REPUBLIC OF TURKEY
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PURIFICATION, CHARACTERIZATION AND INVESTIGATION THE EFFECT OF SOME DRUGS ON GLUTATHIONE S-TRANSFERASE (GST) ENZYME FROM RAT ERYTHROCYTE

MASTER'S THESIS

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I would like to dedicate this scientific study to all persons who like science in the world, especially biochemistry reseachers who specialize himself in science for the humanity. More specifically this thesis is dedicated to Bingol University Institute of Science and Technology and all staffs of biochemistry department. I also dedicate this thesis to Salahaddin Uniersity - College of Science - Chemistry department. Most importantly this thesis is dedicated to my beloved wife, children, father and mother.

Luqman Qader Ahmed KHOSHNAW
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LIST OF ABBREVIATINS

GST	: Glutathione S-transferase
GSH	: Reduced glutathion
GSSG	: Oxidized glutathione
CDNB	: 1- Chloro 2,4- dinitrobenzene
E	: Enzyme
S	: Sbstrate
ES	: Enzyme substate complex
P	: Product
TEMED	: Tetamethylethylenediamine
PER	: Ammonium persulfate
SDS	: Sodium dodycyl sulfate
PAGE	: Polyacrylamide gel electrophoresis
D.W.	: Distilled water
mL	: Milliliter
g	: Gram
mg	: Milligram
kDa	: Kilo Dalton
ROS	: Reactive oxygen species
M	: Molar
mM	: Millimolar
L	: Liter
DNA	: Deoxyribonucleic acid
Tris	: Trihydroxymethylaminomethane
R_f	: Retardation factor
TCA	: Trichloro acetic acid
EDTA	: Ethylene diamine tetra acetic acid
nm	: Nanometer

mmol : Millimole

EC : Enzyme Commission

M.W. : Molecular weight

EU : Enzyme unit

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GLUTATYON S-TRANSFERAZ (GST) ENZİMİNİN SIÇAN ERİTROSİTLERİNDEN SAFLAŞTIRILMASI, KARAKTERİZASYONU VE BAZI İLAÇLARIN ENZİM AKTİVİTESİ ÜZERİNE ETKİSİNİN ARAŞTIRILMASI

ÖZET

Bu çalışmada glutatyon S-transferaz (GST; EC: 2.5.1.18) enzimi, sıçan eritrositlerinden glutatyon agaroz afinite kromatografisi ile tek basamakta 6,3 EU / mg protein spesifik aktivite ile %44 verimle 115 kat saflaştırıldı. Enzimin saflığı, sodyum dodesil sülfat poliakrilamid jel elektroforezi (SDS-PAGE) ile tespit edildi.Saf enzimin alt birimlerine ait molekül ağırlığı 25,2 kDa olarak hesaplandı. Sıçan eritrositlerinden saflaştırılan GST enzimi için yapılan karakterizasyon çalışmalarında; optimum pH potasyum fosfat tamponu pH=8, optimum iyonik şiddet potasyum fosfat tamponu 0,2M, optimum sıcaklık 50^{o}C, stabil pH potasyum fosfat tamponu pH=8 olarak belirlendi. Yapılan kinetik çalışmalarsonucunda; GST enzimine ait iki substrat olan glutatyon (GSH) ve 1-kloro- 2, 4-dinitrobenzen (CDNB) için K_M ve V_{max} değeri sırasıyla GSH için 1,22 mM ve 1,21 EU / mL, CDNB için 0,374 mM ve 3,614 EU / mL olarak bulunmuştur. Son olarak, bazı ilaçların enzim aktivitesi üzerine olan etkileri *in vitro* olarak çalışıldı. Yapılan çalışmalar sonucunda buscopan ve ampisidin antibiyotiklerinin rat eritrositlerinden saflaştırılan GST enziminin aktivitesini artırdığı, gentamisin ve klindamisin' antibyotiklerinin, enzim aktivitisini inhibe ettiği (IC_{50} değerleri sırasıyla 1,69 ve 6,9 mM olarak, K_i değerleri sırasıyla 1,70 ve 2,36 mM bulundu), sefazolin antibiyotiğinin ise enzim aktivitesi üzerinde bir etkisinin olmadığı tespit edildi.

Anahtar Kelimeler: Sıçan eritrosit, glutatyon S-transferaz, saflaştırma, karakterizasyon, antibiyotik, inhibisyon.

PURIFICATION, CHARACTERIZATION AND INVESTIGATION THE EFFECT OF SOME DRUGS ON GLUTATHIONE S-TRANSFERASE (GST) FROM RAT ERYTHROCYTE

ABSTRACT

In this study, glutathione S-transferase (GST; EC: 2.5.1.18) enzyme was purified from rat erythrocyte in a single step by glutathione agarose affinity chromatography. The enzyme was purified with 6.3 EU/mg protein specific activity, with 44% recovery and 115 purification fold. The purity of enzyme was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and produced a single protein band with a calculated molecular weight of 25.2 kDa. The characterization studies showed that the optimum pH=8 in potasium phosphate buffer, optimum temperature is 50^{o}C, optimum ionic strength is 0.2M in potasium phosphate and stable pH=8 in potasium phosphate buffer. Kinetic studies performed for calculating K_M and Vmax for (GST) using both glutathione (GSH), and 1-chloro 2,4- dinitrobenzene (CDNB) as two subsrates, the obtained results are 1.22 mM and 1.21 EU/mL respectively for GSH 0.374 mM and 3.614 EU/mL respectively for (CDNB). Finally inhibition or activation effect of some drugs on the enzyme activity were studied *in vitro.* The results proved that buscopan and ampisid were two activators of the enzyme, gentamicin and clindamicin were two inhibitors (IC_{50} values were found as 1.69 and 6.9 mM respectively and K_i values were found as 1.70 and 2.36 mM respectively), and cefazoline has no effect on the enzyme activity.

Keywords: Rat erythrocyte, glutathione S-transferase, purification, characterization, antibiotic,inhibition.

1. INTRODUCTION

1.1. Enzymes Overview

Enzymes are biological catalysts, they increase the rate of biochemical reactions without being consumed during the reaction. They selectively direct and catalyse all metabolic reactions with 10^3-10^8 times faster than uncatalyzed reaction, by providing them an alternative reaction pathway with a lower activation energy of the transition state (complex) A ⇌ T* ⇌ B as shown in (Figure 1.1) (Harvey and Denise 2016).

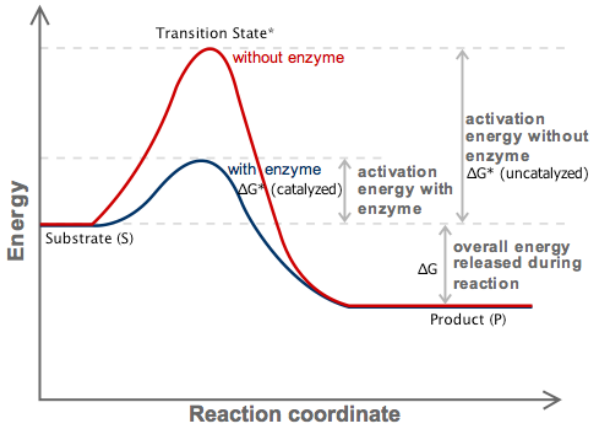

Figure 1.1. Effect of an enzyme on the activation energy of a reaction (https://socratic.org)

The living cell is the center of enormous biochemical activity called metabolism which includes energy production through nutrient, building of new tissue, tissue growth, replacement of old tissue, disposal of waste materials, etc. These building ups and tearing downs are take place in the face of an apparent paradox. Almost all biochemical reactions within human cells are catalysed by the action of enzymes. (Campbell and Farrell 2012).

1.2. Enzyme History

The original of the word enzyme was firstly used by F. W. Kuhne as “enzymos” which is a Greek word that means leavened, and they described as a biological catalyst molecule since the end of 1700s especially after discovery of conversion of starch to sugar. Saliva in 1850 by Pasteur and sugar to alcohol by yeast in 1987 by Buchner. Later in 1926s Sumnur isolated urease and he postulated that all enzymes (except a group of catalytic RNA molecules) are made up by protein (Nelson and Cox 2013). The major development for isolation and purification of the enzyme is the realization of enzymes as proteins and they have biochemical activity, using X-Ray techniques in their characterization studies**,** **e**nzymes have been used in the diagnosis of disease since beginning of 1940's. They have delivered the basis for the environment of clinical chemistry (Mora and Arioli 2014).

1.3. Peptide Bond

Except a small group of catalytic RNA molecules, all enzymes are globular proteins. They are made up mainly of chains of amino acids combined together by peptide (amide) bonds (Figure 1.2).

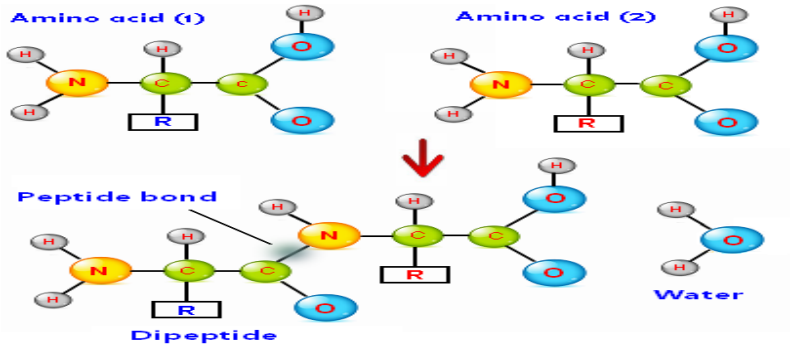

Figure 1.2. Chemical representation of peptide bond (https://en.wikipedia.org)

Rather than having primary and secondary structures, enzymes have a complex tertiary and quaternary structure in which polypeptides are folded around each other to form a globular shape. Enzyme shape is maintained by ionic forces, van der waals forces and hydrogen bonds. The three dimensional shape of an enzyme molecule is very important in which altered enzyme structures, cannot bind to their substrate (Boisseau et al. 2010).

1.4. Enzymes and Cofactors

Some enzymes such as pepsin and trypsin consist of only polypeptides, they require no other factor for activation. In contrast, there are many enzymes that require nonprotein portion in their structure to function properly. The nonprotein part of the enzyme may be inorganic ions, such as Fe^{2+}, Mg^{2+} in this case they are called cofactor, but if its small organic compound that are generally derived from vitamins, are required for the activity of the enzyme in this case they are called coenzyme or cosubstrate (e.g NAD^{+}) (Nelson and Cox 2013). The coenzymes that are tightly bound to the enzymes are referred as prosthetic groups (e.g FAD), while the weakly bound coenzymes are often referred as co-substrates. Inactive enzyme or protein without cofactor is called apoenzyme, while active form with its cofactor is called holoenzyme (Figure 1.3) (Bettelheim et al. 2007). However a group of enzymes that wholly use one coenzyme are all share the similar mechanisms for their actions (Berg et al. 2002).

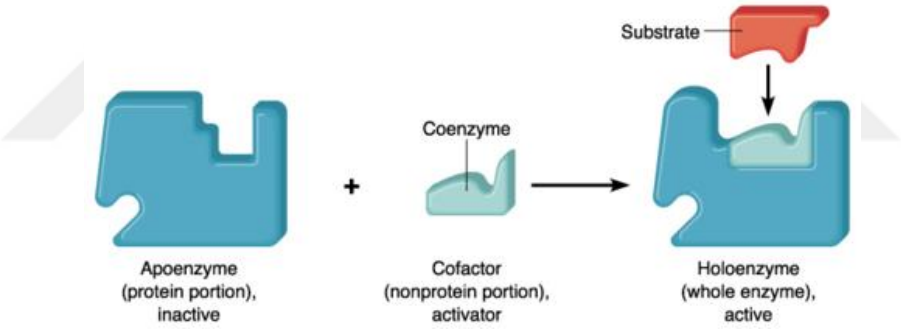

Figure 1.3. Apoenzyme is activated by coenzyme to form holoenzyme (https://www.lifepersona.com)

1.5. Enzymes Nomenclature and Classification

Firstly, the naming of enzymes was in a non-systematic way, like pepsin, trypsin, and chemotrypsin which include no information about enzyme function or the substrate on which the enzyme is catalysing. Later the naming of enzymes included adding the suffix “ase” to the name of substrate molecule in which the enzyme acts on. For example, lactase catalyse the conversion of lactate and produces galactose and glucose. These method is known as “trivial naming” of enzymes. Now enzymes are classified into six functional classes by the International Union of Biochemistry and Molecular Biology (IUBMB). As per the IUBMB system, each enzyme name starts with (Enzyme

Commission; EC number) followed by four digits. The first number represents the class, the second number indicates the subclass, the third number represents the sub-subclass or subgroup and the fourth number provides the particular enzyme.

1. Oxidoreductases, catalyze redox reactions in which oxygen and hydrogen are gained or lost (e.g. Glutathione reductase E.C: 1.8.1.7).

2. Transferases, catalyze transfere of C-, N-, or p- containing functional groups (e.g. Glutathione S-Transferase E.C: 2.5.1.18).

3. Hydrolases, catalyze cleavage of bonds in the presence of water as a reactant (e.g. alfa-amylase E.C: 3.2.1.1).

4. Lyases, catalyze removeable of small molecule such as water and form a double bond, it acts on C-C, C-S, C-O, C-X, and certain C-N bonds (e.g. Carbonic anhydrase E.C: 4.2.1.1).

5. Isomerases, catalyze intramolecular rearrangement to form optical or geometric isomers (e.g. Alanine racemase E.C: 5.1.1.1).

6. Ligases, catalyze formation of bond between two molecules acompany with releasing energy (e.g. Glutathione synthetase E.C: 6.3.1.2) (Tipton and Boyce 2000).

1.6. Enzymes Mechanism

1.6.1. The Lock-key Theory

The method firstly postulated by Emil Fischer in 1894, In this hipotheticl analogy, the lock is the enzyme and the key is the substrate. Only the correct sized key substrate fit into the lock enzyme (Figure 1.4) (Khanna 2010).

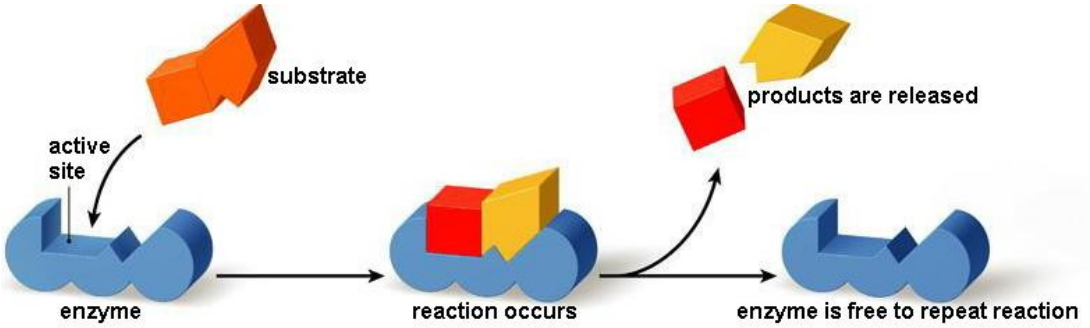

Figure 1.4. The lock-key theory shows only the correct sized key substrate fit into the lock enzyme (http://www.hcrowder.com/uploads)

1.6.2. The Induced-fit Theory

In 1958 Daniel Koshland suggested a modification to the lock and key model. According to this model, the active site (amino acid) of the enzyme can modify itself though interacting with substrates. (Figure 1.5) (Khanna 2010).

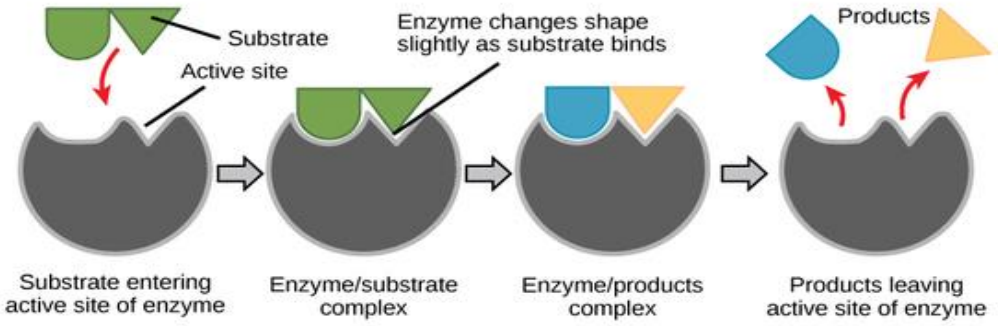

Figure 1.5. Induced-fit Theory of the enzymes, the active site (amino acid) of the enzyme can modify itself though interacting with substrates (https://bio.libretexts.org).

1.7. Enzyme Specificity

Enzymes bind with the substrate molecules through, electrostatic, hydrogen bonding, Van der Waals and hydrophobic interactions. Usually, the active sites amino acid residues on the surface of the cleft are designed in a way that allows them to interact selectively with the substrate molecules. A molecule with different geometrics to the enzyme would not bind with (Voet and Voet 2011; Segel 1968). This specifity inolve stereo specifity (e.g. L-polypeptides are hydrolyzed by trypsin without those of D- polypeptides. And

Geometric specificity is more favorable than the stereo specificity. The geometric specificity of enzyme differs in a great way from one enzyme to another. There are enzymes designed for catalyzing exactly one compound (Voet and Voet 2011). Enzymes are highly specific with one or a few substrates while catalyzing only one kind of reaction. There are many types of enzymes each is specialized to a special function such as anabolic, catabolic, and detoxific enzymes (Martinek 1969).

1.8. Enzyme Kinetic

Enzyme kinetic study include rate of the reactions that are catalyzed with enzymes, by measuring the formation of products concentration or consuming of the substrate concentration per the unit time at 25 ^{o}C to 37 ^{o}C and pH near 7 (Mathews and Van-Holde 1996). In 1913 Leonor Michael and Maud Menten proposed the following mechanism for the combination between enzyme and substrate form product

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

E: enzyme

S: substrate

ES: enzyme-substrate complex

P: product

k_1: rate constant for ES formation

k_{-1}: rate constant for ES dissociation

k_2: rate constant for P formation from ES

The rate of enzymatic catalyzed reaction is gradually increased by an increase in substrate concentration until it reaches maximum velocity, when the substrate concentration is initially high the rate is independent on the substrate concentration but it depends on the enzyme concentration (Figure 6) Nelson and Cox 2013).

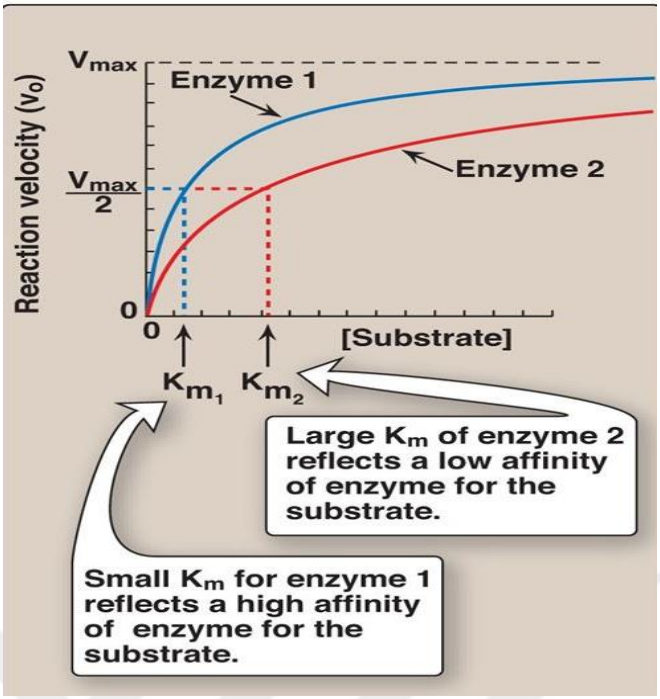

Figure 1.6. Effect of substrate concentration on reaction velocities for two enzymes: enzyme 1 with a small Michaelis constant and enzyme 2 with a large constant (Harvey and Denise 2011)

When the active site of an enzyme is filled with the substrate, the rate of the reaction product is written as follows:

$$V_o = \frac{Vmax.[S]}{Km+[S]} \quad (1.1)$$

V_o: initial velocity

V_{max}: maximum velocity

K_M: Michael's Menten constant

S: substrate

V_{max} is an expression of the enzyme's catalytic activity and it gives information about enzyme catalytic efficiency (Campbell and Farrell 2009).

K_M is determined substrate concentration at which the velocity half maximal, and it's an affinity measurement of an enzyme for a substrate, the values are located between 10^{-6} to 10^{-1}.in which the large value of K_M indicates there is high affinity of enzyme to a substrate and vice versa (Zubay 1998).

1.9. Enzyme,s Property

Enzymes properly work in a definite range of temperature, pH, medium concentration, etc. While in other condition they are denatured and precipitated with salts, solvents and other reagents, They have high molecular weights ranging from about 12000 Da to more than 1000000 Da. (Nelson and cox 2013).

1.10. Factor Affecting Enzyme Activity

1. Substrate concentration; the rate of an enzyme catalyzed reaction increases with gradual increase in substrate concentration until maximum velocity (V_{max}) is reached.

2. Enzyme concentration; in case of the presence of excess substrate concentration, the velocity of enzyme catalyzed reaction increase with increasing of enzyme concentration.

3. Temperature; the reaction rate alters with an increase in temperature until a peak rate is reached.

4. pH; Enzymes have maximum activity in a definite pH value which is also known as optimum pH, any alterations in this value will cause to fall down in their activity.

5. Activators; substances that leads to an increase in the velocity of an enzyme catalyzed reaction, and are more involved in the metabolism control. (Kurland and Pilkis 1995).

6. Inhibitors; substances that are cause to decline the velocity of an enzyme catalyzed reactions, in which are divided into two main kinds of enzyme inhibition first is irreversible which strongly bind with an enzyme and inhibit its work, second is reversible which is weakly bind with an enzyme and subdivided into three types: competitive; an inhibitor competes with the substrate to bind with the active site of an enzyme and cause to arises K_M value while Vmax is remain stable. Noncompetitive; an inhibitor that bind to the enzyme other than the active site and K_M is remain stable while cause to decrease Vmax. Uncompetitive causes to arises K_M and decreases Vmax (Lehninger 2000; Keha and Küfrevioğlu 2004; Berg et al. 2002).

1.11. Free Radicals

Free radicals are substancs that have one or more unpaired electron(s), they are present in free form, and are highly reactive, their reactions include electron transfer reactions (redox reaction) by donating or accepting electron(s), so they are considered as a reductant as well as an oxidant (Buettner 1993). they are introduced into the body tissue through industrial chemicals, environmental pollution, and cigarette smoking as exogenous source, also they endogenously by immune system during metabolism produced to attack and damage pathogen, superoxide $^{*}O_2$ is the most common free radical which produced from the human body. The phagocytic cell generates free radicals that attack pathogen such as bacteria and virus (Droge 2002; Kabel 2014).

1.12. Xenobiotic

Xenobiotic are harmful substances that have no metabolic function (pathway). The accumulation of antibiotics, food additives, natural poisons etc in the living organisms causes production of xenobiotic. More than 200000 xenobiotic have been found and detoxified under xenobiotic metabolizing enzymes (Croom et al. 2012).

1.13. Antioxidants

Antioxidants are any substances that donate electron and capable to full dawn the activity of other harmful molecules such as free radicals, xenobiotics etc before they cause cell damage, and avoid the oxidation of other molecules, and the most of them are reducing agents such as thiols (Durate and Lunec 2005). Antioxidant substances are divided in to two types:

1. Non-enzymatic antioxidant; including Vitamin C, Vitamin E, Carotenoids, Glutathione also is considered as a non-enzymatic antioxidant, which is not essential nutrient from human, since; body can synthesize it from the amino acids

2. Enzymatic antioxidant; are produced in body and are classified into three major groups; Catalase, Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) (Valco et al. 2007).

Drug metabolizing enzymes (DMEs) plays the main role in the metabolism, elimination and detoxification of xenobiotic and drugs that are introduced into the human body. Our bady organs and tissues are well prepared with various DMEs, including phase I, phase II metabolizing enzymes and phase III carriers, which are present in abundance, and can be induced at higher levels after contacting xenobiotics. (Rushmore and Tony Kong 2002).

1.14. Glutathione

Glutathione is an antioxidant molecule identified in plants, animals, fungal species and some of the bacteria. It is reported to be capable of preventing the damage caused by Reactive Oxygen Species (ROS) such as free radicals, heavy metals and peroxides. GSH performs its function by reducing the disulfide bonds formed in cytosolic proteins by acting as an electron donor. During this process reduced glutathione (GSH) is oxidized to GSSG. And reversely GSSG is reduced and restore to GSH by NADPH-dependent Glutathione reductase enzyme (Findlay et al. 2006). Reduced glutathione is a tripeptide consisting of glutamate, cysteine and glycine (Figure 1.7). It has various functions in many metabolic pathways some of which include maintaining amount of glutathione peroxidase, regulating an important component of signaling (nitric oxide cycle) and cell cycle progression (Simony et al. 2002).

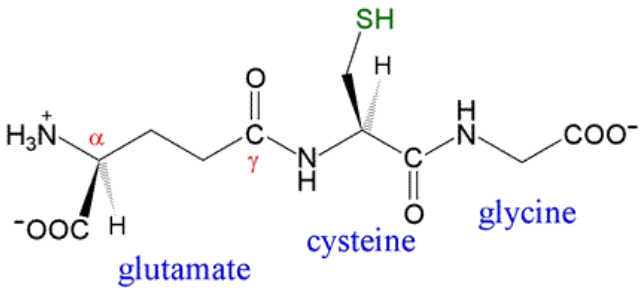

Figure 1.7. Chemical structure of glutathione, reduced glutathione is a tripeptide consisting of glutamate, cysteine and glycine (http://lylishop.net)

1.15. Glutathione S-transferase (GST)

Glutathione S-transferases (GSTs) are important metablic enzyme' the isoenzymes of which are expressed bacteria' human and other organisms. It catalyzes the conjugation of glutathione (GSH) to variety of electrophilic compounds indicating the vital role of GSTs

as housekeepers involved in the detoxification of endogenous as well as exogenous substances and other chemicals (Singh et al. 1987). The first and most important task of GSTs is innolved in the detoxification of mqny compounds such as endogenous and exogenous alkylating agents that include α, β-unsaturated aldehydes and ketones, alkyl and aryl halides (Huang et al. 2008).

GST isoenzymes are important not only for the detoxification of xenobiotics but also it has a key role for the protection of tissues from negative effects of oxidative damage. These enzymes detoxify a variety types of xenobiotics, including chemotherapeutic drugs, environmental carcinogens, and endogenous molecules. Enzymatic detoxification of xenobiotics occurs in 3 different phases. Phase I and II involve lipophilic exchange. In Phase III, nonpolar xenobiotics that are more soluble in water and therefore less toxic metabolites are more easily elicited by the cells. Generally, the reactions of Phase I is catalyzed by the cytochrome P450 system. Phase II enzymes catalyze water soluble substrate conjugation of activated xenobiotics such as reduced glutathione, UDP-glucuronic acid or glycine. They also have important biosynthetic roles, such as transport and oxidation of organic anions (Sherratt and Hayes 2001; Autrup 2000; Sheehan et al 2001).

GST enzyme is predominantly found in mammals, insects, fish, plants, *Crustacea, Planaria*, microorganisms and in some tissues of these organisms especially the organs such as liver, kidney, small intestine, intestine, lung and breast. The amount of GST enzyme is nearly 5% of the total protein content of cytosols of these organs. The reason for the high presence in these organs are that they are directly in contact with the xenobiotics obtained from outside inside the body (Gulcin et al. 2016).

GSTs include are subcategorised in three different types: (1) cytosolic, (2) mitochondrial, and (3) microsomal. In addition to that classification, based upon substrate specificity, amino acid sequence similarities, and immunological cross-reactivity, seven classes of cytosolic GSTs have been classified in mammalian species. These classes include α (alpha), μ (mu), π (pi), σ (sigma), θ (theta), ω (omega), and ζ (zeta) (Strange et al. 2001).

Glutathione S-transferases (GSTs) consist of nearly 223 amino acids. The homodimer or heterodimer subunits of all cytosolic GSTs have molecular weights of around 23-27 kDa. According to X-ray crystallography analyzes, α, μ, π, β, θ GST monomers are structurally similar to each other. They consist of short amino acid residues that connect two domains. Domain I (GSH binding region = G region) is found in the N-terminal region of the protein and is in the α / β structure. Domain II (region H) is the region to which many hydrophobic structures are connected (Bucciarelli et al. 1999).

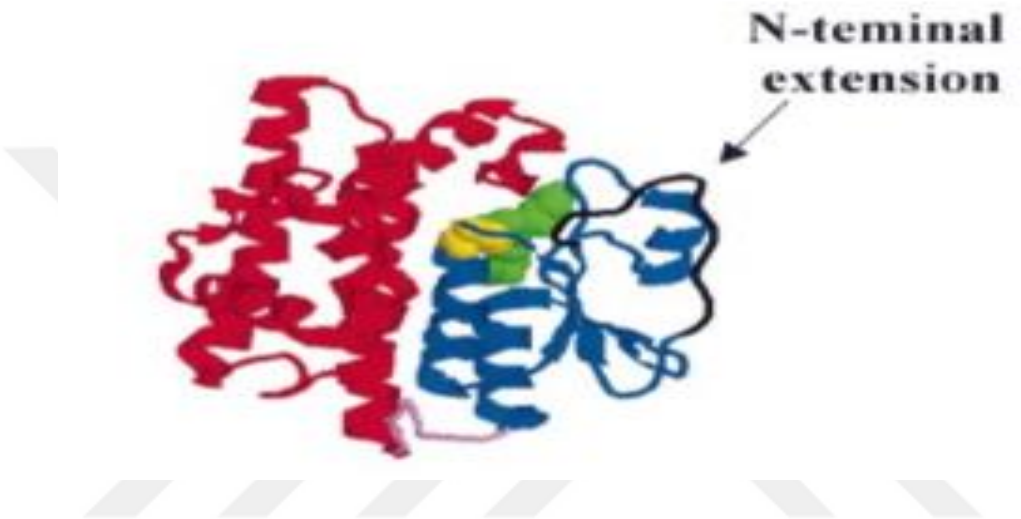

Figure 1.8. Three-dimensional structure of human omega class GST. The N-terminal domain 1 is coloured blue, while the C-terminal domain 2 is red. Catalytically essential cysteine residue is shown as space filling model (sheehan et al. 2001)

As mentioned in previous part, GSTs are multifunctional enzymes for the cellular defence anti xenobiotics and provide protection for organism. They are essential and found in all places of life. GSTs catalyse the conjugation of GSH by a sulfhydryl group (-SH) to electrophilic centres on a wide range of substrates to make the compounds more water soluble in order to detoxified (Gulcin et al. 2016).

The reducing power of GST protects membrane components from lipid peroxidation. In addition, 4-hydroxy alkenals, products of lipid peroxidation in aldehyde structure, are conjugated with GSH. GSTs in the microsomal fraction also provide protection against lipid peroxides with peroxidase activity. GSTs, also considered as one of the natural protective systems, have an important role in the detoxification of electrophilic

xenobiotics such as herbicides, pesticides, anticancer drugs, chemical carcinogens and environmental pollutants (Gyamfi et al. 2004).

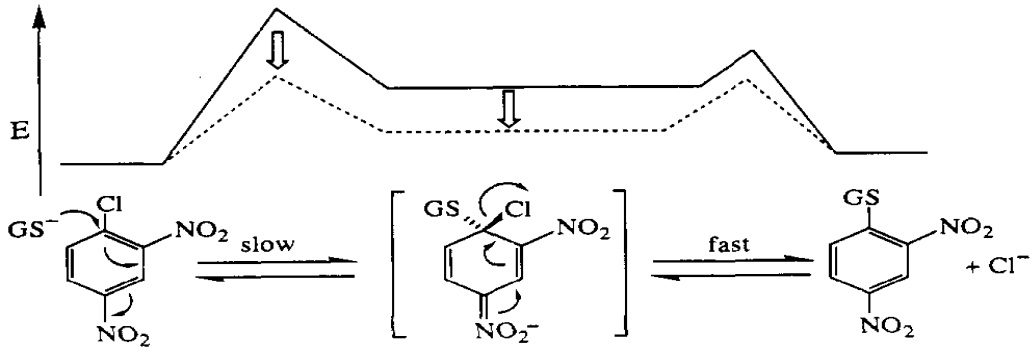

Figure 1.9. GST catalyze the congugation of GSH with CDNB (Armstrong 1991)

GSTs are known for their cell-protective functions and their interest in the improve of anticancer drugs. Recent studies have revealed their non-detoxifying properties. This situation increases the biological significance of GSTs. The elucidation of the interaction of GST enzymes with regulatory kinases, will lead to the design of new GSTs-based cancer treatment modalities and understanding of its role in tumor pathophysiology (Singh 2014).

The aim of the current study is the purification and characterization of Glutathione S-transferase from rat erythrocyte GST which protect cells from the effect of toxic reactive oxygen substances which are dangerous free radicals that contain oxygen. An investigation of the effect of some drugs suchs as buscopan (Hyoscin- N-Butylbromide+paracetamol), ampicid (ampicillin+sulbactam), gentamycin, clindamycin and cefazolin on the activity of the rat erythrocyte GST was also aimed in this master thesis.

2. LITERATURE REVIEW

Glutathione S-Transferasees (GSTs) are very important dimeric proteins that have ability of conjugating reduced glutathione (GSH) with a variety of chemicals containing electrofilic centers**.** The main biological roles of GSTs include the catalysisb of detoxification and protection reactions against oxidative stress. By conjugating glutathione with toxic electrophilic substrates, the resulting molecules commonly become less reactive and more soluble (Moden and Mannervic 2014). Until recently, the activity of different enzymes has been investigated in many living species and plant tissues, and purification, characterization and kinetic studies have been carried out for some enzymes and the obtained results have been reported in the literature. The glutathione S-transferase enzyme is also one of the most important enzymes studied. In the literature investigations, the GST enzyme was purified and characterized in many organisms.

In one study, purification of GST was performed from ovary and testis of the freshwater fish *Clariaslazera* by affinity chromatography. The results revelaed that testis and ovary GST are homodimer of two subunits each with a molecular weight of 27.5 kDa and a heterodimer of two different subunits having molecular weights of 27.5 kDa and 25.1 kDa respectively. The K_M of GSH value (2.5 mM) for testis GST compared to 0.5 mM for ovary GST. On the other hand enzyme activity studies revealed that the activity of ovary GST against para- nitrophenyl acetate, phenthylisothiocyanate, benzyl isothiocyanate and styrene-7, 8-oxide was around 9.4, 7. 5 and 4.5 μmol/min/mg protein respectively. The activity of testis GST was highest against styrene-7, 8-oxide (215 μmol/min/mg protein) (Guneidy et al. 2015).

In another study GST enzyme was obtained from human blood erythrocytes with a specific activity of 5.381 EU· mg−1 and 51.95% yield using a GSH– agarose affinity chromatography in a single step. The effect of some chalcone derivatives were also tested on the enzymatic activity of human erythrocyte GST. The results demonstrated that

K_i values of chalcones were between 7.76– 41.93μM. The results also demonstrated that 4-fluorochalcones were the most powerful inhibitors among all chalcone derivatives tested (Ozaslan et al. 2018).

Temel et al. purified GST enzyme from quail liver tissue with a purification fold of 47.88 and 12.33% recovery by glutathione agarose affinity chromatography. The purity of enzyme was assessed by SDS-PAGE method described by Laemli and showed a single band on the gel. In addition studying molecular weight and specific activity of the enzyme, inhibition effects of (3aR,4S,7R,7aS)-2-(4-((E)-3-(aryl)acryloyl)phenyl)3a,4,7,7a-tetrahydro-1H 4,7methanoiso indole-1,3(2H)-dionderivatives(1a–g) were studied within their research. In their study, the inhibition parameters (IC50 and Ki values) were calculated for these compounds. IC_{50} values of these derivatives (1a–e) were determined as 23.00, 15.75, 115.50, 10.00, and 28.75 μM, respectively. Ki values of these derivatives (1a–e) were estimated in the range of 3.04±0.50 to 131.50±32.50 μM (Temel et al. 2018).

Kufrevioglu and Aksoy purified GST from human erythrocyte with 1654 purification - fold and 19.27% recovery using glutathione agarose affinity chromatography in a single chromatographic step . The purity of the enzyme was assessed by SDS-PAGE, reveasaling a single band, suggesting presence of a homodimer structure. In their study, they investigated the inhibitory effect of some flavonoids such as baicalin, baicalein, phloridzin, and phloretin. It was shown that these compounds inhibit activity of human erythrocyte GST with IC_{50} values of 28.75, 57.50, 769.10, and 99.02lM, respectively. The K_I constants for baicalin, baicalein, phloridzin, and phloretin flavonoids were 14.50±0.71, 24.33±2.08, 762.50±85.97, and 86.49±1.11lM, respectively, suggesting that baicalin is the most powerful inhibitor for human erythrocyte GST (Aksoy and Kufrevioglu 2017).

Comakli et al. Purified Glutathione S-transferase enzyme (EC 2.5.1.18) from rainbow trout erythrocytes, carried out characterisation studies and studied the effects of some metal ions on enzyme activity. For this purpose, the enzyme was purified with a specific activity of 16.54 EU/mg protein and 11,026- purification fold by glutathione-agarose affinity chromatography with 59% yield. The purity of the enzyme purification was checked by SDS-PAGE. Optimal pH, optimum temperature, and K_M and Vmax values

for GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) were 7.3 (0.01M K-phosphate), 30°C, 0.2590 and 0.395mM respectively (Comakli et al. 2013).

Ozaslan et al. purified GST from Van Lake fish (Chalcalburnus tarichii Pallas) gills with a specific activity of 110.664 EU mg-1 and a yield of 79.6% by GSH-agarose affinity chromatography. The inhibition effects of some metal ions were tested at various concentrations on *in vitro* GST enzyme activity. IC50 values were calculated for Cd^{+2}, Cu^{+2}, Zn^{+2}, Ag^{+} as 450.32, 320.25, 1510.13, and 16.43 μM, respectively. On the other hand, K_I values were determined as 197.05 ± 105.23, 333.10 ± 152.76, 1670.21 ± 665.43, and 0.433 ± 0.251 μM, respectively. The inhibition mechanisms of Cd^{2+} and Cu^{2+} were noncompetitive, whereas Zn^{2+} and Ag^{+} inhibited the enzymatic activity competitively. However, the results revealed that Co^{2+}, Cr^{2+}, Pb^{2+}, and Fe^{3+} had no inhibitory activity on GST (Ozaslan et al. 2017).

Ahmed et al. partially purified glutathione S-transferase from the somatic tissue of ruminal amphistome parasite, Gastrothylax crumenifer (Gc) infecting Indian water buffalo. The purified GST subunit appeared as a single band with a molecular weight of 26 kDa. The GST proteins were found to be very stable up to 37°C, Furthermore, the GST purified in this study showed an optimum pH of 7.5 (Ahmed et al. 2017).

Singh et al. reported that alpha, mu and pi classes of GST are present in kidney revealing GST1, GST2 and GST3 gene loci are expressed in this tissue. All the cationic GST isoenzymes of human kidney except for GST 9.1 are demonstrated to be in the form of heterodimers of 26 kDa and 24.5 kDa subunits. GST was found to be a dimer of 24,500-Mr subunits. GST 6.6 and GST 5.5 of kidney were identified to be in the form of dimers of 26,500-Mr subunits and are immunologically similar to GST psi of liver. Unlike other human tissues, kidney has at least two isoenzymes with pI of 4.7 and 4.9 (Singh et al. 1987).

Demirdag et al. purified glutathione S-transferase enzyme from nontumour and tumour human gastric tissue and investigated the effects of heavy metals on the enzyme. In their study, GST was purified with 3089 purification fold, with a specific activity of 20 U/mg and 78% purification yield from gastric tumour tissue; and 1185 fold with a specific activity of 5.69 U/mg and a yield of 50% from nontumour tissue by glutathione−agarose

affinity column. The purity and molecular weight of the enzyme was verified by SDS-PAGE revealing a subunit molecular mass of around 26 kDa. The molecular weight of the enzyme was calculated as 52 kDa by using Sephadex G-75 gel filtration column. Then, inhibitory effects of metal ions on the enzymes were investigated. Mg^{2+} and Cd^{2+} had inhibitory effect on the enzymes activities with Ki of 48 and 2 mM for tumour tissue and 49 and 7 mM for nontumour tissue (Demirdag et al. 2013).

Aksoy et al. purified GST from muscle tissue of *Chalcalburnus tarichii* Pallas with 301.5- purification fold and 19.07% yield by glutathione agarose affinity chromatography. The purity of enzyme was verified by SDS-PAGE, showing two bands, due to presence of heterodimer structure in solution. K_M values were calculated as 1.59 and 0.53 mM for CDNB and GSH respectively. V_{max} values for CDNB and GSH were calculated as 5.58 and 1.88 EU/mL respectively (Aksoy et al. 2016).

GST from the liver and gill tissues of Ağrı Balık Lake Trout (also known as Black Sea Trout) *Salmo trutta labrax* was purified, characterized and the toxic effects of some heavy metals on the enzymeatic activity were studied by Comakli et al**.** In their study, liver GST was purified with a specific activity of 60.87 EU/mg protein, 930 purification fold and 56% yield by glutathione–agarose affinity chromatography while Using GST was purified with specific activity of 46.8 EU/mg protein, 576 purification fold and 60% yield. The purity of the protein was analyzed by SDS-PAGE. Optimal pH, ionic strength for liver tissue were found as 7.5, 0,008 M in potassium phosphate and these values for gill GST were 7.5 and 0.1 M in phospahate buffer. K_M values of liver GST for GSH and CDNB were calculated as 0.385 and 0.231 mM, repectively, and Vmax values were identified as 0.76 and 0.376 EU/mL, respectively. The K_M values of gill GST for GSH and CDNB were calculated as 0.035 and 0.41 mM, respectively, and V_{max} values were identified as 0.203 and 0.62 EU/mL (Comakli et al. 2015).

A cytosolic GST was purified 210-fold from pig lung by Dırr et al. According to their study, this cytosolic enzyme was classified as a class φ isoenzyme on considering itsphysical and chemical properties. It was found to be a homodimeric protein with a subunit M_r of 23 500 having an isoelectronic point of 7.2, and showing a high specific activity against ethacrynic acid. They also identified that glutathione analogues such as *S*-

hexylglutathione and glutathione sulfonate, were powerful inhibitors of the enzyme. The enzyme's primary structure were found to consistsof 203 amino acids and revaling high similarity (82–84% residue identity) to the rat and human class φ isoenzymes (Dirr et al. 1991).

A research carried out by Turk et al. with the aim of understanding the *in vitro* effect of hypericin on human erythrocyte GST-pi (heGST-pi). Their results demonstrated that the enzyme was purified with 71% purification yield and 2550 purification fold by chromatographic methods. The specific activity of the enzyme was calculated to be 51 U/mg protein. It was reported that, hypericin inhibited heGST-pi with noncompetitive inhibition for both substrates, GSH and CDNB with K_I (GSH) of 0.19 ± 0.01 μM and K_I (CDNB) of 0.26 ± 0.03 μM (Turk et al. 2015).

Turkan et al. human erythrocytes GST purified with 6.0 EU/mg proteins specific activity, and 2222.2 purification fold by glutathione-agarose affinity chromatograph column. The enzyme purity control and moleculer weight of subunit was analyzed by SDS-PAGE method. The subunit molecular weight of the enzyme was calculated as approcsimately 28 kDa. Also *in vitro* and *in vivo* effects of some antibiotics such as cefoperazone sodium, cefuroxime and cefazolin were also determined on GST with Ki constants of 0.1392, 1.5179 and 1.006 mM respectively (Turkan et al. 2018).

3. MATERIALS AND METHODS

3.1. Materıals

3.1.1. Chemicals

Ammonium sulfate, glycerol, isopropanol, separation gel, stacking gel, separation buffer, fixing solution, paint solution, Commasie Brilliant Blue R-250 (Fishcer Scientific), ethanol, H_3PO_4, Tris-HCl, magnesium chloride, sodium chloride, sodium bicarbonate, sodium hydroxide, potassium phosphate, potassium chloride, EDTA (ethylene diamine tetra acetic acid), Tris (Trihydroxy methyl amino methane), β-mercaptoethanol, acrylamide, TEMED (N, N, N, N-tetramethylethylenediamine), silver nitrate, hydrochloric acid, acetic acid, GSH, Glutathion agarose gel (Sigma Company). Potassium acetate, bromothymol blue, sodium acetate, potassium hydroxide, glycine amino acid and trichloroacetic acid (Merck Company). Ammonium persulfate (Chem Solute Bio).SDS (sodium dodecyl sulfate), Commasie Brilliant Blue G-250, Commasie Brilliant Blue R-250 (Fishcer scientific). Drugs are; Ampisid (Mustafa Nevzet Ilac Sanayii Istanbul, Turkye), Buscopan (boehringer ingelheim bic. Tic. A. S. Istanbul Turkiye), Gentamycin (i E Ulaqay, Istanbul, Turkiye), Clindamycin (i E Ulaqay, Istanbul, Turkiye), Safazoline (i E Ulaqay, Istanbul, Turkiye).

Table 3.1. The used instruments and their models in this study

Instruments	Models
pH meter	Thermo Orion 3 stars
Vortex	Vortex Lab Companion SK 300
Clinical centrifuge	(Universal 320 R, Hettich) and (Allegra X-30R Beckman Coulter)
Spectrophotometer	(UV-1800SHIMADZU CE)
Electrophoresis tank	Bio Rad
Electrophoresis power supply	Bio Rad
Peristaltic pump	(Bio instruments ATTA SJ-1220)
Shaker	(P Spectra J.P. Selecta, s.a.)
Precision scale	Denver instrument SI-234
GSH-Agarose-Column chromatography	Sigma Aldrich
Automatic pipette	Transferpette, Eppendorf research
Ice maker	Hoshzaki ice maker FM-80EE
Magnetic hotplate stirrers	VMS-C10 Advanced

3.1.2. Enzyme Activity Measurement Solution

1. Enzyme activity buffer solution: 0.1M KH_2PO_4 (pH: 6.5) : 0.68 g of KH_2PO_4 and 0.014g of EDTA was dissolved in 30 ml of distilled water, and the pH of the buffers was adjusted to 6.5 then solution volume was arranged to 50mL by distilled water.

2. 20mM GSH solution: 0.03g of GSH was dissolved in 3ml of distilled water and then final volume was completed to 5ml by distilled water.

3. 25mM CDNB solution: 0.025g of 1-chloro-2, 4-dinitrobenzene was dissolved in ethanol 95% then complete the volume to 5mL by ethanol.

3.1.3. Affinity Chromatography Solutions

1. Regeneration buffer solution 1; consist of 0.1M H_3BO_3 and 0.5M NaCl, pH: 8.5 (borate buffer): The buffer was prepared by dissolving 1.545g of H_3BO_3 and 7.31g of NaCl in 200ml of distilled water, and pH of the buffer was adjusted to 8.5, then the volume was completed to 250ml by D.W.

2. Regeneration buffer solution 2; consist of 0.1M of $NaCH_3COO$ and 0.5M NaCl, pH:4.5 (acetate buffer), was prepared by dissolving 2.05g of $NaCH_3COO$ and 7.31g of NaCl in 200ml of D.W. and adjusted to pH 4.5, then the volume was completed to 250ml by D.W.

3. Azide solution; consist of 0.02% NaN_3 solution: 20mg of NaN_3 was dissolved in 80ml of D.W. Then complete the solution volume was completed to 100ml by D.W. (Solution is used to protect the chromatographic column materials from bacterial contamination).

4. Equilibration solution; 10mM KH_2PO_4 and 150Mm NaCl, (pH: 7.4), solution is used for the packing of the column, equilibration and washing) 0.68g of KH_2PO_4 and 4.38g of NaCl is dissolved in 400ml of D.W. and pH was adjusted to 7.4 then the solution volume was completed to 500ml by D.W.

5. Washing solution; 10mM KH_2PO_4 and 0.1M KCl, pH: 8 (the solution is used to washing column after applied sample): was prepared by dissolving 0.136g of KH_2PO_4 and 0.745g KCl in 80ml D.W. and pH was adjusted to 8.0 then the solution volume was completed to 100ml by D.W.

6. Elution solution; consist of 50mM Tris-HCl buffer and 10mM GSH (pH: 9.5), (buffer used for elution of GST enzyme binding affinity gel): 0.181g of Tris-HCl and 0.092g GSH in some of D.W. then adjust pH to 9.5 and completed the solution volume to 30 ml by D.W.

3.1.4. Solutions to Prepare SDS-PAGE

1. 1M Tris-HCl (pH: 8.8): 12.11g of Tris-HCl was dissolved in 80ml of D.W. and pH was adjusted to 8.8 then completed the solution volume to 100mL by D.W.

2. 1M Tris-HCl (pH: 6.8): 12.11g of Tris-HCl was dissolved in 80ml of D.W. and pH was adjusted to 6.8 then completed the solution volume to 100ml by D.W.

30% acrylamide-0.8% bisacrylamide solution: It was prepared by dissolving 15g of acrylamide and 0.4g of bisacrylamide in 34.6g of D.W. then complete the solution volume to 50mL by D.W.

3. 10% SDS solution: It was prepared by dissolving 1g of sodium dodecyl sulphate in 10Ml D.W.

4. 10% ammonium persulfate solution: was prepared by dissolving 1g of ammonium persulfate in a total volume 10 mL D.W.

5. Running buffer: dissolved 1.51g of Tris-HCl and 7.51g glycine in 450ml of D.W. then added 5ml of 10% SDS and then adjust solution pH to 8.3, finally complete the solution volume to 500ml by D.W.

6. Sample buffer: 0.65mL of 1M Tris-HCl (PH: 6.8) was mixed, 1mL of 10% SDS, 1ml of 100% glycerin, and 1mL of 0.1% brmthymol blue then complete the solution volume to 10mL by D.W. Notice that before using this buffer, for every 950µl of sample solution, 50 µl of β-mercaptoethanol were added (this buffer was used for protein denaturation).

7. Gel fixation solution: was prepared by mixing 50% isopropanol, 10% TCA, and 40% D.W. (this solution was used to stabilize the protein carried in the gel).

8. Gel staining solution: 0.1g of Coomassie Brilliant Blue R-250mL in 50mL methanol then mixed with 10 mL acetic acid and 40mL D.W.

9. Gel washing solution: was prepared by mixing 50mL of methanol, 40mL of D.W. and 10mLof acetic acid.

10. 0.1% bromthymol blue solution: 0.1g of indicator was dissolved in 16mL of 0.01M NaOH, then complete to the 100mL by D.W.

3.1.5. Solution Used for Protein Identification by the Bradford method

1. Coomassie Brilliant Blue solution: 100mg of Coomassie Brilliant Blue G-250 in 50mL of 95% ethanol and added 100mL of phosphoric acid 95% then the solution volume was completed to 1000mL by D.W. and keep the solution in the dark station.

2. Standard protein solution: was prepared by dissolving 1mg of bovine serum albomin (BSA) in 1mL D.W.

3.2. Methods

3.2.1. Preparation of Hemolysate

3 mL of Rat's blood samples from The Bingöl University Experimental Research Center (BÜDAM). Following cold chain rule, the blood were collected in anticoagulant tubes and brought to the laboratory and it was keeping in refrigerator at -20 °C. then they centrifuged at (15 min, 4^{o}C, 2,500 x g), then discarded plasma blood, and packed red blood cells (erythrocyte) were washed with 0.16M KCl solution then centrifuged again at (15 min, 4^{o}C, 2,500 x g) and the previous step was repeated for 3 times. The packed red blood cells were hemolyzed by adding iced water in the ratio of 1:5 volumes, and then centrifuged at (4^{o}C, 10,000 x g, for 60 min), discarded precipitate and kept supernatant (Ninfali et al. 1990).

3.2.2. Purification of Hemolytic Enzyme by Glutathione Agarose Affinity Chromatography

Following hemolysis step, the hemolysates was directly applied to the glutathione-agarose affinity column. The flow rate of the column through the peristaltic pump was adjusted to 20mL/h. The column was then washed with 10mM KH_2PO_4 and 0.1M KCl,

pH: 8 in the column. This washing was followed by spectrophotometer and the absorbance values was determined by aliquoting the fractions in small volumes in eppendorf tubes. After equilibrating the column, the enzyme was purified by gradient elution. The elution solution was from the gradient of the solution containing 50mM Tris-HCl and 1.25-10mM GSH, pH: 9.5 with the help of the fraction collector, the eluates was collect in 1.5mL bottom flat eppendorf tubes and each tubes were examined for checking enzyme activity at 340 nm wavelength. (Guvercin et al. 2008 and Toribio et al. 1996).

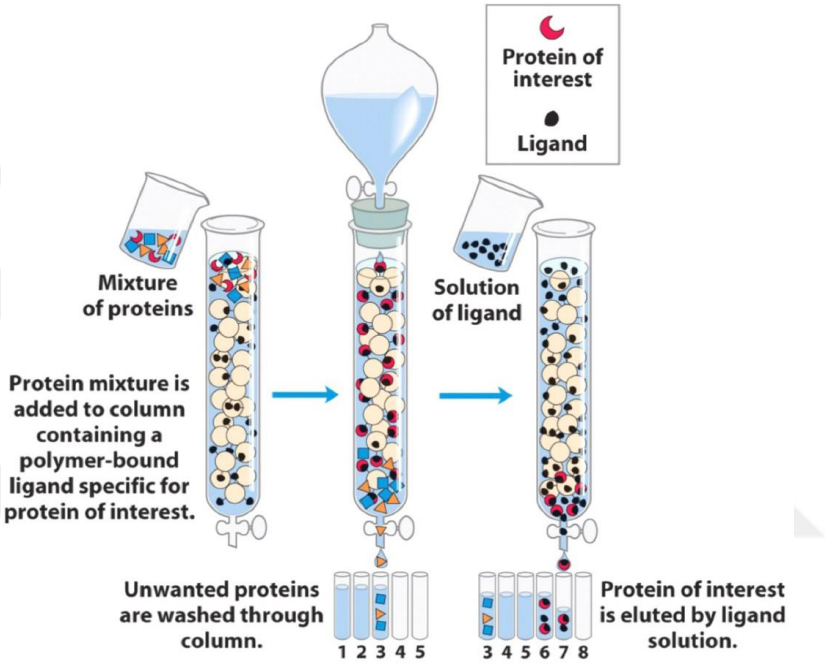

Figure 3.1. Protein purification steps by affinity chromatography (Lehninger 2008)

3.2.3. Protein Assay

3.2.3.1. Determination of Protein

The qualitative protein assay is based on the principle of maximum absorbance of tryptophan and tyrosine in the structure of proteins at 280 nm. With this method, in the chromatography process, the fraction collector will be used to determine the qualitative protein in all fractions taken in equal volume. Fractions are taken in quartz baths, and the absorbance was recorded at 280 nm (Segel 1968**).**

3.2.3.2. Protein Determination by Bradford method

Quantitative protein was determined with following the Bradford method (Bradfored 1976). In this method, Coomassie Brilliant Blue G-250 was as used as the dye, which has a negative charge and binds to the positive charge on the protein. The dye has absorbance in red (λ_{max} = 465 nm) and blue (λ_{max} = 595 nm). Protein binding allows conversion of the red form to the blue form. This method is less sensitive to disturbing factors (between 1 and 100µg). The reaction occurs at high speed and takes place rapidly, completed in two minutes. The assays were performed using the following procedure: 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µl of tubes of standard bovine albumin solution containing 1 mg of protein per ml. With pure water, the volume of all tubes should be 0.1ml and then 4.9ml Coomassie brilliant blue G-250 solution was added, to get a total volume in each tube 5ml and mix with the vortex. After incubation for 10 minutes, the absorbance values against the protein in the 3ml cuvettes at 595nm was measured (Bradford 1976).

Table 3.2. Preparation of the calibration curve for quantitative determinations of proteins

Reagent	**volume (µl)**									
	Tube1	Tube2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
BSA	10	20	30	40	50	60	70	80	90	100
D.W	90	80	70	60	50	40	30	20	10	0.0
Paint	4900	4900	4900	4900	4900	4900	4900	4900	4900	4900

In order to determine the amount of protein in the medium at each stage of purification 3, 50, 100 and 200µl of the diluted solution obtained by diluting the enzyme samples 20 times or more, respectively, were added with 5ml of coloring reagent. After mixing with the vortex, it was left for incubation for 10 minutes. Then absorbance values were read at 595nm.

Table 3.3. Preparation of samples to measure amount of protein

Samples	hemolysate	Ammonium sulfate	pure enzyme
Sample volume	100µl	100µl	100µl
Dye volume	4900µl	4900µl	4900µl

This procedure was repeated 3 times and the mathematics mean of 3 different measurements were taken and protein quantities were determined from the standard graphic. The results obtained the amount of protein in each sample in µg by drawning the graph between the absorbance on y-axis and concentration on x-axis of standard protein solutions. The equation of (y = ax + b) was used for calculation the amount of protein in samples. Y indicate the absorbance, and find x which is the amount of protein. (Bradford 1976).

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

To control the purity of the purified GST enzyme, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiments were carried out at 3-8% cut according to the Laemmli procedure (Leammli 1970). Bio-Rad tools were washed by D.W. and slides were prepaqred in the rack and tested by pure water tob test whether are there any leakage or not between slides and prepared 15% separation gel in a container, and immediately poured into the slides hole nearly full, also another time full the hole by n-butanol to remove bubles and wait about 20 min to allow the gel to polymerize, discard n-butanol and dry the gel. Following that; 3% staking gel immediately prepared and poured into slides hole, then wait 10 min to be plimerized well. After that the slides were taken out and fixed by separation Bio-Rad tool then put it into Bio- Rad tank with full slides pool with running buffer, then the comb was taken from the gel, and poured sample with standard protein into a different wells and add supporting buffer to the Bio- Rad tank according to the gel numbers, then connected to the power supply into two steps 1st 20mA and 80V for 20 min, 2nd 40mA for 120min.

Table 3.4. Preparation of 15% separation gel

Reagents	volume (mL)
Distilled water	2.3
30% acrylamide-80% bisacrylamide	5
1.5M Tris-HCl PH: 8.8	2.5
10% SDS	0.1
PER Ammonium persulfate	0.1
TEMED added at last	0.01

Table 3.5. Preparation of 3% staking gel

Reagents	volume (mL)
Distilled water	3.4
30% acrylamide-80% bisacrylamide	0.83
1.5M Tris-HCl PH: 8.8	0.68
10% SDS	0.05
PER ammonium persulfate	0.05l
TEMED added at last	0.008

In SDS- PAGE proteins are moving through the gel based on the size and the charge under the effect of field in the manner of the larger molecule are moved slower than smaller from the negative toward the positive charge. After separation process were completed, the gel was taken out and standed in fixation solution for 30min with continue and slowly shaking by shaker.

Depending on the amount of protein in the obtain gel, one of the two dyeing method was used. Silver staining or Coomassie Brilliant Blue R-250. In addition, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular mass of the enzyme by following procedure: The molecular mass was calculated using known standard proteins on the gel. As a result, the log Mw-R_f standard graph was plotted by calculating the R_f values of the standard proteins. The R_f value for the GST enzyme from the erythrocytes were then calculated and replaced with the standard graphic to determine the log Mw of the GST enzyme. The molecular mass of the sample was determined by taking the antilogarithm of this value (Laemmli 1970).

$$Rf = \frac{Xe}{Xpaint} \tag{3.1}$$

Rf: values of proteins

Xe: walking distance of protein

X_{paint}: walking distance of paint

3.2.3.4. GST Activity Measurement

The glutathione S-transferase (GST) enzyme catalyzes the conjugation of an aromatic electrophile with a glutathione molecule. The most widely use aromatic electrophile is 1-chloro-2, 4-dinitrobenzene. The reaction yields dinitrobenzene S-glutathione (DNB-SG). The maximum absorbance was measured spctrophotometerically at 340nm. Then measure activity by exploiting the increase in absorbance at this wavelength. In the absence of GSH, CDNB will inactivate GSH. It is necessary to initiate the reaction with CDNB, the volume of solution was 1ml, and the absorbance was taken specrometically in 3 min. (Habig et al. 1974).

Table 3.6. Amounts used for enzyme activity measurement

Reagents	Blank (µl)	Sample (µl)
Distilled water	730	720
0.1M KH_2PO_4	200	200
20mM GSH	50	50
25mM CDNB	20	20
Enzyme sample	0	10

The process was directed in 25^{o}C and pH 6.5 and the following formula was used to determination enzyme unit:

$$EU = \frac{\Delta abs/3}{9.6} \text{ x } \frac{VT}{VE} \text{ X DF} \qquad (3.2)$$

EU: Enzyme unit per 1 ml

Abs: change in absorbance

3: total time (min)

9.6: extiniction coefficient of 1mM DNB-SG at 340 nm

VT: total volume of the cuvette

VE: enzyme volume

DF: dilution factor

3.3. Characterization Studies for GST Enzyme

3.3.1. Studies on the Optimum pH for GST Enzyme

To determine the optimal pH of the GST enzyme, buffer of potassium phosphate with pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 was prepared. With the appropriate substrate solution, the enzyme activity was determined at 340nm.

3.3.2. Studies to Tetermine Optimum Ionic Strength for GST Enzyme

After the optimum pH is determined, the ionic strength of the GST enzyme was investigated. The buffer and substrate solutions were incubated at 0, 100, 200, 300, 400, 500, 600, 700, 800, 1000 mM Tris / HCl or K-phosphate (mM) with the PH of 8 for all solutions. Then activity of the GST enzyme was measured at 340nm by UV-Spectrophotometer. To prepare different concentration of each concentration, 1M KCl and 2M Tris-HCl use the following dilution law:

$$M_1V_1 = M_2V_2 \quad (3.3)$$

M_1: molarity of concentrate solution
V_1: volume of concentrate solution
M_2: molarity of dilute solution
V_2: volume of dilute solution

Table 3.7. To prepare different KCl concentrations from 1M KCl

Concentration (M)	Stock solution (ml)	DW (ml)
0.1	1	9
0.2	2	8
0.4	4	6
0.6	6	4
0.8	8	2
1.0	10	0

Table 3.8. To prepare different Tris-HCl concentrations from 2M Tris-HCl

Concentration (M)	**Stock solution (ml)**	**D.W (ml)**
0.1	0.5	9.5
0.2	1	9
0.4	2	8
0.6	3	7
0.8	4	6
1	5	5
1.2	6	4
1.4	3	7

3.3.3. Studies to Determine Optimum Temperature for GST Enzyme

Water bath used to determine the optimum temperature of the GST enzyme. The mixture in the cuvette containing the buffers and substrates necessary for the activity measurement were stood in the water bath at the required temperature for 3min one-to-one from 0 °C to 80 °C, temperature adjusted in bathtub. Activity after addition enzyme was directly measured at 340nm by spectrophotometer.

3.3.4. Stabilization Studies for GST Enzyme

To determine the pH at which the enzyme is most stable, potassium phosphate with pH of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 was used. 2 ml of the indicate solutions was stored at 4 °C with 1 ml enzyme solution. These solutions were used as an enzyme source. The activity was measured was plotted as % activity against time (days) at 24 hour intervals for 8 days under optimal conditions.

3.3.5. Studies for Finding KM and Vmax Values for GST Substrates

To determine the K_M and V_{max} values for GSH and CDNB substrates of the GST enzyme, activity measurements were made at 5 different concentrations of CDNB at a constant GSH concentration. Line weaver-Burk graph was plotted with the obtained values. From the equation of the obtained graph, the values of K_M and V_{max} for CDNB were determined. In the same way, activity measurements with 5 different concentrations of

GSH at constant concentration of CDNB were made to calculate Line weaver-Burk graph then K_M and V_{max} values for GSH. Activity measurements was performed under optimal conditions (Lineweaver et al. 1934).

3.3.6. Inhition Studies

To determine the effects of some drugs on Rat erythrocyte GST activity, the following concentrations of Amisid (0-38.45mM), Buscopan (0-3.18mM), Gentamicin (0-5mM), Clindamycin (0-21.18mM), Cefazoline (mM) were added in to the measurement cuvette. Activity of the enzyme was measured, and the absence of drougs was used as a control (100% activity). IC_{50} values were obtained from activity (%) drug concentration graph. In order to determine K_i constants in the media with inhibitor, (GSH) substrate concentrations (0.2, 0.5, 1.0, 2.0, 3.0 mM) for drug solutions were added to the reaction medium, and different drug concentrations were used in 1mL of total reaction volume. Lineweaver-Burk graphs (1934) were drawn by using 1/V vs 1/[S] values and K_I constant were calculated from the graphic by Microsoft excel programe.

4. RESULTS

4.1. Preparation of The Hemolysate

Before proceeding with agarose column affinity chromatography purification, 3 mL of rat blood samples from Bingol University Farm were collected in anticoagulant tubes. Foloowing that, the blood was hemolysed according to the procedure explained in material method section (3.2.1).

4.2. Elution from Glutathione-agarose Affinity Column Chromatography

Glutathione S-transferase GST was purified from Rat erythrocyte by glutathione-agarose affinity chromatography. Hemolysate was applied to the column, in which a high ratio of enzyme eluted by the elution solution and the maximum activity was recorded, then 25 eppendorf tubes were available, finally GST activity was measured in each tube at 340nm by spectrophotometer.

Table 4.1. The enzyme activity of the eluted fractions of rat erythrosyte GST enzyme

Fractions Eppendorf tube no.	Activity (EU/mL)
1	0.146
2	0.104
3	0.062
4	0.101
5	0.097
6	0.101
7	0.069
8	0.118
9	0.101
10	0.125
11	0.080
12	0.066
13	0.066
14	0.059
15	0.059
16	0.083
17	0.142
18	0.142
19	0.156
20	0.080
21	0.128
22	0.198
23	0.243
24	0.139
25	0.097

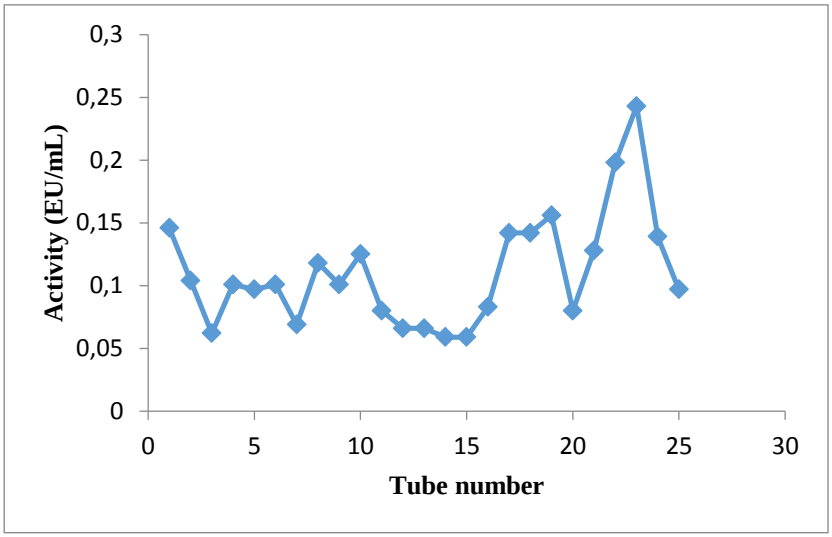

Figure 4.1. The enzyme activity of the eluted fractions of rat erythrocyte GST from the agarose affinity column chromatography

4.3. Sodium Dodecyl Solfate Polyacrylamide Gel Electrophoresis SDS-PAGE

After GST enzyme has been obtained from rat erythrocyte by GSH agarose column chromatography, it was applied to the SDS-PAGE to check the purity of the enzyme. The results demonstrated that single band was observed on the SDS-PAGE. This indicates that the enzyme was purified with high purity in a single chromatographic step (Figure 4.2).

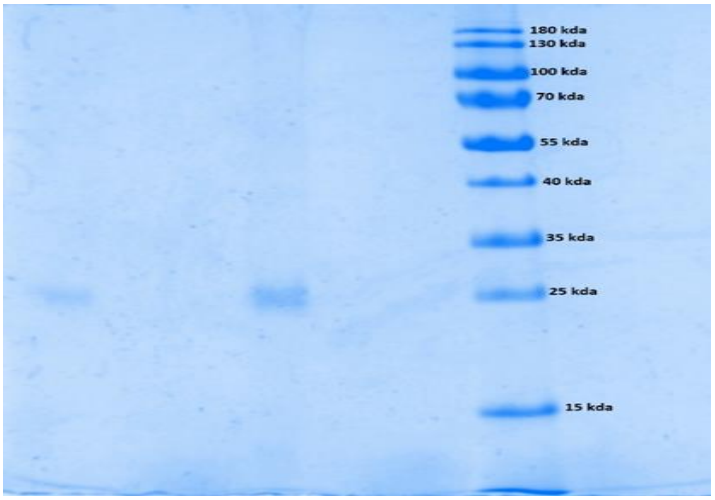

Figure 4.2. SDS-PAGE photograph for the Rat erythrocyte GST enzyme. The molecular weight of each marker protein is given near the proteins. The 25.2 kDa protein is the GST enzyme protein from Rat's erythrocytes

4.4. Enzymes Molar Mass Determination Using SDS-PAGE Method

After determination of the purity of the enzyme the same gel was used to determine molecular weight of the enzyme. The molecular mass of the enzyme was calculated as described in material method chapter in section 3.2.3.4. For this purpose, the retardation factor (the distance the enzyme was travelled on the gel) was plotted against the logarithm of the molecular weight of the each standard protein in the marker. The R_f of the protein hich was purified from rat erythrocyte GST was calculated to be 0.648 on the gel. The molecular weight of the protein was calculated from this plot by using the equation of the graph. From this equation the molecular weight of the enzyme was calculated to be 25.2 kDa.

Table 4.2. Shows the R_f and log M.W. values of the standard proteins and samples of the SDS-PAGE performed for determining the purity of the GST from rat erythrocytes enzyme and determination of its M.W

Rf	**Log (M.W.)**
0,064	5,255
0,096	5,114
0,16	5
0,224	4,845
0,32	4,74
0,392	4,602
0,528	4,544
0,64	4,398
0,904	4,176

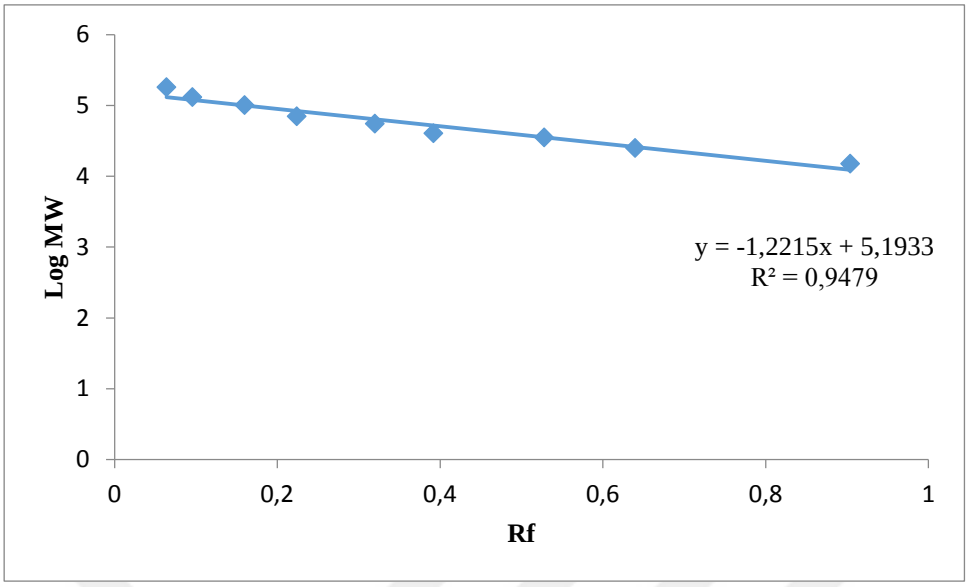

Figure 4.3. The calibration curve between R_f versus log M.W. For determination M.W. of the GST enzyme by Lammelie's method

4.5. Purification Yield, Purification Fold and Specific Activity

After detecting molecular weight and purity of the enzyme, the quality and efficiency of the purification of Rat erythrocyte GST was assessed by using the calibration curve obtained from Bradford method. In order to measure the purification efficiency; the activity, protein concentration, specific activity, purification fold and yield was calculated. The redemonstrated that the enzyme was purified with a specific activity of 6.31 EU/mg protein, in 44 % yield, and 114.7 purification fold (Table 4.3).

Totle activity = Ativity EU x V mL
Totle proteins = Proteins mg x V mL
Specific activity = Totle activity / Totle proteins
Purification folds = Specific activity of purified enzyme / Specific activity of the hemolysate % yield of protein = Total activity of purified enzyme x 100 / Total activity of hemolysate.

Table 4.3. The information about Purification result of Rat erythrocyte GST enzyme using glutathione agaros column affinity gel chromatography

Purification scheme of GST of rat erythrocytes.								
Purification Steps	Activity (EU/ml)	Protein (mg/ml)	Total Activity (EU)	Total Protein (mg)	Specific Activity (EU/mg)	Yield (%)	Purification fold	Total volume (ml)
Hemolysate	0,18	3,22	0.54	9.66	0,055	100	1	3
Glutathione-agarose affinity column	0,12	0,019	0.24	0.038	6,31	44	114,7	2

4.6. Determination of Protein (Qualitatively)

The absorbance of the sample (Rat erythrocyte GST eluates) before, during and after purification steps were measured at 280 nm qualitatively in order to follow the success of the protein concentration. Absorbance was measured until the difference of absorbance was approached from zero. That is mean GST has been attached to the glutathione-agarose affinity gel in the column, while others are eluted. Which indicated that the time of elution process start to purify target enzyme protein in the sample.

4.7. Determination of Protein (Quantitatively)

After qualitative determination of the protein after the purification step, the amount of the protein was measured in both hemolysate and purified protein according to the Bradford method (Bradford 1976) from the equation of the calibration curve. The calculations revealed that, the amount of the protein in hemolysate was 3 .22 mg/ml and concentration of the protein was 0.019 mg/ml after purification (Table 4.4).

Table 4.4. Protein concentration and absorbance for prepare standard calibiration curve

Protein (µg)	Abs at 595nm
10	1.006
20	1.058
30	1.101
40	1.135
50	1.255
60	1.302
70	1.346
80	1.38
90	1.469

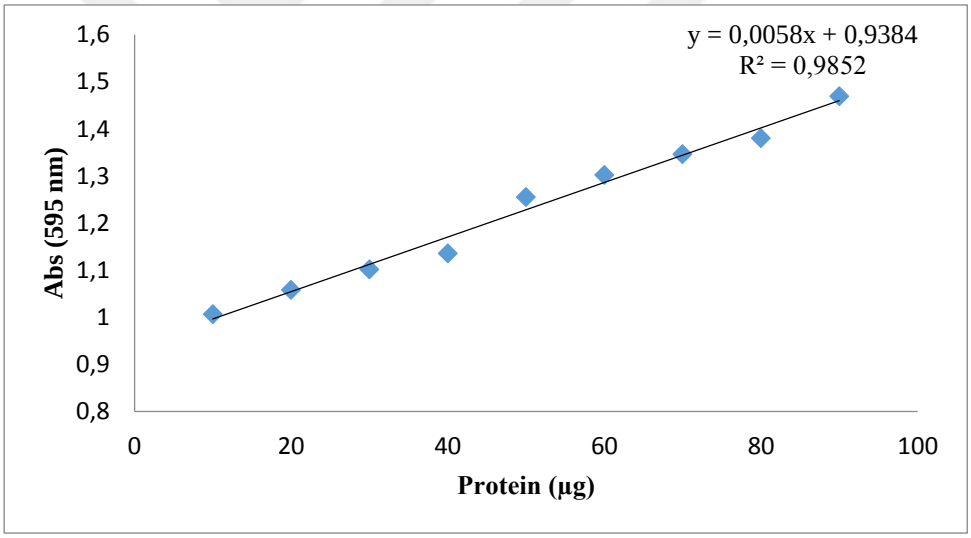

Figure 4.4. The standard calibration curve for quantitative determination of protein by Bradford's method. For determination the amount of protein in the hemolysate and the purified GST Rat's erythrocyte enzyme

4.8. Characterization Studies

After determination molecular weight of the enzyme and calculating some parameters to understand the efficiency of the protein we characterized the enzyme in more detail. For this purpose we determined optimum pH and optimum temperature of the enzyme, ionic strength of the enzyme, pH stability of the enzyme and K_M /V_{max} of the enzyme for both substrates (GSH and CDNB).

4.8.1. Optimum pH

To determine the optimum pH, the activity of GST enzyme was measured at different pH (5.5, 6.0, 6.5, 7.0, and 8.0) using KH_2PO_4 as a buffer. The maximum activity was found at pH 8 indicating that optimum pH of Rat erythrocyte GST was 8 (Table 4.5, Figure 4.5). The similar experiments were carried out in the presence of Tris-HCl at different pH (8, 8.5, 9, and 9.5) however, no results were obtained with Tris as it was conjugating with CDNB and resulting high absorbance at 340 nm.

Table 4.5. The GST enzyme activity using different pH values KH_2PO_4 buffer solution in the enzyme assay

pH values	5.5	6.0	6.5	7.0	8.0
E.A. (EU/mL)	0.01	0.03	0.8	1.0	0.5

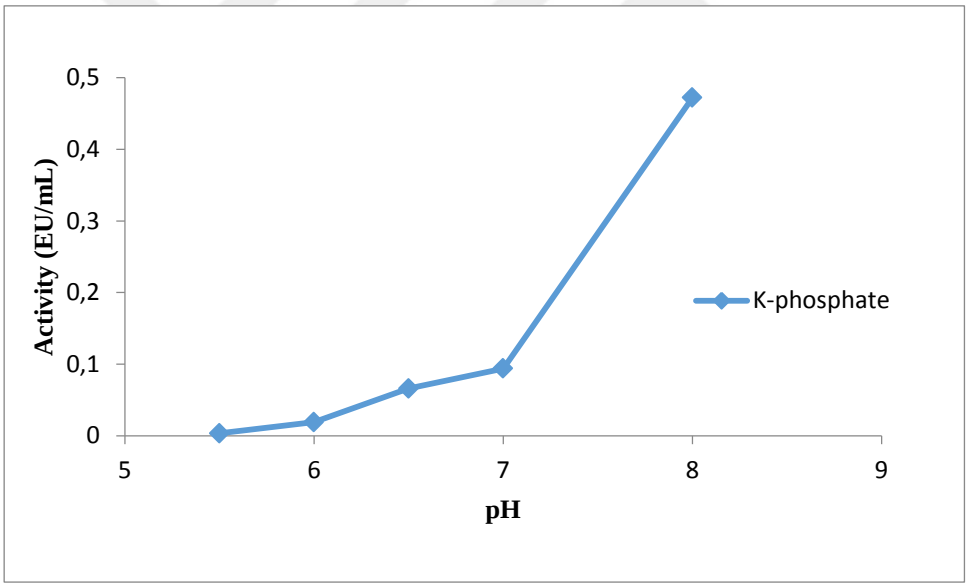

Figure 4.5. The optimum pH value test for the GST enzyme's activity from rat erythrocytes, the test resulted in the best pH value for the enzyme,s activity to be at pH 8.0 by using potassium phosphate buffer

4.8.2. Optimum Temperature

To determine the optimum temperature of Rat erythrocyte GST, the activity of the enzyme was measured at different temperature with 10^{o}C increments (0, 10, 20, 30, 40, and 90^{o}C) in KH_2PO_4 buffer. The maximum activity was found at temperature of 50^{o}C revealing that the optimum temperature of the enzyme was 50^{o}C (Table 4.6, Figure 4.6).

Table 4.6. The effect of temperature on the GST Rat erythrocyte's enzyme activity

Temp ^{o}C	0.0	10	20	30	40	50	60	70	75	80
E.A.(EU/mL)	0.28	0.3	0.38	0.46	0.52	0.67	0.53	0.25	0.21	0.09

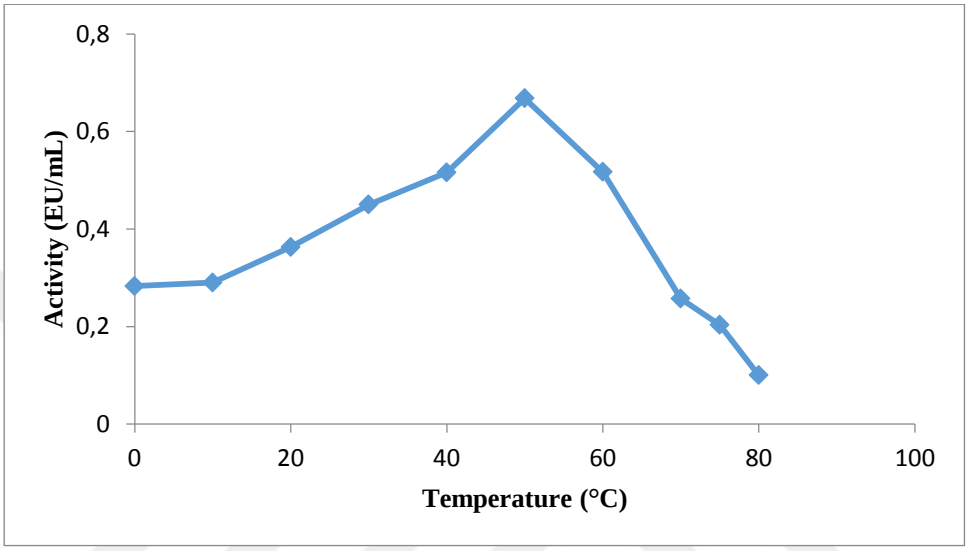

Figure 4.6. The effect of elevating temperature on the GST Rat erythrocyte's enzyme activity. The activity of the enzyme increased with the elevating the temperature from 0.0 ^{o}C 50 ^{o}C and dropped sharply at higher than the above range temperature, due to the decomposition of the structure enzyme's protein

4.8.3. Optimum Ionic Strength

Different concentrations of KH_2PO_4 was prepared, and the activity of GST enzyme was measured by spectrophotometer at 340nm. The result shows that, the maximum enzyme activity mentioned was in the solution concentration of 0.2M phosphate buffer (Table 4.7, Figure 4.7.) Because Tris was conjugating with CDNB and cause high absorbance and noise during activity measurements, the ionic strength of the enzyme in Tris could not be studied.

Table 4.7. The best ionic strength effect on the GST enzyme from Rat erythrocytes

KH_2PO_4(M)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Enzyme activity (EU/mL)	0.42	0.56	0.3	0.5	0.44	0.43	0.46	0.31	0.47	0.37

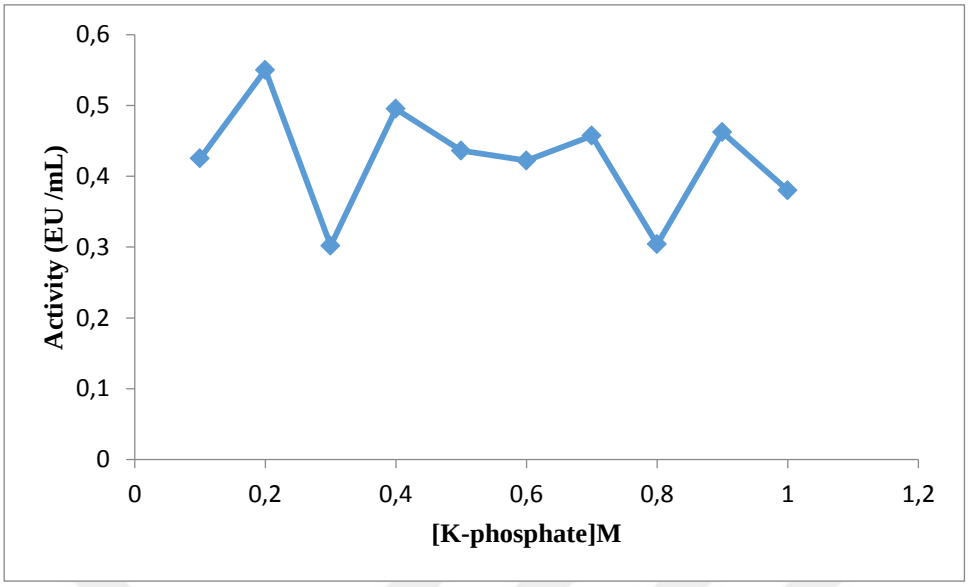

Figure 4.7. The optimum ionic strength effect on GST Rat's erythrocyte enzyme.The best ionic strength effect on the GST Rat's erythrocyte enzyme observed by maximum activity of the enzyme when 0.2M KH_2PO_4 used in the enzyme assay

4.8.4. Stable pH

Stable pH of Glutathione S-transferase was studied in KH_2PO_4 buffer for eight days continuously, the activity of enzyme at 340nm was measured by spectrophotometer in different pH (5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) solutions were prepared, the stable pH for Rat erythrocyte GST was PH: 8 as seen in (Figure 4.8).

Table 4.8. The GST enzymes activity from Rat erythrocytes stored in different pH of KH_2PO_4 buffer

Time in days	pH 5.0 Activity (EU/mL)	pH 5.5 Activity (EU/mL)	pH 6.0 Activity (EU/mL)	pH 6.5 Activity (EU/mL)	pH 7.0 Activity (EU/mL)	pH 7.5 Activity (EU/mL)	pH 8.0 Activity (EU/mL)
1	0.108	0.131	0.106	0.123	0.160	0.138	0.208
2	0.229	0.158	0.202	0.265	0.306	0.290	0.340
3	0.188	0.188	0.227	0.188	0.225	0.292	0.385
4	0.208	0.196	0.198	0.262	0.300	0.394	0.431
5	0.250	0.250	0.271	0.310	0.356	0.402	0.433
6	0.217	0.240	0.288	0.277	0.306	0.419	0.504
7	0.288	0.306	0.340	0.362	0.433	0.506	0.567
8	0.248	0.288	0.285	0.310	0.423	0.533	0.615

Because Tris was conjugating with CDNB and cause high absorbance and noise during activity measurements, the stable pH of the enzyme in Tris could not be studied

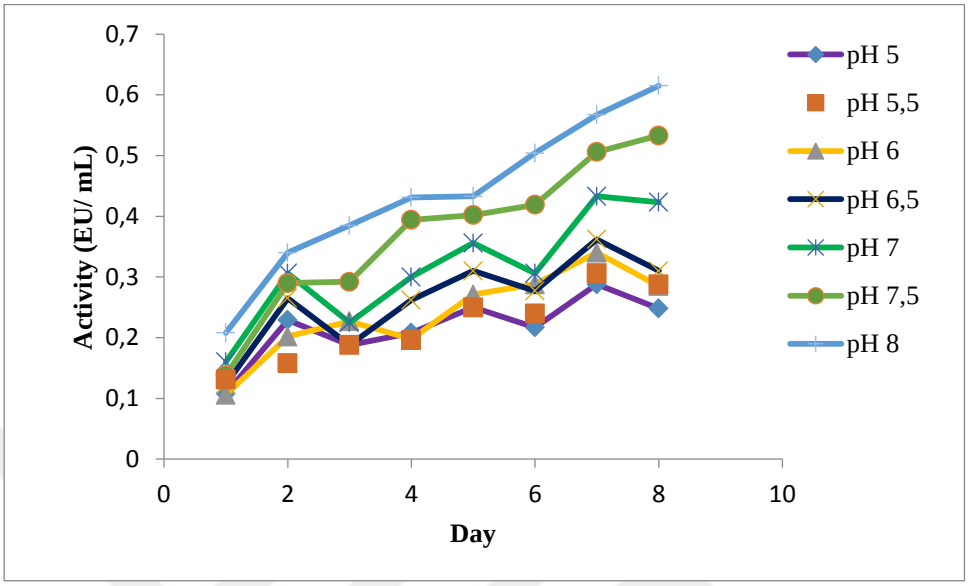

Figure 4.8. The stability of the GST Rat's erythrocytes enzyme in KH_2PO_4 buffer over eight days time. The result showed the optimum buffer for storing the enzyme over eight to be the pH 8, 0.2 M KH_2PO_4 buffer in which the enzyme kept its activity with a minimum decrease comparing to the other pH values

4.8.5. Determination of K_M and V_{max} of Rat Erythrocyte GST Enzyme

The K_M and V_{max} of rat erythrocyte GST was calculated for two substrates (GSH and CDNB) of the enzyme as described in section (3.3.5).

The activity of GST was measured for different concentration of one substrate with the fixed concentration of the other, the results were used for drawing the lineweaver-Burk graphic curve and then determined K_M and V_{max} for GST enzyme from the equation were obtained from the graph, according the following data:. The Km of the enzyme for GSH and CDNB substrates was 1.22 and 0,374mM respectively while V_{max} of the enzyme for GSH and CDNB was 1.21 and 3.614 respectively.

Table 4.9. The activity of GST Rat's erythrocytes enzyme using different concentration of CDNB substrate

CDNB(mM)	Δ Abs	EU/mL	1/[s]	1/V
0,175	0.126	0.219	5,714	14,57
0,25	0.185	0.321	4	3,12
0,325	0.228	0.396	3,077	2,53
0,4	0.270	0.469	2,5	2,16
0,45	0.294	0.510	2,222	1,96
0,575	0.367	0.637	1,739	1,57

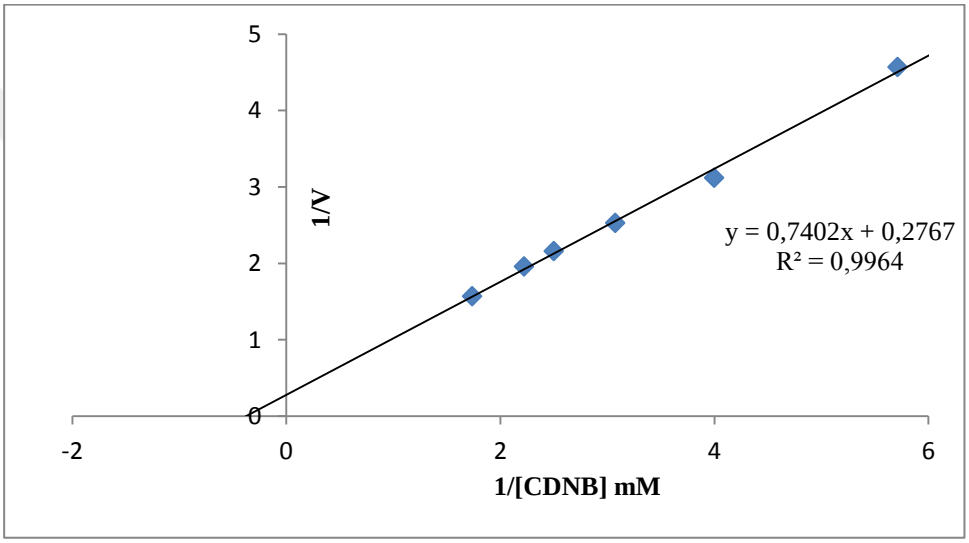

Figure 4.9. The Linweaver-Burk reciprocal plot for the CDNB substrate. Different concentration of the CDNB substrate for GST Rat's erythrocytes enzyme used, in order to find the maximum velocity of the enzyme and the Michealis-Menten constant of the substrate

Table 4.10. The activity of GST Rat's erythrocytes enzyme using different concentration of GSH substrate

[GSH] mM	Δ Abs	EU/mL	1/[S]	1/V
0.200	0.099	0.172	5	5.81
0.400	0.168	0.292	2.5	3.42
0.500	0.190	0.330	2	3.03
0.600	0.238	0.413	1.67	2.42
0.700	0.247	0.429	1.43	2.33
0.800	0.287	0.498	1.25	2
0.900	0.310	0.538	1.11	1.86

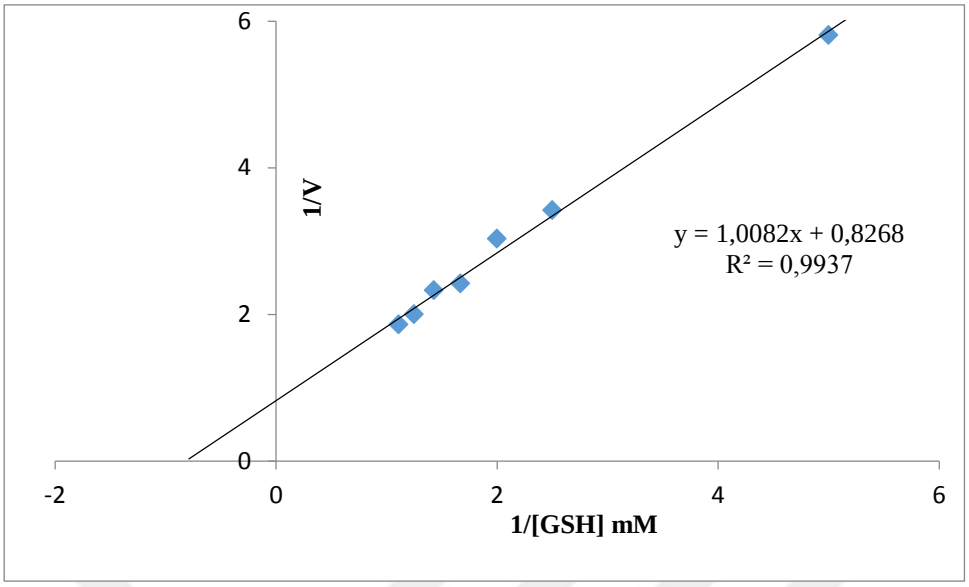

Figure 4.10. The Linweaver-Burk reciprocal plot for the GSH substrate. Different concentration of the GSH substrate for GST Rat's erythrocytes enzyme used, in order to find the maximum velocity of the enzyme and the Michealis-Menten constant for the substrate

The results provided that the value of K_M of GSH (1.22 mM) is greater than the CDNB (0.374 mM). That suggested that GST has high affinity for CDNB While low affinity for GSH.

4.8.6. Investigating the Effect of Some Drugs on the Enzyme Activity

After we purified the GST enzyme from erythrocyte of rats and performed characterization studies, inhibitory effects of some drugs on the enzymatic activity of GST enzyme was studied. To achieve this aim, the effect of some drugs which include gentamicin, clindamycin. Cephalosporin, ampicillin and Hyosın-N-Butilbromür+parasetamol were tested. The inhibition effect of the compounds on enzymes is expressed in IC_{50} values and K_i constants. The inhibition/activation observed with these drugs against GST is shown in Figures 4.11 and 4.12 In this study it was found that the activity of the GST enzyme was got lowered in the presence of clindamycin and gentamicin antibiotics whereas the enzyme activity of GST was higher in the presence of Hyosın-N-Butilbromür+parasetamol and ampicilin. After finding out the effect of these drugs on the GST enzyme IC_{50} and K_i values for the inhibitors were tested. The calculations revealed that clindamycin and gentamicin inhibited the activity of rat

erythrocyte GST with an IC_{50} of 6.9 mM and 1.69 mM respectively (Figure 4.11 and Figure 4.12).

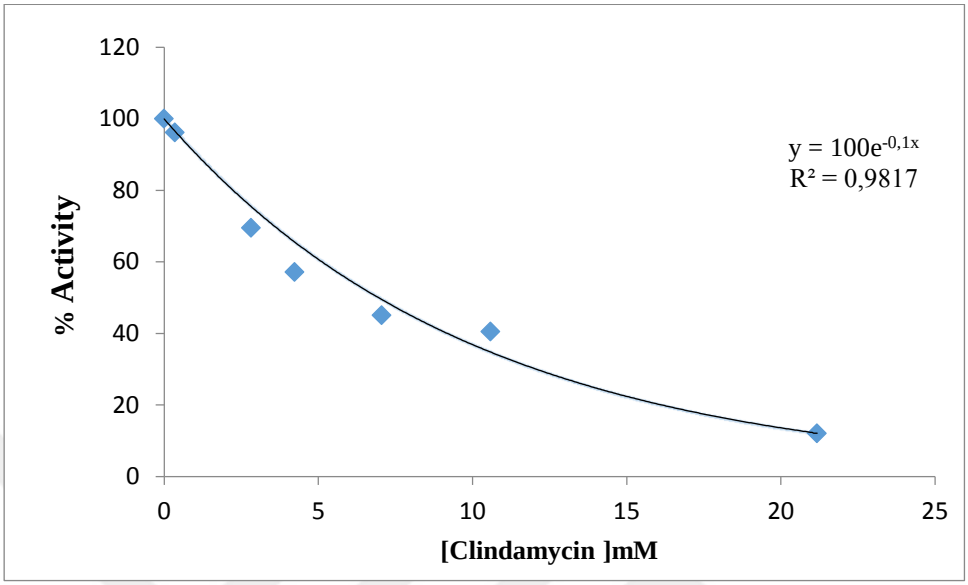

Figure 4.11. Grafic shows inverse proportion between [Clindamycin] and enzyme activity, IC_{50} = 6.9 mM

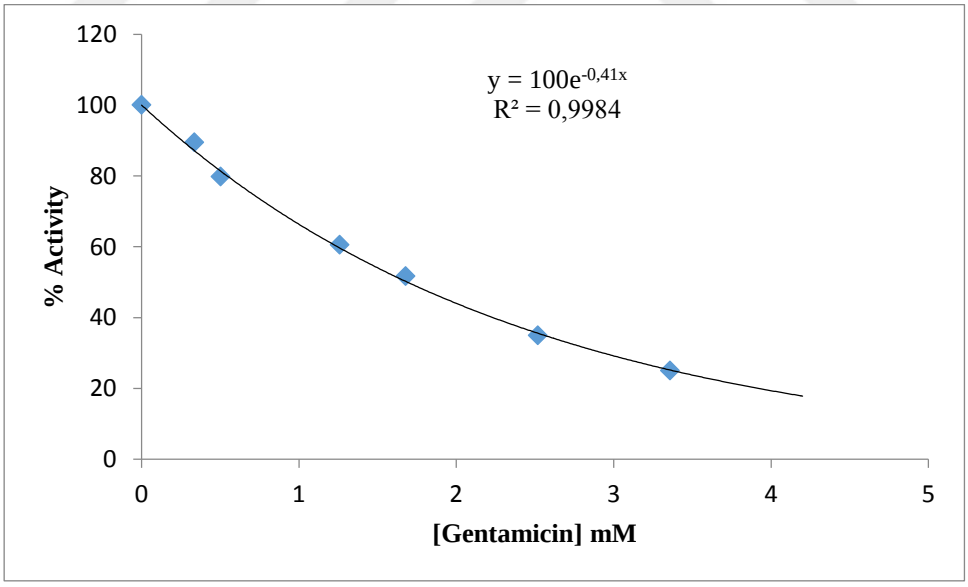

Figure 4.12. Grafic shows inverse proportion between [Gentamicin] and enzyme activity, IC_{50} = 1.69 mM

After determination of IC_{50} values, K_i of the inhibitors were calculated by drawing Lineweaver–Burk diagrams. Their K_i values were calculated based on this diagram to be 2.36 mM and 1.7 mM respectively (Figure 4.13 and Figure 4.14).

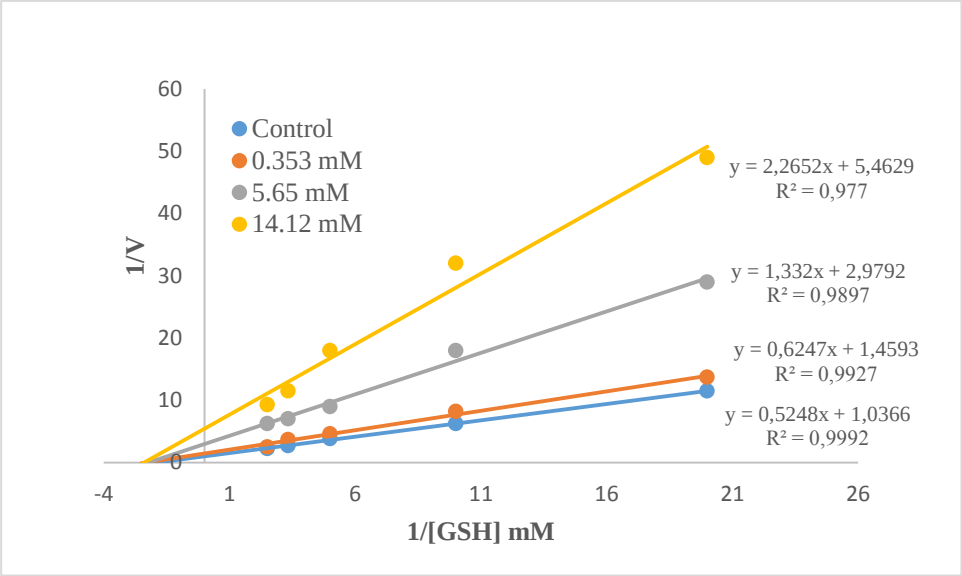

Figure 4.13. Grafic shows [GSH] versus inverted volume to find Ki for clindamycin which is 2.36 mM

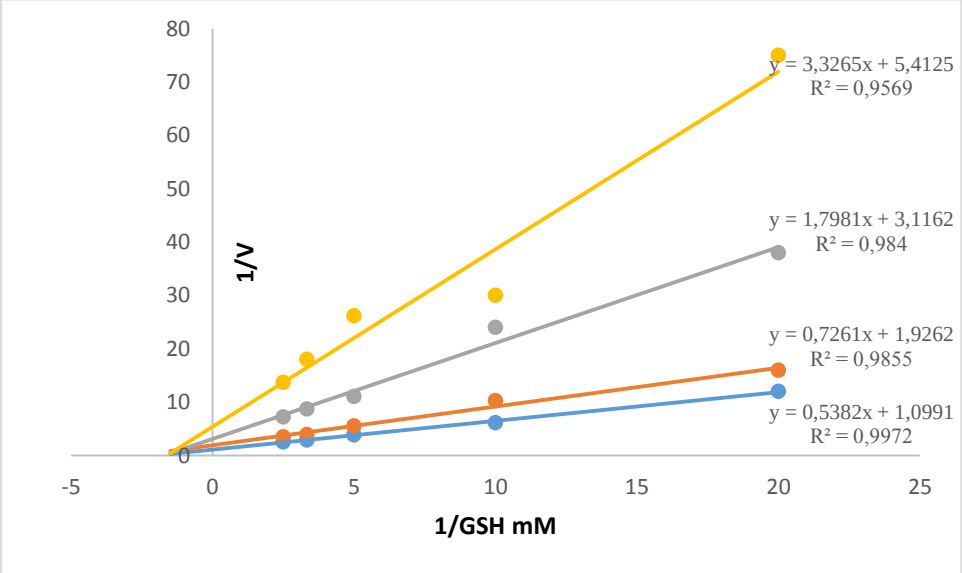

Figure 4.14. Grafic shows [GSH] versus inverted volume to find K_i for gentamicin which is 1.7 mM

While clindamycin and gentamicin inhibited the activity of the rat erythrocyte GST, the activity was increased with Hyoscin-N-Butylbromide+paracetamol and ampicillin (Figure 4.15 and Figure 4.16).

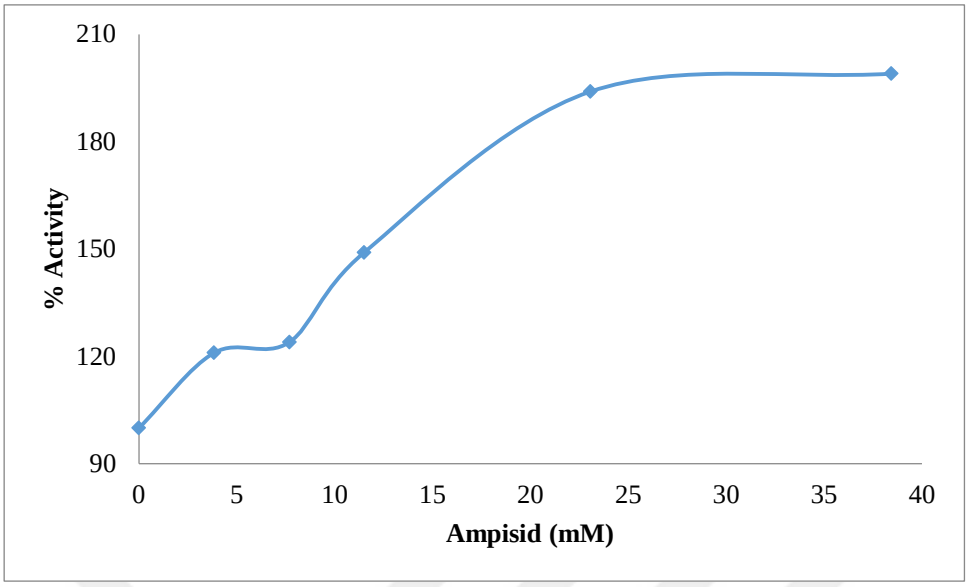

Figure 4.15. Grafic shows direct proportion between [Ampicillin] and enzyme activity

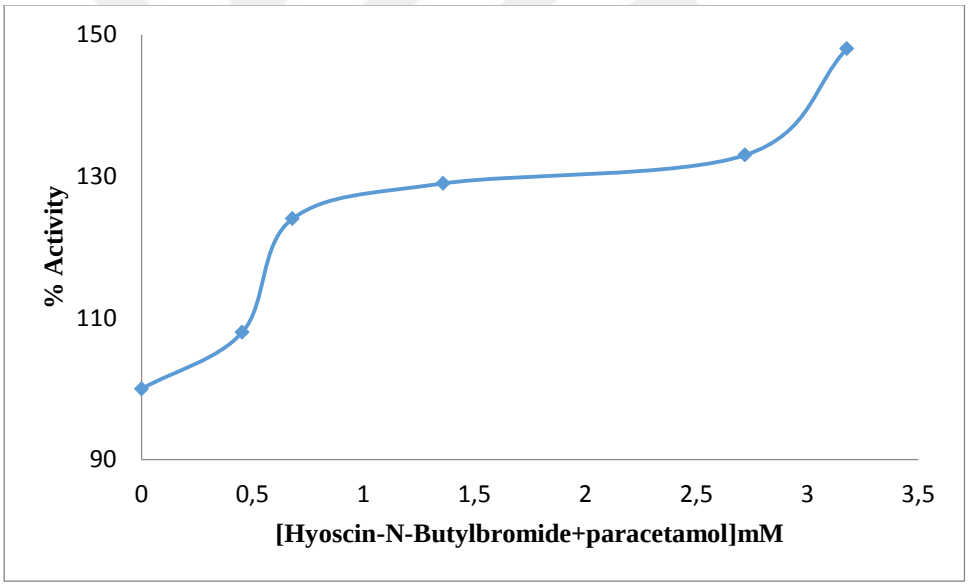

Figure 4.16. Grafic shows direct proportion between Hyoscin-N-Butylbromide + paracetamol and enzyme activity

Cefazoline (iespor): (0.8-8.0 mM) (5-150 mL) of this drug was used, to testing the effected of the drug on the enzyme activity, while there is no gradual an increase or a decrease on the enzyme activity were found.

5. DISCUSSION

The compounds or elements with one or more unpaired electrons in the outer orbit are known as 'free radicals'. Historically, the word 'radical' describes groups of atoms which remain unchanged during the reaction (Staroverov and Davidson 2000). The most vital free radicals in living systems are free oxygen radicals derived from oxygen. These radicals form by activity of oxidase enzymes during oxidation reactions in the energy metabolism in the cell (Koc 2007). The free radicals show their effects against DNA and protein molecules which are very important for cellular life. Various defense mechanisms have been developed to remove the effects of free radicals in living biological systems. These mechanisms are called antioxidant defense mechanisms or antioxidants The antioxidants are divided into two main groups as exogenous and endogenous sources. They are also divided into antioxidants which prevent radical effects (E vitamine, ubiquinone, retinoic acid, glutathion, urate) and radical formation (SOD, catalase, metal chelators and glutathione peroxidase) (Yanbeyi 1999).

One of the most important enzymatic antioxidant molecule is Glutathione S-transferase. It is a well known member of the Phase-II detoxification enzyme family, which protects cellular macromolecules from reactive electrophiles by interacting with electrophilic compounds with glutathione. Molecular weights of these enzymes are between 20,000-28,000 daltons and each subunit of this enzyme is composed of between 200-240 amino acids. (Hayes et al. 2005). GSTs catalyze the nucleophilic attack of glutathione (GSH) tripeptide on electrophilic substrates in enzymatic catalysis reactions. In addition to this role, it also has role in preventing the oxidative products or foreign toxic substances from conjugating with other macromolecules in the body and allows them to be removed without giving damage to the cell components. Due to these reasons, GSTs are known to be one of the most important protective groups of enzymes in human metabolism (Armstrong 1997).

Until recently, a lot of experiments were done on purifying the GST enzyme and characterizing its optimum pH, optimum temperature, ionic strength and pH stability. The K_M and V_{max} of the enzyme was also studied. For example the enzyme was purified and characterized in many organisms including the intestinal mucosa of the catfish (Gadagbui et al. 2000), human stomach (Demirdağ et al. 2013), turkey liver (Akkemik et al. 2012), Rainbow trout (Riol et al. 2001), rice (Chun-hua Zhang et al. 2013), rat liver (Nicholls and Ahokas 1984), human erythrocyte (Beydemir et al. 2018). To date no study focused on purification and characterization of rat erythrocyte GST. The main aim of this thesis was to purify and characterize GST from the rat erythrocytes and study the impact of some drugs such as gentamicin, clindamycin, cefazolin, ampisid (active ingredients: ampicilline + sulbactam), buscopan (active ingredients: Hyoscine N-butyl bromide + paracetamol) on the enzymatic activity of rat erythrocyte GST.

In order to achieve our aim, the GST enzyme was purified by GSH-Agarose affinity column chromatography in a single step. The purity and molecular weight of the enzyme was assessed by SDS-PAGE analysis. Following determination of molecular weight, the optimum pH, optimum temperature, ionic strength and pH stability of the enzyme was determined.

The molecular weight of the rat erythrocyte GST was calculated to be 25.2 kDa in our experiments. In one study, purification of GST was performed from ovary and testis of the freshwater fish Clariaslazera by GSH agarose affinity column chromatography. The results of the SDS-PAGE experiments demosnstrated that the molecular weight of the enezyme was 27.5 kDa for ovary GST and 25.1 kDa for testis GST (Guneidy et al., 2015). GST was also partially purified from somatic tissue of ruminal amphistome parasite, Gastrothylax crumenifer. The SDS PAGE analysis showed that molecular weight of the enzyme was 26 kDa. (Ahmed et al. 2017). A single protein band was obtained on the gel when the GST was purified from human erythrocyte. The subunit molecular mass of enzyme was calculated to be 28 kDa. A cytosolic GST was purified from pig lung with a molecular mass of 23500 Da (Dırr et al. 1991) These comparison demosntrate that molecular weight of rat erythrocyte GST are in the range of molecular weight of other GST enzymes in literature.

Specific activity, percent yield and purification fold of rat erythrocyte GST was calculated as 6.31 EU/mg protein, 44 % and 114.7 respectively. GST enzyme of human blood erythrocytes was purified in a single chromatographic step with a specific activity of 5.381 EU· mg−1 and 51.95% yield (Ozaslan et al. 2018). In another reseach experiment, Temel and his friends obtained GST enzyme from quail liver tissue with a purification fold of 47.88 and 12.33% recovery by glutathione agarose affinity chromatography (Temel et al., 2018). Kufrevioglu and Aksoy did purification experiments on GST from human erythrocyte with 1654 purification -fold and 19.27% yield by glutathione agarose affinity chromatography (Kufrevioglu and Aksoy. 2017). Glutathione S-transferase enzyme (GST) (EC 2.5.1.18) was purified from rainbow trout erythrocytes, with a specific activity of 16.54 EU/mg protein and 11,026- purification fold and 59% yield by glutathione-agarose affinity chromatography (Comakli et al. 2013). GST enzyme was also purified from Van Lake fish (*Chalcalburnus tarichii Pallas*) gills. The specific activity of the enzyme was 110.664 EU/mg and the purification yield was 79.6% (Ozaslan et al., 2017). Demirdag et al. purified glutathione S-transferase enzyme from nontumour and tumour human gastric tissue and researched the impacts of some heavy metals on the activity of the enzyme. The GST enzyme was purified with purification fold of 3089, with a specific activity of 20 EU/mg and 78% yield from gastric tumour tissue; and 1185 fold with a specific activity of 5.69 EU/mg and a yield of 50% from nontumour tissue (Demirdag et al. 2013). In a different study, GST enzyme was purified from muscle tissue of *Chalcalburnus tarichii Pallas* with 301.5- purification fold and 19.07% yield by glutathione agarose affinity chromatography (Aksoy et al., 2016). GST from the liver and gill tissues of Ağrı Fish Lake Trout (also known as Black Sea Trout) *Salmo trutta labrax* was purified by Comakli and his research friends. In their study, liver GST was purified with a specific activity of 60.87 EU/mg protein, 930 purification fold and 56% yield by glutathione–agarose affinity chromatography while, GST was purified with specific activity of 46.8 EU/mg protein, 576 purification fold and 60% yield (Comakli et al. 2015). A study performed by Turk and his friends, revealing that the enzyme was purified with 71% purification yield and 2550 purification fold from human erythrocyte. The specific activity of the enzyme was calculated to be 51 EU/mg protein (Turk et al. 2015). The same enzyme (human erythrocyte GST) was also purified by another group with a specific activity of 6.0 EU/mg proteins, approximately 2222.2 fold by glutathione-agarose affinity colum

chromatography (Turkan et al. 2018). All of these results demonstrate that the specific activity, purification fold and purification yield depend on the source in which the enzyme was purified.

After determination of molecular weight, purity, specific activity, purification fold and purification yield of the enzyme the optimum pH, optimum temperature, ionic strength and K_M-V_{max} values of the rat erythrocyte GST was investigated. It was found that optimum pH of the enzyme was 8 in 500 mM potassium phosphate buffer. The optimum ionic strength for GST enzyme was determined as 200 mM in potassium phosphate at pH 8. The optimum temperature was determined to be 50 ^{O}C. The stable pH for the enzyme was determined as 500 mM potassium phosphate pH 8 buffer. Lineweaver–Burk plots of K_M and V_{max} values were drawn for GSH and CDNB substrates of the enzyme. K_M and V_{max} were calculated as 1.22 mM and 1.21 EU/mL, respectively, for GSH, and K_M and V_{max} were calculated as 0.374 mM and 3.614 EU/mL, respectively, for CDNB. All of these values were also studied for GSTs purified from other tissues and organisms. The K_M value of (2.5 mM) for testis GST compared to 0.5 mM for ovary GST which is different as compared to the K_M found in our study (Guneidy et al. 2015). Glutathione S-transferase enzyme (GST) (EC 2.5.1.18) which was purified from rainbow trout erythrocytes in terms of its optimum pH, optimum temperature, and K_M and V_{max} values for GSH and 1-chloro-2, 4-dinitrobenzene (CDNB). Optimal pH, optimal temperature, and K_M and V_{max} values for GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) were 7.3 (0.01M K-phosphate), 30°C, 0.2590 and 0.395mM respectively (Comakli et al. 2013) which also different as compared to rat erythrocyte GST purified in this work. In a different study partially purified glutathione S-transferase from the somatic tissue of ruminal amphistome parasite, Gastrothylax crumenifer (Gc) infecting Indian water buffalo was characterised in terms of its optimum pH. the GST purified in this study showed an optimum pH of 7.5. (Ahmed S. et al. 2017). GST from muscle tissue of *Chalcalburnus tarichii Pallas* was characterized in terms of its K_M values which were calculated as 1.59 and 0.53 mM for CDNB and GSH respectively. V_{max} values for CDNB and GSH were calculated as 5.58 and 1.88 EU/mL respectively (Aksoy et al. 2016). Optimal pH, ionic strength for liver tissue GST was also studied. These values were found as 7.5, 0.008 M in potassium phosphate and these values for gill GST were 7.5 and 0.1 M in phospahate buffer. K_M values of liver GST for GSH and CDNB were calculated

as 0.385 and 0.231 mM, repectively, and V_{max} values were identified as 0.76 and 0.376 EU/mL, respectively. The K_M values of gill GST for GSH and CDNB were calculated as 0.035 and 0.41 mM, respectively, and V_{max} values were identified as 0.203 and 0.62 EU/mL (Comakli et al. 2015). Considering all of these information it could be concluded that the characterisation studies of GSTs obtained (purified) from different animals or living organism give different results in terms of optimum pH and temperture, K_M and V_{max} values. These differences demosntrate that behaviour of the enzyme in different organism can be different in terms of activity and other characterisation parameters.

After completing purification and characterisation studies the effect of some drugs and antibiotics were tested against the activity of rat erythrocyte GST. The inhibition or sometimes activation effects of the drugs or inhibitors on rat erythrocyte GST was given as IC_{50} values and K_i values. The inhibition/activation observed with these drugs or antibiotics against GST enzyme showed that the enzymatic activity was decreased while two antibiotics including clindamycin and gentamicin were added into the cuvette. However, the activity of rat erythrocyte was shown to increase in the presence of the drug called Buscopan (Hyoscine N-butyl bromide + paracetamol) and ampicid (ampicillin sulbactam). The antibiotics Clindamycin and gentamicin were shown to inhibit the activity of rat erythrocyte GST with an IC_{50} of 6,9 mM and 1,69 mM respectively. The collected data from spectrophotometer and activity calculations were put in Lineweaver–Burk diagrams and indicated that both of these antibiotics inhibited the activity of GST in a noncompetitive manner with respect to GSH as the reaction rate was getting lower but K_M of these compounds did not alter. Their K_i values were estimated based on the Lineweaver–Burk plots to be 2.36 mM and 1.7 mM respectively. The activity of the enzyme was shown to increase in the presence of 0.5 and 3 mM Hyoscine N-butyl bromide + paracetamol while it was increased in the presence of ampicillin sulbactam respectively suggesting Hyoscine N-butyl bromide+ paracetamol is more powerful activator than ampicillin sulbactam.

In the literature there are numerous studies focused on the understandng the effect of GST inhibitors as therapeutic agents, A variety of different organic compounds have been researched as GST inhibitors. For example in one of the studies Ozaslan et al. (2018) studied the inhibition effect of some of the chalcone derivatives on human GST enzymes.

In their study, K_i values of chalcone derivatives were calculated to be between 7.76–41.93 μM which suggests these derivatives are powerful inhibitors of the enzyme. Turk and his friends (2015) also investigated the effect of hypericin on human erythrocyte GST indicating that the compound decrease the activity of the enzyme with a K_i value of 0.19 mM noncompetitively. Their result demosntrate that hypericin is moderate inhibitor of the enzyme. Temel et al. (2018) purified GST from quail liver and studied inhibition effects of some organic compounds such as (3aR,4S,7R,7aS)-2-(4-((E)-3-(aryl)acryloyl)phenyl)-3a,4,7, 7a-tetrahydro-1H-4,7-methanoisoindole-1,3(2H)-dione derivatives on the enzymatic activity. K_i values of these derivatives were calculated to be ranging between 3 to 132 μM which means they are effective inhibitors. In addition to this, the effect of caffeic acid phenethyl ester and rosmarinic acid on the enzymatic activity of GST was researched. The results revealed that activity was effectively inhibited with a K_i of 0.453 nM and 48 nM for caffeic acid phenethyl ester and rosmarinic acid respectively. These inhibition is very effective as nanomolar level of these compounds inhibited the activity of the enzyme (Gulcin et al. 2016). The *in vitro* and *in vivo* effects of some antibiotics such as cefoperazone sodium, cefuroxime and cefazolin were also determined on GST with K_i constants of 0.1392, 1.5179 and 1.006 mM respectively (Turkan et al. 2018). These results indicate that some of these inhibitors amight be effective inhibitors of the enzyme. Among these compounds the effect of cefazolin was also tested in our study demonstrating no effect on the enzymatic activity of rat erythrocyte GST.

6. CONCLUSION

Glutathione S-transferases (GSTs) are family of isoenzymes have a commission number of (EC: 2.5.1.18), It is diversely distributed in the most parts in the (human, animal, bacteria, and plant) tissues. It seen has a major role in detoxification process especially in the phase (II) mechanism against endogenous and exogenous xenobiotic toxic molecules such as free radicals by catalyzing the conjugation them with glutathione and protect cells from the destroying (death). It is also considered as a natural anti-oxidant as same as vitamin C (ascorbic acid) , vitamin E (α-tocopherol), β-carotene, etc. in which plays an important role in neutralization of free radicals due to the capacity of sulfhydryl group (-SH) for donating electron and prevent cells from the damage.

In this study GST was purified from the rat erythrocytes by glutathione-agarose affinity column chromatography in one step, then characterized, and the effect of some drugs was studied. The current study proved that molecular weight was 25.2 kDa, protein concentration specific activity, percent yield and purification fold was calculated. In addition, inhibition effects of some drugs were investigated on the enzyme activity. The results demonstrated that the enzyme was purified with a specific activity of 6.31 EU/mg protein, in 44 % yield, and 114.7 purification fold. During characterization study in KH_2PO_4 buffer of rat erythrocyte GST, we get the following properties: Optimum pH was 8.0, optimum temperature was 50^{o}C, optimum ionic strength was 0.2 M, and stable was 8.0 K_M of GSH and CDNB Was 1.22 mM and 0.374 mM respectively. While Vmax was 1.21 EU/mL and 3.614 EU/mL respectively.

Bascoban and ampicillin activated rat erythrocyte GST, gentamicin and clindamycin inhibited enzyme. IC_{50} values of the drugs (gentamicin and clindamycin) were found as 1.69 and 6.9 mM IC_{50} values of the drugs (gentamicin and clindamycin) were found as 1.69 and 6.9 mM respectively. K_i values of these drugs were calculated as 1.70 and 2.36 mM respectively. Whereas cefazoline has no effected on the activity of the mentioned enzyme.

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PERSONAL BACKGROUND

I have been born on 2 April 1968 in Betwata – Sulaimaniya of Kurdstan region in Iraq. I finished my primary school in Betwata, secondary and high school in Erbil. I started studying chemistry science at University of Salahaddin-Erbil College of science/ chemistry department in 1985 and graduated in 1989 holding a bachelor's degree in chemistry science. I work as a chemistry teacher in the ministry of education. Im married and I have four children. I started my graduated study in February 2015 at Bingol university/ Turkey and awarded the master's degree in biochemistry on July 2018.