PURIFICATION AND CHARACTERIZATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM QUAIL (Coturnix coturnix japonica) LIVER

Salih Awla HAMZAH

Master thesis

Chemistry Department

Supervisor: Prof. Dr. Mehmet ÇIFTCI

2017

T.C.
BİNGÖL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

6-FOSFOGLUKONAT DEHİDROGENAZ ENZİMİNİN JAPON BILDIRCIN (*Coturnix coturnix japonica*) KARACİĞERIRİNDEN SAFLAŞTIRMASI VE KARAKCİĞERİZASYONOU

YÜKSEK LİSANS TEZİ

Salih Awla HAMZAH

Enstitü Anabilim Dalı : Kimya

Tez Danışmanı : Prof. Dr. Mehmet ÇIFTCI

Ocak 2017

REPUBLIC OF TURKEY
BİNGOL UNIVERSITY
INSTITUTE OF SCIENCE

PURIFICATION AND CHARACTERIZATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM QUAIL (Coturnix coturnix japonica) LIVER

MASTER THESIS

Salih Awla HAMZAH

Enstitü Anabilim Dalı : Kimya

Tez Danışmanı : Prof. Dr. Mehmet ÇİFTCİ

January 2017

T.C.
BİNGÖL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

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

MASTER THESIS

Salih Awla HAMZAH

Enstitü Anabilim Dalı : Kimya

Bu tez 25.01.2017 tarihinde aşağıdaki jüri tarafından oy birliği ile kabul edilmiştir.

Prof. Dr. Mehmet ÇİFTCİ	Doç. Dr. Mehtap ÖZÇELİK	Yrd. Doç. Dr. Yusuf TEMEL
Jüri Başkanı	**Üye**	**Üye**

Yukarıdaki sonucu onaylarım

Prof. Dr. İbrahim Y. ERDOĞAN
Enstitü Müdürü

ACKNOWLEDGEMENT

My name is Salih Awla HAMZAH, and I have studied for a Master in Biochemistry in Bingol University. First of all, I would like to express my sincere gratitude to my supervisor Prof. Dr. Mehmet ÇİFTCİ for the continuous support of my master study and research, for his patience, motivation, enthusiasm, and immense knowledge. I could not have imagined having a better advisor and mentor for my master degree study.

I would like to thank Asst. Prof. Dr. Yusuf TEMEL who helped me in the Laboratory, for assistance with my technique, methodology and comments that have greatly improved the manuscript.

I would like to acknowledge Asst. Prof. Dr. İbrahim ERDOĞAN, I would also like to thank the experts who were involved in the validation survey for this research project. Without their passionate participation and input, the validation survey could not have been successfully conducted.

In addition, I would like to thank my family, and especially my mother, my wife and my children.

Thanks to all my friends who have helped me to achieve my dream. Special thanks to my best friend Khalid Ahmad. Thanks to everyone who contributed each word that has enabled me to reach my goal.

Salih Awla HAMZAH

Bingol 2017

CONTENTS

LIST OF ABBREVIATIONS

6-PGD	: 6-Phosphogluconate dehydrogenase
6PG	: 6-Phosphogluconate
PPP	: Pentose phosphate pathway
D	: Dextro-rotator
$NADP^+$	: Nicotine adenine dinucleotide phosphate (oxidized form)
NADPH	: Nicotine adenine dinucleotide phosphate (reduced form)
S	: Substrate
P	: Product
E	: Enzyme
OD	: Optical density
VT	: The total volume measured
VE	: The volume of the enzyme added to the sample cuvette
SF	: The dilution factor (used for sample dilution)
EU	: Enzyme unit
BSA	: Bovine serum albumin
Tris	: Tris (hydroxymethyl) aminomethane
Tris-HCl	: Tris-hydroxymethyl aminomethane hydrochloride
DDT	: Dichlorodiphenyltrichloroethane
EDTA	: Ethylenediaminetetraacetic acid
TEMED	: N, N, N', N'- Tetramethylethylenediamine
SDS	: Sodium dodecyl sulfate
PAGE	: Polyacrylamide gel electrophoresis
Vmax	: Maximal velocity
K_M	: Michaelis constant
nm	: Nanometer
ES	: Enzyme-substrate complex

PER	: Ammonium persulphate
ATP	: Adenosine triphosphate
GR	: Glutathione reductase
GPX	: Glutathione peroxidase
GSSG	: Glutathione disulfide
GSH	: Reduced glutathione
ROS	: Reactive oxygen species
DNA	: Deoxyribonucleic acid
RNA	: Ribonucleic acid

LIST OF FIGURES

LIST OF TABLES

6-FOSFOGLUKONAT DEHİDROGENAZ ENZİMİNİN BILDIRCIN(Coturnix coturnix japonica) KARACİĞERİNDEN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Bu tez çalışmasında 14,6 EÜ/mg.protein spesifik aktivitesine sahip 6-fosfoglukonat dehidrojenaz (6PGD) enzimi bıldırcın karaciğerinden amonyum sülfat çöktürmesi ve 2', 5'-ADP Sefaroz 4B afinite kromatografisi yöntemleri kullanılarak %89 verimle 112 kat saflaştırıldı. Enzim aktivitesi 340 nm'de spektrofotometrik olarak ölçüldü. Enzimin saflığı SDS-PAGE yöntemiyle kontrol edildi. Saflaştırılan enzimin ait alt birimlerinin molekül kütlesi SDS-PAGE yöntemiyle 44 KDa olarak hesaplandı ve protein tayini Bradford yöntemiyle gerçekleştirildi. Ayrıca enzim için optimum pH (8.0), optimum sıcaklık (60°C), optimum iyonik şiddet (600 mM Tris/HCl), stabil pH (8.0) gibi karakterizasyon çalışmaları yapıldı. İlaveten enzim substratları olan 6-PGA ve $NADP^+$ için K_M ve Vmax değerleri sırasıyla 58 ve 0,0015 mM; 0,0078 ve 0,057 EÜ/ml olarak belirlendi.

Anahtar Kelimeler: 6-fosfoglukonat dehidrogenaz, saflaştırma, bıldırcın karaciğeri, karakterizasyon.

PURIFICATION AND CHARACTERISATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM QUAIL (*Coturnix coturnix japonica*) LIVER

ABSTRACT

In this study, 6-phosphogluconate dehydrogenase (6PGD) enzyme was purified from the quail liver by using ammonium sulphate precipitation, and 2', 5'-ADP Sepharose 4B affinity chromatography methods, with specific activities 14.6 EU/ml, 89% yield and 112 purification fold. Enzyme activity was measured spectrophotometrically at 340 nm. SDS-PAGE checked the purity of the enzyme. The molecular weight of purified enzyme by the SDS-PAGE method was calculated to be 44 KDa. Protein determination was performed by the Bradford method. For the enzyme optimal pH (8.0), stable pH (8.0), optimum ionic strength (600 mM in Tris-HCl) optimal temperature (60°C) were determined. In addition K_M and V_{max} values for 6-phosphogluconate (6-PGA) and $NADP^+$ were calculated as 0.058 and 0.0015 mM; 0.0078 and 0.057 EU/ml respectively.

Key Words: 6-Phosphogluconate dehydrogenase, purification, quail liver, characterization.

1. INTRODUCTION

1.1. Enzymes

In living organisms, enzymes are called biological catalysts that accelerate chemical reactions and provide 100% yield without side products. Enzymes are the largest and specialized form of proteins. Except for a small group of RNAs that exhibit catalytic activity, all enzymes are in protein structure (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

Some of the enzymes perform their catalytic functions with protein structures, while others require molecules called cofactors that are not found in the structures of the enzymes. The cofactor may be a metal ion or complex organic molecules called coenzymes. Some enzymes need both. Most of the coenzymes are derived from vitamins, especially those from group B vitamins. Coenzymes are not degraded by the denaturation of the protein and can be used many times. The holoenzymes are called enzymes with cofactor, and the protein part of the enzyme is called apoenzyme (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

The information about the kinetic properties of the enzymes can be understood from the K_M constant expressing the affinity of the enzyme and the Vmax values expressing the catalytic activity of the enzyme involved in the Michaelin-Menten equation. Enzyme activity is expressed as "Enzyme Unit". The Enzyme Unit is defined as the amount of enzyme that converts 1 μmol of substrate to product per minute at 25°C and under optimal conditions. According to the international unit of measure, which is the unit of time in seconds, the enzyme that causes 1 mole change in matter is called 1 katal enzyme. The “specific activity” is defined as the enzyme unit per 1 mg protein. Specific activity is a measure of enzyme purity and it is understood that the enzyme has become pure by reaching a maximum and constant value in the purification steps (Lehninger 2000; Keha

and Küfrevioğlu 2004; Berg et al. 2002). Enzymes are the majority of scientific research topics in history of biochemistry. Enzymes have very important duties and contributions in health, disease, diagnosis and treatment. By qualitative and quantitative determination of enzymes, many hereditary disorders are diagnosed, and clues about the future of the disease are obtained. Today more than 2000 enzymes have been identified, many of them have been purified and characterized and more than 200 enzymes have been crystallized (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

Inhibition is the reduction or elimination of in vivo and in vitro activities of enzymes by substances called inhibitors. These materials are often compounds or ions of small molecular structure. Enzyme inhibition is important because it creates an independent control mechanism in biological systems. Many chemicals, drugs and toxic compounds also show their effects in this way (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

Inhibition can be divided into two as irreversible and reversible. In irreversible inhibition, the inhibitor enzyme either covalently binds or comes to a complex that is difficult to separate. In this inhibition, Vmax decreases while K_M does not change. The enzyme-inhibitor interactions in the reversible inhibition are an equilibrium reaction. This inhibition may be competitive, non-competitive or semi-competitive. In competitive inhibition, the inhibitor and substrate compete to bind to the same active site of the enzyme. The inhibition effect decreases as the substrate concentration increases. K_M value increases in competitive inhibition. The inhibition effect is generally independent of the substrate concentration, as it is bound to the different sites of the enzyme with the inhibitor in the non-competitive inhibition. K_M does not change while Vmax falls in this inhibition. In the semi-competitive inhibition, the inhibitor binds to the ES complex. Vmax and K_M decrease in this inhibition (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

An inhibition event called allosteric inhibition is observed in the case of enzymes called allosteric enzymes that occur in more than one polypeptide chain. In this inhibition, the inhibitors bind to a moiety other than the active site of the enzyme and affect the enzyme activity by modifying the three-dimensional structure (Lehninger 2000; Keha and

Küfrevioğlu 2004; Berg et al. 2002). Enzymes are highly specific to the reaction types they catalyze and substrates they convert to product. These biological catalysts catalyze a single chemical reaction or similar reactions of the same type (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

1.2. Pentose Phosphate Pathway

Pentose byway is also known as hexose monophosphate pathway or phosphogluconate oxidative pathway. It was first introduced by Otto Warburg in 1931, and was fully illuminated by biochemists named Fritz Lipmann, Frank Dinckens, Bernard Horecker and Efrahim Rocher. In this pathway, three, four, five, six and seven carbon sugars are converted into each other by a series of non-oxidative reactions (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

1.2.1. Pentose phosphate pathway reactions

The pentose phosphate pathway consists of two steps, oxidative and non-oxidative. This metabolic pathway occurred in the cytosol is the production of NADPH for the purpose of reductive biosynthesis events and the ribose-5-phosphate in the structure of many compounds. Oxidative reactions start with the conversion of first carbon of glucose-6-phosphate to 6-phosphoglucono-1, 5-lactone as a result of dehydrogenation by the enzyme glucose-6-phosphate dehydrogenase. Secondly, phosphoglucuno-1, 5-lactone is converted to 6-phosphogluconate by the lactonase enzyme, while the product formed here is converted to ribulose-5-phosphate by the enzyme 6-phosphogluconate dehydrogenase, which is converted to ribose-5-phosphate by the phosphopentose isomerase enzyme. These reactions result in D-ribose-5-phosphate and CO_2. NADPH occurs in the first and third reactions. The net equation of the reaction is as follows (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002):

$$\text{Glucose-6-phosphate} + 2H_2O + 2NADP^+ \longrightarrow \text{Ribose-5-phosphate} + 2NADPH + H^+ + CO_2$$

The non-oxidative part starts with formation of glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate from xylulose-5-phosphate and ribose-5-phosphate by

transketolase enzyme. These two products are transformed into erythrose-4-phosphate and fructose-6-phosphate by transaldolase enzyme, and these compounds are converted to glyceroldehyde-3-phosphate and fructose-6-phosphate by transketolase. Thus, the ribose-5-phosphate glycolysis pathway formed by pentose phosphate is converted into its intermediate compounds. The net reaction equation for the non-oxidative part is as follows (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002):

3 Ribose-5-phosphate $\longrightarrow$ 2 Fructose-6-phosphate + Glyceraldehyde-3-phosphate

1.2.2. Metabolic importance of pentose phosphate pathway

The Pentose phosphate pathway has two main functions: synthesis of RNA, DNA and ribose-5-phosphate required for nucleotide synthesis in the cell, and synthesis of NADPH, which is a reducing power in reductive biosynthesis. In addition, phosphorylated carbohydrates such as erythrose-4-phosphate required for aromatic amino acid and vitamin synthesis and sedoheptulose-7-phosphate, which is a component of the bacterial cell wall, are synthesized in this way (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

NADPH plays a role in the reduction of fatty acid, cholesterol, L-ascorbic acid, nitric oxide biosynthesis, reduction of glutathione, drug and xenobiotic detoxification, and reduction of peroxides in the cell. Reduced glutathione (GSH) and GSH-dependent enzymes protect the cell from internal and external toxic compounds and reactive oxygen species (ROS). As the oxygen consumption and H_2O_2 formation in the phagocytes are high, the activity of the pentose phosphate pathway is also high. Xenobiotics are rendered harmless by using NADPH with glutathione peroxidase, cytochrome P-450 detoxification systems. Among the important enzymes of the sorbitol pathway, aldose reductase also uses NADPH. In addition, NADPH allows ribonucleotides to be converted into deoxyribonucleotides for DNA synthesis. NADPH is also required for many water-insoluble compounds (Grunewald 1993; Hollenberg 1992; Borregaard et al. 1984; Keha and Küfrevioğlu 2004; Lehninger 2000; Berg et al. 2002).

The pentose phosphate pathway is an alternative oxidative pathway to glycolysis, which is used for the oxidation of glucose, the main energy source, in some cells, such as red blood cells and brain tissue cells. In this way, 10% of the glucose in the cell is used (Baquer et al. 1988).

2. LITERATURE REVUEW

The 6-Phosphogluconate dehydrogenase enzyme (E.C.1.1.1.44; 6PGD) is the third enzyme of the pentose phosphate pathway that converts the 6-phosphogluconate compound to D-ribulose-5-phosphate in the presence of $NADP^+$. This reaction series result in the formation of the molecules D-ribulose-5-phosphate, which enter the biomolecule structure, and NADPH, which is used as a reducing agent in biochemical reactions. The most important function of the enzyme is to convert $NADP^+$ into NADPH in the metabolism (Yoshida et al. 1997). The enzyme is also extremely important for maintaining the balance of glucose-6-phosphate between the glycolytic and pentose phosphate pathways (Del Mar et al. 1986).

Hemolytic anemia, insufficient reticulocyte count, jaundice and episodic hemolytic events have been identified in the deficiency of the enzyme. Pyruvate kinase activity is increased and a decrease in the GSH level is observed when the enzyme is deficient. This, in turn, shortens lives as a result of early hemolysis of erythrocytes.

Although the 6PGD enzyme can use certain compounds as substrates, its natural substrate is 6-phosphogluconate. The affinity of the 6PGD enzyme in $NADP^+$ is generally higher than in 6-phosphogluconate, which is its substrate (Topham and Dalziel 1986). Also, it has been reported in studies that the NADPH-specific 6PGD enzyme is able to use 2-deoxy-6-phosphocluconate and 3-keto-6-phosphogluconate as substrates in human erythrocytes, lamb liver, Trypanosoma Brucei and Candida utilis (Hanau et al. 1992; Rippa et al. 1998). The coenzyme specificity of the enzyme has been identified as being in three different forms; those that specifically use NAD^+, those that specifically use $NADP^+$ and those that are non-specific (Ohara et al. 1996).

While the use of coenzymes varies by species, mammals and yeasts have generally been identified as $NADP^+$ specific and bacteria as both $NADP^+$ and NAD^+ specific 6PGD.

Today, the amino acid sequence of the enzyme obtained from more than 20 different sources has been identified. It has been found that the enzyme obtained from different sources is made up of an average of about 400 amino acids. Also, the amino acid sequence of enzymes obtained from different sources can vary depending on the source (Hutchison et al. 1984). For example, the human 6PGD enzyme consists of 484 amino acids.

In studies performed on different sources, the structure of the 6PGD gene was elucidated, its nucleotide sequence was determined and genes were cloned. For example, there is 1 gene in humans and 3 genes in tomatoes, found in the 4th, 5th and 12th chromosomes.

X-ray crystallographic examinations have revealed many amino acid residues in the active site that act as proton acceptors and donors. The amino acids of tyrosine 196, glutamate 195 and glutamate 222 were found to be present in a pocket considered to be the active site of the enzyme in sheep liver. It has been demonstrated by laboratory studies that the binding of the enzyme to the substrate is enabled by the ionization of the histidine found in its active region, the binding of the coenzyme is enabled by ionization of tyrosine and also that histidine is required for activity (Topham and Dalziel, 1986).

In X-ray studies, it has been shown that the enzyme is generally crystallized in the form of a rhombus. While the same structure was observed in Lactococcus lactis, the crystal was found to have a triangle form in trypanosoma brucei. In addition, the bovine enzyme was obtained in crystallized form and its three-dimensional structure was examined. It was shown that the enzyme is a homodimer, that each subunit consists of α-β-α-β regions and that these regions bind $NADP^+$ (Adams et al. 1991).

The molecular mass of 6-phosphoglucanate dehydrogenase ranges from 80 to 152 KDa based on its source and the molecular mass of its subunits ranges from 33 to 55 Da. In some organisms, it has a homodimeric structure and in some, it has a homotetrameric structure. It has been shown with experimental data that the molecular mass of the subunit of the enzyme, which has a homodimeric structure in humans, is 53 KDa.

While it varies depending on the species, the optimum pH range of the enzyme varies between 5.5-9.6. Enzyme activity was reported to increase in the range of pH 7.6-9.2 and to be lost after 9.3 (Barenghi et al. 1987). The optimum pH of the natural form of the enzyme is 8.6 in the human brain (Weisz et al. 1985), 7.0 in rat erythrocytes (Beydemir et al. 2003) and 8.0 in the murine renal cortex (Corpas et al. 1995).

The main problem encountered in the purification of the enzyme is that the enzyme is labile and not stable. In general, studies are carried out at +4°C in order to minimize the loss of activity. Stabilization was partially achieved by the addition of 2-mercaptoethanol, EDTA, $NADP^+$ and phenylmethylsulfonylfluoride to the buffer (Toews et al. 1976; Carne 1982; Pearse and Rosemeyer 1974). It was also found that bacteria-derived 6-PGD enzymes are generally more stable than animal-derived enzymes (Menezes et al. 1989).

G6PD and 6PGD were purified together using 2', 5'-ADP Sepharose 4B affinity and DEAE Sepharose fast flow ion exchange columns. Enzymes were eluted by forming a salt gradient from the ion exchange column (Ulusu et al. 1999).

In addition, the enzyme was purified using methods such as ammonium sulphate precipitation, high speed centrifugation, DE-52 column, SP-Sephadex, DEAE cellulose, CM-cellulose, DEAE-Sephadex, CM-Sephadex, hydroxyapatite and $NADP^+$-Sepharose, 2', 5'-ADP Sepharose 4B affinity chromatography, $NADP^+$-Agarose, etc. (Hanua et al. 2004).

3. MATERIAL AND METHOD

3.1. Material

3.1.1. Chemicals

2', 5'-ADP Sepharose 4B was purchased from Pharmacia, 6-Phosphogluconate, nicotine adenine dinucleotide phosphate, ethylene diamine tetraacetic acid, Tris-hydroxymethyl amino ethane hydrochloride Tris, dichlorodiphenyltrichloroethane, ammonium sulfate, acrylamide, bisacrylamide, potassium dihydrogen phosphate, potassium phosphate, potassium acetate, N,N,N',N'-tetramethylethylenediamine, sodium dodecyl sulfate, potassium chloride, Coomassie Brilliant Blue G-250, ammonium per sulfate, glycerol, potassium hydroxide, ethanol, acetic acid, HCl, and sodium chloride were purchased from either Sigma or Merck.

3.1.2. Instruments

Apparatus	Model
Zentrifugen	Universal 320R Hettich
Homogenization	ULTRA-TURRAX
pH meter	Inolab WTW pH 720 series
Automatic pipette	Eppendorf, Volac R 880 / A, 880 / D, 880 / E
Electrophoresis system	BIO-RAD (vertical)
Power supply	Thermo Scientific
Peristaltic pump	Eyeline Micro Tube Pump MP-3
Scrambler (Vortex)	Mixer VX 20
Spectrophotometer	IKA T25 digital (1min*1000)
Electronic balance	Close Avery

3.2. Preparation of Solutions

3.2.1. Homogenate solution:

1. 20 mM Tris-HCl+2 mM DDT+1mM EDTA (PH=7.5); 0.242 g Tris-HCl, 0.068 g DDT, 0.0292 g EDTA were dissolved in 80 mL of distilled water, the pH adjusted to 7.5 by using diluted HCl and KOH solution then the volume completed to 100 mL by distilled water.

3.2.2. Enzyme activity measurement solutions:

1. 1M Tris-HCl+5 mM EDTA; 0.0605 g (5 mmol) Tris-HCl, 0.0292 g (0.1 mmol) EDTA were dissolved in 90 ml distillate water, the pH adjusted to 8.0 with 0.1 M HCl then the total volume completed to 100 mL by distilled water.

2. 6 mM 6PGA solution; 0.0091 g (0.3 mmol) 6PGA was dissolved in 5mL distilled water.

3. 2 mM $NADP^+$ solution; 0.0076 g $NADP^+$ (0.1 mmol) was dissolved in 5 mL distilled water.

4. 50 mM KH_2PO_4: 0.68 g KH_2PO_4 (0.005 mol) was dissolved in 80 ml distilled water then the pH adjusted to 7.5 with 0.1 M KOH and the volume completed to 100 mL.

5. 10 mM Tris-HCl+1mM EDTA; 1.211 g Tris, 0.29225 g EDTA were dissolved in 975 ml of distilled water, then pH adjusted to 7.5 with 0.1 M HCl then the volume completed to 1000 mL.

3.2.3. Preparation of affinity chromatography solutions

1. 50 mM K_2PO_4+1 mM EDTA+1 mM DTT; 3.4 g K_2PO_4, 0.146 g EDTA, 0.077 g DTT were dissolved in 450 mL distilled water, then pH was adjusted to 7.3 and the total volume completed to 500 mL with distilled water.

2. 50 mM KH_2PO_4+1 mM EDTA+1 mM DDT (pH=7.3); 6.8 g KH_2PO_4, 0.292 g EDTA, 0.152 g DDT were dissolved in 950 mL with distilled water, then pH was adjusted 7.3 with 0.1 M KOH and total volume was completed to 1000 mL (This solution was used as buffers, washing and equilibrium).

3. 80 mM K-phosphate+10 mM EDTA+ 80 mM KCl+5 mM $NADP^+$; 0.544 g K-phosphate, 0.121 g EDTA, 0.298 KCl and 0.1913 g $NADP^+$ were dissolved in 40 ml distillate water, pH was adjusted to 7.85 with 0.1 M KOH then total volume was completed to 50 ml with distilled water (Elution solution).

4. 0.1 M Tris-HCl+0.5 M NaCl; 3.03 g Tris-HCl, 7.3 g NaCl were dissolved in 200 mL distilled water then pH was adjusted to 8.5 and the total volume was completed to 250 mL wit distilled water (Regeneration solution 1).

5. (0.1 M CH_3COONa+0.5 M NaCl; 3.4 g Na-acetate, 7.3 g NaCl were dissolved in 200 ml distilled water then pH was adjusted to 4.5 and the total volume completed to 250 ml with distilled water (Regeneration solution 2).

3.2.4. Preparation of SDS-PAGE Solutions

1. 1 M Tris-HCl (pH 8.8); 12.11 mg of Tris was dissolved in 80 mL of distilled water. The pH adjusted to 8.8 and the volume completed to 100 mL with distilled water.

2. 1 M Tris-HCl (pH 6.8); 12.11 mg of Tris was dissolved in 80 mL of distilled water. The pH adjusted to 6.8 and the volume completed to 100 mL with distilled water.

3. 30% Acrylamide+0.8% bisacrylamide; 15 mg of Acrylamide, 0.4 mg bisacrylamide and 34.6 mg of distilled water mixed to form 50 mL mixture solution.

4. 10% Ammonium persulfate; one mg of ammonium persulfate is dissolved in 10 mL of distilled water.

5. 10% SDS; one mg of SDS is mixed with nine mL of distilled water. Total volume was completed to 10 mL.

6. Running buffer for SDS PAGE; 1.51 mg of Tris, 7.51 mg of glycine was mixed and dissolved in 450 mL of distilled water and then five mL of 10% SDS was added to the mixture, the pH adjusted to 8.3 and the volume completed to 500 mL with distilled water.

7. Sample buffer; five mL from 1 M Tris-HCl (pH 6.8) solution, one mL from 10% SDS solution, one mL from 100% glycerin solution, one mL from bromothymol blue solution were added into a tube and the volume completed to 10 mL by distilled water, before using this buffer for each 950 µl of the buffer 50 µl of β-mercaptoethanol should be added.

3.3. Preparation of homogenate

Quail livers (*Coturnix coturnix japonica*) were taken from the Faculty of Aquaculture at Bingol University. Liver samples were first washed three times with 0.9% sodium chloride solution. Then, they were cut into small pieces using a scalpel. 5 g liver pieces were homogenized with the help of liquid nitrogen and suspended in a 20 mM Tris-HCl + 2 mM DDT+ 1mM EDTA, (pH 7.5). The mixture was centrifuged at 12100 rpm at 4℃ for 1 hour. The total volume of the homogenate was 12 mL.

3.4. Measurement of Enzyme Activity

Enzyme activity was measured by monitoring the change in absorbance at 340 nm due to the reduction of $NADP^+$. The reaction medium contained 0.1 mM Tris-HC1 (pH= 8.0), 0.5 mM EDTA, 10 mM $MgCl_2$, 0.2 mM $NADP^+$, and 0.6 mM (G6P) in a total volume of 1 mL. The assay protocol was seen in Table 3.1. One unit of enzyme (EU) activity was defined as the amount of enzyme reducing 1 µmol $NADP^+$ per 1 min at 25ºC (Beutler 1971).

Table 3.1. Protocol of the assay

Solutions	Blank	Test
0.1 M Tris- HCl (pH 8.0)	200 µl	200 µl
0.6 mM 6PGA	100 µl	100 µl
0.2 mM $NADP^+$	100 µl	100 µl
Distilled water	600 µl	570 µl
Enzyme sample	0.00 µl	30 µl
Total volume	1000 µl	

The enzyme activity determined following the equation.

$EU/ml = (\Delta OD/6.22\times V_T/V_E)\ S_F$

The icons have been represented in below;

- ΔOD: Change of absorbance per minute.

- 6.22: The extinction coefficient (1 mm, consisting of NADPH absorbance value).
- V_T: The total volume of the mixture.
- V_E: The volume of the enzyme.
- S_F: The dilution factor.

3.5. Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed at 0-20, 20-30, 30-40, 40-50, 50-60, 60-70% ranges; and the precipitation range of the enzyme was determined. Centrifugation was performed at 13,500 rpm for 15 minutes during each precipitation process. The precipitate, which was obtained after ammonium sulfate, was dissolved in the 50 mM KH_2PO_4 (pH 7.2) buffer. Enzyme activity was checked in the precipitate and supernatant for each time. All these processes were performed at 4^oC. Then, enzyme solution was dialyzed for duration of 2 h against the buffer that contained 10 mM KH_2PO_4 and 1 mM EDTA (pH 7.2).

3. 6. 2', 5'-ADP Sepharose 4B Affinity Chromatography

2', 5'-ADP Sepharose 4B gel was used for affinity chromatography. Two grams gel was taken and packed to the 1x10 cm column. The gel washed and balanced with 0.1 M K-phosphate+0.1 M KCl (pH=7.85) buffer. Then the dialyzed enzyme sample was loaded the column. Afterward the gel washed with 25 ml of 0.1 M potassium acetate + 0.1 M potassium phosphate (pH 6.0), 0.1 M potassium acetate+0.1 M potassium phosphate (pH 7.85), and finally with 25 ml of 0.1 M KCl+0.1 M potassium phosphate buffer (pH 7.85) respectively. After the washing, enzyme eluted with 80 mM K-phosphate+5mM $NADP^+$+10 mM EDTA, (pH=7.85) buffer. The flow rates of all steps were 25 $ml.h^{-1}$. Absorbance (280 nm) and activities (340 nm) were determined in each eluted tube. All experiments carry out at 4°C (Beydemir et al. 2004). After each purification step, the column was regenerated three times with 0.1 M Tris-HCl+0.5 M NaCl (pH 8.5; 45ml); and 0.1 M CH_3COONa+0.5 M NaCl (pH 4.5; 45 ml) solutions respectively.

3.7. Protein Determination

3.7.1. Qualitative protein determination

Qualitative determination of protein is based on the principle of maximum absorbance of the amino acids like tryptophan, tyrosine and phenylalanine, which are found in the structure of proteins, at 280 nm (Segel 1968). Qualitative protein determination in enzyme samples obtained from human erythrocytes was made according to this method. However, no result was obtained since the $NADP^+$ in the enzyme solution at 280 nm masked the absorbance of proteins.

3.7.2. Quantitative protein determination

The amount of protein in the enzyme solutions which were purified via hemolysate, ammonium sulfate precipitation and affinity chromatography was determined by this method. Coomassie Brillant Blue G-250 that is used as the dye in this method is negatively charged and binds to the positive charge on the protein. The dye has red (λmax= 465 nm) and blue (λmax= 595 nm) forms. Upon binding to the protein, the red form turns to the blue from. The reaction rate is high and it is completed in 2 minutes. The stability of the color can last for more than two hours (Bradford 1976). The method has a very low susceptibility to interfering substances (ranging between 1-100 µg).

A standard graphic is required to determine protein with this method. For this purpose, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl of standard bovine albumin solution containing 1 mg protein per 1 ml were taken into the tubes. The volume of all tubes was completed to 0,1 ml with pure water and they were vortexed after adding 5 ml of Coomassie Brilliant Blue G-250 solution. After incubation for 10 minutes, the absorbance values corresponding the blind were read at 595 nm in the 3 ml cuvettes. As the blind, a mixture of 5 ml Coomassie Brilliant Blue G-250 solution and the buffer containing 0.1 ml of the enzyme sample was used. The µg protein values corresponding the absorbance values were presented in a standard graphic.

The enzyme solutions purified by hemolysate, ammonium sulfate precipitation and affinity chromatography were put into the 0.1 ml tubes and 5 ml of Coomassie Brillant

Blue G-250 reagent was added. After vortexing, it was left to be incubated for 10 minutes. Afterwards, the absorbance values were read at 595 nm. Three trials were carried out for each sample and the true value was determined from the arithmetic mean of these three values. Protein quantities were determined according to these values by means of the standard graphic.

3.8. SDS-PAGE Electrophoresis

After purification of the enzyme, its purity was checked by using 3-8% discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli method (1970).

The electrophoresis plates were washed thoroughly first with water, then with alcohol for this process. On both sides, a spacer plate and a flat plate were clamped after they were aligned on top of each other. The stabilized plates were placed in a gel preparation cabinet containing a sponge that prevents leakage. First, the separating gel was prepared and it was filled with a syringe between the plates until 0.5 cm distance was left at the top. It was waited for two hours for the separating gel to polymerize. After making sure that the separating gel was solidified, the stacking gel was prepared. It was filled into the cavity at the top of the gel and the comb was inserted carefully to create the sample wells. While waiting for the stacking gel to polymerize, a wetted filter paper was closed over the system to prevent the desiccation of the gel. Following polymerization of the stacking gel, the comb was removed carefully and the sample wells were designated. It was washed first with pure water then with running buffer and was placed in the electrophoresis tank with the gel plates. Running buffer was filled in the upper and lower parts of the electrophoresis tank. The enzyme samples were prepared as there would be approximately 20 µg protein for each sample. Sample buffer at a ratio of 1:1 was added to get a total volume of 50 µl. It was incubated in boiling water bath for three minutes. The electrophoresis tank was closed. Then (+) anode was placed at the bottom and (-) cathode was placed at the top. First it was run for 20 minutes at 40 volts and the samples came up to the separating gel and agglomerated. Then the voltage was increased up to 80 volts to get the samples to reach the bottom border of the gel. The samples were tracked with the help of bromothymol blue added to sample buffer. After the running process was

over, the voltage was stopped, the gel between the plates were removed carefully and was put into the fixing solution. The gel, which had been left in the fixing solution for 20 minutes, was removed and placed in the dyeing solution and was left on the shaker for 2 hours. Afterwards, the stained gel was put in the washing solution. The gel, which was washed on the shaker until it faded and the protein bands appeared, was taken out and photographed.

3.9. Characterization Studies

3.9.1. Determination of stable pH

In order to determine the pH at which the enzyme was stable, Tris-HCl with the pH values varying between 7.5-9.0 and phosphate buffers with the pH values varying between 5.5-8.0 were used. The buffer solutions with the indicated pH values and the enzyme solutions were mixed at equal volumes and stored at +4°C. The pH at which the enzyme was stabilized was determined by the activity measurements made with 8 hour intervals for 32 hours.

3.9.2. Determination of optimum pH

In order to determine the optimum pH of the enzyme, Tris-HCl with the pH values varying between 7.5-9.0 and phosphate buffers with the pH values varying between 5.5-8.0 were used. The enzyme activity was determined one by one in every buffer with the appropriate substrate solution.

3.9.3. Determination of the optimum temperature

In order to determine the optimum temperature of the enzyme, the activity measurements were carried out between 0°C and 70°C with 10°C intervals.

3.9.4. Determination of ionic strength

In order to determine the ionic strength of the enzyme, at which it has the highest activity, the activity measurements were performed in 200, 400, 800, 1000 mM K-phosphate buffer (pH= 8.0) containing 1mM EDTA, respectively.

3.9.5. Molecular weight Determination

The molecular weight determination was made by SDS-PAGE (Figure 4.5.1). E. Coli ß-galactosidase (120 KDa), bovine serum albumin (85 KDa), ovalbumin white, chicken egg (50 KDa), carbonic anhydrase, and bovine erythrosites (35 KDa) (peqGOLD Protein-Marker III) were used as standards (Yilmaz et al. 2002).

3.10. Kinetic Studies

In order to determine the K_M and Vmax values for 6PGA and $NADP^+$ substrates of the enzyme, activity measurements were carried out at constant $NADP^+$ concentration with 5 different concentrations of 6PGA. Lineweaver-Burk graph was drawn with the obtained values. By means of this graph, the K_M and Vmax values for 6PGA were determined. In the same way, Lineweaver-Burk graph was drawn by measuring activity at a constant concentration of 6PGA with 5 different concentrations of $NADP^+$ and the K_M and Vmax values for GSH were calculated. The activity measurements were conducted under optimal conditions (Lineweaver and Burk 1934).

4. RESULTS

4.1. Measurement of Enzyme Activity

As described in Section 3.4 enzyme activity was measured by monitoring the change in absorbance at 340 nm due to the reduction of $NADP^+$ (Beutler 1971).

4.2. Ammonium Sulfate Precipitation

As described in Section 3.5 ammonium sulfate precipitation was performed at 0-20, 20-30, 30-40, 40-50, 50-60, 60-70% ranges; and the precipitation results were presented in Table 4.1 and Figure 4.1.

Table 4.1. Ammonium sulfate precipitation intervals, precipitate and supernatant activities

Percentage (%)	Supernatant Activity (EU/ml)	Precipitant Activity (EU/ml)
(0-20)	0.066	0
(20-30)	0.064	0
(30-40)	0.085	0
(40-50)	0.066	0.123
(50-60)	0.033	0.173
(60-70)	0.023	0.214

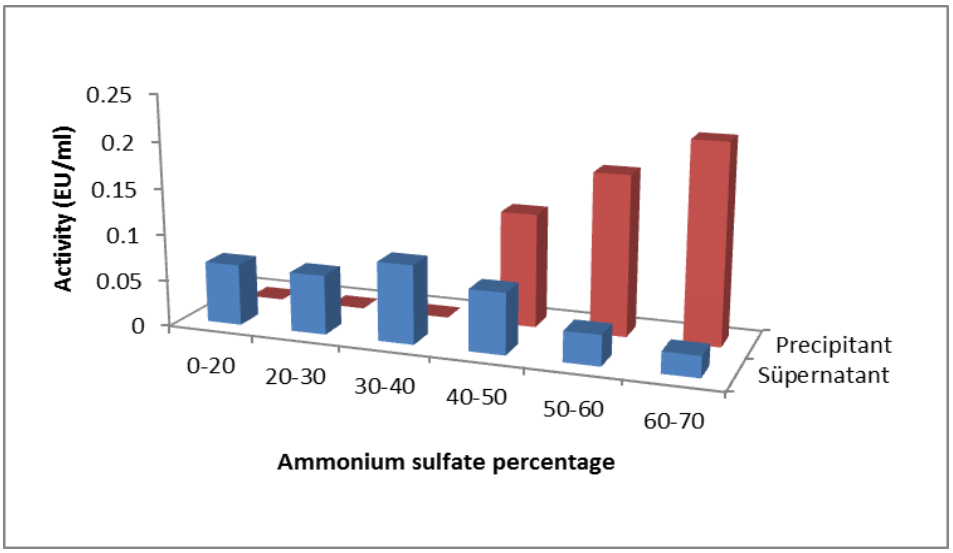

Figure 4.1. Ammonium sulfate precipitation intervals, precipitate and supernatant activities

4.3. Enzyme Purification

As described in Section 3.6 enzyme was purified by 2', 5'-ADP Sepharose 4B affinity chromatography (Beydemir et al. 2004) and results were showed in Table 4.2 and Figure 4.2.

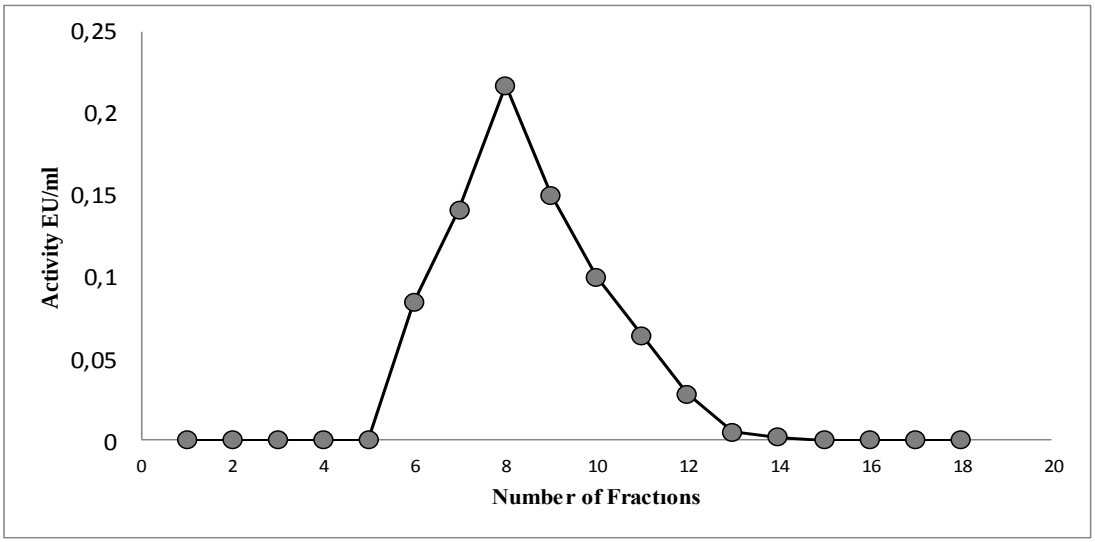

Figure 4.2. Fractions obtained from 2', 5'-ADP Sepharose 4B affinity column

Table 4.2. Purification scheme of 6PGD from quail liver

Purification process	Volume ml	Activity EU/ml	Total Activity EU/ml	Protein mg/ml	Total Proteins	Specific Activity EU/mg	Yield%	Purification folds
Homogenate	12	0.258	3.09	2.15	25.85	0.13	100	1
2', 5'-ADP Affinity Chromatography	3.5	0.79	2.76	0.054	0.190	14.6	89%	112

4.4. Protein Determination

4.4.1. Qualitative protein determination

As described in Section 3.7.1 qualitative protein determinations were calculated in homogenate, ammonium sulfate precipitation and purified enzyme solutions at 280 nm.

4.4.2. Quantitative protein determination

The quantitative protein assay was performed according to Bradford method (1976) Standard graphic (µg Protein *vs* Absorbance**)** was prepared as described in section 3.7.2 (Figure 4.3). Amounts of quantitative protein in the homogenate, ammonium sulfate precipitate and enzyme solution were obtained from this standard plot.

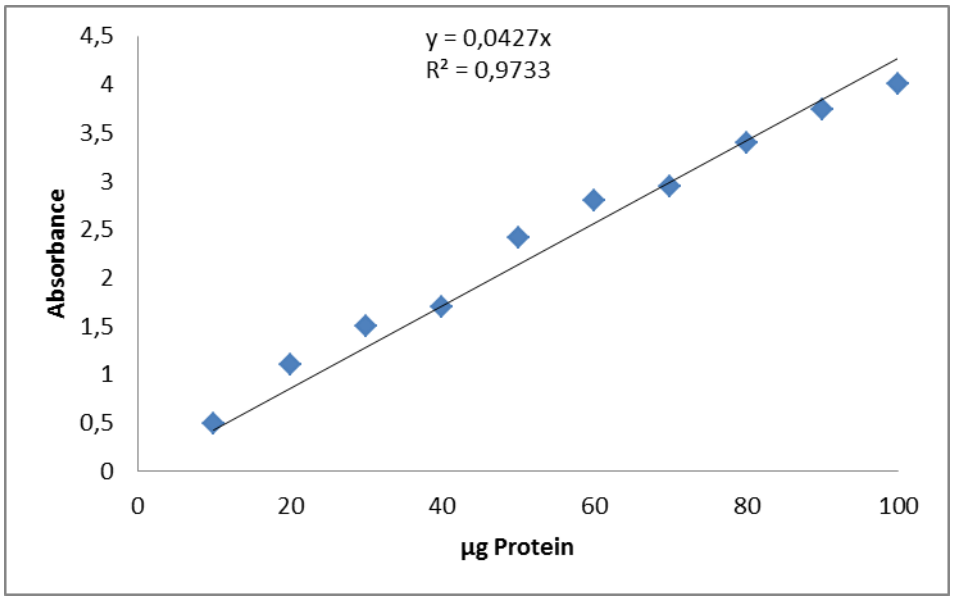

Figure 4.3. Standard curve is for determination of protein by the Bradford method

4.5. SDS-PAGE

As described in Section 3.8, 3-8% SDS-PAGE was done by Laemmli method (1970) and presented in Figure 4.4.

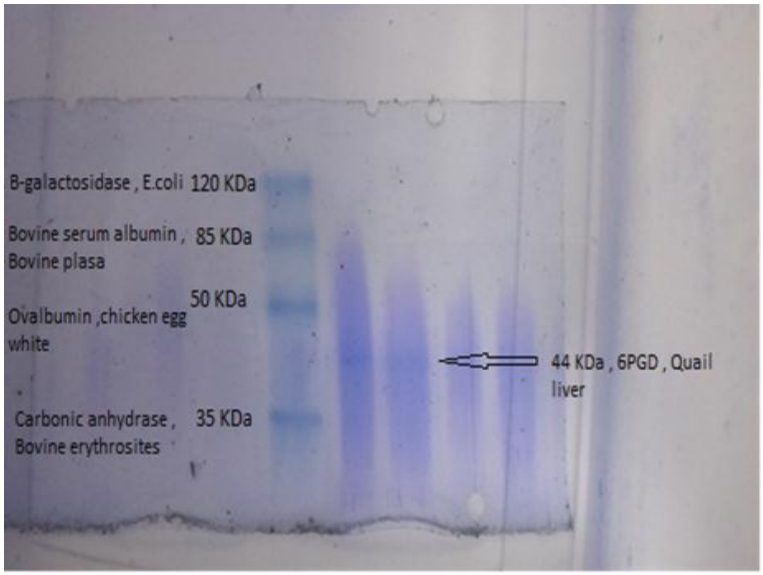

Figure 4.4. SDS-PAGE bands of proteins (Lane 1: Standard proteins; Lane 3: 6PGD) (peqGOLD Protein-Marker III)

4.6. Characterization Studies

4.6.1. Stable pH studies

As described in Section 3.9.1**,** the enzyme activities were determined in Tris-HCl buffer at pH of 7.5, 8.0, 8.5, and 9.0, and in phosphate buffer at pH of 6.0, 7.0, 7.5, and 8.0. Then pH *vs* Activities tables and figures were drawn and presented in Tables 4.3, 4.4 and Figures 4.5, 4.6.

Table 4.3. The results of stable pH studies of quail liver 6PGD in Tris-HCl buffers

Hours / pH	0	8	16	24	32
pH= 7.5	0.0193	0.0182	0.0171	0.0182	0.0150
pH= 8.0	0.0225	0.0214	0.0203	0.0182	0.0182
pH= 8.5	0.0160	0.0150	0.0141	0.0117	0.0117
pH= 9.0	0.0150	0.0128	0.0128	0.0117	0.0107

Table 4.4. The results of stable pH studies of quail liver 6PGD in K-phosphate buffers

Hours / pH	0	8	16	24	32
pH= 6.0	0.0142	0.0133	0.0117	0.0127	0.0118
pH= 7.0	0.0139	0.0128	0.0128	0.0117	0.0115
pH= 7.5	0.0160	0.0150	0.0128	0.0107	0.0107
pH= 8.0	0.0133	0.0118	0.0110	0.0110	0.0105

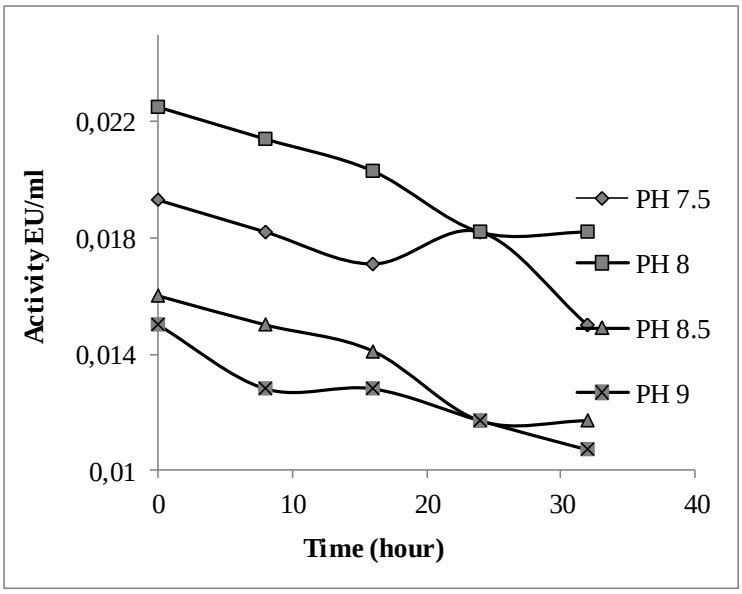

Figure 4.5. Stable pH graph of quail liver 6PGD in Tris-HCl buffer

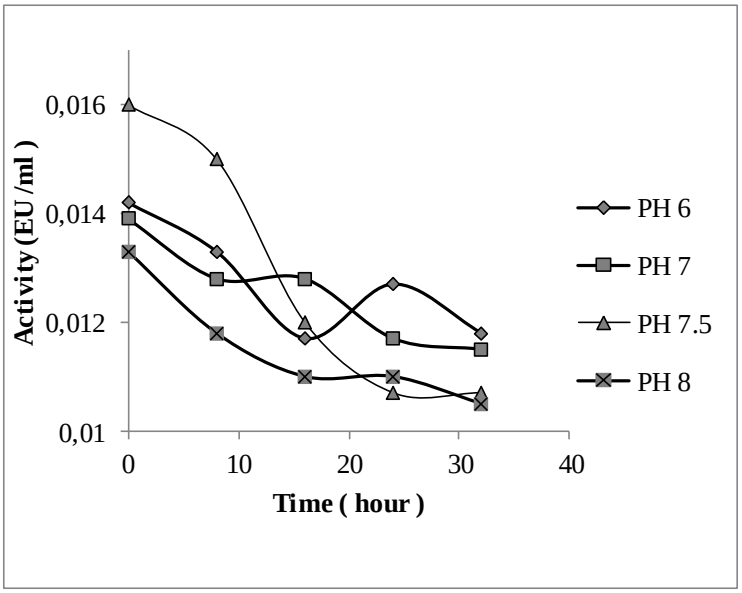

Figure 4.6. Stable pH graph of quail liver 6PGD in K-phosphate buffer

4.6.2. Optimum pH studies

As shown in Chapter 3.9.2, Activity-pH graphs were drawn by measuring the enzyme activity using phosphate buffers for which the pHs are ranging from 5.5 to 8.0 and Tris-HCl buffer for which the pHs are ranging from 7.5 to 9.0 for determine the optimal pH of the enzyme and the corresponding graph is shown in Figure 4.7.

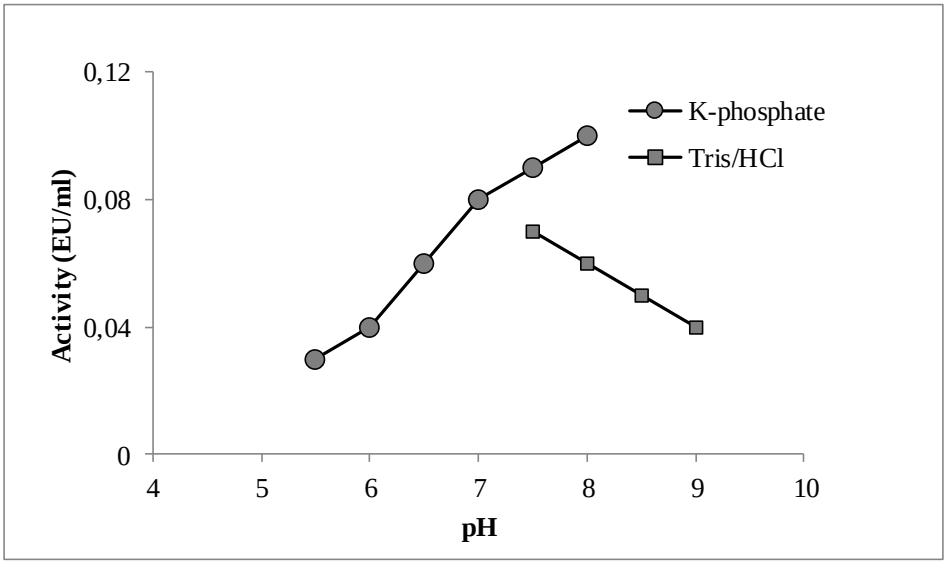

Figure 4.7. PH-Activity graph of enzyme

4.6.3. Optimum temperature studies

As shown in chapter 3.9.3, Temperature-Activity graph was drawn ranging of activity measurements from 0°C to 70°C (Table 4.5) in 10°C intervals for determining the optimum temperature of the enzyme and the graph is shown in Figure 4.8.

Table 4.5. The activities measured at various temperatures to determine the optimum temperature of the 6PGD enzyme

Temperature (°C)	0	10	20	30	40	50	60	70
Activity (EU/ml)	0.01	0.03	0.035	0.04	0.1	0.14	0.16	0.001

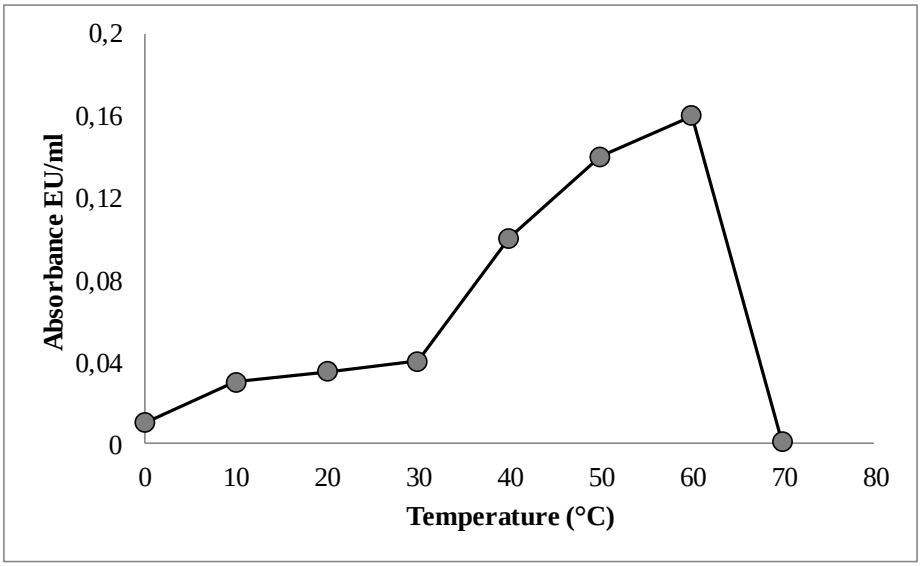

Figure 4.8. Activity-Temperature graph of quail liver 6PGD enzyme

4.6.4. Ionic strength studies

As shown in chapter 3.9.4, activity measurements were performed to determine the ionic strength in the Tris-HCl buffers (pH= 8.0) 0.2, 0.4, 0.8, 1 M intervals, which is including 1 mM EDTA at which the enzyme had the highest activity and the results were shown in table 4.6 and figure 4.9.

Table 4.6. The effects of different concentration of Tris-HCl at pH= 8.0 on the activity of purified 6PGD enzyme

0.1 M Tris-HCl	0.2 M Tris-HCl	0.4 M Tris- HCl	0.6 M Tris-HCl	0.8 M Tris-HCl	1.0M Tris-HCl
Activity EU/ml	0.037	0.05	0.09	0.08	0.04

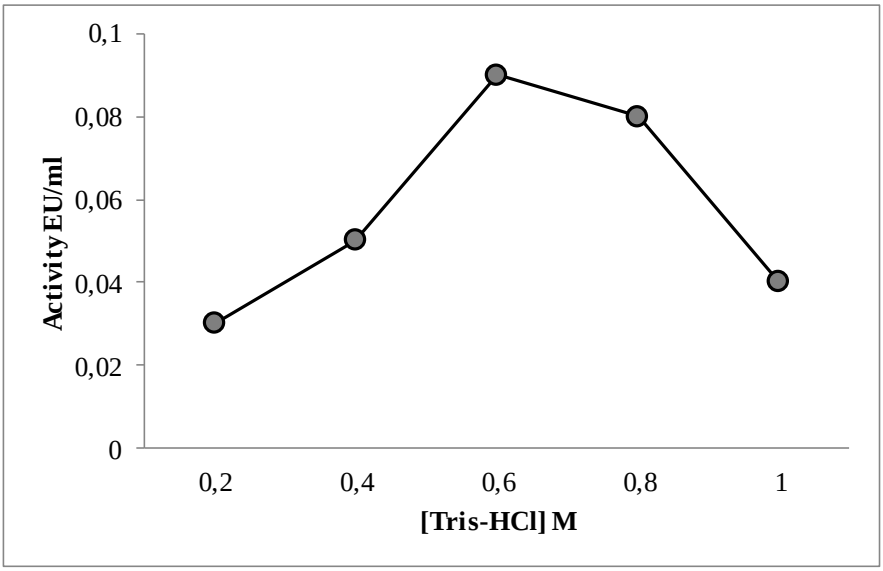

Figure 4.9. Determination of ionic strength of the 6PGD enzyme in Tris-HCl buffer

4.6.5. Molecular weight determination

For the standard proteins and 6PGD, R_f values were calculated from Figure 4.4, and R_f-LogMW graph (Figure 4.10) was obtained according to Laemmli's procedure (1970) showing a molecular weight of 44 KDa for 6PGD.

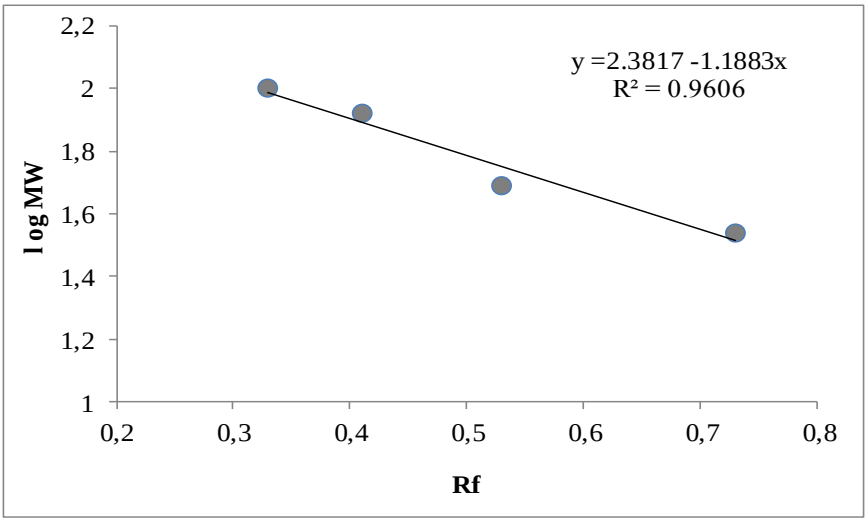

Figure 4.10. Standard Rf-Log MW graph

4.7. Kinetic studies

As shown in chapter 3.10, K_M and Vmax values were determined from Lineweaver-Burk curves (Lineweaver and Burk 1934) and the results were shown in tables 4.7-4.8 and figures 4.11- 4.12.

Table 4.7. 1/V ve 1/[S] values for the determination of K_M and Vmax values of the enzyme in the present of $NADP^+$ as a substrate

1/V	333	250	166	142	125
1/ [$NADP^+$]	0.1	0.04	0.02	0.01	0.005

Table 4.8. 1/V ve 1/[S] values for the determination of K_M and Vmax values of the enzyme in the present of 6PGA as a substrate

1/V	45	55	76	125	250
1/ [6PG]	0.002	0.003	0.005	0.01	0.02

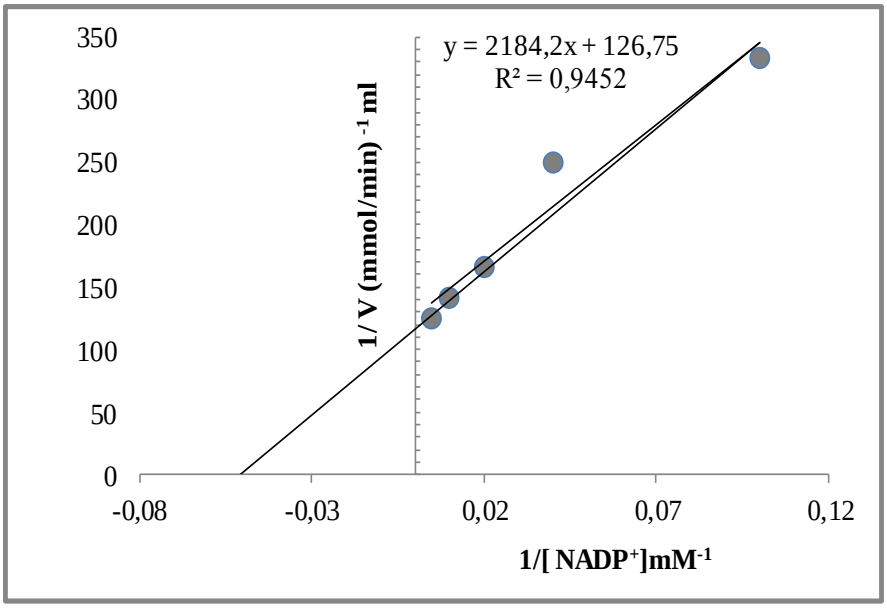

Figure 4.11**.** Lineweaver-Burk graph in 5 different $NADP^+$ concentrations with fixed 6PGA

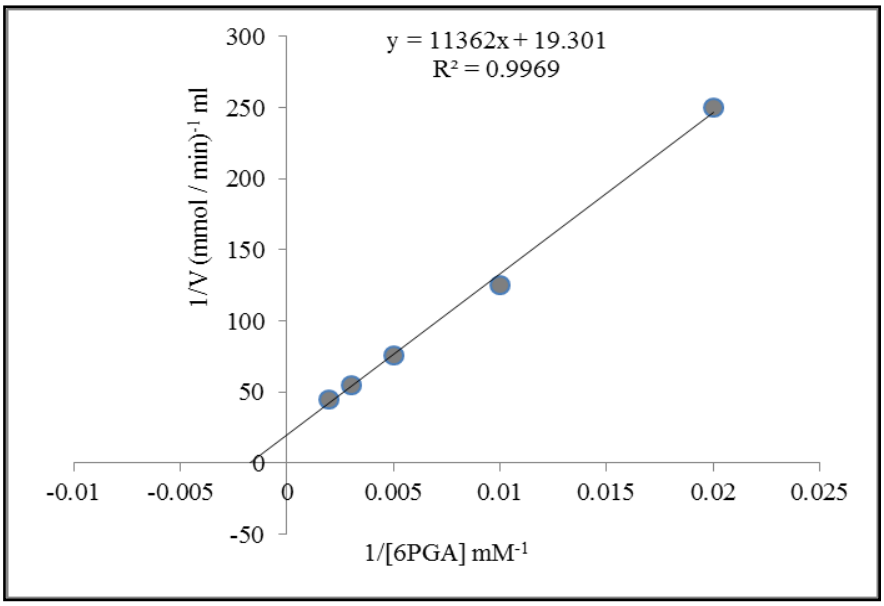

Figure 4.12. Lineweaver-Burk graph in 5 different concentrations 6PGA with fixed $NADP^+$

5. DISCUSSION

6-phosphogluconate dehydrogenase is the third enzyme of the pentose phosphate pathway. 6PGD enzyme catalyzes the conversion of 6PGA and $NADP^+$ into ribulose-5-phosphate and NADPH. NADPH, which is produced in this reaction, is used as a reducing power in metabolism. For example, it facilitates the conversion of oxidized glutathione (GSSG) into reduced glutathione (GSH). GSH is used in antioxidant activity (Yoshida and Beutler 1986). Thus, 6PGD enzyme is considered to be an indirect antioxidant enzyme. The ribulose-5-phosphate produced in this reaction is necessary for the synthesis of nucleotides, some aromatic amino acids and some vitamins (Miclet et al. 2001). Due to these important features, the enzyme has been obtained by purification of the quail liver for the first time by us and some characteristics of the enzyme have been identified.

In the scope of the study, 6PGD enzyme, which has protein specific activity of 14.6 EU/mg, has been purified through 2', 5'-ADP Sepharose 4B affinity chromatography for 112 times with an efficiency of 89% (Figure 4.3.1 and Table 4.3.1**)**. The purification and characterization of 6PGD enzyme from various sources is seen in the literature studies.

For example, the enzyme (4.0 EU/mg protein) has been obtained by purifying sheep liver 315 times with the efficiency of 29% (Beydemir et al. 2004), by purifying rat erythrocytes (5.15 EU/mg.protein) 2775 times with the efficiency of 78.4% (Ciftci et al. 2002), by purifying parsley leaf (18.3 EU/mg.protein) 339 times with the efficiency of 49% (Hulya et al. 2003) and by purifying yellow catfish liver (5.57 EU/mg.protein) 95.8 times with the efficiency of 50% (Zhuo et al. 2015). Most of the specific activities, efficiency rates and purification coefficients in our research seem to be better when compared to the ones obtained in these studies. Ammonium sulfate precipitation is extremely important for elution of concentrated enzyme prior to 2', 5'-ADP Sepharose 4B affinity chromatography. For this purpose, many impurities have behaving the For

This purpose,6PGD enzyme was fully precipitated between 40% and 70% range. Similar results have been obtained by Beydemir et al. (2004), and Hulya et al. (2003). The Bradford method (1976) having consistency of 5-500 μg/ml was preferred for the quantitative protein estimation. This method was preferred for its high sensitivity and because it has been widely used by researchers.

As seen in Figure 4.5.1, a single band was obtained in the third well. Because of this single band, it is understood that the enzyme was fully purified. Also, the R_f-MW graph was plotted (Figure 4.6.5.1) by using this image and the molecular mass of the enzyme under the denaturing conditions was calculated as 44 KDa. According to our literature study, the subunit molecular mass of this enzyme is generally reported in the range of 38-65 KDa (Beydemir et al. 2004, Toews et al. 1976, Villet and Daizail 1969, Tasi and Chen 1998, Krepinsky et al. 2001, Williamson et al. 1980, Bridges et al. 1975, Yoon et al. 1989).

To determine the pH level at which the enzyme is stable, activity measurements at different pHs were made for 32 hours by using Tris-HCl and K-phosphate buffers. As seen in Table 4.6.1.1, Table 4.6.1.2 and Figure 4.6.1.1, Figure 4.6.1.2, the highest and most stable activity was obtained with Tris-HCl buffer at pH = 8.0. Therefore, the pH stability of the enzyme was assumed to be 8.0 in 0.6 mM Tris-HCl buffer. We have determined that similar results were obtained by Beydemir et al. (2004) and Hulya et al. (2003).

Tris-HCl and K-phosphate buffers were also used for optimal pH study. As seen in Figure 4.6.2.1, the highest activity was obtained with the 0.6 M K-phosphate buffer at pH = 8.0. Therefore, the optimal pH of the enzyme was assumed to be 8.0 with the 0.6 M K-phosphate buffer. Similar results were obtained by Beydemir et al. (2004) and Hulya et al. (2003) and Zhuo et al. (2015).

As seen in Table 4.6.3.1 and Figure 4.6.3.1, the activity of the enzyme increased by temperature and reached the maximum value at 60°C. It fell rapidly after reaching 60°C. This situation is related with the denaturation of proteins because most proteins usually lose their activities and three-dimensional structures over the temperature of 50-60°C.

This finding is similar with the results obtained by Beydemir et al. (2004) and Hulya et al. (2003) and Zhuo et al. (2015).

As seen in Table 4.6. 4.1 and Figure 4.6. 4.1, the maximum activity under optimal conditions was obtained by using 0.6 M Tris-HCl buffer. Thus, the optimal ionic strength of the enzyme was assumed to be 0.6 M Tris-HCl. This value is quite different from the one determined by Beydemir et al. (2004). By using Table 4.7.1, Table 4.7.2 and Figure 4.7.1, Figure 4.7.2, K_M and Vmax values were calculated for 6PGA and $NADP^+$, which are substrates of the enzyme. The K_M values for 6PGA and $NADP^+$ were found to be 0.0058 mM and 0.0015 mM respectively. It is seen that the K_M value for $NADP^+$ is smaller when compared to the K_M value for 6PGA. In this case, it can be said that the affinity of $NADP^+$ with enzyme is greater than 6PGA. Similar results were detected also by Beydemir et al. (2004) and Hulya et al. (2003) and Zhuo et al. (2015).

Summary	
Optimum pH	pH= 8.0 in K-Phosphate
Optimum temperature	60 °C
Optimum ionic strength	0.6 M in Tris-HCl buffer (pH= 8.0)
Stable pH	pH= 8.0 in Tris-HCl
Kinetic values of 6PGA	Vmax= 0.0078 EU/ml, K_M= 0.0058 mM
Kinetic values of $NADP^+$	Vmax= 0.057 EU/ml, K_M= 0.0015mM
Molecular weight	44 KDa
Purification folds	112 folds
Specific activity	14.6 EU/mg. Protein
Yield	89%

REFERENCES

Adam, MJ., Gover, S., Leaback, R., Phillips, C., Somers, DO'N., "The structure of 6-phosphogluconate dehydrogenase refined at 2.5 A resolution", Acta Crystallographica Section B Structural Science, 47: 817-820, 1991.

Baquer, NZ., Hothersall, JS., McLean, P., "Function and regulation of the pentose phosphate pathway in brain", Curr Top Cell Regul, 29: 265-89, 1988.

Barenghi, L., Ceriotti, F., Ripamonti, M., Luzzana, M., & Bonini, P., "Erythrocytic glucose-6-phosphate dehydrogenase measured by a differential pH technique", Clinical chemistry, 33(4): 579-582, 1978.

Beutler, E., "Red cell metabolizm Manual of Biochemical Methods", Academic press, London, 12: 68-70, 1971.

Berg, JM., Tymoczko, JL., Stryer, L., "Biochemistry.5th edition, New York: W.H Freeman", 2002.

Beydemir, S., Kulaçoglu, DN., Çiftçi, M., Küfrevioglu, OI., 2003. "The effects of some antibiotics on sheep lens glucose-6-phosphate dehydrogenase in vitro", European journal of ophthalmology, 13: 155–161, 2003.

Beydemir, S., M, çiftci., H, yilmaz., Öİ, kufrevioglu., "6-Phosphogluconate dehydrogenase: Purification, Characterization and Kinetic Properties from Rat Erythrocytes", Turkish Journal of Veterinary and Animal Sciences, 28: 707-714, 2004.

Borregaard, N., Schwartz, JH., Tauber, A., "Proton secretion by stimulated neutrophils", Journal Clinical Investigation, 74(2): 455, 1984.

Bradford, M., “A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding”, Analytical Biochemistry, 72: 248, 1976.

Bridges, RB., Palumbo, MP., Wittenberger, CL., “Purification and properties of an NADP specific 6-phosphogluconate dehydrogenase from streptococcus faecalis”, Journal of Biological Chemistry, 250(15): 6093-6100, 1975.

Carne, A., “Purification by dye-ligand chromatography of an NADP dependent enzyme, 6-phosphogluconate dehydrogenase from lamb’s liver”, Anal Biochemistry, 121: 227-229, 1982.

Çiftci, M., Beydemir, S., Yılmaz, H., Bakan, E., “Effects of some drugs on rat erythrocyte 6-phosphogluconate dehydrogenase: an in vitro and in vivo study”. Pol Journal Pharmacol, 54(3): 275-80, 2002.

Corpas, FJ., Garca-Salguero, L., Barroso, JB., Aranda, F., Lupitez, J.A., “Kinetic properties of hexose-monophosphate dehydrogenases. Isolation and partial purification of 6-phosphogluconate dehydrogenase from rat liver and kidney cortex”, Moll Cell Biochemistry, 144(2): 97-104, 1995.

del Mar, Medina-Puerta., Garrido-Pertierra, A., “Purification and properties of the enzyme 6-phosphogluconate dehydrogenase from Dicentrarchus labrax L. Liver”, Comp Biochem Physiol B, 83(1): 215-20, 1986.

Grunewald, RW., Weber, II., Kinne-Saffran, E., “Control of sorbitol metabolismin renal inner medulla of diabetic rats”, Biochim Biophys Acta, 1225: 39-47, 1993.

Hanau, S., Rippa, M., Dallocchio, F., “NADPH activates a decarboxylation reaction catalysed by lamb liver 6-phosphogluconate dehydrogenase”, Biochimica et Biophysica Acta, 1122(3): 273-277, 1992.

Hanau, S1., Rinaldi, E., Dallocchio, F., Gilbert, IH., Dardonville, C., Adams, MJ., Gover, S., Barrett, MP., "6-phosphogluconate dehydrogenase: a target for drugs in African trypanosomes", Curr Med Chem. Oct, 11(19): 2639-50, 2004.

Hollenberg, PF., "Mechanisms of cytochrome p-450 and peroxidasecatalyzed xenobiothic metabolism", FASEP J, 6: 686-94, 1992.

Hülya, Demir., Mehmet, Çiftci., and ÖIrfan, Küfrevioğlu., "Purification of 6-phosphogluconate dehydrogenase from parsley (Petroselinum hortense) leaves and investigation of some kinetic properties", Prep Biochem Biotechnology Feb, 33(1): 39-52, 2003.

Hutchison, JS., Winberry, L., Nakayama, R., Holten, D., "Kinetics for changes in enzyme synthesis and mRNA content and hormones required for induction of 6-phosphogluconate dehydrogenase in hepatocytes", Biochimica et Biophysica Acta, 781: 30-38, 1984.

Keha, E., ve Küfrevioğlu, ÖÎ., Biyokimya, Aktif Yayınevi, Erzurum. Kesim, MD., "Sigara ve Gebelik", Şişli Etfal Hastanesi Tıp Bülteni, 38(2): 7-14, 2004.

Krepinsky, K., Plaumann, M., Martin, W., Schnarrenberger, C., "Purification and cloning of chloroplast 6-phosphogluconate dehydrogenase from spinach. Cyanobacterial genes for chloroplast and cytosolic isoenzymes encoded in eukaryotic chromosomes", Eur Biochemistry, 268(9): 2678-2686, 2001.

Laemmli, UK., "Cleavage of structural proteins during the assembly of the head of bacteriophage", T4. Nature, 227, 680-685, 1970.

Lehninger, AL., Nelson, DL., Cox, MM., "Principles of Biochemistry", 2nd editions. Worth Publishers Inc., New York, 558-560, 2000.

H, Lineweaver., D, Burk., Journal of the American Chemical Society, 1934, 56(3): 658–666.

Menezes, L., Kelkar, SM., Kaklij, GS., "Glucose 6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from lactobacilius casei", Responses with different modulators. Indian journal of biochemistry & biophysics, 26(5): 329-333, 1989.

Miclet, E., Stoven, V., Michels, PA., Opperdoes, FR., Lallemand, J.Y., Duffieux, F.,. "NMR spectroscopic analysis of the first two steps of the pentose phosphate pathway elucidates the role of 6-phosphogluconolactonase", Journal of Biological Chemistry, 276(37): 34840- 34846, 2001.

Ohara, H., Uchida, K., Yahata, M., Kondo, K., "NAD^+- specific 6-phosphoglueonate dehydrogenase in lactic acid bacteria", Bioscience, biotechnology, and biochemistry, 60(4): 692-693, 1996.

Pearse, BM., Rosemeyer, MA., "The molecular weight and subunit structure of human erythrocytes 6-phosphgluconate dehydrogenase", European Journal of Biochemistry, 42(1): 225-235, 1974.

Rippa, M., Giovannini, PP., Baret, MP., Dallocchiof, H., "6-phosphogluconate dehydrogenase: the mechanism of action investigated by a comparison of the enzyme from different species", Biochimica et Biophysica Acta, 1429(1): 83-92, 1998.

Segel, IH., "Biochemical calculations". Inc., New York, 403, 1968.

Tsai, CS., and Chen, Q., "Biochemistry and cell biology", Biology, 76(4): 637-644, 1998.

Toews, ML., Kanji, MI., Carper, WR., "6-Phosphogluconate dehydrogenase. Purification and kinetics", J Biol Chem, 251(22): 7127-31, 1976.

Topham, CM., Dalziel, K., " The chemical mechanism of sheep liver 6-phosphogluconate dehydrogenase", Biochemistry Journal, 234, 671-677, 1986.

Ulusu, NN., Kus, MS., Acan, NL., Tezcan, EF., "A rapid method for the purification of glucose-6-phosphate dehydrogenase from bovine lens", The international journal of biochemistry & cell biology, 31(7): 787-796, 1999.

Villet, RH., and Dalziel, K. Biochemistry Journal, 115: 633-638, 1969.

Weisz, KS., Schofield, PJ., Edwards, MR., "Human Brain 6-Phosphogluconate Dehydrogenase: Purification and Kinetic Properties", Journal of Neurochemistry, 44(2): 510–517, 1985.

Williamson, JH., Krochko, D., Geer, BW., "6-Phosphogluconate dehydrogenase from Drosophila melanogaster L. Purification and properties of the A isozyme", Biochemistry Genet, 18: 87-95, 1980.

Yılmaz, H., Ciftci, M., Beydemir, Ş., Bakan, E., "Purıfıcatıon of glucose 6-phosphate dehydrogenase from chıcken erythrocytes ınvestıgatıon of some kınetıc propertıes", Preparative Biochemistry and Biotechnology, 32: 3, 287-301, 2002.

Yoon, H., Anderson, CD., Anderson, BM., "Kinetic studies of Haemophilus influenza 6-phosphogluconate dehydrogenase", Biochim Biophys Acta, 994(1): 75-80, 1989.

Yoshida, A., and Beutler, E., "Glucose-6-phosphate Dehydrogenase n.p. Academic", Press, Ltd., London, 1986.

Yoshida, Y., Nakano, Y., Yamashita, Y., Icoga, T., " The gnd gene encoding a novel 6-PGDH and its adjacent region of actinobacillus aclinomycelemcomitants chrosomal DNA", Biochemical and biophysical research communications, 230(1): 220-225, 1997.

Zhuo, M., Luo, Z., Tan, X., Hu, W., Sun, L., Zheng, J., & Chen, Q., "Purification and kinetic characteristics of hepatic 6-phosphogluconate dehydrogenase (6PGD) from yellow catfish Pelteobagrus fulvidraco", Turkish Journal of Biochemistry, 40(1): 15-23, 2015.

CURRICULUM VITAE

I was born in Erbil 1978. I completed my primary, secondary, high school in Erbil. I entered the college of the science department of chemistry Salahhadin University, graduated in 2001. In 2003, I used to work in the laboratory of the hospital doing chemical testing. In February 2015 started post-graduation from Bingol University, Department of Biochemistry Science.