PURIFICATION AND CHARACTERIZATION OF GLUTATHIONEREDUCTASE FROM JAPANESE QUAIL *(Coturnix Coturnix Japonica)* LIVER

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Master thesis

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GLUTATYON REDÜKTAZ ENZİMİNİN BILDIRCIN *(Coturnix Coturnix Japonica)* KARACİĞERİNDEN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

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CONTENT

LIST OF ABBREVIATINS

GR	:	Glutathione reductase
GSH	:	Reduced glutathione
GSSG	:	Oxidized glutathione
NADPH	:	Reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$	:	Nicotinamide adenine dinucleotide phosphate oxıdızed form
ES	:	Enzyme substrate complex
S	:	Substrate
E	:	Enzyme
P	:	Product
TEMED	:	Tetramethylethylenediamine
PER	:	Ammonium persulfate
SDS	:	Sodium dodecyl sulfate
PAGE	:	Polyacrylamide gel electrophoresis
DW	:	Distilled water
mL	:	Milliliter
g	:	Gram
M	:	Molar

mM	:	millimolar
L	:	Liter
mmol	:	Millimole
nm	:	Nanometer
EC	:	Enzyme commission number
EU	:	Enzyme unit
kDa	:	Kilodalton
MW		Molecular weight
DNA	:	Deoxyribonucleic acid
ROS	:	Reactive oxygen species
Tris	:	Tris (hydroxymethyl)aminomethane
R_F	:	Retardation factor
TCA	:	Trichloroacetic acid
EDTA	:	Ethylenediaminetetraacetic acid
PMSF	:	Phenylmethane sulfonyl fluoride
DTT	:	Dithiothreitol
BSA	:	Bovine serum albumin

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GLUTATYON REDÜKTAZ ENZİMİNİN BILDIRCIN (Coturnix Coturnix Japonica) KARACİĞERİNDEN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Yüksek lisans tezi olarak sunuçu bu çalşmadan, glutatyon redüktaz (Glutatyon: $NADP^+$, oksidoredüktaz, E.C 1.8.1.7.; GR) enzimi bıldırcın karaciğerinden amonyum sülfat çöktürmesi ve 2',5'-ADP Sepharose-4B afinite kromatografisi olmak üzere 2 basamakta saflaştırıldı. 22,75 EÜ/mg protin spesifik aktivitesine sahip olan GR enzimi %13,6 verimle, 142,18 kat saflaştırıldı. Enzim aktivitesi spektrofotometrik olarak 340 nm'de Beutler metoduna göre ölçüldü. Enzimin alt biriminin mol kütlesi ise SDS-PAGE ile 59 kDa olarak bulundu. Enzim için optimum pH, optimum iyonik şiddet, optimum sıcaklık, stabil pH, sırasıyla 8, 100 mM, 8, ve 85ºC olarak bulundu. Ayrıca NADPH için K_M değeri 0,109 mM ve V_{max} değeri 0,603 EÜ/mL olarak; GSSG için ise, K_M değeri 0,044 mM ve V_{max} değeri 0,272 EÜ/mL olarak tespit edildi.

Anahtar Kelimeler: Bıldırcın karaciğeri, glutatyon redüktaz, saflaştırma, karakterizasyon.

PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE REDUCTASE ENZYME FROM QUAIL (Coturnix Coturnix Japonica) LIVER

ABSTRACT

In this study, glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, E.C 1.8.1.7; GR) was purified from quail`s liver by using ammonium sulfate precipitation and 2',5'-ADP Sepharose-4B gel affinity chromatography. GR enzyme was obtained having a specific activity of 22.75 EU/mg proteins, with a yield 13.6% and 142.18 purification fold. Enzymatic activity was measured spectrofotometrically according to Beutler`s method at 340 nm. Subunits molecular weight of enzyme was determined by SDS-PAGE as 59 kDa. Optimal pH, optimal ionic strength, stable pH, optimal temperature were determined as 8.0, 0.6 mM, 8.0, 85 °C respectively. Also K_M and V_{max} values were determined for NADPH as 0.109 mM and 0.603 EU/mL; and for GSSG substrates 0.044 mM and 0.272 EU/mL respectively.

Keywords: Quail`s liver, glutathione reductase, purification, characterization.

1. INTRODUCTION

1.1. Enzyme

Catalysis is almost certainly the most important function of protein, most reaction in the absence of catalysis would take place exceedingly slowly to provide product at a sufficient pace for metabolizing organism, the enzymes are the catalysts in the organism cells, and they can accelerate the reaction rate over uncatalyzed reaction by a factor of up to 10^{20} (Campbell and Farrell 2009).

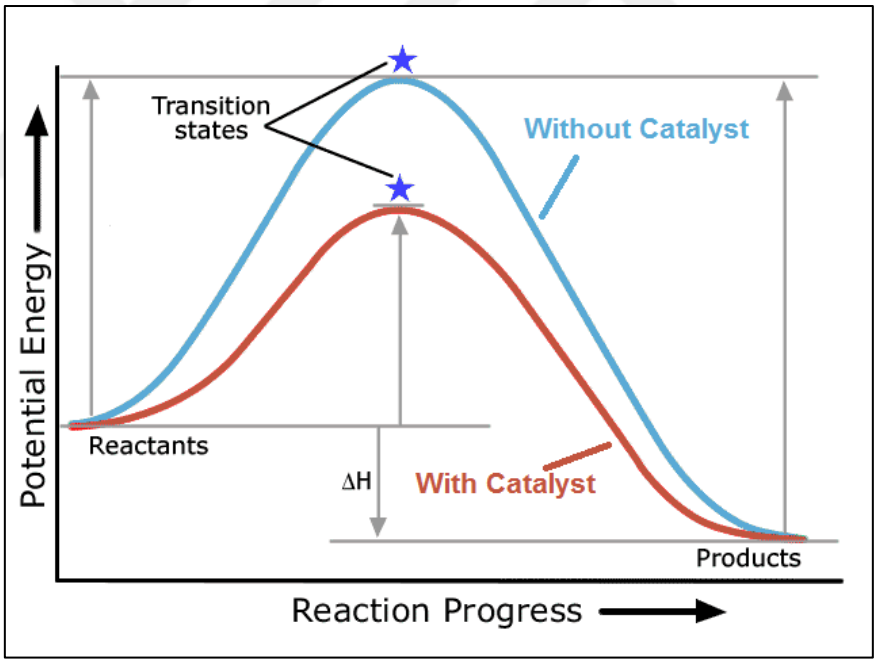

Figure 1.1. Effect of catalyst on the rate of reaction (http://catalystweek.org)

The study of an enzyme has enormous practical importance. In the absence or deficiency of one or more enzymes, may cause some diseases, particularly inheritable genetic disorder. And another disease might be caused by excessive of an enzyme activity. Measurements of enzyme activity in tissue, blood plasma, or erythrocytes sample are

Important in diagnosing assuredly diseases. Also, enzymes are important tools in food technology, chemical engineering, and agriculture. The binding of substrates to enzyme includes interaction between reactive amino acid groups that make up the active site of the enzyme and substrate, this show the appreciable specificity of enzyme for the binding substrate (Nelson and Cox 2013).

1.1.1. History Of Enzymes

Louis Pasteur in 1850 concluded that the "ferments." Catalyzes the fermentation of sugar into alcohol by yeast, he assumed that these ferments were inseparable from the cell structure of living yeast cell. Then Eduard Buchner in 1987 discovered that the fermentation of sugar to alcohol could be ferment by yeast extract. Later F.W. Kuhne gave the name enzyme to the molecules that detected by Buchner, which it means "leavened" from the Greek ''enzymos''. (Nelson and Cox 2013).

1.1.2. Nomenclatures And Classification

Enzymes in generally given names derived from the compound which they act, or the reaction that they catalyze. For example, glutathione reductase speeds up the reduction of glutathione oxidized by reduced nicotinamide adenine dinucleotide phosphate (NADPH). Hydrolysis of phosphate ester bonds are catalyze by acid phosphatase under acidic condition. As can be seen from the mentioned examples, most enzymes name end in ''-ase.'' However, some enzymes from the digestive tract, their names were assigned before clearly understood their actions, which don`t ends with "-ase" among these are trypsin, pepsin, and chymotrypsin. All enzymes have a formal enzyme commission (EC) numbers, for example; glutathione reductase have a commission number of (EC 1.8.1.7), that indicates to: EC 1 oxidoreductase class, EC 1.8 acting on a sulfur group of donors, EC 1.8.1 with $NADP^+$ as acceptor, EC 1.8.1.7 glutathione-disulfide reductase. According to the type of reaction which catalyzed by enzyme, enzyme was classified into six major classes (Rehm and Reed 1995).

Table 1.1.2**.** International nomenclature of enzyme, by; nomenclature committee of the international Union of biochemistry and molecular biology (NC-IUBMB)

Class	Reaction they catalyze	Typical example
1. Oxidoreductases	Oxidation-reduction in which hydrogen and oxygen are gained or lost	Glutathione reductase
2. Transferases	Transfer of functional groups, from one molecular to another	aspartate transaminase
3. Hydrolases	Hydrolysis (addition of water)	Acetylcholinesterase
4. Lyases	Addition of two groups to a double bond or removal of two groups from adjacent molecules to create a double bond.	Aconitase
5. Isomerases	isomerization reactions	Phosphohexose isomerase
6. Ligases or syn - thetases	joining of two molecules	Tyrosine-tRNA synthetase

1.1.3. Enzyme Structure

With exception of small catalytic group of RNA (ribozymes) molecules all enzymes are protein. The catalytic activity of enzyme depends on its native protein, usually enzyme losing their catalytic activity if enzyme denatured to its subunits. Like all other proteins enzymes have molecular weights between 12 to more 1000 kDa. Some enzymes, such as trypsin and pepsin only consist of polypeptide chains, they require no another group for activation, other enzymes require a nonprotein portion of their structure to work properly, these may be inorganic ions, such as Zn^{2+}, Fe^{2+}, Mg^{2+}, or Mn^{2+} which called cofactors, or may be organic or metalloorganic compound which called coenzyme, and most of them are derived from vitamins (Nelson and Cox 2013). A coenzyme or inorganic ions bound to the enzyme protein covalently or very tightly is called a prosthetic group. Inactive enzyme (without its cofactor) is called apoenzyme or apoprotein (Figure 1.2), and an active enzyme (with its cofactors) is called holoenzyme (Bettelheim et al. 2007).

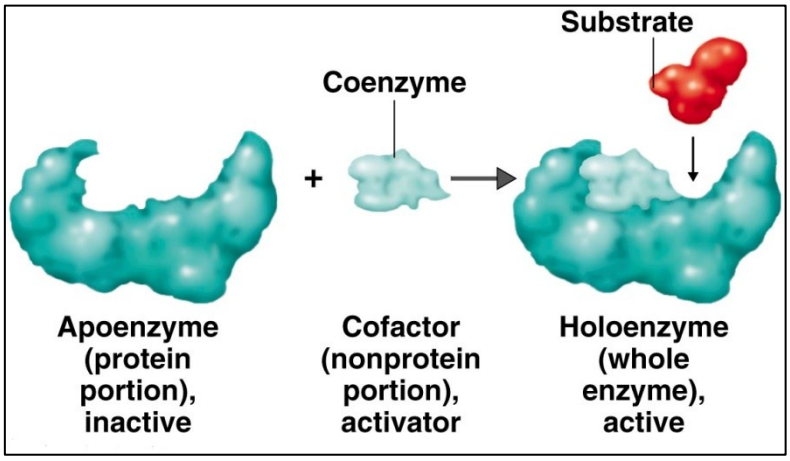

Figure 1.2. Activation of enzyme by coenzyme. Apoenzyme (inactive protein) activated by coenzyme, then formed enzyme-substrate complex http://www.slideshare.net/uweschaeruman/modul-6-biologi- kb-1

1.1.4. Mechanism Of Enzyme Action

The enzyme has a very large molecule, consist typically of 100 to 200 residues of amino acids, but only a few amino acid residues composed the active site of an enzyme. Action of the enzyme was first explained by lock-and-key models. This hypothesis assumed that the enzyme is a three-dimensional rigid body, and contains the active site, which has specific geometric shape to the substrate that fit exactly to each other (Figure 1.3) (Bettelheim et al. 2007).

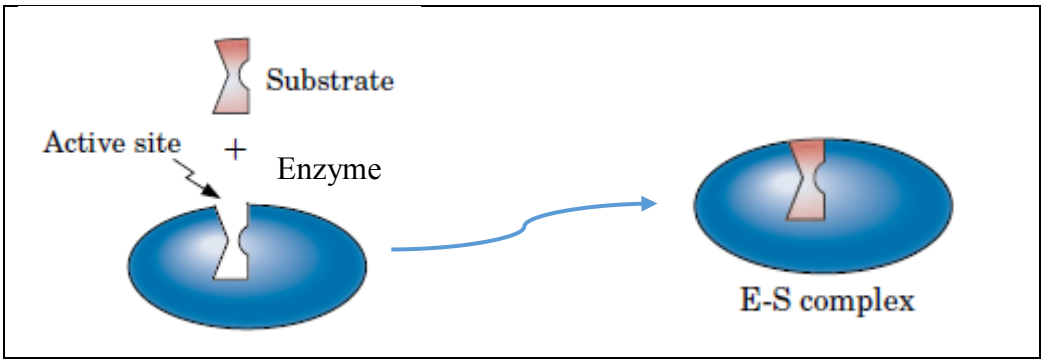

Figure 1.3. Lock and key model, the shape of active site of enzyme and substrate is completely fit to each others

Another model explained the change occurring on the shape of active site of the enzyme during binding the substrate, like a glove when hand is inserted it change its shape (Figure 1.4), this hypothesis called induced-fit model (Bettelheim et al. 2007).

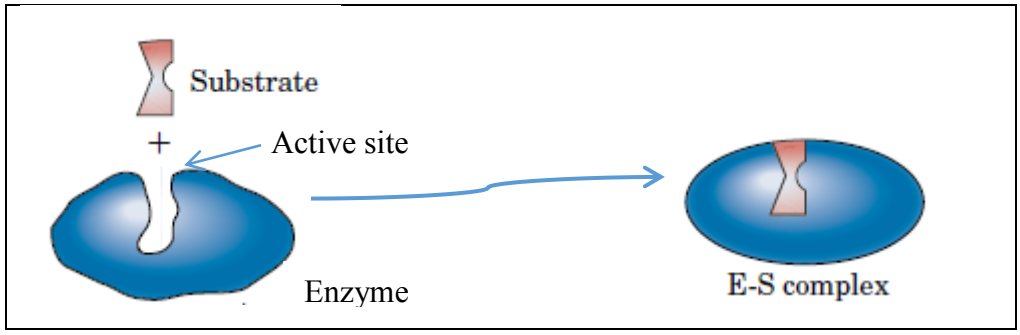

Figure 1.4. Induced fit model, active site of enzyme change its shape after binding substrate

1.1.5. Enzyme Kinetic

Enzyme kinetic is a branch of enzymology that studies the factors affecting the rate of reaction that catalyzed by an enzyme. The most important factors affected the rate of enzyme-catalyzed reaction is ligand concentration (substrates, inhibitors, activators and product), enzyme concentration, temperature, pH, and ionic strength. It is possible to know a great deal about the nature of a reaction that catalyzed by an enzyme when all the above factors analyze properly. All enzymes have an optimal temperature, pH, and ionic strength, at which it has maximum activity (Segel 1968). A key factor affecting the enzyme-catalyzed reaction is substrate concentration. In 1913 Leonor Michael and Maud Menten was the first who postulated the combination of enzyme and substrate to form enzyme-substrate complex and then to product and free enzyme, they describe the enzyme-substrate complex in a general reaction (Equation 1.1).

Equation 1.1. Mekaelis-Menten equation of enzyme-substrate comple

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

Were:

k_1 is a constant rate for the formation of the enzyme–substrate complex ES, from the substrate S and enzyme E, k_{-1} is a constant rate for the reverse reaction of dissociation of the ES complex to substrate and free enzyme, and k_2 is the rate constant for the formation of product P and free enzyme (Suzuki 2015).

When the enzyme first mixed with substrate the reaction rapidly achieves a steady-state (the rate at which forms balance ES complex). As S increases, the activity of steady-state of a fixed enzyme concentration increases in an approach a characteristic maximum rate V_{max}, at which essentially all the enzyme saturated with substrate (Figure 1.5), so further adding of a substrate have no effect on the reaction rate (Nelson and Cox 2013).

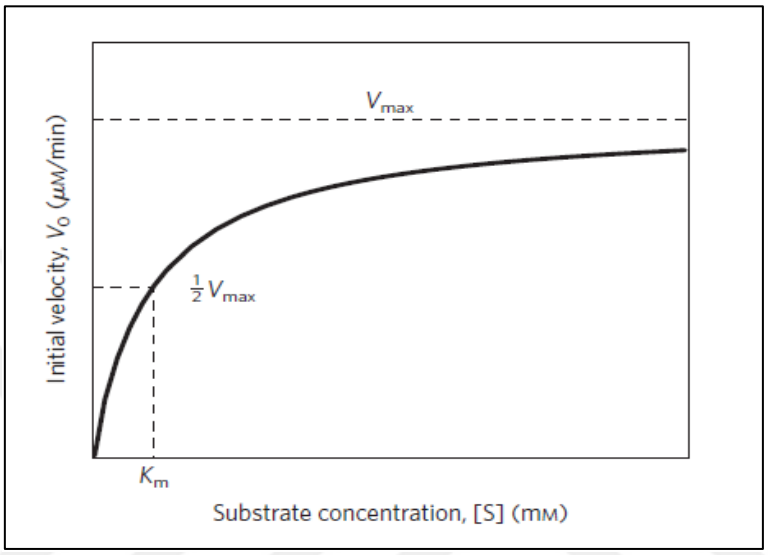

Figure 1.5. Affect of substrate on the rate of enzyme-catalytic reaction, further adding of substrate have no affect on the rata enzyme-catalytic reaction

The Michaelis constant K_M is the substrate concentration that results in a reaction rate equal to 1/2 V_{max}, which is characteristic acting of each enzymes on a given substrate. The Michaelis-Menten equation,

Equation 1.2. Mechaelis-Menten equation, relation between K_M and V_{max}

$$V_0 = \frac{V_{max} \cdot [S]}{K_M + [S]}$$

Relate the initial velocity V_0 to substrate concentration [S] and V_{max} through the constant K_M. Which V_{max} is the velocity of the enzyme-catalyzed reaction when there is a saturating level of the substrate (Campbell and Farrell 2009).

1.1.6. Enzyme İnhibition

An inhibitors can be defined as any substance has capability to decrease the rate of reaction that enzyme act on or prevent the enzyme catalytic. The enzymatic reaction can affect by inhibitors in two ways. An irreversible inhibitor, which of, inhibitor reacts with the enzyme and produce inactive enzyme protein and from which the enzyme cannot be regenerated to its original form. And reversible inhibitor, that can bind to the enzyme and release subsequently, leaving the enzyme in its genuine form. Reversible inhibitors divided into two major groups, they distinguished according to the sites on the enzyme which they bind. One group is competes with the substrate to bind to the active site of enzyme and block the access of substrate, this action modes is called competitive inhibition. Another group of reversible inhibitors binds to the sites of enzyme other than the active site, causes a change of enzyme structure as a result of binding, especially around the active site of enzyme (Figure 1.6). This action modes is called noncompetitive inhibition (Campbell and Farrell 2009).

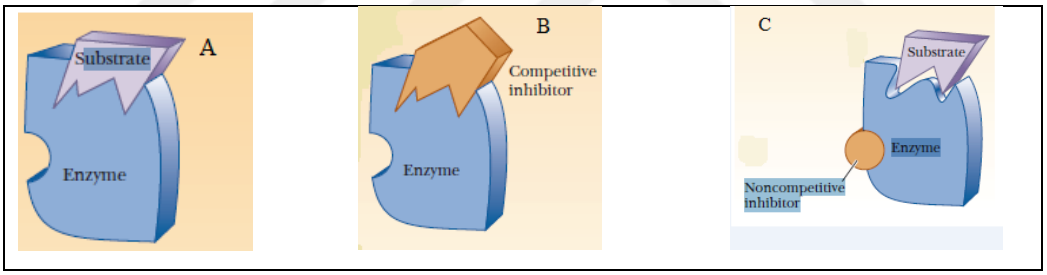

Figure 1.6. Competitive and noncompetitive inhibition. Normal enzyme substrate binding (A), competitive Inhibitor binds active site of enzyme (B), noncompetitive inhibitor binds enzyme other than the active site (C)

1.2. Free Radicals

A free radical can be defined as any molecules or its fragment can exist independently and contain one or more unpaired electron in its outer atomic orbits, thus uncoupled molecules are highly reactive and unstable, which react with other adjacent molecules by accepting or donating electrons, therefore free radical behaves as oxidant and reductant, some free radicals are produced endogenously during metabolism, sometimes immune system create them purposefully to attack and destroy pathogen. However, environmental

pollution, industrial chemicals, and cigarette smoking also an exogenous source for producing free radicals (Kabel 2014).

Free radical, which involving oxygen is refers to as reactive oxygen species (ROS). Superoxide radical ($\cdot O_2$), and hydroxyl radical ($\cdot OH$) are the most commonly formed of ROS. When one electron adds to an oxygen molecule forming superoxide radical and it is the least reactive type of ROS, superoxide is the most common free radical which produced from the human body. The phagocytic cell is prominent sources of superoxide radicals, this cell generates free radicals that attack pathogen such as virus and bacteria (Droge 2002).

Hydrogen peroxide (H_2O_2) forming when oxygen attracts tow molecules of hydrogen, despite the fact that the hydrogen peroxide not considered on free radical, but is a member of ROS family, and may selectivity entered in free radical generation, which is converted a spontaneous reaction catalyzed by Fe^{2+} (Fenton reaction) to the high reactive hydroxyl radical, hydrogen peroxide is a lipid soluble and thus able to diffuse across cell membranes (Lobo et al. 2010).

The most reactive free radical is hydroxyl radical ($\cdot OH$) (Scheibmeir et al. 2005), and has very short half-life approximately 10^{-9} s in vivo, will attack any biological molecule (RH) to abstract hydrogen atom and resulting free radical ($R\cdot$), which is more stable and has longer-live than hydroxyl radical (Rhee and Kil. 2016).

1.3. Antioxidant

An antioxidant is any molecules capable of neutralizing or deactivating free radicals before they cause cell damage. Redox reactions can produce free radical, which is unstable and highly reactive will attack another adjacent molecule to stabilize themselves, that producing chain of reaction that damage cells, antioxidant inhibiting this reaction by donating electron to free radical and neutralizing them without becoming free radical or destabilize themselves (Patckar et al. 2013).

Antioxidant defense systems (ADS) act to protect the body from oxidative stress through suppressing formation of free radicals, antioxidant radical scavenger, and converting the

oxidant to less oxidant compound (Genestra 2007). Antioxidant defense system can categorized in to two types:

- Enzymatic antioxidant

Enzymatic antioxidant present in all cell in the body, they classified into three major classes; superoxide dismutase (SOD), catalase and glutathione peroxidase (GST), body can produces all of them.

- Non enzymatic antioxidant

Human Body get some of the non-enzymatic antioxidants from their diet, which can`t produce them such as, ascorbic acid (Vitamin C), tocopherols and tocotrienols (Vitamin E) and carotenoids. Glutathione also is a nonenzymatic antioxidant, which is not essential nutrient for human, since body can synthesize it from the amino acids (Valko et al. 2007).

1.4. Glutathione

Glutathione is the most abundant thiol prevalence in cellular, synthesized in the cytoplasm and composed of three amino acids L-glutamic, L-cysteine and glycine (Appenzeller-Herzog 2011).

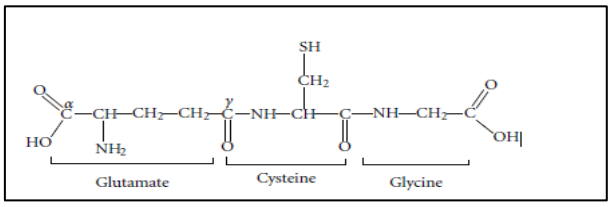

Figure 1.7. Glutathione structure

Glutathione is a water-soluble antioxidant and acts multiple functions at the cellular level (Lushchak 2012). Thiol (-SH) is the characteristic group of glutathione`s structure (Blonska-Sikora et al. 2012), because of the existence of this group, several redox forms of glutathione occurs in human body, which of, oxidized glutathione (GSSG), reduced glutathione (GSH), conjugates of GSSG with protein, and S-nitrosoglutathione (GSNO) (Bilska et al. 2007).

Principle role of GSH in human body is protecting organisms from free radicals, and in gene expression has a signal transduction role (Sies 1999). Moreover, formation of glutathionylated protein by glutathionylation process, which glutathione bound to the protein (Serru et al. 2001), and protect them from oxidative stress and stabilize them, glutathionylation has also a regulation role in transcription process (Blonska-Sikora et al. 2012). GSH reduces various oxidant and reactive species catalytically by glutathione-s-transferase (GST) or glutathione peroxidase (GPX) through formation of oxidized glutathione (GSSG) (Townsend et al. 2003). GSH reduced back by NADPH-dependent enzyme glutathione reductase (GR) that renewed GSH within the cell (Li 2009).

1.5. Glutathione Reductase

Glutathione reductase GR (NADPH: GSSG oxido reductase, EC 1.8.1.7) belong to the pyridine nucleotıde disulfide oxidoreductase family of flavoenzyme (Veine et al. 1998), that includes thıoredoxin reductase and lipoamide dehydrogenase (Bohme et al. 2000), calatyses the reuduction of GSSG concomitant NADPH oxidation

Equation 1.3. GR catalyzes the reduction of GSSG by NADPH

$$GSSG + NADPH \xrightarrow{GR} 2GSH + NADP^{+}$$

This reaction is important to maintain a high GSH/GSSG ratio in the red blood cell (Wong and Blanchard 1989), GR also plays an important role in redox homeostasis (Bauer et al. 2006), and protecting protein oxidation in human cells (Yawata and Tanaka 1974), and it is a key enzyme of the glutathione-dependent antioxidant system (Kanzok et al. 2001).

Throughout studying the behavior of glutathione Hopkin and Illiott (1951) found that the thermolabile system has the capacity to reduce the disulfide from glutathione, and observed that the glutathione reduced more rapidly in livers from well-fed animals than livers from starved animals.

Soon afterward Mann (1932) demonstrated the reducing glutathione by means of glucose in present of glucose dehydrogenase under anaerobic condition, and almost simultaneously rapid reduction and much more slowly oxidation in intact erythrocytes

were observed by Meldrum (1932). Subsequently Meldrum (1933) proved that the hexosemonophosphate has the capability to reduce glutathione under aerobic and anaerobic condition.

The enzyme from wheat-germ which provides the transfer of hydrogen from coenzyme II-dehydrogenases substrate to oxidized glutathione was first termed glutathione reductase GR by Conn and Vennesland (1951). Almost simultaneously this enzyme was demonstrated by Mapson and Goddard (1951) in pea seeds, and Rall and Lehninger (1951) in rat kidney, liver, spleen, heart muscle, brain, and skeletal muscle.

Asnis (1954) proved that GR is a flavoprotein and FAD serve as a prosthetic group in its structure. Later Langdon (1958) reported that the GR extracted from rat liver doesn`t require cofactors and contained no flavin nucleotide. Subsequently Mapson and Isherwood (1963) demonstrated also that GR is a flavoprotein.

The structure of flavoenzyme GR is a dimer of two identical subunits, one FAD moiety is bound to each subunit (figure 1.8) (Schulz et al. 1982), human erythrocytes can synthesiz FAD from riboflavin (Mandula and Beutler. 1970). Polypeptide chain of human GR consist of 478 amino acid residue with MW of 52,4 kDa per subunit (Deonarain et al. 1992). FAD and redox active disulfide bridge (cys 58; cys 63) represent the active center of GR (Sustmann et al. 1989).

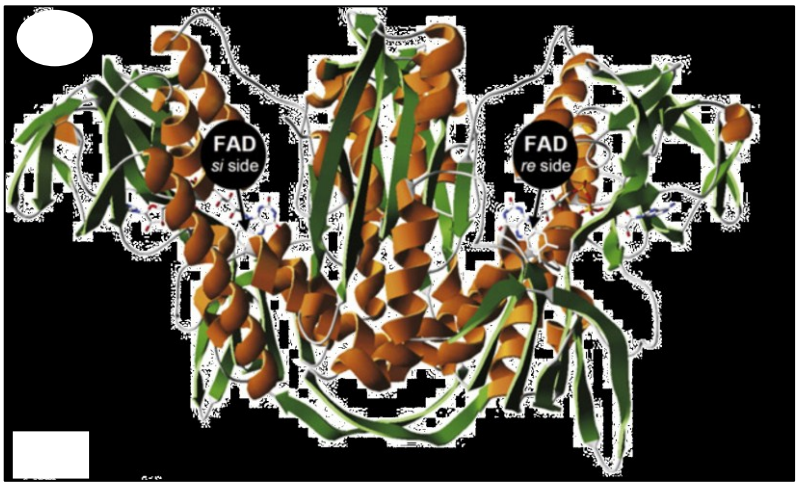

Figure 1.8. Three dimentional structure of glutathione reductase (Deponte 2013)

Kinetic mechanism of GR is ping-pong (Wong et al. 1988), which in the reduction half-reaction NADPH binding to reduce FAD, and electron from FAD pass to redox active-disulfide and reduced enzyme (EH_2), reduction of GSSG by EH_2 in oxidative half-reaction formed 2 mol of GSH concomitant oxidation of enzyme (Figur 1.9) (Rietveld et al.1994; Pai and Shulz. 1983; Deponte 2013).

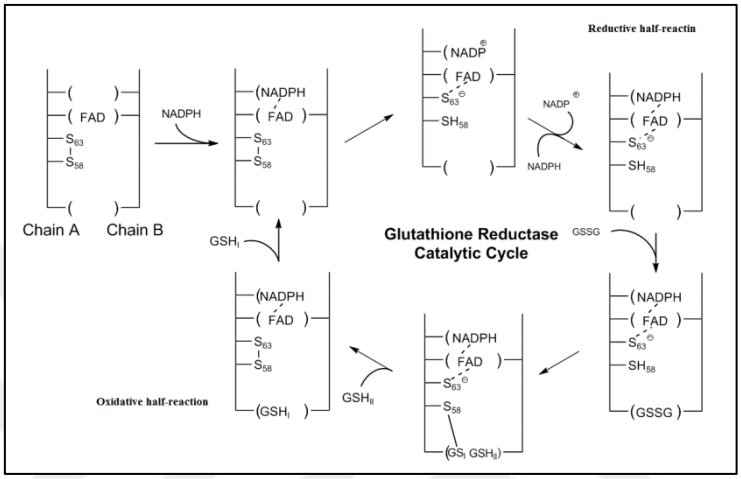

Figure 1.9. Glutathione reductase catalytic cycle. (https//www.wikimedia.org/GSR_catalytic_cycle.PNG)

Mammalian GR is active both in mitochondria and cytoplasms (Tamura et al. 1996; Mbemba et al. 1985), and in plant is active in chloroplast and mitochondria (Creissen et al. 1995). Human GR is lokated on chromosome, encoded by single gene (GSR), consist of 13 exons, and complementary DNA (cDNA) sequence of gene encodes a polypeptide of 522 amino acid residue (Satoh et al. 2010). Likewise, mouse GR gene consist of 13 exons (Kilner and Montoya 2000), differ from GR gene in drosphila melanogaster, which consist of at least two exons (Candas et al. 1997). A singel-copy gene namely GLR1 that encodes 467 amino acid protien (Collinson and Dawes 1995), encodes both the cytoplasms and mitochondrial form of GR in baker`s yeast (Outten and Culotta 2004).

Many scientists reflect the GR deficiency to; riboflavin deficiency, this by dietary supplementation with riboflavin could be easily corrected (Glatze et al. 1968; Eng et al. 1977; Warsy and El-Hazmi 1999), severe hemolytic anemia (Valentine and angles 1968), consanguineous marriage (Loos et al. 1976), cataract and severe neonatal joundice (Frischer 1977), mutation in the apoprotein (Gallo et al. 2009), genetic polymorphisms and inhibition with chemotherapy drug 1,3-bis(chloroethyl)-1-nitrosourea (BCNU)

(Heyob et al. 2008). However, hereditary deficiency of GR is extremely rare (Chang et al. 1978). GR deficiency in newborn mice alter the lung development (Lunchsinger et al. 2010), and in human may cause hemoglobinopathy (Mojzikova et al. 2010).

2. LITERATURE REVIEW

Glutathione reductase (GR, NADPH: oxidized glutathione oxidoreductase, EC 1.8.4.2) is a flavin-containing enzyme required for reducing oxidized glutathione (GSSG) to reduced glutathione (GSH) with the accompaniment oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide phosphate ($NADP^+$).

$$NADPH + H^+ + GSSG \xrightarrow{GR} NADP^+ + 2GSH$$

The main function of GR as seen in the above equation is to upkeep of glutathione in a reduced state (Tandogan and Ulusu 2008; Chang et al. 1978). Glutathione reduced (GSH) is fundamental compound for cell survival, it is also necessary for elimination of reactive oxygen species (ROS), heavy metals and xenobiotics (Garcia-Leiro et al. 2010).

The history of glutathione reductase come back to 1933 when Hopkins and Elliott demonstrated in liver tissue of various animals a heat-labile system has the ability to reduce oxidizing glutathione (Hopkins and Elliott 1933). Soon after, Meldrum and Tarr (1935) confirmed catalyses glutathione reduction by erythrocytes. An enzyme which catalyses the reduction of GSSG for the first time termed GR by Conn and Vennesland (1951). Then Asnis (1955) partially purified GR enzyme from escherichia coli. After that Racker (1955) for the first time isolated GR from yeast in crystalline form.

Glutathione reductase enzyme has been purified and studied in a wide assortment of organisms, including human cells; erythrocytes GR was partially purified by using CG-50 type II resin chromatography, and reduced triphosphopyridine nucleotide (TPNH), or reduced diphosphopyridine nucleotide (DPNH), were used as a coenzymes (Beutler and Yeh 1963). Moroff and Kosow (1978) studied some characteristic on platelets enzyme and Showed that GR include flavin prosthetic group, prefers NADPH over NADH,

Activation by inorganic salts, and inhibition by $NADP^+$. In human leukocytes, purification and partially characterization of GR enzyme were investigated (Ogus and Tezcan 1981). Decreasing of GR activity by 50.1% of the mean value was approved under cataractous condition to normal human lens (Pandya 2012). GR enzyme was purified in human erythrocytes by 2`,5`-ADP- Sepharose-4B gel affinity chromatography (Mannervik et al. 1976).

Mammalian tissues; in rat liver partially purified preparation was able to oxidize $TPNH_2$ in the present of GSSG, this evidence indicated the existence of an enzyme, temporarily called glutathione reductase (Rall and Lehninger 1952). In hepatopancreas of Mytilus Edulis, purification and some properties of GR enzyme was investigated (Ramos-Martinez et al. 1983). GR enzyme activity was studied in isolated chromatin from mouse spleen nuclei (Ochalska-Czepulis and Bitny-Szalchto 1981). Distribution of GR enzyme was determined in murine brain by immunocytochemical techniques (Knollema et al. 1996). Distribution and activity of GR enzyme were investigated in adult rat liver, in order to study the late consequence of exposure to toxic substances during the embryonic period to paraquat (Semenyuk et al. 1991).

Plant tissues; the appearance of stromal GR enzyme in intact but not broken spinach chloroplasts was confirmed by Foyer and Halliwell (1976). Bielawsky and Joy (1986) purified GR from chloroplast, and root of pea, and corroborated the two enzymes had similar MW, but differed in optimum pH and sensitivity to inhibition by copper, ferrous ion, and zinc. Mahan and Burke (1987) investigated the dissimilarity of GR from Corn mesophyll chloroplasts and pea chloroplast in MW and sensitivity to inactivation by NADPH. Properties, synthesis, and activity of GR was examined from leaves of pea subjected to ozone, fumigation, cold, treatment with greening and paraquat of etiolated seedlings (Edwards et al. 1994). Kertulis-Tartar and his friends in (2009), demonstrated the activity of GR in fronds of two Pteris ferns was unchanged after arsenic exposure.

Microorganism cells; in additional to flavin, disulfide as an active center of GR was confirmed from yeast (Massey and Williams 1965). By using suitable method 1.44 g of GR (169 EU/mg) was obtained from 65 g (wet weight) of baker`s yeast cells equivalent to 80% recovery of enzyme (Tsai et al. 1991). Turnover measurement of distribution GR in different compartment of saccharomyces cerevisiae eukaryotic cell for a firs time was

reported by Couto et al. (2013). Friebolin et al. (2008) examined the antimalarial dual drug design based on a short chloroquine (CQ) analogue attached to GR inhibitors in Plasmodium falciparum.

GR has been purified by many methods, such as; in normal human lens by using affinity chromatography of GSSG-NH$(CH_2)_6$NH-agarose gel, GR was purified 33-fold with a specific activity (0.12 EU/mg) (Harding 1973). GR has been purified 40000 fold with an overall yield 36%, and (240 EU/mg) as specific activity from human erythrocytes by using Ion-exchangers on DEAE-Sephadex A-50, CM-Sephadex C-50, and gel filtration sephadex G-200 chromatography (Worthington and Rosemeyer 1974). From human erythrocytes GR was purified 6000 fold with (9 EU/mg) as specific activity by the use of 2′,5′-ADP Sepharose-4B gel affinity chromatography column and $NADP^+$ containing solution used as an elution buffer, (Mannervik et al. 1976). GR bounded to an affinity matrex of GSSG(N)-sTT-Cellulose, was eluted in high concentration with its coenzyme NADPH rather than its substrate GSSG or NADH, GR also eluted by $NADP^+$ but in lower concentration than eluted by NADPH (Danner et al. 1977). GR was purified 275-fold in a yield 33% and specific activity (65 EU/mg), from pea chloroplast by affinity chromatography on ADP-agarose (Connel and Mullet 1986). From rat lung GR was purified 940-fold with an overall yield 95% and specific activity (34.88 EU/mg), by using 2′,5′-ADP Sepharose-4B gel affinity chromatography (Adem and Ciftci 2016).

GR Molecular weight; in spinach leaf was determined as 125 kDa for the dimer (Wirth and Latzko 1978). In gerbil liver was estimated as 120 kDa for the dimeric structure, and 53.4 kDa for each monomer (Trang et al. 1983). In cyanobacterium spirulina maxima the homodimeric and subunits structure were determined as 99.8 and 47 kDa, respectively (Rendon et al. 1986). In pea seedling native MW of was determined as 114 kDa, and 55 kDa per subunit (Madamanchi et al. 1992). 114 kDa and 63 kDa for native and subunit structure, respectively, has been determined in cestode moniezia expansa (Callum and Barrett 1995). In spinach, and yeast, for each monomer 72 and 54 kDa, respectively, were determined (Hou et al. 2004). From e.coli estimated to109 kDa corresponds to a dimer (Mata et al. 1984). From phaeodactylum tricornutum 118 kDa was determined for native enzyme (Arias et al. 2010).

GR gene; Criessen and Mullineaux (1995) confirmed that GR in tobacco encoded by two different genes. Rice GR gene (RGRC2) is primarily localized in cytosol and showed with southern blot that exists as a single copy gene (Kaminaka et al. 1998). Encoded protein of 450 amino acid residues were found in determined DNA sequence GR gene (gor gene) from e. cole (Greer and Perham 1986). A cDNB for pea GR has been cloned, and the squence of amino acid residues were determined to 562 residues (Criessen et al. 1991). Parcial cloning of cDNA to mRNA of mouse GR, and full-length cloning of cDNA to mRNA of human enzyme were described by Tutic et al (1990). Cloning the second cDNA of cytosolic GR gene (GOR2) from pea was confirmed by Stevens et al (1997) Single copy of GR gene in cytosol was found in genome of brassica campestris (Lee et al. 2002). GR gene in cucumber was cloned to have 1995 base pair in length, containing one open reading frame encoding 496 residues of amino acids (Liu et al. 2012).

Inhibition and activation: GR was inhibited by several heavy metals and compounds, such as; β-chain of insulin (Jervell and Vallance-Owen 1966). 4-tert-Butylcatechol (TBC) (Yonemoto et al. 1983).dinitrosyl-iron-dithiolate complex (Boese et al. 1997). Some new organic nitrates (Senturk et al. 2009). Nicotine (Erat et al. 2007). Arsenotriglutathione (Styblo and Thomas 1995). Copper (Rafter 1982). Some antibiotics (Erat and Ciftci 2003; Senturk et al. 2008). Cadmium ion (Ulusu et al. 2003). Plant polyohenols (Zhang et al. 1997). Oncomodulin (Palmer et al. 1990). Quenones (Bironaite et al. 1991). Flavonides (Elliott et al. 1992). Peroxynitrite (Savvides et al. 2002). Acetaminophen (Rousar et al. 2010). Thallium-201 (Sahin et al. 2012). Co^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Cr^{3+}, Sn^{2+} and Mg^{2+} (Ekinci and Senturk 2013). GR also was activated by some another compounds, including; mercury and organomercurial resistance (Ghosh et al. 2006). FAD (Beutler 1969; Ono and Hirano 1984). Melatonin (Erat and Ciftci 2006). Activity of GR in erythrocytes also was shown to increase in diabetes mellitus (Long and Carson1961).

3. MATERIAL AND METHOD

3.1. Chemicals

The chemicals used throughout all this study and their acquired are listed in the table below.

Table 3.1. Chemicals used in whole experiment

Chemicals	Name of 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, bovine serum albumin, GSH, GSSG (6-phosphogluconate monosodium salt), 2', 5'-ADP Sepharose-4B	Sigma
Potassium acetate, bromothymol blue, sodium acetate, potassium hydroxide, glycine amino acid and trichloroacetic acid	Merck
SDS (sodium dodecyl sulfate), Coommasie Brilliant Blue G-250, Coommasie Brilliant Blue R-250	Fishcer Scientific
$NADP^+$ and NADPH	Applichem
Ammonium persulfate	Chem Solute Bio

3.2. Instruments

The instruments used during whole experiments in this study and their models are listed in below table.

Table 3.2. Instruments used conducting whole study

Instruments	Model
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 (P Spectra J.P. Selecta, s.a.)
shaker	SK-300
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

3.3. Preparation Of Solution

3.3.1. Homogenate Solution

50 mM KH_2PO_4/ 1 mM EDTA/ 1 mM DTT/ 1 mM PMSF (pH: 7.0): 2.04 g KH_2PO_4, 0.0876 g EDTA, 0.0462 g DTT and 0.052 g PMSF were dissolved in 250 mL DW, adjusted the pH to 7.0, then completed volume to 300 mL with DW.

3.4. Preparation Of Solution

3.4.1. Homogenate Solution

50 mM KH_2PO_4/ 1 mM EDTA/ 1 mM DTT/ 1 mM PMSF (pH: 7.0): 2.04 g KH_2PO_4, 0.0876 g EDTA, 0.0462 g DTT and 0.052 g PMSF were dissolved in 250 mL DW, adjusted the pH to 7.0, then completed volume to 300 mL with DW.

3.4.2. Enzyme Activity Measurement Solution

1. 0.1 M KH_2PO_4 (pH 7.5): 0.68 g KH_2PO_4, was dissolved in 40 mL DW, adjusted the pH to 7.5, then completed volum to 50 mL with DW.
2. 20 mM GSSG: 61.88 mg glutathione oxidized dissolved in 5 mL DW.
3. 2 mM NADPH: 8.3 mg NADPH was dissolved in 5 mL DW.

3.4.3. Preparation Of Affinity Chromatography Solution

1. Washing buffer A (pH: 7.3). 50 mM KH_2PO_4 + 1 mM EDTA + 1Mm DTT. 3.4 g KH_2PO_4, 0.146 g EDTA and 0.077 g DTT, were dissolved in 450mLDW, and adjusted the pH to 7.3 with 0.1 M KOH, then completed the volume with DW to 500 mL (this buffer solution are used for equilibration and washing column).
2. Washing buffer B (pH: 7.3). 50 mM KH_2PO_4 + 80 mM KCI + 1 mM EDTA + 1mM DTT. 3.4 g KH_2PO_4, 0.146 g EDTA, 0.077 g DTT and 0.149 g KCl were dissolved in 450 mL DW, and adjusted the pH to 7.3 with 0.1 M KOH, then completed the volume with DW to 500 mL.
3. Elution buffer (pH: 7.3). 50 mM KH_2PO_4 + 1 mM EDTA + 1mM GSH + 0,5 mM NADPH: 0.34 g K-phosphate, 0.0146 g EDTA, 0.0307 g GSH and 0.02075 g NADPH were dissolved in 45 mL DW, pH adjusted with 0.1 M KOH to 7.3, then completed the volume with DW to 50 mL.

4. Regeneration buffer A (pH: 4.5). 0.1 M Na-acetate + 0.5 M NaCl: 4.1 g Na-acetate (50 mmol) and 14.61 g NaCl (0.25 mol) were dissolved in 450 mL DW, and pH adjusted to 4.5, then completed the volume to 500 mL with DW.

5. Regeneration buffer B (pH 8.5). 0.1 M Tris + 0.5 M NaCl. 6.05 g Tris and 14.61 g NaCI were dissolved in 450 mL DW, and adjusted the pH to 8.5 with 0.1 M HCl, then the volume was completed to 500 mL with DW.

3.4.4. Preparation Of SDS-PAGE Solution

1. 1.5 M Tris-HCl (pH 8.8): 9.08 g Tris was dissolved in 40 mL DW, adjusted the pH to 8.8 with 0.1 M HCl, then completed the volume with DW to 50 mL.

2. 1.5 M Tris-HCl (pH 6.8): 9.08 g Tris was dissolved in 40 mL DW, and pH adjusted with 0.1 M HCl to 6.8, then completed the volume to 50 mL with DW.

3. 30% Acrylamide 0.8% Bisacrylamide solution: 15 g acrylamide 0.4 g bisacrylamide were dissolved in 50 mL DW.

4. 10% ammonium persulfate solution: 0.1 g of ammonium persulfate was dissolved in 1 mL DW.

5. 10% SDS: 1 g SDS was dissolved in 10 mL DW.

6. Running buffer (pH: 8.3): 1.51 g Tris (12.5 mmol) and 7.51 g glycine (0.1 mol) were dissolved in 450 mL DW, and added 5 mL of 10% SDS, adjusted the pH with 0.1 M HCl to 8.3, then the volume completed with DW to 500 mL.

7. Sample buffer: 0.5 mL from 1M Tris-HCl (pH: 8.0), 1 mL from 10% SDS, 1 mL from 100% glycerin, and 1 mL from 0.1% bromothymol blue were mixed in a beaker, then completed the volume with DW to 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: 50% isopropanol, 10% TCA, and 40% DW were mixed to form stabilization solution (this solution was used to stabilize the protein carried in the gel).

9. Gel staining solution: 0.1 g of Coomassie Brilliant Blue R-250 was dissolved in 50 mL methanol, 10 mL, acetic acid, and 40 mL DW.

10. Gel washing solution: 50 mL, methanol, 10 mL, acetic acid, and 40 mL DW were mixed and obtained.

11. 0.1% bromothymol blue: 0.1 g of bromothymol blue indicator was dissolved in 16 mL, of 1 M NaOH, and the volume was completed with DW to 250 mL.

3.4.5. Preparation Of Solution Used For Quantitative Determination Of Protein By Bradford′s Method

A. Coomassie Brilliant Blue G-250 reagent: 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL, of 95% ethanol. 100 mL, of 95% phosphoric acid was added to the solution. The volume of the solution is completed with DW to 1 L, the solution was stored for one week before using.

B. Standard protein solution: 1 mg of bovine serum albumin (BSA) was dissolved in 1 mL, DW.

3.5. Preparation Of Homogenate

Fresh quail`s liver were taken from agricultural farm of Bingol University and 5 g was cut into small pieces, then suspended with 15 mL, of 50 mM potassium phosphate buffer (pH: 7.0) contain 1 mM EDTA, 1 mM DTT, and 1 mM PMSF by homogenizer, the homogenate supernatant was prepared by centrifugation the solution at 15000 rpm and 4 ºC for one hour, the crude precipitate was discarded.

3.6. Measurement Of Enzyme Activity

Glutathione reductase with its cofactor NADPH together catalyzes the reduction of oxidized glutathione GSSG to glutathione reduced GSH.

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2GSH + 2NADP^{+}$$

The measuring absorbance is based on the amount of NADPH in the solution at 340 nm with a spectrophotometer, decreasing the absorbance refers to the presence of GR enzyme according to the above equations.

Enzyme was assayed by means of Buetler's method (1971), increasing absorbance of $NADP^{+}$ was measured spectrophotometrically at 340 nm depending on time against the blank containing all reagents with exception of enzyme.

Table 3.3. Glutathione reductase activity measurement reagent

Reagent	Sample cuvette (µl)	Blank cuvette (µl)
0.1 M K-phosphate	200	200
20 mM GSSG	100	100
2 mM NADPH	100	100
DW	590	600
Enzyme solution	10	-

The enzyme activity was measured according to the below equation:

Equation 3.1. Buetler`s equation for enzyme activity measurement

$$EU/mL = \frac{\Delta OD}{6.22} \times \frac{V_T}{V_E} \times D_f$$

Were:

EU/mL, is enzyme units in one mL.

ΔOD: is change of absorbance value in one minute

6.22: is absorbance formed in 1 mM NADPH (extension coefficient)

$V_{T:}$ is total volume of cuvette

$V_{E:}$ is enzyme volume in cuvette

$D_{f:}$ is dilution factor (use for dilute sample)

3.7. Ammonium Sulfate Precipitation

Homogenate was brought to 0%-20% saturation with solid ammonium sulfate with fixed and slowly stirrer, the precipitate was collected by centrifugation at 13500 rpm and 4 ºC for 15 min. Precipitate was dissolved in small volume of potassium phosphate (pH 7.5), then enzyme activity was measured for both supernatant and precipitate at 340 nm, the same experiment were performed for supernatant after brought to 20%-30%, 30%-40%, 40%50%, and 50%-60% saturation, respectively, with additional ammonium sulfate, the amount of ammonium sulfate was prepared by the below equation. This method was first performed in 1955 by Green and Hughes (Wingfield 2001)

Equation 3.2. Green and Hughes`s equation to determine the amount of salt (ammonium sulfate)

$$g = \frac{1.77\ V\ (S_2 - S_1)}{3.54 - S_2}$$

Were:

g: is gram of ammonium sulfate

V: is homogenate volume

$S_{1:}$ is initial percent of ammonium sulfate

$S_{2:}$ is final percent of ammonium sulfate

3.8. 2′,5′-ADP Sepharos-4B Gel Affinity Chromatography

2 g of dried 2′,5′-ADP Sepharose-4B was used for a (10 ×1 cm) column The gel was washed with 400 mL, DW to remove foreign materials and air if existed, and suspended with 0.1 M potassium acetate/0.1M potassium phosphate buffer (pH = 6.0) and packed in column. After the gel was precipitated, it was washed and equilibrated with 50 mM potassium phosphate (pH 7.3), contain 1 mM EDTA and 1 mM DTT (washing buffer A), by means of peristaltic pump, and flow rate was settled to 20 mL/h. Then the dialyzed sample was loaded onto column, and washed with 25 mL, of washing buffer A, and 25

mL, of 50 mM potassium phosphate (pH 7.3) including 80 mM KCl, 1 mM EDTA, and 1 mM DTT (washing buffer B). The washing process was continued until difference final absorbance becomes 0.05 at 280 nm. Then GR enzyme was pooled in eppendorf tubes after eluting with 50 mM KH_2PO_4, 1 mM EDTA, 1 mM GSH, and 0.5 mM NADPH (elution buffer pH 7.3), the enzyme activity was measured for all fraction tubes, and the tube has highest activity of GR was applied to SDS-PAGE.

3.9. Protein Determination

3.9.1. Qualitative Determination Of Protein

The fraction tubes which eluted from 2′,5′-ADP Sepharose-4B gel affinity chromatography column were examined for existence of proteins qualitatively. The absorbance of fraction tubes were measured spectrophotometrically at 280 nm against the elution buffer as blank, 280 nm is the maximum wavelength (λmax) of tryptophan, tyrosin and phenyl alanine (Segel 1968).

3.9.2. Quantitative Determination Of Protein (Bradford`S Method)

The amount of protein were determined by Bradford`s method in 595 nm for prepared homogenate, and 2′,5′-ADP Sepharose-4B fraction tube that showed single band in SDS-PAGE (pure enzyme). Bradford`s method involve a binding Coomassie Brilliant Blue G-250 dye negative charge to protein positive charge, causing change in color and maximum absorbance from redder in 465 nm before binding to protein to bluer in 595 nm after binding protein (Bradford 1976).

Protein assay (standard method); from 1 mL of solution contain 1 mg protein (BSA), 10 different concentration were pipetted from 10 µl to 100 µl into test tube, volume completed with DW to 100 µl for all tubes, then 4900 µl of protein reagent (dye) were added to each tube, content mixed by vortex, and incubated at room temperature for 10 min, the absorbance was measured at 595 nm in 3mLdisposed cuvettes for each tube against blank containing 5 mL of protein reagent, the curve of data was plotted to determine the amount of protein in unknown solution.

Table 3.4. Standard protein solution preparation for Bradford`s method

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

Protein assay (sample method); homogenate solution was diluted 10 times with DW, because of high concentration. 100 µL of dilute homogenate pipetted into test tube, completed volume to 5 mL by adding 4900 µL of protein reagent, the same volume for pure enzyme was prepared 100 µL enzyme and 4900 µL protein reagent.

Table 3.5. Homogenate and pure enzyme solution preparation for Bradfor`s method

Sample	Homogenate	Pure enzyme
Sample volume (µL)	100	100
Dye volume (µL)	4900	4900

Tube contents were mixed by vortex, then incubated at room temperature for 10 min, absorbance was measured at 595 nm against blank containing 3 mL of dye in disposed cuvettes, the amount of proteins were determined by the equation that obtained from a standard curve.

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

The purity of enzyme was controlled with SDS-PAGE by LaemmLy`s method (1970). At the beginning tools was washed by DW and glasses plates by ethanol, setted-up the plates to the rack, then separation gel was prepared and poured into sandwich until the indicator mark by pipet, then immediately overlay the solution with water-saturated n-butanol, allowed the gel until polymerized for 15-30 min, dried the area above separation gel with filter paper, filled the sandwich cassette with stacking gel, and immediately placed the comb straight in the cassette, and allowed to polymerize for 10-20 min, assemble the gel with inner chamber then gathered with the electrophoresis cell. Filled

the inner chamber with running buffer and removed the comb by withdrawing it straight up softly and gently. Homogenate sample, denatured enzyme, and standard protein were poured into different wells of gel, then filled the outer buffer chamber to the indicator mark for two gels and connected the electrophoresis cell to the power supply and run the gel with two periods of time, initial time adjusted the current at 20 mA for 15-25 min, and final time adjusted the current at 40 mA for 50-60 min, after electrophoresis is completed the gels were separated from cassettes by floating it off the plates into water, then the gel was fixed for 30 min with slowly and continue shaking by shaker in fixation solution, then stained the gel for 2 hours min in stain solution and abstained the gel with gel washing solution until the single band appearance, then the gel was photographed (figure 4.4)

Table 3.6. Preparation of separating gel

Reagent	Volume (mL)
1.5 M Tris-HCl (pH: 88)	3.8
30% acrylamide- 0.8% bisacrylamide	5
10% SDS	0.15
DW	5.9
PER	0.15
TEMED (at last added)	0.006

Table 3.7. Preparation of stacking gel

Reagent	Volume (mL)
1.5 M Tris-HCl (pH: 6.8)	0.63
30% acrylamide- 0.8% bisacrylamide	0.83
10% SDS	0.05
DW	3.4
PER	0.05
TEMED (at last added)	0.005

Molecular weight of GR enzyme was determined by using a ruler for measuring the migration distance from the top of separation gel to each standard band and dye front, then R_f values was calculated for each band by using following equation

Equation 3.3. Determination of retardation factor R_f.

$$R_f = \frac{X_e \text{ migration distance of the protein}}{X_{dye} \text{ migration distance of the dye front}}$$

repe d, and graphed the data by using excel program, and plotted the log MW as a function of R_f value, by generation the equation y = mx + b, and solving y value the molecular weight of GR enzyme was calculated.

3.11. Characterization Studies

3.11.1. Determination Of Stable Ph

Six different pH (5.5, 6, 6.5, 7, 7.5, 8) of 50 mM potassium phosphate, and four different pH (7.5, 8, 8.5, 9) of 50 mM Tris-HCl were prepared, then from each pH solutions 750 µL were pipetted into eppendorf tube and added 250 µL of purified enzyme into each eppendorf tubes and measured the GR enzyme activity at 340 nm for all tubes, then all tubes were stored at +4 ºC. The same experiment was performed after every 24 hours for one week.

3.11.2. Determination Of Optimum Ph

Different pH of 50 mM potassium phosphate and 50 mM Tris-HCl (5.5, 6, 6.5, 7, 7.5, 8), and (7.5, 8, 8.5, 9), respectively, was prepared and spectrophotometricaly GR enzyme activity was measured at 340 nm in each pH.

3.11.3. Determination Of Optimum Temperature

Placed the prepared cuvette within all reagent with exception of enzyme in 0˚C ice water 3 min incubation, then pipetted the enzyme into cuvette and directly measured the activity of enzyme at 340 nm spectrophotometricaly, the same experiment was performed for increasing temperature to 10, 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, and 95 °C, respectively, in water bath.

3.11.4. Determination Of Optimum İonic Strength

Different concentration (0.1, 0.2, 0.4, 0.6, 0.8, and 1 mM), of potassium phosphate and Tris-HCl at (pH 8.0), were prepared and GR activity was measured spectrophotometricaly in each concentration at 340 nm. At the first 40 mL of 1 mM potassium phosphate was prepared as a stock solution, then by dilution equation another concentrations were prepared.

Equation 3.4. Dilution equation, was used to prepare different concentration of potassium phosphate

$$M_1 \times V_1 = M_2 \times V_2$$

Table 3.8. Preparation of different concentration of potassium phosphate, to determine optimum ionic strength

Concentration (mM)	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

The same method also was repeated for Tris-HCl.

3.12. Kinetic Studies

GR enzyme activity was measured spectrophotometricaly at 340 nm for five different concentration of GSSG (0.04, 0.08, 0.2, 0.6, and 1 mM) and fixed concentration of NADPH 2 mM, the same experiment was done for five different concentration of NADPH (0.02, 0.04, 0.06, 0.08, and 0.1 mM) and constand concentration of GSSG 20 mM, then the data were plotted by Lineweaver-Burk curve, and the K_M and V_{max} value were calculated for both NADPH and GSSG (Lineweaver and Burk 1934).

4. RESULTS

4.1. Enzyme Purification Studies

Purification of an enzyme was performed by three steps. First of all homogenate was prepared, then protein was precipitated by ammonium sulfate precipitation, salt particles was removed by dialyses, finally pure enzyme was obtained after applied dialyzed sample to 2′,5′-ADP Sepharose-4B gel affinity chromatography column, materials and methods described in chapter 3, and the results are shown in next and consecutively paragraphs.

4.1.1. Ammonium Sulfate Precipitation

Protein in homogenate was completely precipitated after brought homogenate to 60% saturation with solid ammonium sulfate, the process are described in section (3.6). The activity of GR was measured for both supernatant and precipitate (Table 4.1) and (Figure 4.1), and lower activity of enzyme in supernatant was measured after brought to 60% saturation with ammonium sulfate, that confirmed the enzyme is completely precipitated.

Table 4.1. Ammonium sulfate precipitate

Ammonium sulfate %	Precipitate (EU/mL)	Supernatant (EU/mL)
0 – 20	0.739	2.652
20 – 30	2.952	2.465
30 – 40	2.518	1.446
40 – 50	2.148	0.128
50 – 60	0.541	0.016

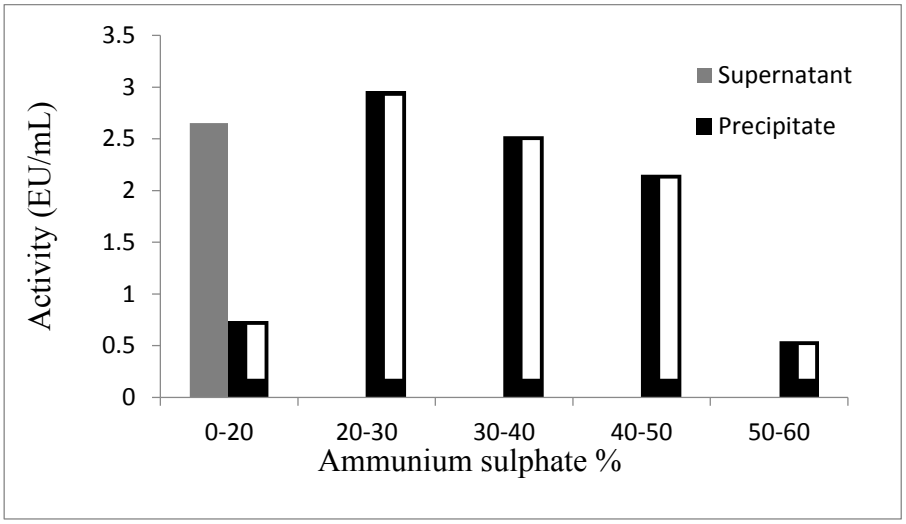

Figure 4.1. Ammonium sulfate precipitation

4.1.2. GR Elution From 2′,5′-ADP Sepharose-4B Gel Affinity Chromatography Column

Dialyzed sample was applied to column, and washed sample two times by two different buffer, after that enzyme was eluted by elution buffer (section 3.7), and enzyme activity was measured spectrophotometrically at 340 nm for all fraction tubes (Figure 4.2).

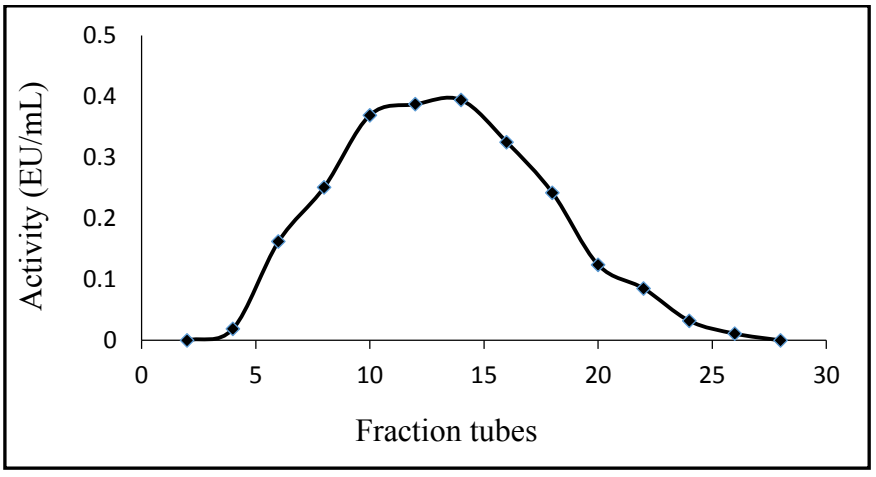

Figure 4.2. 2′,5′-ADP Sepharose-4B affinity column fraction tubes

4.1.3. Purification Yield, Purification Fold And Specific Activity

The amount of protein was determined by Bradford`s method (1976), and by using volume and enzyme activity. Purification yield, purification fold and specific activity were calculated (Table 4.2).

Table 4.2. Quail`s liver glutathione reductase purification results table

Sample type	volume (mL)	Activity (EU/mL)	Protein (mg/mL)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	yield (%)	Purifica-tion fold
Homogenate	11	1.147	7.1	12.61	78.1	0.16	100	1
2′,5′-ADP sepharose-4BAffinity chromatography	4	0.457	0.02	1.82	0.08	22.75	13.6	142.18

4.1.4. Protein Determination

4.1.4.1. Qualitative Protein Determination

The absorbance for eluting solution during washing enzyme before loading GR elution buffer was measured at 280 nm, throughout the washing enzyme final absorbance difference was measured of 0.05, this confirmed the bound of NADP-dependent enzyme to the gel and all others protein eluted throughout washing, these indicate the time of enzyme elution buffer.

4.1.4.2. Quantitative Proteins Determination

Protein quantity was determined by means of Bradford`s method (1976), the process are described in section 4.3, the amount of protein (μg) was measured in both homogenate and pure enzyme by using equation obtained from standard calibration curve (Figure 4.3).

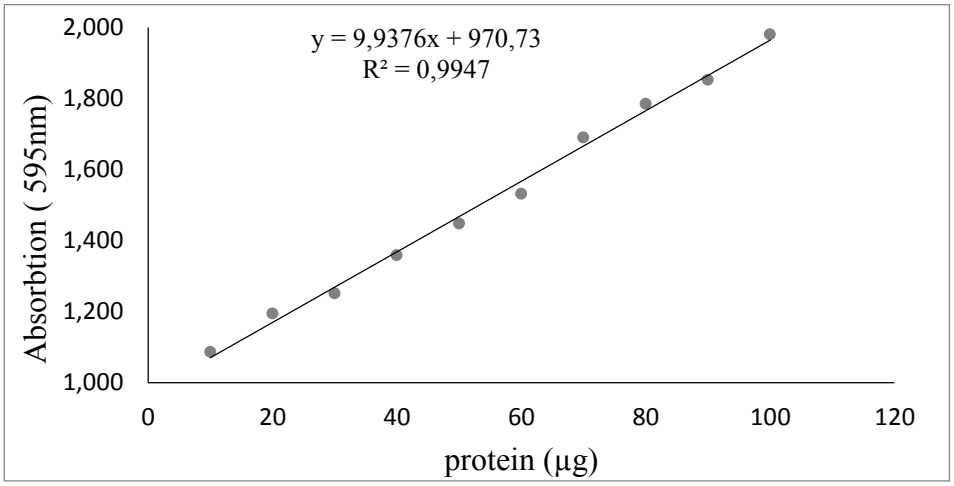

Figure 4.3. Standard protein calibration curve

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

The purity and molecular weight of enzyme eluted from affinity column section 3.9 was cheeked and controlled respectively, by SDS-PAGE. The result showed a single band on the gel (Figure 4.4), this given an evidence for purified enzyme.

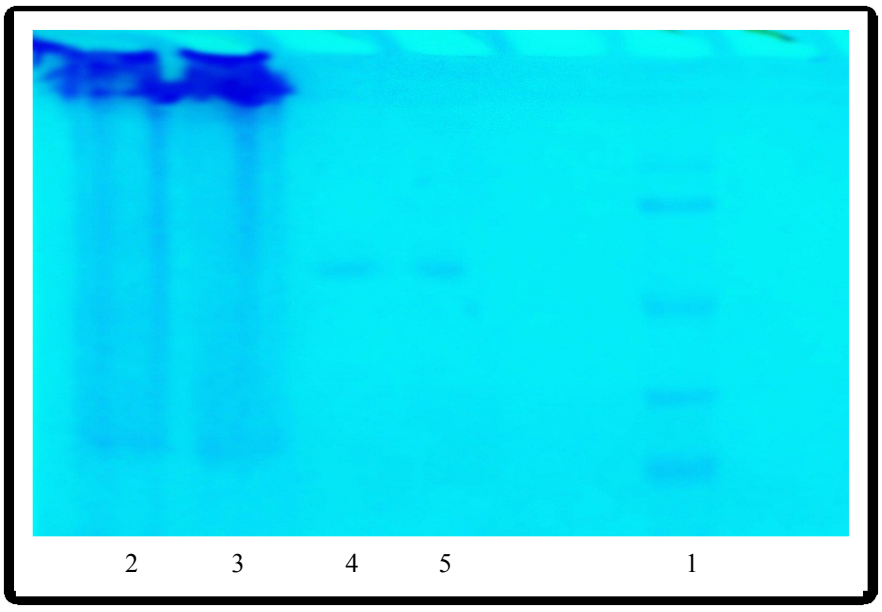

Figure 4.4. SDS-PAGE photograph, lane 1: standard protein (B-galactosidase from E-cole 120 kDa, Bovine serum albumin 85 kDa, chicken ovalbumin 50 kDa, Bovine carbonic anhydrase 35 kDa, bovine milk 25 kDa), lane 2: homogenate sample, lane 3: ammonium sulfate precipitate sample, lane 4, and 5: GR enzyme from quail`s liver

4.1.6. Determination Of Molecular Weight Of GR Enzyme With SDS-PAGE

After run the enzyme in the SDS-PAGE and capturing photograph of gel as shown in (Figure 4.4), The R_f value was measured for standard protein and the enzyme fleeing in the gel, then R_f-log MW graph was drawn (Figure 4.5), and by using the equation that obtained from curve, the molecular weight of GR enzyme was determined, that was 59 kDa.

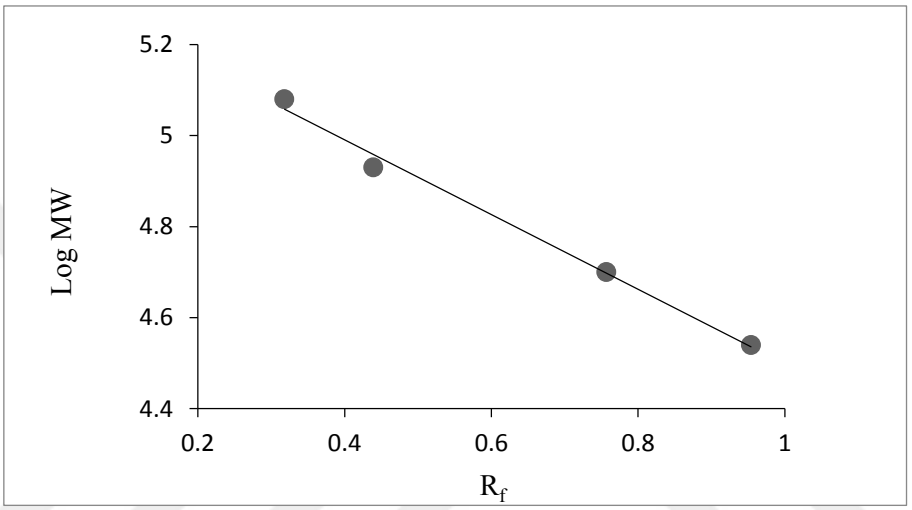

Figure 4.5. R_f-log MW calibration curve to determine MW of enzyme

4.2. Characterization Studies

4.2.1. Stable pH

Stability of enzyme in different pH and two different medium (acid/base medium) was tested against the time section 3.10.1. GR activity was measured every 24 hours for one week and the result showed that GR has highes stability at pH 8.0 and in base medium Tris-HCl buffer (Table 4.3 and 4.4) and (Figure 4.6 and 4.7).

Table 4.3. Results of stable pH studies with K-phosphate buffer

Time (days)	(pH 5.5) Activity (EU/mL)	(pH 6.0) Activity (EU/mL)	(pH 6.5) Activity (EU/mL)	(pH 7.0) Activity (EU/mL)	(pH 7.5) Activity (EU/mL)	(pH 8.0) Activity (EU/mL)
0	0.114	0.130	0.141	0.125	0.114	0.125
1	0.103	0.114	0.119	0.112	0.107	0.126
2	0.092	0.112	0.110	0.110	0.098	0.121
3	0.092	0.092	0.107	0.105	0.096	0.116
4	0.085	0.091	0.101	0.096	0.094	0.107
5	0.075	0.089	0.089	0.091	0.089	0.105
6	0.067	0.083	0.089	0.091	0.085	0.100
7	0.064	0.073	0.073	0.075	0.078	0.085

Table 4.4**.** Results of stable pH studies with Tris-HCl buffer

Time (days)	(pH=7.5) Activity (EU/mL)	(pH=8) Activity (EU/mL)	(pH=8.5) Activity (EU/mL)	(pH=9) Activity (EU/mL)
0	0.051	0.044	0.116	0.112
1	0.053	0.041	0.107	0.094
2	0.050	0.035	0.107	0.094
3	0.044	0.035	0.101	0.092
4	0.041	0.037	0.096	0.091
5	0.044	0.033	0.092	0.082
6	0.041	0.033	0.080	0.069
7	0.035	0.033	0.060	0.050

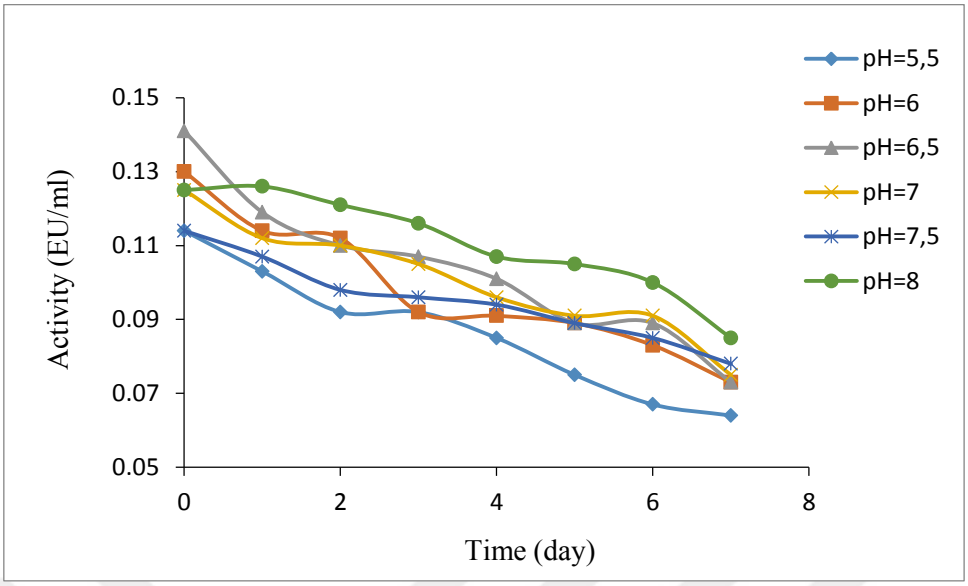

Figure 4.6. Graph of stable pH studies with K-phosphate buffer

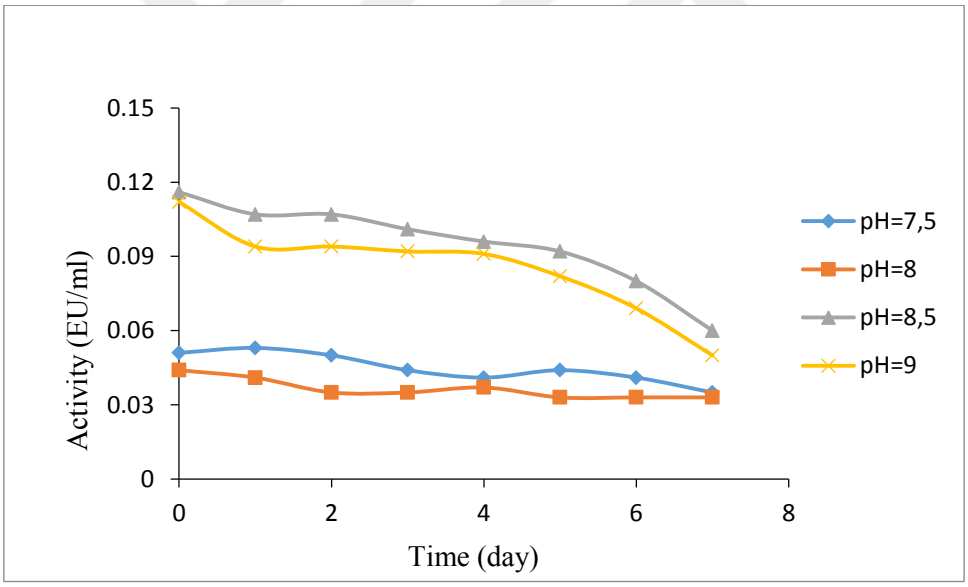

Figure 4.7. Graph of stable pH studies with Tris-HCl buffer

4.2.2. Optimum pH Studies

Preparation of different pH to determine the optimum pH of GR are described in section 3.10.2, The optimum pH of enzyme was determined in potassium phosphate buffer at pH 8.0, as shown as in the (Table 4.5 and 4.6) and (Figure 4.8).

Table 4.5. Results of optimum pH studies with K-phosphate buffer

pH	Activity (UE/mL)
5.5	0.051
6.0	0.071
6.5	0.101
7.0	0.125
7.5	0.128
8.0	0.139

Table 4.6. Results of optimum pH studies with Tris-HCl buffer

pH	Activity (EU/mL)
7.5	0.137
8.0	0.128
8.5	0.125
9.0	0.107

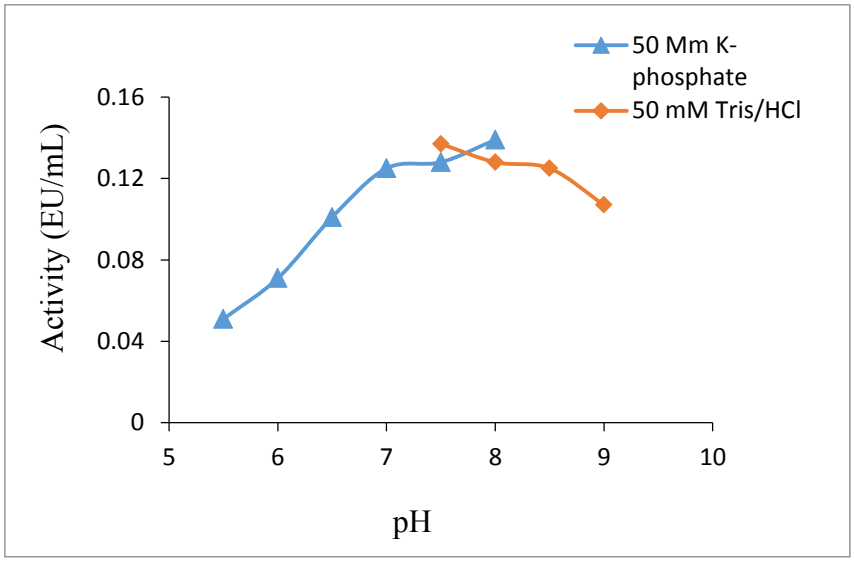

Figure 4.8. The graph of optimum pH results

4.2.3. Optimum Temperature

For determination of the optimum temperature of GR enzyme from quail's liver, the activity of enzyme was assayed in different temperature from 0 to 95 ˚C, as described in section 3.10.3, the enzyme has been showed to has high activity in 85 ˚C, as shown in Table 4.7 and Figure 4.9.

Table 4.7. Results of optimum temperature

Temperature ˚C	Activity (EU/mL)
0	0.044
10	0.092
20	0.114
30	0.171
40	0.198
50	0.266
60	0.325
70	0.359
75	0.373
80	0.384
85	0.401
90	0.353
95	0.051

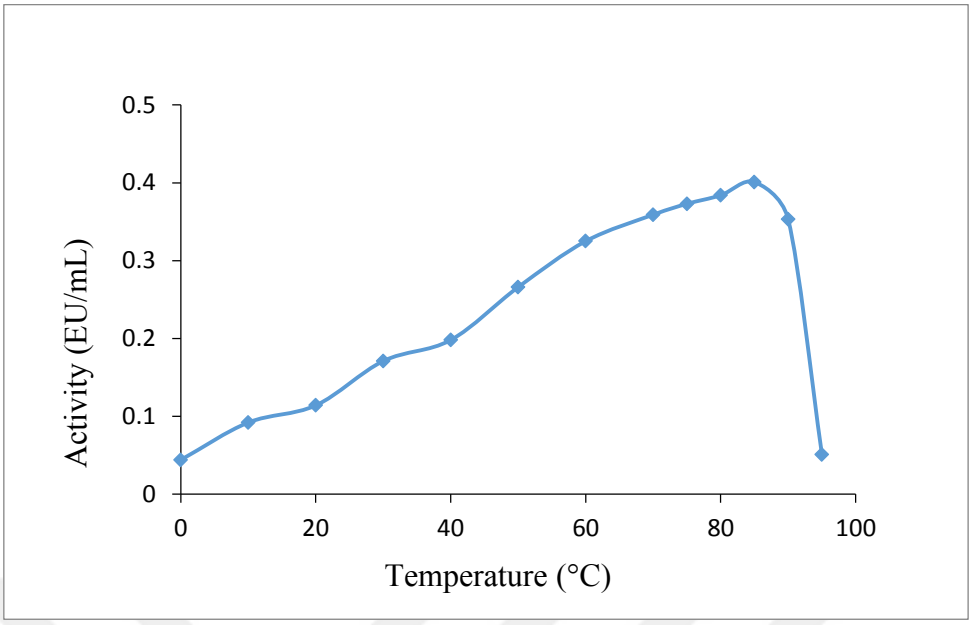

Figure 4.9. Results of optimum temperature

4.2.4. Optimum İonic Strength

Preparation of different concentration to determine the optimum ionic strength of enzyme in is described in section 3.10.4. GR in quail's liver was showed to has high activity in potassium buffer at (0.6 mM), as shown in Table 4.8 and 4.9 and Figure 4.10.

Table 4.8. Results of optimum ionic strength (K-phosphate buffer)

Concentration (mM)	Activity EU/mL
0.1	0.114
0.2	0.160
0.4	0.173
0.6	0.198
0.8	0.166
1.0	0.164

Table 4.9. Results of optimum ionic strength (Tris-HCl buffer)

Concentration (mM)	Activity (EU/mL)
0.1	0.162
0.2	0.178
0.4	0.176
0.6	0.180
0.8	0.175
1.0	0.153

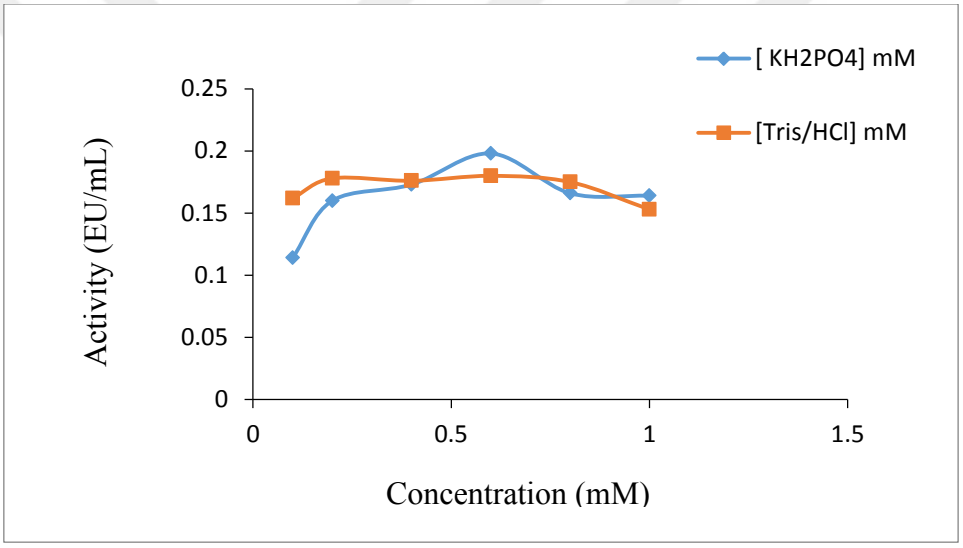

Figure 4.10. The results of optimum ionic strength (K-phosphate and Tris-HCl buffer)

4.3. Kinetic Studies

For NADPH five different concentration of NADPH and constant concentration of GSSG were obtained, and the same experiment was done for five different concentration of GSSG and constant concentration of NADPH section 3.11, then the K_M and V_{max} value for NADPH and GSSG were calculated by using equaition that procured from curve, as shown in Figure 4.11 and 4.12 and Table 4.10.

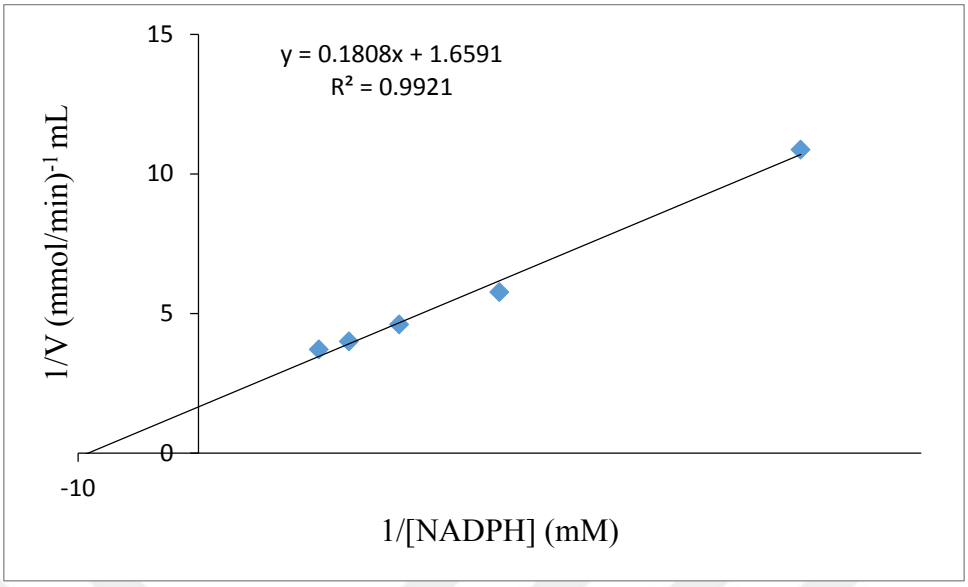

Figure 4.11. Lineweaver-Burk graph with five different concentration of NADPH

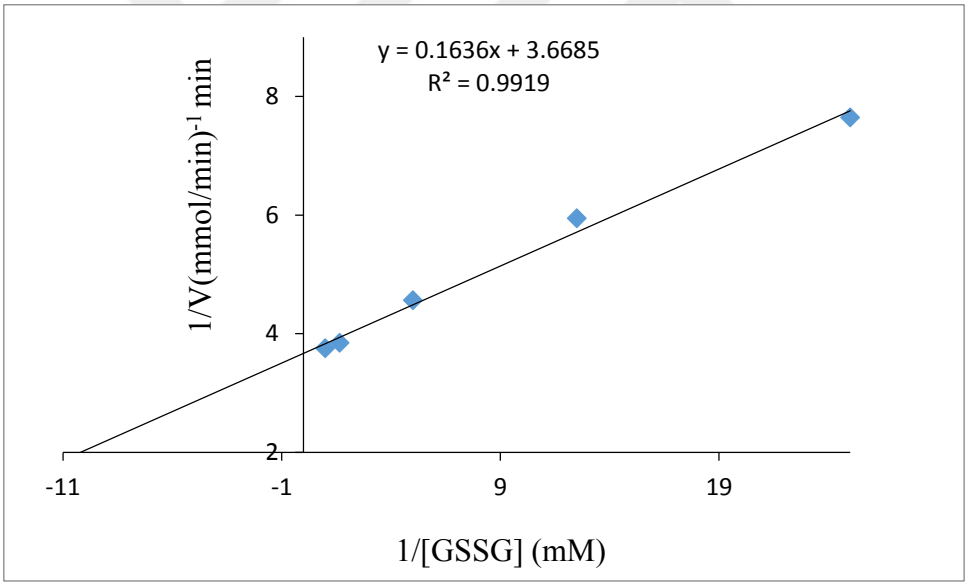

Figure 4.12. Lineweaver-Burk graph with five different concentration of GSSG

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Table 4.10. K_M and V_{max} value for both NADPH and GSSG

Substrate	Vmax	KM
NADPH	0.603	0.109
GSSG	0.272	0.044

K_M value of GSSG is lower than NADPH (Table 4.10), this value gave a mean of the affinity of GR enzyme in quail`s liver to GSSG is greater than to NADPH.

5. DISCUSSION

Glutathione reductase GR (EC: 1.8.1.4) is a ubiquitous flavoenzyme catalyzes the reduction of glutathione disulfide GSSG by NADPH (Staal and Veegel 1969), were the NADPH later being produced from pentose phosphate pathway in oxidative phase by glocuse-6-phosphate dehydrogenase and 6-phospho gluconate dehydrogenase (Sustmann et al.1989).

$$GSSG + NADPH \xrightarrow{GR} NADP^{+} + 2GSH$$

Oxygen consumption in normal cellular metabolism and environmental factors, such as cigarette smoke, air, and water pollutants are producing side product (Birben et al. 2012) or overproduction of reactive oxygen species (ROS) that mainly produced in mitochondria (Gill and Tuteja 2010). ROS are known to be free radical containing oxygen or highly reactive ions, that causing cell damage by impairing cellular function and redox homeostasis (Aoyama and Nakaki 2015).

Various type of human diseases, such as diabetes, Alzheimer, and cancer have been linked to oxidative stress (Winyard et al. 2005).

Intracellular to maintain the normal function of cellular and redox homeostasis has two antioxidant defense system against oxidative stress to neutralize ROS, enzymatic and nonenzymatic defense system (Pradedova et al. 2011). Enzymatic antioxidants, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPO), and glutathione reductase (GR) (Hakiman and Maziah 2009), nonenzymatic antioxidant, involving ascorbic acid (Vitamin C), reduced glutathione (GSH), flavones, and anthocyanins, vitamin A, β-Carotene and Vitamin E (Vaisi-Raygani et al. 2007). All antioxidant require glutathione (reduced glutathione GSH) to functional efficiently. Consequently, glutathione is the master of antioxidant (Quinn 2009), and the most plentiful protein

Containing thiol group in most organs cell (Dickinson and Forman 2002; Ashtiani et al. 2011), and it's exported from the cell and synthesized intracellularly (Meister 1988), also it is a prerequisite for the normal function of the cell (Boggaram et al. 1979).

The formation of peroxide like hydrogen peroxide H_2O_2 is extensively formed in tissue (Chance et al. 1979), which is probably the most stable type of ROS with essential biological function (Shabala 2012), and removed almost throughout reduction by glutathione (Jones et al. 1981). The reduction of H_2O_2 by GSH is catalyzed by glutathione peroxidase GPx.

Equation 5.1. Glutathione peroxidase catalyze the reduction of H_2O_2 by GSH

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$$

That is usually considered the major rout of cellular disulfide generation by enzymatic (Ziegler 1985). The cellular glutathione to be maintain in its reduced form it require glutathione reductase (Boggaram et al. 1979). GR for the first time termed by Conn and Vennesland (1951). After that GR has been purified and characterized on its property in a widely assortment of organisms, including escherichia coli (Asnis 1955; ErmLer and Schulz 1991), Baker`s yeast (Mavis and Stellwagen 1968), psychrophilic antarctic bacterium (Ji et al. 2015), horse liver (Garcia-Alfonso et al. 1993), rat liver (Carlberg and Mannervik 1975), human erythrocytes (Scott et al. 1963), human epidermal tumors (Bersaques 1979).

In human erythrocyte GR enzyme is a homodimer (MW 105 kDa), consist of two identical subunits with 478 residues and one FAD per monomers (Karplus and schulz 1987; Schulz and Karplus 1987), and total MW of apoenzyme FAD-free is 2×51.6 kDa and MW of holoenzyme with FAD is 2×52.4 kDa (Untucht-Grau et al. 1981), in the amino acid sequences Cys-58 are connected Cys-63 that release reduced glutathione and reform redox active disulfide bridge (Krauth-Siegel et al. 1982). This study was undertaken to purify GR from Japanese quail`s liver and characterize some of its properties.

Purification was executed by three steps; preparation of homogenate, ammonium sulfate precipitate, and 2′,5′-ADP Sepharose-4B gel affinity chromatography. All operations were performed at 4 ºC.

Fresh quail`s liver were taken from Bingol university agriculture farm, and five grams were cut into small pieces and homogenized with 15 mL, potassium phosphate buffer (pH 7.0), crude precipitate was removed by centrifugation at 15000 rpm and 4 ºC for one hour, homogenate supernatant was brought to 60% saturation with ammonium sulfate for a reason to precipitate the protein by salting out mechanism. After precipitation was done, and dissolved precipitate in a small volume of potassium phosphate buffer, dialyzed against same buffer at (pH 7.5) containing 1 mM EDTA was performed, for a reason to remove salt particles. The similar percent of ammonium sulfate was used for precipitation of protein in sheep liver (Ulusu et al. 2005), and different percent of ammonium sulfate was used to precipitate protein in rainbow trout liver (Tekman 2007). Ammonium sulfate precipitation is the most common application that used for precipitation of protein (Wingfield 2001).

In order for purification of GR; 8-(6-aminohexyl)-amino-2'-phospho-adenosine di phospho ribose (Lopez-Barea and Lee 1979); FPLC-anion-exchange and FPLC-hydrophobic interaction (Madamanch et al. 1992); Sephadex G-25; CM-cellulose; SephadexG-75, DEAE-cellulose; Hydroxylapatite; Sephacryl S-200 (Carlberg and Mannervik 1975; Carlberg et al. 1981); and Sephadex G-200 (Acan and Tezcan 1989); chromatography has been used.

However, 2′,5′-ADP Sepharose-4B gel affinity chromatography column provided as high rapidly and efficiently process for purification (Carlberg and Mannervik 1981; Madamanchi et al. 1992). Moreover, 2′5′ ADP is an NADP structural analog immobilized on Sepharose-4B, it bind and immobilize NADP-dependent enzymes. Consequently, this gel primarily used for purification of enzymes requiring $NADP^+$ as a cofactors, such as thioredoxin reductase (Temel 2014), and 6-phosphogluconate dehydrogenase 6PGD (Baqi 2016). The same gel also used for purification of GR from quail`s liver.

The dialyzed enzyme solution was applied to 2′,5′-ADP Sepharose-4B gel affinity chromatography column , and washed with solution A and B section 3.7, then the

quantity of GR was determined by Bradford method`s for eluted solution after pouring elution buffer into column. Bradford`s method is the most common method used for quantitative determination of protein, because of easy for using, fast, stability of color near to one hour and less susceptible to destruction effect, the method is based on binding dye negative charge to protein positive charge causing measure of absorbance at 595 nm because of changing color from red to blue (Bradford 1976).

Glutathione reductase in quail`s liver was purified with a specific activity of 22.75 EU/mg protein, in overall yield 13.6%, and 142.18 times. From the literature the similar result were detected; rhodospirillum rubrum (Boll 1969); bovine brain (Gutterer et al. 1999), different results also were reported; human lens (Latta and Augusteyn 1984), cyanobacterium anabaena sp Strain 7119 (Serrano et al. 1984), rat heart (Adem and Ciftci 2016).

MW and purity control of GR was determined and checked by SDS-PAGE, the appearance of single band on the gel represent the purified enzyme, and the molecular weight of enzyme calculated by a curve plotted from R_f-LogMW graph (Figure 4.5).

GR in quail`s liver has subunit molecular weight of 59 kDa (Figure 4.4). The previous experiment demonstrated that GR structure is a dimer with two identical subunits by using gel filtration chromatography to discern non-denatured enzyme, and SDS-PAGE to discern monomers denatured enzyme, for example; native molecular weight of GR from psychrophilic Antarctic bacterium is 95.2 kDa and 48.7 kDa moiety per subunits (Ji et al. 2015). From literature the similar result was reported; human leukocytes (Ogus and Tezcan 1981); anoxia tolerant turtle (Willmore and storey 2007). And a bit far result also was reported; Euglena gracilis (Shigeoka et al. 1987); human erythrocytes (Worthington and Rosemeyer 1975). GR in different source has molecular weight between 70000-150 000 Da (Douglas 1987).

After purification of GR from quail`s liver was performed, some properties of pure enzyme were characterized.

The pH stability of enzyme is detected at 8.0 in Tris-HCl buffer (Table 4.4 and Figure 4.7). From the other studies the pH stable; in Tris-HCL buffer is 8.0 (Tekman 2007), 8.5

(Taser 2010), and 9.0 (Adem 2011); in potassium phosphate buffer 5.5 (Ulusu et al. 2005), and 7.3 (Erat et al. 2003). The GR enzyme in quail`s liver has a similar stable pH to the Takman`s result and approximately near with the Taser and Adam`s result but different than other results.

GR in quail`s liver has an optimum pH of 8.0 in 1 M potassium phosphate buffer (Table 4.5 and Figure 4.8). From some different sources in literature GR has optimum pH in the same buffer, such as turkey liver (Taser and Ciftci 2011); spinach leaves (Halliwell and Foyer 1978); chlamydomonas reinhardtii (Takeda et al. 1993); bovine erythrocytes (Erat et al. 2003); chromatium vino sum (Chung and Hurlbert 1975); bovine filarial worms Setaria Cervi (Arora et al. 2012); rat lung (Adem 2011); bovine kidney cortex (Berivan 2010). And in some another sources GR has an optimum pH in Tris-HCl buffer, such as rainbow trout (Tekman 2007); sheep liver (Ulusu et al. 2005). In general GR enzyme showed to has optimum pH between 6.5-9 in different sources.

The activity of GR increased by increasing temperature as much as at 85 ºC shown the maximum activity and got denaturation at 95 ºC (Table 4.7 and Figure 4.9). from literature the near results were reported in different sources, including; rat heart (Adem 2011); erythrocytes of lake Van pearl mullet (Altun et al. 2015), also a bit different result were reported; rat kidney (Can 2010), rainbow trout liver (Tekman 2007), human jejunal (Ogus and Ozer 1990), human erythrocytes (Ogus and Ozer), bovine erythrocytes (Erat et al. 2003), euglena gracilis Z (Shigeoka et al. 1987), chlamydomonas reinhardtii (Takeda et al. 1993), bovine liver (Ulusu and tandogan 2007), thiobacillus ferrooxidans (Sugio et al. 1995).

Optimum ionic strength of GR enzyme in quail`s liver was determined at 600 mM in potassium phosphate buffer (Table 4.10 and Figure 4.8). From the literature optimum ionic strength was determined at; 100 mM (Taser 2010; Arora et al. 2013); 435 mM (Erat et al. 2003); and 40 mM (Adem 2011) in potassium phosphate buffer. And 50 mM in Tris-HCl buffer (Ulusu et al. 2003; Tekman 2007), 20 mM in Tricine-NaOH (pH 7.8), 0.1 mM in EDTA in the present of 45 mM $MgCl_2$ salt (Kalt-Torres et al. 1984), and 10 mM in phosphate buffer in present of 100 mM of KCl salt (Worthington and Rosemeyer 1976). The optimum ionic strength of GR enzyme in quail`s liver has a little bit similarity with Erat`s result, and extremely different to other results.

For kinetic study of enzyme, the K_M and V_{max} value for both GSSG and NADPH substrates were calculated as 0.044, 0.109 mM and 0.272, 0.603 UE/mL, respectively, from some different source the similar result were reported, such as from sheep liver K_M and V_{max} value for GSSG and NADPH were 0.023, 0.0258 mM and 0.255, 0.266 EU/mL, respectively (Ulusu et al. 2005). From rainbow trout liver, K_M and V_{max} value for GSSG and NADPH were 0.025, 0.098 mM and 0.055, 0.143 EU/mL, respectively (Tekman 2007), and also the different results were reported from several different source, such as from gill tissue of lake Van Fish the K_M and V_{max} value for GSSG nad NADPH were 22.37, 42.14 µM and 9.87, 25.25 EU/mL, respectively (Kuzu et al. 2016), from wheat grain the K_M and V_{max} value for GSSG and NADPH were 3.7, 9.1 mM and 575, 594 EU/ml protein (Lamotte et al. 2000), from turkey liver the value of K_M and V_{max} for GSSG and NADPH were 0.03, 170 mM and 0.228, 0.55 EU/mL, respectively (Taser and Ciftci 2012). Since the K_M value represented the affinity of enzyme to substrate, lower K_M has high affinity and vice versa, therefore, according to the K_M value the affinity of GR enzyme in quail`s liver to GSSG is higher than to the NADPH.

CONCLUSION

Glutathione reductase GR (NADPH: oxidized-glutathione oxidoreductase) catalyze the reduction of GSSG by NADPH as a reducing agent, it is considered as an indirect antioxidant and its main function to maintain glutathione in its reduced form.

GR was purified from Japanese quail`s liver by preparation of homogenate, ammonium sulfate precipitation and 2′,5′-ADP Sepharose-4B gel affinity chromatography column with specific activity of 22.75 EU/mg protein, in an overall yield 13.6% and 142.18 folds. And some property of this enzyme was characterized.

The characterization study results are summarized as bellows:

- Optimum pH: 8.0 (K-phosphate buffer).
- Optimum temperature 85˚C.
- Optimum ionic strength 0.6 M (K-phosphate buffer).
- Stable pH: 8.0 (Tris-HCl buffer).
- Subunit molecular weight 59 kDa.
- K_M and V_{max} value for NADPH were 0.109 mM and 0.603 EU/mL, respectively.
- K_M and V_{max} value for GSSG were 0.044 mM and 0.272 EU/mL, respectively.

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