THE EXPRESSION OF GST GENES IN DIABETIC RAT LIVER TISSUES

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ABSTRACT

THE EXPRESSION OF GST GENES IN DIABETIC RAT LIVER TISSUES

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Free radicals which have critical roles in living systems through their beneficial and detrimental effects play an important role in medical revolution in health. Radicals are produced in the cells and tissues of our bodies by various processes and reactions. Diabetes mellitus is an extremely common disease in the world which seems to be accompanied by a shortage of antioxidants and an increase in free radicals, the end result of oxidative stress. Glutathione S-Transferases (GST; EC 2.5.1.18) are found in enzymatic defense system which has a role in defending cells against potentially toxic and/or carcinogenic compounds.

In this study, the changes in the activities and expressions of various GST isozymes in the liver of diabetic rats related to oxidative stress were studied. The effects of antioxidants, Vitamin C and α-Lipoic acid on GST isozyme activities and mRNA expressions were also investigated. According to our results, diabetic rats exhibited decreased mRNA expressions of both GSTA2 and GSTM1 genes, but the activities of only GST Mu isozyme decreased in diabetic rats, compared to controls and GST Alpha isozyme activity remained unchanged in diabetic animals. Our results also showed that α-Lipoic acid individually has no significant effect on both GSTA2 and GSTM1 gene expressions and activities. Furthermore,

although the administration of Vitamin C alone showed no significant effect on all GST isozyme activities, it decreased GSTA2 mRNA expression significantly. The administration of Vitamin C and α-Lipoic acid together affected both GSTA2 and GSTM1 mRNA expressions in control rats, but only GST Mu activity showed a significant change.

The results of this study showed that, the administration of two antioxidants, α-Lipoic acid and Vitamin C alone and together did not reverse the results of diabetes at the level of both gene expression and activities of GST isozymes.

Key words: Antioxidants, Oxidative stress, Diabetes mellitus, Glutathione S-transferases.

ÖZ

DİYABETLİ SIÇAN KARACİĞER DOKULARINDA GLUTATYON S-TRANSFERAZ ENZİMİNİN GEN EKSPRESYONU

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Yaşayan sistemlerde kritik rolleri olan serbest radikaller, hem yararlı hemde zararlı etkileri ile sağlıktaki tıbbi devrimde önemli rol oynamışlardır. Radikaller vücudumuzun hücre ve dokularında çeşitli yöntemler ve reaksiyonlar sonucunda üretilirler. Dünyada yaygın olarak gözlemlenen şeker hastalığı (Diabetes mellitus) oksidatif stres sonucu oluşan serbest radikallerdeki artış ve antioksidantlardaki eksiklik sonucu oluşmaktadır. Glutatyon S-transferazlar (GST; EC 2.5.1.18) enzimatik savunma mekanizmasında yer alarak, potansiyel toksik ve/veya karsinojenik maddelere karşı hücresel savunmada rol oynarlar.

Bu çalışmada, diyabetik sıçanların karaciğerinde, oksidatif strese bağlı, çeşitli GST izozimlerinin aktivitelerindeki ve ekspresyonlarındaki değişimler çalışılmıştır. Ayrıca antioksidantlardan, C vitamini ve α-lipoik asidin GST izozim aktiviteleri ve mRNA ekspresyonları üzerine etkileri incelenmiştir. Sonuçlarımıza göre, GSTA2 ve GSTM1 gen expresyonlarının ikisinde de diyabetik sıçanlarda kontrole göre düşüş olmasına rağmen, sadece GST Mu izoenzim aktivitesinde düşüş görülmüş ve GST Alpha aktivitesi diyabetli hayvanlarda değişmemiştir. Sonuçlarımız, α-lipoik asidin tek başına GSTA2 ve GSTM1 gen ekspresyonları ve aktiviteleri üzerine

önemli bir etkisinin olmadığını göstermiştir. C vitamini tek başına verildiğinde, tüm GST izozimleri üzerinde önemli bir etki göstermezken sadece GSTA2 mRNA ekspresyonunu önemli oranda azaltmıştır. C vitamini ve α-lipoik asidin birlikte verilmesi kontrol sıçanlarda GSTA2 ve GSTM1 mRNA ekspresyonlarının ikisini de etkilerken, sadece GST Mu aktivitesinde önemli bir değişiklik göstermiştir.

Bu çalışmanın sonuçları, her iki antioksidan α-lipoik asit ve C vitamininin tek başına veya birlikte verilmesinin, GST izozimlerinin gen ekspresyonu ve aktivitelerinde diyabetin sonuçlarını tersine çevirmediğini göstermiştir.

Anahtar kelimeler: Antioksidanlar, Oksidatif stres, Diabetes mellitus, Glutatyon S-transferaz.

Dedicated to my parents,

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ABBREVIATIONS

BSA	Bovine Serum Albumin
CDNB	1-Chloro-2,4-dinitrobenzene
DCNB	1,2-Dichloro-4-nitrobenzene
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-Transferase
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
ROS	Reactive Oxygen Species
TRIS	Tris (hydroxymethyl) aminomethane

CHAPTER I

INTRODUCTION

1.1 Free Radicals

The recent growth in knowledge of free radicals and reactive oxygen species (ROS) in biological systems is producing a medical revolution that promises a new age of health. They play an important role in living systems through their beneficial and detrimental effects (Gutteridge and Halliwell, 2000).

Radicals are produced in the cells and tissues of our bodies by various processes and reactions. For instance; a radical can be produced by high energy radiation, ionizing radiation, visible light with photosensitizes and thermal degradation of organic materials by the loss of a single electron from a non-radical to produce a free radical, or by the gain of a single electron, where the electron pair of a covalent bond is shared between two atoms resulting in each having an odd electron (Halliwell and Gutteridge, 1989). Factors such as site of production, the availability of transition metals, and the action of enzymes determine the fate of each radical species. The consequences of free radicals in biological systems are manifold and include damage to all cell constituents which are prone to oxidative attack.

A free radical can be described as any atom or molecule that lack one or more electrons in the outer orbital. Free radicals attack the nearest stable molecule and steal its electrons and cause oxidative damage (Harmon*, 1956).* They may be positively or negatively charged or neutral and all types are important. Anion radicals are important in biology. The most important of these radicals are the reactive oxygen species. Toxic oxygen free radicals are formed by many sources. Such as mitochondrial and microsomal electron transport chains, activated phagocytes, activation

of arachidonic acid cascade, autoxidation of endogenous compounds, cigarette smoke, ionizing radiation, etc. Five percent of the inhaled oxygen is converted into free radicals. Diatomic oxygen is also a radical because of 2 unpaired electrons. The conversion of diatomic oxygen to water include sequential steps (Bast and Goris, 1989).

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-/H^+} H_2O_2 \xrightarrow{e^-/H^+} OH^\cdot \xrightarrow{e^-/H^+} H_2O^\cdot \qquad [1]$$

First of all one electron is added to oxygen and formed superoxide radicals (O_2^-). Superoxide radicals are converted to the hydrogen peroxides (H_2O_2) by addition of another electron. Addition of one electron and one hydrogen atom to hydrogen peroxide formed extremely destructive hydroxyl radical (Barber and Haris, 1994).

Under normal biological conditions hydrogen peroxides are converted into water and oxygen, but when they react with superoxide radicals, they form hydroxyl radicals ($OH^{\cdot -}$) by the following Haber-Weis Reaction.

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-/H^+} H_2O_2 \xrightarrow{e^-/H^+} OH^\cdot \qquad [2]$$

Metal ion dependent reactions with either ferrous or cupric ions also generate superoxide radicals (McCord and Firdovich, 1969). These reactions are reversible and they can be named as Fenton Reactions.

$$Me^{+n} + H_2O_2 \longleftrightarrow Me^{(n+1)} + OH^\cdot + OH^- \qquad [3]$$

Moreover mitochondria produce superoxide radicals at two sites in the electron chain. The first is ubiquionone to cytochrome c1 step and the second one is NADH-dehydrogenase step (Turrens *et al*, 1985).

Another enzymatic source of superoxide radicals is xantine oxidase. This enzyme is very important when tissue reoxygenation occurs after ischemia or after extreme hypotension (Chambers et al.,1985).

Other group of reactive oxygen species is described by organic peroxides (ROOH) and their hemolytic degradation products such as alkoxyl ($RO^{\cdot}$) and hydroperoxyl ($ROO^{\cdot}$) radicals (Elstner, 1991).

Another physiological free radical is nitrogen oxides $NO^{\cdot}$ and NO_2, which are sufficiently stable to exist at relatively high concentrations. $NO^{\cdot}$ is made by vascular endothelium as a relaxing factor and by phagocytes. Both NO and NO_2 are important pollutant which occur in photochemical smog and computer modelling smog production. Nitric oxide has many good physiological effects but high amount of nitric oxide can be toxic (Halliwell, 1994).

Free radicals can produce large amount of damages on biological components like polyunsatuated fatty acids, proteins and nucleic acids. Carbohydrates are also the targets of ROS. ROS attack can change intrinsic membrane properties, like fluidity, ion transport and can also cause loss of enzyme activity, protein cross-linking, inhibition of protein synthesis and DNA damage (Bandyopadhyay et al.,1999).

The most common effects of free radicals are the oxidative breakdown of polyunsaturated fatty acids. This process can be named as lipid peroxidation. Iron plays a crucial role in the initiation of the chain reaction process of lipid peroxidation (Minotti and Aust, 1987). Decomposition of lipid peroxides may lead to the formation of singlet oxygen (Bast and Goris, 1989).

DNA damage is the other consequence of free radical induced oxidative stress. The DNA damage contains mainly base modification, deoxyribose oxidation, strand breakage, and the formation of DNA-protein crosslink. Hydroxyl radicals generate various products from the DNA bases which mainly contained C-8 hydroxylation of guanine to form 8-oxo-7,8 dehydro-2'-deoxyguanosine, a ring opened product; 2,6-diamino-4-hydroxy-5-formaminodipyrimidine, 8-hydroxy-adenine, 8-OH-guanine, 2-OH-adenine, thymine glycol, cytosine gylcol etc. The structure of some of the

products of ROS attack on DNA are shown in Figure 1.1 (Wiseman and Halliwell,1996).

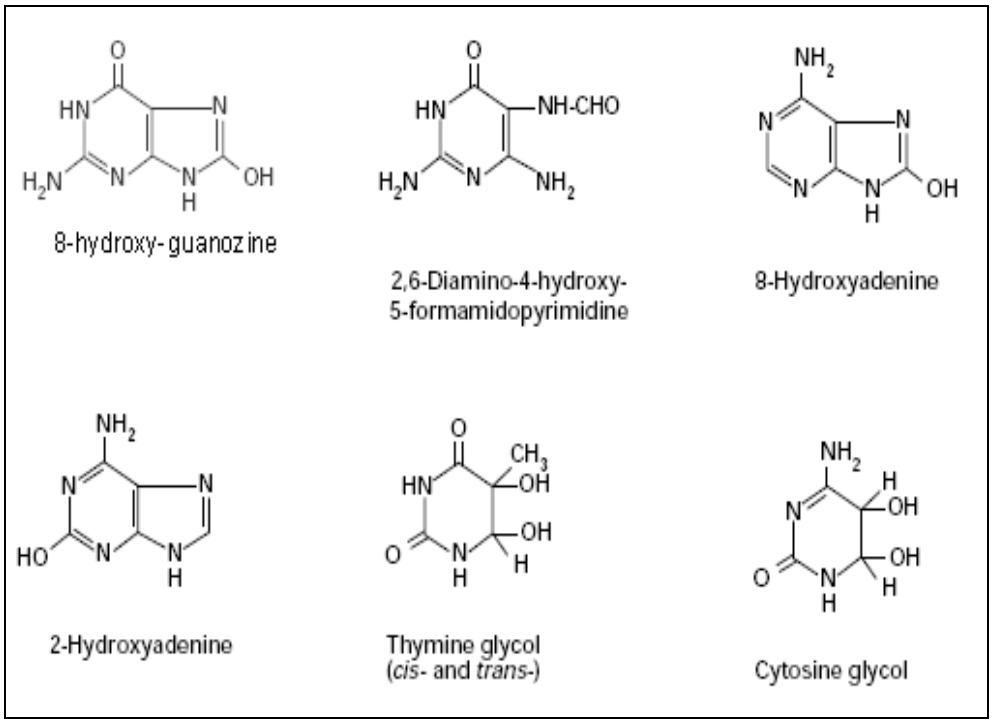


Figure 1.1: The structure of modified DNA bases (Wiseman and Halliwell, 1996).

Proteins are the most possible macromolecules affected from the free radical induced oxidative stress. When proteins are oxidized carbonyl groups (aldehydes and ketones) are generated on protein side chains (especially of Pro, Arg, Lys, and Thr). Protein carbonyl derivatives can also be produced through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by an a-ketoacyl derivative (Berlett et al., 1997).

A variety of natural antioxidants exist to scavenge oxygen free radicals and prevent oxidative damage to biological membranes. These antioxidant defense mechanisms include both enzymatic (intracellular) and non-enzymatic strategies. The enzymatic ones (intracellular) are

superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferases. The major natural antioxidants which are derived from natural sources by dietary intake are vitamin A, vitamin C, vitamin E and carotenoids (Halliwell and Whiteman, 2004; Ramakrishna and Jailkhani, 2007). Also, numerous small molecules has antioxidant capacities. For example glutathione and uric acid, which are synthesized or produced within the body and lipoic acid, mixed carotenoids, coenzyme Q10, several bioflavonoids, antioxidant minerals (copper, zinc, manganase, and selenium) and the cofactors (folic acid, vitamins B1, B2, B6, B12) (Halliwell and Gutteridge, 1999; Maritim et al., 2003; Engler et al., 2003; Heistad, 2005), all show antioxidant properties.

Under normal physiological conditions, there exists crucial balance between the oxygen free radical formation and antioxidant defense systems.

1.2 Diabetes mellitus

Diabetes mellitus, which is associated with “sweet excessive urine (known as glycosuria)”, is an extremely common disease in the world (Kenny et al, 1995). Diabetes mellitus is characterized by either a deficiency in insulin secretion, or resistance to insulin’ effects. The World Health Organization recognizes three main forms of diabetes mellitus: *type 1*, *type 2*, and *gestational diabetes*, which occurs during pregnancy. Ultimately, all forms are due to the beta cells of the pancreas being unable to produce sufficient insulin to prevent hyperglycemia. Type 1 diabetes is usually due to autoimmune destruction of the pancreatic beta cells, which produce insulin. Type 2 diabetes is characterized by insulin resistance in target tissues, but some impairment of beta cell function is necessary for its development. Diabetes also seems to be accompanied by a shortage of antioxidants and an increase in free radicals, the end result of oxidative stress (Report of WHO Consultation,1999).

Diabetes mellitus is a very complex chronic disease with syndrome of hyperglycemia, the primary clinical indicator of diabetes, which has been

accepted as being essential for the development of diabetic complication. Many evidence have shown that some biochemical pathways strictly associated with hyperglycermia. Nonenzymatic glycosylation, glucose autoxidation, polyol pathways and hypergylcemia can raise the production of free radicals as shown in Figure 1.2 (Glugliano et al,1996).

Diabetic patients have an increased level of blood glucose and excess glucose can be toxic to cells in several ways, two of which are free radicals such as $O_2^{\cdot}$-, $OH^{\cdot}$and the formation of advanced glycation end products (AGE).

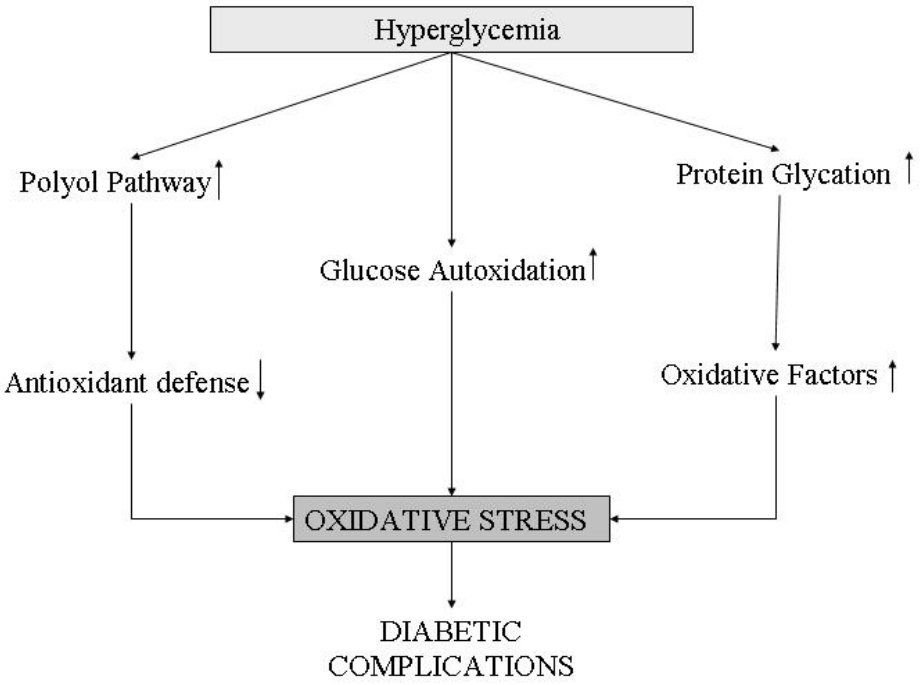


Figure 1.2: The hyperglycemic complications associated with free radical formation (Glugliano et al,1996)

Both types of products can produce diabetic complications. Hyperglycemia can increase the levels of free radicals through protein glycation, autoxidation glycation, protein kinase and also by an increase in the polyol pathway (Pfaffly, 2001).

The oxidizing intermediates formed by autoxidation is suggested to be a cause for some of the structural damage seen in diabetes. This reaction is often catalyzed by transition metals, and even with the catalyst, the reaction is very slow. A sugar aldehyde or ketone can react with an amino group of proteins, and also with phospholipids and nucleic acids, in a non-enzymatic reaction, giving rise to a Schiff base. The reaction is reversible and occurs until reaching the equilibrium. However the Schiff base is slowly rearranged giving the so-called Amadori product, that is fructosamine. Amadori adducts can then decompose to form deoxyglucones, which are considerably more reactive than the sugar they derived (Pfaffly, 2001). This is the early glycation process and the compounds formed are considered early glycation adducts. However, they can undergo a further rearrangement and eventually dehydration, condensation, fragmentation, oxidation and cyclization reactions, giving rise to compounds which bind irreversibly, the so-called advanced glycation end-products (AGEs). Formation of AGEs are shown in Figure 1.3 and the chemical structures are shown in Figure 1.4.

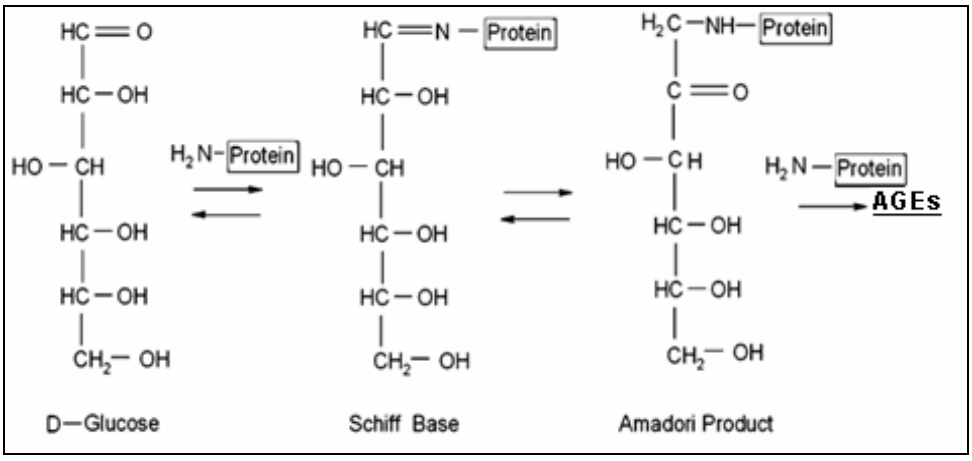


Figure 1.3: The formation of Advanced Glycation End-Products(AGEs).

When blood sugar is increased, more AGEs are formed and therefore these compounds have been initially connected with diabetes.

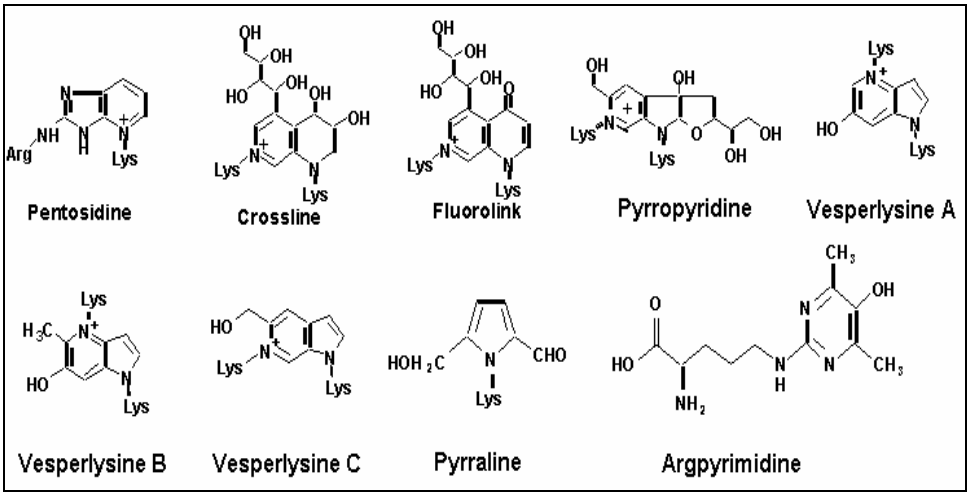


Figure 1.4: The chemical structures of AGEs.

AGEs can propagate free-radical reactions, that may catalyze further damaged proteins, lipids or DNA (Grillo and Colombatto, 2007). Glycation process and subsequent degradation of glycation products are shown in Figure 1.5.

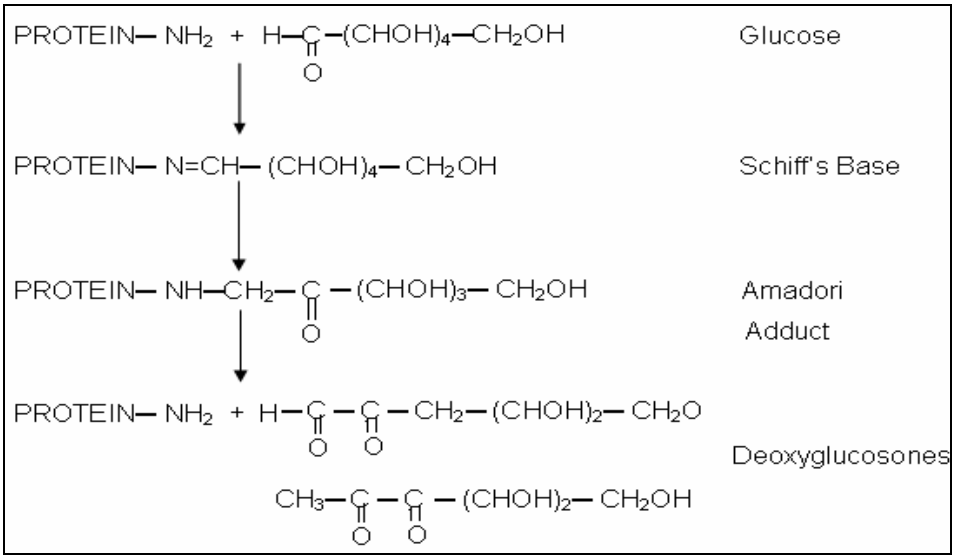


Figure 1.5: Glycation process and subsequent degradation of glycation products (Wolff et al.,1991).

Free radicals can also be produced by polyol pathway (Glugliano et al., 1996). Exposure to elevated glucose levels raise intracellular sorbitol and fructose content because of aldose reductase and sorbitol dehydrogenase activities. In polyol pathway; aldose reductase catalyzes the reduction of glucose by NADPH to sorbitol which can, in turn, be oxidized to fructose by sorbitol dehydrogenase (SDH) leading to redox imbalance (NAD^+/NADH ratio). An increase in NAD^+/ NADH ratio is linked to O_2·- formation via the reduction of prostaglandin G_2 to prostaglandin H_2 (PGH2) by prostaglandin hydroperoxidase which use NADH or NADPH as a reducing cosubstrate. The polyol pathway is given in Figure 1.6. Oxidation of sorbitol to fructose is joined to reduction of $NADP^+$ to NADPH (Baynes, 1991).

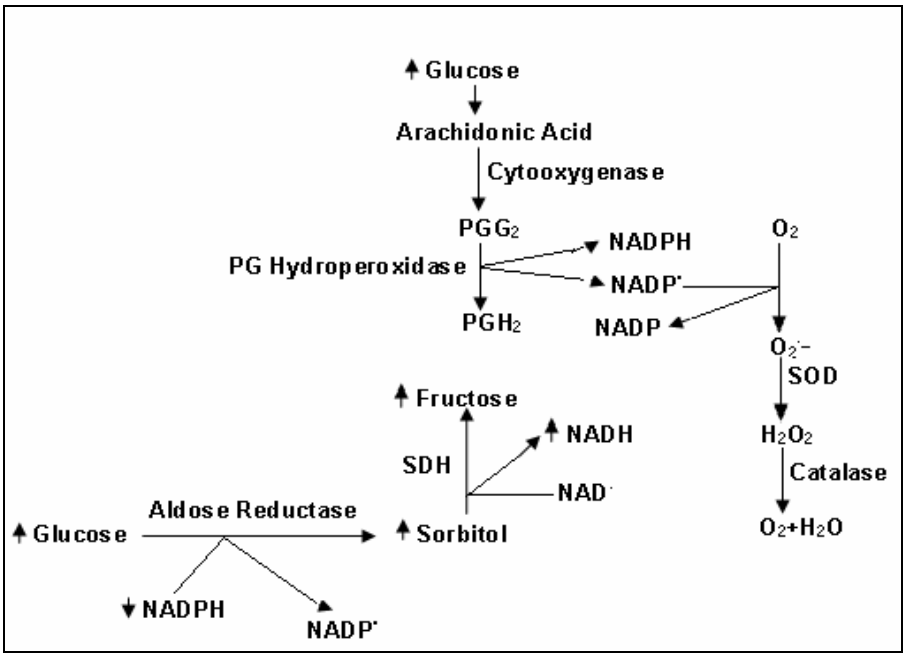


Figure 1.6: The polyol pathway (Tesfamariam, 1993).

1.3 Glutathione S-Transferases (GSTs)

Glutathione S-Transferases (EC 2.5.1.18) have a role in defending cells against potentially toxic and/or carcinogenic compounds by catalyzing the conjugation of glutathione (GSH) to electrophilic metabolites (Boyland

and Chasseaud, 1969; Jacoby, 1978). GSTs have various isoenzymes that are ubiqitiously distributed in nature, being found in organism as varied as microbes, insects, plants, fish, birds, and mammals (Hayes and Pulford, 1995). GSTs were first discovered in animals in the 1960s as a result of their importance in the metabolism and detoxification of drugs (Wilce and Parker, 1994).

The enzymatic detoxification of xenobiotics has been classified into three distinct phases which act in a tightly integrated manner. Phases I and II involve the conversion of a lipophilic, non-polar xenobiotics into a more water-soluble and therefore less toxic metabolite, which can then be eliminated more easily from the cell as shown in Figure 1.7. Phase I is catalyzed mainly by the cytochrome P450 system. This family of microsomal proteins is responsible for a range of reactions, of which oxidation appears to be the most important. Phase II enzymes catalyze the conjugation of activated xenobiotics to an endogenous water-soluble substrate, such as reduced glutathione (GSH), UDP-glucuronic acid or glycine. Quantitatively, conjugation to GSH, which is catalyzed by the GSTs, is the major phase II reaction in many species (Salinas and Wong, 1999).

The addition of GSH to the molecule gives it a molecular ‘flag’ which allows the xenobiotic conjugate to be eliminated from the cell during the Phase III part of the drug metabolism (Hayes and McLellan, 1999). Antiporter activity (p-glycoprotein or multidrug resistance) has been defined as the Phase III detoxification system. Antiporter activity is an important factor in the first pass metabolism of pharmaceuticals and other xenobiotics. The antiporter is an energy-dependent efflux pump, which pumps xenobiotics out of a cell, thereby decreasing the intracellular concentration of xenobiotics. After phase II reactions, the xenobiotic conjugates may be further metabolized. A common example is the processing of glutathione conjugates to acetylcysteine (mercapturic acid) conjugates. The γ-glutamate and glycine residues in the glutathione molecule are removed by gamma-glutamyl transpeptidase and dipeptidases. In the final step, the cystine residue in the conjugate is acetylated. Conjugates and their metabolites can be excreted from cells in

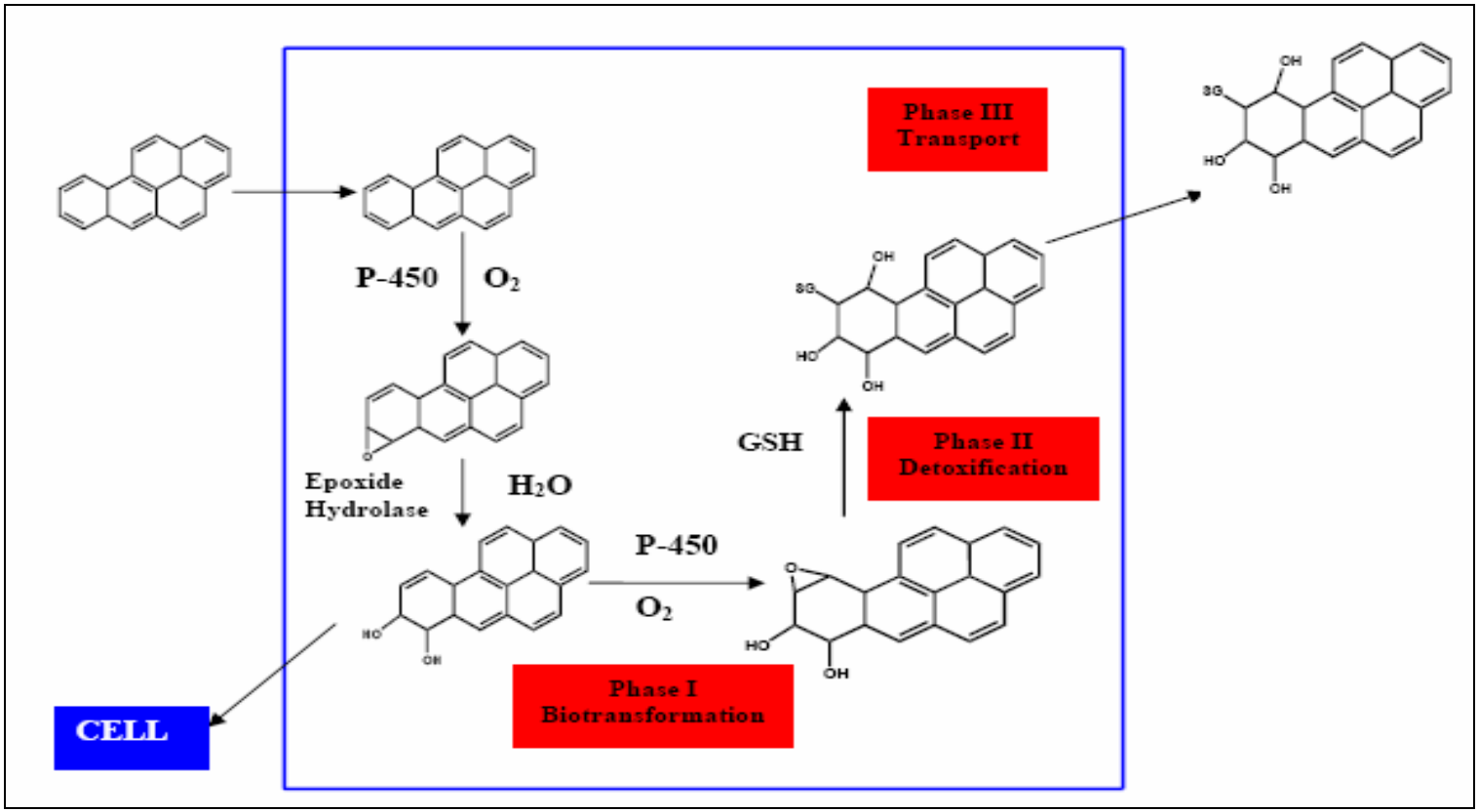


Figure 1.7: Overview of enzymatic biotransformations by phase I (cytochrome P450 and epoxide hydrolase), phase II (glutathione S-transferase) and phase III mechanisms (Sheenan et al., 2001)

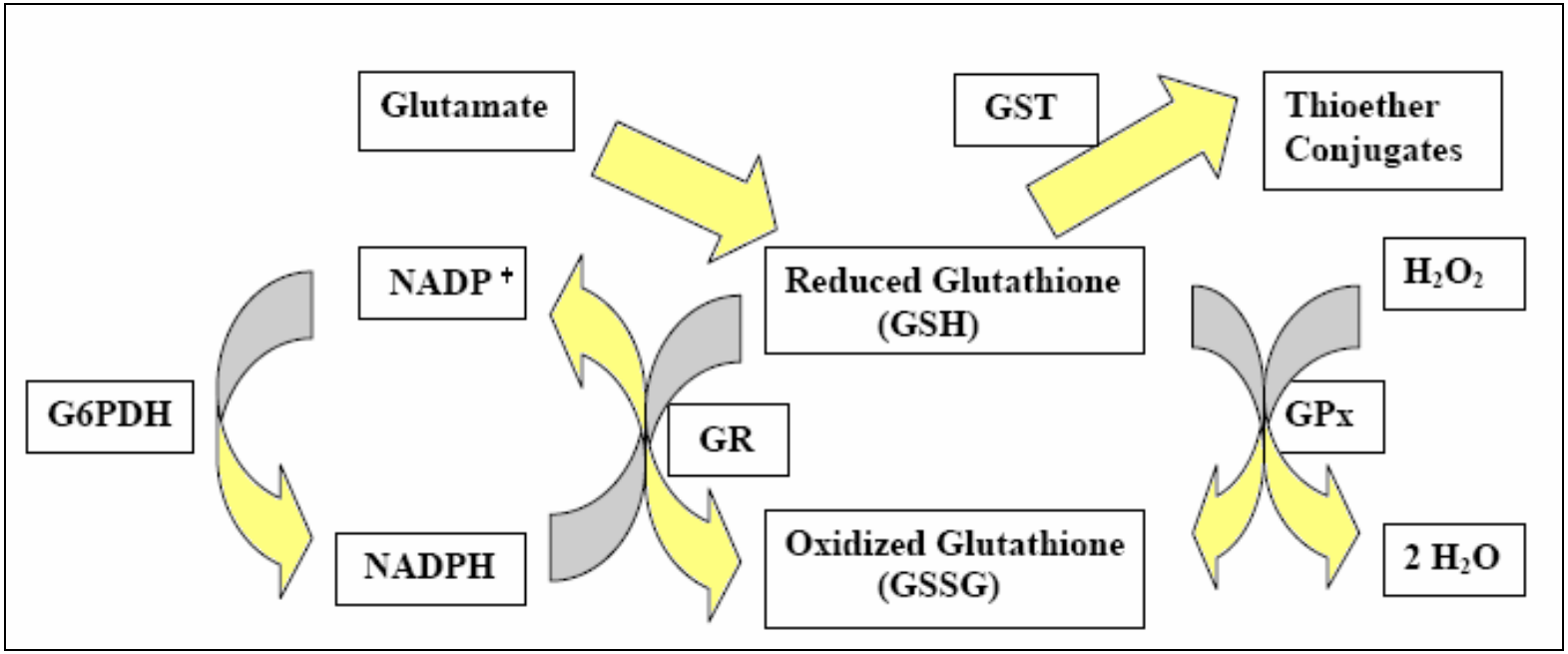
phase III of their metabolism, with the anionic groups acting as affinity tags for a variety of membrane transporters of the multidrug resistance protein (MRP) family. These proteins are members of the family of ATP-binding cassette transporters and can catalyze the ATP-dependent transport of a huge variety of hydrophobic anions, and thus act to remove phase II products to the extracellular medium, where they may be further metabolized or excreted (Liska, 1998).

The glutathione redox cycle is shown in Figure 1.8. In this cycle, most of the enzymes catalyze the conjugation of reduced Glutathione (GSH) with compounds that include an electrophilic center through the formation of a thioether bond between the sulphur atom of GSH and the substrate (Mannervik, 1985).

Glutathione S-transferases are grouped into three major families. Two of these are cytosolic and mitochondrial, which are called soluble GSTs, and the other family is microsomal GST (Mannervik, 1985; Jacobsson et al, 1999).

Based on the degree of sequence identity, the soluble mammalian GST enzymes have been assigned to eight families, or classes, named as Alpha (α), Mu (μ), Pi (π), Sigma (σ), Theta (θ), Zeta (ξ), Omega (ω) and Kappa (κ) (Board *et al*., 2000). Alpha, mu and pi classes are active in drug metabolism. Four additional classes of this superfamily, called Beta (β), Delta (δ), Phi (φ), and Tau (τ) are represented in bacteria, insects and plants (Hayes and McLennan, 1999). The phylogenetic tree illustrating the diversity of GSTs and the relationships between classes are shown in Figure 1.9.

All GST proteins are composed of two subunits and exist as either homo- or heterodimeric proteins (Forkert et al., 1999). These subunits are designated by two nomenclature systems. One of which is class-based nomenclature and other one Arabic numerals which has been proposed by Jakoby and colleagues (1984). In this nomenclature, capital letters are used to represent the alpha (A), mu (M), pi (P), sigma (S), and theta (T) classes, and Arabic numerals are used to represent each of the gene products; for example, class alpha subunits are designated A1, A2, A3, and so forth. Ya, Yb, and Yc are designated by class based nomenclature and



G6PDH : Glucose 6-Phosphate Dehydrogenase
GPx : Glutathione Peroxidase
GR : Glutathione Reductase
GST : Glutathione S-Transferase
$NADP^+$: Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)
NADPH : Nicotinamide Adenine Dinucleotide Phosphate (reduced form)

Figure 1.8 : Glutathione Redox Cycle

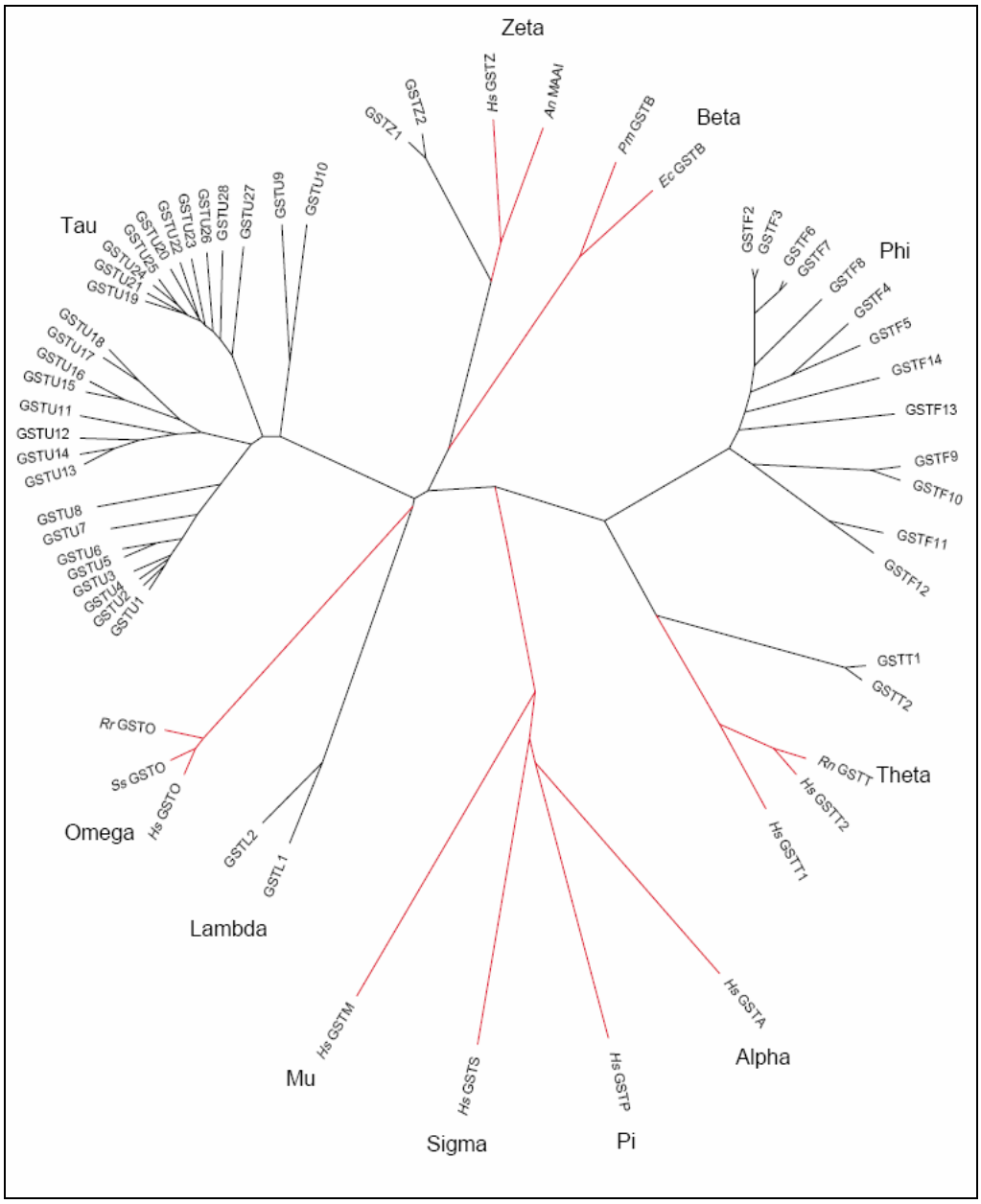


Figure 1.9 : Phylogenetic tree of GSTs (Dixon *et al., 2002)*

the Ya and Yc bands represent class alpha GST, whereas the Yb band represents class mu GST (Bass et al., 1977; Scully and Mantle, 1981; Hayes and Clarkson, 1982).

The overall structures of the enzymes are remarkably similar. These structural relationships are shown in Figure 1.10. Several enzymes are

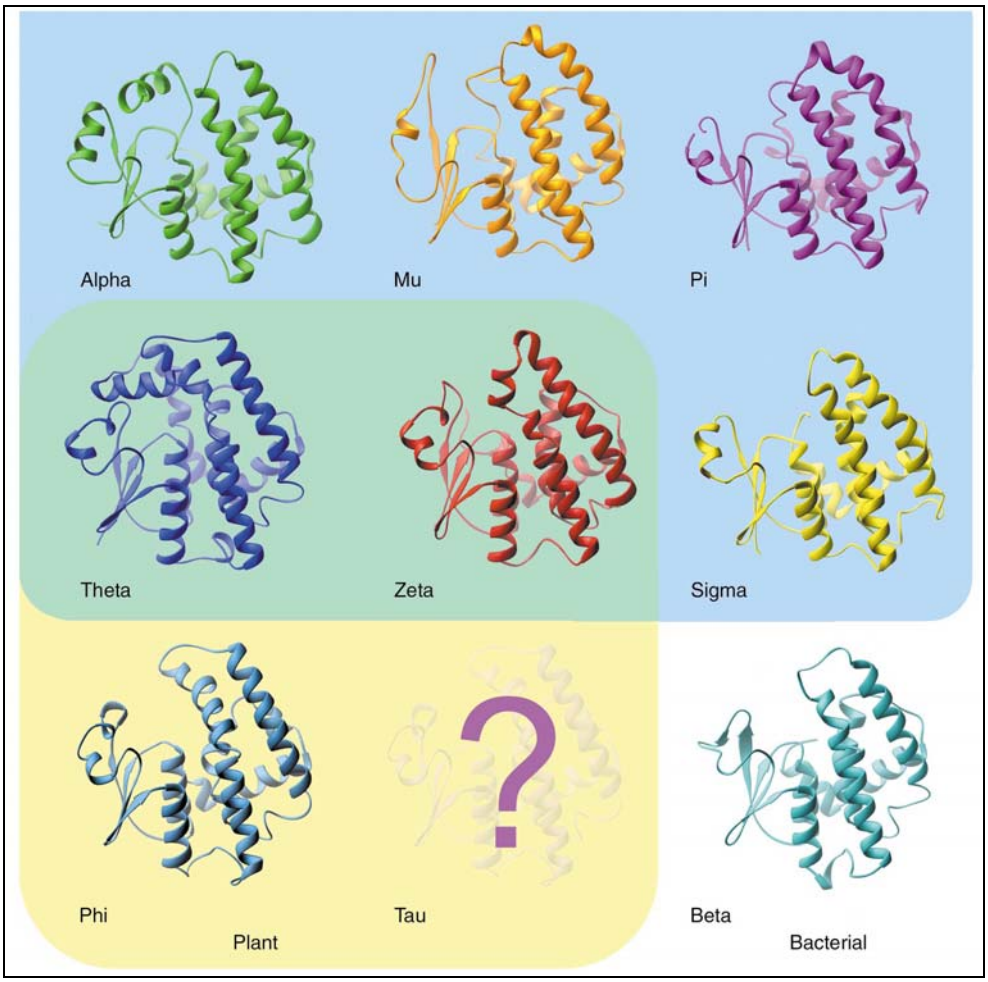


Figure 1.10: Ribbon representations of the structures of GST subunits. The GSTs specific to mammals (alpha, mu, pi and sigma) have a blue background; the plantspecific (phi) and bacteria-specific (beta) GSTs have yellow and white backgrounds, respectively; GSTs (theta and zeta) that have counterparts in both animals and plants have green backgrounds. Although there is little sequence similarity between enzymes of different classes, there is significant conservation in overall structure (Dixon et al., 2002).

recognized as belonging to the Alpha and Mu classes, while the Pi class originally contained only one protein, GST P. At least six distinct Mu-class

subunits (M1-6) have been identified in the rat, with homologous gene loci for the first five of these in humans.

The soluble GSTs are included primarily in the metabolism of foreign chemicals, such as carcinogens, environmental pollutants and cancer chemotheurapatic drugs, as well as the detoxication of potentially harmful endogenously derived reactive compounds (Hayes and Pulford 1995). Many endogenous GST substrates are created as a result of modification macromolecules by reactive oxygen species, and the transferases are therefore considered to serve an antioxidant function (Mannervik 1986; Hayes and McLellan 1999)

Glutathione S-transferases are of interest to pharmacologists and toxicologists as they provide targets for antiasthmatic and antitumor drug therapies (Matsushita *et al*., 1998; Jakobsson *et al*., 1999), and they also metabolize cancer chemotherapeutic agents, insecticides, herbicides, carcinogens, and by-products of oxidative stress. Polymorphisms in genes coding for enzymes involved in protection against oxidative stress have been implicated in the predisposition of individuals to disease states such as cancer (Forsberg et al., 2001). Mu class, may predispose certain individuals to greater risk from toxic xenobiotics (Nakajima *et al*., 1995; To-Figueras *et al*., 2000).

Overexpression of GSTs in mammalian tumor cells has been implicated with resistance to various anticancer agents and chemical carcinogens (Hayes and Pulford, 1995).

The cancer chemotherapeutic agents adriamycin, 1,3- *bis* (2-chloroethyl)-1-nitrosourea (BCNU), busulfan, carmustine, chlorambucil, *cis*-platin, crotonyloxymethyl-2- cyclohexenone (COMC-6), cyclophosphamide, ethacrynic acid, melphalan, mitozantrone, and thiotepa are mainly detoxified by structure of a tau GST (Hayes and Pulford 1995; Hamilton *et al*. 2003; Lien *et al*. 2002).

Furthermore, many environmental chemicals and their metabolites are detoxified by GSTs, like acrolein, atrazine, DDT, inorganic arsenic, lindane, malathion, methyl parathion, muconaldehyde, and tridiphane (Hayes and Pulford, 1995; Abel *et al*., 2004).

GSTs also detoxify a large number of epoxides, such as the antibiotic fosfomycin and those derived from environmental carcinogens. The latter group includes epoxides formed from aflatoxin B1, 1-nitropyrene, 4-nitroquinoline, polycyclic aromatic hydrocarbons (PAHs), and styrene by the actions of cytochromes P450 in the liver, lung, gastrointestinal tract, and other organs. Conjugation of aflatoxin B 1-8, 9-epoxide with GSH is a major mechanism of protection against the mycotoxin, at least in rodents (Kelly *et al.*, 2000).

Glutathione S-transferases play a crucial role in the detoxification of products of oxidative stress, degradation of aromatic amino acids, synthesis of steroid hormones, synthesis and inactivation of eicosanoids, modulation of signaling pathways. GSTs also serve an important role in the isomerization of many biologically important molecules (Benson *et al*., 1977). Together with catalase (CAT), superoxide dismutase (SOD), GSTs and glutathione peroxidase (GPx) are the major enzymes that remove (Chance, 1979) and protect the cells from the free radicals.

As a result, GSTs are a multigene family of isozymes that catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophilic compounds as the first step in a detoxification pathway and detoxify toxic metabolites which are produced within the cell by oxidative stress and protect cells from oxidant injury (Ngo and Nutter, 1994).

Alfa-Lipoic acid (LA) is a naturally occurring compound present as a cofactor in several mitochondrial enzymes that are involved in metabolism and energy production (Maritim et al., 2003). In its free form, LA is a powerful antioxidant, functioning as a ROS scavenging. Antioxidant effects of LA is based on its interaction with peroxyl radicals, which are essential for the initiation of lipid peroxidation; and ascorbyl radicals of vitamin C. Reduced form of lipoic acid, dihydrolipoic acid (DHLA), can recycle ascorbyl radicals and reduce dehydroascorbate generated in the course of ascorbate oxidation by radicals. Therefore, dihydrolipoic acid may act as a strong chain-breaking antioxidant and may enhance the antioxidant potency of other antioxidants like vitamin C in both the aqueous and in hydrophobic membrane phase (Padayatty *et al*., 2002).

1.4 Aim of The Study

The aim of the study is to investigate the changes in STZ-diabetes related to oxidative stress and the activities and expressions of various GST isozymes in the liver of diabetic rats. In order to elucidate the alterations in oxidative stress in animals with diabetes, we have measured the GSH concentration and lipid peroxidation previously. In this study, some of the catalytic activities of GST isozymes by using different substrates and their mRNA expressions by using different primers were first optimized and then both activities and mRNA expressions were calculated for both diabetic, control and treated rat livers. The effects of some antioxidants like α-lipoic acid and vitamin C on different GST isozyme activities and their gene expressions, in liver tissues were also studied in diabetic rats.

The results of this study, will show the effects of hyperglycemia and oxidative stress caused by diabetes, on the gene expressions and activities of different GST isozymes and the possible effects of the antioxidants, vitamin C and lipoic acid.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Streptozotocin (STZ), isopropanol, dithiothreitol (DDT), bovine serum albumin (BSA), ethyldiaminetetraaceticacid (EDTA), sodium-potassium tartarate, phenylmethylsulfonylfloride (PMSF), Folin-Ciocalteu-Phenol reagent, guanidium isothiocyanate, sarcosyl, sodium acetate, phenol, chloroform, isoamyl alcohol, sodium citrate, 1-chloro-2,4-dinitrobenzene (CDNB), paranitrobenzylchloride (PNBC), 1,2-epoxy-3(paranitrophenoxy) propane (EPNP), reduced glutathione (GSH) were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, U.S.A.

Potassium chloride (KCl), dipotassium hydrogen phosphate (K_2HPO_4) and 2-mercaptoethanol were purchased from Merck Chemical Company, Germany.

Sodium carbonate, sodium hydroxide, cuppersulfatepentahydrate and ethyl alcohol were purchased from Riedel de-Haen Chemical Company, Germany.

Diethyl pyrocarbonate (DEPC), deionized formamide, Tris-(hydroxymethyl)-aminomethane (Tris) and morpholinopropane sulfonoic acid (MOPS) were purchased from AppliChem Chemical Company, Darmstadt, Germany.

1,2-dichloro-4-nitrobenzene (DCNB) was purchased from Fluka Chemical Company, U.S.A.

Primers were purchased from Iontec Company, Merter, Istanbul.

All chemicals were analytical grade and were obtained from commercial sources at their highest purity available.

2.2 Animals and Induction of Diabetes

Sixty one male Wistar rats (250-300g), which were divided into 8 groups; control group (n=9), untreated diabetic group (n=9), diabetic group supplemented with Vitamin C (n=12), diabetic group supplemented with α-Lipoic acid (n=8), diabetic group supplemented with combination of Vitamin C and α-Lipoic acid (n=7), and control group treated with Vitamin C (n=4), control group treated with α-Lipoic acid (n=8), control group treated with combination of Vitamin C and α-Lipoic acid (n=4) were obtained from Dicle University, Elazığ. All the animals were fed with standard diet and water. Induction of diabetes was made by a single intraperitoneal dose of streptozotocin (STZ) (50mg/kg) dissolved in 0.05 M Citrate buffer (pH 4.5) for all Vitamin C, α-Lipoic acid, combination treated and diabetic groups. Control group received 50 mg/kg physiological saline solution instead of STZ. One week after the STZ administration, LA groups were started to administer 50 mg/kg LA dissolved in minimum amount of ethanol: saline solution (1:1). Vitamin C groups were administered 50 mg/kg Vitamin C daily dissolved in neutral physiological saline solution. The combination groups were received 50 mg/kg LA and 50 mg/kg Vitamin C. For all groups the injections were done daily. During the four weeks growing period, blood glucose levels were checked weekly and the rats that had blood glucose levels above 200 mg/dL were considered as diabetic. At the end of the four weeks, rats were weighed, decapitated, livers were removed, freezed in liquid nitrogen and transported to the laboratory and stored at -80 ºC until use.

2.3 Methods

2.3.1 Preparation of Rat Liver Cytosolic Fractions

Principles:

Basis of the principles of the preparation of cytosolic fractions for the determination of enzyme activities is to make a homogenate of rat liver tissues by using the Teflon glass homogenizer and destroy cellular boundaries. Then by the help of differential centrifugation cellular fractions

were separated for the determination of enzyme activities (Biochemical Techniques: Theory and Practice book, 1990).

Reagents:

1- Homogenization buffer: 1.15 % w/v KCl
25 mM Potassium phosphate (KPi) Buffer, pH 7.4
5 mM EDTA
0.2 mM PMSF
0.2 mM DTT

Preparation:

Livers were washed twice or more with cold buffer to remove blood, then dried on a filter paper, and fatty tissues were removed. Then livers were cut into small pieces and weighed. Four mL of homogenization buffer was added to each gram wet weight of tissue. All of these steps were carried out at 0-4 ºC. The tissues were homogenized by using Potter-Elvehjem glass homogenizer packed in crush ice, coupled motor driven (Black& Decker, V850, multispeed drill) teflon pestle at 2400 rpm for 3x30 second.

After homogenization, homogenates were centrifuged at 16000*g* (Eppendorf Centrifuge 5810 R) for 20 minutes and then mitochondrial pellets were discarded. The resulting fractions were stored as small aliquots at -80 ºC for the determination of total GST and GST isozymes activities and also for protein determinations.

2.3.2 Protein Determinations

Principles :

The principle of protein determination is based on the reduction of Folin-Ciocalteu-Phenol reagent (phosphomolybdate and phosphotungstate) by Cu^{2+} treated proteins tryptophan and tyrosine residues. The color due to

reduction of molybdate in Folin reagent is directly proportional to protein content and measured at 660 nm. (*Lowry et al*.,1951)

Reagents:

1- Reagent I : 2% w/v $CuSO_4.5H_2O$

2- Reagent II : 2% w/v Na-K Tartarate

3- Reagent A : 2% w/v Na_2CO_3 in 0,1 N NaOH

4- Lowry ACR Reagent (alkaline cupper reagent): Reagent I, Reagent II and Reagent A were mixed respectively with a ratio of 1:1:100 (v/v/v)

5- Folin Phenol Reagent: 2N stock reagent were diluted to 1N with dH_2O and kept in dark.

Assay :

Before preparation of reaction tubes, samples from differential centrifugation were diluted initially 200 fold with distilled water and further 2.5 and 5 fold dilutions were done in test tubes. Volumes of samples are shown in Table 1. Standard BSA solutions were also prepared and the volumes and concentrations of standard BSA are given in Table 2.

Table 1: Preparation of Samples

Tube Number	**Samples**	**H_2O**
S1-S1'	0.1 mL (200X diluted)	0.4 mL
S2-S2'	0.2 mL (200X diluted)	0.3 mL

Table 2: Volumes and Concentration of Standard BSA

Tube Number	**BSA Standards**	**H_2O**
Blank- Blank'	0	0.5 mL
1-1'	0.5 mL (0,02 mg/mL)	0
2-2'	0.5 mL (0,05 mg/mL)	0
3-3'	0.5 mL (0,10 mg/mL)	0
4-4'	0.5 mL (0,15 mg/mL)	0
5-5'	0.5 mL (0,20 mg/mL)	0

Then in separate duplicate tubes, 0.5 mL diluted samples and standard BSA preparations were added. To each tube, 2.5 mL Lowry ACR was added, mixed and incubated for 10 minutes at room temperature for copper reaction in alkaline medium.

After that, 0.25 mL Folin Reagent was added to each tube and mixed within 8 seconds. The tubes were then incubated at room temperature (RT), for further 30 min. The intensity of color developed was measured at 660 nm against blank cuvette containing 0.5 mL dH_2O instead of standard BSA solutions. Finally, standard BSA calibration curve was constructed from the absorbance readings of standard and used for the calculation of protein amounts of samples.

2.3.3 Determination of Rat Liver Glutathione S-Transferase (GST) Activities

Different GST isozyme activities were determined by using their specific substrates according to the general procedure of Habig *et.al*., (1974). 1-Chloro-2,4-dinitrobenzene (CDNB) is a common substrate for all isozymes except Theta class. CDNB was used to determine the total GST activities. 1,2-Dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3- (p-nitro phenoxy) propane (EPNP) and p-nitrobenzyl chloride (PNBC) were used for determination of Mu, Theta and Alpha class GST isozyme activities, respectively (Habig *et.al*., 1974).

2.3.3.1 Determination of Rat Liver GST Activities toward CDNB

Principles:

CDNB is a common substrate for all isozymes. GSTs catalyze glutathione (GSH) oxidation which was monitored by the increase in absorbance at 340 nm due to the 1-glutathione-2,4-dinitrobenzene-(DNB-SG) formation (Habig *et.al*., 1974)

Reagents:

1- 25 mM CDNB in ethanol/dH_2O (3/2 v/v)

Note: CDNB was first dissolved in ethanol and then water was added and the solution should be kept in dark bottle.

2- 50 mM KP*i* buffer, pH 7.0

3- 20 mM Reduced glutathione (GSH)

Assay:

In 3 mL quartz cuvettes, followings were added with the order of 2500 µL KPi Buffer, 200 µL GSH, 150 µL 200 fold diluted cytosolic fraction. All were mixed and incubated for 2 min at RT. Then the reaction was started with the addition of 150 µL of 25 mM CDNB. The components of the reaction mixture for total GST assay are shown in Table 3. During the reaction the thioether formation was measured at 340 nm for 2 minutes. Blank readings (reaction with no enzyme) were carried out in order to detect the non-enzymatic product formation. The assay conditions were optimized previously in the lab, such as substrate concentrations, pH and temperature.

Table 3: The components of the reaction mixture for Total GST Assay

KPi buffer (50 mM, pH 7.0)	**GSH** 20 mM	**Cytosolic Fraction** (1/200 diluted)	**CDNB** 25 mM
2500 µL	200 µL	150 µL	150 µL

Total GST activities were calculated as the amount of thioether formation (µmol) by 1 mg total protein containing cytosol in one minute by using extinction coefficient as 0.0096 μMcm^{-1}. Thus total specific activity was defined as the formed DNB-SG (µmol) / min/ mg protein.

2.3.3.2 Determination of Rat Liver GST Alpha Isozyme Activities

Principles:

Alpha class GST activity was determined spectrophotometrically by monitoring the thioether formation at 310 nm by using p-nitrobenzyl chloride as substrate (Habig *et.al.*, 1974)

Reagents:

1- 10 mM PNBC in ethanol/dH2O (3/2 v/v)

2- 100 mM KPi buffer, pH 7.5

3- 75 mM GSH

Assay:

In 3 mL quartz cuvettes, followings were added with the order of 2500 µL 100 mM KPi Buffer pH 7.5, 200 µL GSH, 150 µL 10 fold diluted cytosolic fraction and they were mixed and incubated for 2 min at RT. Then the reaction was initiated by the addition of 150 µL PNBC. The constituents of the reaction mixture for GST Alpha assay is shown in Table 4.

Table 4: The components of the reaction mixture for GST Alpha Assay

KPi buffer 100 mM, pH 7.5	**GSH** 75 mM	**Cytosolic Fraction** (1/10 diluted)	**PNBC** 10 mM
2500 µL	200 µL	150 µL	150 µL

The thioether formation was measured at 310 nm for 2 minutes. Each time blank readings (reaction with no enzyme) were carried out in order to subtract the non-enzymatic product formation from the GST assay.

The enzyme activity was calculated according to the differences between the thioether formation (µmol) in 1 mg total protein containing cytosol and the blank, using extinction coefficient as 0.0019 μMcm^{-1}.

2.3.3.2.1 Effect of pH on GST Alpha Activity

- Acidic region activities were measured with 100 mM Acetate buffer; pH 4.0, 4.5, 5.0, 5.5, 6.0
- Neutral region activities were measured with 100 mM Potassium Phosphate buffer; pH 6.5, 7.0, 7.5, 8.0
- Basic region activities were measured with 100 mM Tris buffer; pH 7.5, 8.0, 8.5, 9.0, 9.5

2.3.3.2.2 Effect of Temperature on GST Alpha Activity

After mixing all materials of the assay medium, except PNBC, 5 min incubation period was performed in circulatory water bath at different temperatures namely 5, 10, 15, 20, 25, 30, 35, 37, 40, 45, 50, 55, 60 or 63,5 ºC, then the normal procedure was carried out and the effect of temperature on GST Alpha activity was determined.

2.3.3.2.3 Effect of Substrate Concentration and Km and Vmax Determinations

The kinetic parameters, namely, Km and Vmax values for GST alpha were determined by fixing one substrate at saturating concentration and changing the concentration of the second substrate. That is, Km value of GST against GSH was determined by using 5, 10, 20, 30, 50, 70, 75, 80, 100, and 120 mM stock solution of GSH and 5 mM PNBC at which GST Alpha was saturated. Similarly, 0.005, 0.01, 0.025, 0.0375, 0.05, 0.075, 0.1, 0.125, 0.15, 0.2, 0.25, 0.3, 0.4 and 0.5 mM PNBC, and 100 mM stock GSH were used for the determination of Km of GST Alpha for PNBC.

2.3.3.3 Determination of Rat Liver GST Mu Isozyme Activities

Principles:

GST Mu activity was determined spectrophotometrically by monitoring the thioether formation at 345 nm by using 1, 2-dichloro-4-nitro benzene (DCNB) as substrate (Habig *et.al*.,1974).

Reagents:

1- 20 mM DCNB in ethanol/dH2O (3/2 v/v)

2- 100 mM KPi buffer, pH 7.5

3- 75 mM GSH

Assay:

In 3 mL quartz cuvettes, followings were added with the order of 2500 µL 100mM KPi Buffer pH 7.5, 200 µL GSH, 150 µL 10 fold diluted cytosolic fraction and they were mixed and incubated for 2 min at RT. Then the reaction was initiated with the addition of 150 µL DCNB. The components of the reaction mixture for class Mu GST assay are shown in Table 5.

The thioether formation was measured at 345 nm for 2 minutes. Each time blank readings (reaction with no enzyme) were carried out in order to subtract the non-enzymatic product formation from the GST assay. The enzyme activity was calculated according to the differences between the thioether formation (µmol) in 1 mg total protein containing cytosol and the blank in one minute using extinction coefficient as 0.0085 μMcm^{-1}.

Table 5: The components of the reaction mixture for GST Mu Assay

KPi buffer 100 mM, pH 7.5	**GSH** 75 mM	**Cytosolic Fraction** (1/10 diluted)	**DCNB** 20 mM
2500 µL	200 µL	150 µL	150 µL

2.3.3.3.1 Effect of pH on GST Mu Activity

- Acidic region activities were measured with 100 mM Acetate buffer; pH 4.0, 4.5, 5.0, 5.5, 6.0
- Neutral region activities were measured with 100 mM Potassium Phosphate buffer; pH 6.0, 6.5, 7.0, 7.5, 8.0
- Basic region activities were measured with 100 mM Tris buffer; pH.7.5, 8.0, 8.5, 9.0, 9.5

2.3.3.3.2 Effect of Temperature on GST Mu Activity

After mixing all materials of the assay medium, except DCNB, 5 min incubation period was performed in circulatory water bath at different temperatures namely 5, 10, 15, 20, 25, 27, 30, 33, 35, 37, 40, 45, 50, 55 and 60ºC, then the normal procedure was carried out and the effect of temperature on GST Mu activity was determined.

2.3.3.3.3 Effect of Substrate Concentration and Km and Vmax Determinations

The kinetic parameters, namely, Km and Vmax values for GST Mu were determined by fixing one substrate at saturating concentration and changing the concentration of second substrate. Km value of GSH was determined by using 5, 10, 20, 30, 50, 70, 75, 80, 100, 110, 120, 140, 150,and 200 mM stock solution of GSH and 20 mM DCNB at which GST Mu was saturated. Similarly, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.25 and 1,5 mM DCNB, and 100 mM stock GSH were used for the determination of Km of GST Mu for DCNB.

2.3.3.4 Determination of Rat Liver GST Theta Isozyme Activities

Principles:

GST Theta activity was determined spectrophotometrically by monitoring the thioether formation at 360 nm by using 1, 2-epoxy-3(paranitrophenoxy) propane (EPNP) as substrate (Habig *et.al*.,1974).

Reagents:

1- 6 mM EPNP in ethanol/dH2O (3/2 v/v)

2- 100 mM Acetate buffer, pH 6.0

3- 120 mM GSH

Assay:

In 3 mL quartz cuvettes, followings were added with the order of 2500 µL 100mM Acetate Buffer pH 6.0, 200 µL GSH, 150 µL 3 fold diluted cytosolic fraction and they were mixed and incubated for 2 min at RT. Then the reaction was initiated with the addition of 150 µL EPNP. The components of the reaction mixture for class Mu GST assay are shown in Table 6.

Table 6: The components of the reaction mixture for GST Theta Assay

KPi buffer 100 mM, pH 7.5	**GSH** 120 mM	**Cytosolic Fraction** (1/3 diluted)	**EPNP** 6 mM
2500 µL	200 µL	150 µL	150 µL

The thioether formation was measured at 360 nm for 2 minutes. Each time blank readings (reaction with no enzyme and reaction with no substrate) were carried out in order to subtract the non-enzymatic product formation from the GST assay. The enzyme activity was calculated according to the differences between the thioether formation (µmol) in 1 mg total protein containing cytosol and the blank in one minute using extinction coefficient as 0,0085 $\mu M cm^{-1}$.

2.3.3.4.1 Effect of pH on GST Theta Activity

- Acidic region activities were measured with 100 mM Acetate buffer; pH 4.0, 5.0, 5.5, 6.0
- Neutral region activities were measured with 100 mM Potassium Phosphate buffer; pH 6.5, 7.0, 7.5, 8.0
- Basic region activities were measured with 100 mM Tris buffer; pH.7.5, 8.0, 8.5, 9.0, 9.5

2.3.3.4.2 Effect of Temperature on GST Theta Activity

After mixing all materials of the assay medium, except EPNP, 5 min incubation period was performed in circulatory water bath at different temperatures namely 5, 10, 15, 20, 25, 30, 35, 45, 50, 55 and 60 ºC, then the normal procedure was carried out and the effect of temperature on GST Theta activity was determined.

2.3.3.4.3 Effect of Substrate Concentration and Km and Vmax Determinations

The kinetic parameters, namely, Km and Vmax values for GST Theta were determined by fixing one substrate at saturating concentration and changing the concentration of second substrate. Km value for GSH was determined by using 5, 10, 20, 30, 50, 70, 75, 80, 100, 120 and 150 mM stock solution of GSH and 10 mM EPNP at which GST Theta was saturated. Similarly, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.3, 0.375, 0.5 and 0.6 mM EPNP and 150 mM stock GSH were used for the determination of Km of GST Theta for EPNP.

2.3.4 Isolation of Total RNA from Rat Liver Tissues

Principles:

Total RNA was isolated from rat liver according to the method of Chomczynski and Sacchi (1987). This method is based on differential extraction of RNAs by organic solvents. Proteins are denatured by strong denaturating agent, Guanidine isothiocyanide, and DNAs are selectively fractionated from RNAs by phenol at acidic pH.

Reagents:

1- DEPC (Diethyl Pyrocarbonate)
2- Solution D: GTC solution
   4M Guanidine isothiocyanide
   25 mM Sodium Citrate (pH 7.0)
   0.5% (w/v) L-Lauryl Sarcosine
   0.1 M 2-mercaptoethanol
3- 2M Sodium acetate pH 4.0
4- Phenol
5- Chloroform-isoamyl alcohol mixture (49:1 v/v)
6- Isopropanol
7- Deionized Formamide
8- 0.5% Sodium dodecyl sulfate (SDS)
9- 75% Ethanol

Procedure:

In order to decrease the possibility of RNA degradation during the procedure, all glassware and plastics were treated with 0.1% DEPC solution overnight and then autoclaved and oven-dried during which DEPC was converted into CO_2 and ethanol. Furthermore, all solutions were treated with DEPC or prepared with 0.1% DEPC treated water.

Hundred mg liver tissue were minced on ice and homogenized (at RT) with 1 mL of solution D, GTC solution, in a glass-teflon homogenizer and subsequently transferred to a 15 mL polypropylene tube.

Sequentially, 0.1 mL of 2M Sodium acetate pH 4.0, 1 mL of phenol (water saturated, acidic pH) 0.2 mL of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent.

The final suspension were shaken vigorously for 10 second and cooled on ice for 15 minute. Samples were centrifuged at 10000*g* for 20 min at 4°C, and after that, RNA was present in the aqueous phase whereas proteins and DNA were accumulated in the interphase and phenol phase, respectively.

The upper aqueous phase was transferred to a fresh tube, mixed with 1 mL of ice-cold isopropanol, and then placed at -20°C for at least 1 hour to precipitate RNA.

Sedimentation at 10000*g* for 20 minute, was again performed and the resulting RNA pellet was redissolved in 0.3 mL of solution D, transferred into a 1.5-mL eppendorf tube, and precipitated with 1 volume of isopropanol at -20°C for 1 hour.

After centrifuge at 10000*g* for 10 min at 4°C, the RNA pellet were resuspended in 75% ethanol, sedimented, dried in oven (60-65^{o}C, 15 min), and dissolved in 50µL DEPC treated water or 0.5% SDS at 65^{o}C for 10min.

After precipitation with ethanol, RNA might also be dissolved in deionized formamide and stored at -20°C (Chomczynski, 1992). Formamide provided a chemically stable environment that also protects RNA against degradation by RNases. For the RT-PCR reactions, RNA were recovered from formamide by precipitation with 4 volumes of ethanol as described by Chomczynski (1992).

2.3.4.1 Spectrophotometric Analysis of RNA

Seven micro liters of total RNA isolate was diluted with 693 µL of Tris EDTA (TE) buffer (pH 8.0) in a quartz cuvette. The absorbance of the solution was measured at 260 and 280 nm using TE buffer as blank. The purity of the isolated RNA was determined by taking the ratio of A_{260} and A_{280} readings.

The optimal value for RNA purity was accepted between 1.9-2.2 (MacDonald *et al.,* 1987). Forty µg/mL solution of single stranded RNA gives an absorbance reading of 1.000 and we can calculate the concentration of the RNA in our sample as follows:

RNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (40 µg RNA/µL)

2.3.4.2 Agorose Gel Electrophoresis of RNA

Principles:

The integrity of the total RNA isolated from rat liver tissues were analyzed by agarose gel electrophoresis. As single-stranded RNA can form secondary structures, electrophoresis must be carried out under denaturation conditions. RNA must be denatured prior to loading into the gel and the electrophoresis conditions should maintain the denatured state.

RNA electrophoresis was carried out according to the method of Sambrook and Russell (2001), in which formaldehyde agarose gels with Morpholinopropanesulfonoic acid (MOPS) buffer is used to denature the RNA.

In this system, formaldehyde forms unstable Schiff bases with the single imino group of the guanine residues. These adducts maintain RNA in the denatured state by preventing intrastrand Watson-Crick base pairing. Because the Schiff bases are unstable and easily removed by dilution, RNA can be maintained in the denatured state only when formaldehyde is present in the buffer or gel.

Reagents:

1- DEPC
2- 3% Hydrogen peroxide
3- Formaldehyde
4- 10x Morpholinopropanesulfonoic acid (MOPS) electrophoresis buffer

    200 mM MOPS (pH 7.0)

50 mM Sodium acetate

10 mM EDTA

5- Formaldehyde gel-loading buffer

50% Glycerol (diluted in DEPC-treated H_2O)

10 mM EDTA (pH 8.0)

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol FF

6- 0.1 M Ammonium acetate

7- Etidium Bromide (EtBr)

Procedure:

During the electrophoresis, it is very important to minimize the RNase activity. For this purpose, the electrophoresis set was first cleaned with detergent solution, followed by rinsing with distilled water and then the set was dried with ethanol. After that, gel apparatus was filled with 3% hydrogen peroxide and soaked in this solution for 10 min. Then, all the apparatus were rinsed thoroughly with DEPC-treated water.

One percent of agarose gel containing EtBr (0.5 µg/mL) was prepared in 1X MOPS buffer (pH 7.0) and 6% formaldehyde to set the denaturating conditions. A pre-electrophoresis before loading the samples was carried out for 20 min. Then, 10 µLs of total RNA was mixed with 1 µL of loading solution and loaded onto the gel. Electrophoresis was carried out at a maximum of 5V per 1 cm of distance between electrodes until bromophenol blue has traveled at least 80% of the way through the gel.

2.3.4.3 Complementary DNA (cDNA) Preparation

Principles:

Reverse transcriptase-PCR (RT-PCR) is a method used to amplify cDNA copies of RNA. After isolation, RNAs are reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV-RT) in order to produce complete cDNAs of mRNAs having polyA tails, as oligo $(dT)_{15}$ was used as a primer for reverse transcription (Sadi *et al*.,2008).

Reagents:

1- M-MuLV-RT (200 unit/ µL)
2- M-MuLV-reverse transcriptase buffer
3- dNTP mix (10mM each)
4- RNase inhibitor (40 unit/ µL)

Procedure:

Into a 0.2 mL Eppendorf PCR tube, 1 µg of total RNA (1 µL- 10 µL) and 2 µL of oligo $(dT)_{15}$ (0.5 µg/mL) were added and the total volume was completed to 12 µL with pyrogen (nuclease) free sterile water.

Then, tubes were incubated at 70°C for 5 min and chilled on ice. Then, 4 µL of 5X M-MuLV-reverse transcriptase buffer (final concentration 1X), 2 µL of 10 mM dNTP mix (final concentration 1.0 mM), and 1 µL of RNase inhibitor (20 U/µL) were added into the tubes in the indicated order. After incubation at 37°C for 5 min, 1 µL M-MuLV-reverse transcriptase (200 U/µL) was added and reverse transcription was carried out at 42°C for 60 min. Finally, the tubes were heated up to 70°C for 10 min to stop the reverse transcription by denaturing M-MuLV-RT and then chilled on ice. Reverse transcribed samples were stored at -20°C for further use.

2.3.5 Polymerase Chain Reaction

Principles:

Multiplex PCR reaction was performed by the simultaneous amplification of the different genes in the same reaction tube (Sadi *et al.,* 2008)

Reagents:

1- Pyrogen free sterile H_2O
2- Reaction buffer
   100 mM Tris pH 9.0
   500 mM KCl
   1% Triton X-100

3- 25 mM $MgCl_2$
4- dNTP mix (10 mM each)
5- Forward / Reverse primer (100 mM each)

Procedure:

The reaction was performed by simply adding the following items in the indicated order into a 1.5 mL eppendorf tube to produce a master mix as shown at Table 7.

Table 7: Ingredients of PCR master mix

Ingredients	**Stock Solution**	**Final Concentration**
Pyrogen free sterile H_2O	-	up to 20µL
Reaction buffer	10X	1X
$MgCl_2$	25 mM	2.5 mM for GST(Alpha) 1.5 mM for GST (Mu)
dNTP mix	10 mM (each)	0.1 mM (each)
Forward / reverse primer	100 mM (each)	1.0 mM (each)

From the freshly prepared master mix preparation, 19 µL aliquots were added into 0.2 mL PCR tubes. Then, 0,8 µL from the cDNA reaction mixture (no more than 1/10 of volume) and 0.2 µL Taq DNA polymerase (final concentration 2.5 U/ml) was added making a final volume of 20 µL. The thermal cycler was programmed according to our previously optimized results and started with initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec for GST M1, 55°C for GST A2, and extension at 72°C for 45 sec. These steps were repeated for the cycle number at which linear phase of amplification was determined and it was also optimized as 30 cycles for GST A2 and 24 cycles for GST M1. Last extension was carried out at 72°C for 5 min and then finally reaction was kept at 4°C. After the reaction was completed, PCR tubes were stored at -20°C until further use. The following primers were used in the reactions; GST A2 forward primer: 5'-TTGACATGTATTCAGAGGGT-3'; reverse primer: 5'-TTGTTTTGCATCCAT GGCTG-3'. GST M1 forward primer: 5'-

AGAAGCAGAAGCCAGAGTTC-3'; reverse primer: 5'-GGGGTGAGGTTG AGGAGATG-3'. β–Actin forward primer: 5'-CCTGCTTGCTGATCCACA-3'; reverse primer: 5'-CTGACCGAGCGTGGCTAC-3' (Hoen et al., 2002).

2.3.6 Agarose Gel Electrophoresis of PCR Products

Two percent agarose was prepared by using 1X Tris borate EDTA (TBE) buffer containing 0.5 µg/mL EtBr and poured onto a gel tray. The 0.25 µg/µL of DNA Ladder (100 bp-3000 bp) was loaded into the first well. 20 µL of each PCR product were mixed with the 4 µL 6X loading dye and 5 µL of it was loaded into each well. The gel was run at 5 V/cm for 60 min and then visualized under ultraviolet transilluminator and picture was taken.

2.3.7 Data Acquisition

In order to quantify the PCR products on agarose gels, densitometric measurements were done. The photographs of the PCR gels were analyzed using software, called Image J. The intensities of the bands were converted into peaks by the software. GST enzymes' gene expressions were calculated from the area under these peaks.

CHAPTER III

RESULTS AND DISCUSSION

Diabetes mellitus is a disease which has been characterized by a series of complications affecting several tissues in the body. High blood glucose concentrations leading hyperglycemia cause free radicals to be produced via glucose autoxidation (Hunt *et al*., 1990), non-enzymatic protein glycation (Wolff and Dean, 1987), and increased influx toward polyol pathway (Chung *et al.*,2003). The protection of cells from the resulting oxidative stress is essential and under normal conditions adequate protection is provided by the antioxidant systems. A number of major cellular antioxidant defense mechanisms exist to neutralize the damaging effects of these free radicals.

In this study, 8 groups of Wistar rats, Control group (n=9), untreated diabetic group (n=9), diabetic group suplemented with Vitamin C (n=12), diabetic group suplemented with α-lipoic acid (n=8), diabetic group suplemented with combination of Vitamin C and α-lipoic acid (n=7), and control group treated with Vitamin C (n=4), control group treated with α-lipoic acid (n=8), control group treated with combination of Vitamin C and α-lipoic acid (n=4) were used. Blood glucose levels and body weights of 61 animals were measured weekly starting after STZ treatment until the fourth week. During the four weeks growing period, blood glucose levels were checked weekly and the rats who had blood glucose level above 200 mg/dL were considered as diabetic.

The effects of four week diabetes and the effect of antioxidants, Vitamin C and α-lipoic acid on liver GST enzyme activities were studied. First, the conditions of the activity measurements of the isozymes (alpha, mu and theta), were optimized by using control animal liver tissues, and the optimum conditions for each assay (pH, temperature and substrate concentrations) were determined.

3.1 Activity Optimizations

3.1.1 GST Alpha Isozyme

3.1.1.1 Effect of pH on GST Alpha Isozyme Activity

Figure 3.1 shows the effect of varying pH on the GST Alpha isozyme activity. The pH of the reaction medium was varied between 4 and 9 in the presence of acetate, phosphate and Tris-HCl buffers. The maximum activity of GST Alpha isozyme was obtained at pH 7.5 and after that all the GST Alpha activity measurements in cytosolic fractions obtained from 8 groups of animals were carried out by using 100 mM KPi buffer, pH 7.5.

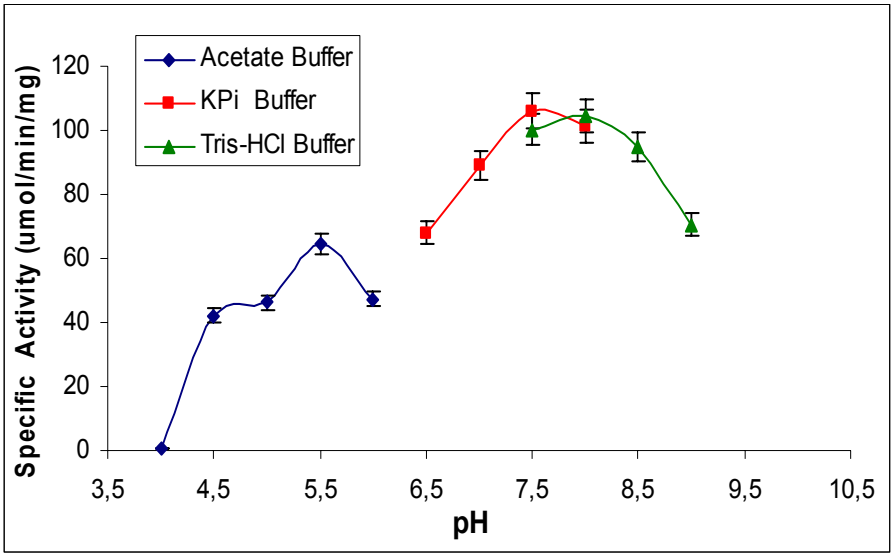


Figure 3.1 The effect of pH on GST Alpha isozyme activity. The reactions were carried out at the indicated pH values, using 100 mM acetic acid-Na acetate buffer for pH 4.0, 4.5, 5.0, 5.5 and 6.0; 100 mM KPi buffer for pH 6.5, 7.0 7.5 and 8.0; 100 mM Tris-HCl buffer for pH 7.5, 8.0, 8.5 and pH 9.0. The points are the means of duplicate measurements.

3.1.1.2 Effect of Temperature on GST Alpha Isozyme Activity

The effect of varying temperature on the GST Alpha activity is shown in Figure 3.2. The temperature of the reaction medium was varied

between 5 to 63.5°C. As the temperature increased, the GST alpha isozyme activity increased and the maximum GST Alpha isozyme activity was observed at 60°C. When the temperature was further increased to 63.5°C, the activity decreased drastically. Alpha isozyme was seemed to be very stable at high temperatures. As this temperature is very high, throughout this study GST Alpha isozyme activity measurements were carried out at RT.

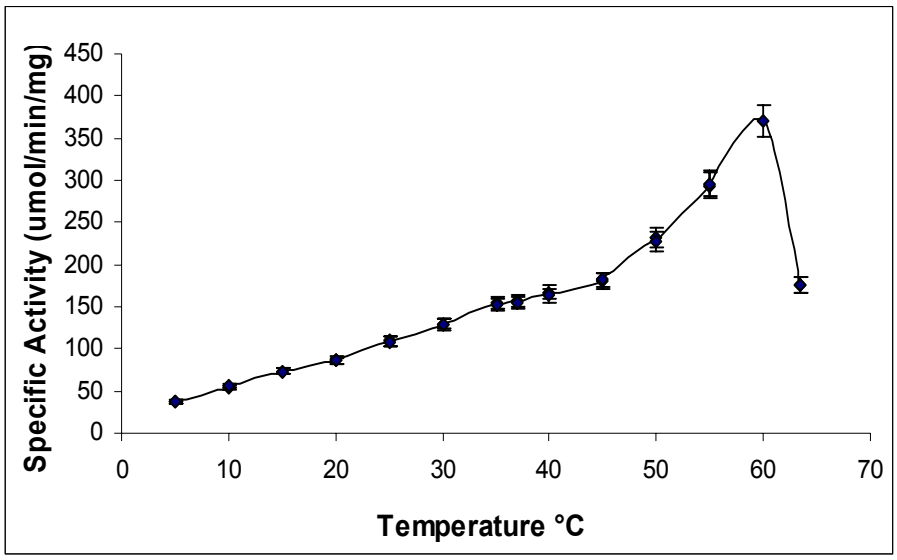


Figure 3.2: The effect of reaction temperature on GST Alpha isozyme activity. The assay mixture was heated to the indicated temperature and the endogenous rate was recorded. The points are the means of duplicate measurements.

3.1.1.3 Effect of Substrate Concentration on GST Alpha isozyme Activity

The effect of substrate concentration on GST Alpha isozyme activity was measured by changing PNBC concentrations between 0.005 to 0.5 mM. The effect of substrate concentration is shown in Figure 3.3 and as it is seen from the figure, GST Alpha isozyme was saturated at approximately 0.25 mM substrate concentration; after that there was little change in

enzyme activity. 0.5 mM PNBC concentration was used throughout the study for measurement of GST Alpha isozyme activity of cytosolic fractions of liver.

Km and Vmax values of GST Alpha isozyme were calculated for PNBC reaction by constructing Lineweaver-Burk plot (Figure 3.4).

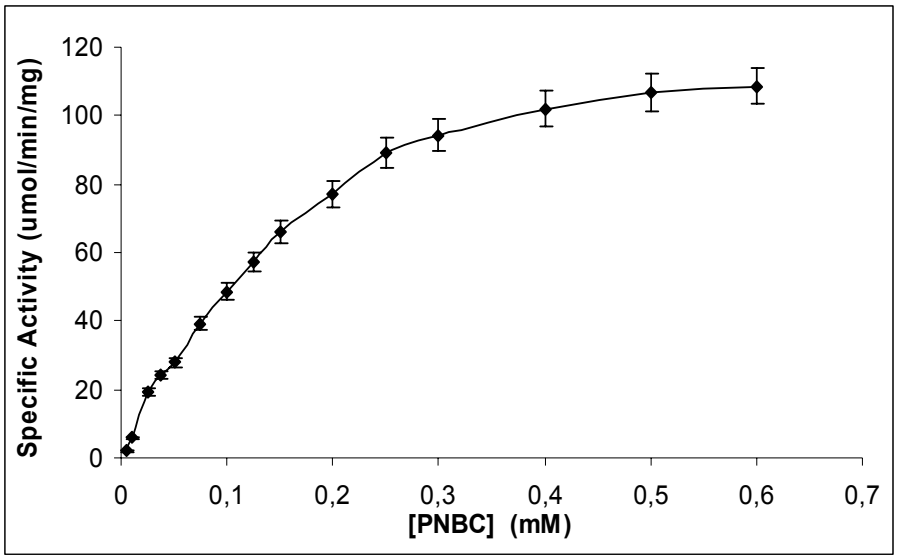


Figure 3.3: The effect of substrate concentration on GST Alpha isozyme activity. Fifteen different concentrations of PNBC, changing from 0.005 to 0.5 mM were used in the reaction medium.

According to Lineweaver-Burk plot, Km and Vmax values of GST Alpha isozyme were calculated as 0,136 mM and 113 µmol/min/mg, respectively.

Km and Vmax values of GST Alpha isozyme were also calculated for GSH reaction by constructing Lineweaver-Burk plot (Figure D1, D2). According to Lineweaver-Burk plot, Km and Vmax values of GST Alpha isozyme were calculated as 0.71 mM and 58 µmol/min/mg, respectively.

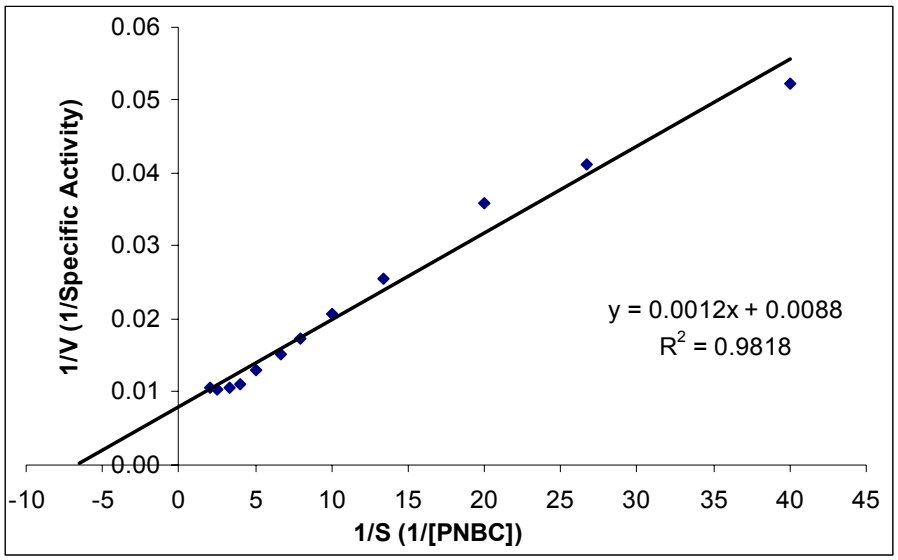


Figure 3.4: Lineweaver-Burk plot for GST Alpha isozyme.

3.1.2 GST Mu

3.1.2.1 Effect of pH on GST Mu isozyme Activity

Figure 3.5 shows the effect of varying pH on the GST Mu isozyme activity. The pH of the reaction medium was varied between 4 and 9 in the presence of acetate, phosphate and Tris-HCl buffers. The maximum activity of GST Mu isozyme was obtained at pH 7.5 and all the other GST Mu isozyme activity measurements were carried out by using 100 mM KPi buffer, pH 7.5.

3.1.2.2 Effect of Temperature on GST Mu Isozyme Activity

The effect of varying temperature on the GST Mu isozyme activity is shown in Figure 3.6. The temperature of the reaction medium was varied between 5 to 60°C. Maximum GST Mu isozyme activity was observed at 50°C. Then the specific activities decreased drastically, most probably due to protein denaturation**.** But as this temperature is too high, throughout this study GST Mu isozyme activity measurements were carried out at RT.

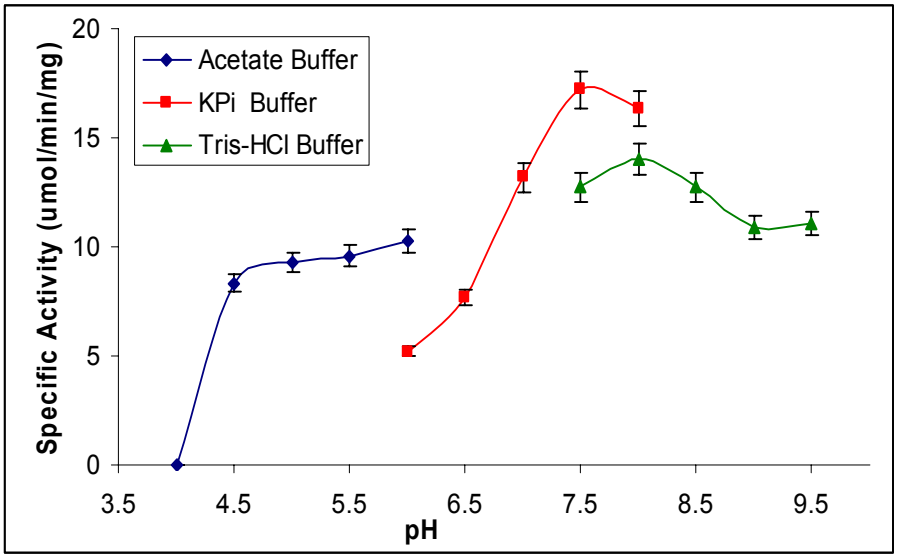


Figure 3.5: The effect of pH on GST Mu isozyme Activity. The reactions were carried out at the indicated pH values, using 100 mM acetate buffer for pH 4.0 to 6.0; 100 mM KPi buffer for pH 6.0 to 8.0; 100 mM Tris-HCl buffer for pH 7.5 to 9.5. The points are the means of duplicate measurements.

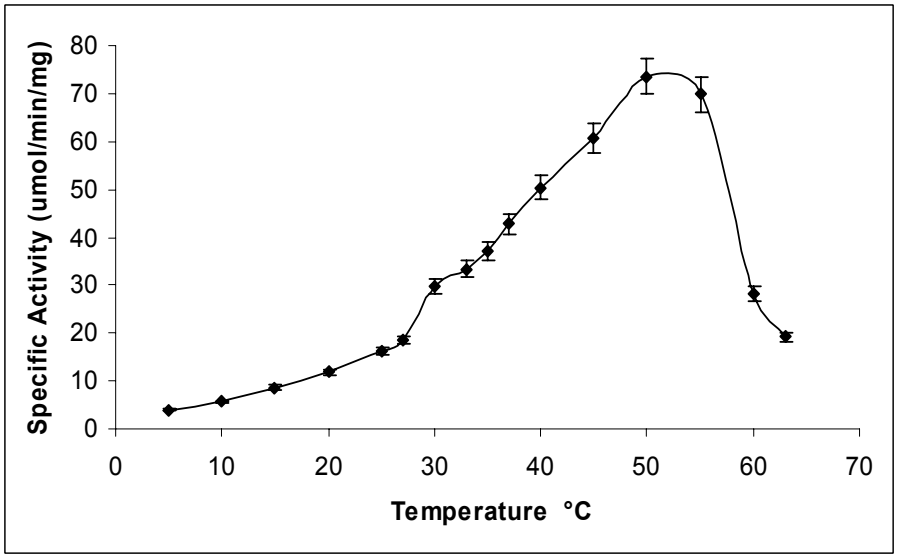


Figure 3.6: The effect of reaction temperature on GST Mu isozyme activity. The points are the means of duplicate measurements.

3.1.2.3 Effect of Substrate Concentration on GST Mu isozyme Activity

The effect of substrate concentration on GST Mu isozyme activity was measured by changing DCNB concentrations between 0.3 and 1.25 mM in the 3 mL reaction mixture. The effect of substrate concentration is shown in Figure 3.7 and as it is seen from the figure, at concentration of approximately 1 mM DCNB, GST Mu isozyme was fully saturated by the substrate. Therefore, 1 mM DCNB concentration was used throughout this study for measurement of GST Mu isozyme activity of cytosolic fractions of liver.

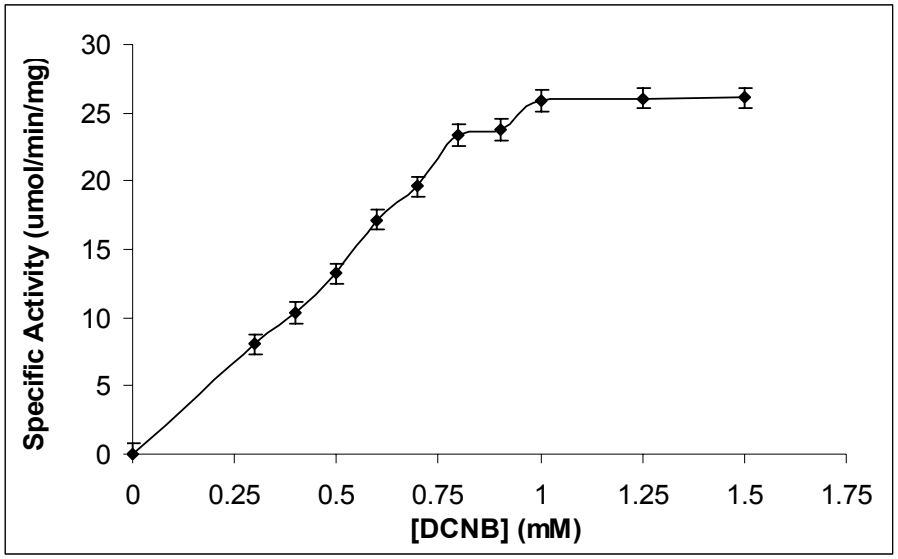


Figure 3.7: The effect of substrate concentration on GST Mu isozyme activity.

Km and Vmax values of GST Mu isozyme were calculated for DCNB reaction by constructing Lineweaver-Burk plot (Figure 3.8). According to Lineweaver-Burk plot, Km and Vmax values of GST Mu isozyme were calculated as 0,6 mM and 27 µmol/min/mg, respectively

Km and Vmax values of GST Mu isozyme were also calculated for GSH reaction by constructing Lineweaver-Burk plot (Figure D3, D4).

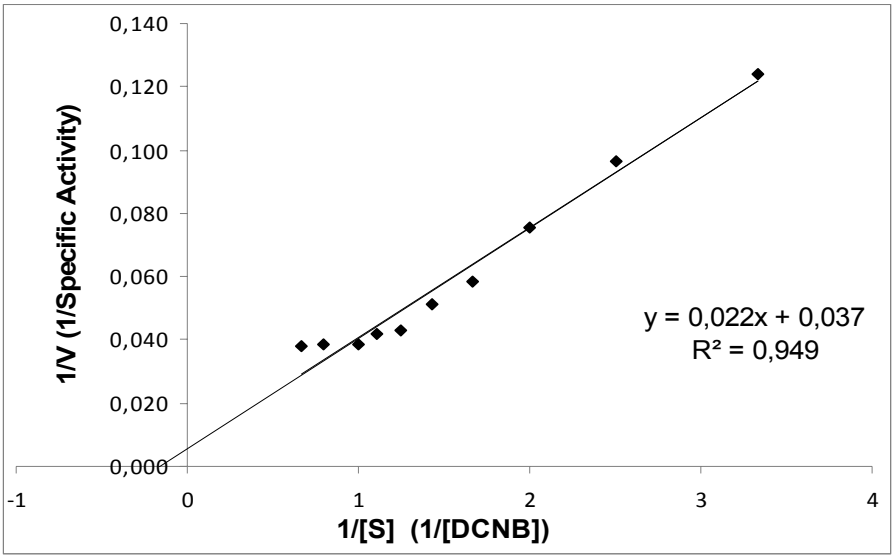


Figure 3.8: Lineweaver-Burk plot for GST Mu isozyme.

According to Lineweaver-Burk plot, Km and Vmax values of GST Mu isozyme were calculated as 0,107 mM and 18 µmol/min/mg, respectively.

3.1.3 GST Theta Isozyme

3.1.3.1 Effect of pH on GST Theta Isozyme Activity

Figure 3.9 shows the effect of varying pH on the GST Theta isozyme activity. The pH of the reaction medium was varied between 4 and 9.5 in the presence of acetate, phosphate and Tris-HCl buffers. Although the maximum activity of GST Theta isozyme was obtained at pH 6.0 with acetate buffer, further activity measurements with acetate buffer gave conflicting results. As we could not solve this fluctuation problem with acetate buffer, we have used 100 mM KPi buffer at 7.5 for the theta isozyme activity measurements in cytosolic fractions.

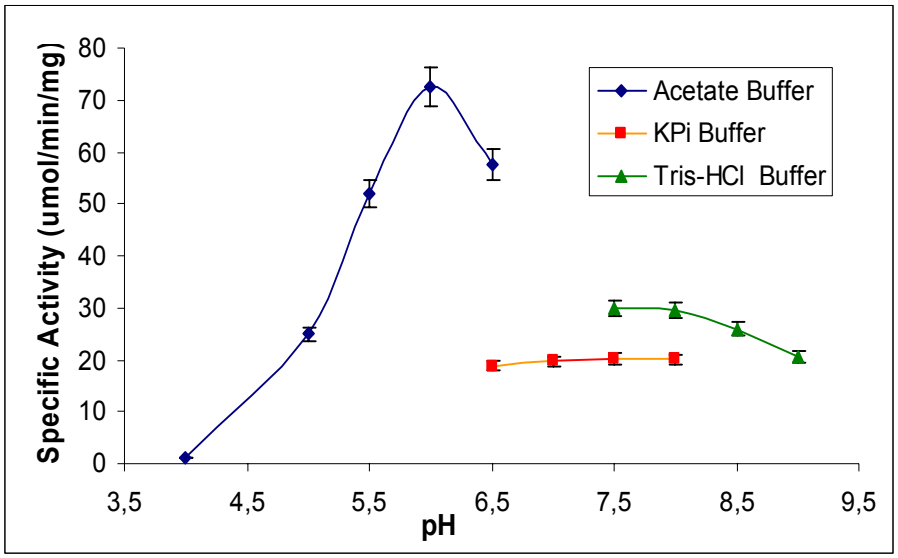


Figure 3.9: The effect of pH on GST Theta isozyme Activity. The reactions were carried out at the indicated pH values, using 100 mM acetate buffer for pH 4.0 to 6.0; 100 mM KPi buffer for pH 6.5 to 8.0; 100 mM Tris-HCl buffer for pH 7.5 to 9.5. The points are the means of duplicate measurements.

3.1.3.2 Effect of Temperature on GST Theta Isozyme Activity

The effect of varying temperature on the GST theta isozyme activity is shown in Figure 3.10. The temperature of the reaction medium was varied between 5 to 60°C. Maximum GST theta isozyme activity was observed at 50°C, but like the other GST isozymes, the theta isozyme activity measurements were also carried out at RT, throughout this study.

3.1.3.3 Effect of Substrate Concentration on GST Theta Isozyme Activity

The effect of substrate concentration on GST Theta isozyme activity was measured by changing EPNP concentrations between 0.025 and 0.6 mM. The effect of substrate concentration is shown in Figure 3.11 and as it

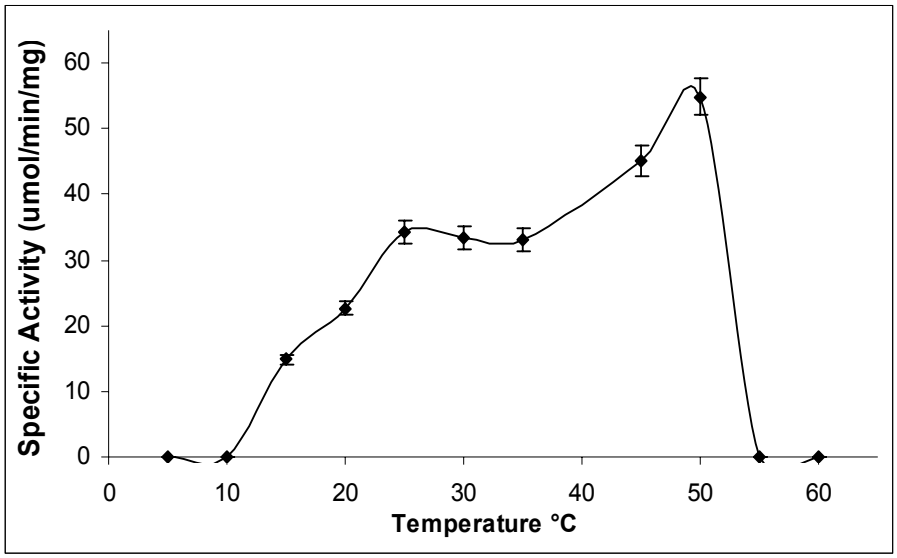


Figure 3.10: The effect of reaction temperature on GST Theta isozyme activity. The points are the means of duplicate measurements.

is seen from the hyperbolic curve, saturation has started approximately at 0.3 mM EPNP and at concentration of approximately 0.4 mM EPNP GST Theta isozyme was fully saturated by its substrate. 0.3 mM EPNP concentration was used throughout this study for measurement of GST theta isozyme activity of cytosolic fractions of liver.

Km and Vmax values of GST theta isozyme were calculated for EPNP reaction by constructing Lineweaver-Burk plot (Figure 3.12). According to Lineweaver-Burk plot, Km and Vmax values of GST theta isozyme were calculated as 0,375 mM and 61 µmol/min/mg, respectively.

Km and Vmax values of GST Theta isozyme were also calculated for GSH reaction by constructing Lineweaver-Burk plot (Figure D5, D6). According to Lineweaver-Burk plot, Km and Vmax values of GST Theta isozyme were calculated as 1.88 mM and 27.9 µmol/min/mg, respectively.

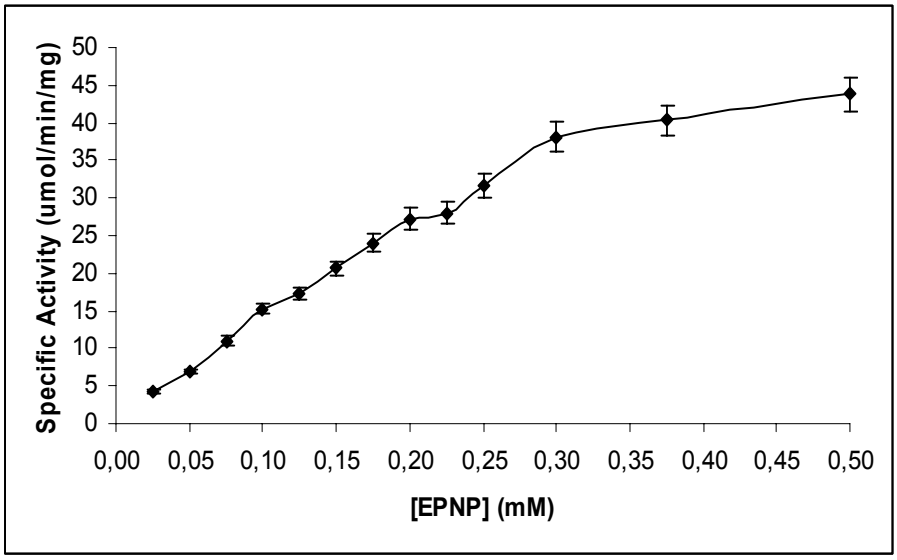


Figure 3.11: The effect of substrate concentration on GST Theta isozyme activity. Reactions were carried out RT as described in methods. The points are the means of two different sets of data and each point is the mean of duplicate determinations.

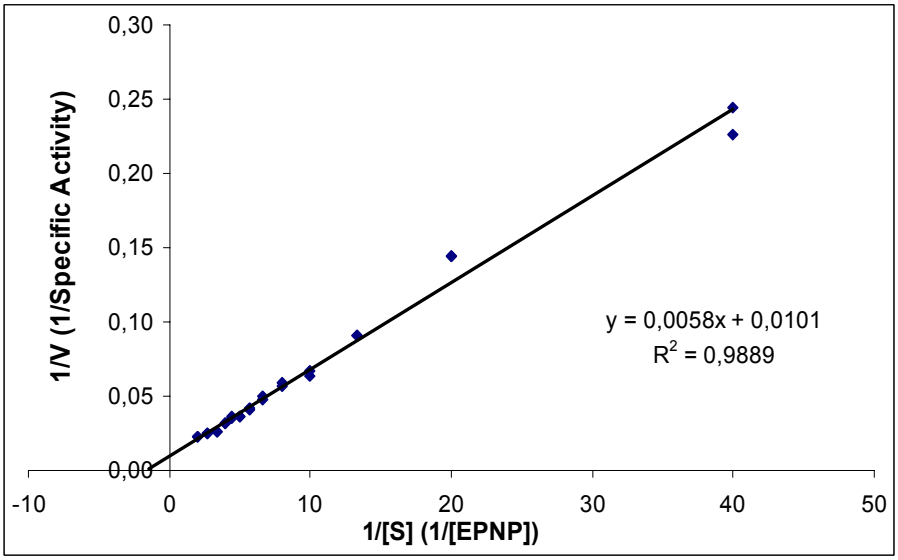


Figure 3.12: Lineweaver-Burk plot for GST Theta isozyme.

3.2 Determination of GST Enzyme Activities

We have investigated the effects of two antioxidants, namely, Vitamin C and Lipoic acid on GST activities in STZ-induced diabetic rat livers.

3.2.1 Total GST Activities

The change in the Total GST activities of control, treated control groups, diabetic and treated diabetic groups are given in the Figure 3.13.

According to the Figure 3.13, there is a little increase in diabetics compared to controls, but, this increase in the mean total GST activities in diabetic animals were not significant. Also Vitamin C and α-Lipoic acid did not change the total GST activities in diabetic animals. Interestingly when the two antioxidants administered together, they reduced GST activities even below the control values.

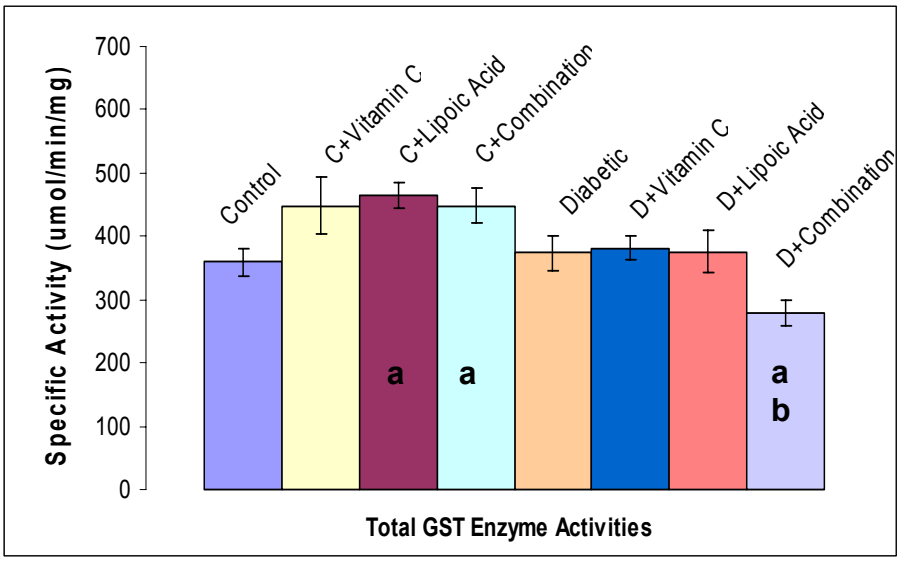


Figure 3.13: Total GST Activities in Control, Control+Treated, Diabetic and Diabetic+Treated Groups Rat Liver Tissues. Values were expressed as mean ± S.E.M. a; represents significance of 95% as compared with control. b; represents significance of 95% as compared with diabetic animals.

On the other hand, treating the control animals with lipoic acid alone or in combination with vitamin C increased the total GST activities significantly ($p < 0.05$).

Vitamin C treatement increased the Total GST activity but this increase was not statistically significant. Alpha lipoic acid increased the Total GST activities significantly ($p < 0.005$). Furthermore, combination of these antioxidants increases total GST activities in control animals significantly ($p < 0.05$). The results of the statistical analysis are given in Table 8.

Table 8: The results of the statistical analysis of Total GST

Sample	**Specific Activity (umol/min/mg)**	***p***
Controls (n=9)	**359.0**	
Diabetic (n=9)	373.8	0.67
C+ Vitamin C (n=4)	448.4	0.15
C+ LA (n=8)	464.6	**0.0025**
C+ Combination (n=4)	448.5	**0.014**
D+ Vitamin C (n=12)	381.3	0.42
D+ LA (n=8)	375.4	0.68
D+ Combination (n=7)	278.3	**0.014**

3.2.2 GST Alpha Isozyme Activities

The change in the GST alpha isozyme activities of control, treated control groups, diabetic and treated diabetic groups are given in the Figure 3.14. It was found that the mean GST Alpha isozyme activity did not change in diabetic animals when compared to controls. Either lipoic acid or combination of the antioxidants did not change the GST Alpha isozyme activities in diabetic animals, but vitamin C increased the GST Alpha isozyme activity significantly.

In control groups, both vitamin C and lipoic acid and their combinations increased GST Alpha isozyme activities. Treatment of rats with both antioxidants, increased the GST Alpha isozyme activities significantly ($p<0.005$). The results of the statistical analysis are given in Table 9.

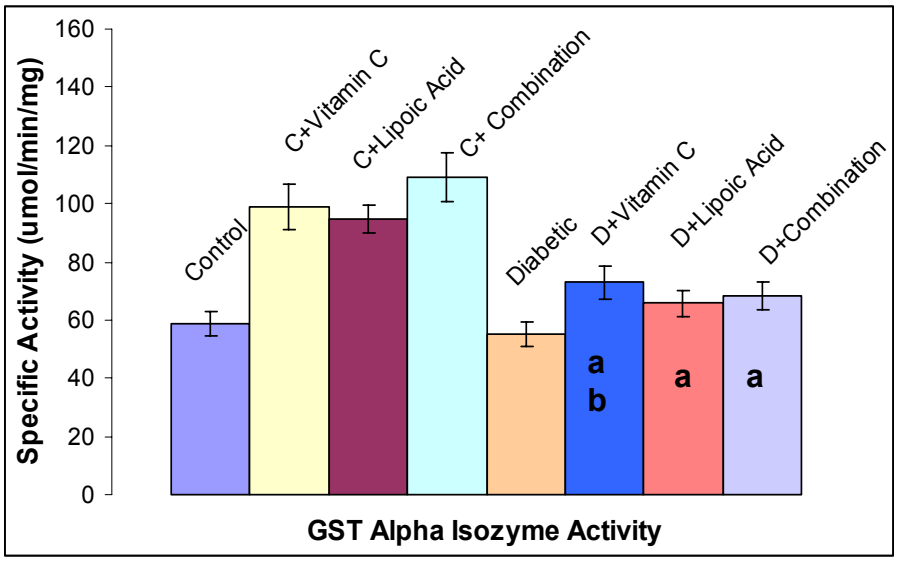


Figure 3.14: GST Alpha isozyme Activities in Control, Control+Treated Groups, Diabetic and Diabetic+Treated Rat Liver Tissues. Values were expressed as mean ± S.E.M. a; represents significance of 95% as compared with control. b; represents significance of 95% as compared with diabetic animals.

Table 9: The results of the statistical analysis of GST Alpha

Sample	**Specific Activity (umol/min/mg)**	***p***
Controls (n=9)	**58.9**	
Diabetic (n=9)	55.2	0.54
C+ Vitamin C (n=4)	98.9	**0.0059**
C+ LA (n=8)	94.7	**0.0001**
C+ Combination (n=4)	108.9	**0.0063**
D+ Vitamin C (n=12)	72.8	0.064
D+ LA (n=8)	65.8	0.28
D+ Combination (n=7)	68.2	0.16

3.2.3 GST Mu Isozyme Activities

The change in the GST Mu isozyme activities of control, treated control groups, diabetic and treated diabetic groups are given in the Figure 3.15.

GST Mu isozyme activity decreased significantly in diabetic animals compared to controls. Although, LA did not change their activities, diabetics treated with vitamin C showed an increase in Mu activity which is statistically significant ($p<0.005$), but this increase still did not reach the control values. When two antioxidants administered together, they reduced the diabetic GST Mu activities even below the control values.

In control groups, vitamin C or Lipoic acid when administered alone, they showed no effect on the control rats, statistically. However combination of vitamin C and lipoic acid increased the Mu isozyme activity, significantly ($p<0,005$). The results of the statistical analysis are given in Table 10.

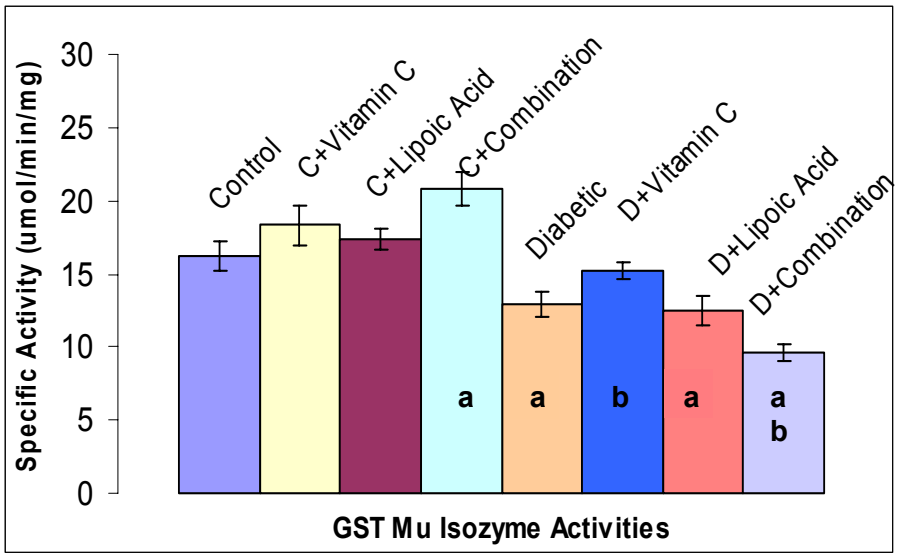


Figure 3.15: GST Mu Activities in Control, Control+Treated Groups, Diabetic and Diabetic+Treated Rat Liver Tissues. Values were expressed as mean ± S.E.M. a; represents significance of 95% as compared to control. b; represents significance of 95% as compared to diabetic animals.

Table 10: The results of the statistical analysis of GST Mu

Sample	Specific Activity (umol/min/mg)	*p*
Controls (n=9)	**16.21**	
Diabetic (n=9)	12.96	**0.031**
C+ Vitamin C (n=4)	18.34	0.11
C+ LA (n=8)	17.41	0.35
C+ Combination (n=4)	20.82	**0.023**
D+ Vitamin C (n=12)	15.26	0.44
D+ LA (n=8)	12.49	**0.023**
D+ Combination (n=7)	9.59	**0.0001**

3.2.4 GST Theta Isozyme Activities

The change in the GST Theta isozyme activities of control, treated control groups, diabetic and treated diabetic groups are given in the Figure 3.16.

According to our results, the mean GST Theta activity increased in diabetic animals but decreased in all diabetic treated groups. However, only the effect of lipoic acid on GST Theta activity was statistically significant ($p<0.05$). Vitamin C, Lipoic acid and combination of these antioxidants, increased the GST activity in control animals, but this increase is also not statistically significant. The results of the statistical analysis are given in Table 11.

3.3 PCR Optimization

In order to see the effect of antioxidants; vitamin C and lipoic acid, on the gene expressions of different GST isozymes, PCR conditions were first optimized. In this study, two GST isozyme GST A2 and GST M1 gene expressions were studied.

Unfortunately, as we could not find an already designed primer for GST theta in data banks; in our future studies, we are planning to design the primer and study GST theta gene expressions, also.

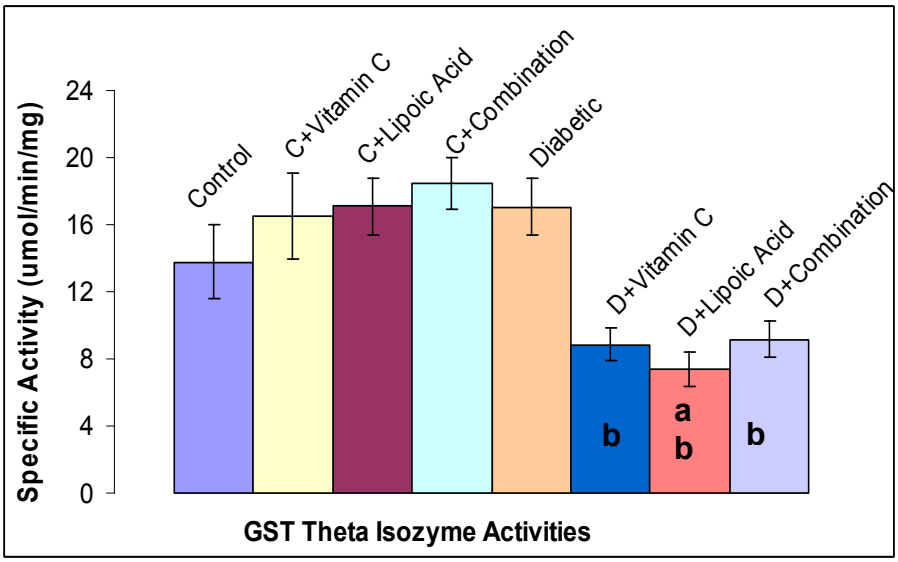


Figure 3.16: GST Theta Activities in Control, Control+Treated, Diabetic and Diabetic+Treated Rat Liver Tissues. Values were expressed as mean ± S.E.M. a; represents significance of 95% as compared to control. b; represents significance of 95% as compared to diabetic animals.

Table 11: The results of the statistical analysis of GST Theta

Sample	Specific Activity (umol/min/mg)	*p*
Controls (n=9)	**13.75**	
Diabetic (n=9)	17.04	0.26
C+ Vitamin C (n=4)	16.52	0.45
C+ LA (n=8)	17.11	0.25
C+ Combination (n=4)	18.46	0.11
D+ Vitamin C (n=12)	8.87	0.074
D+ LA (n=8)	7.40	**0.026**
D+ Combination (n=7)	9.17	0.094

3.3.1 Cycle optimizations of β-Actin and GST cDNA Amplification by PCR

Cycle optimizations are very important in RT-PCR experiments. In order to quantitate, PCR product should be in the linear phase of the amplification process.

As seen in Figure 3.17, at the plateau, no matter how much initial mRNA was present, all the intensities would be the same amount. This non-proportionality is due to a so-called *plateau effect*, which is the attenuation in the exponential rate of the product accumulation in late stages of a PCR. This may be caused by degradation of reactants (dNTPs, enzyme), reactant depletion (primers, dNTPs - former a problem with short products, latter for long products), end-product inhibition (pyrophosphate formation), and competition for reactants by non-specific products, or competition for primer binding by re-annealing of concentrated (10 nM) products (Innis and Gelfand, 1990).

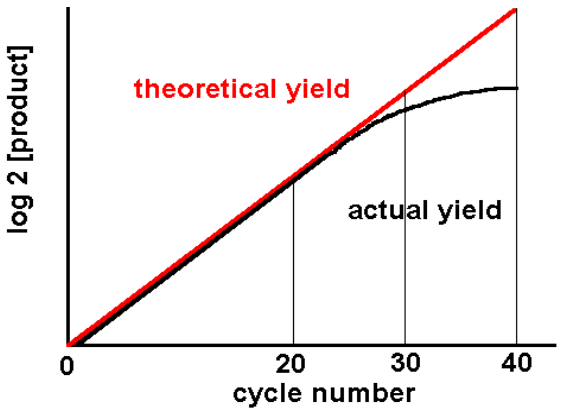


Figure 3.17: Plateau effect in PCR amplification

3.3.1.1 β-Actin Cycle Optimizations

β-Actin is a housekeeping gene used in the experiments in order to calculate the relative expression of GST isozymes in tissues. The gel photograph of the β-Actin cycle optimizations is given at Figure 3.18. The band intensity and the cycle number are given at Table 8. From the table and the figure shown below, we found that at cycle 34, PCR product of β-actin gene reached to its maximum value, and so, we choosed the optimum cycle number of this gene as 28, as shown in Figure 3.19, cycle number versus Intensity graph.

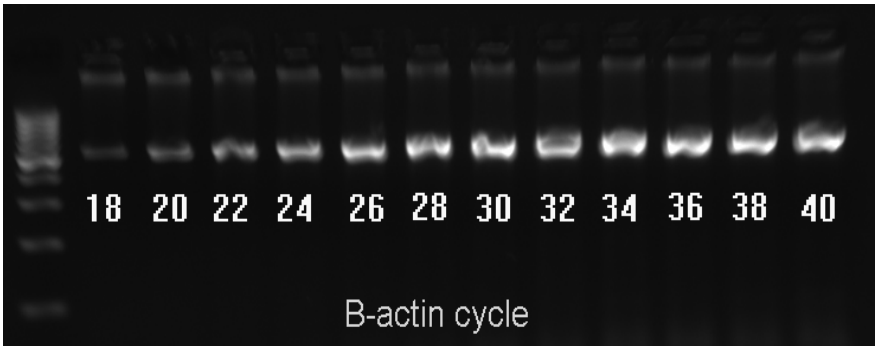


Figure 3.18: β-actin mRNA Amplification Cycle Optimization. Lane 1 contains the molecular weight standards and following lanes contain the same reaction mixture (Materials and Methods) at same annealing temperature, same primer and $MgCl_2$ concentration but different cycle numbers.

3.3.1.2 GST A2 Cycle Optimizations

The GST A2 cycle optimizations were carried out at 11 different cycles and the resulting gel photograph for cycle optimizations is given in Figure 3.20. From this figure, band intensities were found and the data is given at Table 12.

Table 12: Cycle number and β-Actin Band Intensity (arbitrary unit)

Cycle number	Band intensity
18	19424
20	31884
22	48630
24	62044
26	80413
28	91192
30	100258
32	94751
34	108787
36	102528
38	100401
40	100548

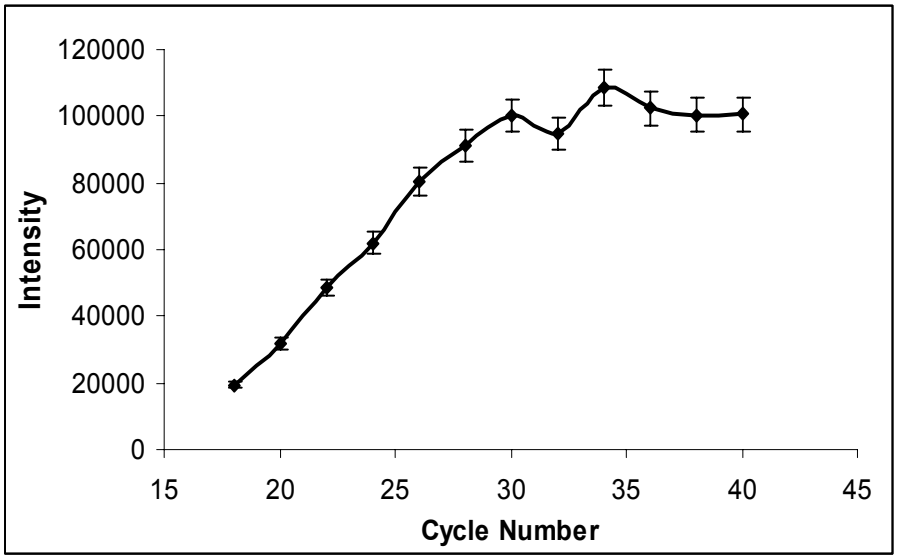


Figure 3.19: β-Actin Intensity versus Cycle Number Graph

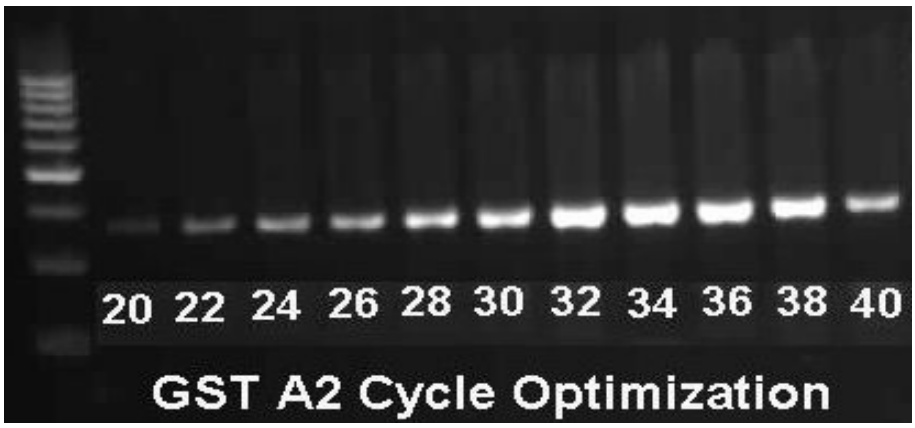


Figure 3.20: GST A2 mRNA Amplification Cycle Optimization. Lane 1 contains the molecular weight standards and following lanes contain the same reaction mixture (Materials and Methods) at same annealing temperature, same primer and $MgCl_2$ concentrations but different cycle numbers.

Table 13: Cycle number and GSTA2 Band Intensity

Cycle number	Band intensity
20	13963
22	22838
24	32538
26	38557
28	51991
30	59610
32	78150
34	77830
36	78313
38	73332
40	50236

As seen from the Table 9 and also from Figure 3.20, at cycle 32 the PCR product of GST A2 has almost reached to the maximum value, so the optimum cycle number for this gene was used as 28, as shown in Figure 3.21, cycle number versus Intensity graph.

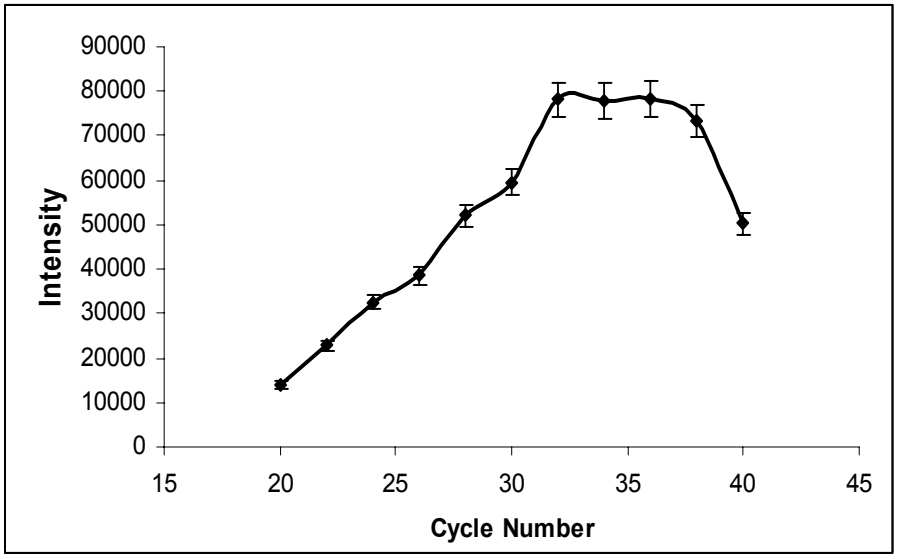


Figure 3.21: GST A2 Intensity versus Cycle Number Graph

3.3.1.3 GST M1 Cycle Optimizations

Cycle optimization for GSTM1 was also carried out with different cycle numbers. The results are given in Figure 3.22, and the band intensities versus cycle numbers are given at Table 14.

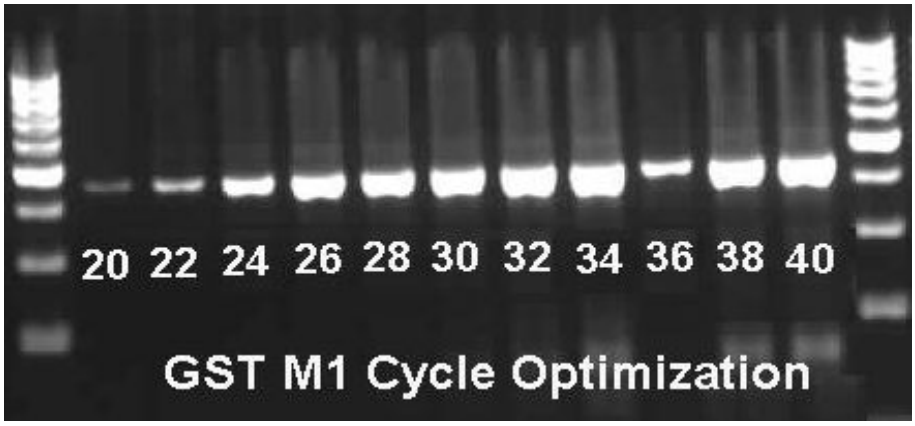


Figure 3.22: GST M1 mRNA Amplification Cycle Optimization. Lane 1 contains the molecular weight standards and following lanes contain the same reaction mixture (Materials and Methods) at same annealing temperature, same primer and $MgCl_2$ concentrations but different cycle number.

Table 14: Cycle number and GSTM1 Band Intensity

Cycle number	Band intensity
20	29571
22	45241
24	75009
26	95532
28	96036
30	102615
32	112310
34	115596
36	70028
38	110251
40	116191

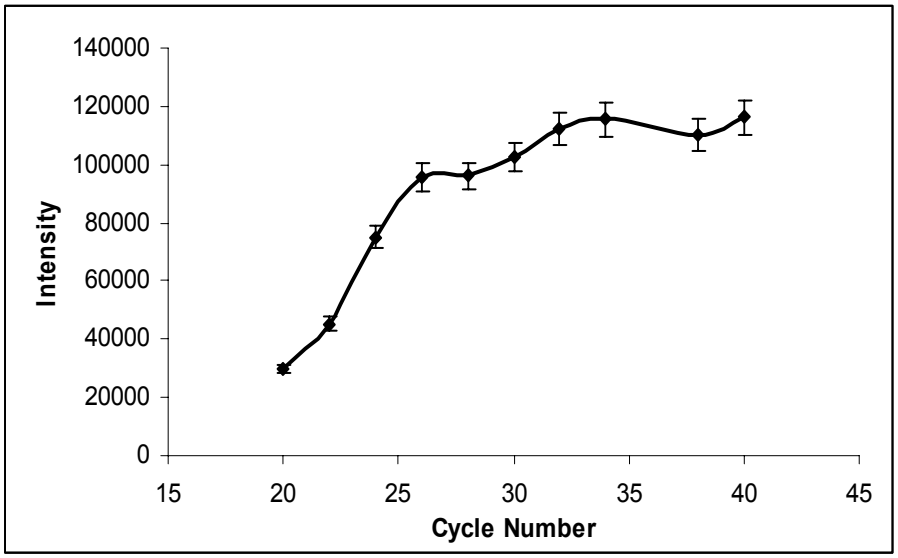


Figure 3.23: GSTM1 Intensity versus Cycle Number Graph

The cycle number versus intensity plot given at Figure 3.23 showed that, the PCR product of GSTM1 gene has reached to its maximum at cycle 34, and cycle number 28 was used as the optimum cycle number

3.3.2 Annealing temperature optimizations of GSTs for PCR reactions

One of the very important parameter in PCR amplification reactions is the annealing temperature, as the formation of DNA/DNA or DNA/RNA complexes, e.g., binding of a primer to a template requires a temperature which should be lower then the extension temperature of 72 ^{o}C. Moreover, the flexibility of this parameter allows the optimization of the reaction in the presence of variable amounts of other ingredients (especially in multiplex PCR). The efficiency of primers to anneal to the cDNA sequence of interests can vary with temperature. Optimizing the annealing temperature is especially critical when long products are synthesized or when total genomic DNA is the substrate for PCR reaction. The optimal annealing temperature depends on the melting temperature of the primer-template

hybrid. If the temperature is too high the primers will not anneal efficiently, and on the other hand if the annealing temperature is too low the primers may anneal nonspecifically.

3.3.2.1 β-Actin Annealing Temperature Optimization

β-Actin annealing temperature optimization was done at 12 different annealing temperatures and the results are given Figure 3.24. From this figure, the band intensities were found and the data is given at Table 15. From the results, we concluded that at 61 ºC, the PCR product of β-Actin gene has reached to the maximum value so we choosed optimum annealing temperature for this gene as 58 ºC, as shown in Figure 3.25, annealing temperature versus Intensity graph.

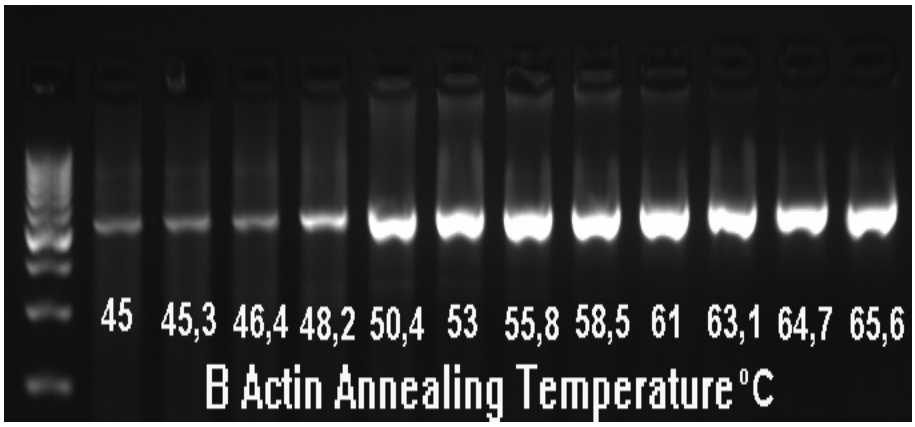


Figure 3.24: β-Actin mRNA Amplification Annealing Temperature Optimization. Lane 1 contains the molecular weight standards and following lanes contain the same reaction mixture as described under Materials and Methods at different annealing temperatures for 30 seconds.

Table 15: β-actin Annealing Temperature and Band Intensity

Annealing Temperature (ºC)	Band intensity
45.0	4.89
45.3	4.92
46.4	5.27
48.2	8.26
50.4	13.39
53.0	14.19
55.8	15.19
58.5	14.59
61.0	15.74
63.1	14.29
64.7	14.33
65.6	14.75

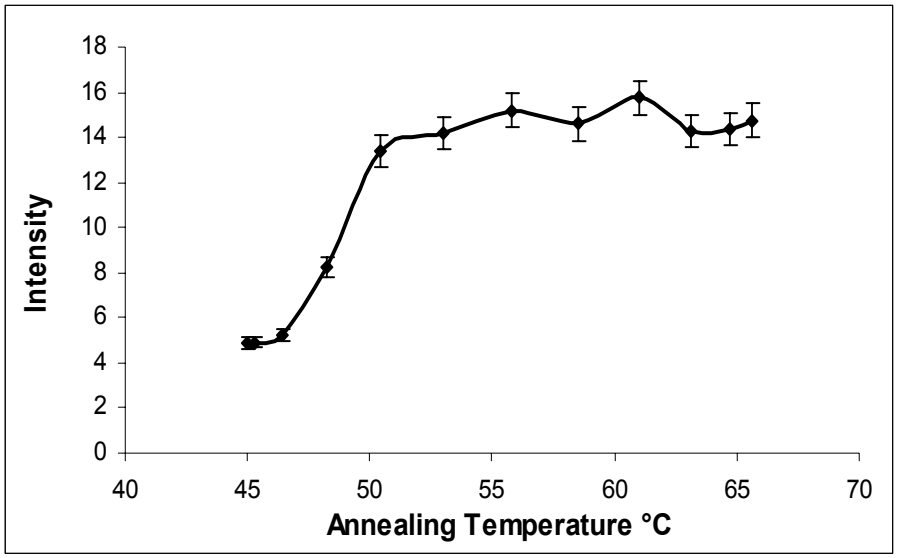


Figure 3.25: β-Actin Intensity versus Annealing Temperature graph.

3.3.2.2 GST A2 Annealing Temperature Optimization

The optimization of annealing temperatures for GSTA2 gene was carried out at 12 different temperatures and the gel photograph is show in Figure 3.26. The band intensities at different temperatures are given at Table 16, and the plot annealing temperature versus band intensities are

shown in Figure 3.27. As seen from both table and the figure, the PCR product of GSTA2 gene has reached to its maximum at around 58.5 ºC and the optimum annealing temperature was selected as 58 ºC.

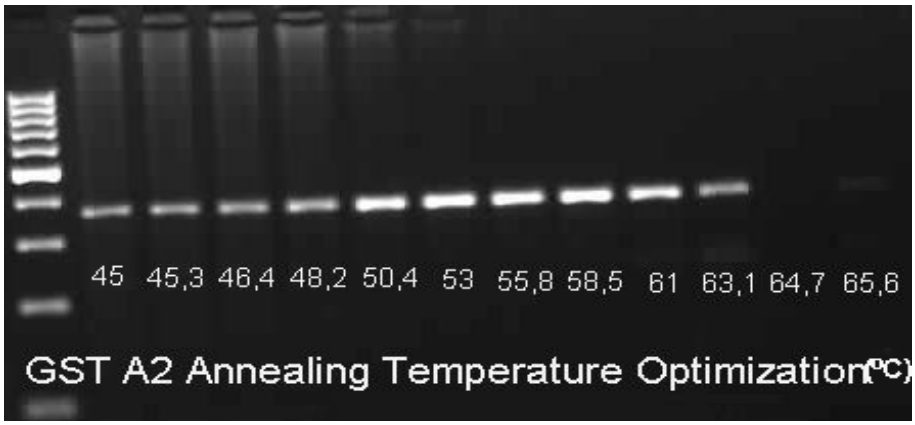


Figure 3.26: GST A2 mRNA Amplification Annealing Temperature ptimization. Lane 1 contains the molecular weight standards and following lanes contain the same reaction mixture as described under materials and methods at different annealing temperatures for 30 seconds.

Table 16: GSTA2 Annealing Temperature and Band Intensity

Annealing Temperature (ºC)	Band intensity
45.0	39521
45.3	39443
46.4	41338
48.2	45668
50.4	59018
53.0	62349
55.8	60587
58.5	62477
61.0	54186
63.1	37391
64.7	14162
65.6	15412

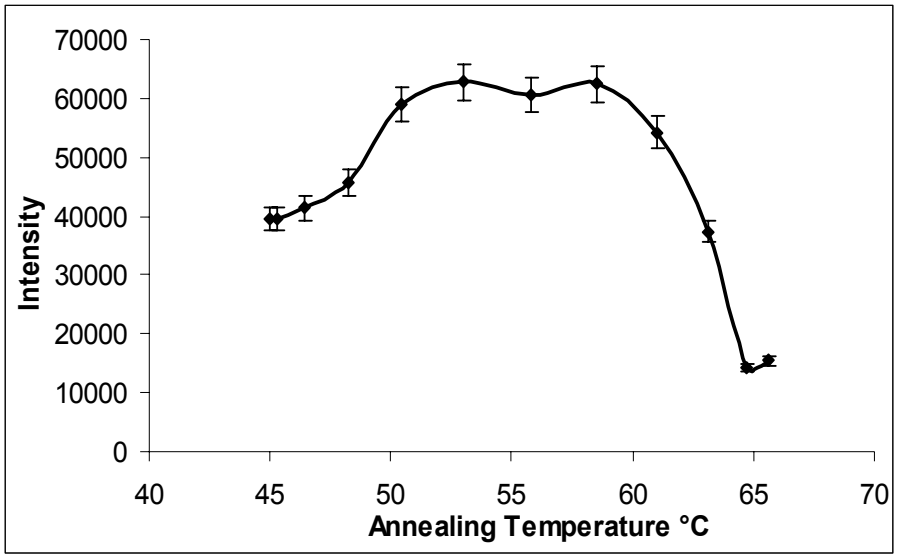


Figure 3.27: GST A2 Intensity versus Annealing Temperature graph.

3.3.2.3 GST M1 Annealing Temperature Optimization

GST M1 annealing temperature optimization was carried out at 12 different annealing temperatures and the gel photograph is shown in Figure 3.28. From this figure, band intensities were found and the data was given at Table 17. From the Table and the intensity versus annealing temperature graph shown in Figure 3.29, we concluded that at 61ºC, the PCR product of GSTM1 gene has reached to its maximum value and as a result 58 ºC was selected as an optimum annealing temperature, like GSTA2 and β-Actin genes.

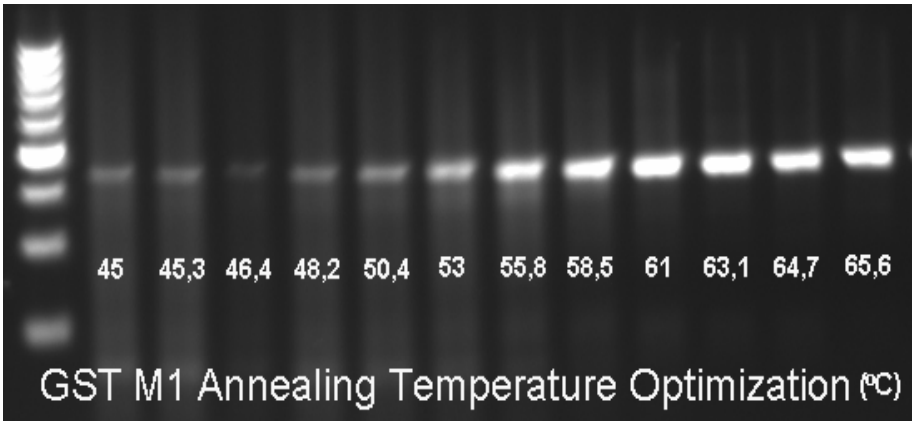


Figure 3.28: GST M1 mRNA Amplification Annealing Temperature Optimizations Lane 1 contains the molecular weight standards and following lanes contain the same reaction mixture (Materials and Methods) at different annealing temperatures for 30 seconds.

Table 17: GST M1 Annealing Temperature and Band Intensity

Annealing Temperature (ºC)	Band intensity
45.0	6249
45.3	6054
46.4	4034
48.2	6153
50.4	7070
53.0	9189
55.8	11498
58.5	11849
61.0	12328
63.1	11876
64.7	10824
65.6	10692

After cycle number and temperature optimizations, all the other conditions for PCR reactions were also optimized. All the concentrations of the ingredients and the appropriate annealing temperatures and reaction times are given at Table 18.

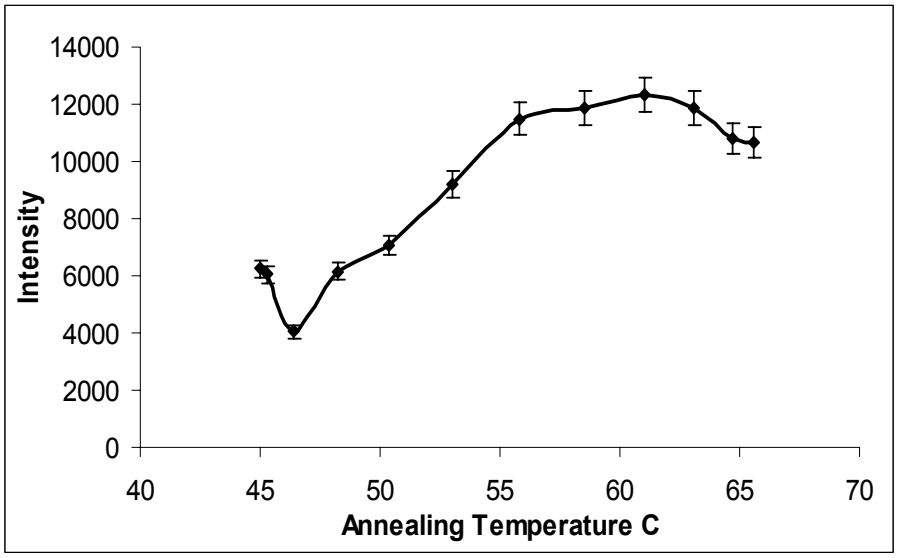


Figure 3.29: GST M1 Intensity versus Annealing Temperature Graph.

Table 18: Optimized PCR Conditions.

	β-actin	**GSTA2**	**GSTM1**
PCR cycle	26-28	28-30	28-30
$MgCl_2$ (mM)	1.5	1.5	1.5
primer (mM)	0.75	0.75	0.75
Anneal Temp (^oC)	58	58- 58.5	59
Anneal Time (sec.)	30	30	30

3.4 Determination of mRNA Expressions

3.4.1 GST A2 mRNA Expression

The change in the GST A2 mRNA expressions of all control, treated control, diabetic and treated diabetic groups are given in Figure 3.30. According to the results, GSTA2 mRNA expression was decreased in diabetic rats compared to controls, significantly ($p< 0,05$). Lipoic acid has no effect on GST A2 mRNA expression. But Vitamin C treated control and diabetic groups' mRNA expressions were significantly lower ($p< 0,05$). R The results of the statistical analysis are given in Table19.

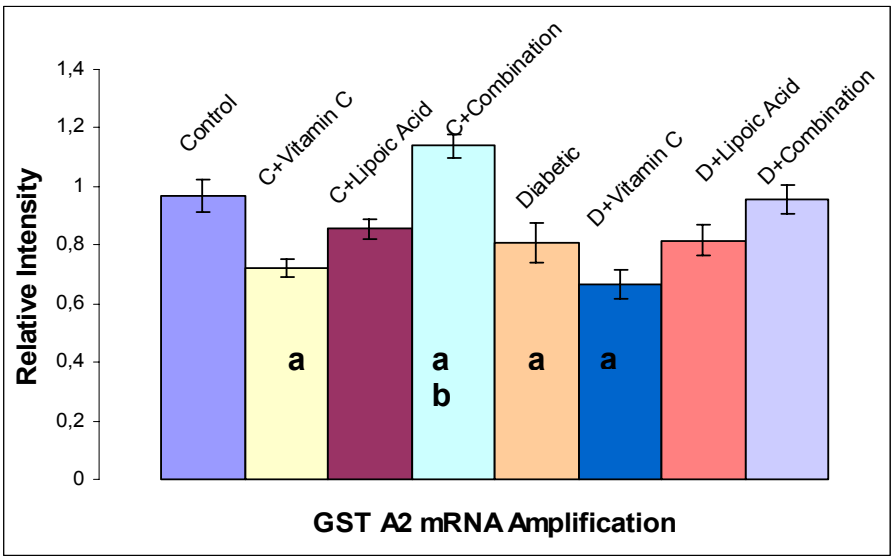


Figure 3.30: GST A2 mRNA expressions of Control, Control+Treated, Diabetic and Diabetic+Treated Groups. Values were expressed as mean ± S.E.M. a; represents significance of 95% as compared to control. b; represents significance of 95% as compared to diabetic animals.

Table 19: Results of the statistical analysis of GSTA2 mRNA Amplification

Sample	**Intensity**	***p***
Controls (n=9)	**0.968**	
Diabetic (n=9)	0.205	0.089
C+ Vitamin C (n=4)	0.721	**0.0027**
C+ LA (n=8)	0.855	0.11
C+ Combination (n=4)	1.139	**0.032**
D+ Vitamin C (n=12)	0.665	**0.0008**
D+ LA (n=8)	0.815	0.062
D+ Combination (n=7)	0.957	0.89

The combination of both antioxidants has increased control group's GST A2 mRNA expression significantly. The combination treatment also increased the mRNA expression of diabetics, but this increase was not statistically significant. Though the individual treatments of both antioxidants alone did not reverse the effects of diabetes, the combination

of both seems to restore the effects of oxidative stress on the expression of GSTA2 gene.

3.4.2 GST M1 mRNA Expression

The change in the GST M1 mRNA expressions of all control, treated control, diabetic and treated diabetic groups are shown in Figure 3.31. As seen from the figure, GSTM1 mRNA expressions were decreased significantly ($p < 0,05$) in diabetic animals compared to controls. Lipoic acid treatment increased GSTM1 mRNA expression in control groups but decreased in diabetic groups.Though the increase was more significant in control tissues, both change was found to be statistically significant ($p < 0,05$). Vitamin C has no effect on control animals but decreased the mRNA expression of diabetic rats, significantly ($p < 0,05$).

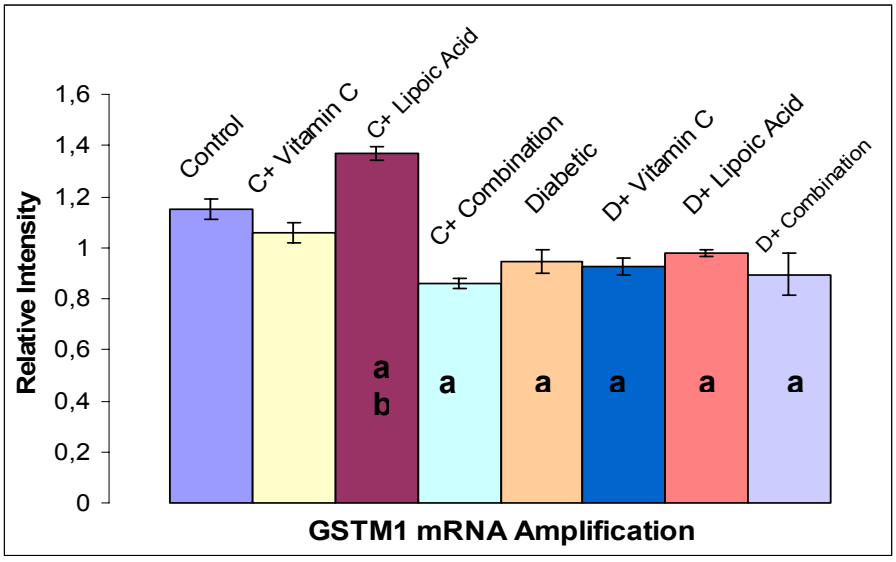


Figure 3.31: GST M1 mRNA expressions of Control, Control+Treated, Diabetic and Diabetic+Treated Groups. Values were expressed as mean ± S.E.M. a; represents significance of 95% as compared to control. b; represents significance of 95% as compared to diabetic animals.

On the other hand, the combination of both antioxidants decreased GSTM1 mRNA expressions in both control and diabetic animals significantly. The results of the statistical analysis are given in Table 20.

Table 20: Results of the statistical analysis of GSTM1 mRNA Amplification

Sample	**Intensity**	***p***
Controls (n=9)	**1.15**	
Diabetic (n=9)	0.94	**0.0055**
C+ Vitamin C (n=4)	1.06	0.15
C+ LA (n=8)	1.37	**0.0005**
C+ Combination (n=4)	0.86	**0.0001**
D+ Vitamin C (n=12)	0.93	**0.0006**
D+ LA (n=8)	0.98	**0.003**
D+ Combination (n=7)	0.89	**0.025**

According to our results, GST M1 mRNA expression was decreased in diabetic animals compared to controls, significantly ($p<0,05$). Lipoic acid increased GSTM1 mRNA expression in control groups but however decreased in diabetic groups. Vitamin C has no effect on control animals but decreased the mRNA expression in diabetic animals. The combination of these antioxidants decreased GST M1 mRNA expression in both control and diabetic groups significantly, showing that, combined effect of these two antioxidants is not efficient in changing the gene expression of GST Mu gene. However in the case of GST A2 mRNA expressions, though the individual treatment of the antioxidants showed no effect, the combination of both LA and vitamin C have a restoring effect on the expression of GST A2 gene.

Antioxidant effects of LA is based on its interaction with peroxyl radicals, which are essential for the initiation of lipid peroxidation; and ascorbyl radicals of vitamin C. Reduced form of lipoic acid, dihydrolipoic acid (DHLA), can recycle ascorbyl radicals and reduce dehydroascorbate generated in the course of ascorbate oxidation by radicals. Therefore, what we expect as a result is, LA will act as a strong chain-breaking antioxidant

and thus enhance the antioxidant potency of other antioxidants like vitamin C in both the aqueous and in hydrophobic membrane phase.

Oxidative stress has different effects on the expressions of different genes. In our study we found that both GST M1 and GST A2 gene expressions were decreased in diabetic animals significantly compared to controls and this decrease is statistically significant. However, the restoring effects of LA and vitamin C are quite different in each gene. Interestingly the combined effect of both antioxidants on GST M1 mRNA expression is not positive.

CHAPTER IV

CONCLUSION

The results of this study showed the effects of hyperglycemia and oxidative stress caused by diabetes, on the gene expressions and activities of different GST isozymes and the possible effects of the antioxidants, vitamin C and lipoic acid.

GST Theta activity increased in diabetic animals but decreased in all diabetic treated groups. However, only the effect of lipoic acid on GST Theta activity was statistically significant ($p<0.05$). Vitamin C, Lipoic acid and combination of these antioxidants, increased the GST activity in control animals, but this increase is also not statistically significant.

Although, diabetic rats exhibited decreased mRNA expressions in both GSTA2 and GSTM1, the activities of only GST Mu isozyme decreased in diabetic rats, compared to controls. GST Alpha isozyme activity remained unchanged in diabetic animals. Either, the rate of translation increased or after protein synthesis further activation of the isozyme took place. This question remains unsolved.

Administration of Lipoic acid did not reverse the results of diabetes. LA, individually have no significant effects on both of the GST isozymes' gene expressions and activities. Also administration of Vitamin C did not reverse the results of diabetes. Vitamin C alone has no significant effects on all GST isozyme activities but decrease only GSTA2 mRNA expression significantly. While the GSTA2 mRNA expression was decreased, there is no difference in the GSTA2 activity as a result of administration of Vitamin C alone.

Although the administration of both antioxidants in control rats affects both GSTA2 and GSTM1 mRNA expressions, only GST Mu activity changed. Interestingly, vitamin C and Lipoic Acid administration alone did not change the GST isozyme activities; only affect is on GST Mu activity.

According to these results, combination of both antioxidants did not reverse the results of diabetes at least at the protein level; combination of both antioxidants decreased all the diabetic GST activities.

As a result, in all GST isozymes, only GST Mu activities and mRNA expressions were changed in the same way by the administration of antioxidants.

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APPENDIX A

GST ALPHA

Table A1: GST Alpha Control Groups

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
Control 1	58.08	69.87	71.46
Control 2	55.15	84.17	86.92
Control 3	50.78	42.29	94.98
Control 4	49.45	58.01	97.52
Control 5	61.13	63.28	62.18
Control 6	59.92	62.28	70.32
Control 7	61.36	49.92	53.39
Control 8	60.15	51.71	46.59
Control 9	74.12	48.14	55.88
C+LA 1	36.95	95.52	87.11
C+LA 2	51.59	94.12	94.68
C+LA 3	47.29	72.63	97.10
C+LA 4	51.53	95.97	80.15
C+LA 5	48.07	87.22	97.38
C+LA 6	35.71	106.11	104.46
C+LA 7	43.77	117.43	116.17
C+LA 8	36.60	88.96	64.95
C+ VC 1	46.57	100.73	72.11
C+ VC 2	52.90	94.78	87.31
C+ VC 3	50.16	118.32	89.09
C+ VC 4	49.38	81.71	77.50
C+(VC+LA) 1	50.93	94.10	83.26
C+(VC+LA) 2	41.98	130.09	101.49
C+(VC+LA) 3	47.83	115.17	93.98
C+(VC+LA) 4	49.49	96.72	113.62

Table A2: GST Alpha Diabetic Groups

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
Diabetic 1	72.40	63.10	68.33
Diabetic 2	64.00	48.60	51.68
Diabetic 3	63.66	65.40	75.66
Diabetic 4	76.59	60.20	80.90
Diabetic 5	76.31	65.18	57.84
Diabetic 6	68.95	36.72	69.61
Diabetic 7	75.39	55.57	36.77
Diabetic 8	49.34	36.37	78.87
Diabetic 9	65.67	65.83	50.47
D+LA 1	63.00	86.05	75.49
D+LA 2	92.35	47.59	84.77
D+LA 3	66.05	68.69	55.71
D+LA 4	66.24	72.07	82.84
D+LA 5	58.19	62.95	81.96
D+LA 6	105.80	53.13	68.64
D+LA 7	90.04	60.38	60.50
D+LA 8	62.74	75.50	64.85
D+ VC 1	60.33	53.83	79.52
D+ VC 2	47.31	56.40	86.58
D+ VC 3	68.27	75.16	93.05
D+ VC 4	48.36	39.07	55.14
D+ VC 5	53.49	74.68	66.43
D+ VC 6	49.04	97.34	62.71
D+ VC 7	42.31	81.48	99.60
D+ VC 8	51.82	67.64	55.29
D+ VC 9	57.54	89.46	72.75
D+ VC 10	73.71	55.34	85.29
D+ VC 11	60.46	106.29	49.03
D+ VC 12	33.66	77.09	118.03

Table A2: GST Alpha Diabetic Groups (Continued)

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
D+(VC+LA) 1	37.54	75.71	60.02
D+(VC+LA) 2	81.83	62.17	59.59
D+(VC+LA) 3	64.69	88.36	61.74
D+(VC+LA) 4	66.50	66.64	57.27
D+(VC+LA) 5	79.36	49.47	50.63
D+(VC+LA) 6	66.70	64.63	67.37
D+(VC+LA) 7	85.43	70.73	57.56

APPENDIX B

GST MU

Table B1: GST Mu Control Groups

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
Control 1	52.71	17.02	71.46
Control 2	50.18	20.74	86.92
Control 3	44.72	12.06	94.98
Control 4	45.02	14.94	97.52
Control 5	57.27	17.96	62.18
Control 6	52.41	19.79	70.32
Control 7	54.03	17.41	53.39
Control 8	74.50	12.67	46.59
Control 9	70.29	13.51	55.88
C+LA 1	39.30	16.46	87.11
C+LA 2	50.25	18.00	94.68
C+LA 3	37.17	18.67	97.10
C+LA 4	49.55	17.68	80.15
C+LA 5	42.00	18.29	97.38
C+LA 6	44.50	14.30	104.46
C+LA 7	33.69	20.49	116.17
C+LA 8	41.62	15.34	64.95
C+ VC 1	44.66	20.15	72.11
C+ VC 2	50.12	18.50	87.31
C+ VC 3	46.29	17.56	89.09
C+ VC 4	47.25	17.13	77.50
C+(VC+LA) 1	48.84	18.50	83.26
C+(VC+LA) 2	39.32	24.09	101.49
C+(VC+LA) 3	44.69	21.11	93.98
C+(VC+LA) 4	43.22	19.60	113.62

Table B2: GST Mu Diabetic Groups

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
Diabetic 1	60.63	14.51	68.33
Diabetic 2	59.13	10.82	51.68
Diabetic 3	43.10	15.74	75.66
Diabetic 4	57.87	15.20	80.90
Diabetic 5	58.77	16.63	57.84
Diabetic 6	52.53	10.29	69.61
Diabetic 7	64.17	13.19	36.77
Diabetic 8	43.88	9.45	78.87
Diabetic 9	65.55	10.83	50.47
D+LA 1	61.04	12.97	75.49
D+LA 2	45.27	15.81	84.77
D+LA 3	61.70	10.06	55.71
D+LA 4	49.84	11.03	82.84
D+LA 5	34.67	17.67	81.96
D+LA 6	65.63	10.60	68.64
D+LA 7	64.78	12.48	60.50
D+LA 8	62.78	9.27	64.85
D+ VC 1	40.17	14.53	79.52
D+ VC 2	36.95	14.68	86.58
D+ VC 3	46.37	15.60	93.05
D+ VC 4	35.71	11.46	55.14
D+ VC 5	41.41	14.19	66.43
D+ VC 6	36.95	18.12	62.71
D+ VC 7	32.98	15.98	99.60
D+ VC 8	41.90	15.86	55.29
D+ VC 9	45.87	13.67	72.75
D+ VC 10	36.95	19.30	85.29
D+ VC 11	51.87	13.97	49.03
D+ VC 12	26.78	15.79	118.03

Table B2: GST Mu Diabetic Groups (Continued)

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
D+(VC+LA) 1	34.54	11.50	60.02
D+(VC+LA) 2	81.83	8.94	59.59
D+(VC+LA) 3	64.69	11.68	61.74
D+(VC+LA) 4	66.50	9.38	57.27
D+(VC+LA) 5	79.36	8.36	50.63
D+(VC+LA) 6	66.70	9.35	67.37
D+(VC+LA) 7	85.43	7.89	57.56

APPENDIX C

GST THETA

Table C1: GST Theta Control Groups

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
Control 1	58.08	26.55	71.46
Control 2	55.15	13.82	86.92
Control 3	50.78	9.33	94.98
Control 4	49.45	14.80	97.52
Control 5	61.13	14.13	62.18
Control 6	59.92	5.71	70.32
Control 7	61.36	21.81	53.39
Control 8	60.15	8.28	46.59
Control 9	74.12	9.31	55.88
C+LA 1	36.95	20.38	87.11
C+LA 2	51.59	20.70	94.68
C+LA 3	47.29	9.77	97.10
C+LA 4	51.53	15.37	80.15
C+LA 5	48.07	20.09	97.38
C+LA 6	35.71	10.92	104.46
C+LA 7	43.77	22.62	116.17
C+LA 8	36.60	17.05	64.95
C+ VC 1	46.57	23.45	72.11
C+ VC 2	52.90	16.45	87.31
C+ VC 3	50.16	10.65	89.09
C+ VC 4	49.38	15.55	77.50
C+(VC+LA) 1	50.93	19.09	83.26
C+(VC+LA) 2	41.98	14.15	101.49
C+(VC+LA) 3	47.83	20.87	93.98
C+(VC+LA) 4	49.49	19.76	113.62

Table C2: GST Theta Diabetic Groups

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
Diabetic 1	72.40	14.51	68.33
Diabetic 2	64.00	10.82	51.68
Diabetic 3	63.66	15.74	75.66
Diabetic 4	76.59	15.20	80.90
Diabetic 5	76.31	16.63	57.84
Diabetic 6	68.95	10.29	69.61
Diabetic 7	75.39	13.19	36.77
Diabetic 8	49.34	9.45	78.87
Diabetic 9	65.67	10.83	50.47
D+LA 1	63.00	12.97	75.49
D+LA 2	92.35	15.81	84.77
D+LA 3	66.05	10.06	55.71
D+LA 4	66.24	11.03	82.84
D+LA 5	58.19	17.67	81.96
D+LA 6	105.80	10.60	68.64
D+LA 7	90.04	12.48	60.50
D+LA 8	62.74	9.27	64.85
D+ VC 1	60.33	14.53	79.52
D+ VC 2	47.31	14.68	86.58
D+ VC 3	68.27	15.60	93.05
D+ VC 4	48.36	11.46	55.14
D+ VC 5	53.49	14.19	66.43
D+ VC 6	49.04	18.12	62.71
D+ VC 7	42.31	15.98	99.60
D+ VC 8	51.82	15.86	55.29
D+ VC 9	57.54	13.67	72.75
D+ VC 10	73.71	19.30	85.29
D+ VC 11	60.46	13.97	49.03
D+ VC 12	33.66	15.79	118.03

Table C2: GST Theta Diabetic Groups (Continued)

Sample	[Protein] (mg)	Specific Activity (Umol/min/mg)	[GSH] (nmol/mg)
D+(VC+LA) 1	37.54	14.87	60.02
D+(VC+LA) 2	81.83	6.75	59.59
D+(VC+LA) 3	64.69	11.50	61.74
D+(VC+LA) 4	66.50	8.94	57.27
D+(VC+LA) 5	79.36	11.68	50.63
D+(VC+LA) 6	66.70	9.38	67.37
D+(VC+LA) 7	85.43	8.36	57.56

APPENDIX D

THE EFFECTS OF SUBSTRATE (GSH) CONCENTRATION

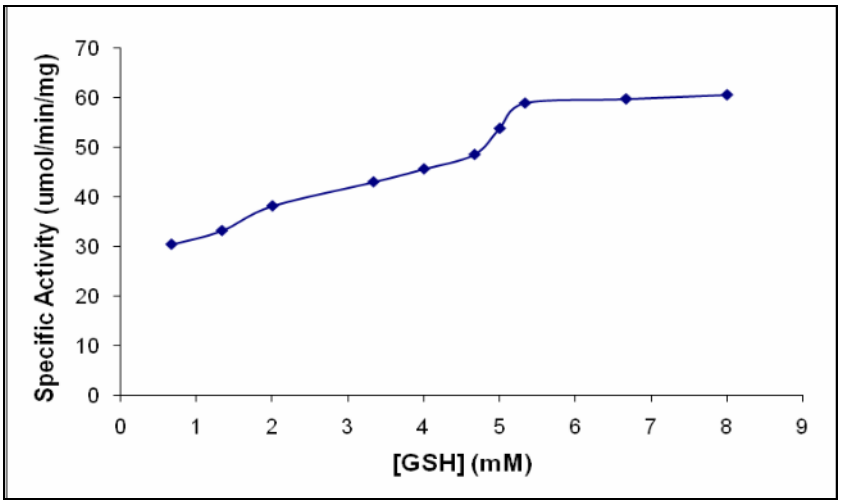

Figure D1: The Effect of Substrate (GSH) Concentrations on GST Alpha isozyme activity

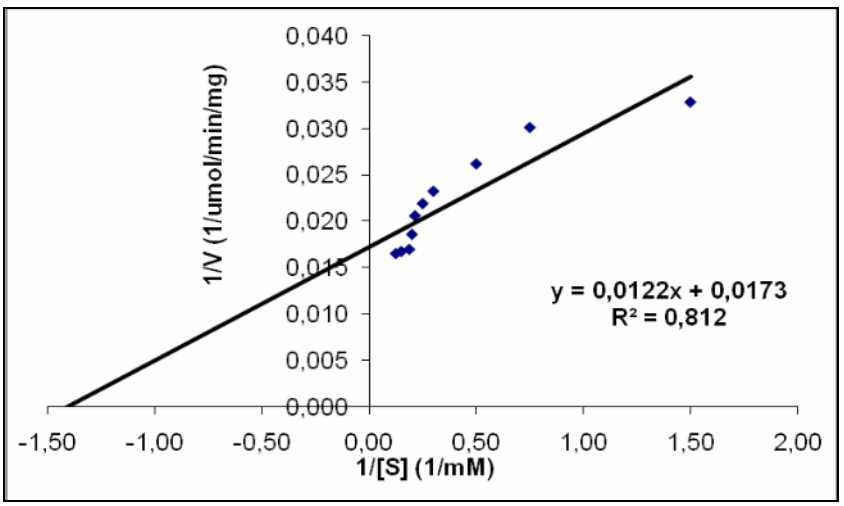

Figure D2: Lineweaver-Burk Plot of GST Alpha isozyme.

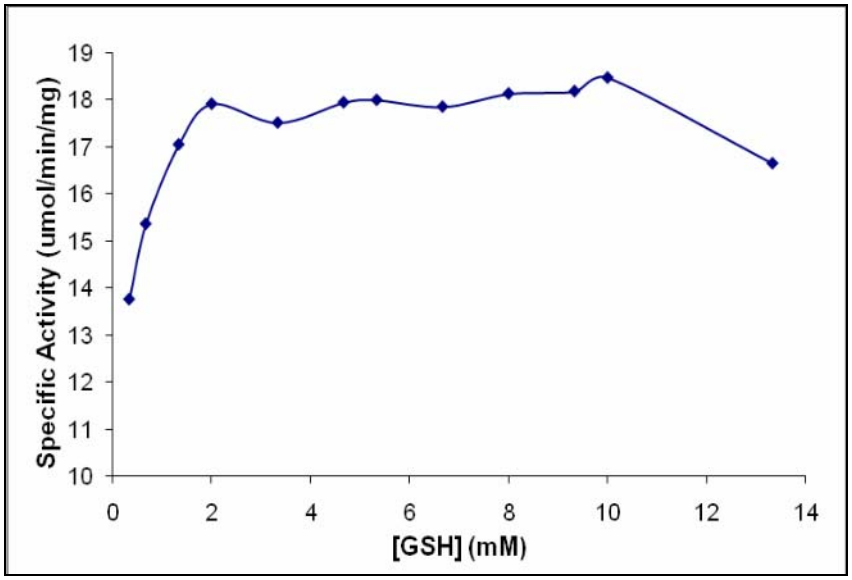

Figure D3: The Effect of Substrate (GSH) Concentrations on GST Mu isozyme activity.

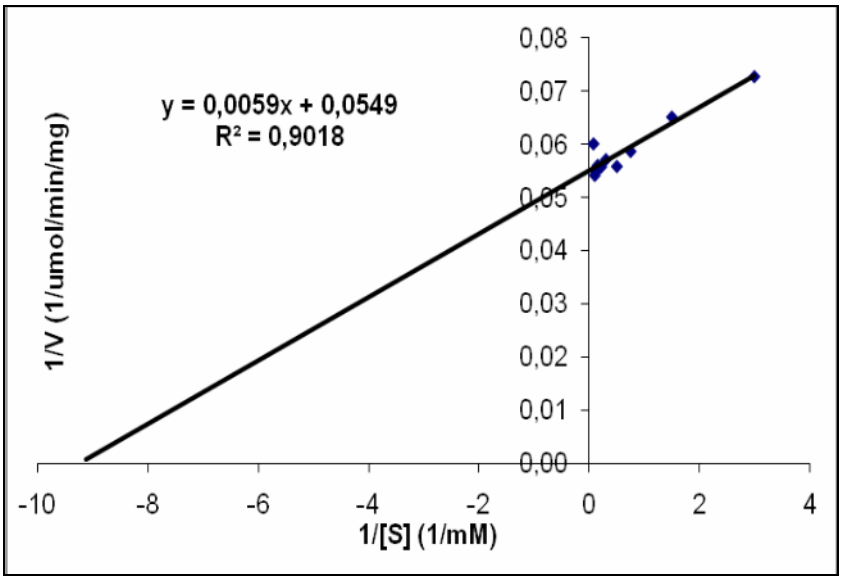

Figure D4: Lineweaver-Burk Plot of GST Mu isozyme.

.

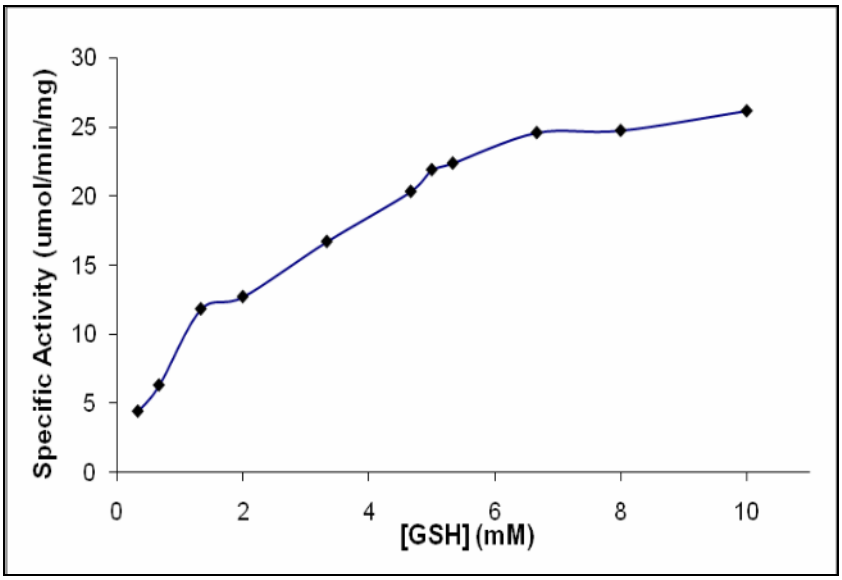

Figure D5: The Effect of Substrate (GSH) Concentrations on GST Theta isozyme activity.

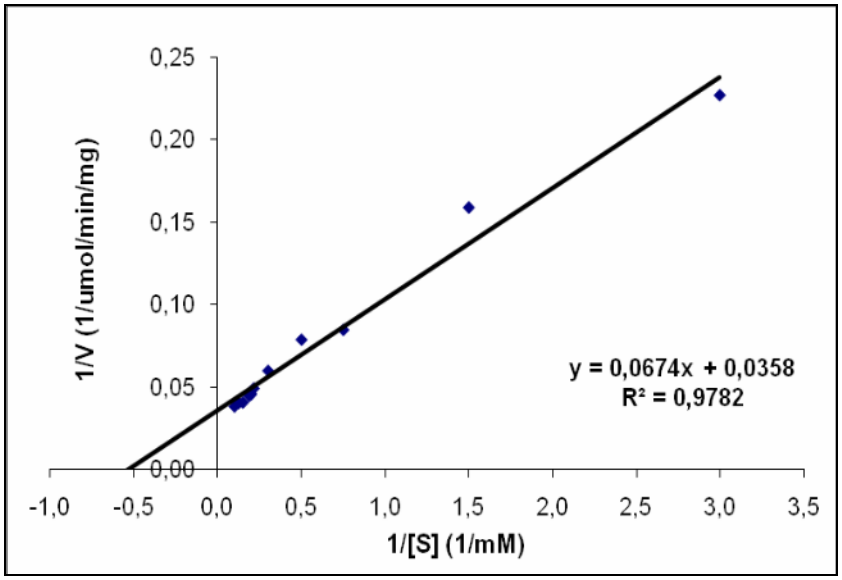

Figure D6: Lineweaver-Burk Plot of GST Theta isozyme.