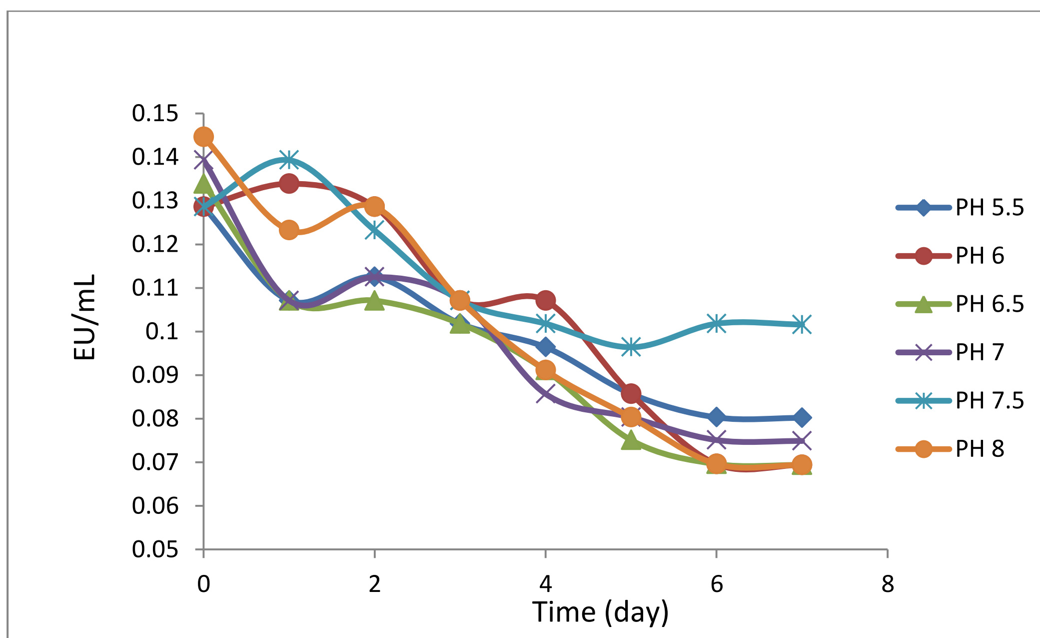

Figure 4.9. The stability of the 6PGD Japanese quail's erythrocytes enzyme in potassium phosphate buffer over seven days time

Table 4.10. The 6PGD enzyme's activity from Japanese quail's erythrocytes stored in different pH values of potassium 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)
0	0.1286	0.1286	0.1339	0.1339	0.1286	0.1446
1	0.1071	0.1339	0.1071	0.1071	0.1393	0.1232
2	0.1125	0.1286	0.1071	0.1125	0.1232	0.1286
3	0.1018	0.1071	0.1018	0.1071	0.1071	0.1071
4	0.0964	0.1071	0.0911	0.0857	0.1018	0.0911
5	0.0857	0.0857	0.0750	0.0803	0.0964	0.0803
6	0.0803	0.0696	0.0696	0.0750	0.1018	0.0696

Table 4.11. The 6PGD enzyme's activity from Japanese quail's erythrocytes 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)
0	0.1393	0.1500	0.1176	0.1446
1	0.1607	0.1393	0.1500	0.1071
2	0.1018	0.1178	0.1232	0.0964
3	0.0911	0.1125	0.1178	0.0803
4	0.0911	0.1018	0.1018	0.0535
5	0.0964	0.1018	0.0911	0.0375
6	0.0803	0.0964	0.0750	0.0321

4.4.6. K_M and V_{max} values

For finding the K_M and V_{max} values for the quail's erythrocyte 6PGD enzyme substrates and coenzyme (6PGA and $NADP^+$) the method as described in section 3.2.7.5 used. At the stable concentrations of the $NADP^+$ the enzyme activity was checked by using of eight different concentration of 6PGA, the collected data from the measurements used for

plotting the Lineweaver-Burk graph between 1/V vs [1/6PGA] mM, and the values of K_M and V_{max} were founded for 6PGA as K_M 0.120 mM and V_{max} 0.1911 EU/mL. Then by using constant concentration of the 6PGA and different concentrations of $NADP^+$ the enzyme activity was checked as well, and the collected data from the measurements used to plot the Lineweaver-Burk graph between 1/V vs [1/$NADP^+$] mM then the K_M and V_{max} values calculated from the graph for $NADP^+$ as K_M 0.0174 mM and V_{max} 0.2288 EU/mL.

By comparing the K_M values for both of the 6PGA and $NADP^+$ we can see that the K_M value of the $NADP^+$ is smaller than the K_M value for 6PGA, which means $NADP^+$ is more favorable than 6PGA for the catalytic reactions of 6PGD enzyme of Japanese quail erythrocytes (Table 4.12, 4.13; Figure 4.10, 4.11).

Table 4.12. The activity of 6PGD Japanese quail erythrocytes enzyme using different concentrations of 6PGA substrate

6PGA (mM)	10	25	50	100	200	300	400	500
Activity (EU/mL)	0.0053	0.0107	0.0267	0.0643	0.0946	0.1071	01232	0.1536

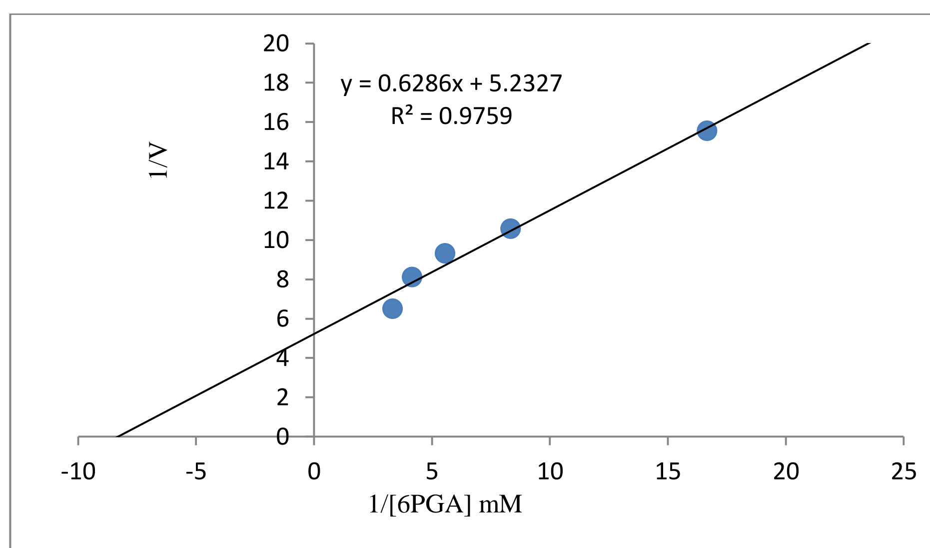

Figure 4.10. The Lineweaver-Burk reciprocal plot for the 6PGA substrate: different concentrations of the 6PGA substrate for 6PGD quail's erythrocytes enzyme used in order to find the maximum velocity of the enzyme and the Michaelis- Menten constant for the substrate

Table 4.13. The activity of 6PGD Japanese quail's erythrocytes enzyme using different concentrations of $NADP^+$ coenzyme

$[NADP^+]$ (mM)	10	25	50	100	200	300	400	500
Activity (EU/mL)	0.803	0.0899	0.1054	0.1232	0.1571	0.1732	0.1875	0.2018

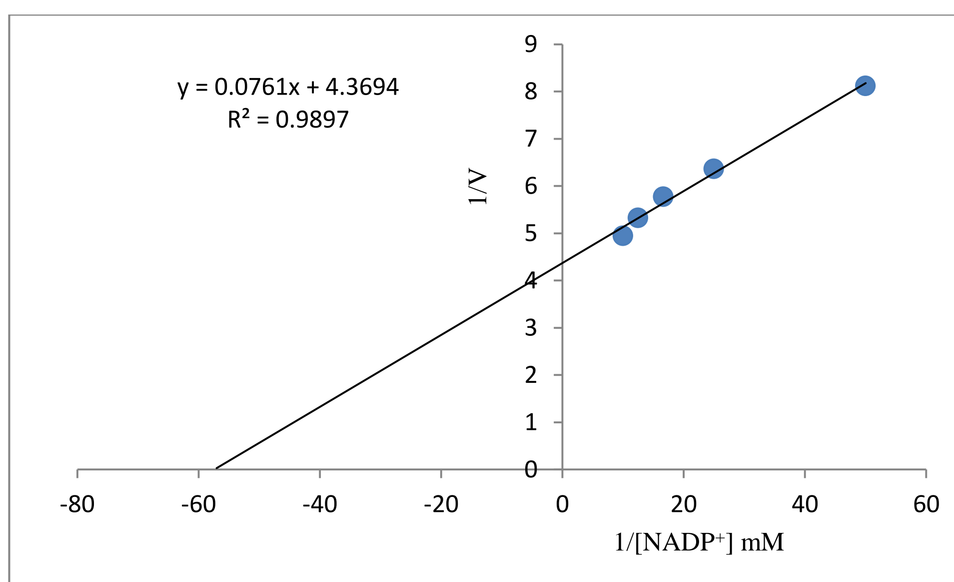

Figure 4.11. The Lineweaver-Burk reciprocal plot for $NADP^+$ coenzyme: different concentrations of $NADP^+$ coenzyme for 6PGD Japanese quail's erythrocytes enzyme used in order to find the maximum velocity of the enzyme and the Michaelis - Menten constant for the coenzyme

5. DISCUSSION

There are a lot of antioxidants in living organisms, they divided into two types. One is the direct anti-oxidant and the other one is the indirect anti-oxidant. 6PGD enzyme is one of the indirect anti-oxidants (Reiter et al. 1997; Jacobasch and Rapoport 1996). This enzyme exists in plentiful amounts in blood (Dallocchio et al. 1985). Any decrease in the amount of the enzyme causes the glutathione to be reduced that allows the peroxides and Fe^{3+} as well as the free radicals to cause damage to the cells (Soysal and Bakan 1984). As a result of 6PGD enzyme deficiency, the 6PGA will be accumulated and completely inhibits the phosphoglucose isomerase and glucose metabolism (Hanau et al. 1996). This condition cause toxicity in the cells and increases the enzyme activity of pyruvate kinase in erythrocytes that changes the number of reticulocytes.

The present study is about the purification and characterization of 6PGD enzyme from Japanese quail (*Coturnix coturnix japonica*) erythrocytes. This purification is the first study to be performed on the 6PGD enzyme of Japanese quail's red blood cells (RBC) s. The method used for the purification of the enzyme was 2', 5'-ADP Sepharose 4B affinity gel chromatography, which is a very powerful, reliable, easy, economic and a single step purification method that gives very good purification yields with an ability to purify bulk amounts of the enzymes. These advantages of the method are some of the characteristics of it which make it by comparison to other purification methods the most suitable to work with.

The blood samples of Japanese quail (*Coturnix coturnix japonica*) were obtained from the Bingol University farm for research and collected freshly into tubes containing anticoagulants then transferred to the laboratory with care at 4°C (section 3.2.2), where the experiments instantly started on the freshly collected blood due to the fact that enzyme activities are decreased by the factor of time and storing conditions, see section 1.6. First step in the purification procedure is the hemolysate preparation, then

preparation of the affinity gel, and later loading the hemolysate into it then washing, eluting-collecting the enzyme fractions. Finally, the measurement of the enzyme activity in each fraction was performed for distinguishing the highest containing enzyme fraction for further characterization experiments. It is important to perform the purification procedure in a smallest amount of time as possible and every equipment should be previously prepared including the affinity column to save time and to guarantee the results of good purification due to the sensitivity of the enzyme's stability by means of time, temperature, pH value and other storing conditions.

The optimization of the purification conditions for each enzyme differs and takes so much time to find the best optimal conditions for each specific experiment. It is crucial to test which buffer solutions should be used in the experiment and the buffers pH values, concentrations used for the eluting the enzymes from the gel inside the affinity column.

For the molecular weight determinations of the 6PGD enzyme of Japanese quail erythrocyte the SDS-PAGE method used in which the enzyme loaded into the same gel with standard proteins, and the electrophoreses ran containing both the purified enzyme and standard proteins. A graph plotted between Log M.W. of standard proteins and R_f values of the travelled protein particles through the gel. The molecular weight of the subunit of the purified enzyme found to be ~81 kDa. The molecular weights of 6PGD enzymes from different sources in literature had been 59.5 kDa from the rat's erythrocytes (Beydemir et al. 2004), 52.6 kDa from Van's cat erythrocyte (Kiliç 2007), 52 kDa from rat's small intestine (Ceyhan et al. 2005), *E. coli* bacteria had 53 kDa 6PGD (Wang and Zhang 2009), 118.34 kDa from rainbow trout by gel filtration chromatography (Taranci 2011) and 24.1 kDa from parsley (Demir et al. 2003). By looking through above molecular weights from different sources, we find that the molecular weight of 6PGD from Japanese quail's is different from all the mentioned sources.

6PGD enzyme had been investigated in many different sources and checked for its kinetic properties and characterized for its functions. The enzyme had been purified from sheep's liver (Villet and Dalziel 1972), parsley's leaves (Demir et al. 2003), chicken's liver (Erat 2005), rat's small intestine (Ceyhan et al. 2005), Van's cat erythrocytes (Kiliç 2007), human's erythrocytes (Özabacigil 2005), human cerebral (Weisz et al. 1985), rabbit

mammary glands (Betts and Mayer 1975) and rat heart and lung tissues (Adem 2010), and now this recent study from the Japanese quail's erythrocytes.

For the purification of the 6PGD enzyme from the Japanese quail erythrocytes. Firstly, the fresh blood samples were collected and then the hemolysate was prepared from them. The method used for preparation of the hemolysate was by using ice water. This method is much easier than other methods to work with. It gives good hemolysis results of the red blood cells. The mechanism in which the ice water hemolysis the erythrocytes is based on cell swelling and breaking up. In the method, a hypotonic environment was prepared for the RBCs in which they swelled then exploded their membrane, and ejected their interior proteins and other constituents into the solution.

In order to partially purify the enzyme after the hemolysate preparation from unwanted proteins, a step of ammonium sulfate precipitation is needed. However, this step in our study has been canceled when we have observed the uselessness of this precipitation for our experiment exclusively, as there were no precipitations while we used 10-60% ammonium sulfate. We refer the reason to the small amounts of the blood samples in which we used for the purification procedure, as the volume of the collected blood from each quail bird is only about 4 mL. On that basis, no longer ammonium sulfate precipitations were carried out throughout the entire purification experiment.

Different methods were used for the purification of 6PGD enzyme throughout the literature of enzymology like DEAE cellulose; C-M cellulose ions exchange chromatography (Villet and Dalziel 1969), DEAE Sephadex, hydroxy apatite (Silverberg and Dalziel 1973), $NADP^+$ Sepharose, $NADP^+$ agarose (Betts and Mayer 1975), matrix gel-A column chromatography (Somers et al. 1991), DEAE Sephadex A-50 ion-exchange chromatography (Demir et al. 2003). In this study, we used 2', 5'- ADP Sepharose 4B affinity gel column chromatography for the purification of the 6PGD enzyme. The preference of this method is because of the structure of its gel. It has an inhibition effect on its structure towards ortho phosphate on the 6PGD enzyme. In addition using affinity chromatography takes much less time for purification with much higher purification yield and much easier to perform. This method is the most appropriate method to purify enzymes for characterizing of their biological functions. Besides that, the used materials in this method are valid for reusing in more similar experiments. The other methods

require multiple complex steps for the purification. This method is a single step purification method. While in other methods only small amounts of enzymes could be purified per each experiment; in this method, big amounts of protein solutions could be loaded into the affinity column, and this lets big amounts of enzymes to be purified in one experiment that makes working by this method more rapid, easier and in high percentage yields. At the end of the study, we could obtain a purified 6PGD enzyme from Japanese quail erythrocytes with a 69% yield, 4984 folds and the specific activity of 52.84 EU/mg.

The 2', 5'- ADP Sepharose 4B affinity gel column chromatography method used for purification of 6PGD enzyme from different sources like human erythrocytes, the purified enzyme had 0.46 EU/mg specific activity, 742 folds and 50% yield of purification (Özabacigil 2005); from rainbow trout erythrocytes, the enzyme had 68.46 EU/mg, 17553 folds and 19.89% yield purification (Taranci 2011), from rat erythrocytes the enzyme had 78% yield and 5.15 EU/mg (Beydemir et al. 2004), from chicken liver along with sephadex G-200 gel filtration chromatography the enzyme had 5.57% yield, and 344 fold protein (Erat 2005), from human erythrocytes the enzyme had 78.37%, 61 EU/mg and 6552 fold proteins (Adem 2006) and the enzyme from erythrocytes of Van's cat had been purified using the same 2', 5'-ADP Sepharose 4B affinity chromatography method had 3.86 EU/mg specific activity and 48% yield (Kiliç 2007).

The 2', 5'-ADP Sepharose 4B affinity gel chromatography method was used for the purification of other $NADP^+$ dependent enzymes effectively. It was used in purifying the thioredoxin reductase enzyme from turkey's (*Meleagris gallopavo*) liver tissue (Temel 2014), the glutathione reductase and glucose 6-phosphate dehydrogenase enzymes from rat's heart and kidney tissues (Adem and Çiftci 2016; Adem and Çiftci 2012). Moreover, multiple $NADP^+$ dependent enzymes could be purified in parallel from a single 2', 5'-ADP Sepharose 4B affinity chromatography by using different elution buffers for eluting each specific enzyme.

The quantitative protein determinations were done using Bradford's method (1976). The dye in this method is Coomassie Brilliant Blue G-250 that has a negative charge on it in which the positively charged proteins attach to it, section 3.2.1.2. The protein-dye bindings shift the λ_{max} of the dye from 465 nm to 595 nm, that increase in the absorbance measured spectrophotometrically at 595 nm. The method is fast, easy, needs small

amounts of reactants and if compared with other protein determination methods is more sensitive, the range of protein determinations through this method starts from 1 to 100 µg s (Bradford 1976). So because of the above advantages of this method we used it for the protein determinations in our research experiment. The hemolysate from the Japanese quail's erythrocytes had 8.097 mg/mL while the purified fraction of the 6PGD enzyme contained 0.005625 mg/mL, see section 4.1 above.

Following the purification of the enzyme from quail's erythrocytes the characterization of the enzyme began by determining the stable and optimal pH values, optimal temperatures and optimal ionic strength upon the enzyme activity.

For finding the optimum pH value for the enzyme's activity two different buffers used which included Tris-HCl buffer pH 7.5 to 9.0, and potassium phosphate buffer pH ranges from 5.5 to 8.0. The optimum pH value for quail's erythrocytes was found to be pH 8.0 using Tris-HCl buffers, section 4.4.2. By looking through other researches from literature, we find that the optimum pH for Van's cat erythrocytes had been at pH 8.0 using 0.1 M Tris-HCl buffer (Kiliç 2007), rainbow trout erythrocyte had its highest activity at optimum pH 7.5 (Taranci 2011), rat erythrocyte's 6PGD enzyme had its optimal pH value using Tris-HCl pH 7.0 (Beydemir et al. 2004), rat liver and kidney enzyme had their maximum activity using pH 8.0 buffers (Corpas et al. 1995) and from yellow catfish, (*Pelteobagrus fulvidraco)* liver enzyme had the optimum pH value at 7.85 (Zhuo et al. 2015). By observing literature the optimum pH value found for the quail's erythrocyte 6PGD enzyme is similar to or slightly differs from the enzymes from different tissues and sources, which is at pH 8.0 using 0.5M Tris-HCl buffer.

Throughout the whole experiments in order to find the best conditions for the enzymatic catalytic reactions, including the best ionic strength effect, various buffer solutions for measurement of the enzyme activity had been prepared, a series of buffers containing different concentrations of Tris-HCl and potassium phosphate were prepared to see the effect of the ionic strength on the enzyme's activity, and the best ionic strength effect of the salts on the enzyme applied to the whole measuring buffer solutions during the entire experiments. For the 6PGD enzyme from erythrocytes of the Japanese quail, the highest activity achieved by using 0.5 M Tris-HCl pH 8.0 buffer, section 4.4.3.

As a result of elevating temperatures of most chemical reactions, the rate of reactions increases due to the elevating of the productive collisions between the reacting particles. This fact is also true for the enzyme catalytic reactions. However, the tertiary structure of the proteins and enzymes highly puts limitations to the amount of temperature elevations to the enzymatic reactions before they begin to disrupt, that leads the enzymes to lose their activity and denature. The disruption of the enzyme structures indicates the delicate nature of enzymes. The enzymes differ in their heating stabilities, this differentiation among many other factors like pH, ionic strength, substrate concentration and ligands, comes from the molecular weight of enzymes in a way that low molecular weight enzymes that are composed of short-chain amino acids bonded by disulfide bonds gain more stability to heat than higher molecular weight enzymes.

For finding the optimum temperature for the 6PGD from Japanese quail's erythrocytes, the enzyme's activity assayed using buffers having optimum ionic strength effect and optimum pH value in different temperatures ranged as 0 to 80℃ and the highest enzyme activity found at 60℃, section 4.4.4. After that, the activity of enzyme dropped down sharply due to the high temperature that caused the denaturation of proteins, and the enzyme lost its activity instantly. The optimum temperature of the 6PGD enzyme from rat erythrocytes was at 45℃ (Beydemir et al. 2004), yellow catfish 6PGD enzyme's optimum temperature was at 60℃ (Zhuo et al. 2015), 6PGD from parsley the enzyme highest activity was at its optimum temperature, which was 50℃ (Demir et al. 2003), chicken's liver 6PGD optimum temperature was at 60℃ (Erat 2005), rainbow trout erythrocyte enzyme's optimum temperature was at 40℃ (Taranci 2011) and Van's cat erythrocytes 6PGD enzymes had its optimum temperature at 50℃ (Kiliç 2007). The result we found from our research is similar to that of chicken's liver and yellow catfish and close to others.

The stable pH for the purified enzyme from quail erythrocytes checked using two different series of buffers: Tris-HCl buffer pH ranges from 7.5 to 9.0 and potassium phosphate buffer pH ranges from 5.5 to 8.0. Through seven days of measurements, two measurements per day performed for the enzymes activities. The best buffer for the 6PGD enzyme in which the 6PGD enzyme kept most of its activity in seven days was Tris-HCl buffer with a pH value of 8.0, section 4.4.5. From the literature, we observe that the enzyme from rat erythrocytes had its stable pH value at a pH 8.0 (Beydemir et al.

2004), chicken's liver enzyme had its stable pH value at 7.5 (Erat 2005) and rainbow trout erythrocyte's enzyme stable pH value had been at pH 7.0 (Taranci 2011). The result from this experiment for the 6PGD enzyme of Japanese quail's erythrocytes is similar to that of rat erythrocytes and close to others.

The natural substrate for the 6PGD enzyme is 6PGA. However, other than the 6PGA the enzyme is also interested in the $NADP^+$ as a coenzyme. For determining the comparison of the enzyme's interest to each of the enzyme's substrate and the coenzyme, the K_M and V_{max} values were found. K_M is described as the binding affinity between the substrates and enzymes, and it is equal to the concentrations of the substrate [S] when the reaction rate is at its half rate of the maximal velocity of the reaction. For finding the K_M value, once 6PGA concentrations stabilized and $NADP^+$ concentrations changed, then $NADP^+$ concentrations stabilized and 6PGA concentrations changed. The activity of the enzyme in both series was checked, and the data used for plotting a Lineweaver-Burk linear curve and K_M and V_{max} values were calculated from it, see section 4.4.6. The K_M value for 6PGA was 0.12 mM, V_{max} for 6PGA was 0.1911 EU/mL, the K_M for $NADP^+$ was 0.01741 mM and V_{max} was 0.2288 EU/mL.

From observing the results, we get over the fact that 6PGD enzyme is more interested in the $NADP^+$ than its natural substrate 6PGA. The Van's cat erythrocytes K_M and V_{max} for $NADP^+$ had found to be 0.048 M and 0.158 EU/mL respectively and for the 6PGA 0.351M and 0.162 EU/mL (Kiliç 2007), the rainbow trout erythrocyte's 6PGD enzyme K_M and V_{max} values for $NADP^+$ and 6PGA had been 0.00279 mM, 0.0417 EU/mL, 0.0207 mM and 0.257 EU/mL respectively (Taranci 2011), the yellow catfish's liver 6PGD enzyme K_M values founded as 169.3 µM and 91.1 µM for 6PGA and $NADP^+$ respectively and V_{max} values are 51.109 EU/mL and 6.6776 EU/mL respectively for 6PGA and $NADP^+$ (Zhuo et al. 2015), the *E. coli* bacteria's 6PGD enzyme K_M values had been 11µM and 10 µM for 6PGA and NADP respectively (Wang and Zhang 2009), rat's liver 6PGD enzyme had its own K_M values for $NADP^+$ and it was 0.258 M and for 6PGA was 0.157 M and for the rat kidneys K_M for $NADP^+$ it was 0.056 M, for the 6PGA was 0.049 M (Corpas et al. 1995). For the rat erythrocytes 6PGD the K_M and V_{max} values for $NADP^+$ were 0.059 M and 0.063 EU/mL and for 6PGA were 0.194 M and 0.054 EU/mL (Beydemir et al. 2004).

For the preparation of the sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), two chemicals have very important roles, which are ammonium per sulfate (PER) and tetramethylethylenediamine (TEMED), section 3.1.3.5. These two chemicals polymerize the acrylamide gel by a free-radical mechanism that results from the reaction of PER with TEMED generating sulfate free radical and initiating the acrylamide bisacrylamide polymerization process. The gel structure composed of acrylamide as polymer chains and bisacrylamide contains crosslinks between the polymers that form the gel matrix in which its pore sizes differ according to the concentration of the bisacrylamide inside the gel composition. Also, the SDS component in the gel has the ability of denaturation of proteins and making the proteins to possess same mass to charge ratio that allows the proteins inside the gel to travel by means of their molecular weights through the gel. The β-mercaptoethanol breaks the disulfide bonds between the amino acids.

Two methods used for staining of the sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). First was Coomassie Brilliant Blue R-250 and the second one was silver nitrate. However, because of the clarity of the single band in the gel only the photograph of the Coomassie Brilliant Blue G- R-250 staining was putted in the results of this thesis (section 4.2.2).

The assay cuvette for the 6PGD Japanese quail erythrocyte's enzyme conducted by Beutler's method (Beutler 1971), containing more than one compounds that have optical density at 340 nm, which are $NADP^+$ and NADPH (section 3.2.6). The optical density of NADPH is 0.311 at 340 nm in 1 cm cuvette. However at 340 nm only NADPH absorbs light. Therefore the value of extinction coefficient of NADPH; (6.22) is considered in calculations of 6PGD Japanese quail's erythrocytes enzyme.

The reason why the enzymes were assayed for their activity rather than the determination of their molar concentrations is due to the fact that they could not be tested for determining their actual molar concentrations easily. Instead of their molar concentrations, their catalytic activities are checked biochemically, in which interpreted the actual abundance of the enzyme assayed in any specified sample or tissue

CONCLUSION

The 6-phosphogluconate dehydrogenase (6PGD) enzyme from the erythrocytes of Japanese quail (*Coturnix coturnix japonica*) was purified at the end of this study.

The enzyme is the third 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 removing the oxidant effects of the free radicals in the cells.

The enzyme purification method was single step purification by the 2', 5'-ADP Sepharose 4B affinity gel chromatography and the purified 6PGD enzyme at the end of the process 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 6PGD enzyme of the Japanese quail erythrocytes was characterized for determining its behaviors 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 6PGD enzyme of Japanese 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

Enzyme characterizations	**Results**
Optimum pH value	pH 8.0, Tris-HCl buffer
Stable pH value	pH 8.0, Tris-HCl buffer
Optimum Temperature	60°C
Optimum ionic strength	0.5 M, Tris-HCl buffer
K_M value for 6PGA	0.12 mM
V_{max} value for 6PGA	0.1911EU/mL
K_M value for $NADP^+$	0.01741mM
V_{max} value for $NADP^+$	0.2288 EU/mL
Molecular weight determination for the 6PGD enzyme from Japanese quail's erythrocytes	
SDS-PAGE method	~81 kDa
Information about the purification process and the purified enzyme	
Purification yield %	69%
Purification folds	4984 folds
Specific activity of enzyme	52.84 EU/mg

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