REPUBLIC OF TURKEY
BİNGÖL UNIVERSITY
INSTITUTE OF SCIENCE

PURIFICATION AND CHARACTERIZATION GLUTATHIONE S-TRANSFERASE ENZYME FROM JAPANESE QUAIL *(Coturnix, coturnix japonica)* HEART

MASTER THESIS

BARZAN MIRZA AHMED

CHEMISTRY

SUPERVISOR OF THESIS
Prof. Dr. Mehmet CIFTCI

BİNGÖL-2017

ACKNOWLEDGEMENT

First of all, I always have so thanks for my GOD (**ALLAH**) for gave me this opportunity to Master study and successfully finished, so for make facility and blessed me always in my life and giving me hope to success. Also I am so thankful for my supervisor Prof. Dr. Mehmet CIFTCI to accept me by his student and helped me during my study with his experience in Biochemistry. So thanks so much to my advisor assist. Prof. Dr. Yusuf TEMEL for advised me with his knowledge during my study. I would like to have thanks to the manager of the Institute science Prof. Dr. İbrahim ERDOĞAN for his support and helped me with sincere in his response's.

I would like thank my family for helping and supporting me always espcially my parents, my loyal mother, my sisters and brothers, in the other hand I am so thankful for my lovely friends whom sincere with me and thanks for anybody which helped and encuraged me to success.

DEDICATION

This study will dedicate to scientific researchers from anywhere and for anybody like science in the world, so dedicate to the Bingöl University whom study in science range, especially for biochemistry staff. Also I dedicate to my parents and best friends in my life.

Barzan Mirza AHMED

Bingöl University 2017

CONTENTS

LIST OF ABBREVIATIONS

GST	:	Glutathione S-transferase
GSH	:	Reduced glutathione
GSSG	:	Oxidized glutathione
CDNB		Chloro-2,4-dinitrobenzen
ES	:	Enzyme substrate complex
S	:	Substrate
E	:	Enzyme
P	:	Product
TEMED	:	Tetramethylethylenediamine
PER	:	Ammonium persulfate
SDS	:	Sodium dodecyl sulfate
PAGE	:	Polyacrylamide gel electrophoresis
DW	:	Distillate water
mL	:	milliliter
g	:	Gram
mg	:	Milligram
M	:	Molar
mM	:	Millimolar
L	:	Liter

mmol	:	Millimole
nm	:	Nanometer
EC	:	Enzyme commission
EU	:	Enzyme unit
kDa	:	Kilo Dalton
MW	:	Molecular weight
DNA	:	Deoxyribonucleic acid
ROS	:	Reactive oxygen species
Tris	:	Trihydroxymethylaminomethane
R_f	:	Retardation factor
TCA	:	Trichloro acetic acid
EDTA	:	Ethylene diamine tetra acetic acid
PMSF	:	Phenyl methane sulfonyl fluoride
DTT	:	Dithiothreitol
BSA	:	Bovine serum albumin

LIST OF TABLES

LIST OF FIGURES

GLUTATYON S-TRANSFERAZ ENZİMİNİN BILDIRCIN (Coturnix coturnix japonica) YÜREK DOKUSUNDAN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Bu çalışmada; glutatyon S-transferaz enzimi (GST) bıldırcın yürek dokusundan 34,0 EÜ/mg spesifik aktivite ile %10,44 verimle 78,29 kat saflaştırıldı ve karakterize edildi. Saflaştırma işlemi homojenat hazırlanması, amonyum sülfat çöktürmesi ve glutatyon-agaroz afinite kromatografisi olmak üzere üç basamakta gerçekleştirildi. Bıldırcın yürek GST enziminin saflığını test etmek ve molekül ağırlığını belirlemek için SDS-PAGE metodu kullanıldı. Saf enzime ait altbirim molekül kütlesi 26,3 kDa olarak hesaplandı. GST enziminin aktivite ölçümü 340 nm'de spektrofometrik olarak Beutler metoduna göre yapıldı. Enzime ait karakterizasyon özellikleri sırasıyla; optimum iyonik şiddet 1,2 M Tris/HCl tamponu pH=8,0, optimum pH Tris/HCl tamponu pH=8,0, optimum sıcaklık 60 ºC ve stabil pH Tris/HCl tamponu pH=9,0 olarak belirlendi. Daha sonra bıldırcın yürek GST enzimine ait substratlar olan glutatyon ve 1-kloro 2,4-dinitrobenzen'e ait K_M ve V_{max} değerleri glutatyon için K_M değeri 1,64 mM V_{max} değeri 0,50 EÜ/mL, 1-kloro 2,4-Dinitrobenzen için K_M değeri 3,88 mM V_{max} değeri 0,59 EÜ/mL olarak belirlendi.

Anahtar kelimeler: Bıldırcın yürek, glutatyon S-transferaz, saflaştırma, karakterizasyon.

PURIFICATION AND CHARACTERIZATION GLUTATHIONE S-TRANSFERASE (GST) ENZYME FROM QUAIL (Coturnix, coturnix japonica) HEART

ABSTRACT

In this study glutathione S-transferase enzyme (EC: 2.5.1.18) from the heart of Japanese quail was purified with 34.0 EU/mg specific activity, 10.44% purification yield and 78.29 purification folds and characterized. Firstly homogenate was prepared and ammonium sulfate precipitation process was performed, then the sample applied to the glutathione-agarose gel affinity column chromatography. To check the purity GST enzyme used SDS-PAGE method. Then calculated the molar mass as 26.3 kDa by SDS-PAGE method. Enzymatic activity was measured spectrofotometrically according to Beutler`s method at 340 nm. Also characterizations study done, and the results obtained are stability-pH = 9.0 in Tris/HCL buffer, optimum pH = 8.0 in Tris/HCl buffer, optimum temperature 60 °C, optimum ionic strength was 1.2 M in Tris/HCl buffer and kinetic studies performed for determined K_M and V_{max} for GST purified enzyme by used both glutathione and 1-chloro 2,4-dinitrobenzen as substrate, results obtained are 1.642 mM and 0.502 EU/mL respectively for GSH substrate with 3.880 mM and 0.588 EU/mL respectively for CDNB substrate.

Keywords: Quail's heart, glutathione S-transferase, purification, characterizations.

1. INTRODUCTION

1.1. Enzyme

A catalyst is a substance that affects the rate of a chemical reaction without being consumed or permanently changed in the reaction. Catalysts increase the rate of the reaction by lowering activation energy of chemical reaction. Catalysts in biological systems belong to a special class of proteins called (enzymes). The substance upon which an enzyme operates is known as its substrate (Seah and Kaplan 1973).

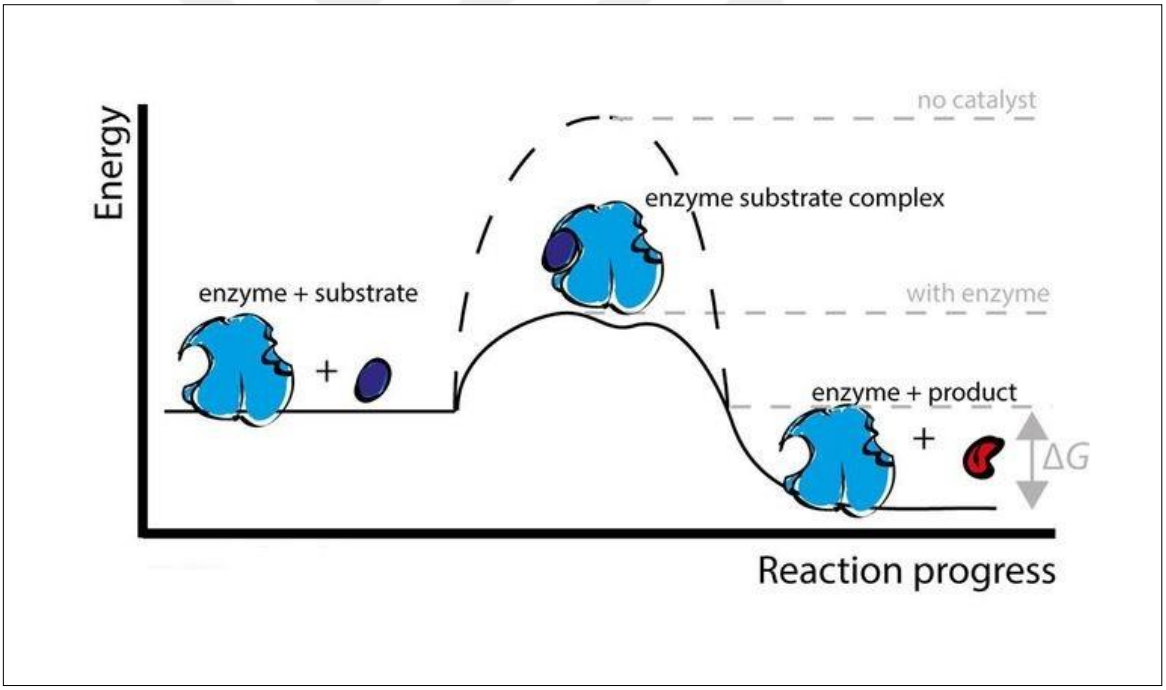

Figure 1.1. Enzyme catalysis chemical reaction convert substrate to product without side product (https://upload.wikimedia.org/wikipedia/commons/thumb/1/1f/Enzyme_action.png/400px Enzyme_ action. png)

Enzymes consists of the proteins that responsible for the catalysis of life, enzyme key of life. Enzymes are catalysts of chemical reaction in living organism by lowering activation energy and increase chemical reactions speeds without side product. Enzymes sharing a common ancestor as defined by sequence and structure similarity are grouped into families and super families. Enzymes molecular function are defined as their ability to catalyze biochemical reactions (Cuesta et al. 2015).

1.1.1. History

The enzyme's nation as biocatalysts was discovered in 1833 originally with the discovery of the conversion of starch into sugars by diastase catalyze (Payen and Persoz 1833). However, it not realized all potential scientists in the medicine and technology context until the 20th century. Major landmarks methods were developed for isolate and purify of enzymes, the realization that enzymes are proteins with biochemical activity and using x-ray diffraction techniques in their enzymes characterization (Mora and Arioli 2014). Dynamic studies nature of the ribonuclease structure and efforts in decipher the catalytic mechanism of lysozyme revealed enzymology as an emerging scientific discipline (De Simone et al. 2015).

1.1.2. Names and enzymes classification

Only as taxonomic classification proved too useful in dissect and identify the diversity of living organisms during the 18th century, in 1956 enzymologists and biochemists launched an initiative to gather all available information about the overall reaction catalyzed to names and enzymes classification. This was lead by experts in the enzyme commission (E.C) of the Nomenclscture Committe of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) who presented a limited to name old enzyme according to new enzymes must be classified. Now enzymes are named and identified systematically with an EC number enzyme commission consist of four- level description that is used to enzymes classify basing on the overall chemical change of substrates into products (Tipton and Boyce 2000). Enzymes were classified to six different class according to the type of chemistry being carried out.

Table 1.1. International nomenclature of enzyme, by nomenclature committee of the international union of biochemistry and molecular biology (nc-iubmb)

Classes	Functions	Instances
EC.1. Oxidoreductases	Catalyze oxidation/reduction reactions.	Glutathione reductase EC: 1.8.1.7
EC.2. Transferases	Transfer a chemical group for example, a methyl or glycosyl moiety.	Glutathione S-transferase EC: 2.5.1.18
EC.3. Hydrolases	Perform hydrolysis of chemical bonds.	α-amylase EC.3.2.1.1
EC.4. Lyases	Cleave chemical bonds by other means than by oxidation or hydrolysis.	Carbonic anhydrase EC.4.2.1.1
EC.5. Isomerases	Catalyze geometric and structural changes between isomers.	Alanine racemase EC: 5.1.1.1
EC 6. Ligases	Join two compounds with associated hydrolysis of a nucleoside triphosphate molecule.	Glutamine synthetase EC.6.3.1.2

These EC classes are further divided into subclasses and sub-subclasses (second and third level, in turn) in line with a diversity of criteria such as the cleaved or formed chemical bond, the center of the reaction, the chemical group transferred, and used cofactor for catalysis. The final level of the classification is substrate specificity defines such as, alanine racemase is an isomerase (EC 5), in specific racemase (EC 5.1) proceeding on the amino acid (EC 5.1.1) alanine (EC 5.1.1.1) (Boyce et al. 2004).

1.1.3. Factors effect on enzymes activity

The situations selected to measure the activity of an enzyme would not be the same as those selected to measure the concentration of its substrate. Some factors affect the speed at which enzymatic reactions proceed such as concentration of substrate, concentration of enzyme, pH, temperature and the presence of any inhibitors or activators (Martinek 1969).

1.1.3.1. Substrate concentration

If the quantity of the enzyme is kept constant and the substrate concentration is then slowly increased, the reaction velocity will increase till it reaches at a maximum point. Later this point, increases in substrate concentration will not increase the speed.

1.1.3.2. Enzyme concentration

Study the influence of increasing the enzyme concentration upon the reaction speed, in the presence additional amount substrate, any conversion in the amount of product produced over a specified period of time must depend upon the level of enzyme present. The reaction must be independent of substrate (Kurland and Pilkis 1995).

1.1.3.3. Effect of pH

Enzymes are affected by changes in pH. In which each enzymes work in specific pH. The most optimistic pH-value is the point where the enzymes have maximum activity is known as the optimum pH (Kurland and Pilkis 1995).

1.1.3.4. Temperature effect

Similar to most chemical reactions, the speed of an enzyme-catalyzed reaction increases as the temperature is raised. A 10 °C rise in temperature will increase the activity of greatest enzymes by 50 to 100%. Variants in reaction temperature as small as 1 or 2 degrees could lead changes of 10 to 20% in the results. In the situation of enzymatic reactions, this is problematical by the fact that several enzymes are unfavorably affected by high temperatures, the reaction speed rises with temperature to a maximum level, then sharply declines with further increase of temperature. Because most animal enzymes quickly become denatured at temperatures above 40 °C, many enzyme determinations are carried out rather under that temperature (Kurland and Pilkis 1995).

1.1.3.5. Effect of inhibitors

Enzyme inhibitors are ingredients which change the catalytic action of the enzyme and subsequently slow down, or in several cases, stop catalysis. There are three common sorts of enzyme inhibition competitive, non-competitive and un-competitive enzymes inhibition. Most theories about inhibition mechanisms are based on the presence of the enzyme substrate complex ES (Kurland and Pilkis 1995).

1.1.3.6. Effect of activators

Enzyme activators are particles that combine to enzymes and increase their activity. They are the reverse of enzyme inhibitors. These molecules are more involved in the allosteric regulation of enzymes in the metabolism control (Kurland and Pilkis 1995).

1.1.4. Enzyme kinetic

Enzyme kinetics study, is an issue that studied the speed of the reactions catalysed with enzymes by using the purified or unpurified enzyme, the always to measure the speed of an enzymatic reaction is mix the enzyme with substrate and record the produce of the product or disappearance of substrate at temperature from 25 °C to 37 °C and near to neutral pH, the product formation or substrate disappearance is commonly measure as a function of time (Mathews and Van 1996). Leonor Michael and Maud Menten first time in 1913 were postulated the formation of enzyme-substrate complex by combination enzyme with substrate and reaction occurred then product and free enzyme will formed. Enzyme-substrate were described in general reaction.

$$E + S \underset{k_{-1}}{\overset{k}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1.1)$$

When:

k_1: rate constant for ES complex formation.

k_{-1}: rate constant for dissociation ES complex.

k_2: rate constant for product and free enzyme formation from ES complexes.

The rate of enzymatic reaction always increased gradually with the concentration of substrate at low concentration, but at high substrate concentration the reaction rate becomes independent of the substrate concentration (Nelson and Cox 2013).

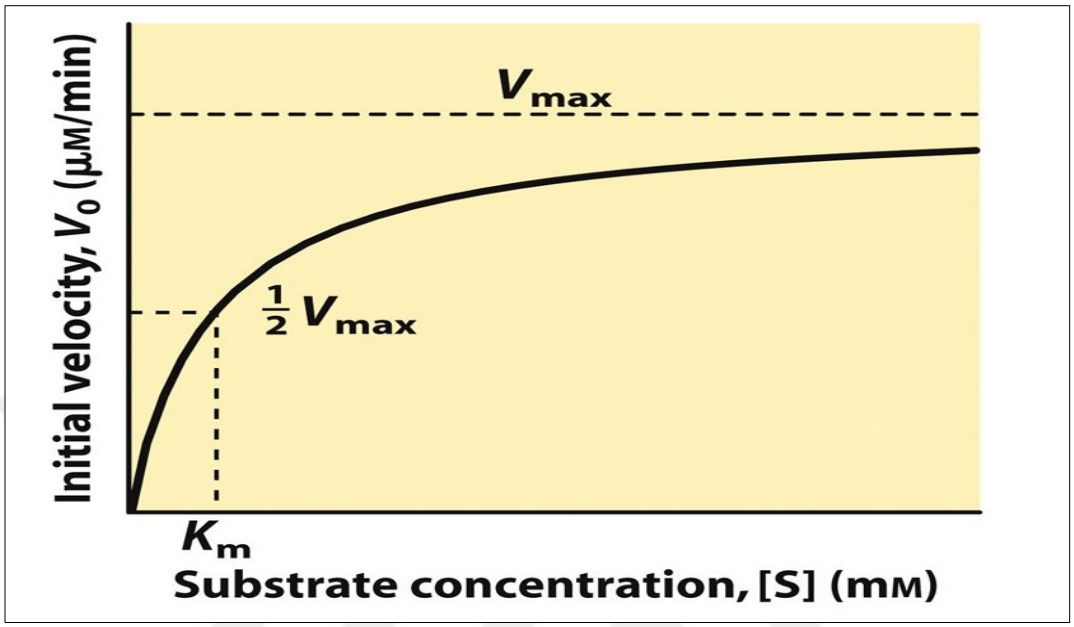

Figure 1.2. Effect of substrate on the rate of enzyme-catalytic reaction, further adding of substrate have no effect on the rate enzyme-catalytic reaction (http://slideplayer.com/slide/4813971/)

At the saturation level active sites of enzyme molecules are occupied. The rate of formation product could be written as.

$$V_o = \frac{V_{max} \,.\, [S]}{K_M + [S]} \qquad (1.2)$$

When

V_0: initial velocity of enzymatic reactions

V_{max}: maximum velocity reached

K_M: Michael's Menten constant

S: Substrate

This equation is known as the Michael's Menten equation, the V_{max} value for any enzymatic reaction different with the enzyme concentration. The more enzyme molecules are present the reaction will faster proceed at saturation, so V_{max} provide information about the enzyme catalytic efficiency and V_{max} is an expression of the enzyme's catalytic activity. Because $V_{max} = k_2$ [ET] with specific concentrations of the maximum speed is reached saturation with the substrate of the enzyme in enzymes catalytic reaction (Campbell and Farrell 2009). In which each enzyme has at typically turnover number. This number refers to the number of substrate molecules that the enzyme molecule saturated with the substrate can converted to product per unit time. The turnover number of the most efficient enzyme are several hundred thousand and the less efficient enzymes have turnover numbers less than one molecule per second (Northrop 1999). The K_M constant is called Michael's constant, it determined the concentration of substrate at which the velocity half maximal. The Michael-constant is measure of affinity of an enzyme for substrate and the value of the constant varies over a wide range but generally falling in the range of 10^{-6} to 10^{-1}. Enzyme with large K_M value shows a reluctance to enzyme dissociate from the substrate. This enzyme generally less active than the enzyme with low K_M values. Enzyme that catalyse reactions involved more than one substrate have K_M values for each substrate. K_M is a measure of the strength enzyme substrate complex. While high-K_M mean weak binding between the enzyme and substrate, it refers to a low-K_M strong binding between the enzyme and substrate complex (Zubay 1998).

1.2. Glutathione S-transferase

Glutathione S-transeferase (EC: 2.5.1.18) isoenzymes consist of approximately 223 amino acids are ubiquitously distributed from the nature, can be detect in both prokaryotes and eukaryotes. Being found in organism as diverse as microbes, insect, plants, fish, birds and mammals (Sherratt and Hayes 2001, hayes and pulford 1995). The transferase possess various activity and participate in several different types of reaction. Most of these enzyme Can catalyze the conjugation of reduced glutathione (GSH) with compounds that contain an electrophilic center through the formation of a thioester bond between the sulfur atom of GSH and the substrate (Mannervik 1985). In accumulation to conjugation reactions, a number of GST isoenzymes reveal other GSH dependent catalytic activities including the reduction of organic hydroperoxides (Ketterer et al. 1990). And isomerization of various unsaturated compounds (Benson et al. 1977).

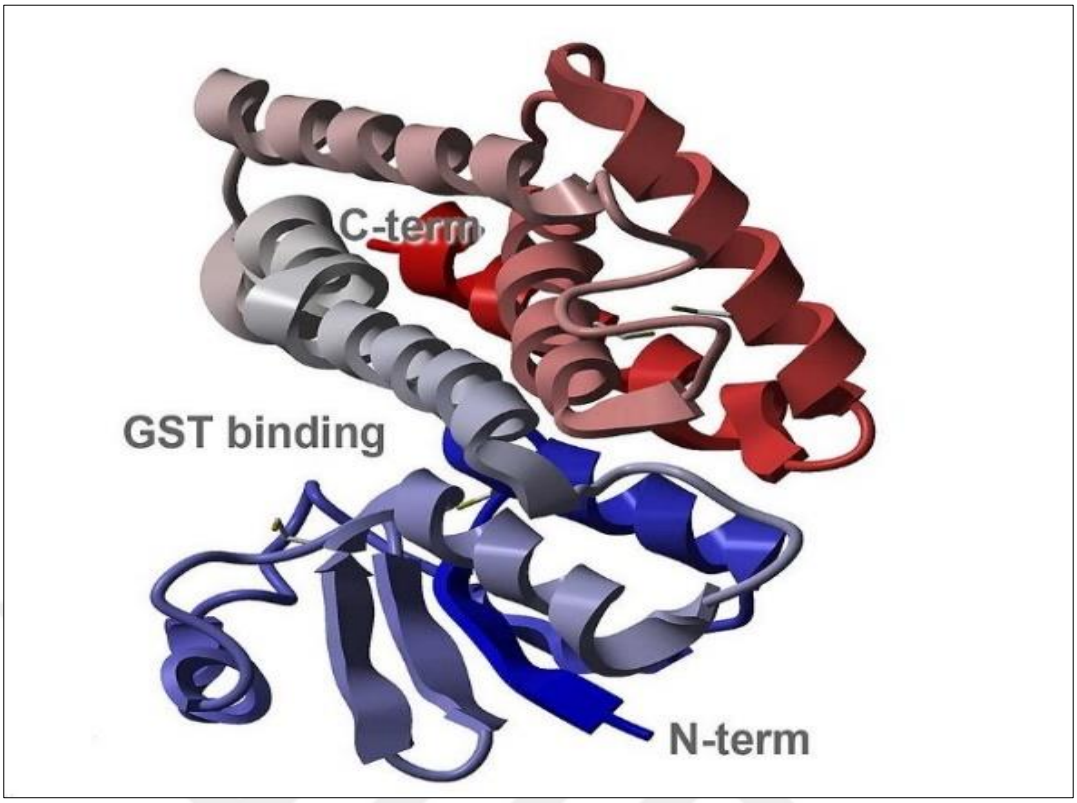

Figure 1.3. Structure of glutathione S-transferase enzyme (https:/ /upload. wikimedia. org/wikipedia/ commons /d/d2/GST-wiki.jpg)

GSTs catalysis the conjugation of GSH by lowering activation energy and increase speed of conjugation that participate in deprotonation of GSH to GS by a tyrosine residue in which as the base catalyst function. The first step from the mercapturic acid pathway is the glutathione conjugation due to elimination of xenobiotic compounds. Evolved GSH by GSTs and are abundant throughout most life forms. GSTs isozyme are divided into two distinct super-family members: the cytosolic family members and membrane bound microsomal based upon the similarity of amino acid sequence. In which five classes are cytosolic (designated α, μ, π, θ, and κ), so two are membrane-band. The cytosolic family of GSTs are subject to genetic significantly polymorphisms in human populations (Townsend and Tew 2003).

These enzymes also have several non-catalytic functions that relate to the sequestering of poisons, intracellular transport of an extensive spectrum of hydrophobic ligands, and change of signal transduction pathways (Takahashi et al. 1993). Glutathione S-transferase (GST) family of enzymes is one of the larger protein super-families with the major role in

Cellular detoxification. Enzyme multifunctional involved in phase II detoxification pathway attacked being the macromolecules cellular product by reactive electrophiles (Arakawa et al. 2012).

Because hydrophobic toxic compounds are harmful, GST catalyzes the conjugation of tripeptide GSH of electrophilic compounds into a wide range. The enzyme catalyzes the nucleophilic attack of the sulfur atom of glutathione (-SH) on the electrophilic groups of hydrophobic toxic compounds, cause the increasing their solubility and rendering them water soluble. The process promotes their excretion out of the cell (Habig et al. 1974).

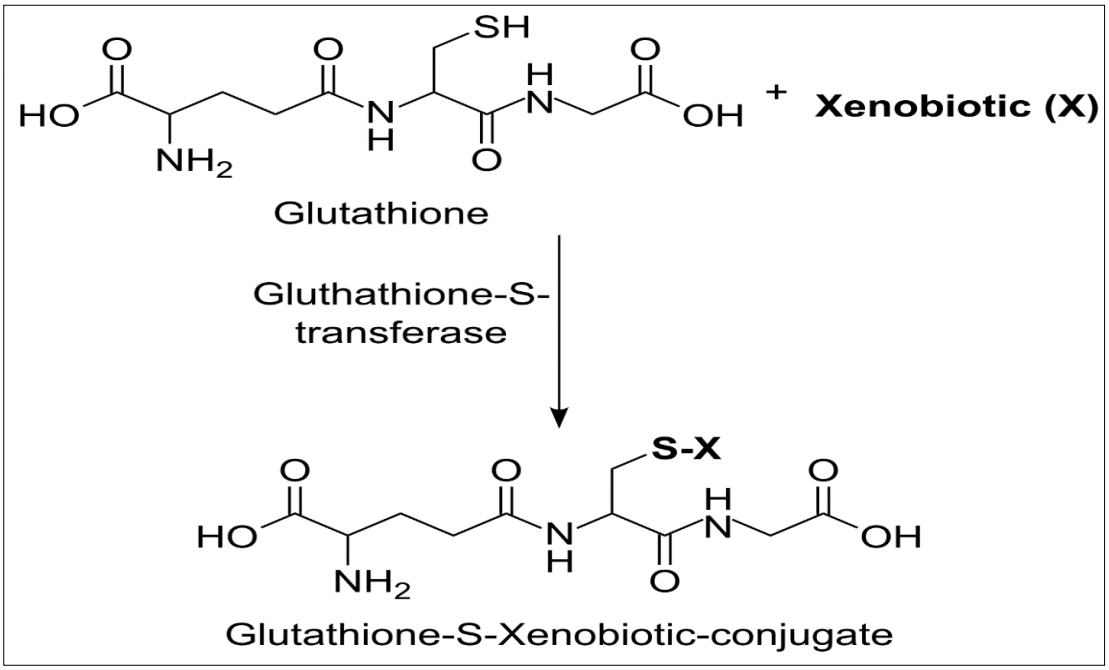

Figure 1.4. Mechanism of glutathione conjugated with xenobiotic toxic compounds catalysis by glutathione S-transferase enzyme (http://2013.igem.org/Team:TU-Munich/Project/Bioaccumulation)

1.2.1. Glutathione

Glutathione molecule is the central oxidation reduction agent of most aerobic organisms. It is reduced from glutathione (GSH≡γ-L-glutamyl-L-cysteinylglycine) functions as a ubiquitous nucleophile because convert a variety of electrophilic molecule substances under physiological conditions. Accelerate most of these chemical reactions in numerous metabolic pathways GSH depend on enzymes significantly (Simoni et al. 2002).

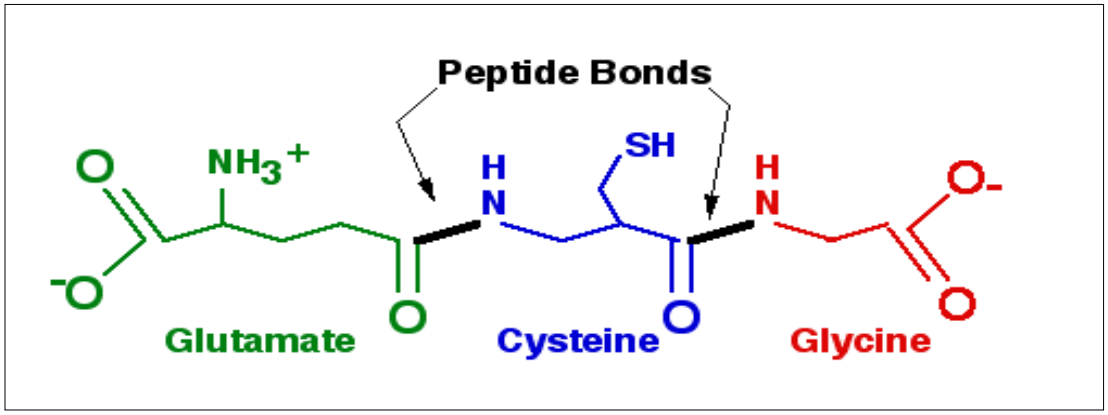

Figure 1.5. Chemical structure of reduce glutathione consist of three amino acids. (http://www.madsci.org/posts/archives/2003-10/1066260582.Bc.r.html)

GSH involve in most cellular reactions proportionally like a free radical and ROS scavenger so non-proportionally like a cofactor in enzymatic reactions. While in these process oxidized GSH to GSSG. To restore homeostasis, subsequently GSSG reduced by the NADPH-dependent glutathione reductase enzyme. Also GSH reacts with substrates of exogenous like the aforementioned drugs that are removed subsequently in the cellular milieu via efflux by the multidrug protein-associated resistance, the ATP binding cassette transporter superfamily member. In this capable GSH has play important role in the survivals cell to commonly utilize agents of chemotherapeutic (Findlay et al. 2006).

1.2.2. Xenobiotic

Foods and drugs are the most materials and compounds are ingesting. Some of these cannot be utilize by the body as foods, if they accumulate in the living organism cells may be harmful, as they have no metabolic function these are called xenobiotic. The xenobiotic term was derived from the Greek words of “xenos” meaning stranger and foreigner so “bios” meaning of life and “tic” is the Greek suffix added for adjective. Xenobiotic example include: (food additives, synthetic drugs, environmental pollutants, natural poisons, antibiotics etc.) There are above 200,000 xenobiotic have been found all are metabolized and detoxified under the xenobiotic-metabolizing enzymes (Croom et al. 2012).

1.2.3. Antioxidants

Any substances or compounds are capable to slowing or preventing the oxidation of other molecules is an antioxidant. Free radical can produce from oxidation reactions that cause the start chain reactions and damage living organism cells. Antioxidant cause the terminate these chain reactions by eliminating free radical intermediates and other oxidation reactions are inhibiting by starting oxidized themselves. The most antioxidants are reducing agents such as polyphenols or thiols (Duarte and Lunec 2005).

Glutathione is a cysteine-containing peptide detect in a lot of forms of aerobic life. It is synthesized by the body cells so does not required in the diet. It has antioxidant properties because there is the thiol group from cysteine amino acid it work as reducing agent and can be revrsible oxidized and reduced. Glutathione reduced by glutathione reductase and oxidized by glutathione S-transferase in the cells also it is reacting directly with oxidants. It is central role in maintaining the cells redox state due to its high level concentration. Glutathione is the one of the most important cellular antioxidants (Ulusu and Tandoğan 2007).

1.2.4. Enzymatic antioxidants

Powerful antioxidant enzymes are present from all cells in the body. The superoxide dismutase, catalases and glutathione (GSH) peroxidases are three major classes of antioxidant enzymes. In addition there are numerous specialized antioxidant enzymes reacting with and detoxifying oxidants (Valko el al. 2007). The glutathione system includes glutathione, glutathione S-transferase, and glutathione reductase and glutathione peroxidase. In which glutathione reductase is the enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl from (GSH) by dependent NADPH coenzyme mechanism, major cellular antioxidant system. So glutathione peroxidase is an enzyme that catalyzes destroy hydrogen peroxide and organic hydro peroxides. But glutathione S-transferases are another class antioxidant enzymes of glutathione-dependent that show high activity with lipid peroxidase (Sharma et al. 2004). Glutathione S-transferases antioxidant enzymes are at high levels present in the liver and also play important role in detoxification metabolism (Hayes et al. 2005).

1.2.5. Free radical

Free radical is the molecules that have one or more unpaired electrons. The unpaired electron of this molecule cause the highly reactive. It has a wide range of reactions; free radical reactions are include electron transfer and addition reactions in results covalent bond are formed (Buettner 1993). Free radicals are normally formed in the body cells as one of the defense mechanisms against toxic compounds. When the antioxidant capacity of the body less than the speed of the production of free radicals, oxidative stress will occurs. Oxidative stress carries harmful influence to all the body systems and is involve from pathogenesis of many diseases like atherosclerosis, hypertension, cancer and diabetes mellitus etc. enzymatic and non-enzymatic antioxidants have major role protection of the body against the damaging influence of free radicals (Ahmed 2014).

1.3. Biochemistry of GST Enzyme

The fundamental bases to all GST catalytic activities is the capable of these enzymes to decrease the pKa of reduce glutathione sulfhydryl group to approximately 6.5 from the 9.0 in aqueous solution while the GSH is binding to active site (Armstrong 2006). In natural pH GSH exists as the thiolate (GS^-) anion whereas with the GST enzyme it produce complex. GST catalysis take place through the capable combined of the enzyme to promote (GS^-) production and to bind xenobiotic compounds at the strongly adjacent site (Hayes and Pulford 1995). The GSH-binding and the xenobiotic substrate-binding sites have been called the G- and H-sites, respectively (Mannervik 1985). GST able to catalysis both forward and backward reaction in the certain substrate case (e.g. phenethyl, benzyl, alkyl dihalides and isothiocyanates), due to increased toxicity rather than detoxification. The active cytosolic enzyme exist as a dimer two subunits (Sheehan et al. 2001).

1.3.1. Evolution of the GST genes

GSTs are distributed widely in nature, from animals, plants, bacteria and yeast. Animal GSTs are theta and zeta also the sigma and theta classes in non-vertebrate animal are abundant (Sheehan et al. 2001 and Dixon et al. 2002). The plant GSTs include phi, tau, theta, zeta and lambda classes. Between the theta class GST and a dichloromethane dehalogenase enzyme have a significant homology from the prokaryote methyl bacterium

(La Roche et al. 1990). Suggesting that the inherited progenitor for mammalian GSTs perhaps arose in the theta class. Human GSTs absence activities, one can see how this might cause to decrease detoxification of carcinogenic of environmental or chemotherapeutic agents so thus for clinical problems in patients absent these genes. Also GST genes emerging is the evidence in the several pathogens might exert immunomodulatory serves towards the immune system, participate separate cytokine profile gene transcription and different patterns of cell growth (Ouaissi et al. 2002). Antioxidants, other than oxidative stress, induce transcription of several of the GST genes (Owuor et al. 2002), cause to raise protection of the organism cell against insult by environmental chemicals and drugs (Rinaldi et al. 2002).

1.3.2. Cytosolic versus membrane-bound GSTs

Most of the GSTs reviews include membrane-bound with cytosolic enzymes (Hayes and Pulford 1995). Also microsomal GST and leukotriene C4 syntheses described as members of the family of GST, however it has been noted that not be shared sequence identity with the cytosolic GSTs (Morgenstern and Depierre 1983). Therefore maybe appear it likely that these GST enzyme represent in membrane-bound like of convergent, rather than divergent, evolution during evolution time at a specific point, that required mother nature of an enzyme to carry out such catalytic reaction a membrane-bound so assigned that have the two domains GST_C and GST_N in the number of other proteins one or the other domains appear. Since that explain why some other protein has activity exhibit GST (Nicholson et al. 1993).

1.4. Glutathione S-transferase Functions

Glutathione S-transferase have been consider in play an important part in phase II of drug metabolism by detoxication of foreign compounds they contribute to the survival cells (Klaassen 1996). Compounds undergo GST-catalyzed conjugation with GSH that include isothiocyante, alkyl, aryl-halides, epoxide containing compounds, and α,β-unsaturated carbonyl and quinones (Hayes and pulford 1995). In addition to synthesizing glutathione S-conjugates, GSTs catalyze the reduction of peroxide-containing compounds that may otherwise be toxic to the cell (Mannervik 1986) So GSTs serve a major role in many biological important molecules isomerization. The transferase can catalyze the movement

of double bond within a polycyclic molecules or cis-trans isomerization reactions (Benson et al. 1977). GST isoenzymes in no enzymatic ligand-binding capacity serve a variety of involved factions in intracellular transport and carcinogen-detoxification of a wide spectrum of substances (Litwack et al. 1971).

The aim of the present work is to purify and characterize cytosolic glutathione S-transferase from heart of quail, where different cells populations are present, in order to determine if the enzymatic forms from isolated cells of quail may provide a useful system in toxicological research. Among the series of enzymes deliver protection from harmful injury by toxic chemicals. Glutathione S-transferase (GST) is most imperative antioxidant enzyme for detoxifying exogenous and endogenous substances to protect cells from the toxic effects of ROS. Reactive oxygen free radicals are implicated in the pathogenesis of a multistage process of head and neck carcinogenesis, which are proposed to cause DNA base alterations, strand breaks, damage to tumor suppressor genes and an enhanced expression of proto-oncogenes.

2. LITERATURE REVIEW

Senjo and Ishibashi purified Glutathione S-Transferase from the rat brain cytosol. Which obtained three enzyme functions, functions were apparently homogeneous as judged. That is the native form was a protein dimeric with a MW 45 kDa and enzyme chromatofocusing gave four peaks at pI 6.7, 7.6, 8.9 and 10.1. GSTs of rat liver cytosol raised against by antibodies were used to detect the purified enzyme. It has been proved that rat brain cytosol has no ligand in (GST-L2 = YaYa), which is the liver GST major component (Senjo and Ishibashi 1986). Singh S, et al. studied for isolate and purify of Glutathione S-Transferase in the humane kidney. Some type of GSTs are present from the human kidney, so the isoenzyme pattern present in the kidney differs significantly from other human tissues. Total cationic GST isoenzymes of human kidney except for GST 9.1 are heterodimers of MW 26.5 kDa and MW 24.5 kDa subunits. GST 9.1 is a dimer of MW 22.5 kDa subunits. Total kidney GST cationic isoenzyme cross-react with antibodies against raised a mixture of GSTα,β, γ, δ and ε isoenzymes of liver. GST 5.5 and GST 6.6 of kidney are dimer of MW26.5 kDa subunits and similar immunologically to GST φ of liver. Unlike the other human tissues, kidney has at less two isoenzymes (pI 4.7 and pI 4.9) with GST3 locus associated. Both isoenzymes are dimer of MW 22.5 kDa subunits and are immunologically similar to GST π of placenta (Singh et al. 1987).

Kwak and Park purified and characterized glutathione S-tranferase from the human placental tissues with the methods are salting-out, S-hexylglutathione Sepharose-6B affinity column, (SDS-PAGE) and kinetic studies. Results obtained was 11% yield, 1107 purification folds, K_M 0.085 mM for glutathione (GSH) and 2.0 mM for (CDNB) substrates, GST MW was 25 kDa and isoelectric point was 4.48 (Kwak and Park 1988). Dierckx studied to isolate of glutathione S-transferase from PLC/PRF/5 cells, an established cell line derived from Human hepatoma, were cultured in a cell factory to large numbers. The sole transferase was found as a human glutathione S-transferase π by its

pl (pH 4.4 on chromatofocusing), as determined by electrophoresis (one band at MW 26 kDa). The result is described from the view that glutathione S-transferase π is considered as a tumor marker: in order to PLC/PRF/5 cells certainly contain the glutathione S-transferase π isoenzyme, these cells could not be used as an alternative model to intact human liver (Dierickx 1989).

Irzyk et al. studied of glutaathione S-transferase in maize (zea mays pioneer hybrid 3906) with the dichloroacetamide herbicide safener benoxacor (CGA-154281) for purified homogeneity and characterized partially. The enzyme was purified about 200-fold. The purified protein shows a single band as 27 kDa MW. Native protein was determined with a MW approximately 57 kDa and the protein exists as a dimer by using monodenaturing PAGE. Two-dimentinal electrophoresis revealed just single protein has 5.75 isoelectric point and 27 kD MW. Suggest on the results the protein exists as a homodimer of two identical 27 kDa sub-units. The activity of enzyme was high with a chloroacetamide structure as substrate possessing, l-chloro-2, 4-dinitrobenzene and trans-cinnamic acid were not effective substrates. Apparently K_M values for the enzyme were 292 µM to glutathione and 10.8 µM to chloroacetamide metolachlor. So enzyme was active between the pH 6 to 9, with optimum pH among 7.5 to 8 (Irzyk et al. 1993).

Gronwald and Plaisance purified two type A1/A1 and B1/B2 of glutathione S-transferase (GST) isozymes from the *sorghum cereal.* Results was obtained for GST A1/A1was homodimer isozymes had a sub-unit MW of 26 kDa and an isoelectric point of 4.9 with high activity to (CDNB) substrate also GST B1/B2 was heterodimer isozymes with sub-unit MW of 26 kDa (designated the B1 subunit) and 28 kDa (designated the B2 subunit) and native isoelectric point of 4.8 with low activity to (CDNB) but high activity to metachlor as the substrate (Gronwald and Plaisance 1998). Gadagbui and James studied to isolate and purify of glutathione S-transferase in the cytosol prepared from catfish intestinal mucosa by GSH-agarose affinity chromatography and enzyme molecular weighted by gel electrophoresis. There was obtained results in intestinal GSTs were purified 100-fold with respect to cytosolic activity with (CDNB) and obtained a single band in SDS-PAGE in 26.7 kDa MW (Gadagbui and James 2000).

Riol et al. studied on glutathione S-tranferase in the rainbow trout hepatocytes so the aimed of this study to compare the different characteristics of (GST) from freshly isolated rainbow

trout hepatocytes with those corresponding to the total liver of some fish, with establish the similarities. GST enzyme was purified in the total liver samples were represented in the isolated cells also conformable to isoforms with MW of nearly 25.5 and 23.0 kDa (Riol et al. 2001). Cha et al**.** were worked to isolate and purify of glutathione S-transferas in the fungus Cunningham Ella elegans, the enzyme was purified 172-fold from the fraction of cytosolic (120,000 x g) of the extract in the culture of C. The native purified enzyme was homodimeric with a subunit of MW 27kDa. (Cha et al. 2001). Huang et al. purified and partial characterized cytosolic glutathione S-transferase (GST) from the liver of the freshwater fish Monopterus albus by affinity chromatography. The maGST indicated to be a homodaimer consist of two subunits with MW 26 kDa for each subunit and showed high enzyme activity to the CDNB and NBD-Cl substrate. Also kinetic study with CDNB substrate revealed a K_M and V_{max} are 0.28 mM and 15.68 μmol/min per mg of protein respectively and showed maximum activity in pH range 7.0-7.5 with optimum temperature in range 30 °C– 55 °C, the temperature stability at 45 °C (Huang et al. 2008).

Ahmad et al. purified and biochemical characterized cytosolic glutathione S-transferase from the filarial worms *Setaria cervi*. GST purified by prepared homogenate, salting out process, centrifugation, cat ion exchange and glutahione-sepharose affinity chromatography followed by ultrafiltration. Also purified GST showed single band on SDS-PAGE then gel filtration and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOFMS) illustrated the native enzyme was homodimer with MW 24.6 kDa. Comparted kinetic properties of the mammalian and parasitic GST enzymes revealed significant variation between them and substrate specificity with inhibitor profile of cytosolic GST in *S. cervi* indicated to be differ from GST in mammalian sources (Ahmad et al. 2008). Essam et al. isolated and purified glutathione S-transferase (EC 2.5.1.18) from the rat liver. Resulted of enzyme purified approximately 419.88 fold with a 56.43% yield. Specific activity of purified enzyme is 1250 x 10^{-3} unit/mg protein, indicated that enzyme is monomer of MW 50 kDa. SDS gel electrophoresis studies (Essam et al. 2009).

Dou et al. studied to purified glutathione S-transferase from susceptible and two resistant strains (DDVP-R for dichlorvos-resistant strain and PH_3-R for phosphine-resistant strain) of *L. Bostrychophila* by glutathione–agarose gel affinity chromatography then characterized by their Michaels–Menten kinetics study towards artificial substrates, such as, 1-chloro-2, 4-dinitrobenzene (CDNB), in a photometric microplate assay. The specific

activities of GSTs purified from two resistant strains were significantly higher than their susceptible counterpart. For the resistant strains, GSTs both showed a significantly higher affinity to the substrate GSH while a declined affinity to CDNB than those of susceptible strain (Dou et al. 2009).

Hamed et al. glutathione S-transferase purified and biochemical characterized from Down syndrome (DS) and normal children erythrocytes by affinity chromatography with specific activity of 23.7% and 7.9%, respectively. Then comparative studied was performed to illustrate the difference in the role of GST enzyme in the cell. İn which GST and GSH was determined in 10 DS and 10 healthy children matched for age (3–10 years). DS group indicated significantly lower GST value (2.7 units/g Hb) as compared to controls (6.6 units/g Hb) (40.9%). The activity of GST was significantly decreased to 40.9% in the DS group with compared to control. Also concentration of GSH was significantly decreased to 60.6% in the DS group compared to the control. The influence of freezing and melting, storage time of freezing and concentration of GSH on the stability of the enzyme were examined. Normal GST enzyme exhibited a pH optimum at pH = 7 followed by risk decreased, however DS GST enzyme exhibited pH, optimum pH between 7.5 and 8. The K_M values for GSH and CDNB were 0.786 mM and 0.205 mM, respectively, in which normal GST, and CDNB 0.318 mM and 1.307 mM, respectively for DS GST. The activation energy were calculated for normal GST and 3.8 cal/mol for DS GST and 2.25 and 4.25 cal/mol for normal GST (Hamed et al. 2011).

Lebda et al. studied to isolate and purify of glutathione S-transferase from the rat livers and effect of carbon tetracloride and Camels Milk. For both GSH and CDND substrates CCl_4 inhibitor cause the decrease V_{max} and increase K_M then control showed the CCl_4 uncompetitive inhibitor of GST enzyme while V_{max} increase and K_M decrease in camel's milk for both GSH and CDNB substrates that means affinity of enzyme in camel's milk increase to both substrate the activity of GST is proportionally to the concentration of substrate till reaching the maximum activity after that activity of GST forming a plateau. Measuring the temperature effect on GST activity, the GST activity was maximum observed between 25-35ºC and inactivated above 40 ºC. The GST activity increased when pH increased progressively till reach the maximum activity at pH value 7.1 and decreased when the pH increased (Lebda et al. 2012). Ezeji et al. were experimented to determine the effect of permethrin insecticide on glutathione S-transferase and Glutathione (GSH)

activity from the serum and liver of poultry birds. Weighted poultry birds (broilers) have six weeks old between 400-600 g, five birds, each birds was divided to four groups. Exposed the poultry birds for 30 days with different concentrations (1, 5 and 10%) of (Rambo) locally manufactured insect powder which contains 0.6% permethrin. The experiment results confirmed the use of glutathione S-transferase in ecological evaluation of environmental pollutants as a biomarker (Ezeji et al. 2012).

Akkemik et al. purified and characterized glutathione S-transferase enzyme from the turkey liver and inhibition influence of several metal ions on enzyme activity, results GST enzyme was purified 252.7-fold with a yield of 45%, with a specific activity of 164.31 EU/mL, The purity of the enzyme was checked by SDS-PAGE and showed two bands with molar MW nearly 26 kDa and 24 kDa on the gel and native molar mass approximately 53 kDa by Sephadex G-100 gel filtration chromatography determined. Optimal pH, stable pH, optimal temperature, optimum ionic strength, K_M and V_{max} values for CDNB and GSH were also determined for the enzyme as 7.3, 8.5, 50 °C, 600 mM, 0.380 mM, 0.154 mM, 2.125 EU/mL and 1.803 EU/mL, respectively. Additionally, inhibitory influences of metal ions (Cu^{2+}, Hg^{2+}, Fe^{2+}, Zn^{2+}, Ag^{+}, Mg^{2+}, Ni^{2+}, and Mn^{2+}) were examined the enzyme's activity in vitro by performing Line weaver–Burk graphs and plotting activity% *vs* concentration, respectively (Akkemik et al. 2012).

Ozaslan and Ciftci purified and characterized glutathione S-trasferase from the liver of the Van Lake fish, Purification was carried out in two steps, preparation of the homogenate and glutathione Agarose affinity chromatography with the results specific activity 29.304 EU / mg protein, purification fold 316.11 folds and purification yield 37.36%. Also calculate MW as 62.35 kDa by gel filtration chromatography but molar mass determined by SDS-PAGE two subunits as 28 kDa and 33.8 kDa. So optimum pH = 7.8, optimum ionic strength = 100 mM Tris-HCl buffer, optimum temperature = 30 °C and stable pH = 5.5 in KH_2PO_4 buffer. İn addition K_M and V_{max} determined as 0.891 mM and 0.060 mM, 1.245 EU / ml and 0.562 EU / ml for CDNB and GSH respectively (Ozaslan and Ciftci 2014).
Simarani et al. were studied an attempt for isolate glutathione S-transferas –expressing bacteria in the rhizospheric soil of herbal plants selected. İn our soil collection screening showed nine positive and isolates of twelve bacterial samples from large microbial population. Purification glutathione S-transferase using GST trap yielded two distinct subunits with MW of 23 and 24 kDa as visualized on SDS-polyacrylamide gel

electrophoresis. GST purified show reactivity to words 1,2-dichloro-4-nitrobenzene,1-chloro-2,4-dinitrobenzene and ethacrynic acid with a specific activity of 0.264 ± 0.038 nmol/min/mg and 0.056 ± 0.002 nmol/min/mg and 10.500 ± 3.130 nmol/min/mg, respectively. Whereas no activity was detected in the study against trans-hepta-2, sulfobromophthalein, p-nitrobenzyl chloride, hexa-2, 4-dienal, trans-4-phenyl-3-butene-2-one, 4-dienal and trans-oct-2-enal (Simarani et al. 2016).

Wan et al. Were identified and functional characterized glutathione S-transferase from the beet armyworm (*Spodoptera exigua*), result obtained *Spodoptera exigua* (SeGSTe) gene consists of 746 bp full-length cDNA and a 669 bp open reading frame encoding a predicted GST protein is 223 amino acid with MW 24.517 kDa and an isoelectric point of 6.44. As well as, sequence analysis of SeGSTe indicated 90% sequence identity to *Spodoptera litura* GSTe2 and 47–36% to other insect GSTs (Wan et al. 2016). Aksoy et al. Purified and characterized glutathione S-transferase from the muscle tissue of Van Lake fish (Chalcalburnus tarichii Pallas) by glutathione agarose affinity chromatography with specific activity of 35,583 EU/mg protein, purification fold 301.5-folds, a purification yield 19.07% and The purity of enzyme was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis, two band appeared on SDS-PAGE since have heterodimer structure. So K_M and V_{max} determined as 1.59 Mm, 5.58 EU/mL and 0.53mM, 1.88 EU/mL for CDND and GSH respectively (Aksoy et al. 2016).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Table 3.1. The chemicals used in this studied

Chemicals	Company
Ammonium sulfate, magnesium chloride, sodium chloride, sodium bicarbonate, sodium hydroxide, potassium phosphate, potassium chloride, EDTA ethylene diamine tetra acetic acid, Tris (Trihydroxy methyl amino methane), isopropanol, β-mercaptoethanol, acrylamide, TEMED (N, N, N, N tetramethylethylenediamine), silver nitrate, hydrochloric acid, phosphoric acid, glycerine, ethanol, methanol, acetic acid, GSH, glutathione-agarose gel	Sigma company
Potassium acetate, bromothymol blue, sodium acetate, potassium hydroxide, glycine amino acid and trichloroacetic acid.	Merck
SDS (sodium dodecyl sulfate), Commasie Brilliant Blue G-250, Commasie Brilliant Blue R-250	Fishcer Scientific
Ammonium persulfate	Chem Solute Bio

3.1.2. Instruments

Table 3.2. The instruments used in this studied

Instruments	Models
Clinical centrifuge	(Universal 320 R, Hettich) and (Allegra X-30R Beckman Coulter)
Spectrophotometer	(Shimadzu CC-10) UV 1800)
pH meter	Thermo Orion 3 stars
Homogenizer	IKA T25 ultra-turrax
Electrophoresis tank	Bio Rad
Electrophoresis power supply	Bio Rad
Peristatic pump	(Bio instruments ATTA SJ-1220) and shaker (P Spectra J.P. Selecta, s.a.)
Vortex	Vortex Lab Companion SK 300
Sensitive balance	Denver instrument SI 234
Column chromatography	Sigma Aldrich
Automatic pipette	Transferpette, Eppendorf research
Ice maker	Hoshzaki ice maker FM-80EE
Magnetic hotplate stirrers	VMS-C10 Advanced

3.1.3. Preparation solutions

3.1.3.1. Homogenate solution

Homogenate solution used to prepare homogenate from quail heart tissue consist of 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF buffer: It prepared by dissolved 0.605 g of Tris in 80 ml distillate water, then add 0.015 g of DTT, 0.027 g of EDTA and 0.017 g of PMSF dissolved in 5 mL n-butanol was added and the pH was adjusted to 7.2 and the solution complete to 100 mL by distillate water.

3.1.3.2. Enzyme activity measurement solutions

1. 0.1 M KH_2PO_4 (pH: 6.5): prepared by dissoved 0.68 g KH_2PO_4 And 0.014 g of EDTA in 30 ml of distillate water and the pH was adjusted to 6.5 then complete solution volume to 50 mL by distillate water.
2. 20 mM GSH solution: prepared by dissolved 0.03 g of reduced glutathione in 3 mL distillate water and complete volume to 5 mL by distillate water.
3. 25 mM CDNB solution: prepared by dissolved 0.025 g of 1-chloro 2,4 dinitrobenzene in some 95% ethanol then complete solution to 5 mL by 95% ethanol.

3.1.3.3. Affinity chromatography solutions

1. Regeneration 1 consist of 0.1 M H_3BO_3 and 0.5 M NaCl, pH: 8.5 (borate buffer) prepared by disolved 1,545 g of H_3BO_3 And 7.31 g of NaCl in 200 mL distillate water was also pH was adjusted to 8.5, then complete solution volume to 250 mL by distillate water.
2. Regeneration 2 consist of 0.1 M $NaCH_3COO$ and 0.5 M NaCl, pH: 4.5 (acetate buffer) prepared by dissolved 2.05 g of $NaCH_3COO$ and 7.31 g NaCl in 200 mL of distillate water and adjusted to pH: 4.5, the volume was adjusted to 250 ml with distillate water completed.
3. Azide solution consist of 0.02% NaN_3 solution: prepared by disolved 20 mg NaN_3 in 80 mL distillate water then complete volume to 100 mL by distillate water (Solution used to protect the chromatographic column materials from bacteria).

4. Equilibration solution 10 mM KH_2PO_4 And 150 mM NaCl, (pH: 7.4) (solution used for packing of the column, equilibration and washinig): prepared by dissolved 0.68 g KH_2PO_4 and 4.38 g of NaCl in 400 mL distillate water and pH was adjusted to 7.4 then complete solution volume to 500 mL by distillate water.
5. Washing solution 10 mM KH_2PO_4 and 0.1M KCl (pH=8.0),(solution used to washing column after applied sample): prepared by disolved 0.136 g KH_2PO_4 and 0.745 g KCl in 80 mL distillate water and pH was adjusted to 8.0 then complete solution volume to 100 mL by distillate water.
6. Elution solution 50 mM Tris-HCl buffer and 10 mM GSH (pH: 9.5), (buffer used for elution of GST enzyme binding affinity gel): Prepared by dissolved 0.181g Tris-HCl and 0.092 g GSH (reduce glutathione) in some distillate water and pH was adjusted to 9.5 then complete solution to 30 mL by distillate water.

3.1.3.4. SDS-PAGE solutions

1. 1 M Tris-HCl (pH: 8.8): prepared by disolved 12.11 g of Tris in 80 ml of distillate water and adjusted to pH 8.8 then complete solution volume to 100 mL by distillate water.
2. 1 M Tris-HCl (pH: 6.8): prepared by dissolved 12.11 g Tris in 80 mL distillate water and adjusted to pH 6.8 then coplete solution volume to 100 mL by distillate water.
3. 30% acrylamide-0.8% bisacrylamide solution: prepared by dissolved 15 g acrylamide and 0.4 gm bisacrylamide in 34.6 g of distillate water then complete solution volume to 50 mL by distillate water.
4. 10% SDS solution: Prepared by dissolved 1 g of sodium dodecyl sulphate in 9 ml distillate water to obtained 10 mL.
5. 10% ammonium persulfate solution: Prepared by dissolved 1 g of ammonium persulfate in 9 ml distillate water to obtained 10 mL.
6. Running buffer: Prepared by dissolved 1.51 g Tris and 7.51 g glycine in 450 mL distillate water and add 5 ml of 10% SDS after that adjust the pH to 8.3 then complete solution volume to 500 mL by distillate water.
7. Sample buffer: Prepered by mixed 0.65 mL of 1 M Tris-HCl (pH 6.8), 1 mL of 10% SDS 1 mL of 100% glycerin and 1 mL of 0.1% bromothymol blue with distillate water the total solution volume was 10 mL. before using this buffer for every 950 µL of sample solution, 50 µL of β-mercaptoethanol were added (this buffer was used for protein denaturation).

8. Gel fixation solution: Prepared by mixed 50% isopropanol, 10% TCA, and 40% DW (this solution was used to stabilize the protein carried in the gel).
9. Gel staining solution: Prepared by dissolved 0.1 g of Coomassie Brilliant Blue R-250 in 50 mL methanol then mixed with 10 mL acetic acid and 40 mL distillate water.
10. Gel washing solution: Prepared by mixed 50 ml of methanol, 40 ml of distillate water and 10 ml of acetic acid.
11. 0.1% bromothymol blue solution: prepared by dissolved 0.1 gm indicator in 16 mL of 0.01 M NaOH then complete volume to 100 mL by distillate water.

3.1.3.5. Solutions used for protein identification by the Bradford method

1. Coommassie Brilliant Blue solution: Prepared by dissolved 100 mg Coommassie Brillant Blue G-250 in 50 mL of 95% ethanol and added 100 mL of phosphoric acid 95% Then complete solution volume to 1000 mL by distillate water. The solution kept in dark place.
2. Standard protein solution: Prepared by dissolved 1 mg of bovine serum albumin (BSA) in 1 mL distillate water.

3.2. Methods

3.2.1. Preparing quail heart and homogenate

The quails used in this experiment was supplied fresh from the Bingöl University application farm. According to the cold chain rule, the quails were brought to the biochemistry laboratory and cut them carefully with separated heart tissue quickly. Hearts was frozen in deep freeze at -20 °C. In the experiments, the frozen hearts 4 g was cut into small pieces and suspended in 12 mL of homogenate buffer by ultra-turrax homogenizer until the heart tissue was completely destroyed and the cell membrane destroyed to enzymes come out from the cytosolic of cell. Next debris and unbroken proteins removed by centrifuged. Finally the suspension was centrifuged at 13,000 rpm for one hours at 4 °C and separated precipitate from supernatant then precipitate was discarded and Homogenate saved.

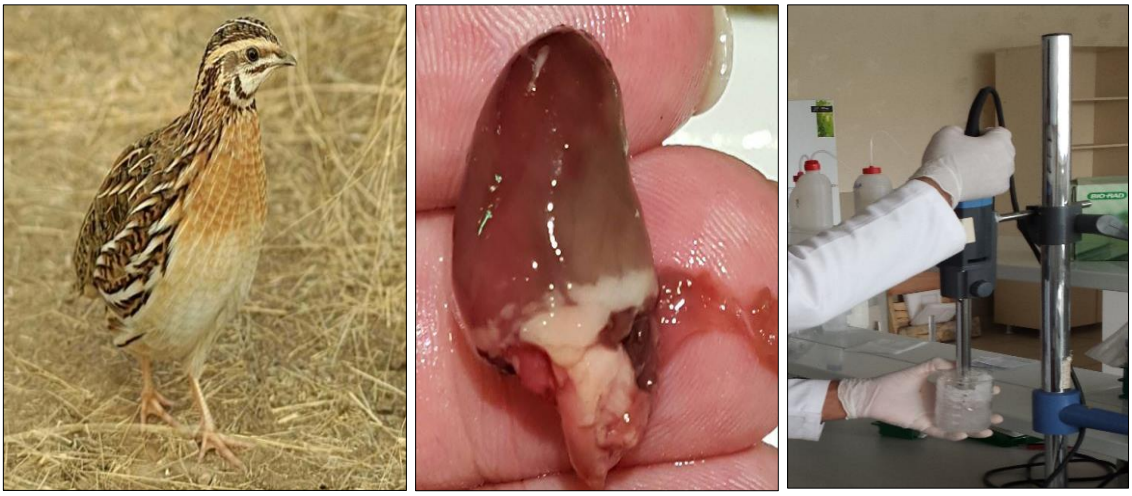

Figure 3.1. Separated heart of quail bird and prepared homogenate used ulta-turax device (http:// www.roysfarm. com/quail-farming/)

3.2.2. Enzyme activity measurement

The glutathione S-transferase (GST) enzyme catalyzes the conjugation of an aromatic electrophilic with a glutathione molecule. The most widely used aromatic electrophile is 1-chloro-2, 4-dinitrobenzene (CDNB). The dinitrobenzene 5-glutathione (DNB-SG) product formed. The maximum absorbance spectrophotometrically measured at 340 nm, was using this substrate in the basic of the process.

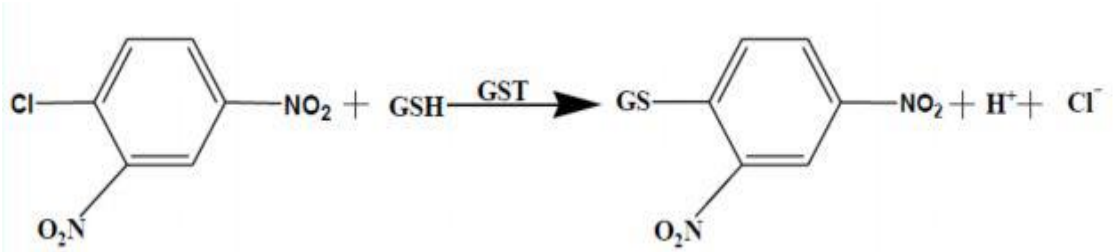

Figure 3.2. Reaction between 1−chloro−2, 4−dinitrobenzene (CDNB) and glutathione in the presence of glutathione S−transferase enzyme

Then measure activity by exploiting the increase in absorbance at this wavelength. In the absence of GSH, CDNB rapidly inactivates glutathione S-transferase. It is necessary to initiate the reaction with CDNB. For activity measurement the solution as a total volume of 1 mL was prepared. The cuvette were placed on a spectrophotometer and reading started. Measured absorbance values in three minutes (Habig et al. 1974).

Table 3.3. Procedure of measured GST enzyme activity

Reagents	Blank	Sample
Distillate water	730 µL	720 µL
0.1 M KH_2PO_4 buffer	200 µL	200 µL
20 mM GSH	50 µL	50 µL
25 mM CDNB	20 µL	20 µL
Enzyme sample	-	10 µL

One enzyme unit is in one minute, the enzyme was converting substrate to the product at 25 °C and pH of 6.5 activity. The following formula was used when the enzyme unit was calculated.

$$EU = (\Delta OD/3/ 9.6) \times (V_T/V_E) \times D_F \quad (3.1)$$

When

EU: enzyme unit in 1 mL.

ΔOD: Change in absorbance in a minute.

3: total times of measurement

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

V_T: Total volume of the bathtub (cuvette) where the measurement was made.

V_E: The volume of the enzyme sample added to the cuvette being measured.

D_F: Dilution factor (used for diluted samples).

3.2.3. Ammonium sulfate and dialysis (salting out)

Solid ammonium sulfate precipitation was performed at 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% to the prepared homogenate and the settling interval was determined. The solid $(NH_4)_2SO_4$ used for precipitation must be slowly added to dissolved thoroughly on the magnetic stirrer with the bar. This process is the sample saturations with different

percentage of ammonium sulfate salt and centrifuged sample from each steps. In which the ammonium sulfate is the more soluble salt cause the precipitate or collapse protein molecules based on solubility of protein molecules in the sample solution, Because ammonium sulfate is dissociate in sample solution to strength ions and have influence to attraction the solvent molecules to surrounding the ions than proteins, since proteins are not ions. While the salt ions surrounding by solvent proteins are accumulated together and precipitated because of less soluble and heavy than other molecules. Ammonium sulfate precipitation had done first at 20% saturation. The saline hemolyzate will place in centrifuge tubes and centrifuged at 13,500 rpm for 15 minutes (thus allowing precipitation of foreign proteins).

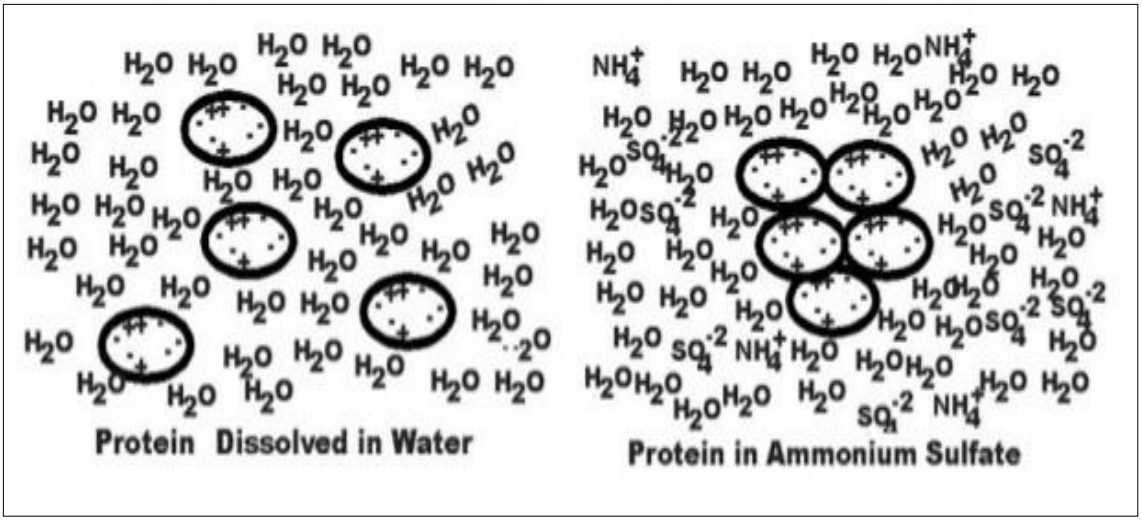

Figure 3.3. Salting out process to precipitate proteins (https://www.slideshare.net/ouopened/protein-purification-lecture)

Subsequently, ammonium sulfate precipitation at 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% saturation were carry out in the same way. The dissolve saline hemolysate was placed in a centrifuge tube and centrifuged at 13,500 rpm for 15 minutes. The precipitate portion was dissolved in the minimum homogenate buffer (50 mM Tris-HCl, pH = 7.2) then enzyme activity was measured for both supernatant and precipitate at 340 nm. The amount of ammonium sulfate was added to the homogenate in each steps prepared by the below equation. This method was first performed in 1955 by Green and Hughes (Wingfield 2001).

$$\text{Gram of } (NH_4)_2SO_4 = \frac{1.77.\ V.\ (S_2 - S_1)}{3.54 - S_2} \qquad (3.2)$$

When

Gram: is gram of ammonium sulfate

V: homogenate volume

S_1: initial percent of ammonium sulfate

S_2: final percent of ammonium sulfate

The ammonium sulfate precipitate was dialysis by placing the mixture in a dialysis bag and sunk it in a beaker filled with dialysis buffer (10 mM K-phosphate, 1 mM EDTA pH = 7.5) then put the beaker in ice-fill container after that put all upon the magnetic stirrer with bar (slowly stirring) changing it twice against for two hours.

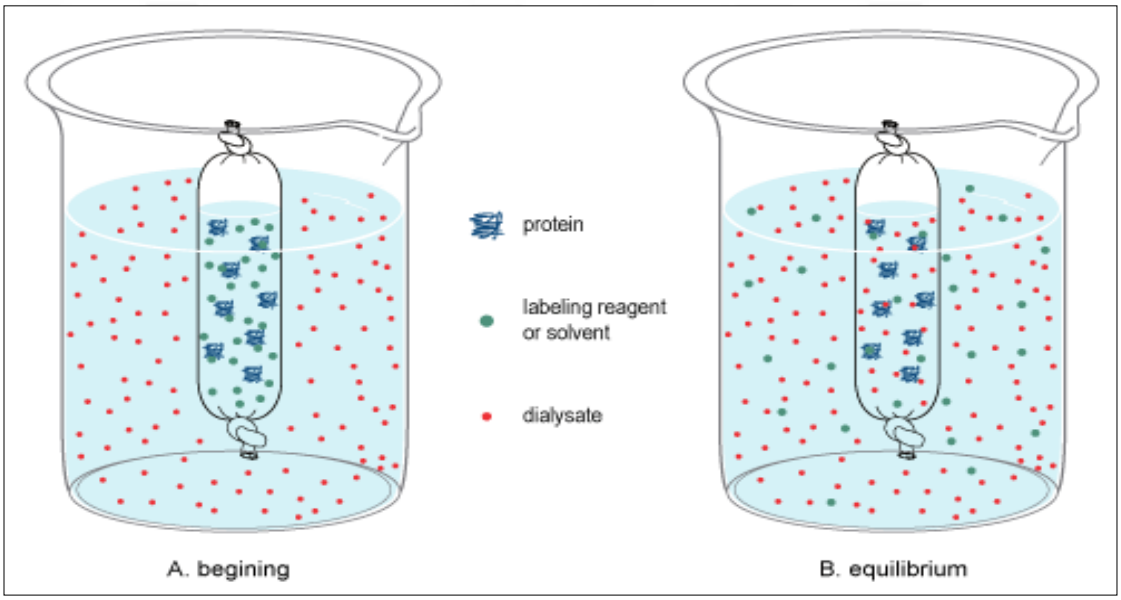

Figure 3.4. Dialysis process to eliminate unwanted small molecules (https:// upload.wikimedia. org/wikipedia/commons/2/2c/Dialysis_Figure.png)

This process done to eliminate small molecules in the sample based on the diffusion through the semi-permeable membrane between the two same buffers in which small molecules able to path through the sac membrane, but larger molecules (proteins) cannot able to path and remained in the sac, like the dilution process to the small molecules in the sample. Dialysis is the continuing process to more purification or increased enzyme activity also prepared sample to apply load to the column.

3.2.4. Purification of quail GST by affinity chromatography

First prepared gel by weighted 1 gram of dry glutathione agarose gel to use in purification enzyme and gel washed by distilled water for some times to remove impurities also dissolved gel in 200 mL distilled water or equilibration buffer and kept it overnight between 2 ºC and 4 ºC. The gel was swollen, the inflated gelatin air was removed vacuumed by used water tromp. The prepared gel was cooled in the (1 x 10 cm) column and closed system packed. After the gel precipitated in the column, the column flow rate was controlled by peristaltic pump and equilibration column with the buffers called first regeneration and second regeneration, in which first regeneration consist of 0.1 M boric acid and 0.5 M sodium chloride also second regeneration consist of 0.1 M sodium acetate and 0.5 M sodium chloride both buffer used to regeneration of column by three steps for each buffer 15 mL added to the column respectively. After that equilibrated column by equilibration solution consist of 10 mM KH_2PO_4 and 150 mM NaCl, (pH = 7.4) until the pH in the column equal to the 7.4. After equilibrated column ammonium sulfate precipitation was prepared before, it hand and applied directly to the glutathione-agarose affinity column. Then the column was washed with 10 mM KH_2PO_4 and 0.1M KCl, pH = 8.0 in the column. This washing was continue with measure the presence of activity from the flow eluate column by taking absorbance with the UV-Spectrophotometer. The washing process was continued until difference final absorbance becomes 0.05 at 280 nm. After washed process the enzyme was purified by gradient elution. The elution solution was formed from the gradient of the solution containing 50 mM Tris-HCl and 1.25-10 mM GSH, pH = 9.5. With the help of the fraction collector, the eluates were collected in 1mL tubes and exam for absorbance at 340 nm. The flow rate of the column through the peristaltic pump was adjust to 20 mL / h (Güvercin et al. 2008 and Toribio et al. 1996).

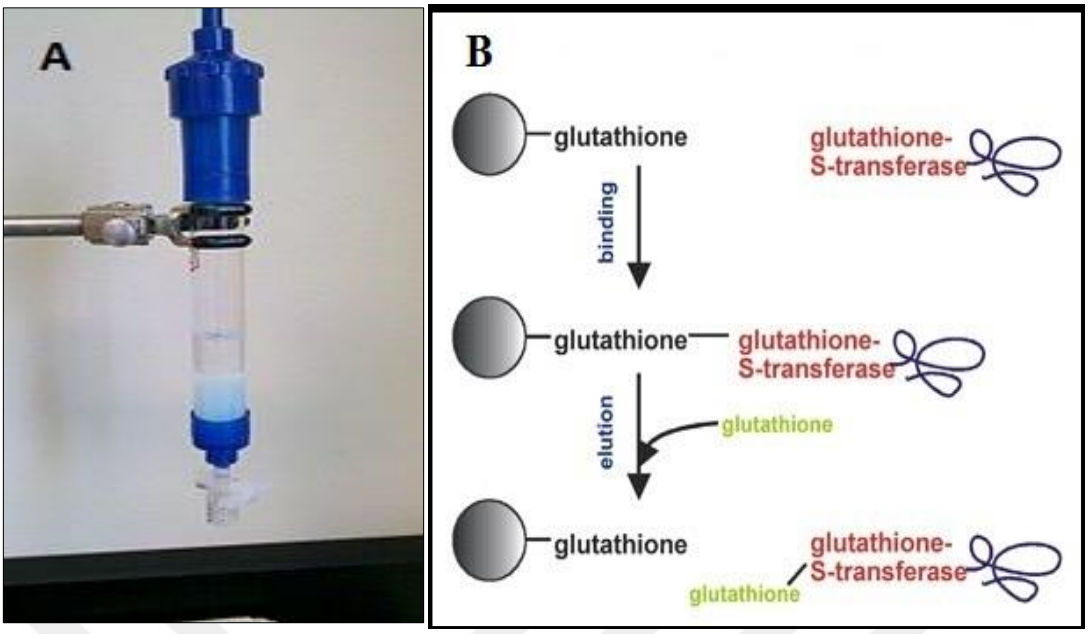

Figure 3.5. **A**. Glutathione-agarose affinity column **B**. Prencipile of GST-tag protein purification (https:// upload. wikimedia.org/wikipedia/ commons/ thumb/6/6d/Nickel_resin.jpg/ 150px-Nickel_resin.jpg), (https:/ /www. bioke . com / webshop/mn/745500.html)

The GST enzyme was banded to the glutathione on the agarose gel in the column and other enzymes were flow out through the column except GST remained in the column and washed process used to flow other enzymes through the column. After using elution to flow GST enzyme in which elution contain free glutathione molecules with high molarity cause the flow GST enzyme through the column, because GST had more affinity to free glutathione than glutathione on the gel. Then eluate collected which contain GST enzyme in Eppendorf tubes, after that activities measured all pure enzyme tubes then load pure enzyme to the gel electrophoresis process to check it.

3.2.5. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

To control the purity of the GST enzyme, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) should be performed at 3% to 15% cut according to the Laemmle procedure. Bio-Rad tools were washed by distilled water and make slides in the rack and tested by pure water and prepared 15% separation gel in a container, then immediately poured into the slides hole nearly full. Also full between slides with n-butanol to remove bulbs in the gel then waiting for 15 to 20 minutes to polymerized gel after that discarded n-butanol and dried the above gel by tissue paper to be clear from n-butanol.

Next 3% stacking gel prepared immediately and poured into the slides hole with putting the comb in the stacking gel to be created wells to the sample, then waiting for 10 minutes to the stacking gel to be polymerized. Next, slides from rack were taken out and fixed in the separation Bio-Rad tool then put it into Bio-Rad tank with full slides pool by running buffer, after that the comb was taken out carefully from the gel, and poured sample with standard protein into the different wells on the gel carefully and added supporting buffer to the Bio-Rad tank according to the gel numbers, then connected to the power supply into two steps first step loaded process with 20 mA and 80 volts for 20 minutes in this step the samples were stacked and started to separated. The second step loaded process with 40 mA and 120 volts for 120 minutes.

Table 3.4. Preparation 15% of separating gel

Reagents	Volume (mL)
Distilled water	2.3
30% acrylamide- 0.8% bisacrylamide	5
1.5 M Tris-HCl (pH: 88)	2.5
10% SDS	0.1
PER Ammonium persulfate	0.1
TEMED (at last added)	0.010

Table 3.5. Preparation 3% of stacking gel

Reagents	Volume (mL)
Distilled water	3.4
30% acrylamide- 0.8% bisacrylamide	0.83
1.5 M Tris-HCl (pH: 88)	0.63
10% SDS	0.05
PER Ammonium persulfate	0.05
TEMED (at last added)	0.008

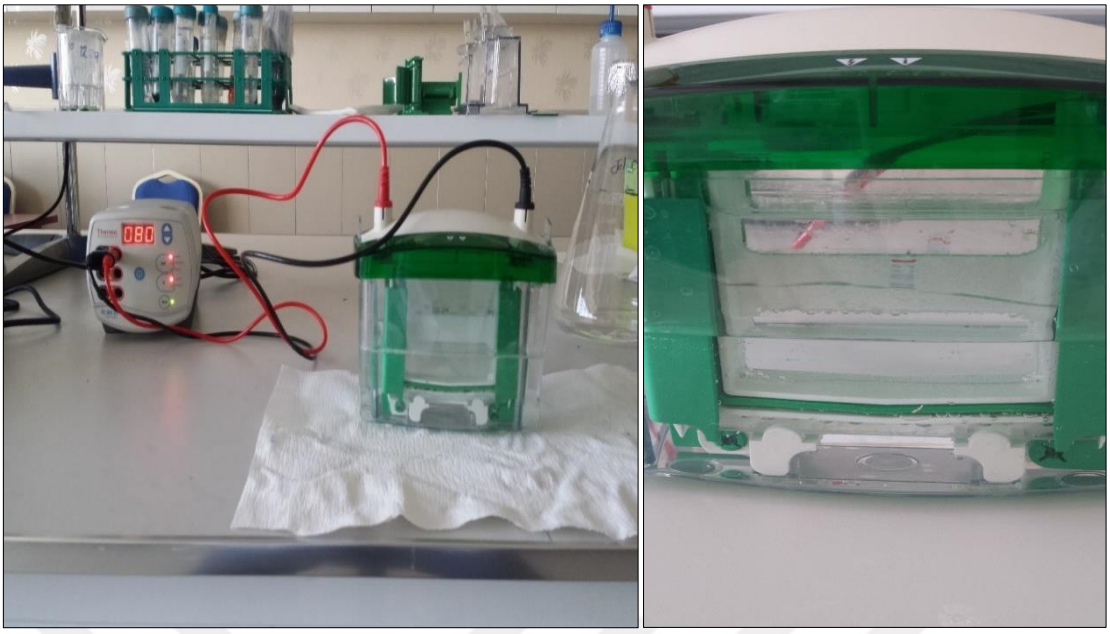

Figure 3.6. Loading sample on SDS-PAGE of Bio-Rad technology

In SDS-PAGE the proteins are moving through the gel based on the size and the charge under the effect of electric field in which the larger proteins slowly moved than small proteins through the gel from the negative charge toward the positive charge. When the separated process were completed, the gel taken out carefully and stand in fixation solution for 30 minutes to be fixed proteins on the gel with slowly and continue shaking by shaker, after that the gel was dyed for two hours in Coomassie Brilliant Blue R-250 or silver staining solution depending on the amount of protein in the obtained gel, and distained the gel with gel washing solution until the single band was appeared, then the gel was photographed by scanning device. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was determined the molar mass of the enzyme as follows; the molecular mass was electrophoresis used to know standard proteins. As a result, the log MW-R_f standard graph will be plot by calculated the R_f values of the standard proteins. The R_f value for the GST enzyme from the heart obtained, and then calculated and replaced with the standard graphic to determine the log M.W of the GST enzyme. The molecular mass of the sample was determined by taking the antilogarithm of this value (Laemmli et al. 1970).

$$R_f = \frac{X_e}{Xdye} \tag{3.3}$$

Xe = walking distance of protein, Xdye = walking distance of dye

3.3.Protein Assay

3.3.1. Qualitative determination of protein

The qualitative protein assay is bases on the principle of maximum absorbance of tryptophan, tyrosine and phenyl alanine in the structure of proteins at 280 nm. Because those amino acids are able to absorbs UV-VIS light that attributed to contain the aromatic branch in amino acids. Aromatic compound have able to absorb light due to the resonance of double bound in the ring structure. With this method, in the glutathione-agarose affinity chromatography process, the fraction collector tubes was used to determine the qualitative protein in all fractions taken in equal volume. Fractions are taken in quartz cuvette, the absorbance was reading against the blank in the spectrophotometer (Segel 1968).

3.3.2. Quantitative determination of protein (Bradford's method)

Quantitative protein was determined according to Bradford method 1976. In this method, Coomassie Brilliant Blue G-250 was used as a dye, which has a negative charge and binds to the positive charge on the protein. The dye is available in red (λmax = 465 nm) and blue (λmax = 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 100 μg). The reaction was repeated at high speed and takes place rapidly, completed in two minutes. The assays was carried out according to the following procedure: prepare ten different concentration solutions from standard bovine albumin solution containing 1 mg of protein per ml, by taking 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μL in ten tubes and complete to 100 μL with distilled water also add 4900 μL of Coomessie Brilliant Blue G-250 solution total volume equal to 5 mL in each tubes. The solutions were mixed with the vortex. After that incubation for 10 minutes, the absorbance values against the protein in the 3 mL cuvettes at 595 nm were measured.

Table 3.6. Preparation of standards in Bradford method for protein determination

Reagents	Volume (µL)									
BSA	10	20	30	40	50	60	70	80	90	100
DW	90	80	70	60	50	40	30	20	10	0
Dye	4900	4900	4900	4900	4900	4900	4900	4900	4900	4900

In order to detect the amount of protein in the sample from each steps of purification, prepared three sample solution from the homogenate, ammonium sulfate and pure enzymes according to the above procedure. Brought three tubes each tubes added 100 µL homogenate, 100 µL ammonium sulfate and 100 µL pure enzyme respectively. Also added 4900 µL Coomessie Brilliant Blue G-250 solution to each tubes total volume of solutions must be equal to 5 mL in each tubes. After that mixed samples with the vortex and left to incubated for 10 minutes. Then absorbance values reading at 595 nm on UV-spectrophotometer. If there was high absorbance of samples without standard proteins, the samples must be diluted until the range of standard proteins.

Table 3.7. Preparation of samples to measure amount of protein contain

Samples	Homogenate	Ammonium sulfate	Pure enzyme
Samples volume	100 µL	100 µL	100 µL
Dye volume	4900 µL	4900 µL	4900 µL

The results obtained amount of protein in samples in µg by drawn the graphic between the absorbance in Y-axis and concentration in X-axis of standards protein solutions. The graphic curve gave an equation ($y = a x + b$). Used to calculate the amount of protein in samples. The absorbance of samples were known and put in state of Y value in equation and calculate X value, that is the value of protein contained in the samples, repeat it for each sample (Bradford 1976).

3.4. Characterization Study of GST Enzyme

3.4.1. Optimum pH

To determine the optimal pH of the GST enzyme, in both buffers are potassium phosphate with pHs 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and Tris / HCl with pHs 7.8, 8.0, 8.5, 9.0 were prepared. With appropriate substrate solution, the GST enzyme activity were determined at 340 nm by spectrophotometer separately in each buffer.

3.4.2. Stable-pH

To detect the GST enzyme at which pH is stable, from the 50 mM potassium phosphate buffer prepared six solutions with pHs of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Also from the 50 mM Tris-HCl buffer prepared four solutions with pHs of 7.5, 8.0, 8.5, and 9.0. Then from those indicated solutions 750 µL and from purified enzyme 250 µL added to Eppendorf tubes for each buffer solution after that stored all tubes at + 4 °C. These solutions were used as an enzyme source. The GST enzyme activity measured at 340 nm on spectrophotometer. % activity were found as against time (days) at 24 hour intervals for 7 days under optimal conditions.

3.4.3. Optimum ionic strength

To determine the optimum ionic strength to the GST enzyme, from the 1.0 M potassium phosphate prepared 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 M and from the 2.0 M Tris-HCl prepared 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 M with the pH = 8.0 for all solutions. Then measures GST enzyme activity from each solution at 340 nm by spectrophotometer. To prepare different concentration solution from 1 M potassium phosphate and 2 M Tris-HCl prepared used dilution equation.

$$M_1 \times V_1 = M_2 \times V_2 \quad (3.4)$$

M_1: Molar of concentrate solution
M_2: Molar of dilution solution
V_1: Volume of concentrate solution
V_2: Volume of dilution solution

Table 3.8. Procedure to prepared different concentration solution from 1 M potassium phosphate stock solution in 10 mL to determined ionic strength

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 3.9. Procedure to prepared different concentration solution from 2 M Tris-HCl stock solution in 10 mL to determined optimum ionic strength

Concentration (M)	Stock solution (mL)	DW (mL)
0.1	0.5	9.5
0.2	1	9
0.4	2	8
0.6	3	7
0.8	4	6
1.0	5	5
1.2	6	4
1.4	7	3

3.4.4. Optimum temperature

To determine optimum temperature to GST enzyme used water bath in which prepared cuvette contained all reagent except enzyme stood in water bath at 0 °C for three minutes after that cuvette taken out and added enzyme immediately measured activity at 340 nm by spectrophotometer. Repeat above process as increased temperature at 10, 20, 30, 40, 50, 60, 70, 75, and 80 °C, respectively, in water bath.

3.4.5. K_M and V_{max} values for GST enzyme

To determine the K_M and V_{max} values for GSH and CDNB substrates with GST enzyme, activity of GST enzyme was measuring. The process should be made at 5 different concentrations of CDNB and constant GSH concentration. Line weaver-Burk graph plotted with the obtain data. From the curve of graph obtained an equation, the values of K_M and V_{max} for CDNB substrate were determined by using that equation. In the same way, activity measurement with 5 different concentrations of GSH and constant CDNB concentration. Draw the Line Weaver-Burk graph between concentration and activity data obtained then calculated K_M and V_{max} values for GSH. Activity measurements was performed under optimal condition (Line Weaver et al. 1934).

4. RESULTS

4.1. Enzyme Purification Studies

Glutathione S-transferase enzyme from quail heart tissue was purified based on protein purification process. The process was performed in three steps to purify GST enzyme: The first step homogenate was preparing from quail heart tissue. The second step ammonium sulfate precipitation process and the third step glutathione-agarose affinity column chromatography.

4.1.1. Homogenate preparation

Homogenate sample was prepared from heart tissue of quail bird (Section 3.2.1).In which quails brought into the lab then the heart separated. 4 gram of heart tissue was cut to small piece and suspended in 12 mL buffer solution by ultra-turrax device then centrifuged for one hour with 13,000 rpm at 4 °C. After that precipitate discarded and supernatant kept, it was homogenate sample that obtained with 10 mL volume and 0.632 EU/mL enzyme activities.

4.1.2. Ammonium sulfate precipitation (salting out)

Enzyme in homogenate sample was precipitated or collapsed by ammonium sulfate precipitation process (Section 3.2.3). In which subsequently performed, start from 0% to 100% ratio of ammonium sulfate were added to the homogenate. The maximum GST enzyme was precipitated at 60-70% ratio of ammonium sulfate with indicate the maximum GST enzyme activity than others. So selected that to continue study. The results were showed in Table 4.1 and Figure 4.1.

Table 4.1. Ammonium sulfate precipitated results

Ammonium sulfate%	Precipitate (EU/mL)	Supernatant (EU/mL)
0-20	0	0.565
20-30	0.170	0.569
30-40	0.177	0.562
40-50	0.225	0.531
50-60	0.770	0.423
60-70	1.753	0.138
70-80	0.5	0.052
80-90	0.211	0.041
90-100	0.079	0.027

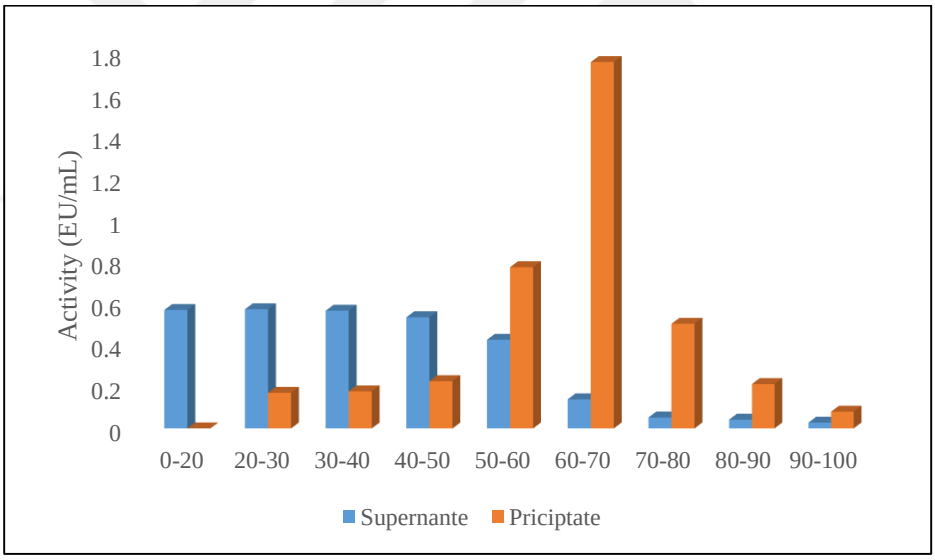

Figure 4.1. Results of ammonium sulfate precipitation

4.1.3. GST elution from glutathione-agarose affinity column chromatography

Purification on glutathione-agarose affinity column was performed to 60-70% ratio added ammonium sulfate salt in to homogenate (Section 3.2.4).That ratio precipitated maximum amount of GST enzyme in homogenate sample and gave maximum activity than others. Since it used as a sample, after dialyzed then applied to the column to purification GST

enzyme. Then accumulated 20 Eppendorf tubes. Finally GST activity were measured to all tubes at 340 nm by spectrophotometer. The followed results were obtained in Table 4.2 and Figure 4.2.

Table 4.2. Pure enzyme tubes from glutathione-agarose affinity column results

No. Tubes	EU/mL
1	0.097
2	0.131
3	0.118
4	0.329
5	0.170
6	0.145
7	0.118
8	0.190
9	0.156
10	0.145
11	0.180
12	0.201
13	0.225
14	0.125
15	0.177
16	0.145
17	0.118
18	0.121
19	0.104
20	0.055

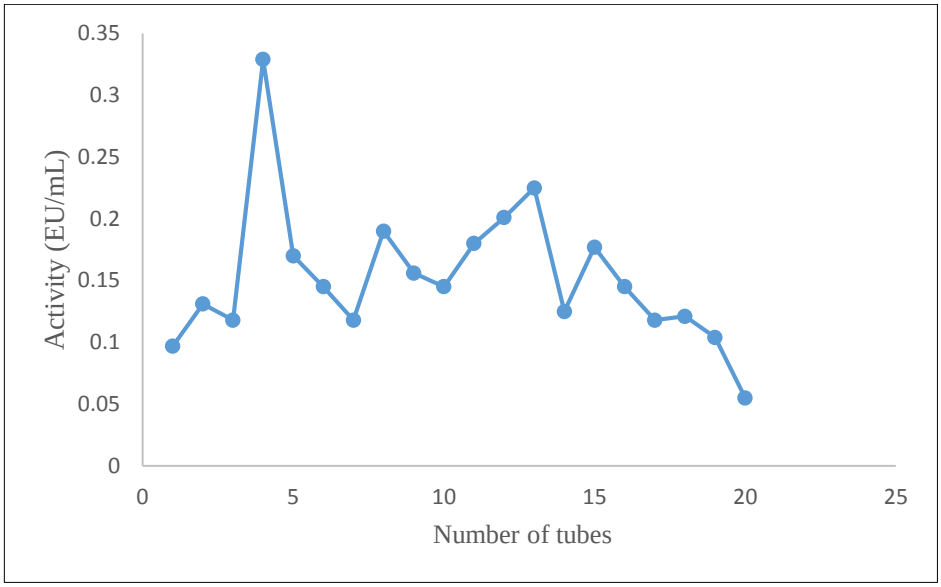

Figure 4.2. Pure enzyme tubes from glutathione agarose affinity column

4.1.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis results

After got purified GST enzyme by glutathione-agarose affinity column, then applied purified enzyme to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) process to check purified GST enzyme (Section 3.2.5). The results gave a single band on the SDS-PAGE photograph, it means that enzyme was purified. There was an evidence for realized it. Result was showed from Figure 4.3.

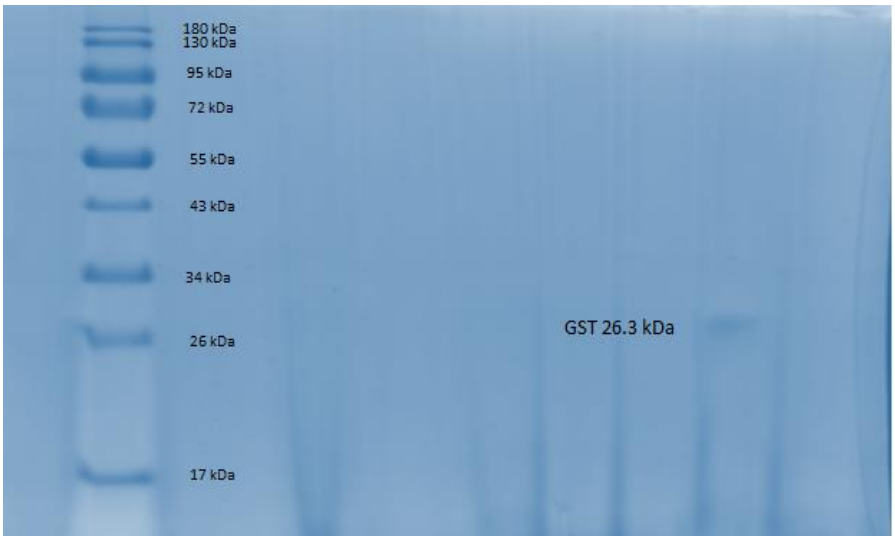

Figure 4.3. SDS-PAGE photograph show a single band

4.1.5. Determination of molar mass of GST enzyme with SDS-PAGE method

Single band was obtained on SDS-PAGE after applied purified enzyme also there were a standard protein bands on the SDS-PAGE, logarithm took to standard protein molecular weights also R_f-value took for each bands of standard proteins and R_f-value GST pure enzyme band on SDS-PAGE. Then graphic drawn between the logarithm-value of MW versus R_f-value. There was obtained an equation from the curve. Used this equation to determined GST enzyme molecular weight as 26.3 kDa. Result were shown from Table 4.3 and Figure 4.4.

Table 4.3. R_f and log MW results

R_f-of bands	Log-MW
0.21	4.85
0.3	4.74
0.386	4.63
0.51	4.53
0.63	4.41

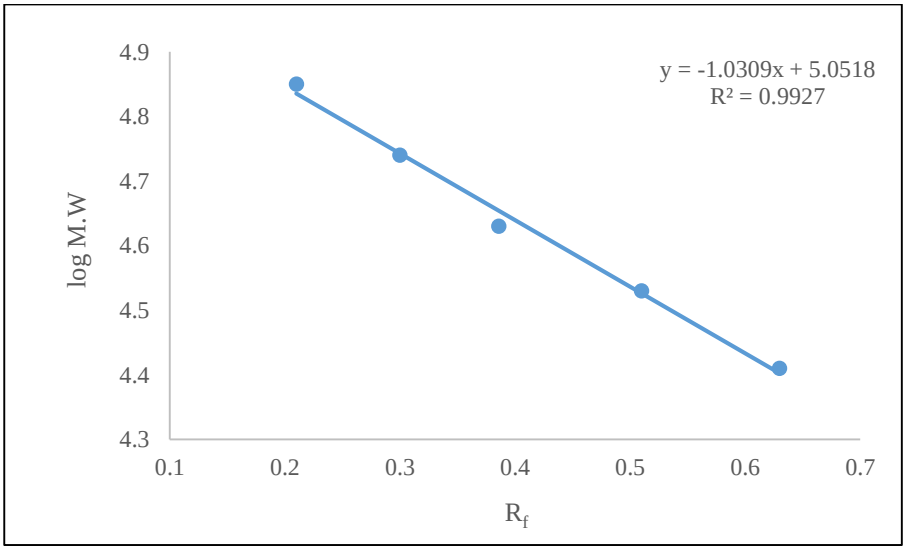

Figure 4.4. R_f-log MW calibration curve to determine MW of GST enzyme

4.1.6. Purification yield, purification fold and specific activity

Determined the amount of protein in the samples from each steps of purification by Bradford methods (Bradford 1976), these results with enzyme activities and volume samples in each steps were used to calculate the purification yields, purification folds and specific activity to purified glutathione S-transferase enzyme from the heart tissue of quail in each steps of purification process. The results showed in Table 4.4.

Table 4.4. Quail`s heart tissue glutathione S-transferase purification results table

Samples	Volume mL	Activity EU/mL	Total activity EU	Protein content mg/mL	Total protein mg	Specific activity EU/mg	Purification yield%	Purification fold
Homogenate	10	0.632	6.319	1.455	14.55	0.434	100	1
Ammonium sulfate precipitation	2	1.753	3.506	1.189	2.378	1.474	55.494	3.395
Glutathione agarose Affinity column chromatography	2	0.329	0.659	0.0097	0.0194	34.0	10.439	78.296

4.2. Protein Determination

4.2.1. Qualitative protein determination

Absorbance for eluate measured from the glutathione-agarose affinity during the washing process at 280 nm by spectrophotometer, without added GST elution (Section 3.3.1). Until the last difference in absorbance was 0.05. It means that GST enzymes had banded to the glutathione-agarose affinity gel in the column and other proteins were eluate through the column under the washing process and this indicated that it was the time to elution process on the column to purification target protein in the sample.

4.2.2. Quantitative proteins determination

The quantity of protein was determined according to the Bradford method (Bradford 1976) and the amount of protein was determined as microgram 335.5 µg, 308.7 µg and 9.7 µg in homogenate, ammonium sulfate and pure enzyme samples respectively (Section 3.3.2). To calculate protein quantity in each step of purification process the equation obtained from the curve in the graphic between the concentrations against absorbance was used. Results showed in Table 4.5 and Figure 4.5.

Table 4.5. Absorbance and amounts of protein results

Content protein as (µg)		Absorbance at 595 nm
Standards	10	1.181
	20	1.253
	30	1.319
	40	1.352
	50	1.436
	60	1.464
	70	1.559
	80	1.642
	90	1.690
	100	1.768
Samples	Homogenate	3.260
	Ammonium sulfate	3.089
	Pure enzyme	1.175

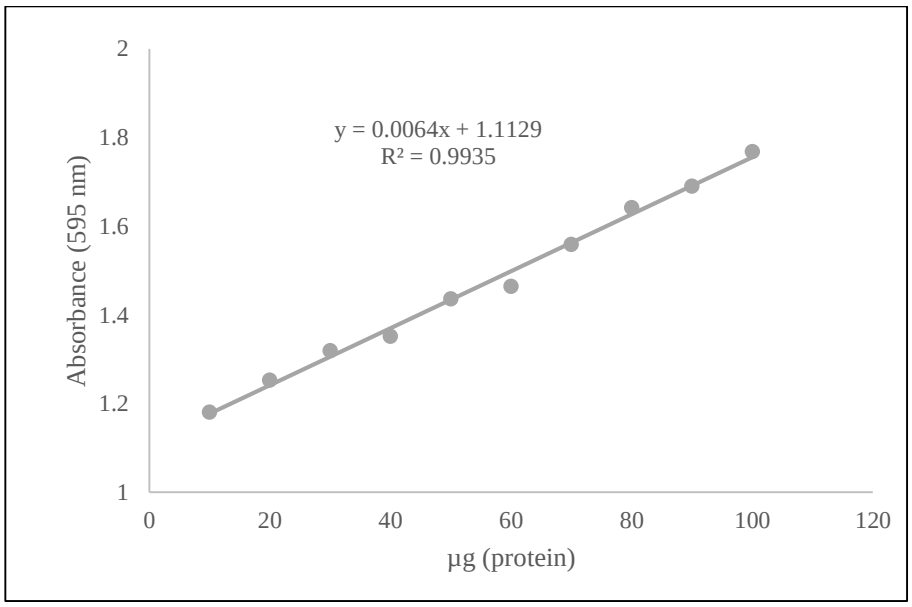

Figure 4.5. Standard protein calibration curve

4.3. Characterization Studies

4.3.1. Stable pH

Stable pH determine for GST enzyme in two different buffer solution (Section 3.4.2). In which the activity of GST enzyme was measured at 340 nm by spectrophotometer at 24 hours interval and during one week in those different pH solutions were prepared of both different buffer, the pH stability was determined for GST enzyme from the quail heart tissue as 9.0 in Tris-HCl buffer. Since the GST enzyme was more stable and show high enzyme activity in pH = 9.0 in Tris-HCl than other pH of buffers. Results were shown in Table 4.6, 4.7 and Figure 4.6, 4.7.

Table 4.6. Results of stable pH studied with KH_2PO_4 buffer

Time (days)	pH=5.5 Activity (EU/mL)	pH=6.0 Activity (EU/mL)	pH=6.5 Activity (EU/mL)	pH=7.0 Activity (EU/mL)	pH=7.5 Activity (EU/mL)	pH=8.0 Activity (EU/mL)
1	0.031	0.055	0.069	0.118	0.184	0.211
2	0.034	0.027	0.052	0.107	0.156	0.225
3	0.048	0.048	0.062	0.100	0.204	0.253
4	0.020	0.038	0.069	0.090	0.170	0.222
5	0.013	0.086	0.065	0.114	0.118	0.138
6	0.072	0.065	0.093	0.159	0.260	0.326
7	0.041	0.059	0.100	0.149	0.260	0.309

Table 4.7. Results of stable pH studied with Tris-HCl buffer

Time (days)	pH=7.5 Activity (EU/mL)	pH=8.0 Activity (EU/mL)	pH=8.5 Activity (EU/mL)	pH=9.0 Activity (EU/mL)
1	0.152	0.326	0.743	1.236
2	0.121	0.302	0.729	1.142
3	0.159	0.361	0.788	1.260
4	0.173	0.371	0.857	1.253
5	0.145	0.291	0.920	1.298
6	0.239	0.458	1.020	1.600
7	0.204	0.420	1.079	1.694

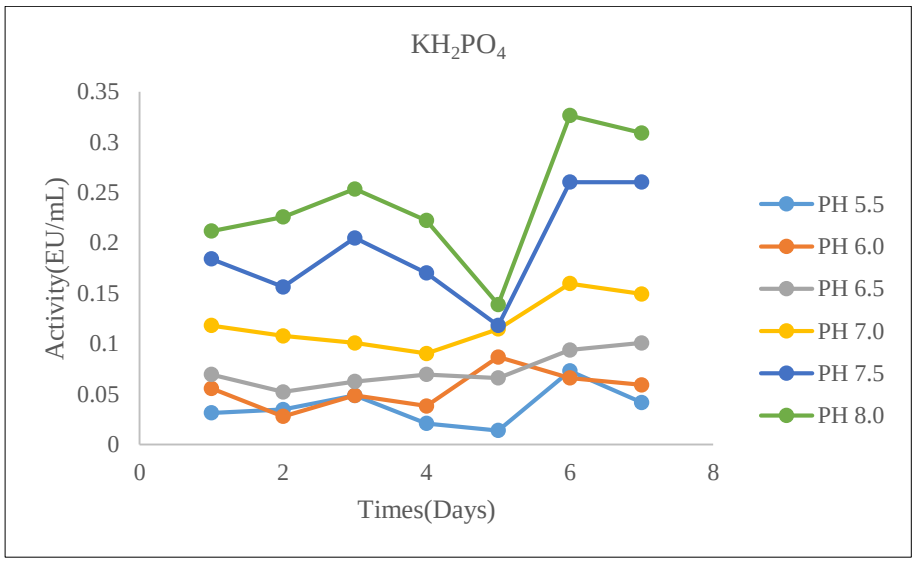

Figure 4.6. Graph of stable pH studied with KH_2PO_4 buffer

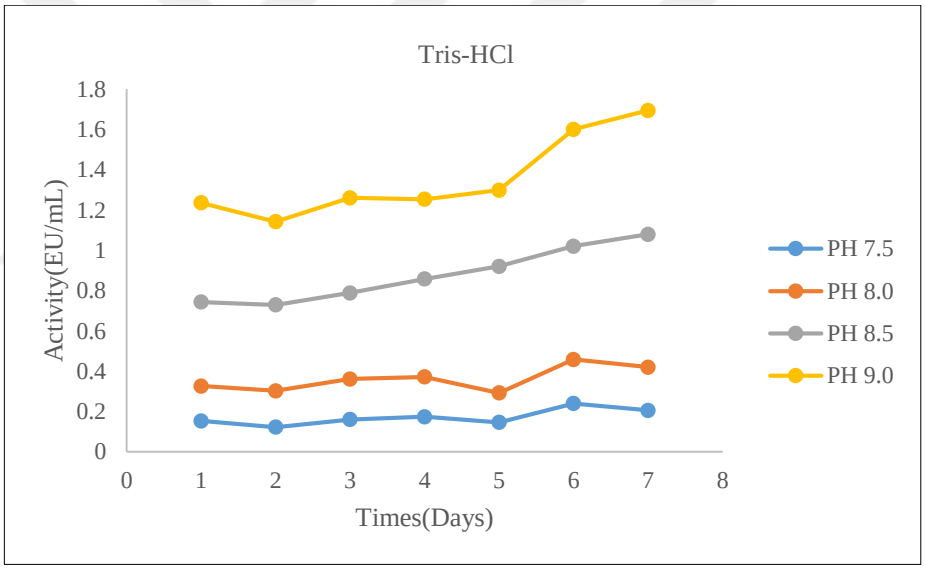

Figure 4.7. Graph of stable pH studied with Tris-HCl buffer

4.3.2. Optimum pH

Optimum pH determine for GST enzyme in two different buffer solutions (Section 3.4.1). In which the activity of GST enzyme measured in those different pH-solutions prepared from both buffer. The obtained results showed that the optimum pH was equal to 8.0 because GST enzyme have maximum activity in pH = 8.0 in Tris-HCl buffer. Result were shown in Table 4.8, 4.9 and Figure 4.8.

Table 4.8. Results of optimum pH studied with KH_2PO_4 buffer

pH	Activity (UE/mL)
5.5	0.059
6.0	0.128
6.5	0.232
7.0	0.440
7.5	1.062
8.0	2.711

Table 4.9. Results of optimum pH studied with Tris-HCl buffer

pH	Activity (UE/mL)
7.5	1.951
8.0	3.222
8.5	3.034
9.0	1.246

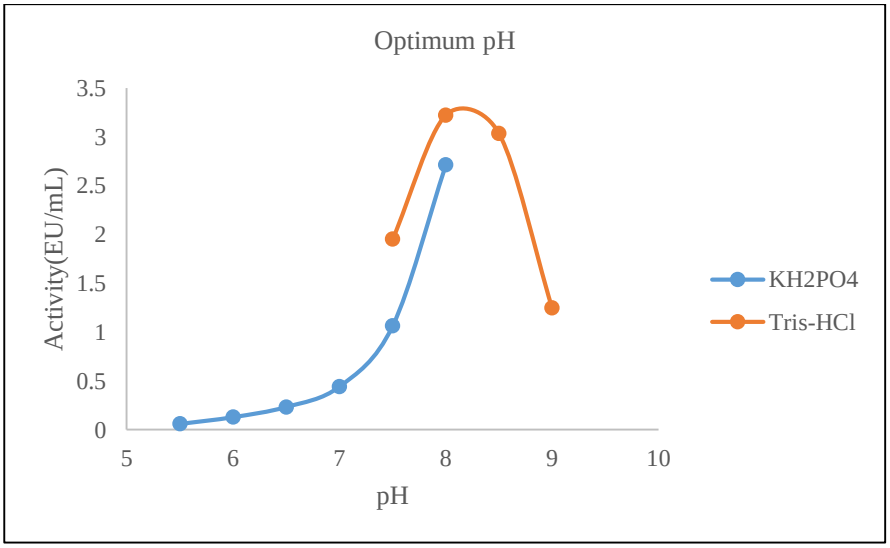

Figure 4.8 the graph of optimum pH results

4.3.3. Optimum temperature

Optimum temperature for GST enzyme was determined by measuring the activity for GST enzyme in different temperature started from 0 °C to 80 °C (Section 3.4.4). The obtained result showed the optimum temperature equal to 60 °C because GST enzyme have maximum activity at 60 °C than others temperature. Result were shownn in Table 4.10 and Figure 4.9.

Table 4.10. Results of optimum temperature

Temperature °C	Activity (EU/mL)
0	0.284
10	0.503
20	0.659
30	0.777
40	0.857
50	0.909
60	0.927
65	0.805
70	0.052
75	0.027
80	0.007

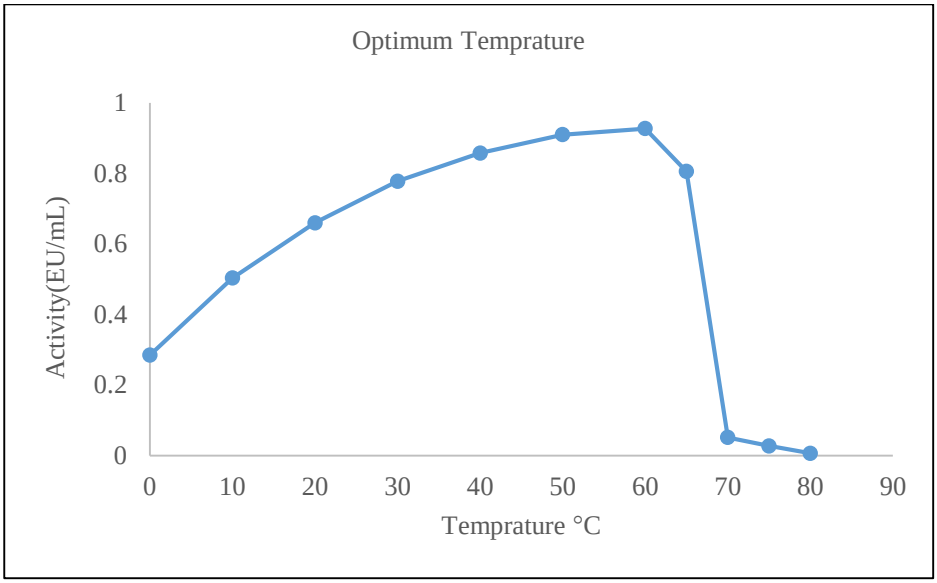

Figure 4.9. Results of optimum temperature

4.3.4. Optimum ionic strength

The effect of ionic strength on GST enzyme from the quail heart tissue was determined by the measured the activity of GST enzyme in several different concentrations of two buffer solutions which were prepared (Section 3.4.3). The obtained results showed, the optimum ionic strength equal to 1.2 M of Tris/HCl buffer, since GST enzyme have maximum activity in this concentrations than others. Results were shown in Table 4.11, 4.12 and Figure 4.10.

Table 4.11. Results of optimum ionic strength KH_2PO_4

Concentration (M)	Activity EU/mL
0.2	0.520
0.4	0.534
0.6	0.604
0.8	0.739
1.0	0.677

Table 4.12. Results of optimum ionic strength Tris-HCl buffer

Concentration (M)	Activity EU/mL
0.2	0.482
0.4	0.604
0.6	0.642
0.8	0.663
1	0.756
1.2	1.416
1.4	1.194

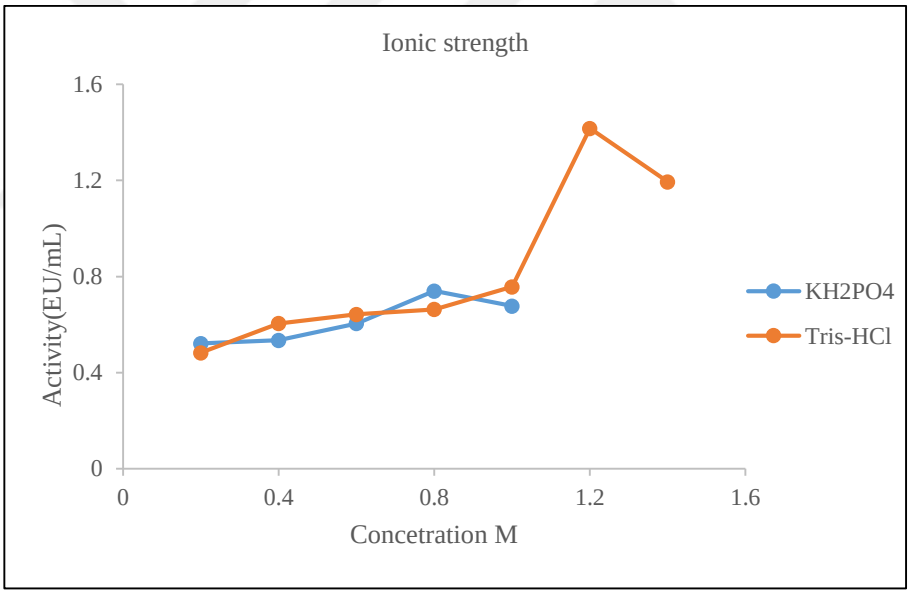

Figure 4.10. The results of optimum ionic strength (KH_2PO_4 and Tris-HCl buffer)

4.4. Kinetic Studies

Kinetic study had done to determine K_M and V_{max} for GST enzyme from the quail heart tissue (Section 3.4.5). GST enzyme activity was measured in five different concetrations of Glutathione reduced with constant CDNB concetration. Also the perocess were repeating in five different concntrations of CDNB with constant GSH concetration. Results

were used to draw line weaver-Burk graphic. An equation obtained from graphic curve used to calculate the K_M and V_{max}. Result were shown in Table 4.13, 414 and Figure 4.11, 4.12.

Table 4.13. Results studied of five different CDNB with constant GSH

Substrate [CDNB] 1/mM	1/velocity $(mmol/min)^{-1}$ mL
0.8	7.02439
0.4	4
0.266	3.6
0.228	3.555
0.133	2.360

Table 4.14. Results studied of five different GSH with constant CDNB

Substrate [GSH] 1/mM	1/velocity $(mmol/min)^{-1}$ mL
2	8.470
1	5.333
0.5	3.740
0.333	3.2
0.25	2.548

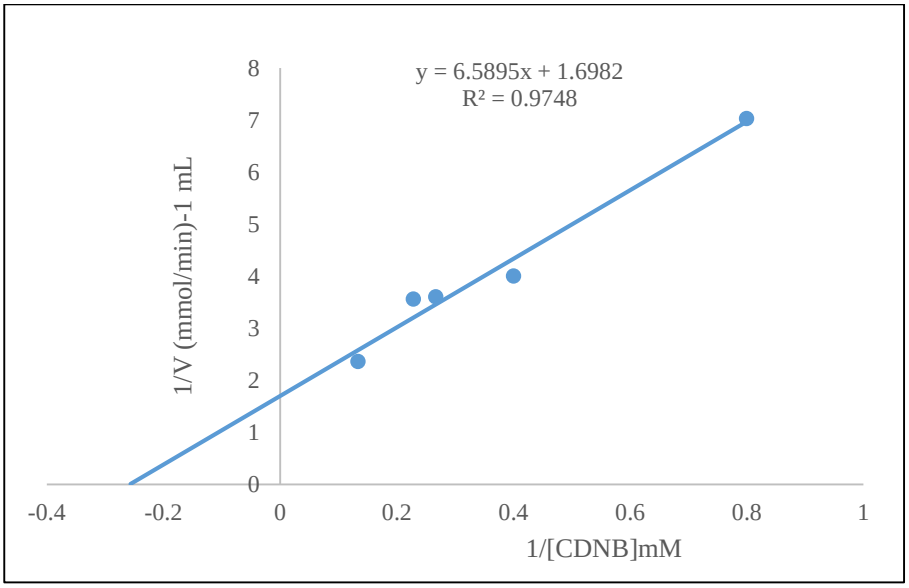

Figure 4.11. Lineweaver-Burk graph with five different CDNB concentration

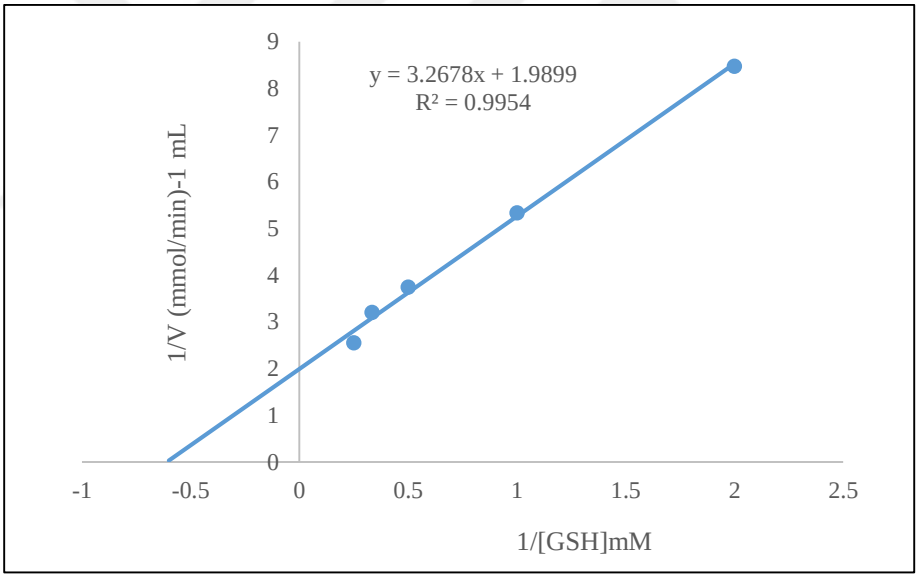

Figure 4.12. Lineweaver-Burk graph with five different GSH concentration

Table 4.15. K_M and V_{max} value for both CDNB and GSH

Substrates	V_{max}	K_M
CDNB	0.588	3.880
GSH	0.502	1.642

The K_M value was greater for CDNB substrate than K_M value for GSH substrate that indicated the glutathione S-transferase have low affinity to CDNB substrate than the affinity to GSH substrate. Results were shown in Table 4.15.

5. DISCUSSION

Glutathione *S*-transferase (GST, EC 2.5.1.18) is an enzyme that was discovered in 1961 (Booth et al. 1961). There are abundant proteins encoded by a highly divergent, ancient gene family. These cellular detoxification enzymes exist mostly from kidney and liver as well as intestine also present in other tissue such as heart, brain and erythrocyte. GSTs are intracellular proteins, play important role in the intracellular transport of endogenous compounds, able to metabolizes various electrophilic xenobiotic compounds, ligand transport and thus are protects living organism cells against toxic influence (George 1994). GSTs can catalysis the conjugation of glutathione to different electrophiles and reverse conjugation of various electrophiles compounds with glutathione (Yu 1996). Because of GST response of detoxifying both endogenously and exogenously derived toxic compounds (Cancado et al. 2005).

Glutathione S-transferase enzymes superfamily were classified into at least four major families of proteins, which include soluble or cytosolic GSTs, microsomal GSTs, mitochondrial GSTs and bacterial fosfomycin-resistance proteins (Hayes et al. 2005 and Scarcella et al. 2012). Which the cytosolic GSTs (cGSTs) have been sub-grouped for numerous divergent classes based on their physical and chemical property of structure (Sheehan et al. 2001). The mitochondrial GSTs was known as kappa GSTs class, it is soluble enzymes that have been characterized in eukaryotes cell (Robinson et al. 2004). The microsomal GSTs are third family class includes membrane-bound transferase known as membrane-associated proteins that participate in glutathione and eicosanoids metabolism, but these endure no similarity to soluble GSTs (Jakobsson et al. 1999). Representative of above three GST families class also exist in prokaryotes cells but the four GSTs family was only found in bacteria (Allocati et al. 2009). The soluble mammalian GSTs enzyme are classified based on their amino acid sequences into eight classes, those includes (α, κ, μ, ω, π, σ, θ and ζ) (Umasuthan et al. 2012).

Detoxification pathway consist of phases6: phase I is the transformation reactions which are performed by a family of enzymes called cytochrome P450s (CYPs), Phase II enzymes are involving protect against xenobiotic compounds and Phase III is the transport phase. This phase of detoxification participate the elimination of toxins from organism cells. The side products formed from phase I and II reactions are transported out of the living organism cells into the bloodstream and remove xenobiotic compounds to excretion from the body (Ziglari and Allameh 2013). In detoxification pathway Phase II conjugation consist of reactions in which metabolism process containing appropriate functional groups are conjugated with substrate such as reduced glutathione, sulfated, glutamate, and glucoronate. Commonly conjugation process cause the improve solubility then promote rapidly excretion. Between the many types of conjugation reactions which are exist in the body, include glutathione, sulfation, amino acid and glucuronidation conjugations. In which glutathione conjugation is catalyzed by glutathione S-transferase enzyme. Phase II is the major reaction in most species (Liska 1998). The biotransformation strategy of phase II is to convert a xenobiotic compounds to a more hydrophilic form via the attachment of a chemical moiety which is ionizable at physiological pH results of metabolic transformation is reduced affinity of xenobiotic compounds for its cellular target (Sacco 2006). Between the detoxification pathways, glutathione conjugation pathway process is the prominent route of Aflatoxins B1 (AFB1) inactivation in body tissue of mammalians, based on available of GSH in cellular and the activation of glutathione S-transferase enzyme subclasses, detoxification of AFB1 is facilitated (Eaton and Gallagher 1994).

$$GSH + RX \xrightarrow{GST} GSR + HX \qquad (5.1)$$

GSTs catalyze the reaction between a reduced glutathione (GSH; glutamyl–cysteinyl–glycine) and a reactive electrophilic center for various substrates, as a result a polar S glutathionylated product formed to reducing toxicity (Dixon et al. 2002)

Glutathione is a ubiquitous thiol-containing isodipeptide (γ-glu-cys-gly), consisting of glutamate, cysteine and glycine amino acids. It is a water soluble antioxidant. It is in the same family with α-tocopherol (vitamin E), ascorbic acid (vitamin C), β-carotene, and flavonoids *etc.* GSH is referred as the common antioxidant (Cotgreave and Gerdes 1998). Neutralizes free radicals by donating electron of sulfhydryl (-SH) group and prevent damage to the major tissue cellular components by associated in the defense of cellular against toxicity (Cancado et al. 2005). GSH is most sensitive detector of cell functionality

and sustainability. It is concentrating in human tissue range from 0.1 to 10 mM especially in liver tissue up to 10 mM and in the heart , kidney, spleen, erythrocyte , lens and leukocytes and it is empting be joined to variety of diseases. In normal condition GSH is predominantly exist as reduced glutathione form with less proportion present as fully oxidized state (Dixon et al. 1998). In addition the GSH/GSSG joint with their high reduction potential involved in maintaining other cellular thiol in reduced state. At the end GSH tends as a substrate or cofactor from several enzyme GSH linked. There are some GSH linked enzymes those are participate into protection cellular contrast toxic compounds (Thornalley 1993). These enzymes are glutathione reductase, glutathione S-transferase, glutathione peroxidase and glyoxalase (Arthur 2000). Also GSH dependent enzymes with most properties that catalyzed the GSH conjugation with deferent electrophilic substances and is the one of the most investigated function (Edalat 2002).

The glutathione antioxidant system in enzymatic antioxidant includes glutathione S-transferase, glutathione reductase and glutathione peroxidase. Glutathione S-tansferase enzymes are the major class of glutathione-dependent antioxidant enzymes. It exists in high level especially in the liver tissue (Sharma et al. 2004). GST enzyme is the proteins with multifunctional family along with various gene families have play an important role in body protection process from xenobiotic compounds as antioxidant enzymes. It protects cells from toxins via biotransformation reactions in the cytoplasm (Awasthi et al. 1994). Additionally glutathione S-transferase enzymes are working as antioxidant in detoxification xenobiotic compounds from endogenous and exogenous of the living organism cell like other neutral antioxidant participate in the protection of body from free radical and other toxic substance by catalyzing the conjugation of glutathione with the toxic compound cause the improvement detoxification process (Hayes et al. 2005).

Study on glutathione S-transferase enzymes from the various tissue of organisms or plants include the isolation, purification, investigation, estimation, characterization and inhibition studies by a variety of methods that are available to determine the quantity and quality of enzymes, on the other hand to know enzymes property.

In this master study, glutathione S-transferase enzyme for the first time was purified from the Japanese quails heart tissue based on the protein purification process with three steps. After purification, characterization work were carried out. Glutathione agarose affinity

column chromatography method was used to purify the GST enzyme. It is available and powerful method because it is an easy process to apply, low cost, less time, single step, potable for bulk amounts of enzymes purified and gives very good purification yields.

Initially homogenate was prepared from the fresh heart tissue of Japanese quail. Review literature homogenate were prepared for GST purified from rat brain (Senjo and Ishibashi 1986), human kidney for (Singh et al. 1987), human placental tissue (Kwak and Park 1988), filarial worms *Setaria cervi.* (Ahmad et al. 2008), rat liver (Essam et al. 2009), turkey liver (Akkemik et al. 2012), liver of the Van Lake fish (Ozaslan and Ciftci 2014) and muscle tissue of Van Lake fish (Aksoy et al. 2016).

Ammonium sulfate precipitation (salting out) process was performed to the homogenate sample that is the purification step in process. The ammonium sulfate is the more soluble salt cause the precipitate or collapse protein molecules based on solubility of protein molecules in the sample solution, because ammonium sulfate is dissociate in sample solution to strength ions and have influence to attraction the solvent molecules to surrounding the ions than proteins, since proteins are not ions. While the salt ions surrounding by solvent proteins are accumulated together and precipitated because of less soluble and heavy than other molecules. Salting out process increased enzyme activity approximately two to five folds and riddance from the unwanted Materials. Review in literature salting out performed for GST purified from human placental tissue (Kwak and Park 1988), filarial worms *Setaria cervi.* (Ahmad et al. 2008), turkey liver (Akkemik et al. 2012). Dialysis process was performed. This process like a dilution process to riddance from the ammonium sulfate salts in this sample because of the dialysis sac is semepermeable membrane allow to pass out the small molecules from the sample to the dialysis buffer solution, but larger molecules like proteins remained in the sac. After that enzyme sample is ready to apply the column.

Finally enzymes mixture sample applied to the glutathione-agarose affinity column to purified and separated target enzyme from others based on the affinity of target molecule to the glutathione liganding agarose gel bed in the column. The column was prepared and equlibrated. Then apply the sample to the column and the column was washed to complete flow out other unbinded enzymes through the column. During elution process fill several tubes of eluate of column that contains pure target enzyme and kept them for further

studies. (Güvercin et al. 2008 and Toribio et al. 1996). The same method was used for purification of GST enzyme from human hepatoma (Dierickx 1989), catfish intestinal mucosa (Gadagbui and James 2000), liver of the freshwater fish *Monopterus albus* (Huang et al. 2008), *L. Bostrychophila* (Dou et al. 2009), Down syndrome (DS) and normal children erythrocytes (Hamed et al. 2011), rat liver (Lebda et al. 2012), liver of the Van Lake fish (Ozaslan and Ciftci 2014) and muscle tissue of Van Lake fish (Aksoy et al. 2016). Also different method were used for purification of GST enzyme such as Sephadex G-75, glutathione-linked Sepharose 6B and CM-cellulose column chromatography from rat brain (Senjo and Ishibashi 1986), S-hexylglutathione Sepharose 6B affinity column from human placental tissues (Kwak and Park 1988), using Q-Sepharose ion exchange chromatography and glutathione affinity chromatography in the fungus Cunningham Ella elegans (Cha et al. 2001), cation exchange and glutahione-sepharose affinity chromatography from filarial worms *Setaria cervi* (Ahmad et al. 2008) and anion exchange chromatographic step used DEAE-Cellulose, then cation exchange chromatographic CM-Cellulose was used, followed by subsequent chromatography on Sephacryl S-200 in the rat liver (Essam et al. 2009). In which the glutathione agarose affinity column chromatography most powerful and one step to purification with the great result than others.

Glutathione S-transferase enzyme was purified by glutathione-agarose gel affinity column and to check the result to know that the enzyme was purified or not, applied pure enzyme to the sodium doduecyl sulfate-polyacrylamaide gel electrophorsis (SDS-PAGE). In which prepared gel and pure enzyme was denatured by heating for 30 minutes and a sample solution used to cause the negativity charge of proteins. Then apply negative denaturated pure enzyme sample into the different wells of gel with the standard protein, then connected to the electric field. According to the Laemmli method 1970. In SDS-PAGE the proteins are moving through the gel based on the size and the charge under the effect of electric field in which the larger proteins slowly moved than small proteins through the gel from the negative charge toward the positive charge. Then SDS-PAGE were stained and distained and single band was appeared on the gel. If there was not appeared single band or appeared two or more band on the SDS-PAGE it means that the enzyme was not purified but appearing a single band on the SDS-PAGE is the evidence to purified enzymes (Laemmli et al. 1970). The same method were used in rat brain cytosol (Senjo and Ishibashi 1986), human placental (Kwak and Park 1988), human hepatoma (Dierickx 1989), and rainbow trout hepatocytes (Riol et al. 2001), filarial worms *Setaria cervi* (Ahmad et al.

2008), rat liver (Essam et al. 2009), turkey liver (Akkemik et al. 2012), liver of the Van Lake fish (Ozaslan and Ciftci 2014), and muscle tissue of Van Lake fish (Aksoy et al. 2016). SDS-PAGE method is suitable and powerful to check purity of enzymes with good results by less time and easy to work. In this study the single band was appeared on SDS-PAGE that is used to calculate the molar mass of glutathione S-transferase enzyme by taking the R_f-value for standard protein bands and GST enzyme single band, the result value was a drawn plot between R_f-value and logarithm of molar mass of standard proteins. The molar mass was determined as 26.3kDa for GST enzyme from the heart tissue of Japanese quail in which compared with heterodimers of MW 26.5 kDa and MW 24.5 kDa subunit in human kidney (Singh et al. 1987), 25 kDa from the human placental tissues (Kwak And Park 1988), 27 kDa in maize (zea mays pioneer hybrid 3906) (Irzyk et al. 1993), 26 kDa from the sorghum cereal (Gronwald and Plaisance 1998), 26.7 kDa from catfish intestinal mucosa (Gadagbui and James 2000) 26 kDa and 24 kDa from the turkey liver (Akkemik et al. 2012) and two subunits as 28 kDa and 33.8 kDa from liver of the van lake fish (Ozaslan and Ciftci 2014) in literature**.** This comparison illustrates that the different molar mass and dimeric protein attributed to the GSTs are isozyme and ubiquitous distributed. In which GST molar mass in quail heart near to the GST in human kidney, turkey liver, sorghum cereal than human placental, liver of the Van Lake fish, mazie (zea mays pioneer hybrid 3906), and catfish intestinal mucosa.

The protein concentration of GST enzyme was calculated as 1.455, 1.189 and 0.0097 mg/mL for homogenate, ammonium sulfate and pure enzyme respectively according to the Bradford methods 1976, another protein assay was performed for GST enzyme in which maximum absorbance was measured at 280 nm spectrophotometrically based on presence of tryptophan, tyrosine and phenyl alanine amino acids in the structure of protein. Because those amino acids are able to absorbs UV-VIS light that attributed to contain the aromatic branch in amino acids. Aromatic compound have able to absorb light due to the resonance of double bound in the ring structure. Also calculated the total protein, total activity, specific activity, purification yield and purification fold from each step of purification process in this study, by using activities, volumes and protein contents of enzyme sample. In which the total activity results are 6.319, 3.506 and 0.659 EU/mL, results of total protein are 14.55, 2.378 and 0.0194 mg, result of specific activity are 0.434, 1.474 and 34.0 EU/mg, result of purification yield are 100, 55.494 and 10.439% and result of purification fold are 1.0, 3.395, and 78.296 folds for homogenate, ammonium sulfate and pure enzyme

respectively. To pure enzyme got specific activity 34.0 EU/mg, purification yield 10.439% and purification fold 78.296 folds in which compared with specific activity of 23.7 EU/mg, 11% yield, 1107-fold purification from the human placental tissues (Kwak and Park 1988), specific activity 1.250 x 10^{-6} unit/mg protein, 56.43% yield and 419.88 fold from the rat liver (Essam et al. 2009), specific activity of 164.31 U/mg, a yield of 45%, with 252.7-folds from the turkey liver (Akkemik et al. 2012), specific activity 29,304 EU / mg protein, purification fold 316,11 folds and purification yield 37.36% from the liver of the Van Lake fish (Ozaslan and Ciftci 2014) and specific activity of 35,583 EU/mg protein, purification fold 301.5-folds, a purification yield 19.07% from the muscle tissue of Van Lake fish (Aksoy et al. 2016) in literature. Variation in results due to the amount of GSTs present from different parameters or different tissues with study techniques.

After purifying GST enzymes from the heart tissue of Japanese quail, characterization study was performed to determine the optimum pH, optimum ionic strength, optimum temperature, pH-stability and K_M and V_{max} values. First, pH-stability study was determined. The result obtained from study illustrated GST enzyme from quails heart are more stable and show high enzyme activity at pH = 9.0 in the Tris/HCl buffer than other pH. The result was compared with stable-pH =7.5 in Down syndrome (DS) and normal children erythrocytes (Hamed et al. 2011), stability-pH = 8.5 in turkey liver (Akkemik et al. 2012) and stable-pH = 5.5 liver of the Van Lake fish (Ozaslan and Ciftci 2014), the result was closest to the stable pH in turkey liver in literature**,** pH-stability is most important point during this study on all enzymes because of keeping enzymes in stable pH help to have a best results and continues study for a longest interval on target enzymes, since enzymes are not denatured, loss activity and biological functions to a long time. By continuing the study optimum pH was determined, results obtained that illustrated the optimum pH for GST enzyme from quails heart tissue equal to 8.0 in Tris-HCl buffer which GST enzyme has maximum activity at pH = 8.0. Compare to optimum pH = 7.5 – 8.0 from the maize (zea mays pioneer hybrid 3906) (Irzyk et al. 1993), optimum pH = 7 from the Down syndrome (DS) and normal children erythrocytes (Hamed et al. 2011), optimum pH = 7.3 from the turkey liver (Akkemik et al. 2012) and optimum pH = 7.8 from the liver of the Van Lake fish (Ozaslan and Ciftci 2014) in the literature in which result was similar to the optimum pH of GST from the liver of the Van Lake fish and maize (zea mays pioneer hybrid 3906). In the living organism cell each enzyme works in specific pH called optimum pH in which it has maximum activity. Since pH is the important factor that have

influence on enzyme activity at below and above optimum pH all enzymes loss activity and denatured especially in high acidic and basic medium.

Also temperature is another factor that have effect on enzyme activities, like all reaction enzyme activities increase with increase temperature but for enzyme increasing temperature is limited because at very high temperature all enzymes are denatured this is the lose biological function and lose enzymes activity. In this study optimum temperature was determined, suggesting the optimum temperature for GST enzyme is equal to 60 ºC. In which above that degree GST loses activity until the 80 ºC the activity becomes zero. Compared with optimum temperature in range 30 °C– 55 °C from the liver of the freshwater fish Monopterus albus (Huang et al. 2008), optimum temperature between 25-35ºC from the rat livers (Lebda et al. 2012), optimum temperature = 50 °C in turkey liver (Akkemik et al. 2012) and optimum temperature = 30 °C from the liver of the Van Lake fish (Ozaslan and Ciftci 2014) in the literature in which result nearest to the optimum temperature of GST from the turkey liver and liver of the freshwater fish Monopterus albus. In continuous study, the effect of ionic-strength on glutathione S-transferase was studied, and the results obtained illustrated that the optimum ionic-strength for this enzyme is 1.2 M of Tris-HCl buffer pH = 8.0. The optimum ionic strength for GST 600 mM turkey liver (Akkemik et al. 2012), 100 mM in liver Van Lake fish (Ozaslan and Ciftci 2014), in which the result in different from the literature, the GST enzyme has maximum enzyme activity at that concentration, in below and above that concentration, the activity was less.

In addition, enzyme kinetic study was performed to determine K_M and V_{max} for glutathione S-transferase from the Quails heart tissue. The results obtained are 1.642 mM and 0.502 EU/mL respectively for GSH substrate and 3.880 mM and 0.588 EU/mL respectively for CDNB substrate. The results is compare with K_M 0.085 mM for GSH and 2.0 mM for CDNB from the human placental tissues (Singh et al. 1987), K_M and V_{max} for CDNB are 0.28 mM and 15.68 EU/ml, respectively from the liver of the freshwater fish Monopterus albus (Huang et al. 2008), K_M values 0.786 mM for GSH and 0.205 mM for CDNB from human erythrocyte (Hamed et al. 2011), K_M and V_{max} values 0.154 mM, 1.803 EU/mL for GSH and 0.380 mM, 2.125 EU/mL for CDNB substrate, respectively for GST enzyme in turkey liver (Akkemik et al. 2012), K_M and V_{max} determined as 0.060 mM, 0.562 EU / mL for GSH and 0.891 mM, 1.245 EU / mL for CDNB, respectively from the liver of the Van Lake fish (Ozaslan and Ciftci 2014), K_M and V_{max} determined as 0.53mM, 1.88 EU/mL

for GSH and 1.59 mM, 5.58 EU/mL for CDND, respectively from the muscle tissue of Van Lake fish (Aksoy et al. 2016), In the literature. Results illustrated that the GST enzyme has low affinity for CDNB substrate than GSH substrate because the K_M value for CDNB substrate greater than K_M value for GSH substrate. The result was similar to the GST enzyme in liver and muscle tissue of the Van Lake fish, turkey liver and human placental tissues, but vice versa with human erythrocyte. Also the results illustrated that the V_{max} for both GSH and CDNB are closest value but V_{max} for CDNB substrate was greater than V_{max} for GSH substrate it mean the CDNB substrate concentration has the more influence on the rate of reaction that catalyzed by GST enzyme than GSH substrate concentration, in which results was similar to the GST from turkey liver, muscle tissue of Van Lake fish and liver of the Van Lake fish.

6. CONCLUSION

Glutathione S-transferase enzyme is the enzyme that ubiquitously distributed from the nature present in animals, plants and in bacteria with the Enzyme Commission (EC: 2.5.1.18) from the family of transferase class in enzyme classification, that has an important role from detoxification process of cellular against the effects of xenobiotic compounds to protect cells by catalyzing the conjugation of glutathione molecule into the toxic compounds. GSTs represent an important group of enzymes that detoxify both endogenous compounds and foreign chemicals, such as pharmaceuticals and environmental pollutants and GSTs expressed as antioxidant enzyme as the glutathione is the natural antioxidant like α-tocopherol (vitamin E), ascorbic acid (vitamin C), β-carotene and others play major in neutralizing free radicals attributed to the capacity of sulfhydryl (-SH) group donating more electron and prevent damage to the major tissue cells components by associated in the defense of cellular against toxicity.

In this study glutathione S-transferase for the first time was purified and characterized from the Japanese quail heart tissue. The heart tissue was successfully isolated from the Japanese quail, homogenate was prepared and ammonium sulfate precipitation performed then using glutathione-agarose gel affinity column chromatography. The enzyme was purified. The present study showed that the GST enzyme exist in the heart tissue with molar mass 26.3 kDa and activity towards glutathione and CDNB substrates. Also obtained results are 34 EU/mL, 78.29 folds and 10.439 % of specific activity, purification folds and purification yields respectively for purified GST enzyme.

According to obtained results from the characterization of purified GST enzyme in this study, that illustrated the glutathione S-transferase from the quail heart have the following property.

- Stable-pH = 9.0 in Tris/HCl buffer
- Optimum pH = 8.0 in Tris-HCl buffer
- Optimum temperature = 60℃
- Optimum ionic strength = 1.2 M Tris-HCl buffer
- K_M and V_{max} value determined as 1.642 mM, 3.880 mM and 0.502 EU/mL, 0.588 EU/mL for Glutathione and 1-chloro 2, 4-dinitrobenzen as substrate respectively.

GSTs are the best to study of their evolutionary biochemistry, and how an adaptable protein stand can be adapted to support multiple and useful activities which can then undergo selective fine alteration over generations. In turn, such study investigation also helps to answer major questions in yield science relating to the very high titer and diversity of GSTs from the various tissue. Research is in progress to further characterize the purified protein and to study the potential use of the enzyme.

It has been demonstrated both GSTs and GSH substrate have extended major roles that much further than simple detoxification reactions. Indeed, it is not unreasonable to predict that glutathionylation may provide regulatory control complementary to other well-studied and established posttranslational modifications. Future studies will extra experience with an advanced knowledge of the proteins participated in the cells response to “stress” and the interplay of proteins within the cells, with not only themselves in an enzymatic manner, but with other proteins in a regulatory fashion.

REFERENCES

Arakawa S, Maejima T, Kiyosawa N, Yamaguchi T, Shibaya Y, Aida Y, Kawai R, Fujimoto K, Manabe S Takasaki W (2012) Methemoglobinemia induced by 1,2-dichloro-4-nitrobenzene in mice with a disrupted glutathione S-transferase mu 1 gene. The American Society for Pharmacology and Experimental Therapeutics 38: 1545-1552

Ahmed M, Kabel (2014) Free Radicals and Antioxidants: Role of Enzymes and Nutrition. World Journal of Nutrition and Health 2(3): 35-38

Ahmad R, Srivastava A, Walter R (2008) Purification and biochemical characterization of cytosolic glutathione-S-transferase from filarial worms Setaria cervi. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 151(3): 237-245

Aksoy M, Ozaslan M, Kufrevioglu O (2016) Purification of glutathione S-transferase from Van Lake fish (Chalcalburnus tarichii Pallas) muscle and investigation of some metal ions effect on enzyme activity. Journal of enzyme inhibition and medicinal chemistry 31(4): 546-550

Armstrong R (2006) Glutathione S-Transferases: Structure and Mechanism of an Archetypical Detoxication Enzyme. Advances in Enzymology and Related Areas of Molecular Biology Volume 69: 1-44

Allocati N, Federici l, Masulli M, Dillio C (2009) Glutathione transferases in bacteria. The FEBS Journal 276: 58-75

Arthur JR (2000) the glutathione peroxidases Cell Mol Life Sci 57: 1825-35

Akkemik E, Taser P, Bayindir A, Budak H, Ciftci M (2012) Purification and characterization of glutathione S-transferase from turkey liver and inhibition effects of some metal ions on enzyme activity Environmental toxicology and pharmacology 34(3): 888-894

Awasthi YC, Sharma R, Singhal SS (1994) Human glutathione S-transferase. International Journal of Bio- chemistry 26: 295-308

Boyce S, Tipton K, McDonald AG (2004) Extending enzyme classification with metabolic and kinetic data: some difficulties to be resolved In Proceedings of the 1st International Beilstein Workshop on Experimental Standard Conditions of Enzyme Characterizations. Beilstein Institut Frankfurt Germany: 17-43

Benson AM, Talalay P, Keen JH, Jakoby WB (1977) Relationship between the soluble glutathione-dependent Dalta 5-3-ketosteroid isomerase and the glutathione S-transferase of the liver proceedings of the National Academy of Sciences USA 74: 158-162

Bradford MM, (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analitical Biochemistry 72, 248

Buettner GR (1993) the pecking order of free radicals and antioxidants Lipid peroxidation, a-tocopherol, and ascorbate. Arch Biochem Biophys 300: 535-543

Bradford MM (1976) a rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. analytical biochemistry 72(1–2): 248–54

Booth J, Boyl E, Sims P (1961) An enzyme from rat liver catalyzing conjugations with glutathione Biochem J 79: 516-24

Croom E (2012) 3 Metabolism of Xenobiotics of Human Environments Progress in molecular biology and translational science 112, 31

Cuesta SM, Syed AR, icholas Furnham, Janet MT (2015) The classification and evolution of enzyme function. Biophysical journal 109 no. 6: 1082-1086

Cha CJ, Coles BF, Cerniglia CE (2001) Purification and characterization of a glutathione S-transferase from the fungus Cunninghamella elegans. FEMS microbiology letters 203(2): 257-261

Campbell MK, Farrell SO (2009) biochemistry thomson brooks/cole usa sixth edit: 141-160

Cancado GM, De Rosa Jr VE, Fernandez JH, Maron LG, Jorge RA, Menossi M (2005) Glutathione S-transferase and aluminum toxicity in maize. Functional Plant Biology 32: 1045-1055

Cotgreave IA, Gerdes RG (1998) recent trends in glutathione biochemistry glutathione-protein interactions: a molecular link between oxidative stress and cell proliferation. Biochem Biophys Res Commun 242: 1-9

Duarte TL, Lunec J (2005) When is an antioxidant not an antioxidant A review of novel actions and reactions of vitamin C Free Radic Res 39 (7): 671-86

De Simone A, Aprile FA, Dhulesia A, Dobson CM, Vendruscolo M (2015) Structure of a low-population intermediate state in the release of an enzyme product. Elife 4 e02777

Dierickx PJ (1989) Purification and characterization of glutathione S-transferase from the human hepatoma derived PLC/PRF/5 cell line. Biomedical research 10(4): 301-306

Dixon D, Lapthorn A, Edwards R (2002) Plant glutathione transferases. Genome Biology 3: reviews3004

Dou W, Wu S, Hassan MW, Wang JJ (2009) Purification and biochemical characterization of glutathione S-transferases from three strains of Liposcelis bostrychophila Badonnel (Psocoptera: Liposcelididae) Implication of insecticide resistance. Pesticide biochemistry and physiology 94(1): 10-14

Dixon DP, Cummins L, Cole DJ, Edwards R (1998) Glutathione-mediated detoxification systems in plants. Current opinion in Plant Biology 258-266

Dixon DP, Lapthorn A, Edwards R (2002) Plant glutathione transferases. Genome biology 3(3): reviews3004-1

Essam FA, Al-Jumaily Zahraa F, Ameen Ayad H, Jassim Dece (2009) Isoalaion and purification of glutathion s.transferase from the rat liver. Biological department geneticEngineering and Biotechnology Institute,Baghdad University Baghdad-Iraq 12(4): 137-144

Ezeji EU, Anyalogbu EA, Ezejiofor TN, Udensi JU (2012) Determination of reduced glutathione and glutathione S-transferase of poultry birds exposed to permethrin insecticide. American Journal of Biochemistry 2(3): 21-24

Eaton DL, Gallagher EP (1994) Mechanisms of aflatoxin carcinogenesis. Annu Rev Pharmacol Toxicol 34: 135-172

Edalat M (2002) Multiple functions of glutathione transferase. Dissertation for the Degree of Doctor of Philosophy in Biochemistry presented at Uppsala University

Findlay VJ, Townsend DM, Tew KD (2006) Glutathione and Glutathione S-Transferases in Drug Resistance. In Cancer Drug Resistance: 213-221 Humana Press

Ferruzzi E, Franceschini R, Cazzolato G, Geroni C, Fowst C, Pastorino U, Gion M (2003) Blood glutathione as a surrogate marker of cancer tissue glutathione S-transferase activity in non-small cell lung cancer and squamous cell carcinoma of the head and neck. European Journal of Cancer 39(7): 1019-1029

Güvercin S, Erat M, Şakiroğlu H, (2008) Determination of Some Kinetic and Characteristic Properties of Glutathione S-transferase from Bovine Erythrocytes Protein & Peptide Letters 15(1): 6-12

Gadagbui BK, James MO (2000) Activities of affinity-isolated glutathione S-transferase (GST) from channel catfish whole intestine. Aquatic Toxicology 49(1): 27-37

Gronwald JW, Plaisance KL (1998) Isolation and Characterization of GlutathioneS-Transferase Isozymes from Sorghum. Plant physiology 117(3): 877-892

George SG (1994) Enzymology and molecular biology of phase II xenobiotic conjugating enzymes in fish. Aquatic toxicology, molecular biochemical and cellular perspectives (Eds: D Malins and GKOstrander) Lewis Publishers Ann Arbor, 27-86

Hayes JD, Pulford DJ (1995) The glutathione S-transferase supergene family regulation of GST and the contribution of the lsoenzymes to cancer chemoprotection and drug resistance part II. Critical reviews in biochemistry and molecular biology 30(6): 521-600

Hayes JD, Pulford DJ, (1995) the glutathione S-transferase supergene family regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and durg resistance. Critical Reviews in Biochemistry and Molecular Biology 30: 445-600

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases The first enzymatic step in mercapturic acid formation. The Journal of Biological Chemistry 249(22): 7130-7139 Iersel ML Plo

Huang Q, Liang L, Wei T, Zhang D, Zeng QY (2008) Purification and partial characterization of glutathione transferase from the teleost Monopterus albus. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 147(1): 96-100

Hamed RR, Maharem TM, Abdel-Meguid N, Sabry GM, Abdalla AM, Guneidy RA (2011) Purification and biochemical characterization of glutathione S-transferase from Down syndrome and normal children erythrocytes A comparative study. Research in developmental disabilities 32(5): 1470-1482

Huang Q, Liang L, Wei T, Zhang D, Zeng QY (2008) Purification and partial characterization of glutathione transferase from the teleost Monopterus albus. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 147(1): 96-100

Habig WH, Pabst MJ, Jakoby WB, (1974) Glutathione S- transferases The first enzymmatic step in mercapturic acid formation J Biol Chem 246: 7130-7139

Hayes JD, Flanagan JU, Jowsey IR (2005) Glutathione transferases. Annu Rev Pharmacol Toxicol 45: 51-88

Irzyk GP, Fuerst EP (1993) Purification and characterization of a glutathione S-transferase from benoxacor-treated maize (Zea mays). Plant physiology 102(3): 803-810

Jakobsson PJ, Morgenstern R, Mancini J, Ford-Hutchinson A, Persson B (1999) Common structural features of MAPEG- a widespread superfamily of membrane-associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. Protein Sci 8: 689-692

Kwak SJ, Park SC (1988) Purification and Characterization of Glutathione S-transferase π from Human Placental Tissues

Ketterer B, Meyer DJ, Taylor JB, Pemble S, Coles B, Fraser G (1990) GSTs and protection against oxidative stress In Glutathione S-Transferase and Drug Resistance. Hayes JD, Mantle TJ, Pickett CB (eds) Taylor and Francis Bristol: 97-109

Kurland IJ, Pilkis SJ (1995) Covalent control of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase insights into autoregulation of a bifunctional enzyme Protein. Sci 4 (6): 1023–37

Klaassen CD (1996) Casarett and Doulls Toxicology The Basic Sciece of Poisons 5th edition McGraw-Hill New YORK

La Roche SD, Leisinger TH (1990) Sequence analysis and expression of the bacterial dichloromethane dehalogenase structural gene, a member of the glutathione S-transferase supergene family. Journal of bacteriology 172(1): 164-171

Litwack G, Ketterer B, Arias IM (1971) Ligandin a hepatic protein which binds steroids bilirubin carcinogens and a number of exogenous anions Nature (London) 234: 466-467

Lebda M, Taha N, Noeman S, Korshom M, Mandour A, El-Leboudy A (2012) Purification and Characterization of Glutathione S-transferase from Rat′ s Liver Effect of Carbon Tetrachloride and Camel′ s Milk. J Chromat Separation Techniq 3(133): 2

Laemmli DK (1970) Cleavage of structural proteins during in assembly of the heat of bacteriophage T4. Nature London 227-680

Lineweaver H, Burk D (1934) The determination of enzyme dissocation constants. J Am Chem Soc 57-685

Laemmli UK, (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature 227: 680–85

Liska DJ (1998) The detoxification enzyme system. Alternative Medicine Review 3(3): 187-198

Mannervik B, Alin P, Guthenbergg C, Jensson H, Tahir MK.Warholm M, Jornvall H (1985) Identification of three classes of cytosolic glutathione transferase common to several mammalian species correlation between stracture dataand enzymatic properties Proceedings. National academy of sciences USA 82: 7202-206

Morgenstern R, DePIERRE JW (1983) Microsomal glutathione transferase. European Journal of Biochemistry 134(3): 591-597

Mora D, Arioli S (2014) Microbial urease in health and disease. PLoS Pathog 10(12): e1004472

Mathwes KC, VanHolde KE (1996) Enzyme kinetic enzyme catalystic and specifity Benjamin/Gummings publishing Company Biochemistry, pp 360-409

Martinek R (1969) Practical clinical enzymology. J Am Med Tech 31-162

Mannervik B (1986) Glutahione and the evalution of enzymes for detoxication of products of oxygen metabolism Chemica Scripta 263: 281-284

Mannervik BE, NGT (1985) The isoenzymes of glutathione transferase. Adv Enzymol Relat Areas Mol Biol 57: 357-417

Nelson D, Cox M (2013) lehninger principles of biochemistry wh freeman new york sixth edit: 180-200

Nicholson DW, Ali A, Vaillancourt JP, Calaycay JR, Mumford RA, Zamboni RJ, Ford-Hutchinson AW (1993) Purification to homogeneity and the N-terminal sequence of human leukotriene C4 synthase a homodimeric glutathione S-transferase composed of 18-kDa subunits *Proceedings of the National Academy of Sciences 90*(5): 2015-2019

Northrop DB, (1999) Enzyme kinetics enzyme cataylysis and specificity Rethinking fundamentals of enzyme action Advanced in enzymology 73: 25-55

Ozaslan MS, Ciftci M (2014) Purification of glutathione S-transferase from Van Lake fish (Chalcalburnus tarichii Pallas) liver and investigation of some metal ions effect on enzyme activity. Journal of enzyme inhibition and medicinal chemistry 31(4): 546-550

Ouaissi A, Ouaissi M, Sereno D (2002) Glutathione S-transferases and related proteins from pathogenic human parasites behave as immunomodulatory factors. Immunology letters 81(3): 159-164

Owuor ED, Kong ANT (2002) Antioxidants and oxidants regulated signal transduction pathways. Biochemical pharmacology 64(5): 765-770

Payen A, Persoz J (1833) Me´moire surla Diastase les principaux Produits de ses Re´actions et leurs applications aux arts industriels. Ann Chim Phys 53:73–92

Pabst MJ, Habig WH, and Jakoby WB, (1974) Glutathione S-transferase A. The Journal of Biological Chemistry 7140-7150

Riol MM, Valinas MN, Fernandez MG, Lopez MP (2001) Glutathione S-transferases from rainbow trout liver and freshly isolated hepatocytes purification and characterization. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 128(2): 227-235

Robinson A, Huttley GA, Booth HS, Board PG (2004) Modelling and bioinformatics studies of the human kappa-class glutathione transferase predict a novel third glutathione transferase family with similarity to prokaryotic 2-hydroxychromene-2-carboxylate isomerases. Biochem J 379: 541-552

Rinaldi R, Eliasson E, Swedmark S, Morgenstern R (2002) Reactive intermediates and the dynamics of glutathione transferases. Drug Metabolism and Disposition 30(10): 1053-1058

Simoni RD, Hill RL, Vaughan M (2002) The discovery of glutathione by F Gowland Hopkins and the beginning of biochemistry at Cambridge University. Journal of Biological Chemistry 277(24): e13-e13

Sharma R, Yang Y, Sharma A, Awasthi S, Awasthi Y (2004) Antioxidant role of glutathione S-transferases protection against oxidant toxicity and regulation of stress-mediated apoptosis Antioxid Redox Signal 6(2): 289-300

Sheehan D, Meade G, Foley VM (2001) Structure, function and evolution of glutathione transferases implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochemical Journal 360(1): 1-16

Seah TMC, Kaplan JG (1973) Purification and Properties of the Catalase of Bakers' Yeast. Journal of Biological Chemistry 248(8): 2889-2893

Sherratt PJ Hayes JD (2001) Glutathione S-transferases. In Enzymes Systems that Metabolise Drugs and Other Xenobiotics edited by C Ioannides University of Dundee UK John Wiley and Sons Ltd: 320-352

Simarani K, Yusoff WHA, Alias Z (2016) Purification of Glutathione Transferases (GSTs) from Identified Rhizospheric Bacteria Sains Malaysiana 45(7): 1057-1062

Senjo M, Ishibashi T (1986) Purification and characterization of glutathione S-transferase from rat brain cytosol identification of four isozymes and evidence for absence of the Ya subunit. Biomedical research 7(1): 19-26

Singh SV, Leal T, Ansari GA, Awasthi YC (1987) Purification and characterization of glutathione S-transferases of human kidney. Biochemical Journal 246(1): 179-186

Segel IH (1968) Biochemical Caculations Inc New York p: 40

Segel IH (1968) biochemical calculations how to solve mathematical problems in general biochemistry Wiley New York: 230

Scarcella S, Lamenza P, Virkel G, Solana H (2012) Expression differential of microsomal and cytosolic glutathione S-transferases in Fasciola hepatica resistant at triclabendazole. Molecular and Biochemical Parasitology 181(1): 37-39

Sheehan D, Meade G, Foley VM, Dowd CA (2001) Structure function and evolution of glutathione transferases implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochemical Journal 360: 1-16

Sacco JC (2006) Phase II biotransformation of xenobiotics in polar bear (Ursus maritimus) and channel catfish (Ictalurus punctatus). A dissertation presented to the graduate school of the University of Florida in partial fulfillment of the requirements for the degree of doctor of philosophy Waxman

Townsend DM, Tew KD (2003) The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene 22(47): 7369

Tipton K, Boyce S (2000) History of the enzyme nomenclature system. Bioinformatics16: 34–40

Takahashi Y, Campblell EA, Hirata Y, Takahyama T, Listowsky I (1993) A basis for differentiatingg among the multiple human Mu-glutathione S,transferase and molecular cloning of brain GSTM5. The Jornal of Biological Chemistry 286: 8893-8898

Toribio F, Martinez-Lara E, Pascual P, Lopez-Barea J (1996) Methods for purification of glutathione peroksidase and related enzymes. Journal of Chromatography B 684: 77-97

Thornalley PJ (1993) the glyoxalase system in health and disease. Mol Aspects Med 14: 287-371

Ulusu NN, Tandoğan B (2007) Purification and kinetic properties of glutathione reductase from bovine liver. Mol Cell Biochem 303(1-2): 45-51

Umasuthan N, Revathy KS, Lee Y, Whang I, Choi CY, Lee J (2012) A novel molluscan sigma-like glutathione S-transferase from Manila clam, Rudi tapes philippinarum Cloning characterization and transcriptional profiling. Toxicology & Pharmacology 155(4): 539-550

Valko M, Leibfritz D, Moncol J, Cronin M, Mazur M (2007) Free radicals and antioxidants in normal physiological functions and human disease. Biochem Cell Biol 39 (1): 44-84

Wingfield P (2001) protein precipitation using ammonium sulfate current protocols in protein science 53(4): a 3f 1-a 3f 8

Wan H, Zhan S, Xia X, Xu P, You H, Jin BR, Li J (2016) Identification and functional characterization of an epsilon glutathione S-transferase from the beet armyworm (Spodoptera exigua). Pesticide biochemistry and physiology 132: 81-88

Yu SJ (1996) Insect glutathione S-transferases. Review article Zoological Studies 35: 9-19

Zubay GL (1998) Basic prencipales of enzyme kinetics and catalysis and cofactors. Biochemistry 159-261

Ziglari T, Allameh A (2013) The Significance of Glutathione Conjugation in Aflatoxin Metabolism. In Aflatoxins-Recent Advances and Future Prospects InTech

PERSONAL BACKGRAOUND

I was born from Sulaymaniyah city in Iraq at February 20th of 1988, finished primary, secondary and high school from the Saidsadiq District in Sulaymaniyah, so started study in chemistry science at 2009 from the Garmian University in Iraq and graduated at 2013, held bachelor certificate, started work in chemistry department faculty of science from Garmian University at September 2013 until February 2016 as assistant research in chemistry lab, started master study in biochemistry at February 2016 from Bingol University in Turkey, and awarded master degree in biochemistry at December 2017.