T.R.
VAN YUZUNCU YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF CHEMISTRY

EXAMINATION OF THE EFFECT OF SOME VITAMINS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE ENZYME PURIFIED FROM SHEEP LIVER

M.Sc. THESIS

Warvin H. M. Salim MOHAMMED SALIM
Supervisor: Assoc. Prof. Dr. Zehra BAŞ

VAN – 2024

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Jury Members of the Thesis Defence Exam

Prof. Dr. Vedat TÜRKOĞLU (Chair)

Assoc. Prof. Dr. Zehra BAŞ (Supervisor)

Prof. Dr. Fatih Çağlar ÇELİKEZEN (Member)

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ACCEPTANCE and APPROVAL PAGE

This thesis entitled "Examination of the Effect of Some Vitamins on Glucose-6-Phosphate Dehydrogenase Enzyme Purified from Sheep Liver" presented by Warvin H. M. Salim MOHAMMED SALIM under supervision of Assoc. Prof. Dr. Zehra BAŞ in the department of Chemistry has been accepted as a M.Sc. thesis according to Legislations of Graduate Higher Education on 23/09/2024 with unanimity of votes members of jury.

Chair: Prof. Dr. Vedat TÜRKOĞLU	Signature:
Member: Prof. Dr. Fatih Çağlar ÇELİKEZEN	Signature:
Member: Assoc. Prof. Dr. Zehra BAŞ	Signature:

This thesis has been approved by the committee of The Institute of Natural and Applied Science on ..……../...../…...….... with decision number .....

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ETHICAL DECLARATION

I declare that all the information in this thesis has been obtained and presented within the framework of ethical behavior and academic rules, and that in this thesis, which has been prepared in accordance with the thesis writing rules, all kinds of statements and information that do not belong to me have been fully cited.

Signature

Warvin H. M. Salim MOHAMMED SALIM

ABSTRACT

EXAMINATION OF THE EFFECT OF SOME VITAMINS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE ENZYME PURIFIED FROM SHEEP LIVER

MOHAMMED SALIM, Warvin H. M. Salim
M.Sc. Thesis, Department of Chemistry
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The enzyme glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) is the first and rate-limiting enzyme of the pentose phosphate pathway, which is essential for the integrity and function of red blood cells. In this study, G6PD was purified 6308-fold in a single step by affinity chromatography from sheep liver. The molecular weight of the G6PD enzyme was found to be 93 kDa with SDS-PAGE. V_{max} and K_M values were calculated as 0.58 (μmol/min).mL^{-1} and 1.44 mM, respectively. The effect of biotin (vitamin B_7) and folic acid (vitamin B_9) vitamins on G6PD activity was examined. Biotin and folic acid had a significantly inhibitory effect on G6PD activity. The IC_{50} values of biotin and folic acid vitamins were calculated as 61.64 μM and 17.21 μM, respectively. K_i values and inhibitory types of these vitamins were found by Lineweaver-Burk graphs. The inhibitory type of these vitamins was determined as non-competitive inhibitory. K_i values for biotin and folic acid were calculated as 121.13 μM and 22.73 μM, respectively. The increase in the activity of the G6PD enzyme in various cancer diseases causes the rapid development of cancer cells. Therefore, G6PD inhibitors can be a very important treatment method in cancer treatment. In this study, it was concluded that biotin and folic acid vitamins may be effective in cancer treatment with their inhibitory effect on G6PD.

Keywords: Activation, Glucose-6-phosphate dehydrogenase, Inhibition, Purification, Vitamins

ÖZET

KOYUN KARACİĞERİNDEN SAFLAŞTIRILAN GLUKOZ-6-FOSFAT DEHİDROGENAZ ENZİMİ ÜZERİNE BAZI VİTAMİNLERİN ETKİSİNİN İNCELENMESİ

MOHAMMED SALIM, Warvin H. M. Salim
Yüksek Lisans Tezi, Kimya Anabilim Dalı
Danışman: Doç. Dr. Zehra BAŞ
Eylül 2024, 53 sayfa

Glikoz-6-fosfat dehidrogenaz (G6PD, EC 1.1.1.49) enzimi kırmızı kan hücrelerinin bütünlüğü ve işlevi için gerekli olan pentoz fosfat yolunun ilk ve hız sınırlayıcı enzimidir. Bu çalışmada G6PD, koyun karaciğerinden afinite kromatografisi ile tek basamakta 6308 kat saflaştırıldı. SDS-PAGE ile G6PD enziminin molekül ağırlığı 93 kDa olarak bulundu. V_{max} ve K_M ve değerleri sırasıyla 0.58 (µmol/dk).mL^{-1} ve 1.44 mM olarak hesaplandı. G6PD aktivitesi üzerine biotin (B_7 vitamini) ve folik asit (B_9 vitamini) vitaminlerinin etkisi incelendi. Biotin ve folik asit G6PD aktivitesi üzerine önemli ölçüde inhibitör etkisi gösterdi. Biotin ve folik asit vitaminlerinin IC_{50} değerleri sırasıyla 61.64 µM ve 17.21 µM olarak hesaplandı. Lineweaver-Burk grafikleri ile bu vitaminlerin K_i değerleri ve inhibitör türleri bulundu. Bu vitaminlerin inhibitör türü yarışmasız inhibitör olarak belirlendi. Biotin ve folik asit için K_i değerleri sırasıyla 121.13 µM ve 22.73 µM olarak hesaplandı. Çeşitli kanser hastalıklarında G6PD enziminin aktivitesinin artması kanser hücrelerinin hızlı gelişmesine sebep olmaktadır. Bu nedenle G6PD inhibitörleri kanser tedavisinde çok önemli bir tedavi yöntemi olabilir. Bu çalışmada, biotin ve folik asit vitaminlerinin G6PD üzerine inhibitör etkisi göstermesi ile kanser tedavisinde etkili olabileceği sonucuna varıldı.

Anahtar kelimeler: Aktivasyon, Glukoz-6-fosfat dehidrogenaz, İnhibisyon, Vitaminler, Saflaştırma

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2024

Warvin H. M. Salim MOHAMMED SALIM

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SYMBOLS AND ABBREVIATIONS

Some symbols and abbreviations used in this study are presented below with their descriptions.

Symbols	Description
µg/mL	Microgram/milliliter
µL	Microliter
µM	Micromolar
Da	Dalton
min	Minute
EU	Enzyme unit
g	Gram
IC_{50}	Inhibitor concentration that causes 50% inhibition
kcal	Kilocalories
kDa	Kilodalton
K_i	Inhibition constant
K_M	Michaelis Menten constant
L	Liter
M	Molar
mg	Milligram
mg/mL	Milligram/milliliter
mL	Milliliter
mM	Millimolar
nM	Nanomolar
nm	Nanometer
^oC	Degrees celsius
V_{max}	Maximum speed

Abbreviations	Description
2', 5'-ADP	2', 5'-Adenosine Diphosphate
5-MTHF	5-Methyltetrahydrofolate
6-PGA	6-Phosphogluconate
ACCα	Acetyl-Coenzyme A Carboxylase-α
APS	Ammonium Persulfate
ATP	Adenosine Triphosphate
$CaCl_2$	Calcium Chloride
CoA	Coenzyme A
$CoCl_2$	Cobalt (II) Chloride
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
FAD	Flavin Adenine Dinucleotide
G6P	Glucose-6-Phosphate
G6PD	Glucose-6-Phosphate Dehydrogenase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
H_2O_2	Hydrogen Peroxide
$KMnO_4$	Potassium Permanganate
$MgCl_2$	Magnesium Chloride
$MnCl_2$	Manganese (II) Chloride
$Na_2HPO_4.2H_2O$	Di-Sodium Hydrogen Phosphate Dihydrate
NAC	N-Acetylcysteine
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Oxidized Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
PPP	Pentose Phosphate Pathway
PS	Phosphatidylserine
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species

Abbreviations	Description
SAM	Adenosylmethionine
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TEMED	N,N,N′,N′ -Tetramethylethylenediamine

1. INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) enzyme is the first and rate-limiting enzyme of the pentose phosphate pathway, which is necessary for the integrity and function of red blood cells. The more the reaction catalyzed by the G6PD is activated, the more NADPH is produced. Thus, the integrity of erythrocyte cells is preserved. At the same time, dysfunction of the G6PD plays a critical role in many diseases. Among them, the most common disease is cancer. The increase in the activity of the G6PD enzyme in various cancer diseases causes the rapid development of cancer cells. Therefore, inhibitors of G6PD constitute an important area of research in cancer treatment.

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that has many basic biochemical functions. In various cancer diseases, increased activity of G6PD leads to an increase in cancer cells. Therefore, G6PD inhibitors can be a very important treatment method in cancer treatment. Here, the inhibition impact of some vitamins on G6PD purified from sheep liver was researched (Song et al., 2022). Vitamins are essential organic compounds taken from natural foods for the growth and development of living things. In many recent studies, it has been determined that vitamins have both therapeutic and protective effects against diseases such as diabetes, cancer, cardiovascular diseases, atherosclerosis, and hypertension. Therefore, it is also recommended as a supplementary nutrient against many diseases. In this work, the inhibitory effect of biotin and folic acid vitamins on the G6PD enzyme purified by affinity chromatography was investigated and a significant inhibitory effect was observed.

1.1 Pentose Phosphate Pathway

The pentose phosphate pathway, also called the phosphogluconate pathway, produces ribose 5-phosphate and NADPH. NADPH transports chemical energy in the form of decreasing power and is mostly utilized as a reducer in anabolic pathways. The role of NADPH in mammals is particularly predominant in tissues such as breast tissue, liver, adrenal cortex, and adipose tissue that actively synthesize fatty acids and steroids. These tissues use NADPH to decrease carbonyl groups and double bonds of intermediates

in synthetic processes. The second task of the pentose phosphate pathway is to produce pentoses, especially the D-ribose compound, which is necessary for nucleic acid biosynthesis (Nelson and Cox, 2005).

The first reaction in the pentose phosphate pathway is the dehydrogenation of glucose 6-phosphate (G6P) to an intramolecular ester of 6 phosphoglucono-δ-lactone by the enzyme glucose 6-phosphate dehydrogenase. In this reaction, $NADP^+$ acts as an electron acceptor and the entire equilibrium takes place in the direction of NADPH production. Lactone is hydrolyzed by a specific lactonase to the free fatty acid 6-phosphogluconate (6-PGA). The 6-phosphogluconate then undergoes dehydrogenation and decarboxylation to form D-ribulose 5-phosphate, which is ketopentose by 6-phosphogluconate dehydrogenase. In this reaction, the second production of NADPH takes place. Phosphopentose isomerase converts ribulose 5-phosphate to ribose 5-phosphate, an aldose isomer of it (Nelson and Cox, 2005; Luzzatto et al., 2020). In some tissues, the pentose phosphate pathway ends in this reaction and the whole reaction occurs as follows:

Equation 1.1 Hydrolysis of G6P and $NADP^+$ to Ribose 5-Phosphate and NADPH (Nelson and Cox, 2005).

$$G6P + 2NADP^+ + H_2O \longrightarrow \text{Ribose 5-Phosphate} + CO_2 + 2NADPH + 2H^+ \quad (1.1)$$

The pentose phosphate pathway is sometimes referred to as the phosphogluconate oxidative pathway or the hexose monophosphate pathway. The first step in the elucidation of this series of reactions was taken by Otto Warburg in 1931 and was completely demonstrated by biochemists Fritz Lipmann, Frank Dickens, Bernard Horecker, and Efraim Racker. The five-carbon sugar and its derivatives produced in this pathway are components of important biomolecules such as DNA, RNA, CoA, ATP, NAD^+, and FAD. At the same time, the conversion of three-, four-, five-, six- and seven-carbon sugars to each other through a series of non-oxidative reactions is catalyzed in the pentose phosphate pathway (Nelson and Cox, 2005; Luzzatto et al., 2020).

The pentose phosphate pathway is a glycolysis reaction that starts with G6P and ends with glyceraldehyde-3-phosphate and fructose-6-phosphate. The primary

physiological significance of this pathway is to provide nucleotides and NADPH for vital activities such as DNA synthesis and defense of reactive oxygen species (ROS) (Nelson and Cox, 2005; Song et al., 2022).

The pentose phosphate pathway begins with the dehydrogenation of carbon 1 of G6P. This reaction is catalyzed by the G6PD enzyme. G6PD is exceedingly specific to $NADP^+$, and the K_M value for NAD^+ is about 1000 times higher than for $NADP^+$. The dehydrogenation of glucose 6-phosphate, which is the first reaction of the pentose phosphate pathway, is an irreversible event. Indeed, this step is the control point of this path under physiological conditions. $NADP^+$ is also the most important regulatory factor. NADPH competes with $NADP^+$ and ATP competes with G6P to bind to the enzyme (Keha and Küfrevioğlu, 2012).

The effect of $NADP^+$ level on the oxidative reactions of the pentose phosphate pathway thus regulates the production of NADPH, which is used in reductive biosynthesis events. G6PD is also a very important enzyme from a clinical point of view. A disease of genetic anemia is observed, in which there is hemolysis of erythrocytes, in its deficiency (Nelson and Cox, 2005; Keha and Küfrevioğlu, 2012; Song et al., 2022).

Experiments with glucose labeled radioactive ^{14}C related to the pentose phosphate pathway have shown that this pathway is more active in adipose tissue than in muscle tissues. This result supports the claim that the first task of the pentose phosphate pathway is to synthesize NADPH for use in reductive biosynthesis events. Because a large amount of NADPH is used to synthesize fatty acids from the acetyl CoA compound in adipose tissue cells (Nelson and Cox, 2005; Keha and Küfrevioğlu, 2012).

The pentose phosphate pathway has a very vital role in erythrocytes. Because the NADPH produced is used in the reduction of oxidized glutathione. Glutathione is a tripeptide with a free sulfhydryl group. In red blood cells, the reduced form of glutathione (GSH) acts as a sulfhydryl buffer by keeping cysteine residues found in hemoglobin and other erythrocyte proteins in a decreased state. The GSH/GSSG ratio in red blood cells is about 500. Reduced glutathione reacts with organic peroxides and hydrogen peroxide under the catalyst of glutathione peroxidase enzyme and exhibits an antioxidant effect (Nelson and Cox, 2005; Keha and Küfrevioğlu, 2012).

1.2 Glucose-6-Phosphate Dehydrogenase Enzyme

The enzyme glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that is essential for the function and integrity of red blood cells. The G6PD enzyme is a very important component that protects erythrocytes from oxidative stress. The pentose phosphate pathway begins with the dehydrogenation of carbon 1 of glucose 6-phosphate. This reaction is synthesized by G6PD (Luzzatto et al., 2020; Abdelwahab et al., 2023). G6PD deficiency is an inherited disease that is estimated to occur in at least 329 million people worldwide. G6PD deficiency causes hemolysis of red blood cells in response to oxidative stress. G6PD deficiency has generally been observed in two ways. The first is type A, which manifests as a healthy person with mild deficiency and recurrent hemolysis attacks, and the second is type B, which presents as chronic hemolytic anemia with significant deficiency and recurrent hemolysis attacks (Nkhoma et al., 2009; Gómez-Manzo et al., 2017; Abdelwahab et al., 2023). In the metabolism in the pentose phosphate pathway, the enzyme G6PD performs the conversion of $NADP^+$ to NADPH. This conversion is necessary for the synthesis of glutathione (GSH) tripeptide, a crucial antioxidant that protects erythrocytes against oxidative stress (Sparrow, 2017; Abdelwahab et al., 2023). G6PD deficiency causes hemolysis to occur in response to reactive oxygen agents (ROS and RNS) and free oxygen radicals produced by stresses such as infections, medications, certain foods, or life habits (Bubp et al., 2015).

Antioxidant vitamins (C and E) are considered one of the systems of protection against reactive oxygen species. In many in vitro and in vivo studies, it has been observed that antioxidant vitamins E and C significantly reduce hemolysis in patients with G6PD enzyme deficiency. For example; in one study, vitamin E supplementation to preterm infants with hyperbilirubinemia resulted in a significant decrease in plasma bilirubin levels. This suggests that it may be due to a decrease in red cell hemolysis (Pacher et al., 2007; Pham-Huy et al., 2008). As an antioxidant, vitamin E can protect the GSH compound from free radical and peroxide-forming oxidation and significantly benefit G6PD-deficient erythrocytes (Tappel et al., 1974; Corash et al., 1982). In an in vivo study, the G6PD enzyme inactivated in nicotine-treated rats regained its activity with vitamin E administration. At the same time, in vitro study, vitamin E increased the activity of the purified G6PD enzyme (Gumustekin et al., 2005). Pharmacologically, taking vitamin C

has been observed to greatly alleviate hemolysis in patients with G6PD deficiency (Fontes et al., 2015).

Erythrocyte membranes are the parts most affected by oxidative stress. Erythrocytes are highly prone to oxidative reactions due to hemoglobin, relatively high oxygenation, and plasma membrane rich in polyunsaturated fatty acids (Turgut et al., 2004). It has been determined that the size of hemolysis is directly proportional to the amount of free radicals that can be inhibited by antioxidant substances. Newborns with G6PD deficiency may have red blood hemolysis (Frank, 2005). G6PD deficiency as a genetic disorder predisposes red blood cells to oxidative stress. Therefore, the balance of oxidant species and antioxidants can result in hyperbilirubinemia and red-cell hemolysis. For example, vitamin C (ascorbic acid) is known as a very important antioxidant that donates an electron to reduce oxidizing radicals and can be protective in G6PD deficiency at mild supraphysiological levels (Winterbourn, 1979; Quinn et al., 2017).

G6PD catalyzes the first reaction that produces NADPH in the pentose phosphate pathway. This reaction, which is required in many biosynthetic pathways, protects cells from oxidative damage by the hydrogen peroxide (H_2O_2) radical. During normal detoxification, the H_2O_2 compound is converted to H_2O by GSH and glutathione peroxidase. Oxidized glutathione (GSSG) is converted back to GSH by NADPH and glutathione reductase. In G6PD-deficient individuals, detoxification of the H_2O_2 radical is inhibited and NADPH production is reduced. Because of this, cellular damage, breakdown of the erythrocyte membrane, lipid peroxidation, and DNA oxidation occur (Nelson and Cox, 2005).

1.3 Biotin (Vitamin B_7)

Biotin (vitamin B_7), a water-soluble vitamin, is a white, crystalline compound. It dissolves in water as well as in alcohol but does not dissolve in ether, chloroform, and petroleum ether. In addition to being resistant to temperature, it is also highly resistant to acid and alkali reactions. It is damaged by oxygen and ultraviolet rays. This vitamin helps the utilization of protein, folic acid, and vitamin B_{12}. Since biotin is dissolved in water, the excess amount is excreted in the urine. It has a condensed ring system consisting of two five-atom rings. It is a monocarboxylic acid of a heterocyclic structure containing

sulfur. It is soluble in water and is resistant to heat and oxidation (Zempleni et al., 2008; Agrawal et al., 2016; Saleem and Soos, 2023).

Biotin is a vitamin that acts as an essential coenzyme for propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase, and acetyl-CoA carboxylase 1 and 2, which are five carboxylases. These carboxylases help in various chemical processes in the cell, gluconeogenesis, including fatty acid synthesis and amino acid metabolism. A daily intake of 30 mcg/day of biotin is recommended by experts to maintain good health. Biotin deficiency has been observed very rarely in people who eat a normal balanced diet. Foods rich in biotin include egg yolks, vegetables, cereals, liver, and rice. Breast milk and dairy products also contain biotin (Kim, 1997; Camporeale et al., 2006; Zempleni et al., 2008; Saleem and Soos, 2023).

Biotin-dependent carboxylases catalyze pathways involved in gluconeogenesis, fatty acid biosynthesis, tricarboxylic acid cycle regulation, especially for genes in carbohydrate metabolism. Acetyl-CoA carboxylase-α (ACCα) present in the cytoplasm catalyzes the binding of bicarbonate to acetyl-CoA, carrying out the production of malonyl-CoA for fatty acid synthesis (Kim, 1997; Camporeale et al., 2006; Zempleni et al., 2008).

Biotin is the coenzyme of the enzyme in the reaction of pyruvate carboxylase. Biotin is an important component that plays a key role in many carboxylase reactions. Biotin is a vitamin that is a specific transporter of CO_2, the most common oxidized form of single carbon groups. The carboxyl groups are activated by a reaction in which ATP and CO_2 are added to the enzyme bond biotin. This activated CO_2 then passes into a receptor in a carboxylation reaction (Nelson and Cox, 2005; Camporeale et al., 2006; Zempleni et al., 2008; Keha and Küfrevioğlu, 2012).

It cannot be stored in the body for biotin, a water-soluble vitamin. Biotin plays a role in many metabolic processes like protein, carbohydrate, and fat metabolism, as well as cell growth, DNA synthesis, and protein synthesis. Biotin level is determined by a blood test. Biotin is synthesized by many plants and microorganisms. Biotin can be stored in the liver and kidney to a limited extent (Nelson and Cox, 2005; Camporeale et al., 2006; Zempleni et al., 2008; Keha and Küfrevioğlu, 2012).

Biotin deficiency occurs by sterilizing the intestinal tract, administering biotin antimetabolites, or eating raw egg whites. Raw egg whites contain a basic glycoprotein

called avidin. Since avidine is not affected by complex proteolytic enzymes formed by combining with biotin, avidin prevents resorption. Avidine has a molecular weight of 70 kDa. Each subunit can bind biotin (Nelson and Cox, 2005; Keha and Küfrevioğlu, 2012).

Since biotin is widely found in nature and is synthesized sufficiently in the intestines, it is practically impossible to talk about biotin deficiency, but if people are fed a diet containing 30% raw egg whites, deficiency symptoms appear after 5-7 weeks. These are dermatitis, drowsiness, nausea, muscle aches, swelling of the skin and mucous membranes. In addition, the binding capacity of CO_2 to oxalocetate decreases and fatty acid synthesis decreases (Nelson and Cox, 2005; Keha and Küfrevioğlu, 2012).

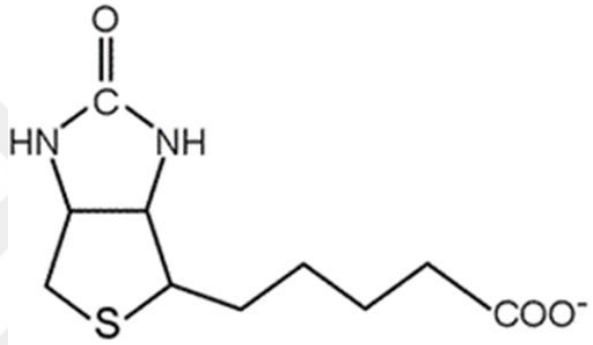

Figure 1.1 Biotin (vitamin B_7) molecular structure

1.4 Folic Acid (Vitamin B_9)

Folic acid has been designated as a synthetic form of vitamin B_9, also known as folate. Folate originates from the word folium, which means green leaf in Latin. Folate is the naturally occurring form of vitamin B_9 in foods. Folic acid, on the other hand, is a synthetically produced folate derivative. Folate, which is taken into the body with food, is synthesized into its active form, 5-methyltetrahydrofolate (5-MTHF), before passing into the bloodstream. In addition to the digestive system, the liver and other tissues are also involved in the activation of folic acid. Folic acid plays an important role in many different functions and reactions of the body. This vitamin is involved in the synthesis of nitrogenous organic bases, which are necessary for the production of various cells and the replication of DNA and RNA during cell division. In addition, folic acid is also effective in the maturation of red blood cells. Since folic acid plays a substantial role in cell

division, it is highly significant to consume it adequately during fast growth periods like infancy, adolescence, and pregnancy (Crider et al., 2011; Pieroth et al., 2018).

Folic acid is a substituted ptteridine derivative, composed of glutamic acid and p-amino benzoic acid. Folic acid is slightly soluble in water and very soluble in aqueous alcohol. It is in the form of yellow crystals. Folic acid is found in metabolism in the form of tetrahydrofolic acid (THF; 5,6,7,8 tetrahydropteraylglutamic acid) and is effectively so. That's why there are those who look at folic acid as a provitamin. As such, it is not biologically active. Pteromonoglutamic acid first becomes free. Then, thanks to this enzyme, it is converted to 7,8-dihydroxypteraylglutamic acid and finally to 5,6,7,8-tetrahydrofolic acid. Ascorbic acid also participates in this enzymatic reaction. THF is also called Coenzyme F (CoF). CoF is a coenzyme of enzymes required for the oxidative and reductive exchange of one-carbon units to each other and for transport to various acceptors (Nelson and Cox, 2005; Keha and Küfrevioğlu, 2012).

Folic acid deficiency can lead to macrocytic megaloblastic anemia in children and adults. Humans cannot synthesize folic acid. For this reason, living things can get folic acid, which is necessary for their bodies, through supplements and foods (Crider et al., 2011; Pieroth et al., 2018).

Folic acid is converted to tetrahydrofolate in the liver by the enzyme dihydrofolate reductase (DHFR). Folic acid intake above 400 mcg can saturate the DHFR. In this case, it has been shown that it can produce unreduced folic acid, which is suggested to be a potential mechanism for carcinogenesis. Folate is a very important vitamin due to its important role in one-carbon metabolism. This is why folate levels have been extensively investigated as a possible mechanism for the development of cancer. 5-MTHF and cobalamin are essential components for the conversion of homocysteine to methionine. Methionine is synthesized into the compound S-adenosylmtionine (SAM). SAM is the main methyl additive component for DNA and RNA methylation and many reactions in the body. Insufficient production of SAM can lead to the alteration and reduction of the expression of proto-oncogenes and tumor suppressor genes (Rai, 2014; Liew, 2016; Pieroth et al., 2018).

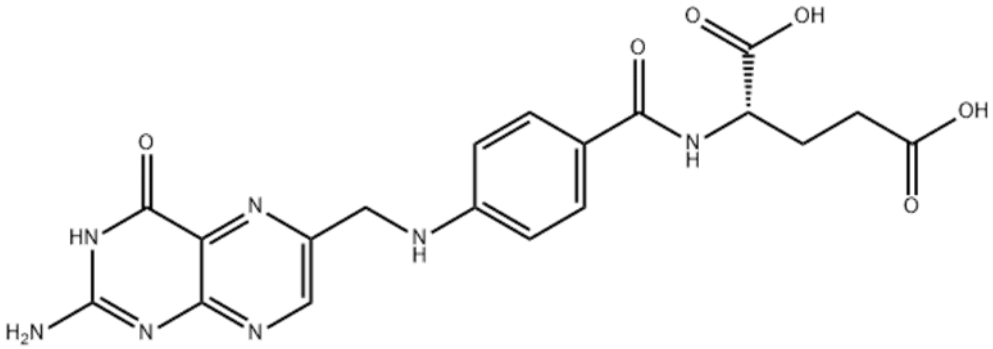

Figure 1.2 Folic acid (vitamin B_9) molecular structure

2. LITERATURE REVIEW

Glucose-6-phosphate dehydrogenase (G6PD) was partially purified from *Bacillus* sp. (BA-142) bacteria. The purification processes consisted of homogenate and ammonium sulfate precipitation stages. As a result of these two steps, the G6PD was purified with an 82-fold yield of 79%. Optimum temperature and optimum pH values were determined for the enzyme (Çiftçi et al., 2011).

In one study, G6PD was purified from sheep liver. For the purification process, the homogenate was first prepared. Purification was performed by affinity chromatography. The G6PD enzyme was purified 1920-fold. K_M and V_{max} values for G6P were calculated as 0.176 mM and 0.0179 EU/mL, respectively. K_M and V_{max} values for $NADP^+$ were determined as 0.0194 mM and 0.0223 EU/mL, respectively. The type of inhibition and K_i value for NADPH were described as non-competitive inhibition and 4.707 ± 0.49 μM (Türkoğlu et al., 2003).

The inhibitor effect of some drugs on hepatic G6PD purified from Lake Van fish was examined. G6PD was purified 899-fold from the liver of fish by 4B affinity chromatography. The purity of G6PD was determined by SDS-PAGE. Vancomycin, sulfanilamide, sulfanyl acetamide, nidazole, ciprofloxacin, amoxicillin, and $KMnO_4$ drugs have shown inhibitory effects on G6PD activity. IC_{50} values of vancomycin, sulfanyl acetamide, sulfanilamide, nidazole, ciprofloxacin, amoxicillin, and $KMnO_4$ were 1.88, 0.032, 0.037, 1.178, 643.5, 2.26, and 0.0002 mM, respectively. Nidazole and vancomycin showed a competitive inhibitory effect. Other drugs showed non-competitive inhibitory effects (Ciftci et al., 2007).

G6PD was purified from camel liver by ammonium sulfate precipitation, DEAE-cellulose, Sephacryl S-300 gel filtration, and 2',5'-ADP Sepharose 4B affinity chromatography steps. The purity of the G6PD enzyme was determined by both nativ-PAGE and SDS-PAGE, and its molecular weight was designated as 64 kDa. The molecular weight of the natural form of G6PD was found to be 194 kDa by gel filtration chromatography. The K_M value for $NADP^+$ is 0.081 mM. It showed an inhibitory effect of $CaCl_2$, $NiCl_2$, $CuCl_2$, $FeCl_2$, and $ZnCl_2$ on G6PD activity (Ibrahim et al., 2014).

In one study, the effect of furosemide, cefuroxime, gentamicin, cefazolin, and clindamycin antibiotics on rat erythrocyte G6PD was explored. G6PD enzyme was

purified 1825-fold by affinity chromatography. The IC_{50} values of clindamycin, gentamicin, and furosemide, which have inhibitory effects, were calculated as 34.65, 1.75, and 0.526 mM, respectively. K_i values were 39.8, 0.7, and 0.860 mM, respectively (Temel et al., 2020).

In one study, it was determined that polydatin, a natural molecule, inhibits the G6PD enzyme. Polydatin inhibits the enzyme G6PD, resulting in a strong increase in endoplasmic reticulum stress and accumulation of reactive oxygen species. These effects are followed by approximately 50% apoptosis, cell cycle blockade in the S phase, and 60% inhibition of invasion in vitro. Polydatin is well tolerated in humans by a phase II clinical study and is also non-toxic in animals up to doses of up to 200 mg/kg. Therefore, polydatin compound can be used as a reliable ingredient to limit the metastatic spread and growth of human cancer (Mele et al., 2018).

Samples from healthy male subjects with G6PD enzyme deficiency were examined. Hemolysates were prepared to measure G6PD enzyme activities by the Beutler method (Beutler, 1971). Next, the impact of different glyphosate concentrations ($5.3x10^{-3}$, $5.3x10^{-4}$, $5.3x10^{-5}$, $5.3x10^{-6}$ mmol/mL) on G6PD activity in mutant and normal enzymes was evaluated. Also, the in vitro impact of the antioxidant N-acetylcysteine (NAC) on G6PD in the presence and without glyphosate has been investigated. The result of normal erythrocyte G6PD activity was 12U/g for healthy subjects and 2.5U/g Hb for enzyme-deficient subjects. The highest inhibition effect on G6PD was determined at a glyphosate concentration of $5.3x10^{-3}$ mmol/mL. It partially increased the inhibition of glyphosate on the G6PD in healthy subjects with N-Acetylcysteine compound at a concentration of $4.7x10^{-7}$ mmol/mL. The glyphosate compound is speculated to affect G6PD activity in humans in vitro (Kartlaşmış and Dikmen, 2022).

In a study, G6PD enzyme purified from sheep spleen tissue were investigated. The purity level of the G6PD was controlled by SDS-PAGE. The effect of diclofenac sodium on the activity of the G6PD enzyme obtained in pure form was investigated. IC_{50} and K_i values of diclofenac sodium, which shows an inhibitory effect on the G6PD enzyme, were calculated. IC_{50} value of diclofenac sodium was determined as 0.51 mM and K_i value as 0.48 ± 0.01 mM. The type of inhibition was found to be non-competitive inhibition (Çoban et al., 2022).

The in vivo and in vitro impacts of nicotine and nicotine + vitamin E on G6PD activity in rat muscle, heart, kidney, lungs, testis, brain, stomach, and liver were investigated. The duration of supplementation (n = 8 rats per group) was 3 weeks with the control group, nicotine, and nicotine + vitamin E. Nicotine (0.5 mg/kg, i.p.) inhibited G6PD activity in the brain, lung, testis, stomach, and kidney by 23.35%, 12.5%, 48%, 13%, and 20.8%, respectively. Nicotine showed no effect on heart, muscle, and liver G6PD. Furthermore, nicotine + vitamin E inhibited G6PD activity in the brain, testes, and liver by 21.5%, 32.5%, and 16.5%, respectively. Nicotine + vitamin E activated stomach and muscle G6PD activity by 20% and 36%, respectively. Also, nicotine + vitamin E showed no effect on the G6PD activity of the lungs, heart, and kidneys. In addition, in vitro studies conducted to elucidate the impacts of nicotine and vitamin E on G6PD also showed similar effects as in vivo experimental results. The results here suggest that in vivo and in vitro administration of vitamin E in various rat tissues again increases the nicotine-induced decreased G6PD activity (Gumustekin et al., 2005).

The antioxidant vitamin E was administered to a group of 68 subjects with G6PD deficiency to reduce chronic hemolysis and repair normal cell types in the blood film. It has been observed that some abnormal red cells in peripheral blood films and the rate of hemolysis decrease after vitamin E supplementation in patients with G6PD deficiency (Sultana et al., 2011).

One study examined the effects of folic acid and vitamin E in ameliorating acute hemolysis caused by G6PD deficiency in patients with G6PD deficiency. Patients were divided into 4 groups as control groups folic acid, vitamin E, and a combination of both supplements. All patients received the same treatment for acute hemolysis. As a result, a significant increase in hematocrit and hemoglobin levels was observed in patients receiving supplements compared to the control group (Darbandi et al., 2017).

In another study, it was investigated whether vitamin C has a protective impact on the development of G6PD deficiency and H_2O_2-induced eryptosis. Erythrocytes with different G6PD activity were divided into several groups that were treated with H_2O_2 and Vitamin C. Compared to the empty group, the rate of phosphatidylserine (PS) rotation of erythrocytes, active caspase 3 expression level, and intracellular Ca^{2+} concentration increased importantly after 0.05% H_2O_2 treatment. After, the erythrocyte index reduced highly after being treated with Vitamin C for 30 min. Here, vitamin C significantly

inhibited erythrosis, which is increased by oxidative stress caused by H_2O_2 (Shan et al., 2016).

3. MATERIALS AND METHODS

3.1 Material

55 g of fresh sheep liver sample was cut with a knife. Excess blood, foreign tissue, and membranes were removed from the samples. Liver tissue was suspended in 500 mL of 5 mM phosphate buffer (pH 7.4) containing 458 mM of sucrose and homogenized using a stirrer at the highest speed for 3 min. Then the material was homogenized for 40 min in an ultrasonic homogenizer. Primarily, tubes with equal amounts of sample deposits were centrifuged at 1500 rpm for 20 minutes each, and the precipitate containing blood cells and cellular debris was discarded. After, the homogenate was centrifuged at 9000 rpm for 60 min and the supernatant was separated. The separated supernatant was centrifuged again at 9000 rpm for 60 minutes. This process was repeated 3 times. During the homogenization process, the temperature was maintained at 4 ^{o}C (Mohammed et al., 1976).

3.1.1 Chemicals Used

Glucose-6-phosphate, 2',5'-ADP Sepharose 4B, $NADP^{+}$, EDTA, biotin, folic acid, $MgCl_2$, $K_2HPO_4.3H_2O$, $NADP^{+}$, serum albumin, trihydroxymethyl aminomethane (Tris), β-mercapto ethanol were obtained from Sigma Chemical Comp. N,N,N',N'-tetramethyl ethylenediamine (TEMED), acrylamide, N,N′-methylene bisacrylamide, bromine thymol blue, bromphenol blue, sodium dodecyl sulfate (SDS), ammonium persulfate were procured from AppliChem GmbH. Sodium chloride, hydrochloric acid, phosphoric acid, sodium azide, glycerin, ethanol, methanol, acetic acid, and Coomassie brilliant blue G-250 were purchased from E. Merck AG. Electrophoresis marker 26616 fermentas was obtained from Life Science Thermo Fisher Scientific.

3.1.2 Tools and Devices Used

Spectrophotometer: Shimadzu UV Spectrophotometer UV-1800
Incubator: G24 Environmental Incubatur Shaker

Refrigerated clinical centrifuge: Hettich Zentrifugen Universal 320 R
Clinical centrifuge: Hettich Zentrifugen Universal 320 R
Electrophoresis tank: P8DS Vertical Electrophoresis System (vertical)
Peristaltic bomb: Eyela Micro Tube Pump MP-3
Peristaltic bomb: Easy-load master flex model 7518-10
Power supply: Thermo Scientific
pH meter: Inolab WTW series pH 720
Mixer (Vorteks): Mixer VM 20
Precision balance: Gec Avery
Precision balance: Shimadzu Uni Bloc AUW 220D
Affinity colon chromatography: Sigma 1 cm x 10 cm
Automatic pipettes: Eppendorf, Volac (5-50 μL, 10-100 μL, 100-1000 μL)
Homogenizer: Wiggen Hauser 220-240 volt 50-60 Hz
Shredder mixer: Warning Commercial Laboratory Blender. Country of origin U.S.A.
Constant temperature bath: Julabo Labortechnik GMBH D-7633
Constant temperature circulation bath: Grand Technical. Specification LTD 6G-20 to 100^{o}C

3.1.3 Preparation of the Solutions Used

All solutions were prepared using bidistilled water.

3.1.3.1 Solutions Used in Protein Determination

1) Coomassie brilliant blue G-250 color reagent (The stock solution used in protein determination): 100 mg coomassie brilliant blue G-250 dissolved in 50 mL of 95% ethanol. 100 mL of 95% orthophosphoric acid was slowly added to this solution. The volume of the prepared solution is topped up to 1 L with bidistile water.
2) Standard serum albumin solution (the standard solution used for protein determination): 10 mg of bovine serum albumin was taken and slowly dissolved in some distilled water without foaming, and its volume was topped up to 10 mL with distilled water.

3.1.3.2 Solutions Used in Activity Measurement

1) Buffer E (buffer used to measure enzyme activity): 6.05 g of tris and 0.073 g of EDTA were dissolved in some distilled water. It was adjusted to pH 8 with 1 N HCl and its volume was topped up to 50 mL.
2) 0.1 M $MgCl_2$ solution (activator solution used in enzyme activity): 0.475 g of magnesium chloride ($MgCl_2$) was taken and dissolved with some distilled water and the volume was topped up to 50 mL with distilled water.
3) 2 mM $NADP^+$ solution (solution used in enzyme activity): 0.0765 g of $NADP^+$ was taken and dissolved in some distilled water and its volume was completed to 50 mL with distilled water.
4) 6 mM G6P solution (solution used in enzyme activity): 0.091 g of G6P was taken and dissolved with some distilled water and the volume was completed to 50 mL with distilled water.

3.1.3.3 Solutions Used for Column Filler

1) Buffer A (buffer used for balancing and flushing the affinity column): A mixture of 8.2 g of sodium acetate and 17.4 g of potassium phosphate ($K_2HPO_4.3H_2O$) was dissolved in 800 mL of distilled water. The pH was adjusted to 6 with a 1 N HCl solution and its volume was completed to 1L.

3.1.3.4 Solutions Used in Affinity Chromatography Column

1) Buffer A (buffer used for balancing and flushing the affinity column): A mixture of 8.2 g of sodium acetate and 17.4 g of potassium phosphate ($K_2HPO_4.3H_2O$) was dissolved in 800 mL of distilled water. The pH was adjusted to 6 with a 1 N HCl solution and its volume was completed to 1L.
2) Buffer B (1st wash buffer used for washing the affinity column after applying the sample): A mixture of 8.2 g of sodium acetate and 17.41 g of potassium phosphate ($K_2HPO_4.3H_2O$) was dissolved in 800 mL of distilled water. The pH was adjusted to 7.85 with a 1 N HCl solution and its volume was completed to l L.

3) Buffer C (2nd wash buffer used for washing the affinity column after applying the sample): A mixture of 17.41 g of potassium phosphate ($K_2HPO_4.3H_2O$) and 7.45 g of potassium chloride (KCl) was dissolved in 800 mL of distilled water. The pH was adjusted to 7.85 with a 1N HCl solution and its volume was topped up to 1 L.

4) Buffer D (Buffer used for elution of glucose-6-phosphate dehydrogenase (G6PD) enzyme attached to affinity gel): A mixture of 3.48 potassium phosphate ($K_2HPO_4.3H_2O$), 1.49 g potassium chloride (KCl), 0.096 g $NADP^+$ and 0.605 g EDTA was dissolved in 200 ml of distilled water. The pH was adjusted to 7.85 with a 1 N HCl solution and the volume was completed to 250 mL.

5) 0.02% NaN_3 (Sodium azide) solution (to protect chromatography column materials from bacteria): 10 mg NaN_3 was taken and dissolved in 50 mL of bidistilled water.

3.1.3.5 SDS-PAGE Solutions Used for Electrophoresis

1) 1.5 M Tris-HCl (pH= 8.8): 18.17 g Tris (0.1 mol) was weighed and dissolved in 80 mL of bidistilled water, the volume was completed to 100 mL with bidistilled water after pH adjustment.

2) 0.5 M Tris-HCl (pH= 6.8): 6.057 g Tris (0.1 mol) was weighed and dissolved in 80 mL of bidistilled water, the volume was topped up to 100 mL with bidistilled water after pH adjustment.

3) 30% Acrylamide - 2.7% Bisacrylamide solution: 14.6 g of acrylamide, 0.4 g of bisacrylamide was dissolved in 40 mL of bidistilled water, and the volume was topped up to 50 mL with bidistilled water.

4) 10% ammonium persulfate solution: 1 g of ammonium persulfate was weighed and the volume was completed to 10 mL with bidistilled water.

5) 10% SDS: 1 g of SDS was weighed and completed with 10 mL of bidistilled water.

6) Execution buffer: 3.028 g of Tris and 14.410 g of glycine were weighed and dissolved in 950 mL of bidistilled water, then 10 mL of the 10% SDS was added to adjust to pH= 8.3, and the total volume was topped up to 1000 mL with bidistilled water.

7) Sample buffer solution: 2.5 mL of 1.5 M Tris-HCl (pH= 6.8), 4 mL of 10% SDS, 2 mL of 100% glycerin, and 1 mL of β-mercaptoethanol were taken and the volume was completed to 10 mL with bidistilled water.

8) Gel staining solution: dissolve 0.025 g of Coomassie brillant blue R-250 reagent in 40 mL of methanol, and 7 mL of acetic acid to complete the volume with bidistilled water to 100 mL.
9) I. Gel wash solution: Prepared with 50 mL of methanol - 10 mL of acetic acid - 40 mL of bidistilled water.
10) II. Gel wash solution: Prepared with 5 mL of methanol - 7 mL of acetic acid - 88 mL of bidistilled water.
11) Water-saturated n-butanol: Prepared by mixing 10 mL of n-butanol and 1 mL of bidistilled water.
12) 0.1% bromthymol blue solution: 0.01 g of the indicator was dissolved in 1 mL of bidistilled water and the total volume was topped up to 10 mL with bidistilled water.

3.2 Method

3.2.1 Determination of Protein

3.2.1.1 Qualitative Protein Determination

Qualitative protein determinations of each of the fractions obtained from the affinity column were performed. Qualitative protein determination is performed based on the maximum absorbance of tryptophan and tyrosine in the structure of proteins at 280 nm (Carlberg and Mannervik, 1981). Qualitative protein determination in enzyme samples obtained from sheep liver was determined according to this method. The fractions were taken into quartz cuvettes and their absorbances were read against the blind. Elution buffer was used blindly.

3.2.1.2 Quantitative Protein Determination by Bradford Method

Quantitative protein determinations of each fraction obtained from the affinity column were performed. In this method, proteins form a complex with Coomassie Brillant Blue G-250 reagent in an o-phosphoric acid environment and this complex shows maximum absorbance at 595 nm. Coomassie Brillant Blue G-250, which has a negative

charge, is used as a dye in this method and binds to the positive charge on the protein. The dye is available in red (maximum wavelength 465 nm) and blue (maximum wavelength 596 nm) form. By binding the protein to the negative charge on the dye, the red form changes to the blue form. The protein-dye complex persists in solutions for a long time. The sensitivity of this method is 1-100 μg. The reaction is very reproducible and occurs quickly. The reaction is completed in approximately two minutes. Color stability continues for over two hours. It has been determined that the amino acid composition of the protein (especially basic amino acids such as arginine and aromatic amino acids) is effective in the reaction of color formation (Bradford, 1976).

The measurement was made as follows: 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μL were taken into tubes in standard bovine albumin solution containing 1 mg of protein per 1 mL. The volume of all tubes was topped up to 0.1 mL with bidistilled water. 5 mL of Coomassie Brilliant Blue G-250 reagent was added to the tubes and mixed with a vortex mixer. After 10 min incubation, absorbance values against the blind were read in 3 mL cuvettes at 595 nm. A solution consisting of 0.1 ml of the same buffer and 5 mL of Coomassie Brilliant Blue G-250 reagent was used blindly. A standard graph was drawn using μg protein values corresponding to the absorbance values read.

0.1 mL of prepared enzyme solutions (from each fraction) were taken separately. 5 mL of Coomassie reagent was added and mixed with the vortex and left to incubate for 10 min. Absorbance values were read at 595 nm using a solution consisting of 0.1 mL of the same buffer and 5 mL of Coomassie reagent, again blindly. The absorbance values for each fraction were converted from the standard graph to μg.protein.

3.2.2 Preparation of the Affinity Column

Weighed 2 g of dry 2′-5′ ADP Sepharose 4B gel for a bed volume of 10 mL. It was washed several times with 400 mL of distilled water to remove impurities. During washing, the gel swelled. All these procedures were carried out under laboratory conditions. After the swollen gel was deaerated, the gel was suspended with 25% balancing buffer (0.1M Na-Acetate / 0.1M K-Phosphate) and 75% gel. The suspended gel (1x10) was packed in the colon. It was then stabilized with a stabilization buffer. After

the gel was settled, it was washed off with a balancing buffer using a peristaltic pump. In equilibrium and washing, the flow rate was applied as 50 mL/h.

3.2.3 Purification of Sheep Liver Glucose-6-Phosphate Dehydrogenase Enzyme

3.2.3.1 Sheep Liver Production

For the experiments, freshly slaughtered sheep liver was taken from the slaughterhouse and brought to our biochemistry laboratory after being kept in a container filled with ice.

First of all, the pieces taken from different parts of the sheep liver were cut into very small cubic shapes and these pieces were further reduced with the help of a thin knife. In this way, around 55 g of liver was obtained. For the preparation of liver aqueous solution; Thinned liver was added to 0.02 M Sodium phosphate buffer (pH: 7.4) containing 0.356 g $Na_2HPO_4.2H_2O$ and 8.25 g sucrose in a 500 mL volume. This process was repeated 3 times to obtain sufficient homogenate. In other words, a liver mixture of 500 mL of 0.02 M sodium phosphate buffer was obtained. This mixture was subjected to disintegration for 3 minutes with the help of a mixer, during which time it was prevented from heating; it was carried out by cooling the mixer with ice. The resulting homogenate was treated for 40 min in an ultrasonic homogenizer to achieve further disintegration. In this process, the bottom and sides of the homogenate container were cooled with ice to prevent heating. After that, the centrifugation process was started.

Tubes with equal amounts of sample deposits were centrifuged at 1500 rpm for 20 minutes each, and the precipitate containing blood cells and cellular debris was discarded. The resulting supernatants were collected and centrifuged at +4 ^oC and 9000 rpm for 60 minutes with a high-speed and cooled centrifuge. The upper clear homogenate was separated, and the lower sediment consisting of cell debris was discarded. This process was repeated 3 times, the goal was to completely remove substances that could damage the colon (Mohammed et al., 1976). The pH of the homogenate to be applied to the colon was adjusted to pH= 6, which is the pH of the balancing buffer, with solid NaH_2PO_4. Thus, the homogenate became to be applied to the colon.

3.2.3.2 Administration of Sheep Liver To the Affinity Column and Enzyme Elution

The prepared affinity gel was packaged in a 1 cm x 10 cm column and balanced with buffer A for 4 days. The homogenate prepared above was applied to the column. After the entire liver homogenate passed through the colon, it was waited for half an hour. At the end of this period, the balancing buffer (pH:6 Buffer A) was given to the colon. The input and output absorbance difference was observed in the spectrophotometer at 280 nm, and this process was continued until the difference decreased below 0.1. Then, 25 mL of wash buffer (pH :7.85. Buffer B), and finally 25 mL of other wash buffer (pH:7.85. Buffer C) were sent. After the procedures, the absorbance difference at 280 nm approached 0.05. Thus, a large part of the enzyme was attached to the gel and other impurities were removed. All these procedures were performed at +4 ^{o}C and with a flow rate of 50 mL/h (Ninfali et al., 1990; Morelli et al., 1978). Elution buffer (pH :7.85 Buffer D) was sent to the colon with a flow rate of 20 mL/h, while fractions of 2 mL were collected. Qualitative protein assays of all fractions at 280 nm, glucose-6-phosphate dehydrogenase activities, and quantitative protein quantities were determined. By combining the active fractions, the total amount of protein in the pure enzyme and homogenate and the G6PD enzyme activity were determined. Then, the purification rate was found by calculating their specific activities separately.

3.2.4 Control of Purity of G6PD by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After G6PD was purified, the purity of the enzyme was checked using the 4-10% batch sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method (Laemmli, 1970). First, the electrophoresis plates were thoroughly washed with water and then with alcohol. Using spacing bars on both sides of the plates, the plates were overlapped and attached with clamps. After the plates were fixed, they were placed in the gel preparation cabinet containing a sponge that prevents leakage. First, the separation gel was prepared and filled between the injector and the plates until 0.3 cm remained in the uppercut. While waiting for this gel to freeze, a thin layer of n-butanol was poured over it to ensure that the gel was smooth (pouring is done by tilting to the side). After waiting for the separation gel to freeze for an hour and determining that the separation

gel had solidified, the deposition gel was prepared. The prepared deposition gel was filled into the cavity at the top of the separation gel, and the comb was carefully inserted into the gel to form the sample wells. Care was taken to avoid the formation of air bubbles on the comb tips (if air bubbles form, oxygen will inhibit polymerization and cause a defect on the gel surface on the underside of the wells). While waiting for the deposition gel to solidify, the soaked strainer paper was covered over the system and the top of the gel was prevented from drying out too much. After it was determined that the deposition gel had solidified, the comb was carefully removed to determine the sample wells. Each well was washed with distilled water, then the wells were filled with execution buffer and placed in the electrophoresis tank along with the gel plates. The upper and lower part of the electrophoresis tank was filled with an execution buffer. After the sample buffer was added to the enzyme samples, 0.1% bromthymol blue was dripped on it as a follow-up dye. A 1/1 sample buffer was added for a total volume of 200 µL and this mixture was kept in a water bath at 65 ^{o}C for 90 seconds. 20, 30, 40, and 50 µL of enzyme samples and markers (26616) were added to each well. The samples were injected into wells in the gel with a thin syringe. The electrophoresis tank was closed and the (+) anode was placed on the bottom and the (-) cathode was placed on the top. First, it was carried out at 60 volts for 20 minutes and the samples came up to the separation gel and accumulated there. Then the current was increased to 80 volts and the execution process was continued until the samples reached the lower limit of the gel. The sample bands were followed with bromthymol blue added to the sample buffer and the current was cut off when the follow-up dye reached the bottom. After the execution process was finished, the current was cut off and the gel between the plates was carefully removed. The gel was left in the staining solution and left on the shaker for 2 hours to stain. After The gel was stained, it was removed from the solution and left in the I. washing solution. The gel was then left in the second wash solution to better define the tapes. The gel was washed on a shaker until the color of the gel lightened and the protein bands became evident and the gel was removed and photographed (Figure 4.3).

Separation gel: 5 mL of 1.5M Tris-HCl (pH= 8.8), 6.6 mL of 30% acrylamide-2.7% bisacrylamide, 0.2 mL of 10% SDS, 6.6 µL of TEMED, 100 µL of 10% ammonium persulfate (APS) and 8 mL of bidistilled water were mixed.

Deposition gel: 2.5 mL of 0.5 M Tris-HCl (pH= 6.8), 1.33 mL of 30% acrylamide 2.7% bisacrylamide, 0.1 mL of 10% SDS, 5 µL of TEMED, 50 µL of 10% APS, and 6.1 mL of bidistilled water were mixed. The APS solution was freshly prepared and APS was added immediately while the gel solution was mixed (Table 3.1).

Table 3.1 Separation gel and deposition gel concentrations for SDS-PAGE electrophoresis

	Separation Gel	**Deposition Gel**
Tris-HCl (pH= 8.8)	5 mL	-
Tris-HCl (pH= 6.8)	-	2.5 mL
30% acrylamide-2.7% bisacrylamide	6.6 mL	1.33 mL
10% SDS	0.2 mL	0.1 mL
TEMED	6.6 µL	5 µL
10% APS	100 µL	50 µL
Bidistilled water	8 mL	6.1 mL

3.2.5 Determination of Molecular Weights of Subunits of Sheep Liver G6PD by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed to determine the molecular weight of subunits of sheep liver G6PD enzyme. SDS denatures proteins and breaks them down into their subunits. The band observed in the electrophoresis picture is not the total molecular mass, but the molecular weight of its subunits. Electrophoresis for this purpose was performed as described in 3.2.4. to determine the molecular weight of the subunits of the enzyme, it is also necessary to carry out the standard protein mixture (protein marker). For this reason, the commercial marker Fermentas 26616, which consists of standard proteins with molecular weights ranging from 10 kDa to 180 kDa, was used. After the gel was prepared and placed in the electrophoresis tank, one of the wells was loaded with 5 µL of standard solution. Enzyme samples were applied to other wells. After the execution process was finished, the gel was painted and the gel was photographed after washing. The R_f values of the enzymes and standards were calculated from the formula R_f = walking distance of the enzyme/walking distance of the dye and the R_f = log M_W standard graph was created. Using this graph and the equation of the graph, the molecular weight of the glucose-6-phosphate

dehydrogenase enzyme in sheep liver whose Rf value was calculated was calculated (Figure 4.4) (Laemmli, 1970).

3.2.6 Determination of V_{max} and K_M Values for Sheep Liver G6PD

Its biochemical parameters were found to further characterize the G6PD enzyme purified from sheep liver. Measurements were made at 5 different G6P concentrations for the purified G6PD enzyme. Then, using these values, the Lineweaver-Burk graph was drawn. V_{max} and K_M values were calculated from this graph.

3.2.7 Measurement of Enzyme Activity

The G6PD enzyme, for which we have purified it, catalyzes the oxidation of Glucose-6-Phosphate (G6P) to 6-phosphogluconate (6-PGA).

Equation 3.1 Hydrolysis of G6P and $NADP^+$ to 6-PGA and NADPH compounds (Nelson and Cox, 2005).

$$G6P + NADP^+ \xrightarrow{\text{Glucose-6-Phosphate Dehydrogenase}} 6\text{-PGA} + NADPH + H^+ \qquad (3.1)$$

In activity measurement studies, NADPH formed as a result of the reaction is taken into account. Since NADPH gives maximum absorbance at 340 nm, the activity of the enzyme is measured as a result of the increase in absorption of $NADPH^+$ by spectrophotometric method, which occurs as a result of the reduction of $NADP^+$ at this wavelength and at 37°C. When 1mM $NADP^+$ is reduced at 37°C at a wavelength of 340 nm, an optical density (OD) value of 6.22 is read on the spectrophotometer.

Nicotinamide adenine dinucleotide phosphate ($NADP^+$) is reduced by Glucose-6-Phosphate Dehydrogenase in the presence of Glucose-6-Phosphate (G6P). G6PD activity was determined spectrophotometrically at 37°C according to Beutler's study (Beutler, 1971). Accordingly, the formed incubation mixture of 2.5 mL (final volume); it contains 1 M Tris-HCl + 5 mM EDTA (pH = 8.0), 0.1 M $MgCl_2$, 2 mM $NADP^+$, and 6 mM G6P. The determination procedure applied can be briefly summarized as follows:

The prepared solutions were incubated at 37°C for 10 minutes. Then, the sample was monitored for a total of 5 minutes, every half minute (30 seconds) against the control, and absorbance increases were recorded.

Equation 3.2 was used for enzyme unit calculations.

$$A = \frac{\Delta OD}{6.22} \times \frac{Vc}{Ve} \times f \qquad (3.2)$$

A: mL Per enzyme unit (EU/mL)

ΔOD: Absorbance change in one minute

V_c: The total volume of cuvettes for which the measurement was made

V_e: The volume of the enzyme sample added to the cuvette where the measurement was made

f: Dilution factor

6.22 mM^{-1} cm^{-1}: Extinction coefficient

3.2.8 Preparation of Solutions of Biotin and Folic Acid Vitamins

Biotin and folic acid vitamins were used in our study.

Folic acid; 5 milligram of folic acid vitamin was weighed and taken, dissolved in some pure water, and then completed to 5 mL in volume with pure water.

Biotin; 5 milligram of biotin vitamin was weighed, dissolved in some pure water, and then completed to 5 mL in volume with pure water.

3.2.9 Kinetic Studies with G6PD

3.2.9.1 Determination of The Effects of Biotin and Folic Acid Vitamins on Sheep Liver G6PD Activity

Stock solution was first prepared to determine the effect of biotin and folic acid vitamins on G6PD activity purified from sheep liver. This stock solution was used in the

measurement to determine the optimal concentration range. Enzyme activity was measured by diluting the stock solution many times to determine the concentration range. G6PD enzyme activity was measured using the normal procedure. However, the compounds to be placed in the measuring cuvette were added by deducting the same volume from the buffer amount so that they did not exceed the total volume of the cuvette (1 mL). This process was applied in all kinetic studies.

The effect of biotin and folic acid vitamins on G6PD activity purified from sheep liver was determined. The results from these compounds are shown in Tables 4.2-4.3 and Figures 4.5-4.6.

Table 3.2 Contents of the cuvette measuring the activity of sheep liver G6PD containing biotin and folic acid

	Control Cuvette	**Sample Cuvette**
1 M Tris-HCI,5 mM EDTA	250 µL	250 µL
0.1 M $MgCI_2$	250 µL	250 µL
2 mM $NADP^+$	250 µL	250 µL
Homogenate	50 µL	50 µL
Bidistilled water	1700 µL	1450 µL
6 mM G6P	-	250 µL
Incubation at 37 ºC for 15 minutes		

3.2.10 Studies to Determine the K_i Value, Inhibition Type, and IC_{50} Value of Biotin and Folic Acid Vitamins, Whose Effects were Examined on Sheep Liver G6PD Activity

Biotin and folic acid vitamins were added to the cuvette content at different concentrations and G6PD activities were measured. Graphs were drawn with the % Activity – [Inhibitor] value from these activities. IC_{50} values were calculated from the obtained graph equations.

The concentration of biotin and folic acid vitamins by halving the activity of G6PD purified from sheep liver to determine the type of inhibition of biotin and folic acid vitamins and 3 substrate concentrations at constant concentrations of biotin and folic acid vitamins below and above this value Activity measurements were made with. Lineweaver-Burk graphs were drawn for each inhibitor with the obtained values. The type of inhibition was determined from the graphs. The results are given in Table 4.4.

4. RESULTS

4.1 Standard Chart for Quantitative Protein Determination

Bradford method was utilized to designate quantitative proteins. With this method, a standard graph was created in advance to determine proteins. A standard graph was drawn using protein solutions in the concentration range of 10-100 μg protein/mL and their absorbances at 595 nm. The amount of protein in both hemolysate and pure enzyme solutions was determined by this standard graph.

The concentrations of protein solutions used in the creation of the standard graph and the absorbance values at 595 nm corresponding to these concentrations are indicated in Figure 4.1.

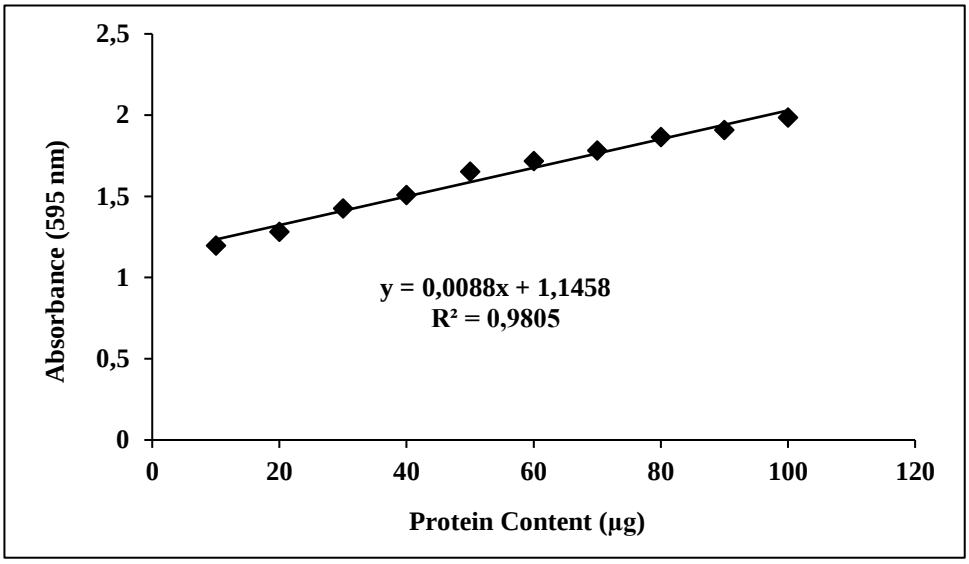

Figure 4.1 Standard graphic used in the quantitative determination of proteins by the Bradford method

4.2 Results of Purification of Sheep Liver G6PD Enzyme by Affinity Chromatography Method

In this study, the values found for G6PD enzyme tubes purified by sheep liver affinity chromatography are shown in Figure 4.2.

4.2.1 Purification Results for Sheep Liver G6PD Enzyme

For sheep liver, the enzyme unit in mL was determined as 0.161 EU/mL, and the amount of protein in mL was determined as 21.159 mg protein/mL.

The specific activity for sheep liver is specified in Equation 4.1 below.

$$\frac{\text{EU/mL}}{\text{mg protein/mL}} = \frac{0.161}{21.159} = 0.0076 \tag{4.1}$$

Quantitative protein determination of G6PD enzyme solution purified from sheep liver by the Bradford method was performed. At the same time, G6PD activity was determined and the enzyme unit was determined in EU/mL. The enzyme unit for G6PD was calculated both in hemolysate and in elutions. For the G6PD solution purified from sheep liver, the enzyme unit in mL was found to be 0.338 EU/mL, and the amount of protein in mL was found to be 0.00705 mg/mL.

The specific activity for pure liver G6PD was found to be in Equation 4.2.

$$\frac{\text{EU/mL}}{\text{mg protein/mL}} = \frac{0.338}{0.00705} = 47.943 \tag{4.2}$$

The purification coefficient is;

$$\frac{\text{Specific Activity 2}}{\text{Specific Activity 1}} = \frac{47.943}{0.0076} = 6308 \tag{4.3}$$

was determined in Equation 4.3.

According to this result, as a result of the direct delivery of sheep liver to the affinity column, the G6PD enzyme was purified 6308 times from sheep liver.

The amount of protein in the tubes with pure enzyme was found by the regression equation of the standard curve created at 595 nm, and the amount of protein μg/mL for each tube. As can be seen from the graph, it was observed that tubes 7-13 showed both high protein content and high G6PD activity (Figure 4.2).

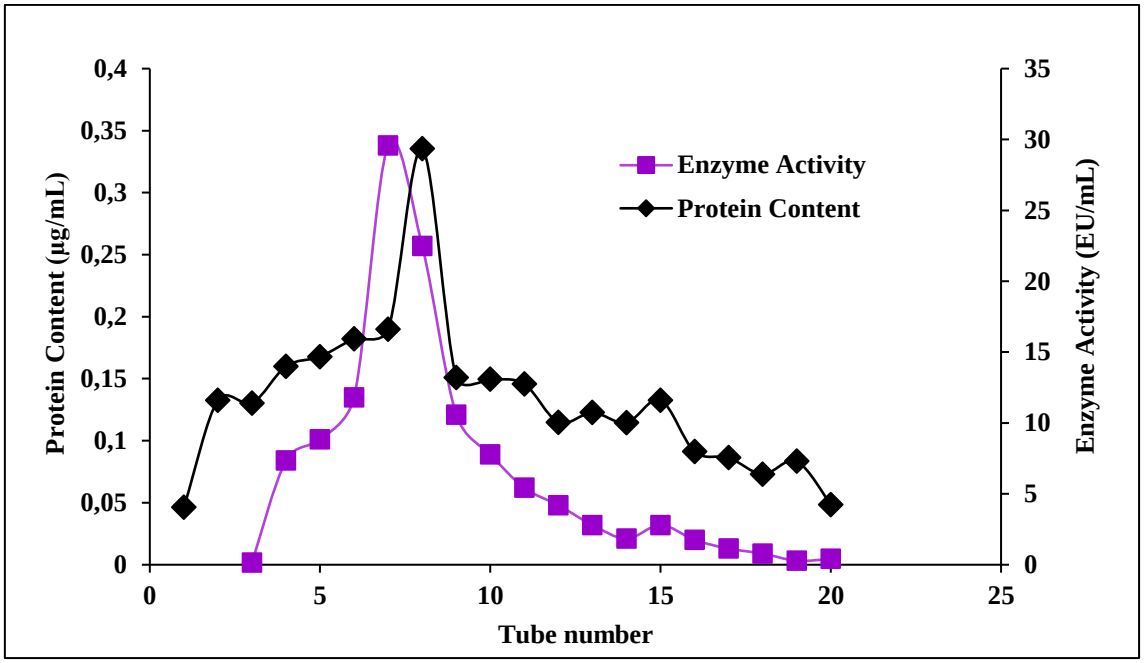

Figure 4.2 Qualitative protein determinations and activity values of sheep liver tubes obtained from affinity column

Table 4.1 Purification steps of G6PD enzyme in sheep liver

Purification Steps	Activity (EU/mL)	Total Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg)	Yield (%)	Purification Coefficient
Sheep liver	**0.161**	**60**	**21.159**	**1269.54**	**9.66**	**0.0076**	**100**	**1.0**
2',5'-ADP Sepharose 4B	**0.338**	**20**	**0.00705**	**0.141**	**6.76**	**47.943**	**33.33**	**6308**

4.3 Control of Purity of Sheep Liver G6PD Enzyme by SDS-PAGE Method

The SDS-PAGE was used to determine the molecular weight of G6PD purified from sheep liver and to check its purity. With this method, proteins were executed by adding pure G6PD enzyme to several wells and protein marker in the far well with a vertical SDS-PAGE electrophoresis system with 4-10% batches. The photograph showing the bands located on the electrophoresis gel is indicated in Figure 4.3.

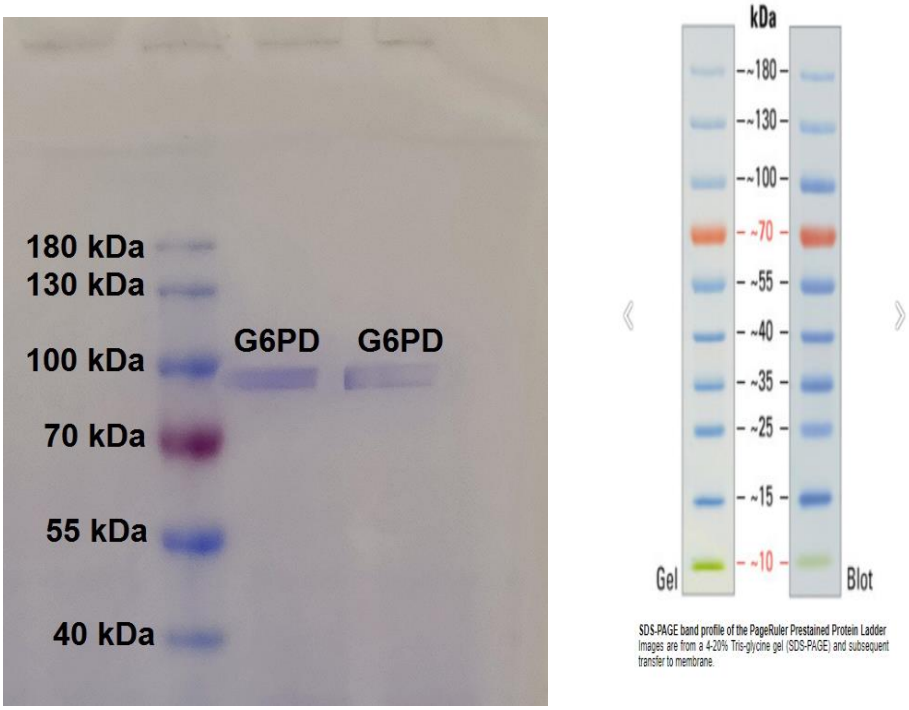

Figure 4.3 SDS-PAGE photograph of G6PD purified from sheep liver and original indication of the standard marker

4.4 Results of Finding the Molecular Weight of Subunits of G6PD in Sheep Liver by SDS-PAGE Method

The designation of the molecular weights of sheep liver G6PD was done as specified in section 3.2.4. Marker and G6PD enzyme samples were obtained with SDS-PAGE. The tapes on the gel were photographed. By finding the R_f values of the standards and enzymes carried out, the R_f (Relative Migration) – log M_W standard graph was created. The molecular weight of the G6PD purified from sheep liver was calculated as 93 kDa utilizing the generated graphical equation.

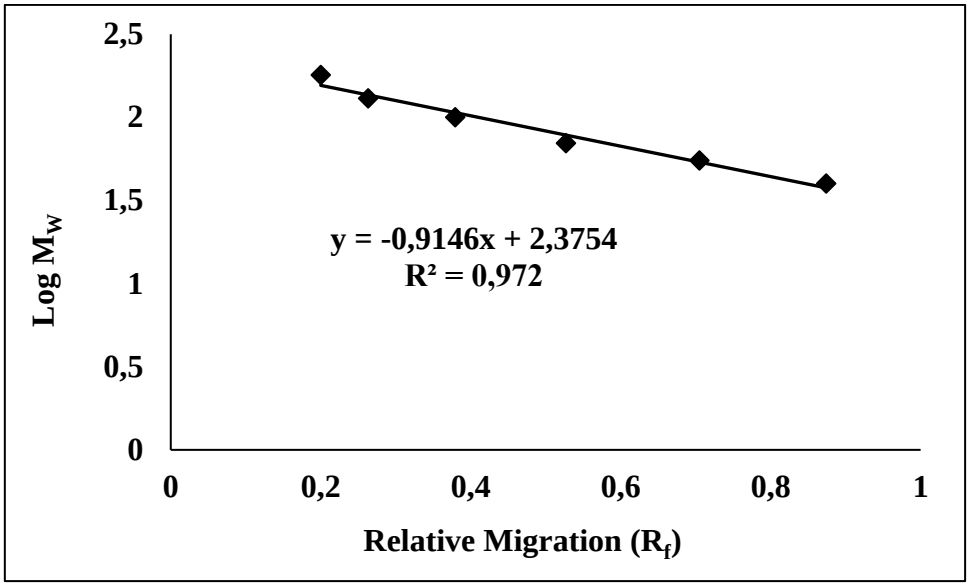

Figure 4.4 Standard graph used to determine the molecular weights of sheep liver G6PD with SDS-PAGE

4.5 Results of Determination of V_{max} and K_M Values for Sheep Liver G6PD

Measurements were made at 5 different G6P concentrations for the purified G6PD enzyme. Then, using these values, the Lineweaver-Burk chart was created. From this graphic, the V_{max} and K_M values were found as 0.58 (μmol/min).mL^{-1} and 1.44 mM, respectively (Figure 4.8).

4.6 Study Results on the Effects of Biotin and Folic Acid Vitamins on Sheep Liver G6PD Enzyme

Activity measurements were made to find the impacts of biotin and folic acid vitamins on purified G6PD obtained from sheep liver. The values obtained from these activity results were indicated in Tables 4.2-4.3 and Figures 4.5-4.6.

Table 4.2 Activity and % activity values of G6PD enzyme in sheep liver varying with biotin concentration

I (µM)	0	16.37	32.74	49.12	65.49
EU	0.177	0.154	0.127	0.119	0.076
%Akt.	100	87.14	71.79	67.12	42.71

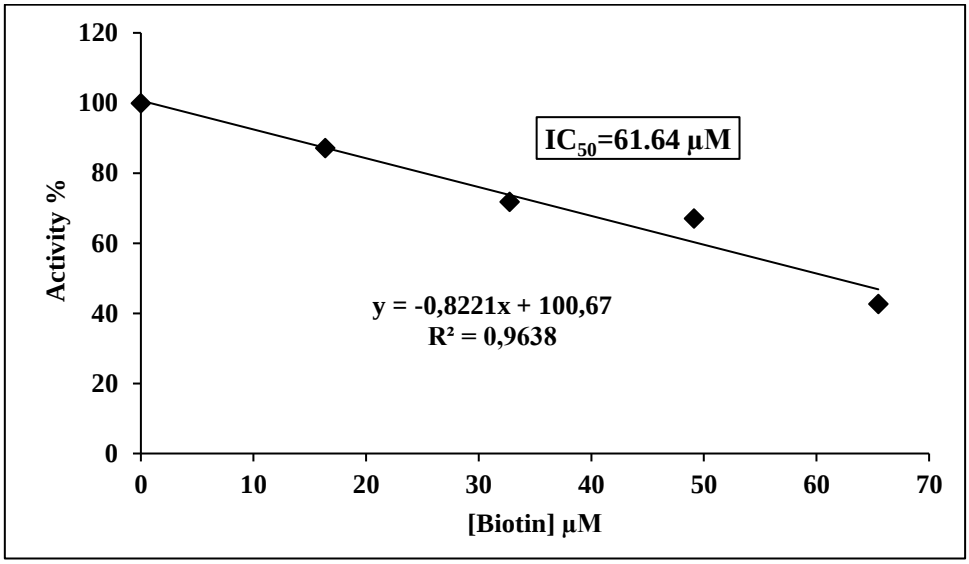

Figure 4.5 Effect of biotin vitamin on G6PD enzyme in sheep liver

Table 4.3 Activity and % activity values of G6PD enzyme in sheep liver varying with folic acid concentration

I (µM)	0	4.54	9.08	13.62	18.16
EU	0.187	0.153	0.145	0.117	0.083
%Akt.	100	81.82	77.54	62.59	44.56

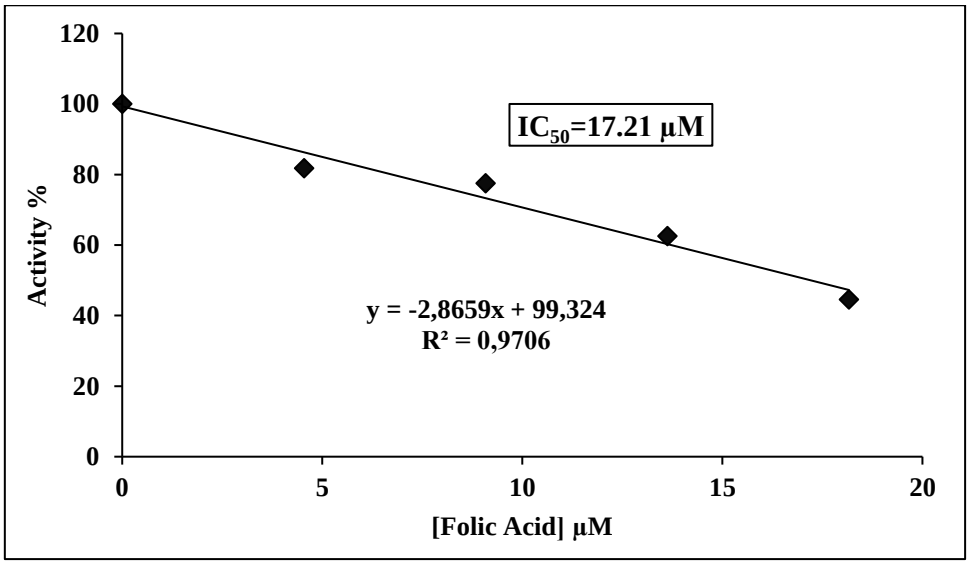

Figure 4.6 Effect of folic acid vitamin on G6PD enzyme in sheep liver

Table 4.4 IC_{50}, K_i values, and types of inhibition found for sheep liver G6PD

İnhibitor	**IC_{50}**	**Ki**	**İnhibition type**
Biotin	61.64 µM	121.13 µM	Non-competitive
Folic acid	17.21 µM	22.73 µM	Non-competitive

4.7 Study Results of Finding the Inhibition Type of Biotin and Folic acid Vitamins Showing Inhibitory Effect on Sheep Liver G6PD Enzyme

To find the type of inhibition of biotin and folic acid vitamins, which exerts inhibitory effects on purified G6PD, activity values were determined by finding 3 appropriate inhibitor concentrations and 5 substrate concentrations of the same inhibitor compound as specified in section 3.2.10. Lineweaver-Burk graphs were created with these values. The type of inhibition for biotin and folic acid, which is the inhibitor that

gives the graph formed by the intersecting lines on the x-axis, was determined as non-competitive inhibition.

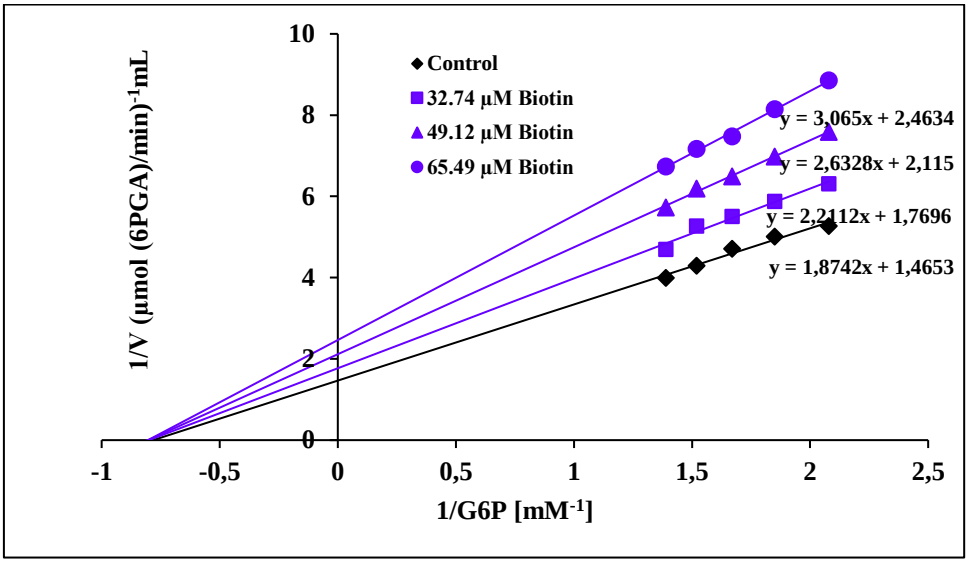

Figure 4.7 Lineweaver-Burk graph of biotin vitamin on sheep liver G6PD enzyme

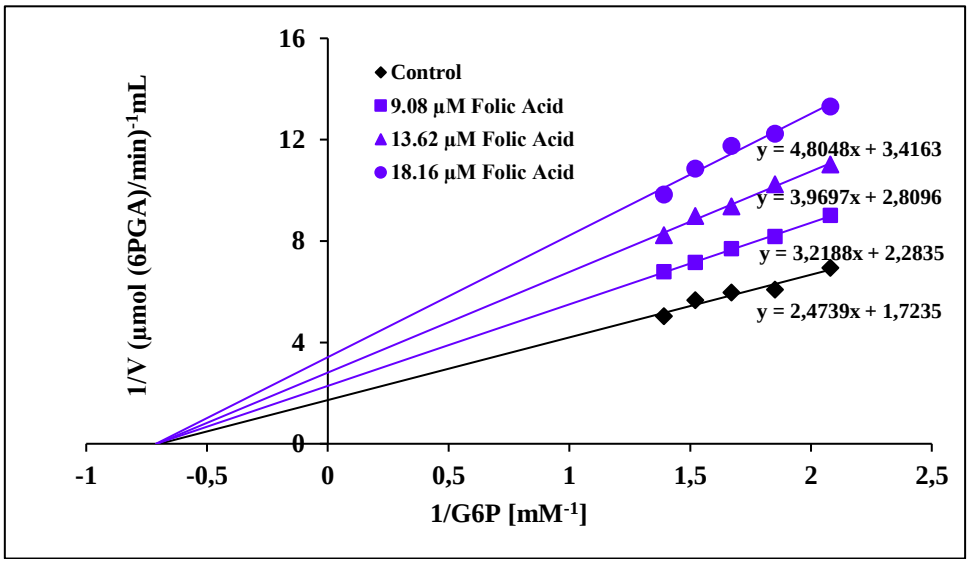

Figure 4.8 Lineweaver-Burk graph of folic acid vitamin on sheep liver G6PD enzyme

5. DISCUSSION AND CONCLUSION

The enzyme glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that is essential for the function and integrity of erythrocyte cells. The G6PD enzyme is a very important component that protects erythrocytes from oxidative stress. Thus, the integrity of erythrocyte cells is preserved. At the same time, dysfunction of the G6PD plays a role in many diseases. The most common of these diseases is cancer. Therefore, inhibitors of G6PD in cancer treatment constitute an important area of research. Herein, the inhibition effect of water-soluble biotin (vitamin B_7) and folic acid (vitamin B_9) on purified G6PD activity was investigated.

In our study, G6PD was purified and characterized from sheep liver. G6PD was purified 6308-fold in a single step by affinity chromatography. 2',5'-ADP Sepharose 4B was used as column filler. In a work, G6PD was partially purified from *Bacillus* sp. (BA-142) bacteria by ammonium sulfate precipitation As a result of these two steps, the specific activity was found as 0.23 EU/mg protein and the purification coefficient was calculated as 82-fold (Çiftçi et al., 2011). In another work, G6PD was purified from camel liver by ammonium sulfate precipitation, Sephacryl S-300 gel filtration, DEAE-cellulose, and affinity chromatography steps (Ibrahim et al., 2014). In one work, the G6PD enzyme was purified 1825-fold by 2',5'- ADP Sepharose 4B affinity chromatography from rat erythrocyte. Its specific activity was calculated as 29.2 EU/mg protein (Temel et al., 2020). Compared to similar studies, the high purification coefficient in our study proves that a successful purification was carried out in a single step.

In this study, the molecular weight and purity of the G6PD enzyme purified from sheep liver were determined by SDS-PAGE. The molecular weight of the G6PD was defined as 93 kDa. In our study, the purity of the G6PD enzyme was determined as a single band by the SDS-PAGE method and its molecular weight was calculated.

Its biochemical parameters were found to further characterize the G6PD purified from sheep liver. Measurements were made at 5 different G6P concentrations for the purified G6PD enzyme. Then, using these values, the Lineweaver-Burk chart was created. Using this graph, K_M and V_{max} values were computed as 1.44 mM and 0.58 (μmol/min).mL^{-1}, respectively.

The G6PD enzyme in the pentose phosphate pathway is the rate-limiting and the first enzyme required for the integrity and function of red blood cells. The more the reaction synthesized by G6PD is activated, the more NADPH is produced. Thus, the integrity of erythrocyte cells is preserved. The G6PD enzyme is a very important enzyme that protects erythrocytes from oxidative stress. At the same time, the decreased activity of the G6PD enzyme is an important factor in many diseases. Especially in diseases such as cancer, G6PD dysfunction is seen. Therefore, the investigation of G6PD inhibitors in cancer treatment is a very important issue. In studies, the inhibitory effect of some compounds on G6PD has been investigated. For example, in one study, the inhibition type and K_i value of the NADPH compound, which has an inhibitor impact on the G6PD enzyme purified from sheep liver, were determined as non-competitive inhibition and 4.707 ± 0.49 μM (Türkoğlu et al., 2003). In another study, the inhibition effect of furosemide, gentamicin, and clindamycin antibiotics on the G6PD enzyme obtained from rat erythrocytes was investigated. The IC_{50} values of the antibiotics gentamicin, clindamycin, and furosemide were found to be 1.75, 34.65, and 0.526 mM, respectively. K_i values were calculated as 0.7, 39.8, and 0.860 mM, respectively (Temel et al., 2020). In one study, the effect of diclofenac sodium on G6PD purified from sheep spleen tissue was investigated. IC_{50} and K_i values of diclofenac sodium, which has an inhibitory effect on the G6PD enzyme, were calculated. The IC_{50} value of diclofenac sodium was 0.51 mM and the K_i value was 0.48 ± 0.01 mM. The type of inhibition was found to be non-competitive inhibition (Çoban et al., 2022).

In this work, the inhibitor effect of biotin and folic acid vitamins on G6PD purified from sheep liver was investigated. These vitamins had an inhibitor impact on pure G6PD. % Activity and Inhibitor concentrations graphs were plotted using different concentrations of biotin and folic acid vitamins. From the equations of these graphs, the IC_{50} values of biotin and folic acid vitamins were designated as 61.64 μM and 17.21 μM, respectively. Lineweaver-Burk graphs were created with 5 substrates and 3 inhibitors and their concentrations to find the type of inhibition and K_i values of these compounds. For biotin and folic acid, the type of inhibition was alternately non-competitive and the K_i values were 121.13 μM and 22.73 μM. Based on these results, it was observed that biotin and folic acid vitamins have a largely inhibitory effect on the pure G6PD enzyme.

Vitamins are essential microorganic molecules that are needed in small amounts for the proper functioning of an organism's metabolism. Vitamins are essential organic catalysts required for the normal functions, growth, and healthy living of the body (Nelson and Cox, 2005; Keha and Küfrevioğlu, 2012). At the same time, vitamins of group B participate in the structure of enzymes as coenzymes for the regular proceeding of reactions in metabolism. It has been observed in experimental and clinical studies that vitamins are also effective in the treatment of many diseases. In many in vivo and in vitro works, antioxidant vitamins E and C have been observed to importantly decrease hemolysis in patients with G6PD enzyme deficiency. In an in vivo study, the G6PD enzyme inactivated in nicotine-treated rats regained its activity with vitamin E administration. At the same time, in vitro study, vitamin E increased the activity of the purified G6PD enzyme (Gumustekin et al., 2005). Pharmacological doses of vitamin C have been found to alleviate hemolysis in patients with G6PD deficiency (Fontes et al., 2015). In another study, vitamin E supplementation to infants with preterm hyperbilirubinemia resulted in an important decrease in plasma bilirubin levels (Pacher et al., 2007; Pham-Huy et al., 2008).

In this work, G6PD was purified from sheep liver by affinity chromatography method. A successful purification was carried out with a high purification coefficient of 6308 times in a single step without the use of processes such as precipitation with neutral salts, dialysis, and ultrafiltration. The inhibition effect of biotin and folic acid on pure G6PD activity was investigated. These vitamins showed a great inhibitory effect with low IC_{50} values. G6PD is an enzyme that has many basic biochemical functions. In various cancer diseases, increased activity of G6PD leads to an increase in cancer cells. Therefore, G6PD inhibitors can be a very important treatment method in cancer treatment. Vitamins are essential organic compounds taken from natural foods for the growth and development of living things. In many recent studies, it has been determined that vitamins have both therapeutic and protective impacts against diseases like cancer, diabetes, cardiovascular diseases, atherosclerosis, and hypertension. Therefore, it is also recommended as a supplementary nutrient against many diseases.

The pentose phosphate pathway is a very important metabolic pathway that produces NADPH, synthesizes DNA and RNA precursors necessary for proliferation, and provides intracellular ROS detoxification in healthy organisms. However, in patients with

cancer, increased activity of some enzymes such as G6PD in the pentose phosphate pathway may cause cancer cells to increase nutrition. In particular, inhibition of key enzymes of the pentose phosphate pathway, including G6PD, can strongly reduce cancer cell proliferation both in vitro and in vivo. Therefore, inhibition of G6PD activity has been proposed as an attractive therapeutic strategy against cancer. In this study, the inhibitory effect of biotin and folic acid vitamins on G6PD may have a protective and therapeutic effect against cancer.

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EXTENDED TURKISH SUMMARY
(GENİŞLETİLMİŞ TÜRKÇE ÖZET)

KOYUN KARACİĞERİNDEN SAFLAŞTIRILAN GLUKOZ-6-FOSFAT DEHİDROGENAZ ENZİMİ ÜZERİNE BAZI VİTAMİNLERİN ETKİSİNİN İNCELENMESİ

MOHAMMED SALIM, Warvin H. M. Salim
Yüksek Lisans Tezi, Kimya Anabilim Dalı
Danışman: Doç. Dr. Zehra BAŞ
Eylül 2024, 53 sayfa

Glikoz-6-fosfat dehidrogenaz (G6PD, EC 1.1.1.49) enzimi kırmızı kan hücrelerinin bütünlüğü ve işlevi için gerekli olan pentoz fosfat yolunun ilk ve hız sınırlayıcı enzimidir. Bu çalışmada G6PD, koyun karaciğerinden afinite kromatografisi ile tek basamakta 6308 kat saflaştırıldı. SDS-PAGE ile G6PD enziminin molekül ağırlığı 93 kDa olarak bulundu. V_{max} ve K_M ve değerleri sırasıyla 0.58 (µmol/dk).mL^{-1} ve 1.44 mM olarak hesaplandı. G6PD aktivitesi üzerine biotin (B_7 vitamini) ve folik asit (B_9 vitamini) vitaminlerinin etkisi incelendi. Biotin ve folik asit G6PD aktivitesi üzerine önemli ölçüde inhibitör etkisi gösterdi. Biotin ve folik asit vitaminlerinin IC_{50} değerleri sırasıyla 61.64 µM ve 17.21 µM olarak hesaplandı. Lineweaver-Burk grafikleri ile bu vitaminlerin K_i değerleri ve inhibitör türleri bulundu. Bu vitaminlerin inhibitör türü yarışmasız inhibitör olarak belirlendi. Biotin ve folik asit için K_i değerleri sırasıyla 121.13 µM ve 22.73 µM olarak hesaplandı. Çeşitli kanser hastalıklarında G6PD enziminin aktivitesinin artması kanser hücrelerinin hızlı gelişmesine sebep olmaktadır. Bu nedenle G6PD inhibitörleri kanser tedavisinde çok önemli bir tedavi yöntemi olabilir. Bu çalışmada, biotin ve folik asit vitaminlerinin G6PD üzerine inhibitör etkisi göstermesi ile kanser tedavisinde etkili olabileceği sonucuna varıldı.

Anahtar kelimeler: Aktivasyon, Glukoz-6-fosfat dehidrogenaz, İnhibisyon, Vitaminler, Saflaştırma

1. GİRİŞ

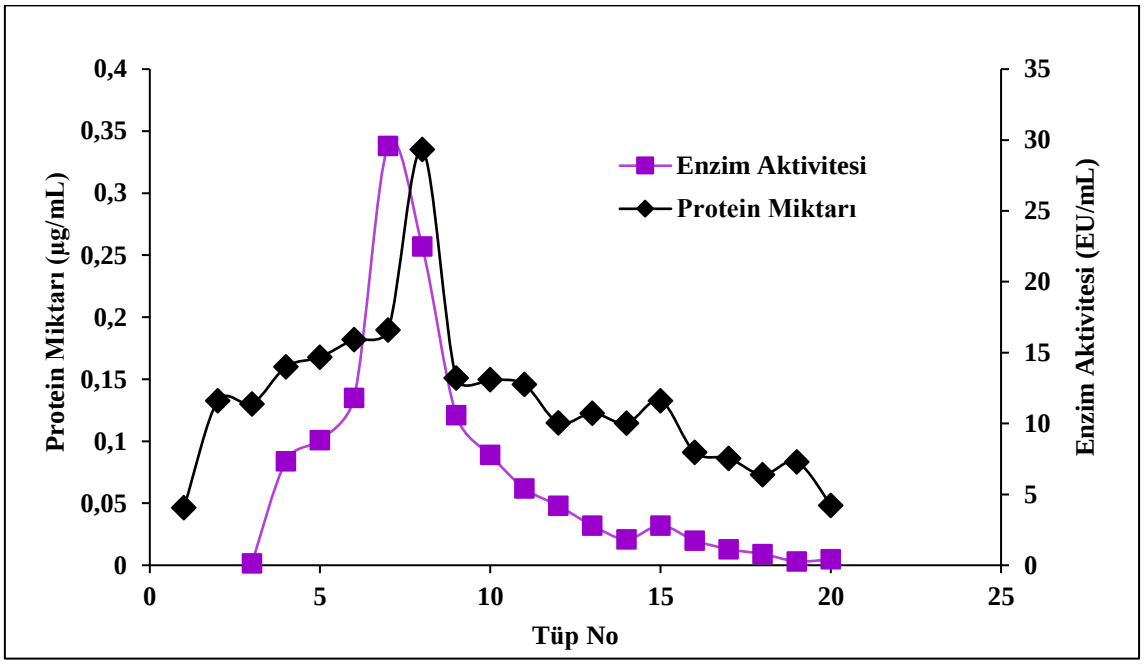
Pentoz fosfat yolundaki glukoz 6-fosfat dehidrogenaz (G6PD, EC 1.1.1.49) enzimi kırmızı kan hücrelerinin bütünlüğü ve işlevi için gerekli olan hız sınırlayıcı ve ilk enzimdir. G6PD enzimi tarafından katalize edilen reaksiyon ne kadar çok aktive edilirse, o kadar fazla NADPH üretilir. Böylece eritrosit hücrelerinin bütünlüğü korunmuş olur. Aynı zamanda, G6PD enziminin disfonksiyonu birçok hastalıkta kritik bir rol oynar. Bunlar arasında en sık görülen hastalık kanserdir. Çeşitli kanser hastalıklarında G6PD enziminin aktivitesinin artması kanser hücrelerinin hızlı gelişmesine sebep olmaktadır. Bu nedenle, G6PD inhibitörleri kanser tedavisinde önemli bir araştırma alanı oluşturmaktadır.

G6PD enzimi, birçok temel biyokimyasal fonksiyona sahip olan bir enzimdir. Çeşitli kanser hastalıklarında G6PD enziminin aktivitesinin artması kanser hücrelerinin artmasına neden olur. Bu nedenle G6PD inhibitörleri kanser tedavisinde çok önemli bir tedavi yöntemi olabilir. Bu çalışmada, koyun karaciğerinden saflaştırılan G6PD enzimi aktivitesi üzerine bazı vitaminlerin inhibisyon etkisi araştırıldı (Song ve ark., 2022). Vitaminler, canlıların büyüme ve gelişmesi için doğal gıdalardan alınan esansiyel organik bileşiklerdir. Son zamanlarda yapılan birçok çalışmada vitaminlerin diyabet, kanser, kardiyovasküler hastalıklar, ateroskleroz ve hipertansiyon gibi hastalıklara karşı hem tedavi edici hem de koruyucu etkileri olduğu tespit edilmiştir. Bu nedenle birçok hastalığa karşı takviye besin olarak da önerilmektedir. Bu çalışmada, afinite kromatografisi ile saflaştırılan G6PD enzimi üzerine biotin ve folik asit vitaminlerinin inhibitör etkisi araştırıldı ve önemli ölçüde inhibitör etkisi gözlemlendi.

2. BULGULAR

2.1 Koyun Karaciğeri G6PD Enziminin Saflaştırılması Sonuçları

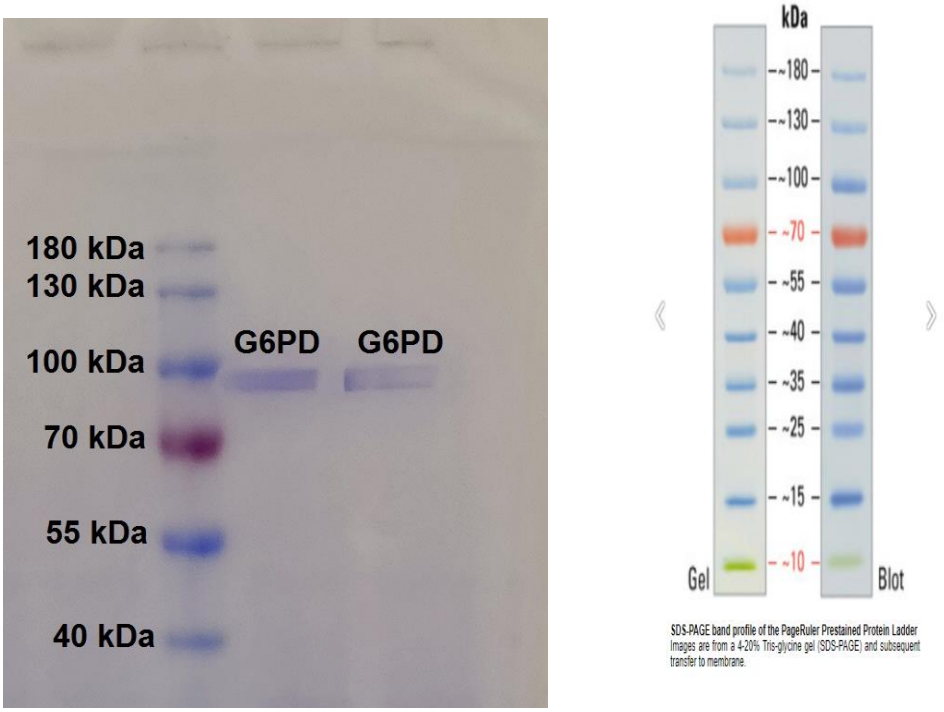
Bu çalışmada saflaştırılan G6PD enzimi tüpleri için bulunan değerler Şekil 2.1'de belirtilmiştir.



Şekil 2.1 Afinite kolonu ile elde edilen koyun karaciğeri tüplerinin kalitatif protein tayinleri ve aktivite değerleri

2.2 SDS-PAGE Yöntemi ile Koyun Karaciğeri G6PD Enziminin Saflığının Kontrolü

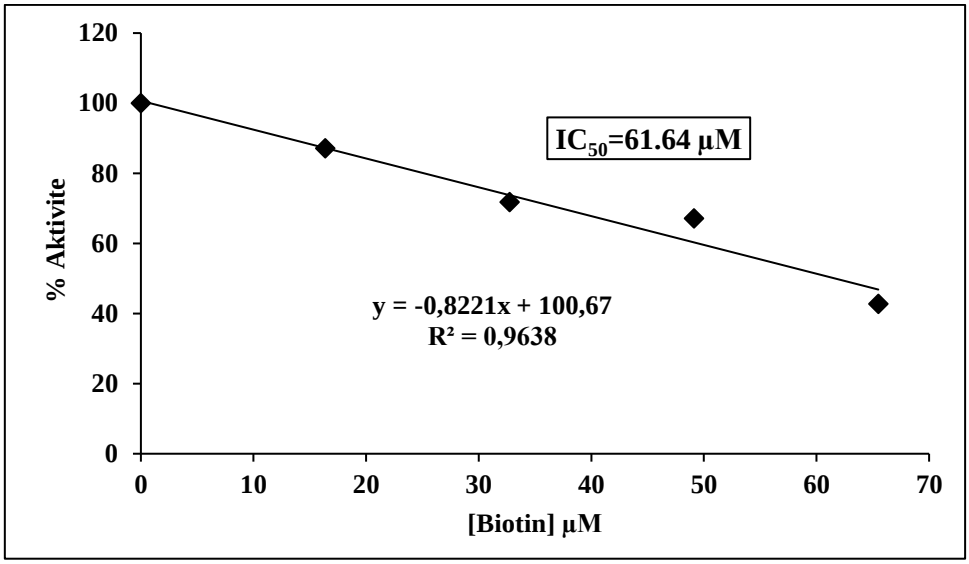
SDS-PAGE metodu ile saflaştırılan G6PD enziminin saflığını kontrol edildi ve molekül ağırlığı bulundu.



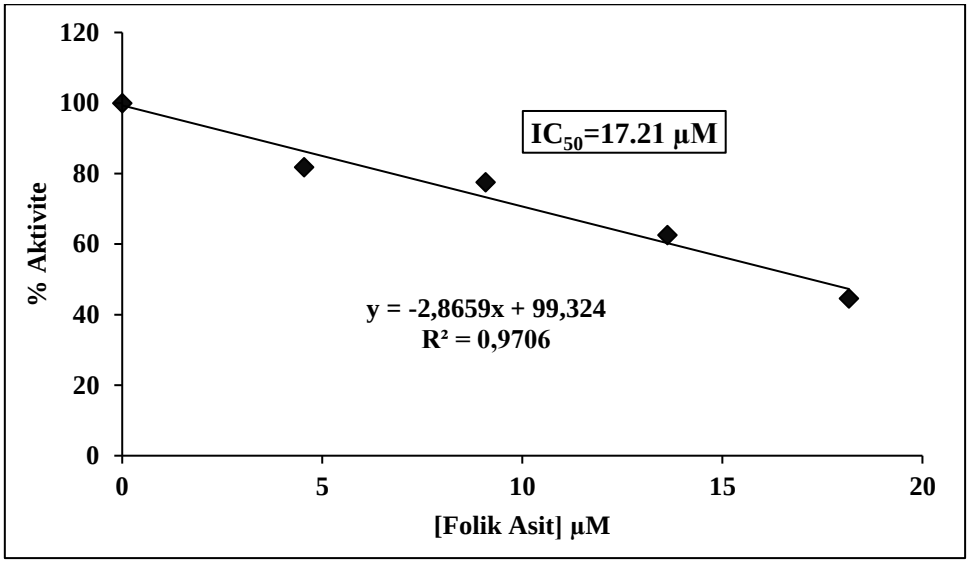
Şekil 2.2 Koyun karaciğerinden saflaştırılan G6PD enziminin SDS-PAGE fotoğrafı ve standart markırın orijinal göstergesi

2.3 Koyun Karaciğeri G6PD Enzimi Üzerine Biotin ve Folik Asit Vitaminlerinin Etkilerinin Bulunmasına Ait Çalışma Sonuçları

Saflaştırılan G6PD enzimi üzerine biotin ve folik asit vitaminlerinin etkilerini bulmak için aktivite ölçümleri ile grafikler çizildi. Bu değerler ile çizilen grafikler Şekil 2.3-2.4'te gösterildi.



Şekil 2.3 Koyun karaciğerindeki G6PD enzimi üzerine biotin vitamininin etkisi



Şekil 2.4 Koyun karaciğerindeki G6PD enzimi üzerine folik asit vitamininin etkisi

3. TARTIŞMA VE SONUÇ

Glikoz-6-fosfat dehidrogenaz enzimi (G6PD), kırmızı kan hücrelerinin bütünlüğü ve işlevi için gerekli olan bir protein kompleksi enzimidir. G6PD enzimi, eritrositleri oksidatif stresten korumak için kritik öneme sahiptir. Böylece eritrosit hücrelerinin bütünlüğü korunmuş olur. Aynı zamanda, G6PD enziminin aktivite bozukluğu birçok hastalıkta gözlemlenmiştir. Bu hastalıklar arasında en yaygın olanı kanserdir. Bu yüzden, kanser tedavisinde G6PD enziminin inhibitörleri önemli bir araştırma alanı oluşturmaktadır.

Çalışmamızda, G6PD koyun karaciğerinden saflaştırıldı ve karakterize edildi. G6PD afinite kromatografisi ile 6308 kat saflaştırıldı. Spesifik aktivite 47.943 EU/mg protein olarak hesaplandı. Kolon dolgu maddesi olarak 2',5'-ADP Sepharose 4B kullanıldı. Saflaştırılan G6PD aktivitesi üzerine biotin (B_7 vitamini) ve folik asit (B_9 vitamini) vitaminlerinin inhibisyon etkisi araştırıldı.

Bu çalışmada koyun karaciğerinden saflaştırılan G6PD enziminin saflığı ve molekül ağırlığı SDS-PAGE yöntemi ile bulundu. Elektroforez jeli üzerinde 93 kDa olmak üzere tek bant oluştuğu görüldü. Burada SDS-PAGE yöntemi ile belirlenen molekül ağırlığı, saflaştırılan G6PD enziminin molekül ağırlığıdır.

Koyun karaciğerinden saflaştırılan G6PD enziminin karakterizasyon için biyokimyasal parametreleri belirlendi. G6PD için 5 farklı G6P konsantrasyonunda ölçümler alındı. Daha sonra ölçülen konsantrasyon değerleri ile Lineweaver-Burk grafiği çizildi. Bu grafikten K_M ve V_{max} değerleri sırasıyla 1.44 mM ve 0.58 (μmol/dk).mL^{-1} olarak bulundu.

G6PD enzimi kırmızı kan hücrelerinin bütünlüğü ve işlevi için gerekli olan pentoz fosfat yolunun hız sınırlayıcı enzimidir. G6PD enziminin katalizlediği reaksiyon ne kadar fazla aktive olursa o kadar fazla NADPH üretilir. Böylece eritrosit hücrelerinin bütünlüğü korunmuş olur. G6PD enzimi, eritrositleri oksidatif stresten korumak için kritik öneme sahiptir. Aynı zamanda, G6PD enziminin aktivitesinin azalması birçok hastalıkta önemli bir etkendir.

Bu çalışmamızda ise koyun karaciğerinden saflaştırılan G6PD üzerine biotin ve folik asit vitaminlerinin inhibitör etkisi belirlendi. G6PD enzimi üzerine her iki vitaminde inhibitör etkisi gösterdi. Biotin ve folik asit vitaminlerinin farklı konsantrasyonları ile

inhibitör grafikleri çizildi. Bu grafiklerin denklemleri kullanılarak biotin ve folik asit vitaminlerinin IC_{50} değerleri sırasıyla 61.64 μM ve 17.21 μM olarak hesaplandı. Bu bileşiklerin K_i değerlerini ve inhibisyon türünü belirlemek için Lineweaver-Burk grafikleri çizildi. Biotin ve folik asit için K_i değerleri sırasıyla 121.13 μM ve 22.73 μM ve inhibisyon çeşidi dönüşümlü yarışmasız olarak bulundu. Bu sonuçlar ile biotin ve folik asit vitaminlerinin saf G6PD enzimi üzerine büyük oranda inhibitör etkisi gösterdiği belirlendi.

Bu çalışmada G6PD koyun karaciğerinden afinite kromatografisi yöntemi ile saflaştırıldı. Tek basamakta 6308 kat bir yüksek saflaştırma katsayısı ile başarılı bir saflaştırma gerçekleştirildi. Saf G6PD aktivitesi üzerine biotin ve folik asit vitaminleri yüksek oranda inhibisyon etkisi gösterdi. Bu vitaminlerin G6PD enzimi üzerine büyük ölçüde inhibisyon etkisi gösterdiği düşük IC_{50} değerleri ile kanıtlandı. Çünkü bir inhibitörün IC_{50} değeri ne kadar küçük olursa inhibisyon etkisi o kadar büyük olur. Glukoz-6-fosfat dehidrogenaz (G6PD), birçok temel biyokimyasal fonksiyona sahip olan bir enzimdir. Çeşitli kanser hastalıklarında G6PD'nin aktivitesinin artması kanser hücrelerinin artmasına neden olur. Bu nedenle G6PD inhibitörleri kanser tedavisinde çok önemli bir tedavi yöntemi olabilir. Vitaminler canlıların büyümesi ve gelişmesi için doğal besinlerden alınan esansiyel organik bileşiklerdir. Son zamanlarda yapılan birçok çalışmada vitaminlerin diyabet, kanser, kardiyovasküler hastalıklar, aterosklerosis ve hipertansiyon gibi hastalıklara karşı hem tedavi edici hem de koruyucu etkileri olduğu belirlenmiştir. Bu yüzden birçok hastalığa karşı takviye besin elemanı olarak da tavsiye edilmektedir.

Pentoz fosfat yolu sağlıklı canlılarda NADPH üreten, proliferasyon için gerekli olan DNA ve RNA öncüllerini sentezleyen ve hücre içi ROS detoksifikasyonunu sağlayan çok önemli metabolik bir yoldur. Ancak kanserli hastalarda pentoz fosfat yolundaki G6PD gibi bazı enzimlerin aktivitesinin artması kanser hücrelerinin beslenerek artmasına neden olabilir. Özellikle G6PD dahil olmak üzere pentoz fosfat yolu anahtar enzimlerinin inhibisyonu hem in vitro hem de in vivo kanser hücresi proliferasyonunu güçlü bir şekilde azaltabilir. Bu nedenle, G6PD aktivitesinin inhibisyonu kansere karşı çekici bir tedavi stratejisi olarak önerilmiştir. Bu çalışmada biotin ve folik asit vitaminlerinin G6PD üzerindeki inhibitör etkisi kansere karşı koruyucu ve tedavi edici etkisi olabileceği sonucunu ortaya çıkarmıştır.

CURRICULUM VITAE

Personal Information

Name Surname : Warvin H. M. Salim MOHAMMED SALIM

Education Information

Undergraduate

University : Duhok University
Faculty : Faculty of Science
Department : Chemistry
Graduation Year : 2012

ETHICS COMMITTEE FINAL RESULT APPROVAL CERTIFICATE

VAN YÜHADYEK
VAN YÜZÜNCÜ YIL ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu

ÇALIŞMA ONAY BELGESİ

VAN YUZUNCU YIL UNIVERSITY (TURKEY)
ANIMAL RESEARCHES LOCAL ETHIC COMMITTEE
APPROVAL CERTIFICATE

Araştırmanın Adı: ***Research Title:***	Koyun karaciğerinden saflaştırılan glukoz-6-fosfat dehidrogenaz enzimi üzerine bazı vitaminlerin etkisinin incelenmesi. Examination of the effect of some vitamins on glucose-6-phosphate dehydrogenase enzyme purified from sheep liver.	
Araştırıcı(lar): ***Investigator(s)***	**Yürütücü / *Chief investigator:***	Doç. Dr. Zehra BAŞ
	Yardımcı Araştırıcı(lar) / *Co-investigator(s):*	Warvin H. M. Salim MOHAMMED SALIM

Araştırmada kullanılacak hayvanlar / *Animals to be used in the research:*

Tür / species: Koyun / Sheep **Yaş /*Age:***	**Sayı / *Numbers:*** Mezbahaneden kesim esnasında sağlıklı bir koyundan karaciğer alınacaktır. **Cinsiyet / *Sex:***
Araştırmanın Öngörülen Başlama Tarihi / *Proposed Research Starting Date:*29.02.2024	
Araştırmanın Öngörülen Bitiş Tarihi / *Proposed Research Completion Date:*31.09.2024	

Karar:
Yukarıda bilgileri verilen planlanan araştırma projesi için Hayvan Deneyleri Etik Kurul Onayı gerekmemektedir. Tarih: 01/02/2024..; Karar No: .2024/01-01

Decision:
*The proposed research project detailed above does not need Animal Researches Ethic Committee Approval. Date:*01/02/2024. *Decision number:* 2024/01-01

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Date: 02/10/2024

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Name and Surname: Warvin H. M. Salim MOHAMMED SALIM
Student ID: 22910001165
Deparment: Chemistry
Program: Biochemistry
Status: (X) M.Sc. ( ) Ph.D.

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