ISOLATION AND IMMUNOLOGICAL CHARACTERIZATION OF THETA CLASS GLUTATHIONE -S-TRANSFERASE GSTT2-2 FROM BOVINE LIVER

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Approval of the Graduate School of Natural and Applied Sciences.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

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The glutathione-S-transferases (GSTs) (EC.2.5.1.18) are enzymes that participate in cellular detoxification of endogenous as well as foreign electrophilic compounds, function in the cellular detoxification systems and are evolved to protect cells against reactive oxygen metabolites by conjugating the reactive molecules to the nucleophile scavenging tripeptide glutathione (GSH, γ-glu-cys-gly). The GSTs are found in all eukaryotes and prokaryotic systems, in the cytoplasm, on the microsomes, and in the mitochondria. Cytosolic GSTs have been grouped into seven distinct classes as: alpha (α), mu (μ), pi (π), sigma (σ), omega, theta (θ) and zeta (δ).

In comparison with other GSTs, class theta enzymes have proven difficult to isolate and characterize. Two distinct theta GSTs have been identified in man, GSTT1-1 and GSTT2-2 three in the rat rGST1-1, rGSTT2-2 and 13-13 and one in

the mouse. this study, a class theta GST (GSTT2-2), with high activity towards 1-MS was isolated and purified from bovine liver in 3% yield with a purification factor of 3-fold. The purification protocol included a sequential DEAE cellulose anion exchanger liquid chromatography column, S-hexylglutathione agarose affinity column, dye binding orange A and chromatofocusing columns. The enzyme activity and protein content decreased rapidly after the last step of purification. The purified GSTT2-2 showed significant activity only towards 1-MS as 77 nmole/min/mg. The GSTT2-2 purified from bovine liver had a molecular weigth (Mr) value of about 28,200 which was also confirmed by Western Blott Analysis. The purified farctions of GSTT2-2 with other kolon farctions were tested with anti GSTT2-2, antiGST alfa, antiGST mu and antiGST pi antibodies. The enzyme activities towards CDNB, 4-nitrobenzylchloride (NBC) and 1-menapthyl sulfate were measured as described by Habig and Jacoby.

Key Words: Glutathione-*S*-transferases, Theta class GSTs, Purification, Western blotting, characterization.

ÖZ

SIĞIR KARACİĞERİNDEKİ TETA-SINIFI GLUTATYON S-TRANSFERAZ: GSTT2-2'İN İZOLASYONU VE İMMÜNOLOJİK KARAKTERİZASYONU

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Glutatyon-S-transferazlar (GST) elektrofilik yapıdaki ksenobiyotikler ve bazı endojenlerin detoksifikasyonlarında rol oynayan aynı zamanda reaktif oksijen metabolitlerinin nükleofil tutan tripeptid yapısındaki glutatyona konjugasyonunu sağlayarak vucudu koruyan bir enzim grubudur. Ökaryotlarda ve prokaryotlarda , sitoplazmada, mikrozomlarda ve mitokondride bulunurlar. Sitozolik GST ler altı grupta toplanırlar; alfa, mu, pi, sigma, zeta ve teta.

Diğer GST'lerle karşılaştırıldığında, teta sınıfı GST'lerin izolasyonuy ve karakterizasyonu oldukça zordur. İnsanda; GSTT1-1 ve GSTT2-2 olmak üzere iki, siçanda rGSTT1-1 , rGSTT2-2 ve 13-13 olmak üzewre üç, ve fare de bir tane teta sınıfı GST' leri tanımlanmıştır.

Bu çalışmada, 1-MS ' a karşı yüksek aktivite içeren teta sınıfı GSTT2-2 izoziminin sığır karaciğerinden %3 lük ürün ve yine 3 kat saf olarak izolasyonu ve saflaştırılması gerçekleştirilmiştir. Saflaştırma protokolü 1-MS' a karşı yüksek

aktiviteye sahip sitozolik fraksiyonun sırasıyla DEAE anyon değişim, S-hexylglutatyon afinite, Oranj A ve kromatofokuslama kolonlarına uygulanmasıyla elde edilmiş, protein miktarı ve 1-MS' a karşı enzim aktivitesi son kolondan sonra hızla düşmüştür. Saflaştırılan numune sadece 1-MS'a karşı 77 nmole/min/mg değerinde aktivite göstermiştir. Saflaştırılan sığır karaciğeri GSTT2-2 izoziminin SDS-PAGE ile molekül ağırlığı (Mr) 28,200 olarak bulunmuş bu sonuç Western Blot sonucu ile de desteklenmiştir. Saflaştırılan numune diğer kolon fraksiyonları ile beraber anti GSTT2-2, antiGST alfa, antiGST mu ve antiGST pi antikorları ile test edilmiştir. Enzim aktivitesleri 1-MS, CDNB ve 4-NBC substartları kullanılarak tayin edilmiştir.

Anahtar kelimeler: Glutatyon *S*-transferazler, Teta sınıfı GST'ler, Saflaştırma, Western Blot, Karakterizasyon.

TO MY LOVELY PARENTS

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NOMENCLATURE

Δ^5AD	Δ^5-Androstene-3,17-dione
AP	Alkaline phosphatase
APS	Ammonium persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
Bis	N,N′-methylene bisacrylamide
BSA	Bovine serum albumin
CDNB	1-Chloro-2,4-dinitrobenzene
CYP	Cytochrome P450 enzyme family
DDT	Dichlorodiphenyltrichloroethane
DEAE	Diethylaminoethyl
DHBA	dihydroxymethylbenz[a]antracene
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EA	Ethacrynic acid
EDTA	Ethylenediaminetetraacetic acid
EO	Ethylene oxide
EPNP	1,2-Epoxy-3-(*p*-nitrophenoxy)propane
FTIR	Infrared spectroscopy
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione-S-transferase
HMBA	Hydroxymethylbenz[a]antracene
HPLC	High-pressure liquid chromatography
LTC_4	Leukotriene C_4
MS	1-Menaphthyl sulfate
4-NBC	4-Nitrobenzyl chloride
NBT	Nitro blue tetrazolium

NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbons
PB 96	Polybuffer 96
PBE 94	Polybuffer exchanger 94
PMSF	Phenylmethanesulphonyl fluoride
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TEMED	N,N,N′,N′-tetramethylenediamine
Tris	Tris(hydroxymethyl) aminomethane
TTBS	Tris-buffered saline containing Tween-20

CHAPTER I

INTRODUCTION

Exposure to toxic chemicals has been an important problem for almost all organisms from the beginning of the life. Many of the toxic chemicals we encounter are found naturally into environment. Toxic compounds, occurring naturally, include plant and fungal toxins (plant phenols and aflatoxins) and reactive oxygen species, such as the superoxide radical and hydrogen peroxide (H_2O_2). Such a threat caused by endogenously produced and/or xenobiotic originated ones represents a biological adaptation which is fundamental to survival (Hayes McLellan *et.al.,* 1999). Against to the wide range of xenobiotics, organisms have developed several defense mechanisms such as drug efflux pumps, drug sequestration, drug metabolism, and repair of drug-target sites. The vital function of the chemical defense is known as detoxification and is affected principally by the liver. Secondary sites of detoxification are located in the other tissues, such as the lungs, the gastrointestinal tract, the kidneys and the skin. Along with strategies such as sequestration, scavenging and binding, catalytic biotransformation is evolved as an important biochemical protection mechanism against toxic chemical species.

Cells possess an impressive array of enzymes capable of biotransforming a wide range of different chemical structures and functionalities. These enzymes are called biotransformation enzymes and they play an important role in the metabolism of xenobiotics in which they may be either bioactivated or bioinactivated. Among humans there is a wide inter-individual variability in the levels and activities of

biotransformation enzymes. The reactions characterized by those enzymes are generally divided into two groups called Phase I and Phase II enzymatic reactions.

In two major categories of enzymes involved in the metabolism of drugs and xenobiotics, CYP and MFO isoforms are considered as the major phase I enzymes, responsible for the functionalization of xenobiotics by either introduction of a polar functional group or the unmasking of a polar functionality; and GST, ST, UDPGT, NAT and various methyltransferases are considered as the phase II drug metabolizing enzymes, responsible for conjugating a xenobiotic or its metabolite , thus resulting in a further enhancement of polarity. The two sets of enzymes, either alone or in concert, are responsible for the generation of readily excretable metabolites (Iyer, 1999) (**Figure 1**).

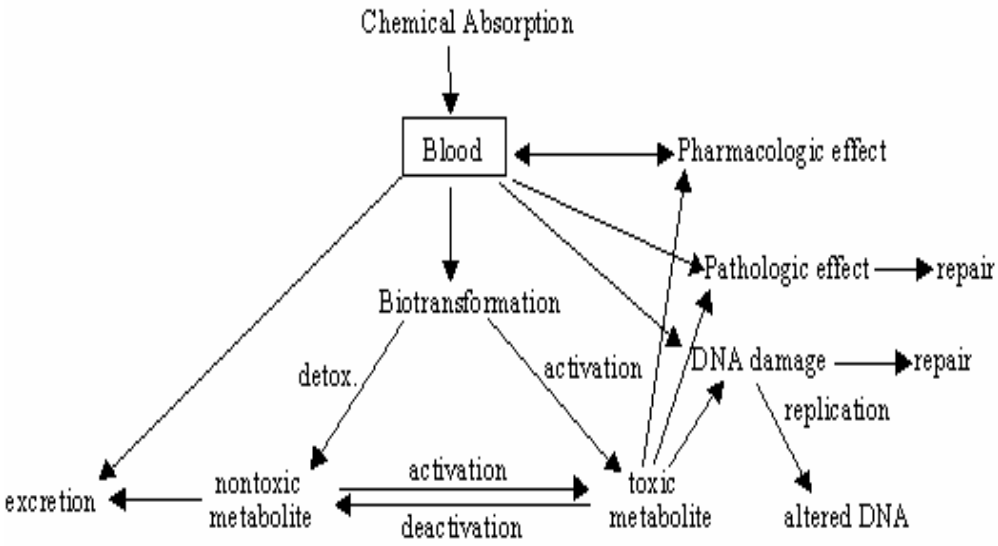


Figure 1: The variety of effects exerted on biological systems as a consequence of the biotransformation of xenobiotics (Clement, 1998).

Detoxification of xenobiotics is one of the major functions of GSH. Toxic electrophiles conjugate with GSH, either spontaneously or enzymatically, in Glutathione S-transferases catalyzed reactions.

Glutathione-S-transferases are a family of enzymes expressed in all human tissues, though the expression of their different isoenzymes is variable (Mainwaring *et.al.,*. 1996a). Thus, transferase reactions are the major pathway for GSH utilization in the liver.

The conjugation of electrophiles with GSH is irreversible, and the resulting conjugates are excreted from the cell. GSH conjugates, then, can be used for resynthesis of GSH through the mercapturic pathway. In addition to exogenous substances many endogenously formed compounds, such as prostaglandins and leukotrienes, conjugate with GSH by a similar mechanism.

Glutathione *S*-Transferase Enzyme Family:

The GSTs (EC.2.5.1.18) are enzymes that participate in cellular detoxication of endogenous as well as foreign electrophilic compounds.

$$GSH + RX \longrightarrow GSR + HX$$

GSTs as a part of detoxication systems are evolved to protect cells against reactive oxygen metabolites by conjugating the reactive molecules to the nucleophile scavenging tripeptide glutathione (GSH, γ-glu-cys-gly) (Coles and Ketterer 1990). With different electrophilic species, GSTs serve as transporters of potentially harmful substances out of the cell (Jemth and Mannervik .,e*t.al.,*, 1999). Following

conjugation, these generally harmless GSH adducts, or their mercapturic metabolites, are secreted into the bile or urine .

GSH, first described in 1888 as philothion, is the most ubiquitous and abundant non-protein thiol in mammal cells and serves as a necessary nucleophile in a number of detoxification reactions (Tew 1994). In addition to its role in intracellular detoxification, it participates in interconversions of arachidonic acid pathway metabolites (prostoglandins and leukotrienes) (Flatgoord *et.al*., 1993) and contributes to regulation of protein and DNA synthesis (Ross 1988) (**Figure 2**).

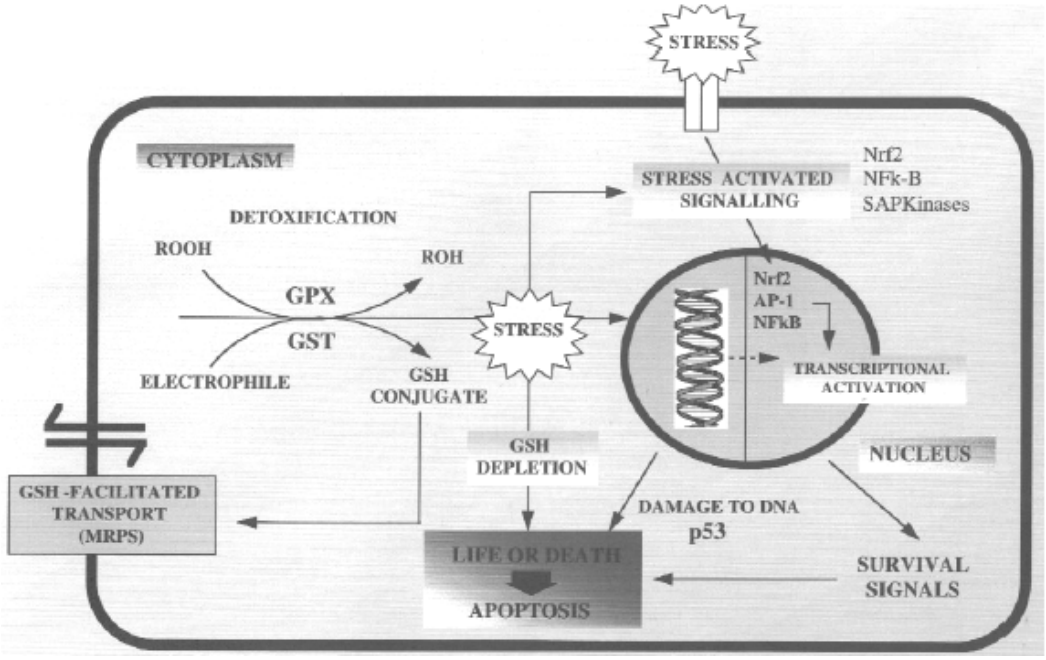


Figure 2. Mechanism for Cellular Protection by Glutathione (McLellan 1999).

Normal levels of GSH in humans are 10-30 μM (plasma), 1-3 μM (urine), 3mM (kidney proximal tubule) and 1-10 mM (tumors of various organ sites) (Tew., 1994).

Maintenance of a homeostatic GSH content is achieved by both de novo synthesis and salvage synthesis and a number of interrelated pathways are also involved (**Figure 3**).

All GST isozymes used reduced GSH as an acceptor species, but they differ in the specificity with which different substrates are transferred to the cysteine thiol of GSH. The GSTs are found in all eukaryotes and prokaryotic systems, in the cytoplasm, in the microsomes, and in the mitochondria.

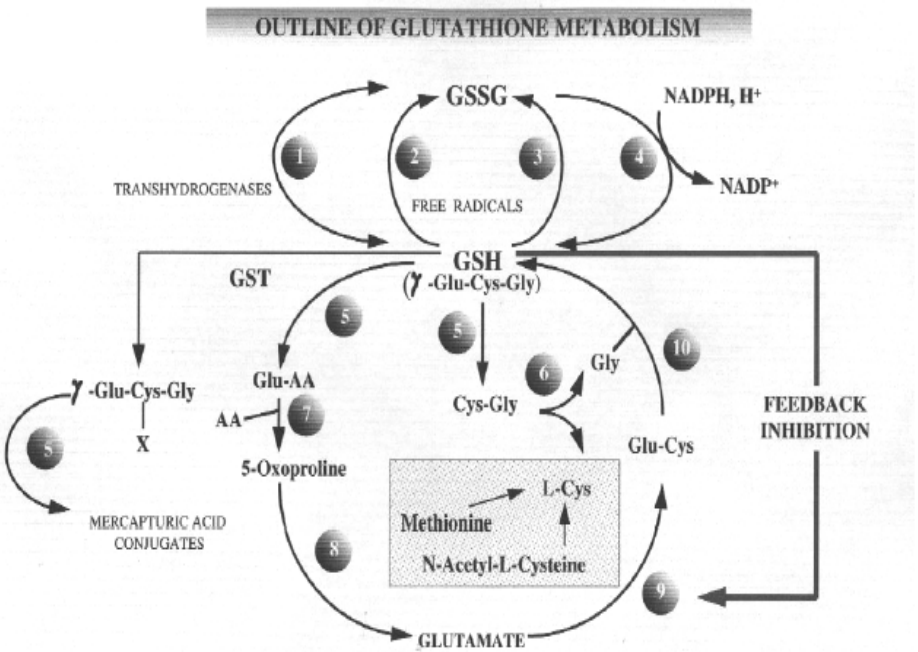


Figure 3: Glutathione metabolism (McLellan 1999).

In those species so far investigated, soluble forms of GSTs are homo or heterodimers of different subunits with distinct substrate specificities (Mannervik and Jenssen 1982) having molecular weight from 20000 to 25000. Sequences and the known three-dimensional structures suggest that these proteins share a common ancestry, though the precise details of their evolution remain obscure. They are expressed at high levels in mammalian liver constituting up to 4% of the total soluble proteins (Eaton *et.al.,*1999) and at least six distinct classes of soluble GSTs have been identified thus far: alpha (α), mu (μ), pi (π), sigma (σ), theta (θ), and zeta (δ). This classification is in accordance with the substrate specificity, chemical affinity, structure, amino acid sequence and kinetic behavior of the enzyme (Landi 2000). The amino acid sequence identitiy within class is greater than 70% , whereas the interclass identity is usually less than 30% (Rossjohn *et.al.,*1998).

The GSTs in addition to their enzymatic activities, bind with high affinity a variety of hydrophobic compounds such as heme, bilirubin, hormones and drugs, which suggests that they may serve as intracellular carrier proteins for the transport of various ligands. A marked increase in GST activity has been observed in tumor cells resistant to anticancer drugs (Daniel 1993). It has also been shown that alterations in GSH and GST levels are related not only in vitro drug resistance but also to clinical response to chemotherapy (Kearns *et.al.,* 1998).

In addition to the roles of GST in drug metabolism, detoxication, and resistance, there are several reports suggesting that a particular GST isozyme, GST-π for example may serve as a biochemical marker for neoplastic transformation (Shea., 1990).

A wide interindividual variability in the levels and activities of biotransformation enzymes exists among humans (Wormhoudt *et.al.* 1999). These interindividual differences may arise from exposure by therapeutically, occupationally and through the diet.

The expression level of the different classes of GST is also tissue specific; in human liver the GST alpha class forms 80% of the total GST expressed, and the GST A1-1 isoform predominates.

In contrast, human colonic tissue expresses GST pi as the major class of GST. Differential expression also occurs within an organ. For example, in the kidney, GST alphas predominate in the proximal tubules, whereas GST pi and GST mu are the major isoforms in the thin loop of Henle, the distal tubules and the collecting ducts (Kearns *et.al* 1998).

The presence of GSTs was first demonstrated in rat tissues (Booth *et.al.,* 1961).The presence of a wide range of GSTs isozymes with a differential and overlapping substrate specificity has been detected in a wide variety of species, including man (Kamisaka *et.al.,*., 1975), rat (Pabst *et.al.,*., 1973; Askelof, 1975; Igarashi *et.al.,*., 1986), mouse (Clark *et.al.,*., 1973; Lee *et.al.,*., 1981; Igarashi *et.al.,*., 1986), rabbit (Igarashi *et.al.,*., 1986) hamster (Smith *et.al.,*, 1980; Igarashi *et.al.,*., 1986), guinea pig (Irwin *et.al.,*., 1980; Di Ilio *et.al.,*., 1982; Igarashi *et.al.,*., 1986; Oshino *et.al.,*., 1990), chicken (Yeung and Gidari, 1980), chick (Chang *et.al.,*., 1990), cow (Saneto *et.al.,*., 1980), monkey (Asaoka *et.al.,*., 1977), trout (Nimmo and Spalding, 1985), shark (Sugiyama *et.al.,*., 1981), little skate (Foureman and Bend, 1984), grass grub (Clark *et.al.,*., 1973), house fly (Clark *et.al.,*., 1973; Clark and Dauterman, 1982), American cockroach (Clark *et.al.,*., 1973), corn (Mozer *et.al.,*., 1983) and sheep (Clark *et.al.,*., 1973; Reddy *et.al.,*., 1983; Ünsal and Öğüş, 1991; Abu-Hijleh, 1993).

1.2 Nomenculature And Classification Of Glutathione-S-Transferases:

By means of using various substrates, inhibitors, and antisera, it has been proposed that the cytosolic GSTs of rat, mouse, and man may be divided into six principal gene classes designated alpha, mu, pi, (Mannervik *et.al.*,, 1985), sigma, zeta and theta (Meyer *et.al.*,, 1991; Zhang *et.al.*,1992).

On the basis of sequence similarity, plant GSTs can be divided into four classes: phi, zeta, tau and theta (Edward., 2000) . The phi and tau classes are unique to plants and are relatively well characterized, being encoded by large gene families in all plant species studied to date (Dixon., 2000) In contrast, the theta and zeta GSTs are less well represented in plants and the presence of homologues in animals and fungi suggests common essential functions in all eukaryotes (Hayes and McLellan., 1999). Although the enzyme has been isolated from numerous animal and plant sources the most thoroughly studied group of isozymes are those purified from rat liver cytosol. In spite of the presence of the membrane bound forms of GSTs (Morgenstein and De Pierre 1988) known as kappa class GST (Pemble *et.al*. 1996), most of the studies were done with the soluble forms.

After enriching the SDS/PAGE of rat hepatic cytosol for GST (reffered to as a "Y" fraction or a "ligandin-containing" fraction), Bass et al., in 1977 resoled three electrophoretic bands for GST that were designated Ya,Yb,Yc according to their decreasing anodal mobility. Later it was found with the other researchers (Hayes 1979 and 1982) that the Ya and Yc bands represent class alpha GST, whereas the Yb band represents class mu .

The first studies with the human liver GTSs resulted with the separation of cationic forms (Kamisaka *et.al.,* 1975) which are now known to be related to the alpha family of the rat GSTs.

In later studies of human liver from various sources, 'near-neutral' and 'anionic' forms were found and shown to be homologues with the μ (Warholm

et.al.,1981). and π families in rat respectively (Mannervik et. al. 1985a; Hussey et.al.1986). The classification of cytosolic GSTs from rat and man, according to this nomenclature system is shown in **Table 1**.

Several nomenclatures have been proposed for rat GST subunits over the years.The most widely used one was proposed by Jackoby et al., using Arabic numeral nomenclature.

This system is of value because it is unambiguous and allows simple displaying of subunit combinations. But this system has disadvantage that it is not clear to show which gene family each subunit belongs. The most important advantage of this system is its usefulness to group GSTs by subfamily and immediate identification of subunits that will dimerize. A class-based subunit nomenclature has been proposed. In this system subunits are grouped by gene family and then numbered according to their order of discovery; this system for defining GST was originally devised for the human transferases (Mannervik 1992), but it is generally applicable. In this nomenclature system, single capital letter abbreviations are used to signify the alpha (A), the mu (M), the pi (P), the sigma (S), and the theta (T)classes, and Arabic numerals are employed for numbering each of the separate gene products; for example, the class alpha subunits are called A1, A2, A3, etc. The dimeric GST isoenzymes are represented by the single letter suffix (signifying class) followed by hyphenated Arabic numerals (signifying each of the two subunits). Hence the class alpha heterodimer formed betweenYa1 (A1) and Yc1 (A3), are GST Ya1Yc1, is designated GSTA1-3 (Hayes and Pulford 1995). (**Table 2**)

Table 1: Classification of cytosolic GSTs from rat and man according to the old nomenclature system (Meyer, 1991; Husey,1992)

	Species	
Class	**Rat**	**Human**
Alpha	1-1 [Ya] 1-2 [YaYc] 2-2 [Yc] 8-8 [Yk]	α, β, γ [B_2B_2] δ [B_1B_2] ε [B_1B_1]
Mu	3-3 [Yb_1] 3-4 [Yb_1Yb_2] 4-4 [Yb_2] 3-6 4-6 6-6 [Yn]	μ
Pi	7-7 [Yf]	π
Theta	5-5 12-12 (Yrs-Yrs) Yrs-Yrs′ Yrs′-Yrs′	θ (T1-1) θ (T2-2)

The more recent and commonly used nomenclature for the mammalian subunit GSTs uses a lower case letter preceding 'GST' to name the species, and an upper case letter denotes the subfamily. According to this system the human theta subunits are named "hGSTT1" and " hGSTT2" whereas the rat GST5 or GST12 subunits were renamed according to their homologies with the human subunits as "rGSTT1" and"rGSTT2" respectively. The mouse subunit Yrs is now named "mGSTT2" (Hussey *et.al* 1992, Lin *et.al.,* 1994, Jemth *et.al* 1996). When the enzyme, instead of the subunit, are named, they are named with the repeated number of their subunits (i.e. hGSTT1-1, mGSTT2-2, rGSTT1-1, etc) according to the homodimeric structures. Finally the gene coding or each subunit adopt the names of the respective subunit in italics; for example *hGSTT1* for hGSTT1, *hGSTT2* for hGSTT2 (Landi 2000).

Table 2. Classification of Cytosolic GSTs from Rat and Man According to the New Nomenclature System (Hayes and Pulford, 1995).

Species	Class			
	Alpha	**Mu**	**Pi**	**Theta**
Rat	rGSTA1-2 [Ya_1Ya_2]	rGSTM1-1 [Yb_1Yb_1]	rGSTP1-1 [YfYf]	rGSTT1-1 [GST 5-5]
	rGSTA1-3 [Ya_1Yc_1]	rGSTM1-2 [Yb_1Yb_2]		rGSTT2-2 [Yrs-Yrs] [12-12]
	rGSTA2-3 [Ya_2Yc_1]	rGSTM2-2 [Yb_2Yb_2]		rGSTT2-2´ [Yrs-Yrs´]
	rGSTA3-3 [Yc_1Yc_1]	rGSTM1-3 [Yb_1Yb_3]		rGSTT2´-2´ [Yrs´-Yrs´]
	rGSTA4-4 [YkYk]	rGSTM2-3 [Yb_2Yb_3]		
	rGSTA1-5 [Ya_1Yc_2]	rGSTM3-3 [Yb_3Yb_3]		
	rGSTA2-5 [Ya_2Yc_2]	rGSTM4-4 [Yb_4Yb_4]		
	rGSTA3-5 [Yc_1Yc_2]	rGSTM3-5* [Yb_3Yn_2]		
	rGSTA2-2 [Ya_2Ya_2]	rGSTM6*-6* [YoYo]		
Man	hGSTA1-1 [ε] [B_1B_1]	hGSTM1a-1a	hGSTP1-1 [π]	hGSTT1-1
	hGSTA1-2 [δ] [B_1B_2]	hGSTM1a-1b		hGSTT2-2
	hGSTA2-2 [α, β, λ] [B_2B_2]	hGSTM1b-1b		
	hGSTA3-3*	hGSTM1b-2		
	hGSTA4-4*	hGSTM2-2		
		hGSTM2-3		
		hGSTM3-3		
		hGSTM4-4		
		hGSTM5-5		

1.3 Functions and Structure of GSTs

1.3.1 Catalytic Activities of GSTs

GSTs catalyze the nucleophilic addition of the tripeptide glutathione to the substrates of exogenous or endogenous origins that have electrophilic functional groups. In addition to their ability to catalyze the formation of conjugates, GSTs can also serve as peroxidases and isomerases (Mannervik 1988).

GSH is synthesized within the cytosol and depleted in it by conjugation reactions, and by the reaction of H_2O_2 and biologically generated radicals across the cell membrane. Oxidation of GSH results in the formation of glutathione disulphide (GSSG), but this is rapidly returned to the reduced state by glutathione reductase, thus maintaining the GSH: GSSG ratio at around 99:1 (**Figure 3**). The third group of GSH can participate in two main types of reaction involving either a one- or a two-electron transfer. These reactions allow GSH to perform key roles within a normal cell, including conservation of the redox status of a cell and participation in certain detoxification reactions. (Kearns 1998).

All GST classes function to lower the pKa of thiol group of bound GSH from 9.0 to between 6.0 and 6.9 thus enhancing the rate of the nucleophilic attack of GSH towards the electrophilic co-substrates. This deprotonation causes a 200-300 fold rate acceleration at physiological pH; representing a crucial step in the enzymatic catalysis. (Caccuri *et al*.,1999). Evidence suggests that glutathione exists as the thiolate (GS^-) anion at neutral pH when complexes with GST acting as a nucleophile to attack the electrophile centers of xenobiotic or endogenous substrates.

Therefore, catalysis by GSTs occurs through the combined ability of the enzyme to promote the formation of GS^- and to bind hydrophobic electrophilic compounds at a closely adjacent site (Jakoby, 1978; Chen *et.al.,*1988; Graminski *et.al.,*1989; Huskey *et.al.,*1991).

Structure of the GSTs:

For all cytosolic GST classes representative crystal structures are available with the exception of the kappa class. Including those structures of the members of mammalian GSTs are from classes α [hGSTA1-A from human liver (Sinning and Armstrong 93); μ [rGSTM1-1 from rat liver (Ji 1992); hGSTM2-2 from human muscle (Rahgunathan 1994)]; π [pGSTP1-1 from pig lung (Reinemer 1991); hGOTP1-1 from human placenta (Reinemer 1992.)]; δ [s GSTS1-1 from squid digestive gland (Ji 1995) and θ [from *Lucilia cuprina* (Wilce 1995), from *Anabidopsis thaliana* (Reinemer 1996), human (Rossjohn et al. ,1998) and a GST from *Schistosoma japanicum* (SjGST) (Lin 1994). Providing the structural basis for investigations of the enzyme active site. A comparison of some structures is provided in **figure 4**.

Although there is low level of sequence identitiy across the classes, all the structures follow a similar folding, with each subunit consisting of two domains of different structure (**Figure 5**). Domain I, the N-terminal domain functions to provide the binding site for glutathione, called as G-site, and a Domain II contains essentially all of the H-site known as xenobiotic substrate binding domain. In addition, it has been also shown that the glutathione binding domain (G-site) is highly conserved (not identical) in all classes.

The N- terminal domain consists of 4β sheets with 3 flanking α-helices (**Figure 5**) .This domain (approx. residues 1-80) adopts a topology similar to that of thioredoxin fold (Wilce 1995). The fold consists of distinct N-terminal and C-terminal motifs which have β α β and β β α arrangement respectively and which are linked by an α-helix (α-2 in **Figure 5**).

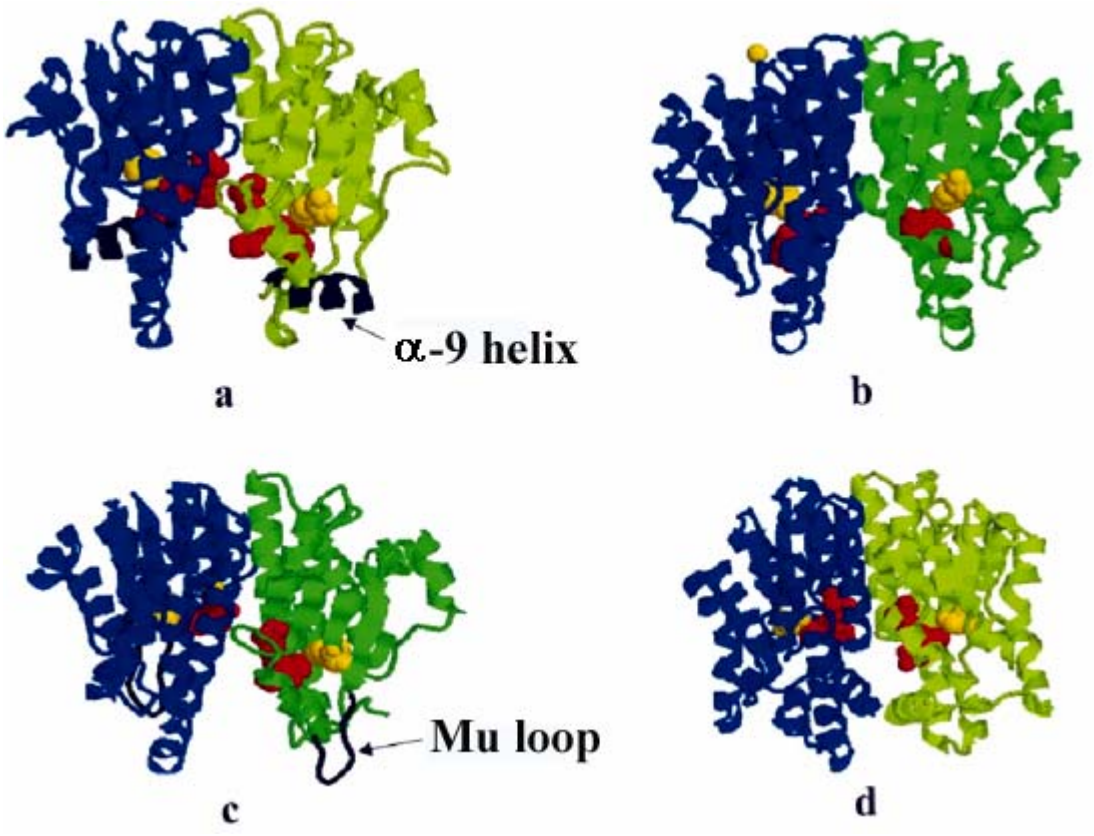


Figure 4: Mammalian GST structures (Sheehan 2001)
Subunits are distinguished by colour (green and blue), and structures are represented to emphasize the relative arrangements around the active site of the right-hand subunit and the inter-subunit cleft. Catalytically essential tyrosine or serine residues are represented in space-filling mode and highlighted in yellow, while the ligand with which the enzyme was co-crystallized is shown in red, identifying the location of the active site. Class-specific features of the Alpha and Mu structures are shown in black. Protein database codes are given in parentheses : (a) human Alpha class (1GUH; [Sinning 1993]) ; (b) human Pi class (1GSS ; [Reinemer 1992]) ; (c) rat Mu class (6GST ; [Xiao 1996]) ; (d) human Theta class (1LJR ; [Rossjohn 1998]).

β α β begins with an N terminal β – strand (β -1), followed by an α- helix (α -1) and then a second β strand (β -2) which is parallel to β -1. A loop region leads into a second α-helix (α -2), which connects with the C terminal motif. This motif consists of two sequential β-strands (β-3 and β-4), which are antiparallel and which are followed by a third α helix (α-3) at the c terminus of the fold. The four sheets are in the same plane, with two helices (α-1 and α-3) below this plane and α-2 above it, facing the solvent. The loop connecting α-2 and β-3 shows characteristic proline residue which is in the less favored cis conformation and is highly conserved in all

GSTs and known as the cis-pro loop. Although it does not play direct role in catalysis, this loop appears to be important in maintaining the protein in a catalytically competent structure. (Allocati *etal* ., 1999). In GSTs, domain I is highly conserved and provides most of the GSH binding site. It is connected to Domain by a short linkert sequence (**Figure 6**).

Domain II (approx. residues 87-210) begins at the C terminus of the linker sequence and consists of five α helices in the case of pi and mu classes. (Ji 1992 and Reinemer 1991) and six α helices in the case of the α class (Sinning *et al*., 1993). The number of helices in domain II varies widely between classes. The C terminal domain is less similar between the three mammalian classes than the N-terminal domain (**Figure 4** and **6**). (Dirr 1994 and Wilce 1995). It contributes most of the residues that interact with the hydrophobic second substrate, as well as contributing a highly conserved aspartic acid residue (occurring in helix α -4) to the GSH binding site. Differences in the C terminal domain may be responsible for the differences in substrate specificity between the three classes (Wilce 1995).

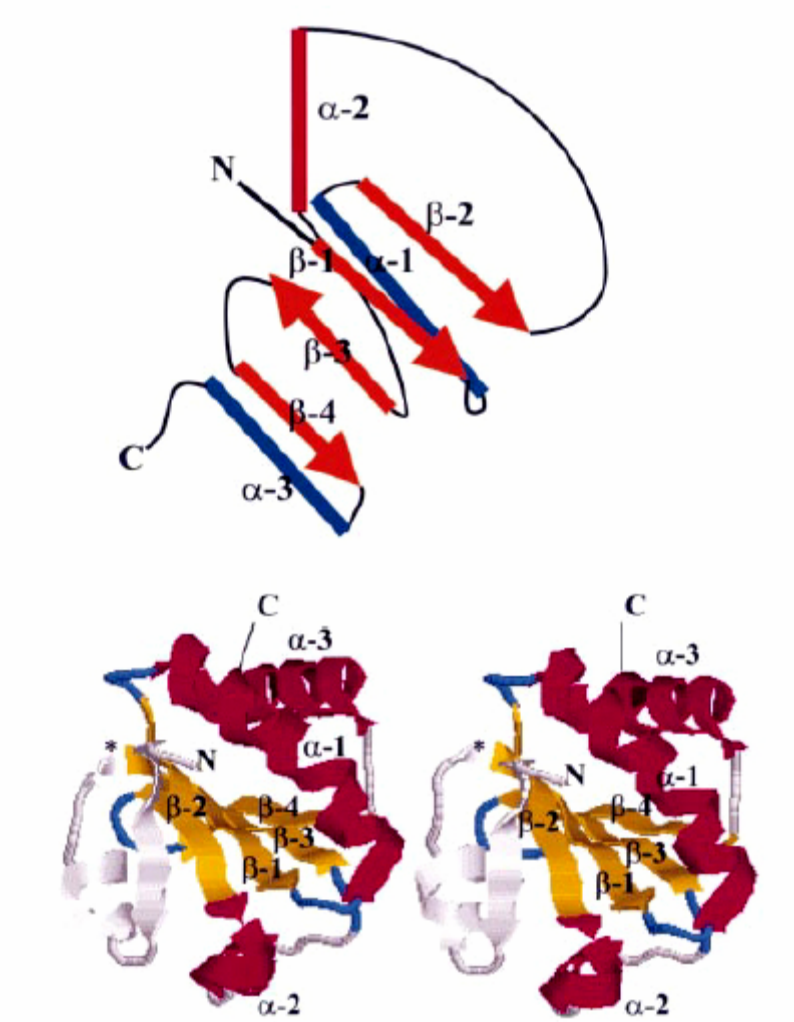


Figure 5: The thioredoxin fold (Sheehan 2001)
A schematic diagram representing the thioredoxin fold is shown above a RasMol depiction of the thioredoxin dimer (Katti 1990). In the diagram, α-helices are shown as cylinders, while β-sheets are shown as orange arrows. The four β-sheets are essentially co-planar, with one helix (α- 2) shown in red above this plane and the other two α-helices (α-1 and α-3) shown in blue below the plane. The cis-Pro loop links α-2 to β-3. In GSTs, domain 2 is connected to the C-terminus by a short linker peptide. In thioredoxin itself, β-sheets are coloured yellow, while α-helices are magenta. The thioredoxin fold has an extra β-sheet and α-helix at the N-terminus (residues 1±21) ending at the point denoted by * where the fold proper begins. These additional N-terminal features are coloured grey.

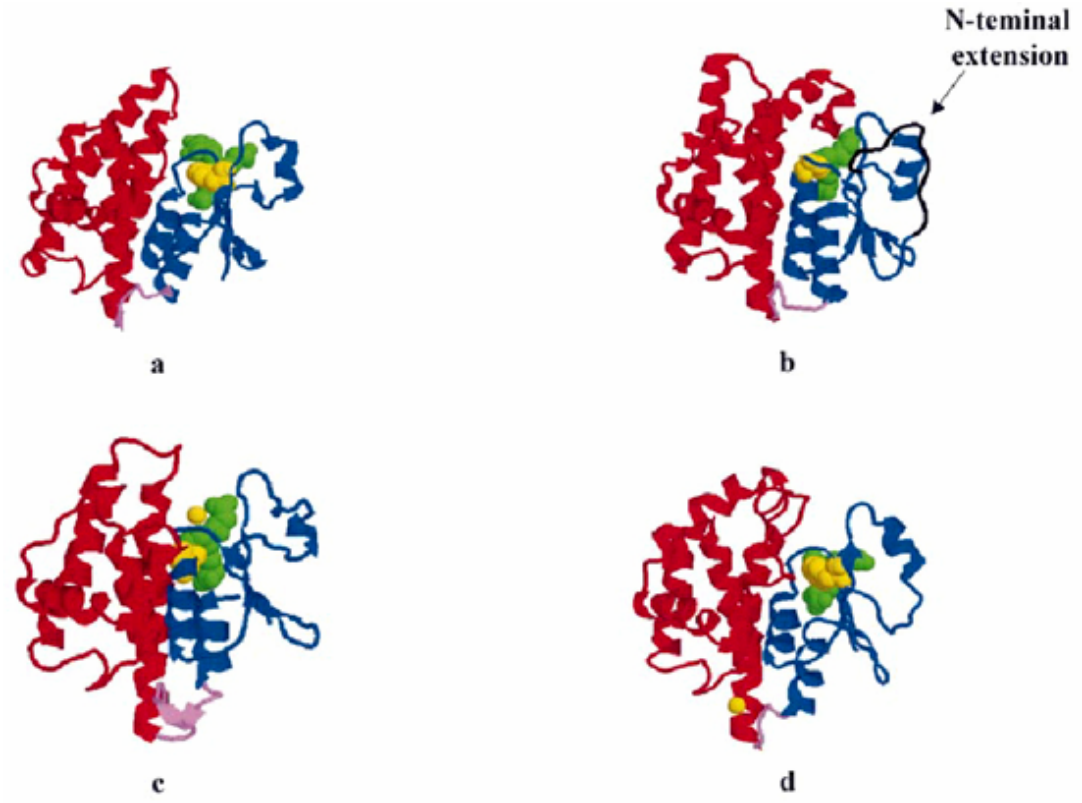


Figure 6: Domain structure of GST subunits (Sheehan 2001).
Three-dimensional structures of individual GST subunits are shown. The N-terminal domain 1 is coloured blue, while the C-terminal domain 2 is red. Catalytically essential residues (tyrosine in a and d; cysteine in b and c) are coloured yellow and presented in space-filling mode, while ligands with which the protein was co-crystallized are shown in green. Linker strands connecting the two domains are shown in violet. Protein database codes and references are given in parentheses : (a) squid Sigma class (1GSQ ; (Ji 1995)) ; (b) human Omega class (1EEM; (Board 2000)) [the C-terminal extension (residues 1±19) unique to this class is shown in black] ; (c) bacterial (Proteus mirabilis) Beta class (1PM7; [Rossjohn J., *et al*., 1998]) ; (d) Fasciola hepatica Mu class (1FHE ; [Rossjohn J., *et al*., 1997]).

It has been observed that GSH binds to the enzyme with three different modes; Class μ GSTs and SJ GST share a common GSH binding mode with the cysteinyl carbonyl hydrogen bonded to the indonyl nitrogen of Trp7. (Ji 1992, Lin 1994). In class α, π, and σ of GSTs the cystenyl carbonyl makes hydrogen bond with a backbone amide group of Phe 8. (Sinning 1993, Reinemer 1991 and Ji 1995). A third binding mode of GSH is presented by class theta GSTs, although an invariant tyrosine residue forms a hydrogen bond with the cysteinyl sulfur in class α, μ, π, σ and SJ GSTs, in class θ GSTs, however, instead of the invariant tyrosine residue nearby serine or another tyrosine residues located in the C-terminal domain of the enzyme interact with the sulphydryl group of GSH. These results suggested the separation of the soluble GSTs into two major subfamilies characterized by either serine residue (ser 9) (Theta and insect Delta classes; the *L.cuprina* GST was presumably termed a theta or theta like GST and has recently been reclassified as a member of the insect Delta classes (Board 1997) or a " Tyr" residue (Tyr 8 in α, Tyr 6 in μ, Tyr 7 in π and Sigma classes) as the key residue in GSH activation. A conserved G-site aspartate (Asp101 in class **Alpha**, Asp105 in class **Mu**, Asp98 in class **Pi**, Asp96 in class **Sigma**) is also involved in catalysis by aiding proton release from certain transition-state conjugates, occurs for example during conjugation between CDNB and GSH (Widersten *et.al.,* 1992; Kolm *et.al.,* 1992).

Although GSH binding site is very well defined for these cytosolic classes of GSTs, only a general description of the H-Site is available, primarily because for most GST enzymes there are no H-site defining product complex structures.

In their biologically active form, cytosolic GSTs are either homodimers or heterodimers in which each subunit functions independently. The two subunits contact each other primarily by ball-and-socket hydrophobic interaction established by wedging the side chain of Phe residue (Ball) (Phe 52 α; Phe 56 μ; Phe 47 π) from domain I of one monomer, into a hydrophobic socket of domain II of its partner monomer of the class α, μ, and π enzymes.

This particular interaction is not observed in the class sigma and theta enzymes due to the absence of Phe residue and the hydrophobic socket between the helices 4 and 5 (Armstrong 1997). The most conserved reagion of structure in all of the cytosolic enzymes is a core ββα motif that is responsible for recognition of the γ- glutamuyl portion of the peptide (**Figure 7)** The cis prolyl residue that precedes this reagion helps to conserve the overall fold of the domain. The only other highly conserved interaction is between the carbonyl and NH groups of the cysteinyl residue and the main chain of the protein just preceding the conserved cis-prolyl residue. The similarity in this region of structure for the two most divergent classes of cytosolic enzyme, theta and alpha, is obvious and much more highly conserved (**Figure 7)** than are the interactions between the proteins and sulphur of GSH (**Figure 8).** The most fundamental difference among the GSH binding sites of the various enzyme classes involves the interaction of protein with the sulfur of the peptide (**Figure 8).**

The theta class enzymes thought to be the evolutionary precursor of the alpha, mu, pi, and sigma class proteins, utilizes the hydroxyl group of a serine residue located near the N-terminus of the polypeptide to activate the sulphydryl group of bound GSH. Whereas, the class alpha, mu, pi, and sigma enzymes have hydroxyl group of tyrosyl residue in a slightly different position to act as a hydrogen bond donor to the sulphur which lowers the pKa of the thiol in the E.GSH complex so that it is ionized at physiological pH. In class alpha additional residue except from seryl or tyrosyl group gathers additional stabilization from its positive charge of Arg15 (Armstrong 1997).

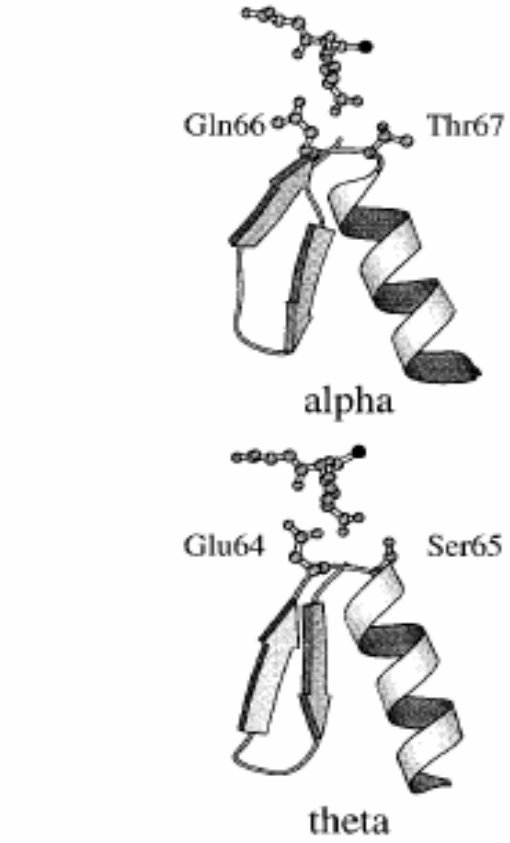


Figure 7: Representation of the highly conserved core ββα motif which is responsible for the recognition of the γ-glutamyl residue of GSH. Glutathione and the side chains located at the turn between the β4-strand and α3-helix involved in hydrogen-bonding interactions with the α-amino and α-carboxyl moieties of the γ-glutamyl residue are illustrated in ball-and-stick. The two motifs represented are from what are considered to be the most divergent classes of cytosolic GSH transferases, the theta and alpha classes (Armstrong 1997).

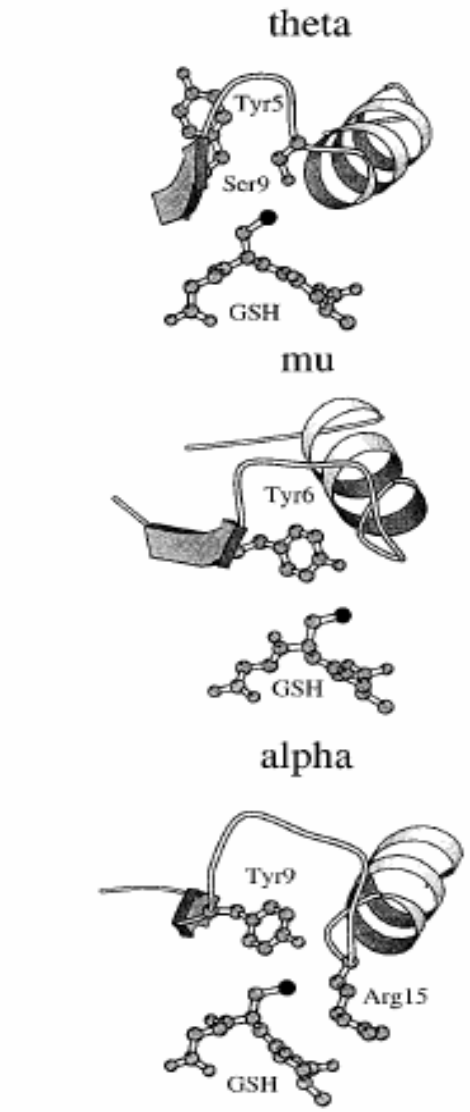


Figure 8: Evolution of first-sphere interactions between the active sites of class theta, mu, and alpha enzymes and the sulfur of GSH. It is possible that the tyrosine residue often, but not always, found near the N-terminus of the class theta enzymes was recruited in the evolution of the protein to the other classes. The most recently evolved enzyme, class alpha, has an additional residue (Arg15) conscripted into the first sphere of the sulfur of GSH (Armstrong 1997).

Three membrane bound glutathione transferases are known, one of which appears to be involved in xenobiotic mechanism. Microsomal GSH transferase I is an integral membrane protein that has been characterized from both rats and humans where it is found in large amounts in liver and is distributed in both microsomal and outer mitochondrial membrane. This protein has no relationship with any of the known cytosolic enzymes with respect to sequence. However, it shows some similarity to two other membrane bound GSH transferases, leukotrienes C_4 synthase and micosomal GSH transferase II, in which the three proteins are about the same size, share a small amount of sequence identity and are all membrane bound. There is no so much information about the xenobiotic mechanisms of microsomal GSTs.

A proposed membrane topology for the microsomal enzyme is illustrated in **figure 9** (Armstrong 1997)**.** Each subunit consists of five membrane spanning regions, at least two of which are likely to be α-helices perpendicular to the membrane surface. The N terminus of the enzyme appears to be located on the luminal side of the endoplasmic reticulum while the C- terminus and the active site face the cytosol. The overall topology is most simply described as a N terminal in, C-terminal out orientation with five membrane spanning regions. Although it is unclear what the GSH-binding motif looks like, the enzyme lowers the pKa of the thiol of bound GSH. as in the case of all cytosolic GSTs.

1.4 GSTs Catalyzed Reactions and Substrates:

1.4.1 Glutathione Conjugation and Detoxification:

All GSTs have the ability to conjugate GSH with compounds containing an electrophilic center.

The electrophilic functional group for conjugation reactions can be provided by a carbon, a nitrogen, or a sulfur atom. Such groups are present in arene oxides, aliphatic and aromatic halides, α,β- unsaturated carbonyls, organic nitrate esters, organic thiocyanates, olefins, organic peroxides, quinines and sulfate esters (Mannervik 1985, Mannervik and Danielson 1988). The range of compounds that contain electrophilic center is extremely large and includes the parent chemical or metabolite of the carcinogens (Table 2) benzo[a]pyrene, 5-methylcrysene, aflatoxin B1, 7,12-dimethybenz[a]anthracene, and 4-nitroquinoline-N-oxide (**Figure 10**).

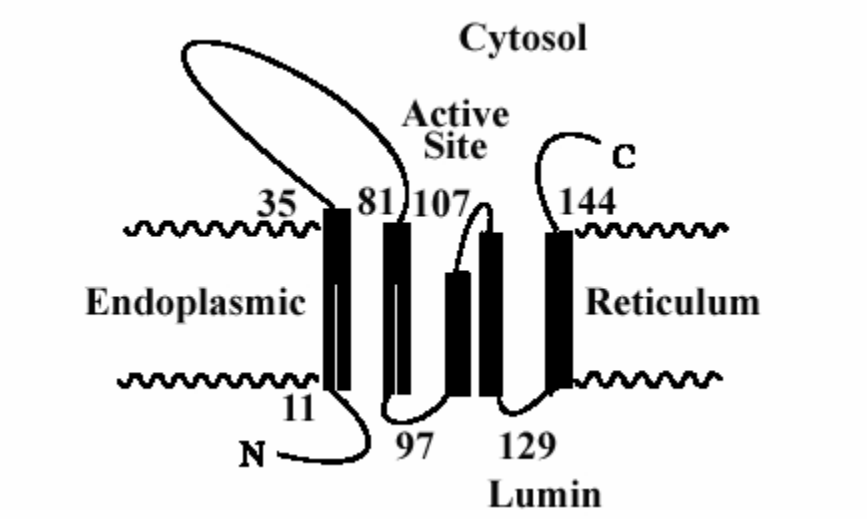


Figure 9: A proposed membrane topology for the microsomal GSH transferase subunit derived from sites of proteolysis, chemical modification and mutagenesis (Andersson 1994). Experimental evidence and hydropathy analysis indicate that there are at least three membrane spanning reagions: residues 11-35,81-97, and 129.Two of the membrane spanning reagions are thought to be α-helices (Hebert 1995).

Table 3: Examples of GSTs Substrates from Different Compound Categories (Hayes and Pulford, 1995).

Compound Type	Substrates
Metabolites of Carcinogens	Aflatoxin B_1-8,9-epoxide Benzo[*a*]pyrene-7,8-diol-9,10-oxide 5-hydroxymethylchrysene sulfate 7-hydroxymethylbenz[*a*]anthracene sulfate 4-nitroquinoline *N*-oxide
Pesticides	Alachlor Atrazine Dichlorodiphenyltrichloroethane (DDT) Lindane Methyl parathion
Oxidative-damage products	Acrolein Base propenals Cholesterol α-oxide Fatty acid hydroperoxides 4-hydroxynonenal
Anticancer drugs	1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU) Chlorambucil Cyclophosphamide Melphalan Thiotepa Fosfomycin

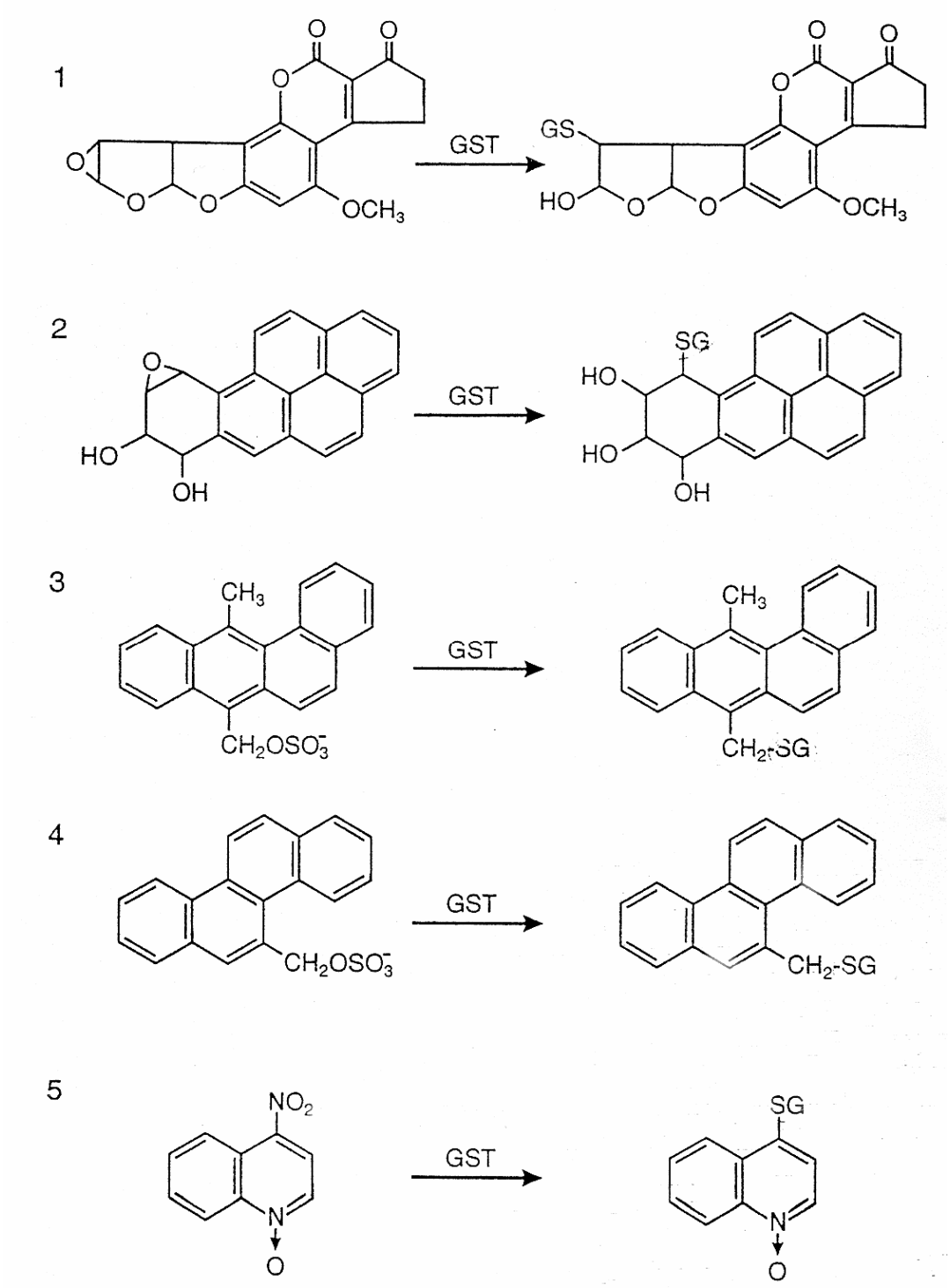


Figure 10 . Detoxification of carcinogens by GSTs. The following reactions are catalyzed by GST: (1) aflatoxin B_1-8,9-epoxide; (2) benzo[a]pyrene-7,8-diol-9,10-oxide;(3) 7-hydroxymethylbenz[a]anthracene sulfate; (4) 5-hydroxymethyl-chrysene sulfate; (5) 4-nitroquinoline *N*-oxide (Hayes and Pulford, 1995).

GST also detoxifies the pesticides, alachlor, atrazine, dichloro-diphenyltrichloroethane (DDT), lindane, and methyl parathion (**Figure 11**), the oxidative-damage products acrolein, base propenals, cholesterol α-oxide, fatty acid hydroperoxides, and 4-hydroxynonenal (**Figure 12**), the anticancer drugs chlorambucil, melphalan, thiotepa, 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU), and cyclophosphamide, as well as the antibiotic fosfomycin (**Figure 13**).

As a result of the conjugation reaction between electrophiles and GSH catalyzed by GSTs a conjugate that is less reactive than the parental compound is formed and the solubility of hydrophobic xenobiotics is increased. Another biological value of GSH conjugation is providing a molecular "flag", which signals export of the conjugate from the cell by ATP-dependent glutathione *S*-conjugate efflux pumps (Phase III) (Ishikawa, 1989 and 1992; Saxena *et.al.,* 1992).

The conjugation reaction between GSH and xenobiotics represents the first step in the synthesis of mercapturic acids, an important group of excretion products that were first identified more than 100 years ago in the urine of animals treated with bromobenzene. In that reaction, after the conjugation with GSH, the glutathione conjugate is converted to the corresponding cysteine conjugate following sequential removal of glutamate and glycine. Cysteine conjugate then is either metabolized to a mercapturate by acetylation or cleaved to a mercaptan by β-lyase (C-S lyase). In addition to the mercapturic acid pathway, methylation of the thiol to form the methylthio-containing metabolite and the glucuronidation of the mercaptan to form the thioglucuronide represent important metabolic steps for the biotransformation of the cysteine conjugate. (Boyland and Chasseaud, 1969; Pickett and Lu, 1989) (**Figure 14).**

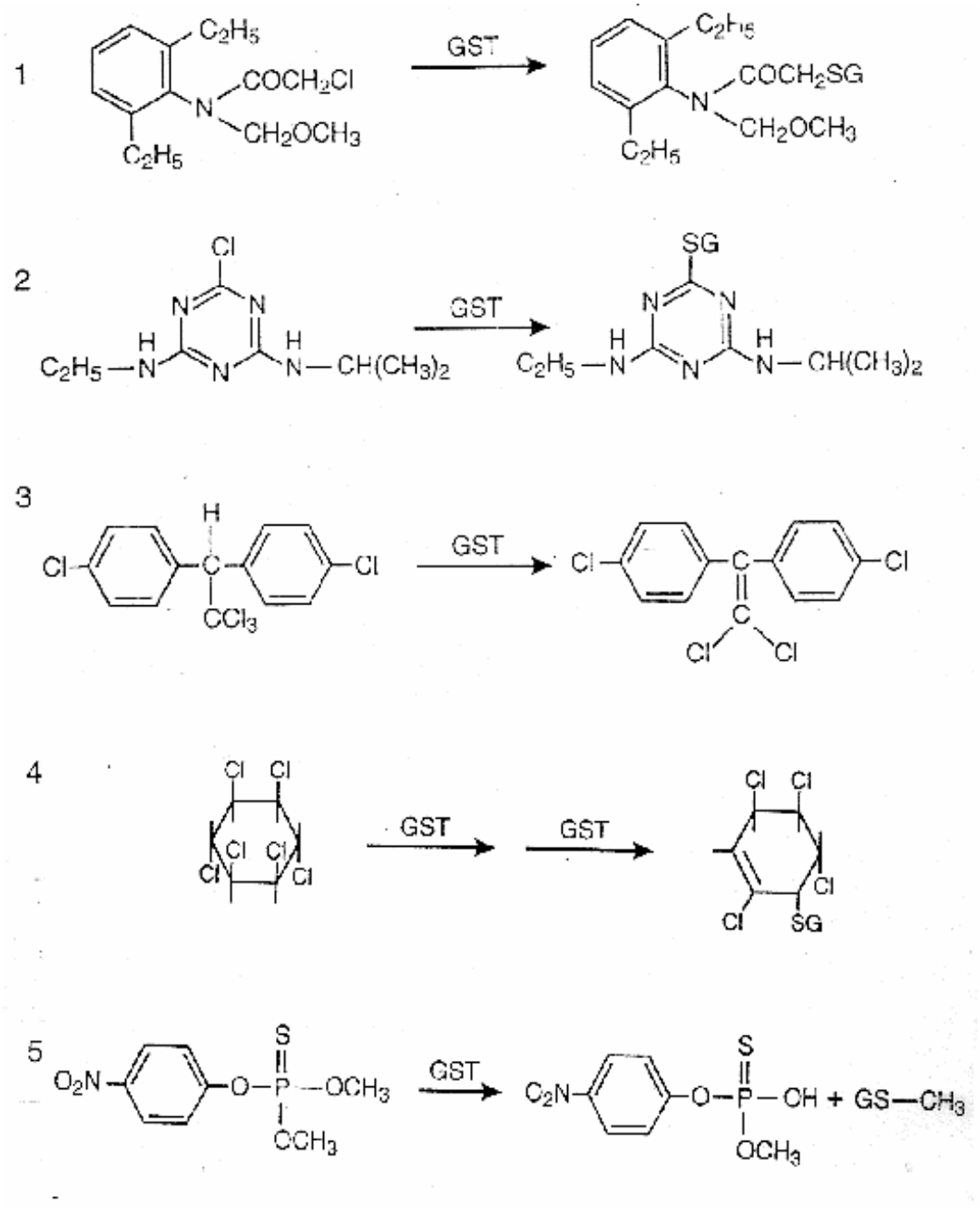


Figure 11: Metabolism of pesticides by GSTs: (1) alachlor; (2) atrazine; (3) DDT; (4) lindane; (5) methyl parathion (Hayes and Pulford, 1995).

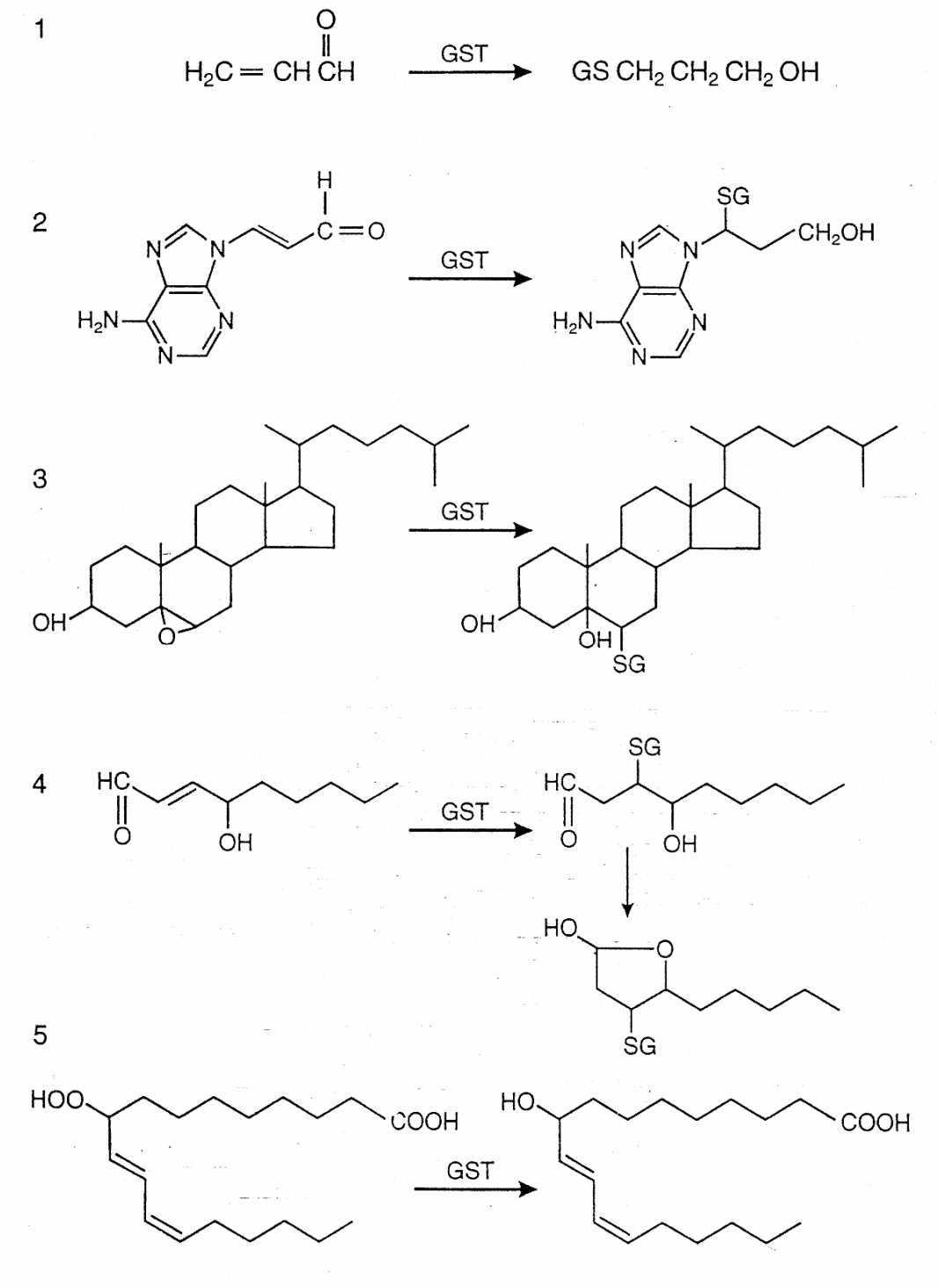


Figure 12: Examples of GST substrates that are produced by oxidative stress: (1) acrolein; (2) adenine propenal; (3) cholesterol-5,6-oxide; (4) 4-hydroxynon-2-enal (5) 9-hydroperoxy-linoleic acid (Hayes and Pulford, 1995).

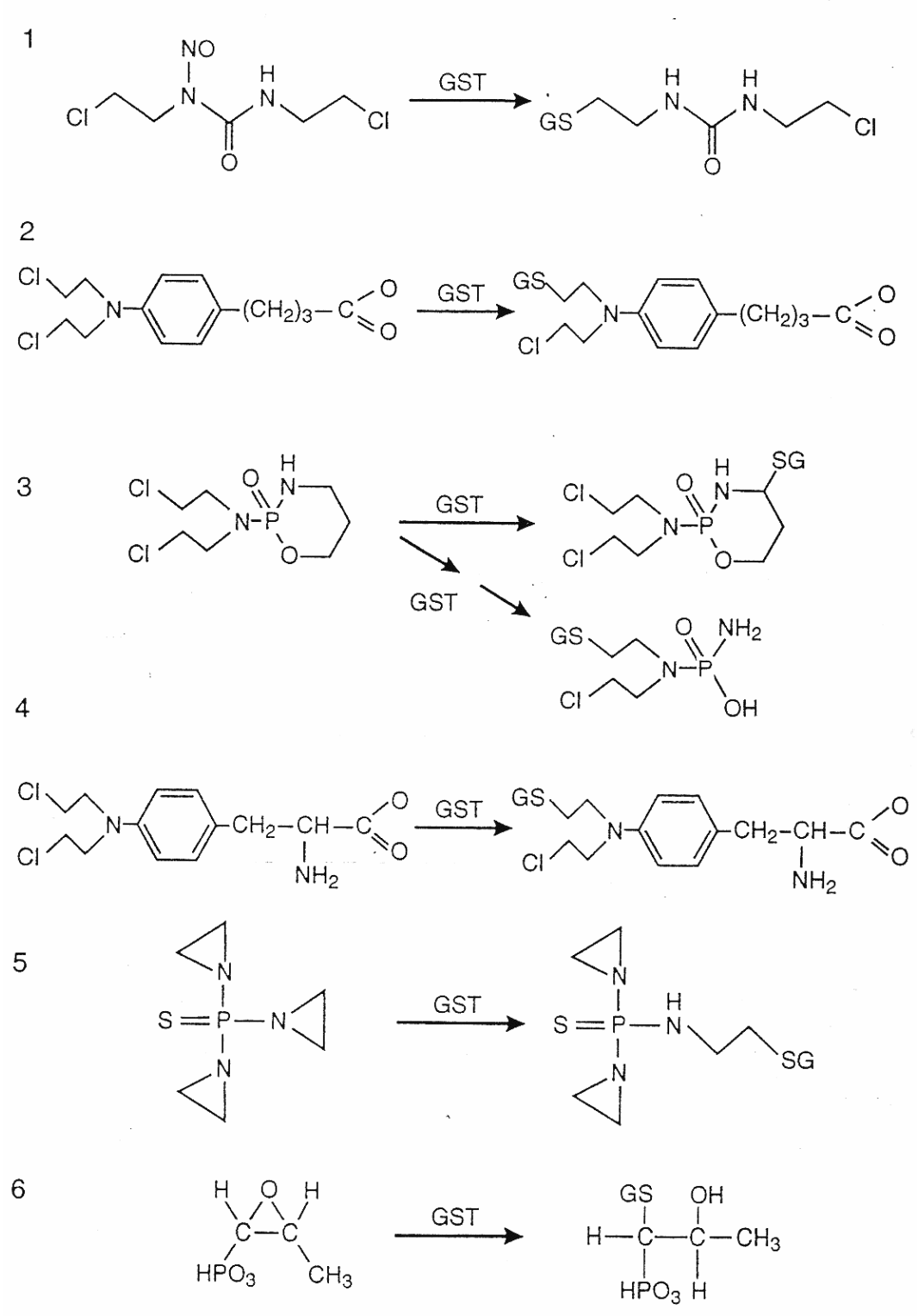


Figure 13: Examples of chemotherapeutic agents that are GST substrates: (1) BCNU; (2) chlorambucil; (3) cyclophosphamide; (4) melphalan; (5) thiotepa; (6) fosfomycin (Hayes and Pulford, 1995).

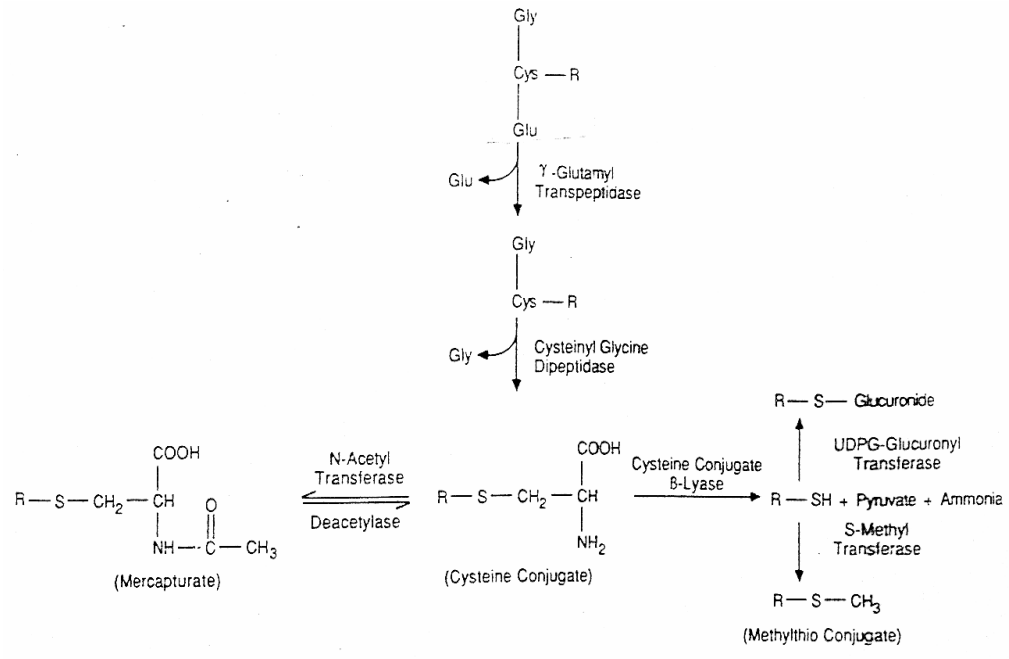


Figure 14: Metabolism of glutathione conjugates (Pickett and Lu, 1989)

1.4.2 Glutathione Conjugation and Toxification :

Although most of the GSH conjugates show detoxification products, several instances exist in which GST activity does not result in the detoxification of xenobiotics. For example, a few GSH conjugates are relatively unstable and the reaction product is either cleaved to liberate an unconjugated metabolite that requires further detoxification , or the reaction is reversible allowing regeneration of the original electrophile.

A potentially more serious situation can arise with a small number GST substrates that yield aGSH conjugate, or a metabolite of the conjugate, that is more reactive than the parental compound:These two groups of compounds have been referred to as directly acting toxic GSH conjugates and indirectly acting toxic GSH conjugates.

Directly acting toxic GSH conjugates are formed from a number of alkyldihalides (Rannug 1978) as in the case of dihaloethanes and dihalomethanes toxification. The conjugates from dihaloethanes may rearrange spontaneously prior to interaction with DNA, but those formed from dihalomethanes, probably do not rearrange (**Figure 15**). GST- catalyzed reactions between 1,2 dihaloalkenes and GSH may yield S-(2-haloalkyl) glutathiones, which can form episulfonium ions intermediates are potent electrophiles and act as alkylating agents (Van Bladeren 1979) . In the case of dihalomethanes the s-methylglutathione conjugate appears to be the ultimate mutagen.

Indirectly acting toxic GSH conjugates are formed from various halogenated alkenes and alkynes. They include hexachlorobutadiene, tetrachloroethane, trichlorotrifluoropropane, and dichloroacetylene (**Figure 16**), all of which are nephrotoxic and also possibly nephrocarcinogenic.(Dekant 1994) These compounds are all preferentially metabolized by membrane bound GST, rather than by the soluble GST (Andersson *et.al.,*1994).

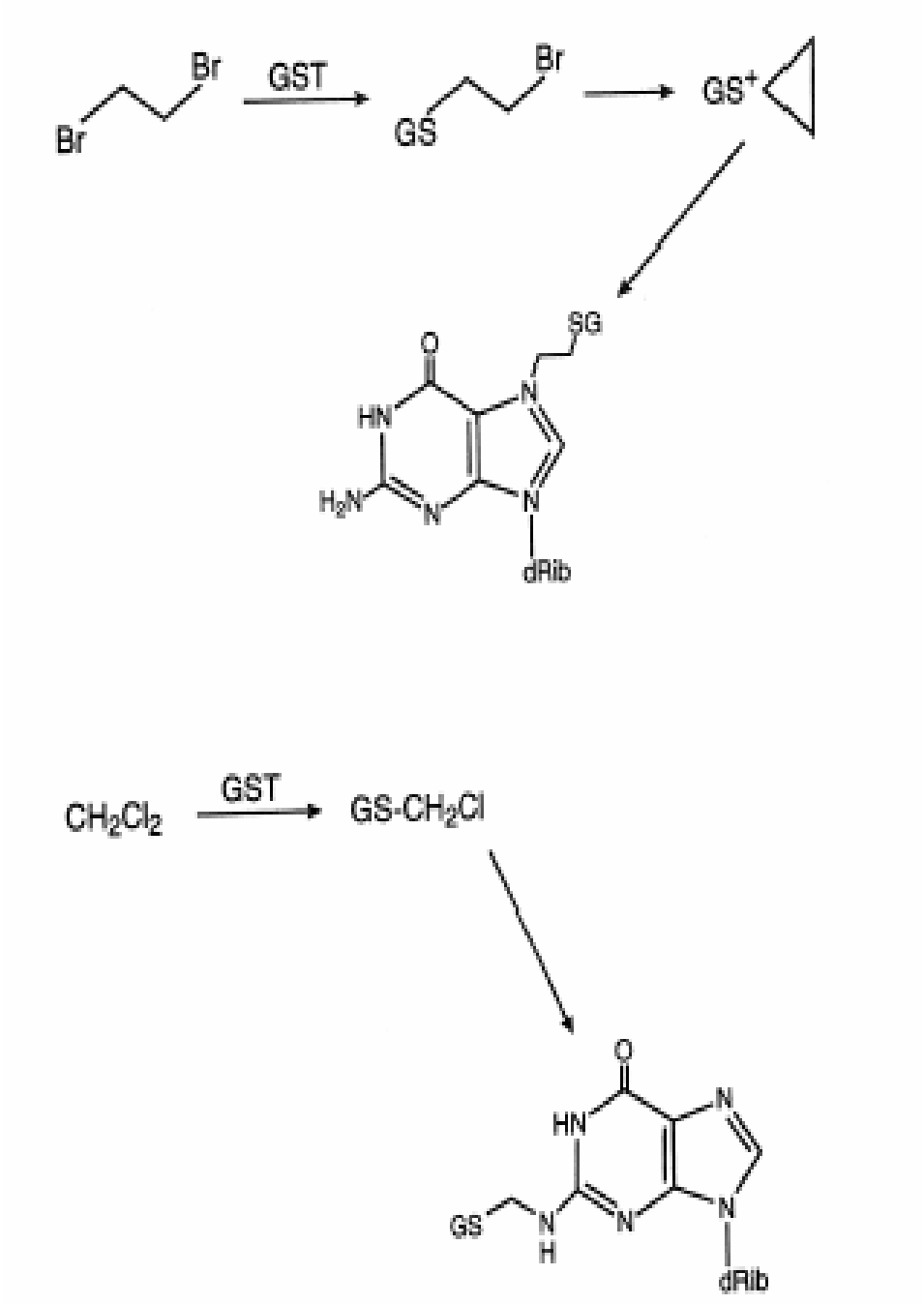


Figure 15: Reaction between GS conjugates formed between (1) ethylene dibromide and DNA and (2) methylene chloride and DNA.

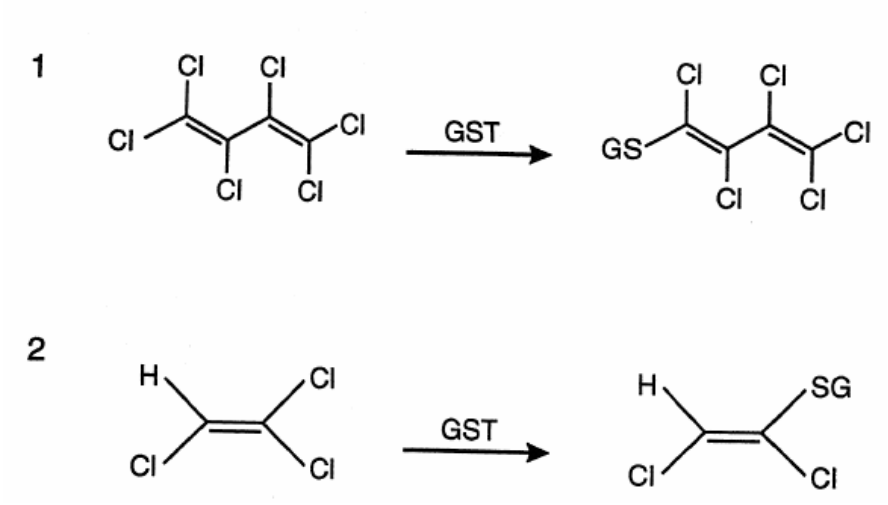


Figure 16: Examples of compounds that form indirectly acting toxic GSH conjugates: (1) hexachloro-1,3-butadiene; (2) trichloroethene. (Hayes and Pulford 1995).

1.4.3 Peroxidase Activity of GSTs

Although it is known that GSTs catalyze the formation of thioether bond between GSH and electrophilic xenobiotics, a significant number of the GST isoenzymes also exhibit glutathione peroxidase activity and catalyze thereduction of organic hydroperoxides to their corresponding alcohols. This type of reaction is thougth to represent nucleophilic attack by GSH on electrophilic oxygen. It is involved in two steps, only one of which is catalytic, and to proceed via formation of the sulfenic acid of glutathione as follows;

i. ROOH + GSH ⟶ ROH + [GSOH]……enzymatic

ii. [GSOH] + GSH ⟶ GSSG + H2O……..spontaneous;

to give the overall reaction,

iii. ROOH + 2 GSH ⟶ ROH + GSSG + H2O

The substrates that GST reduce include fatty acid, phospholipid and DNA hydroperoxides. As these compounds are generated by lipid peroxidation, and oxidative damage to DNA, it has been proposed that GST, as well as other GSH dependent enzymes help combat oxidative stress (Mannervik 1986). An important difference exist between the membrane bound (microsomal) GST and cytosolic GST in their respective roles in protection against reactive oxygen species (Mosialou *et.al.,*,, 1993).

1.4.4. Isomerase Activity of GST

In addition to their role as detoxication enzymes, the GSTs have been suggested to be involved in different facets of biological signalling such as synthesis of various prostaglandins, in the 5-lipoxygenase pathway, and in the interactions with protein kinases of signal transduction systems (Johansson 2002). GST A3-3 is the most recent example of GSTs with a connection to biological signalling (Johansson 2001). GST A3-3 efficiently catalyzes double-bond isomerizations of Δ^5- androstene 3,17 dione (Δ^5-AD) and of Δ^5-pregnene-3,20-dione, intermediates in the biosynthesis of the steriod hormones progesterone and testosterone (Johansson 2001) (**Figure 17).** There are other isomerization reactions catalyzed by GSTs. The cis-trans conversion of retinoic acid is a GSH independent reactions catalyzed by GSTP1-1 (Chen *et al*., 1998).

Zeta class GSTs are involved in the catabolic pathway of tyrosine and phenylalanine by catalyzing the GSH-dependent cis trans isomerization of maleyacetone to fumaryacetone and malay aceto acetic acid to fumarylaceto acetic acid (**Figure 18**). An even smaller number of GST isoenzymes possess ketosteroid isomerase activity and catalyze the conversion of delta 5-3 ketosteroids to delta 4- 3-ketosteroids. The physiological significance of these isomerization of maleylacetoacetic acid occurs in the pathway of tyrosine degredation in mamallian liver (Keen 1978) (**Figure 18**).

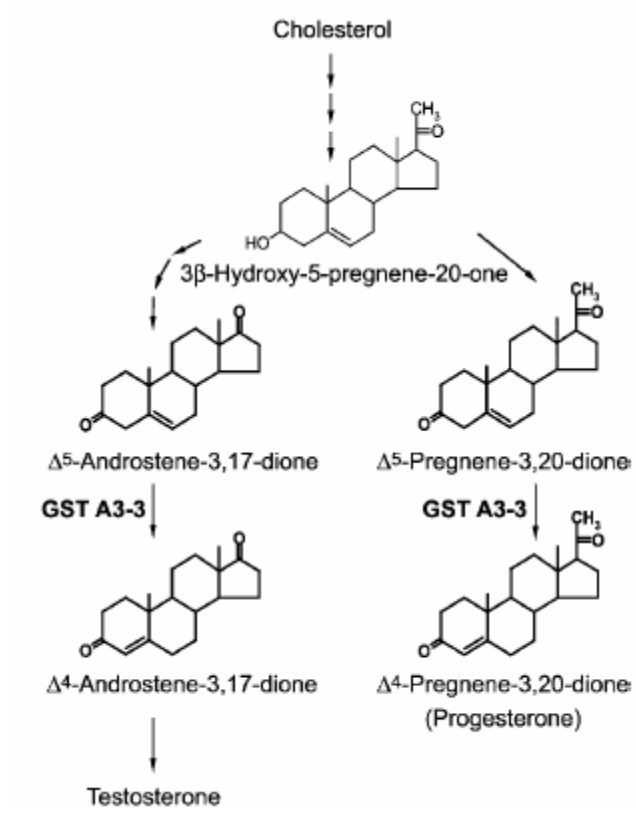


Figure 17: Two biosynthetic pathways leading to the steroid hormones testosterone and progesterone. The obligatory Δ^5- Δ^4 isomerization of the 3-ketosteroids is catalyzed efficiently by human glutathione transferase A3-3 present in steroidogenic tissues (Johansson 2001).

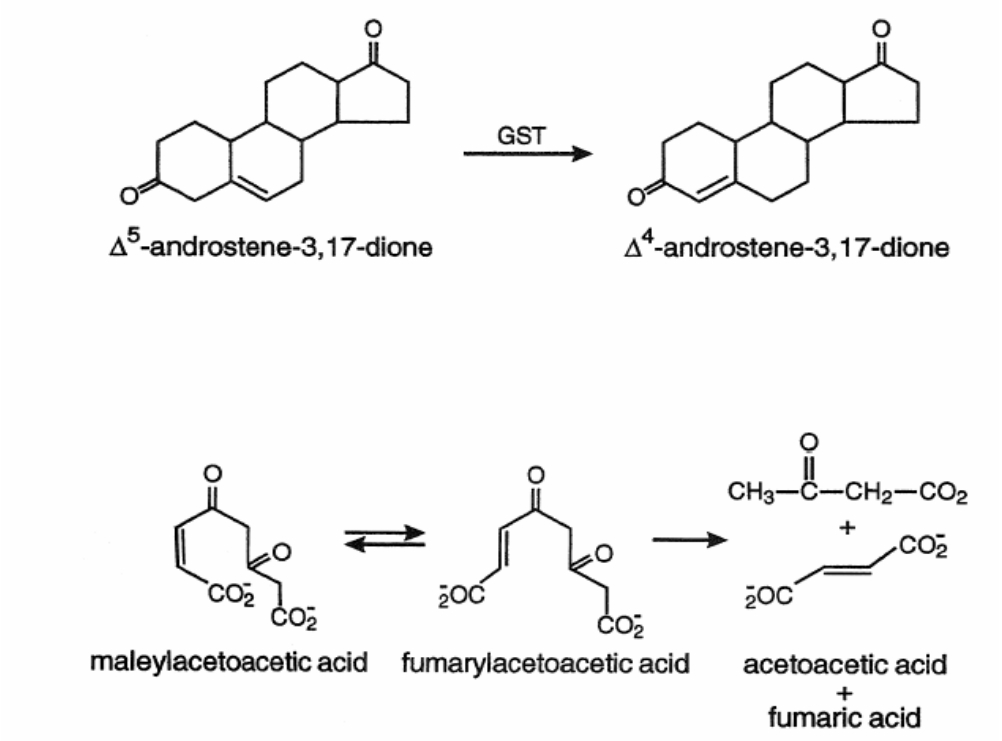


Figure:18 : Isomerization of Δ 5- androstene-3,17-dione and maleylacetoacetic acid, both of which are catalyzed by GST (Hayes and Pulford 1995).

1.4.5 Model Substrates for the Characterization of GST Isoenzymes

The GST isoenzymes display marked differences in their abilities to conjugate GSH with various electrophiles. 1-chloro-2,4-dinitrobenzene (CDNB) is known as the universal substrate for GSTs since it is used for the demonstration of multiple forms of GSTs in various biological species.

When conjugated with GSH it gives *S*-(2,4-dinitrophenyl) glutathione, a compound possessing an absorbance spectrum sufficiently different from that of CDNB to allow a simple spectrophotometric assay at 340 nm (Habig *et.al.,* 1974).

Although CDNB is known as a general substate for GSTs it is also known that certain forms of the enzyme express low activity with this substrate. Class **Theta** GST isoenzymes are examples of the forms that was shown not to display any activity with CDNB and are best characterized by other substrates like 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), 4-nitrobenzyl-chloride (4-NBC), and 4-nitrophenylbromide (4-NPB) for class **Theta** GSTT1-1 (Meyer *et.al.*, 1991) and 1-menaphthyl sulfate (MS) for class **Theta** GSTT2-2 (Hussey and Hayes, 1992). Although different GST isoenzyme forms in the same tissue display overlapping substrate specificities, the activity profiles are, in most of the cases, clearly distinct (Clark *et.al.*,., 1973; Habig and Jakoby, 1981). Reactions of the model substrates, that have been proved to be useful in classifying the GST isoenzymes, are shown in **Figure 19**.

The model GSTs substrates that display selectivity for particular subunits are often used in a "diagnostic" sense to identify GST isoenzymes. The catalytic activities of rat and human GST isoenzymes are given in **Table 4** and **Table 5,** respectively (Hayes and Pulford, 1995).

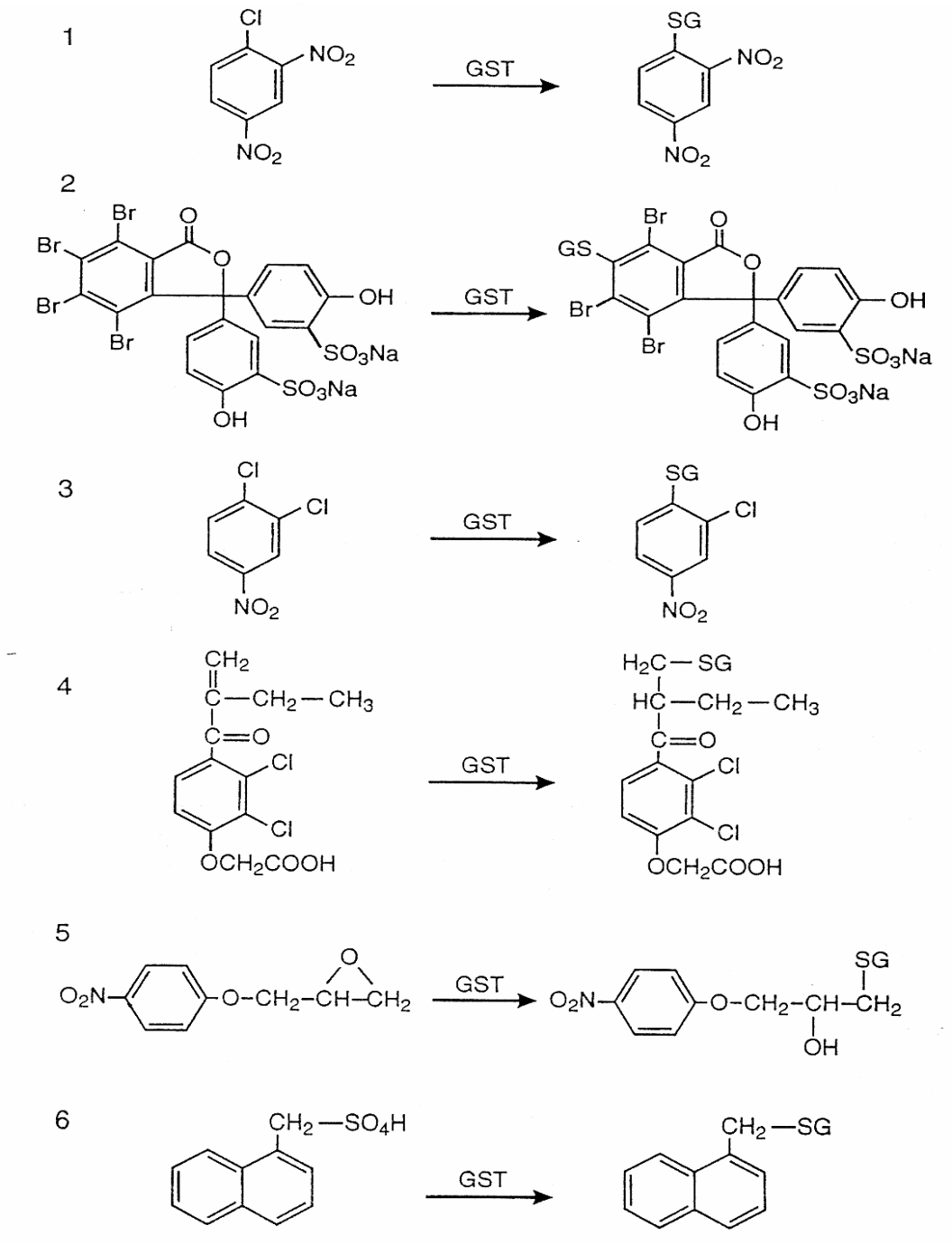


Figure 19: Model substrates used for characterization of GSTs: (1) CDNB; (2) bromosulfophthalein; (3) DCNB; (4) ethacrynic acid; (5) EPNP; (6) 1-menaphthyl sulfate; (7) 4-NBC; (8) 4-nitrophenylacetate; (9) 4-NPB; (10) trans-4-phenyl-3-buten-2-one; (11) styrene-7,8-oxide; (12) cumen hydroperoxide (Hayes and Pulford, 1995).

Figure 19 (continued)

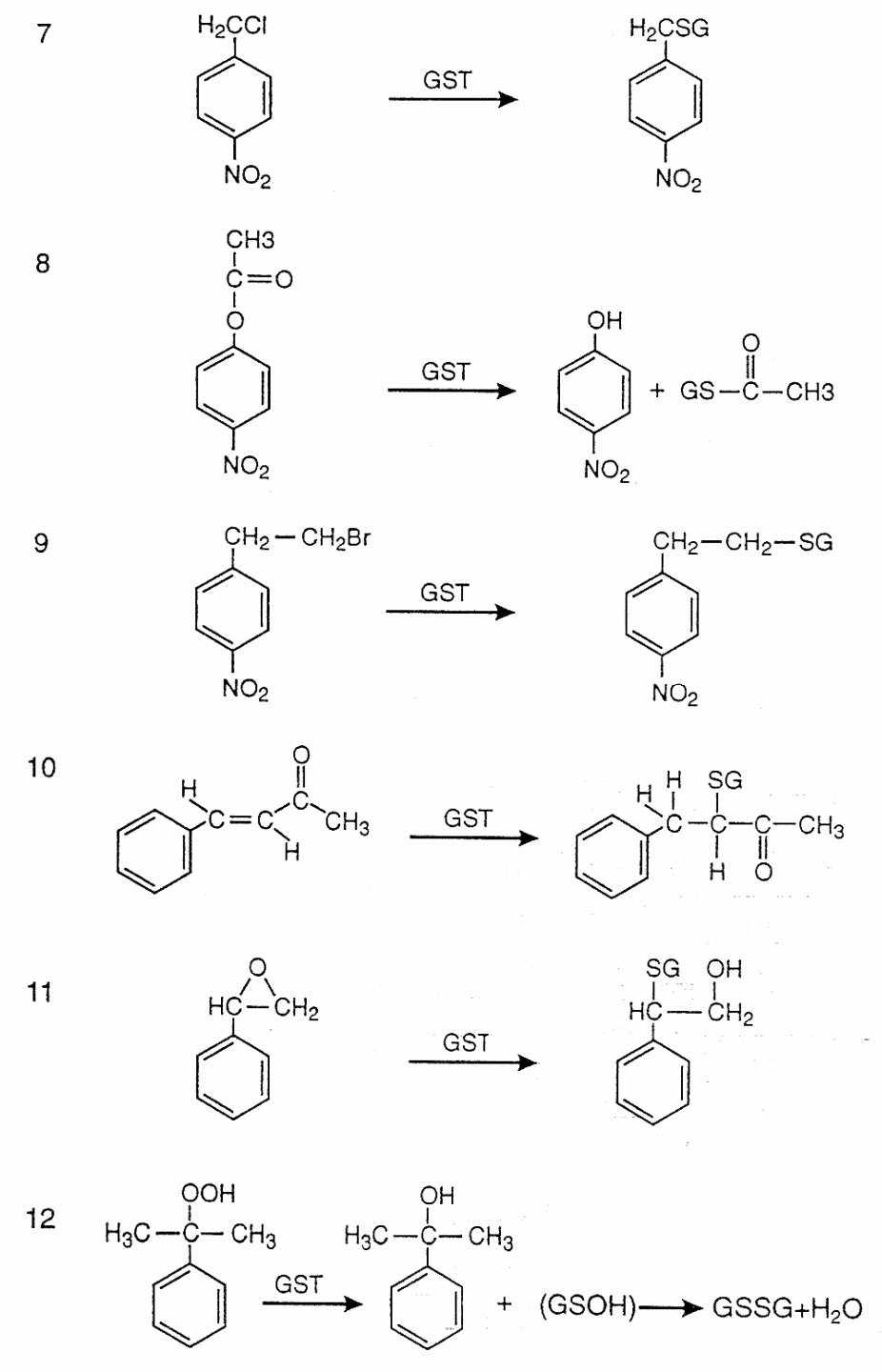


Table 4. Catalytic Activities of Rat GST Isoenzymes (Hayes and Pulford, 1995).

Isoenzyme	Specific Activity (μM/min/mg protein)														
	CDNB	**Δ^5AD**	**BSP**	**DCNB**	**EA**	**EPNP**	**4-HNE**	**MS**	**4-NBC**	**4-NPA**	**4-NPB**	**tPBO**	**SO**	**CuOOH**	**LiOOH**
rGSTA1-2	50.0	2.3	0	0	0.1	<0.1	2.6	–	1.1	0.8	–	0	3.3	3.4	3.0
rGSTA1-3	25.0	0.9	0	0	0.5	<0.1	1.6	–	–	0.3	–	0	–	8.9	–
rGSTA2-3	25.0	0.9	0	0	0.5	<0.1	1.6	–	–	0.3	–	0	–	8.9	–
rGSTA3-3	18.0	<0.01	0	0	1.2	<0.1	0.7	–	0.3	0.2	–	0	3.3	13.7	1.6
rGSTA3-5	14.0	<0.01	–	0	0.8	–	0.4	–	–	–	–	0.005	–	15.5	–
rGSTA4-4	10.0	0.06	0	0.1	7.0	–	170.0	–	1.1	0.1	–	0.1	–	1.1	0.2
rGSTM1-1	58.0	0.02	0.8	5.3	0.1	0.5	2.7	–	14.0	1.0	–	0.1	133.0	0.4	0.2
rGSTM1-2	45.0	0.01	0.3	3.2	0.3	0.9	2.9	–	–	0.6	–	0.6	–	0.5	–
rGSTM2-2	17.0	0.002	0.04	0.2	0.6	1.4	6.9	–	14.0	0.3	–	1.2	104.0	0.7	0.2
rGSTM1-3	64.0	–	0.7	4.9	0.5	<0.1	–	–	–	0.6	–	0.2	–	<0.2	–
rGSTM2-3	45.0	–	–	2.3	0.6	–	0	–	–	–	–	0.6	–	0.45	–
rGSTM3-3	190.0	–	–	2.9	0.06	–	–	–	–	0.2	–	0.02	–	0.19	–
rGSTM3-5*	170.0	–	0	2.4	0	0	–	–	1.7	0.05	–	0.2	–	0.04	0.06
rGSTM6*-6*	30.0	–	–	0.3	0	0.1	–	–	0.5	–	–	–	–	–	–
rGSTP1-1	19.0	–	0.02	0.2	4.2	0.1	1.6	–	–	–	–	0.05	55.0	0.06	1.5
rGSTS1*-1*	70.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rGSTT1-1	<0.5	–	–	–	–	180.0	–	–	86.0	–	65.0	–	–	41.0	–
rGSTT2-2	<0.1	–	–	0	0.4	<0.1	–	0.4	0	0	–	0.02	–	2.0	9.7
rGSTT2-2´	<0.1	–	–	0	1.6	<0.1	–	0.4	0	0	–	–	–	2.0	–
rGSTT2´-2´	<0.1	–	–	0	2.5	<0.1	–	0.4	0	0	–	–	–	2.0	0.9

Table 5: CatalyticActivities of Human GST Isoenzymes (Hayes and Pulford, 1995).

Isoenzyme	Specific Activity (μM/min/mg protein)														
	CDNB	**Δ^5AD**	**BSP**	**DCNB**	**EA**	**EPNP**	**4-HNE**	**MS**	**4-NBC**	**4-NPA**	**4-NPB**	**tPBO**	**SO**	**CuOOH**	**LiOOH**
hGSTA1-1	82.0	4.0	–	0.25	0.1	0	–	–	–	0.7	–	0	0.02	3.1	–
hGSTA1-2	–	–	–	0.8	–	–	–	–	–	–	–	0	–	9.2	–
hGSTA2-2	80.0	–	–	0.9	0.1	0	–	–	–	0.2	–	0	–	10.4	–
hGSTA4-4*	12.5	–	0.07	0.91	2.8	2.4	168.0	–	0.6	–	–	0.03	–	0.6	0.3
hGSTM1a-1a	190.0	0.12	0	0	0.1	0.1	3.3	–	2.7	0	–	0.21	–	0.3	–
hGSTM1a-1b	161.0	–	0	0	–	–	2.3	–	2.2	–	–	0.13	–	0.3	–
hGSTM1b-1b	172.0	–	0	0	–	–	2.5	–	2.2	–	–	0.16	–	0.3	–
hGSTM1b-2	203.0	–	0	1.7	–	–	3.0	–	2.6	–	–	0.13	–	0.04	–
hGSTM2-2	276.01	–	0	2.0	0.2	0	3.6	–	0	1.7	–	0	–	0.1	–
hGSTM2-3	172.0	–	0	2.1	–	–	3.3	–	0	–	–	0	–	0.1	–
hGSTM3-3	15.2	–	0	0	0.2	0	1.8	–	0	0.2	–	0	–	0.05	–
hGSTM4-4	1.4	0	–	0	0.1	0	–	–	–	0.03	–	–	–	–	–
hGSTP1-1	103.0	–	<0.02	0.14	1.22	0.5	1.6	–	–	–	–	0.02	0.14	0.03	–
hGSTT1-1	0	–	–	–	–	>1.9	–	–	–	–	>0.5	–	–	–	–
hGSTT2-2	0	–	–	–	–	0	–	0.5	0	–	–	–	–	6.9	–

1.5 Chromosomal location and Evolution of GSTs in mammals.

1.5.1 Chromosomal Location

Human GSTT1 And GSTT2 genes were colocolized by cell-cell hydridization, in the same chromosomic reagion on human chromosome 22 and, by in situ hybridization, to the subband 22q11.2 (Tan 1995, Webb 1996). The mGSTT1 gene was found to be clustered with mGSTT2 on chromosome 10 by using in situ hybridization method (Whittington 1999). However, depending on the express sequence tags database , in mouse, it is suggested that, different from humans, there may be two additional members of the theta class that share 70% and 88% protein sequence identity with mGSTT1, and less than 55% sequence identity with mGSTT2 genes (Whittington 1999).

GSTT1 and GSTT2 genes were cloned and sequenced in rats, humans and mice. In humans, they are separated by approximately 50kb and show a similar structure, being composed of five exons with identical exon/intron boundaries. (Coggan 1998). The encoded GSTT1 human subunit is about 25.300Da (Juronen 1996) and the gene is 8.1 kb in length (Coggan 1998). The human GSTT2 gene is 3.7 kb coding for 244 amino acids. In *hGSTT2* gene the TATA and CAT boxes are absent, but there are multiple transcription initiation sites, distributed over 58 bp in the upstream sequence from the translation initiation codon (Ogura 1994). The hGSTT2-encoded protein has 78.3% sequence identity with the rGSTT2 and only 55.0% identity with hGSTT1 (Jemth 1996, Tan 1995, Ogura 1994). In the rat, the cDNA of the *GSTT1* gene is 1258 bp in length with an ORF of 732 bp coding for 244 aminoacids (Pemble 1992). It shares 82% of sequence identity with the human homologous gene (Pemble 1996).

The *mGSTT1* cDNA has an open reading frame of 720 bp encoding a peptide of 240 amino acids and a calculated molecular mass of 27,356 Da, an open reading frame of *mGSTT2* has 732 bp encoding a peptide of 244 amino acids with a molecular weight of 26,676 Da (Mainwaring 1996). The *mGSTT2* gene spans approximately 3.1 kb and is composed of five exons interrupted by four introns. The encoded murine GSTT1 subunit shares only 51% amino acid sequence identity with mGSTT2, but greater than 80% deduced amino acid sequence identity with rGSTT1 and hGSTT1. The encoded GSTT2 protein shares amino acid sequence identities of 92% with rGSTT2, 77% with hGSTT2, 51% with rGSTT1, and 55% with hGSTT1 (Whittington 1996). The amino acidic homologies among the mammalian subunits are summarized in Figure 20. The cDNA of the chicken GST-theta was also cloned and sequenced. The chicken protein has 70–73% sequence similarity with mammalian class-theta GSTs. The protein is 261 amino acids in length and its crystal structure is similar to the other theta GSTs (Hsiao 1995). The closer homology among GSTT1 in human, rat, mouse, and chicken than between GSTT1 and GSTT2 within the same species suggests that an ancestral GST theta gene duplicated to form *GSTT2* and *GSTT1* prior to the evolutive divergence of mammals (Landi 2000) (**Figure 20**).

1.5.2 Evolution Of The GSTs:

Drug detoxification enzymes have existed in both prokaryotes and eukaryotes for more than 2.5 billion years (Nerbert 2000). GSTs constitute a very ancient protein superfamily that is thought to evolved from a thioredoxin-like ancestor in response to the development of oxidative stress (Martin 1999). Other GSH-and cysteine- binding proteins share the thioredoxin-like fold it is becoming clear that GSTs share sequence and structural similarities with several stress related proteins in a wide range of organisms (Rossjohn 1996). It is thought that following the divergence, the multiple GST classes arose by a process of gene amplification which is resulted in novel catalytic activities (Armstrong 1998).

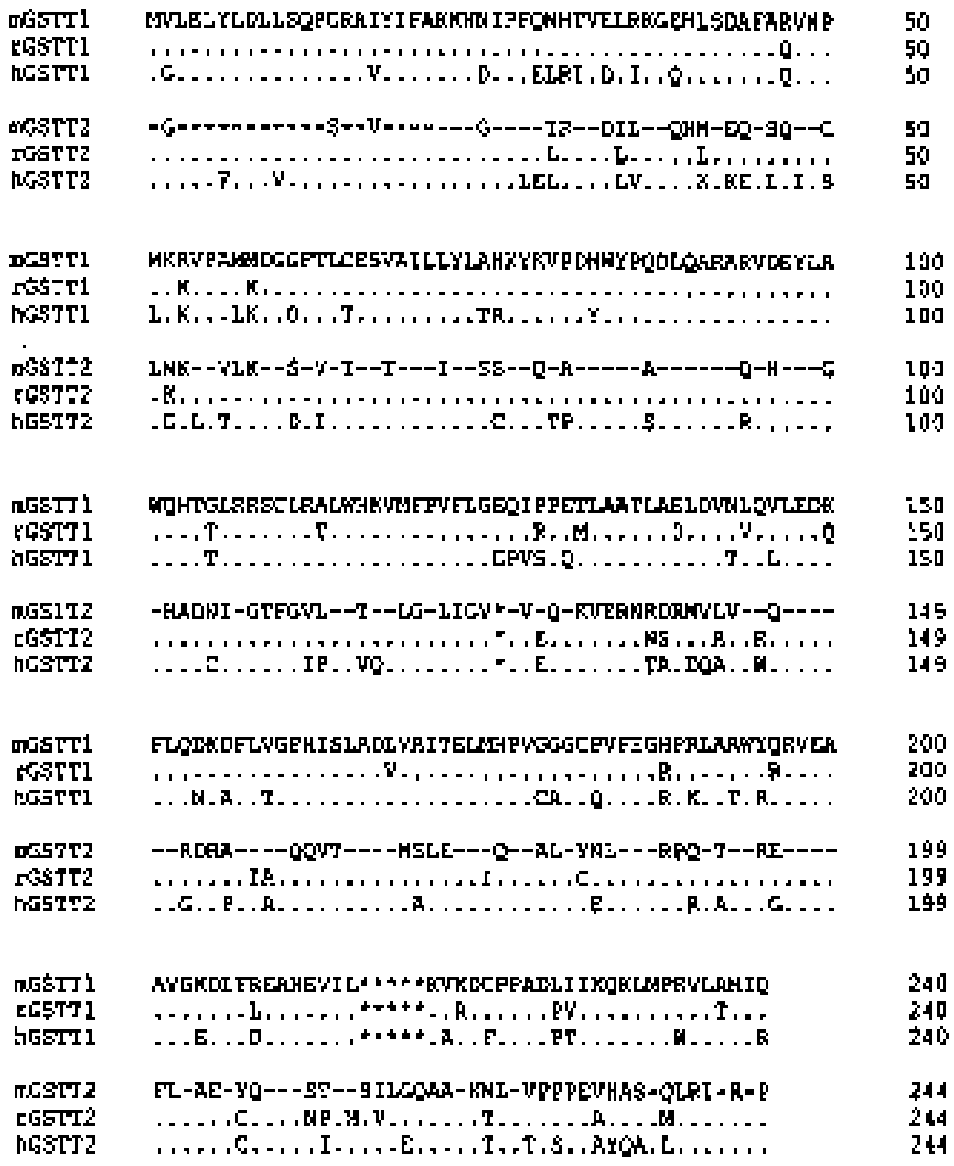


Figure 20:. Homologies among rat, human, and mouse GSTT1 and GSTT2 subunits.

All members of the GST superfamily catalyze the nucleophilic addition of GSH to bind a variety of electrophilic compounds, thus favoring their excretion (Armstrong 1997). Despite their low sequence homology, all of GST isoenzymes have very similar three dimensional structures and a very similar G-site topology as it is explained above (Armstrong 1997, Rossjohn 1997, Wilce 1995). For example, the theta and sigma class enzymes have relatively flat and more hydrophilic interphase but α, μ and π classes have characteristic ball and socket interaction. However, on the other hand, sigma class enzyme uses the same catalytic tyrosine residues as α, μ and π enzymes.

Many of the researchers have used sequence comparisons to generate phylogenetic tree to identify likely patterns of divergence. Alignment of mammalian cytosolic GST, amino acid sequences show that members of the theta class seem to be the closest to the GSTs found in many less advanced species, suggesting that this class might represent the ancestral class (Pemble 1992). On the basis of sequence identity of Kappa class N-terminal with that of class theta, is consistent with the proposed evolutionary path that theta GST should might have arisen from the ancestral mitochondrial GST Kappa (Pemble 1996). Alternatively the theta class may be only older than Alpha, Pi and Mu GSTs and have all diverged from a common ancestor (Board 1997). Ideally it should be possible to compare all full-length sequences known to code for GSTs, but in practice a subset of sequences is usually used to avoid misleading results (**Figure 21**), (Sheehan 2001).

The highly conserved 3'- noncoding sequences of the mu and theta genes also suggest that the mu gene diverged from this precursor before the pi or alpha gene. It is also concluded that the progenitor of the theta class may be kappa class gene which encodes the mitochondrial enzyme (Pemble 1996).

Studies with human GSTT2-2 showed that this enzyme is a homodimeric protein characterized by an additional 40 odd residues at the C-terminus and by a

specific sulfatase reaction not found in more recently evolved α, μ and π GSTs (Caccuri 2001-1).

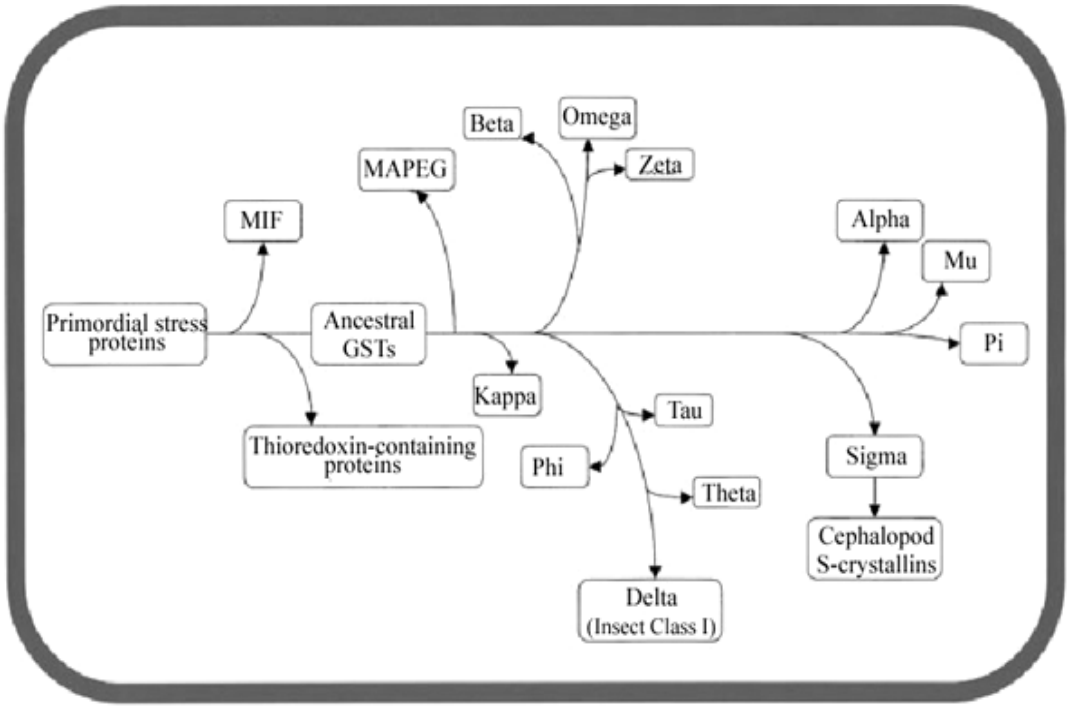


Figure 21: Possible pattern of divergence in the GST superfamily (Sheehan 2001).

In 1999 caccuri and coworkers have also reported that, α, μ and π GSTs display a very similar multi-step mechanism for the binding of the substrate and yield a similar fate for the thiol proton but hGSTT2-2 binds GSH with a different and less efficient mechanism. This result is reasonable because, ıf those enzymes diverged from a common ancestral protein (Board 1997), the molecular architechture, catalytic outcome and kinetic mechanism for GSH binding should be preserved during the evolutionary development. The results obtained from chrystallographic studies was

supported this suggestion that with the crystal structure study it is concluded that the mechanism of losing one molecule of water when GSH binds to the active site and a second water molecule is released when the GSH conjugate is bound, is preserved in α, μ and π enzymes (Caccuri 1999).

The thermodynamic and kinetic efficiency of substrate binding to GSTT2-2 resulted a low affinity for GSH as an apperent K_d of 0.8 mM, a value at least four times higher than that found in the more recent evolved GSTs. It appears with this result that alpha, mu, and pi GSTs are under an evolutionary pressure in the direction of lower K_d values (Caccuri 2001).

Other important factors must be considered is the dynamics of this enzyme family. The mobility profiles derived from the crystallogiraphic temperature factors along the polypeptide chain of α, μ, π and θ GSTs showed that α, μ and π GSTs have a similar and well defined flexibility pattern with a high mobility, but the hGSTT2-2 enzyme showed a completely different flexibility concluding that GSTS have utilized flexibility in terms of an evolutionary progression (Caccuri 2001).

Although most of the data are consistent with class Kappa's being the progenitor of class theta and thus of the soluble GSTs, this point should be resolved when the crystal structures and enzyme mechanisms of several bacterial GSTs and of class Kappa GSTs are characterized (Pemble 1996).

1.6 Purification of Cytosolic GSTs and Identification of Their Subunits :

The first cytosolic GST activity was reported in 1961 in rat liver. The characterization of cytosolic GST has been greatly facilitated by the availability of affinity chromatography gels to which these enzymes bind.

A variety of affinity matrices have been designed to isolate GSTs, these include agarose containing immobilized bromosulfopthalein, cholic acid, glutathione,s-hexylglutathione, s-octyglutathione, thyroxine, and triazine dye (Pulford 1995). Among those matrices the glutathione-agarose (Simons and Vander Jagt, 1977) and *S*-hexylglutathione-agarose (Guthenberg and Mannervik, 1979) are the most widely used (**Figure 20**) to purify esspecially class **Alpha**, **Mu**, **Pi**, and **Sigma** GSTs. Class mu, pi, and sigma GST are absorbed efficiently by both glutathione agarose and s-hexylglutathione agarose. By contrast, the class alpha GST do not display strong affinity for s-hexylglutathione agarose but most isoenzymes of this class are efficiently adsorbed by glutathione-agarose (Hayes 1986). A potentially useful feature of the *S*-hexylglutathione-agarose affinity chromatography is that gradient elution allows resolution of different GST isoenzymes, where the elution order is dependent on K_m value for GSH (i.e., the lower the K_m value of the enzyme the higher the concentration of *S*-hexylglutathione required to elute it from the gel (Hayes, 1988).

The class **Theta** GST isoenzymes have proven to be more difficult to purify than the other classes of GSTs because they are labile and are the least abundant family. Class **Theta** GSTs are not retained neither by glutathione-agarose nor *S*-hexylglutathione agarose, but can be purified by affinity chromatography on the triazinyl dye gels, Orange A matrix and Blue Sepharose (Hiratsuka *et.al.,*., 1990; Meyer *et.al.,*., 1991; Mainwaring *et.al.,*., 1996a).

The reason for the failure of currently used glutathione-affinity chromatography matrices to bind **Theta** class GSTs may be because this class possesses a much deeper active site than the other GST isoenzymes (Wilce *et.al.,* 1995). Therefore, GSH immobilized to an agarose support via a longer spacer arm may provide an effective affinity gel for class **Theta** GST (Lopez *et.al.,* 1994).

Following affinity purification of GSTs, the individual isoenzymes are normally resolved by exploiting differences in their charge using ion-exchange chromatography, chromatofocusing, or isoelectric focusing (Jakoby, 1978; Mannervik, 1985a).

It is advised to use adsorption chromatography on hydroxyapatite as an highly effective purification step when a homogenous protein is not obtained via ion-exchange chromatography (Hayes *et.al.,* 1987).

The analytical methods that have proved valuable in the identification of the GST isoenzymes and the subunits they comprise include SDS-PAGE (Hayes and Mantle, 1986a), isoelectric focusing (Hales *et.al.,*., 1978; Aceto *et.al.,*., 1989), reversed-phase high-pressure liquid chromatography (HPLC) (Ostlund Farrants *et.al.,*., 1987), electrospray mass spectrophotometry (Yeh *et.al.,* 1995), Western blotting (Hayes and Mantle, 1986b), and immunoassay with either polyclonal antibody against purified GST, specific GST peptides, or monoclonal antibodies (McCusker *et.al.,*1989; Peters *et.al.,* 1990; Juronen *et.al.,* 1994, 1996a and 1996b, Sherratt *et.al.,* 1997).

Recently, reversed-phase HPLC has been increasingly employed to identify GST subunits. Satisfactory resolution of GST subunits can be obtained using a μ-Bondapak C_{18} column (particle size less than 10μm) developed with a 41 to 51% gradient of acetonitrile in 0.1% trifluoroacetic (Johnson *et.al.,*., 1992).

1.7 Purification and Characterization of Class Theta GSTs:

By contrast with the other multigene families of GSTs, relatively little is known about the class **Theta** isoenzymes. Although it was first discovered by Gillham in 1973 from rat urine this enzyme was recently studied GST.

In 1973 Gillham has proved the existence of an enzyme catalyzing GSH conjugation of benzyl and menapthyl sulfates on putative precursors of the benzyl and menapthyl mercapturic acids excreted into the urine of rats given the corresponding aryl methanols (Gillham 1973) and called this enzyme as M. In the same year Fjellstedt purified a GST from rat and called E because of its activity with epoxdes. Later this E and M transferases have also been called 5-5 (Meyer *et al.,* 1991) and Yrs-Yrs (Hiratsuka *et al.,* 1990), they are now called as rGST1 and rGSTT2 respectively.

The development of a general purification scheme by Habig and co-workers (Habig *et.al.,*. 1974), allowed the isolation and characterization of several of the major rat liver GSTs and it is found that many isoenzymes are inactive with either EPNP or MS. By contrast, it was also found that most GSTs catalyze the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH and therefore CDNB was adopted as the substrate of choice to monitor GST purification (Clark *et.al.,* 1973 ; Habig *et.al.,* 1976) and later the glutathione affinity matrices were widely used in the purification of GSTs. However, the enzymes that detoxify EPNP and MS do not utilize the model substrate CDNB and do not bind to glutathione or *S*-hexyl-glutathione affinity matrices. Consequently, the GSTs that metabolize EPNP and MS have been largely ignored until relatively recently (Hiratsuka *et.al.,* 1990; Meyer *et.al.,* 1991; Hussey and Hayes, 1992).

To date , two GST theta T1-1 and T2-2 have been isolated from human liver. Meyer *et.al.,*in 1991 have reported that hGSTT1-1 have activity towards 4-nitrobenzyl-chloride (4-NBC), 4-nitrophenyl bromide (4-NPB) and 1,2-epoxy-(*p*-nitrophenoxy) propane (EPNP) but the enzyme was inactive with CDNB, cumene hydroperoxides and 1-MS. Hepatic hGST2-2 has found to have no detectable activity towards CDNB or EPNP but has activity with 1-menaphthyl sulfate and a high glutathione peroxidase activity towards cumene hydroperoxide (Hussey and Hayes, 1992).

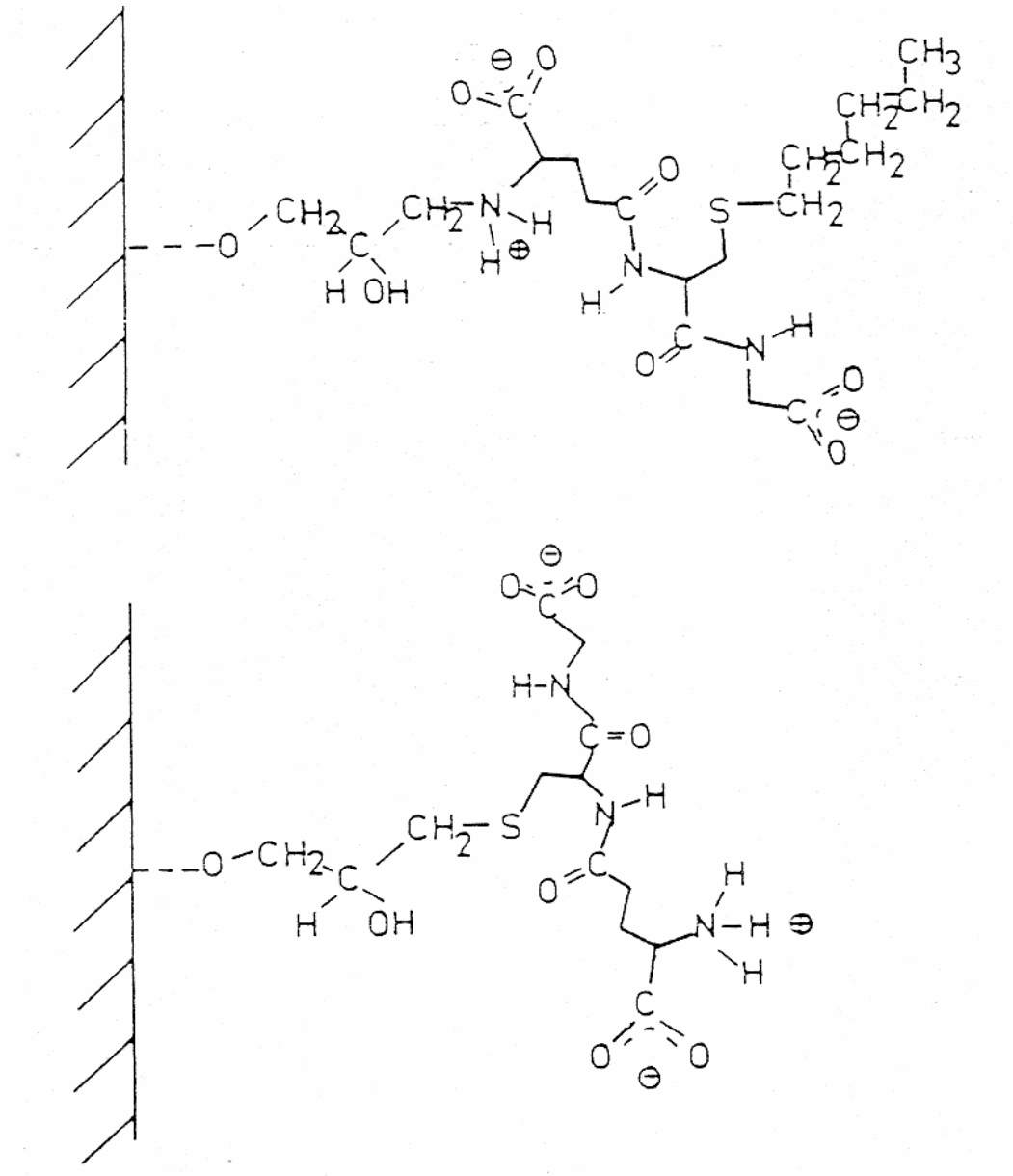


Figure 22: Structure and possible conformation of affinity ligands used for purification GSTs. (a) Immobilized S-hexylglutathione; (b) Immobilized glutathione. In both cases the ligand is attached by coupling with the oxirane group of epoxy-activated Sepharose. It is proposed that the conformations of the ligands are complementary to the glutathione binding site (G-site) of a GST subunit.

In another study in 1996 Juronen *et.al* purified hGSTT1-1 from human liver and erythrocytes using DCM as a substrate. Immunoblot analysis, using monoclonal antibodies raised against the purified hGSTT1-1 (Juronen *et.al.,*., 1996a), revealed that GSTT1 is present in lung, kidney, brain, skeletal muscle, heart, small intestine and spleen, but not in lymphocytes. The N-terminal amino acid sequences from liver and red blood cells were found to be identical.

Later, recombinant hGSTT1-1, purified by Sherratt *et.al.,*. (1997), was found to be catalytically active with EPNP, 4-NBC, 4-NPB and DCM but inactive towards CDNB, EA and MS. Immunoblotting, using antibodies raised against the recombinant hGSTT1-1, showed that hGSTT1 subunit is highly expressed in kidney and liver, with lower levels also observed in cerebrum, pancreas, prostate, skeletal muscle and small intestine, and in trace amounts in lung.

The earliest study for purification of GSTT2-2 (Yrs-Yrs) from rat was performed by Hiratsuka *et.al*., in 1990 by using 5-HCR as substrate. In this study GST couldn't bind to s-hexylglutathione column and did not show any activity towards DCNB, 4-NBC and EPNP. CDNB was a very poor substrate for this GST. But the enzyme had higher activity towards ethacrynic acid and cumene hydroperoxides. The study performed again by Hiratsuka in 1997 was the first comparative study on the purification and properties of both subfamilies of the theta class GSTs.

While the human GSTT1 and hGSTT2 proteins shares 48% sequence identity, hGSTT1 and rGSTT1 share 79% identity and hGSTT2 and rGSTT2 share 78% identity (Pemble *et.al.,*1994; Hayes and Pulford, 1995).

Since they are not retained on GSH- or *S*-hexyl-GSH affinity matrices and they lack activity towards CDNB, the purification of the **Theta** class GSTs,

are much more difficult compared to the other GSTs. For this reason, several chromatographic steps were used to purify the enzymes to apparent homogeneity. Several research groups have suggested different protocols, for the purification of class **Theta** GSTs.

Meyer and co-workers have described the purification of two **Theta** class isoenzymes from rat liver; GST 5-5 (rGSTT1-1) and GST 12-12 (rGSTT2-2), and one isoenzyme from human liver; GST θ (hGSTT1-1), using EPNP as a substrate. The purification protocol included a sequential GSH-agarose affinity, Orange A, hydroxyapatite, anion-exchange or chromatofocusing and hydrophobic interaction chromatography. Since the purified isoenzymes, especially GST12-12 and GST θ, lose activity towards EPNP during the latter stages of purification, but did not obviously disappear in terms of protein so it is concluded that to calculate the yield based on protein are reasonably accurate (Meyer *et.al.*,.1991).

The other study related with the purification of hepatic GSTT1-1 was performed by Juronen and co-workers in 1996. In this study the enzyme was also purified from erythrocytes using DCM as a substrate. Different protocol from Meyer was used to overcome the activity loss. Six subsequent chromatographic steps were; CM-Sephadex C-50, DEAE-Sepharose Fast Flow, salt promoted thiophilic adsorption on the T-gel, Matrex gel Orange A, chromatofocusing and Q-Sepharose Fast Flow . Using this a hGSTT1-1 with high activity towards DCM was isolated from human liver cytosol and purified to homogenity in 18.5% yield with a purification factor of 4400-fold. The GSTT1-1 was also purified from erythrocytes, but the enzyme activity decreased rapidly in the final stages of purification. The obtained data indicated that hGSTT1-1 represents approximately 0.02 % of the soluble liver protein, much higher than it was suggested earlier (0.003%) by Meyer and co-workers (Meyer

et.al.,,1991). The purified hGSTT1-1 was homodimeric with a subunit M_r value of 25,000 and pI 6.64, as confirmed by SDS-PAGE, IEF and Western blot analysis.

The first purification of GSTT2-2 from rat liver cytosol, was performed by Hiratsuka and co-workers (Hiratsuka *et.al.,* 1990). The purification steps contained subsequent column chromatography on DEAE-cellulose (DE-52), S-hexyl-glutathion agarose, chromatofocusing, blue Sepharose and TSK gel G3000 SW. The purified GST was a homodimeric protein with subunit M_r 26,000 and pI 7.9 and it was designated as Yrs-Yrs because of its enzyme activity toward "reactive sulfate esters". Immunoblot analysis of various tissue cytosols of the male rat, using anti-Yrs-IgG preparation suggested that liver and testis have high concentration of rGSTT2-2. The concentration in adrenal and kidney was low and much lower in lung. rGSTT2-2. however found at extremely low concentrations in all the cytosols of skin, heart, small intestine and spleen, so that only much larger amount of protein were applied to be determined. Again in this study, GST conjugation of three different reactive sulfate esters of arylmethanols namely; 1-MS, 7-HMBA sulfate, DHBA7-sulfate activities were tested and among those substrates 1-MS was found the best substrate for this GST.

Hepatic hGSTT2-2 form human was described by Hussey and Hayes (1992). The method devised results in an approximately 500-fold increase in specific activity towards MS after subsequent column chromatography on DEAE-cellulose (DE-52), hydroxyapatite, Orange A and Mono Q anion-exchange. The purified GST was a homodimeric protein with subunit M_r 25,100 and had no detectable activity towards CDNB, EPNP and 4-NBC. However, besides its transferase activity towards MS, the purified GST was found to have considerable glutathione peroxidase activity towards cumene hydroperoxide. In this study Hussey and Hayes have reported that the specific activity of human liver cytosol with 1-MS as a substrate was found to decrease to about 20% of

that recovered in freshly prepared liver cytosol with storage at 4ºC. Because of this dramatic loss of activity the sample should be applied immediately to the subsequent columns.

Later, a method was established for simultaneously isolating GSTT1-1 and GSTT2-2 as homogeneous proteins from rat liver cytosol by Hiratsuka *et.al.,* in 1997. The established method of using an 8-aminooctyl Sepharose 4B column to separate rGSTT1-1 from rGSTT2-2 at the final stage of their purification was a modification of the method previously reported (described above) for the isolation of rGSTT2-2 (Hiratsuka *et.al.,* 1990). Specific substrates used for purification of the **Theta**-class rGSTs were DCM for T1-1 and 5-sulfoxymethyl-chrysene (SMCR) for T2-2. The data obtained revealed that rGSTs T1-1 and T2-2 exist at a ratio of 1:7 at a total concentration of 0.5% of that of the cytosolic protein. Purified rGSTsT1-1 and T2-2 were separated as single bands at 28 and 26.5 kDa by SDS-PAGE and as single peaks at retention times of 36 and 34 min, respectively, by reverse-phase HPLC on a μBondasphere column eluted with a linear gradient of acetonitrile in water containing trifluoroacetic acid. Western blot analysis indicated that rabbit antisera raised against rGSTsT1-1 and T2-2 intensely reacted with the corresponding antigens, but showed no detectable reactivity with the different isoforms of **Theta**-class rGSTs as well as with representative hepatic rGSTs of other classes. The **Theta**-class rGSTs showed high GSH peroxidase activity toward hydroperoxides of cumene, arachidonic acid, and linoleic acid. Cumene hydroperoxide was a better substrate for rGST T1-1 than for rGST T2-2, while the fatty acid hydroperoxides were the better substrates for rGST T2-2 than for rGST T1-1.

1.8 Scope of This Work:

Although GSTs activity has been detected in a wide variety of species, including birds, insects, plants, and microorganisms, the mammalian liver, especially rat liver and human liver, has received much of the attention due to its high concentration of the enzyme and its well recognized role in detoxification.

In spite of their toxicological significance and important role in carcinogenesis, relatively little is known about the class **Theta** enzymes because very few investigations on the purification and characterization of the class **Theta** GSTs have been performed due to low amounts and instability of the purified isoenzymes.

The class **Theta** GST isoenzymes have proven to be more difficult to purify than the other classes of GSTs because they are labile and are the least abundant family. Moreover, class **Theta** GSTs show very low or lack activity with the 1-chloro-2,4-dinitrobenzene (CDNB), a typical and versatile substrate for the other classes of GSTs, and are, unlike the other GSTs, not retained by GSH and *S*-hexylglutathione affinity matrices making it difficult to purify them.

The substrate 1-MS, that is not commercially available, was previously prepared and checked using several techniques including proton-NMR, FTIR and UV-Spectra. This substrate was later used in characterization of the purified isoenzyme(s).

The aim of this study is to purify theta class GST T2-2 isoform from bovine liver. The purification protocol that was adopted from the method of Hiratsuka *et al.,* (1997). In this method the purification performed by the sequential use of Anion exchange DEAE cellulose column, *S*-hexylglutathione agarose affinity, dye-ligand Matrex Gel Orange A and chromatofocusing column chromatography.

After each purification step, the purity of the fractions was checked on silver stained SDS-PAGE, and the class **Theta** GSTs from the cytosol and purified fractions of the bovine liver were compared in terms of their immunological properties using Western blotting with polyclonal antibodies.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials:

1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), 4-nitrobenzyl chloride (4-NBC), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), SDS-PAGE molecular weight markers, ammonium persulphate (APS), bromophenol blue, diethylaminoethyl cellulose (DE-52), coomassie brilliant blue R-250, coomassie brilliant blue G, sucrose, N,N'-methylene-bisacrylamide (Bis), hydroxymethyl aminomethane (Tris), N,N,N',N'-tetramethylenediamine (TEMED), acrylamide, silver nitrate, glycine, glycerol, sodium carbonate, sodium thiosulfate, Tween-20, guanidine hydrochloride, dithiothreitol (DTT), nitro blue tetrazolium (NBT) 5-bromo-4-chloro-3-indolyl phosphate (BCIP) premixed solution, S-hexylglutathione agarose, formaldehyde, phenylmethanesulphonyl fluoride (PMSF), Polybuffer exchanger (PBE 94), Polybuffer 96 (PB 96), S-hexylglutathione agarose column, sodium azide, and cellulose membrane dialysis tubing were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

1-naphthalene methanol, and chlorosulfonic acid were from Aldrich Chemical Company, St. Louis, MO, U.S.A;

2-mercaptoethanol, ether, ultra pure methanol, glacial acetic acid and Ciocalteu's Folin phenol reagent were from Merck, Darmstadt, Germany.

Amplified alkaline phosphatase goat anti-rabbit immunoblot assay kit, biotinylated SDS-PAGE standards, sequi-blot polyvinylidene difluoride membrane (PVDF) were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Matrex Gel Orange A was purchased from Amicon, Lexington, MS, U.S.A.

Polyclonal antibodies against hGST alpha, mu, pi raised in rabbit were purchased from Biotrin International Limited, Dublin, Ireland.

Polyclonal antibody against recombinant rGSTT2-2 were the kind gift of Dr. P.J. Sherratt and Prof. Dr. J.D. Hayes, Dundee, U.K.

All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2 Methods

2.2.1 Preparation of Cytosols from bovine liver:

The livers from well bled bovine about 6-12 months were obtained from Kazan slaughter house - ANKARA. Connective and fatty tissues from

sample weighing 20-25g were removed. Then, whole livers were cut into small pieces with scissors and washed again with cold distilled water, containing 1mM EDTA in order to remove excess blood. After draining the tissue pieces on filter paper, the liver pieces were ground using meat grinder. All the subsequent steps were carried out at 4°C.

The resulting tissues were homogenized in 2 vol. (v/w) of 0.15M KCl solution, containing 10 mM potassium phosphate buffer, pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) using a Potter-Elvehjem homogenizer coupled with a motor (Black and Decker, V850, multi-speed drill)-driven Teflon pestle at 2400 rpm (5 to 10 passes). The homogenate was centrifuged at 12,000g for 25 minutes. The supernatant fraction was passed through two layers of cheesecloth and the pellets were discarded. The filtrates were centrifuged at 134,000g for 50 minutes and the supernatants obtained were passed through cheesecloth to remove floating lipid materials. The filtrate thus obtained was referred to as the cytosol. Most frequently, the protein determination, enzyme activity measurement and purification process were started immediately after the preparation of the cytosol without any further storage. Otherwise, the cytosol in small aliquots of 0.5 ml were stored at -80°C to be used later in SDS-PAGE and Western blotting.

2.2.2 Protein Determinations:

The protein concentrations in the prepared cytosol were determined by the method of Lowry and co-workers (Lowry *et al.,* 1951) with crystalline bovine serum albumin (BSA) as a standard. Aliquots of 0.1 to 0.5 ml of 1:50

diluted liver cytosols were taken into test tubes and were completed to a final volume of 0.5 ml with distilled water. Then, alkaline copper reagent was

prepared by mixing 2% copper sulfate, 2% sodium potassium tartarate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 2.5 ml of the alkaline copper reagent was added to each tube, mixed by vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.25 ml of 1 N Folin Phenol reagent was added to each test tube, mixed immediately within 8 seconds by vortex and incubated 10 minutes at 50°C in water bath. The intensity of color developed in each tube was measured at 660 nm.

The protein concentrations in the crude extracts were calculated from a standard calibration curve that was constructed from the corresponding $O.D_{660nm}$ values of BSA standards (0 to 200 μg). The average protein amount of bovine liver cytosols used throughout this study was found as 25 ∓ 5.3 mg/ml. The protein concentrations in the column chromatography fractions were also determined using Lowry method.

2.2.3 Determination of Cytosolic GSTs Activity:

2.2.3.1 GSTs Activity Assays with the Common GSTs Substrates:

The GST activities were determined against the substrates CDNB, 4-NBC and 1-MS.

All enzyme activity assays were conducted at 37°C except that against CDNB, which was conducted at room temperature.

GST activities against the substrates CDNB, MS, 4-NBC were determined spectrophotometrically by monitoring the formation of the conjugation product under the conditions given in **Table 5** (Gillham, 1971; Habig *et al*., 1974; Habig and Jakoby, 1981).

Table 6. Conditions for spectrophotometric GSTs enzyme assays with different substrates.

Substrate	Stock [Substrate] (mM)	Final [Substrate] (mM)	Final [GSH] (mM)	Final [Buffer] (M) "pH" @	λ_{max} (nm)	ε ($\mu M^{-1}cm^{-1}$)
CDNB	20.0	1.0	1.0	0.1 "6.5"	340	0.0096
4-NBC	20.0	1.0	5.0	0.1 "6.5"	310	0.0019
MS	10.0	0.5	5.0	0.05 "8.3"	298	0.0039

@ 0.10 M Triethanolamine-HCl (TEA) buffer was used with MS,
0.20 M Potassium phosphate buffer was used with CDNB and 4NBC

The constituents of the incubation mixture for GSTs enzyme assays are shown in **Table 6**. The reactions were started by the addition of cytosol. Incubation mixtures without the enzyme source were used as blanks (nonenzymatic reactions), and concentrations of the formed conjugation products were determined from the slopes of initial reaction rates. The GSTs activities were expressed as unit/mg protein. One unit is equal to one nmole of substrate consumed or product formed per minute.

2.2.4 Preparation and Characterization of 1-Menaphthyl Sulfate:

The substrate 1-menaphthyl sulfate (MS) was prepared according to the method of Clapp and Young (1970) starting with 1-naphthalene methanol. A solution was prepared by dissolving 2.0 g of 1-naphthalene methanol in 10 ml pyridine and cooled in ice-bath. Chlorosulfonic acid (0.9 ml) was added slowly with continuous stirring. The reaction was allowed to take place in a closed flask for 2 hours during which the temperature of the reaction mixture was allowed to rise to room temperature. On the next day the reaction mixture was cooled in an ice-bath and saturated NaOH was added with rapid stirring until the mixture was just alkaline to litmus.

Afterwards, ether (10 volumes) was added with continuous stirring and the precipitate that formed was separated by filtration, washed several times with ether, filtered and then suspended in ethanol (~20 ml). Warm distilled water was added slowly with continuous stirring until the solution was just clear and ether (10 volumes) was added with continuous stirring. The formed residue was separated by filtration, washed with ether, filtered again and dissolved in warm 90% ethanol (~ 20 ml). The ethanol-insoluble inorganic salts were removed by filtration. Finally, the sodium salt of the sulfate ester was precipitated from the ethanol solution by the addition of ether (10 volumes), collected by filtration washed with ether and carefully dried under vacuum. The freshly prepared and dried MS was stored under an argon atmosphere at -20°C.

The qualitative and quantitative analysis of the synthesized MS was performed using UV-spectroscopy, FTIR and proton-NMR. The analysis results were compared with those found in the literature (Clapp and Young, 1970; Watabe *et al.*, 1982; Enders *et al*., 1991) to check the purity of the prepared MS.

The UV spectrum taken for a solution of MS in ethanol is shown in **Figure 23**. The maximum absorbance was at λ_{max} of 224 nm with other three small peaks at 270, 280 and 290 nm.

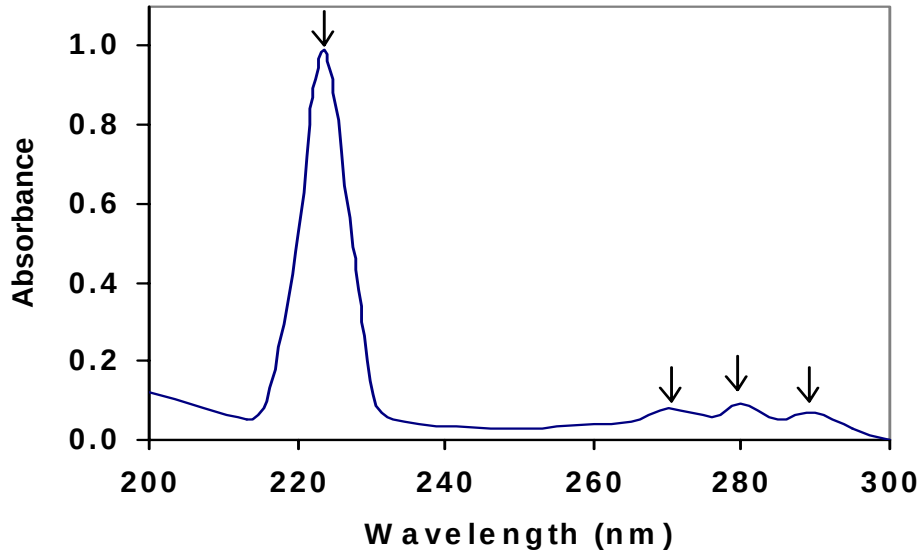


Figure 23: The absorption spectrum of the synthesized MS.

Figure 24 shows the FTIR (ν_{max}^{KBr} vs. %T) profiles obtained for both of the starting material, 1-naphthalene methanol, and the synthesized MS. It is clear that the broad absorption peak, between 3100-3500 cm^{-1}, of the -OH group of the starting material is totally absent in the profile of MS, which means that the product is not contaminated with any portion of the starting material.

The best criterion for the purity of the prepared MS is to follow the down field-shift of the benzylic protons of the sulfate ester in the proton-NMR

spectrum compared to that of the menaphthyl alcohol (1-naphthalene methanol) used as starting material. The proton-NMR spectra for both the starting material and product, using DMSO as a solvent, are shown in **Figure 25** where it is clear that the resonance signal of the alcohol is not detected any more and the ratio of the integrated signals of the aromatic protons to the benzylic methylene group of MS is as expected. The proton-NMR spectrum for MS gave NMR $\delta_{ppm,}$, in DMSO, of 8.23 – 7.46 (7H, arom) and 5.25 (2H, $-CH_2$) which are the same as the values reported in the literature. The TLC of synthesized MS with 1/6 ethanol/ethylacetate as a solvent resulted in a single spot also means that the synthesized product is not contaminated with any portion of the starting material. Consequently, the results of the UV-spectroscopy, FTIR and proton-NMR all show that MS was successfully prepared with a quality good enough to perform a GSTs assay.

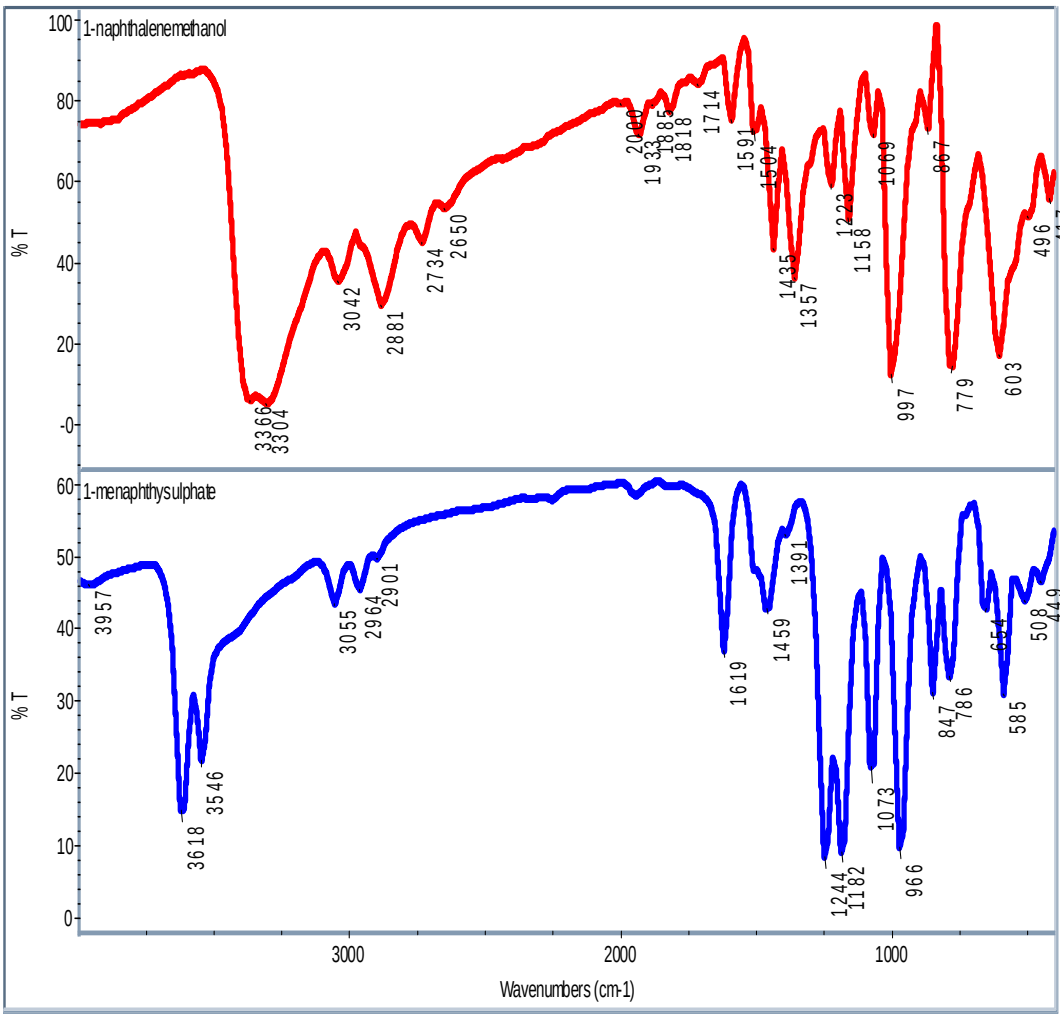
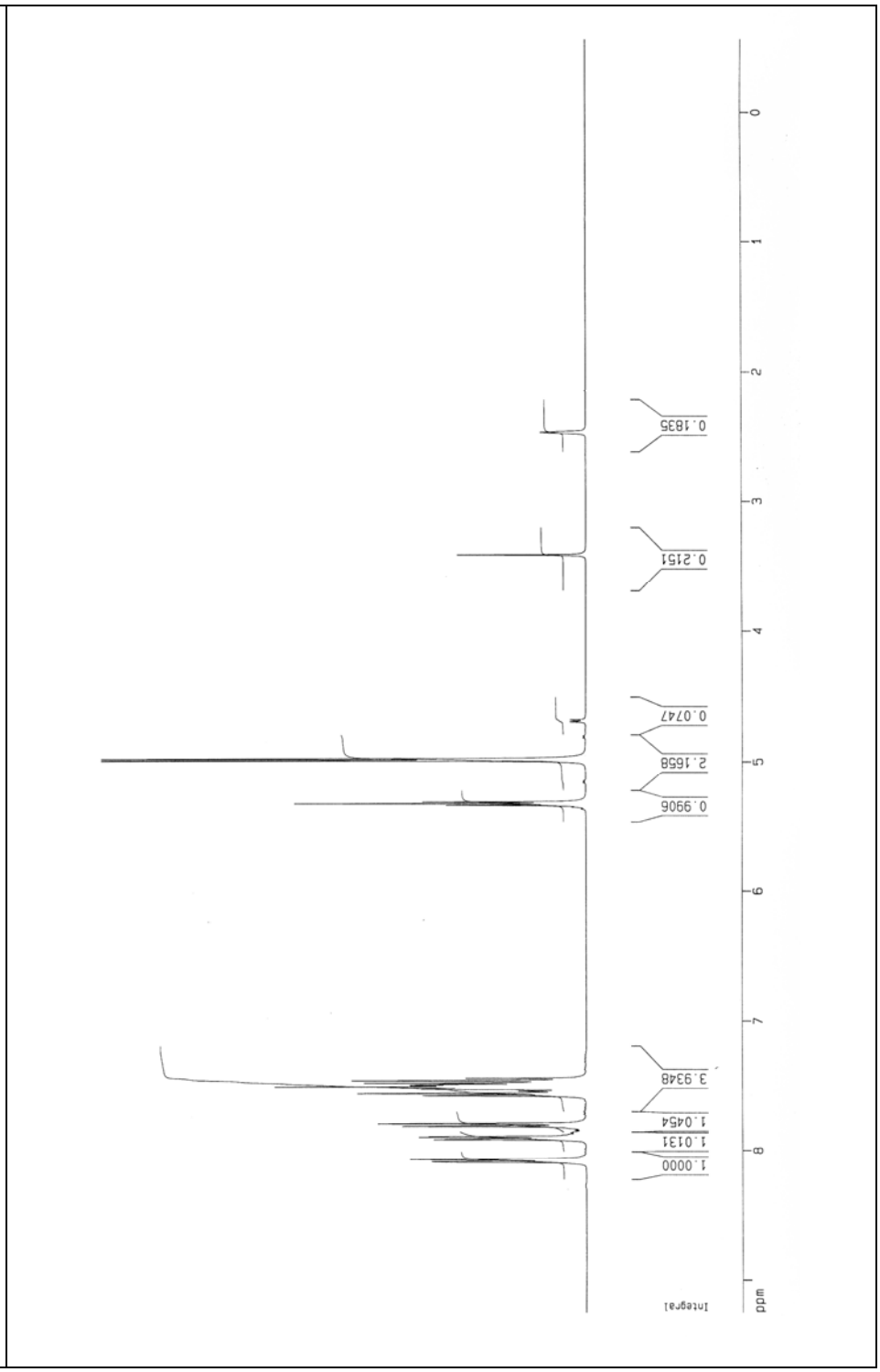


Figure 24: The FTIR profiles for both of the starting material, 1-naphthalene methanol and the synthesized MS .

a)


0.1835
0.2151
0.0747
2.1658
0.9906
3.9348
1.0454
1.0131
1.0000
Integral
ppm

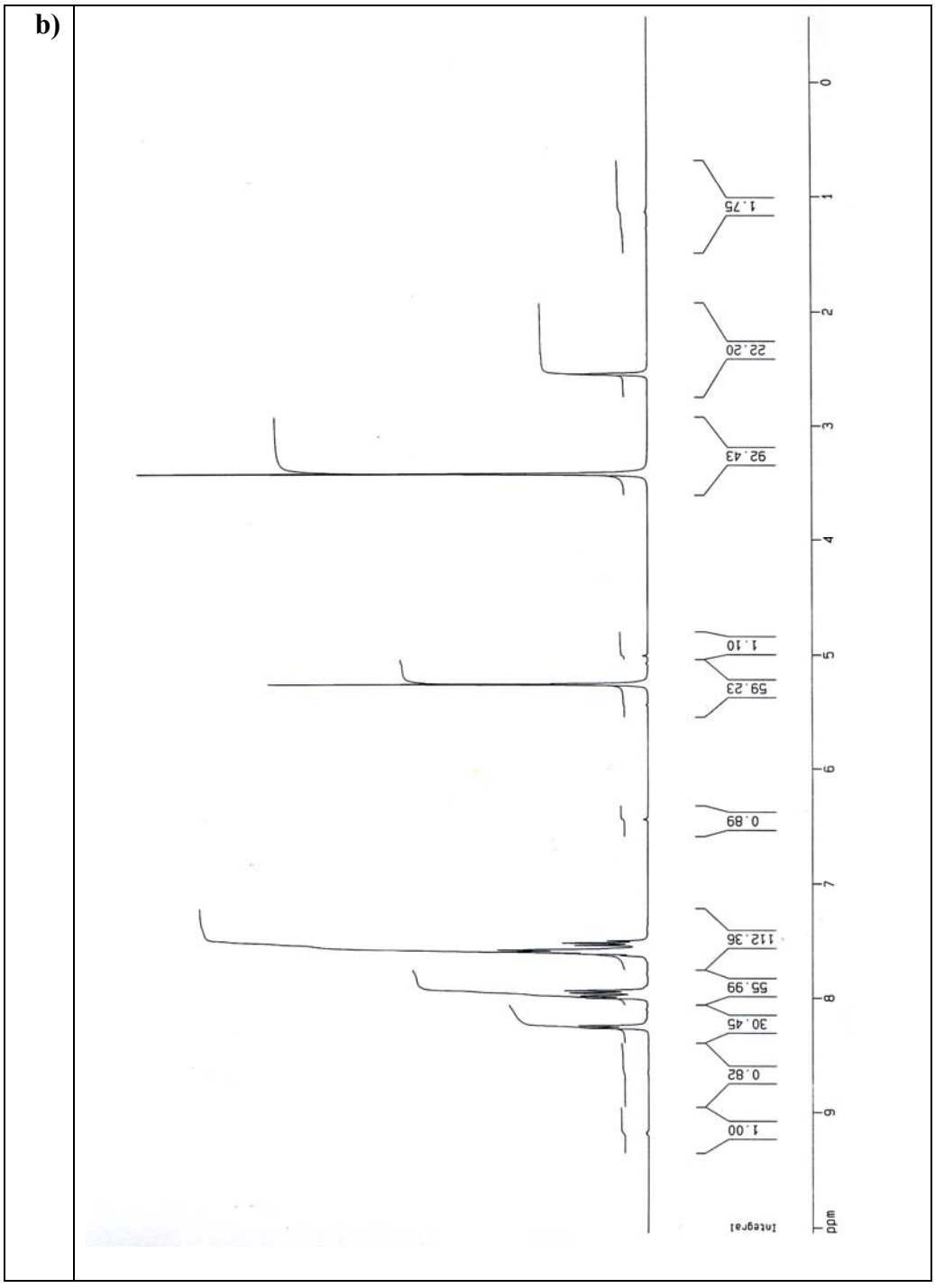


Figure 25: Proton NMR result of a) starting material 1-napthalene methanol b) the synthesized MS both in DMSO.

2.2.5 Purification of GSTT2-2 from Bovine liver Cytosols:

The purification scheme, for obtaining GSTT2-2 form bovine liver or cytosolic fractions, consisted of a sequential DEAE cellulose anion exchange, *S*-hexylglutathione agarose affinity, dye-ligand (Matrex Gel Orange A) and chromatofocusing column chromatography using 1-MS as a substrate to follow the activity in the eluted fractions.

Most of the time, the total protein decreased rapidly after the dye-ligand (Matrex Orange A) chromatography step. For this reason, the pooled fractions of this step with activity against 1-MS were stored at -80°C after checking the purity and determining the molecular weight and immunological properties of the obtained GSTT2-2 isoenzyme using SDS-PAGE and Western blotting.

2.2.5.1 DEAE Anion Exchange Column Chromatography:

The column (1.5 X 15.0 cm) packed with DEAE cellulose and equilibrated in the cold room with 10 mM potassium phosphate buffer pH: 7.0 containing 2mM 2-mercaptoethanol, 2mM EDTA and 10% (v/v) glycerol. The freshly prepared cytosol having protein of 30 mg and 25.6 unit/mg 1-MS activity, 370 unit/mg CDNB activity and 195 unit/mg 4-NBC activity was applied to the column with the flow rate of 12ml/hour. The column was then washed with the equilibration buffer until no absorption of effluent at 280 nm was detected. The flow through fractions from this column having activity towards 1-MS were pooled collected and applied to S-hexylglutathione affinity column. The bound proteins were eluted from the column with a linear NaCl gradient (0-1.0 M) consisting of 100ml of the equilibration buffer and 100 ml of the same buffer containing 1.0 M NaCl.

2.2.5.2 Affinity Column Chromatography on S-Hexylglutathione Agarose:

The column (1.0 cm X 5.0 cm) packed with *S*-hexylglutathione agarose was equilibrated in the cold room with 10 mM potassium phosphate buffer, pH 7.0 containing 2 mM EDTA, 2 mM 2-mercaptoethanol and 10% (v/v) glycerol. The combined fraction, from the previous step, containing a total of 10 mg protein with 39 unit/mg of GSTs activity towards MS and 836 unit/mg of GSTs activity towards CDNB was applied to the column at a flow rate of 8 ml/hour. Afterwards, the column was washed with the equilibration buffer at a flow rate of about 12 ml/hour until no absorption of effluent at 280nm was detected. The column was further washed with the same buffer containing 0.2 M NaCl. Afterwards, the bound proteins were eluted with 20 ml of 50 mM Tris-HCl buffer, pH 9.5 containing 0.2 M NaCl and 10 mM GSH at a flow rate of 15 ml/hr.

GSTs activities against MS and CDNB were measured in the fractions collected from the column. The washing unbound fractions with the highest activity against MS were combined in one fraction for further purification. The elution fractions with the highest activity against CDNB were also collected and stored at -80°C. The elution fractions activity against CDNB were determined as 25000 unit/mg.

The *S*-hexylglutathione agarose affinity gel was regenerated in the column, without repacking, by washing with 1.0 – 2.0 M NaCl or 3.0 – 6.0 M guanidine – HCl (about 5 bed volumes) to remove the bound substances. The column was then washed extensively with distilled water (more than 10 bed volumes) and equilibrated again with the equilibration buffer. The resin is stored at 4°C in neutral buffer containing 0.02 % sodium azide to avoid microbial growth

2.2.5.5 Dye-Ligand Column Chromatography on Matrex Gel Orange A:

The column (1.0 cm X 15 cm) packed with Matrex Gel Orange A was equilibrated in the cold room with 10 mM potassium phosphate buffer, pH 7.0 containing 2 mM 2-mercaptoethanol and 10% (V/V) glycerol. The fractions from the previous step, containing a total of 4,5 mg protein with 27 unit/mg of GSTs activity towards MS was applied to the column at a flow rate of 12 ml/hour. Afterwards, the column was washed with the equilibration buffer at a flow rate of about 15 ml/hour until no absorption of effluent at 280 nm was detected. The bound proteins were eluted from the column with a linear NaCl gradient (0-1.0 M) consisting of 100 ml of the equilibration buffer and 100 ml of the same buffer containing 1.0 M NaCl at a flow rate of 25 ml/hour.

GSTs activity against MS was measured in the fractions (1.5 ml each) collected from the column. The fractions with the highest activity against MS (eluted in the gradient at about 0.2M KCl) were pooled and stored at -80°C.

The Matrex Gel Orange A was regenerated in the column, without repacking, by washing with 6.0 M guanidine – HCl (more than 5 bed volumes) until no residual dye appeared in the eluant. The column was then washed extensively with distilled water (more than 10 bed volumes) and equilibrated again with the equilibration buffer. The resin is stored at 4°C in neutral buffer containing 0.02 % sodium azide to avoid microbial growth.

2.2.5.6 Chromatofocusing Column Chromatography:

The column (1.0 cm X 17 cm) was packed with Polybuffer exchanger PBE 94 and layered with about 1 cm Sephadex G-25 to ensure homogenous sample application. The column was equilibrated in the cold room with 25 mM ethanolamine-acetic acid buffer, pH 9.6, containing 2 mM 2-mercaptoethanol. The combined fractions from the previous Orange A column chromatography steps, dialyzed against the equilibration buffer and concentrated. Dialized and concentrated sample containing a total of 0.3 mg protein with 137 unit/mg of GSTs activity towards MS was loaded to the column at a flow rate of 10 ml/hour. Afterwards, the column was washed with the equilibration buffer at a flow rate of about 15 ml/hour until no absorption of effluent at 280nm was detected.

The pH gradient was developed with about 120 ml of a 10 % (v/v) Polybuffer 96 – acetic acid, pH 6.0, containing 2 mM 2-mercaptoethanol. Fractions of 1.0 ml each were collected and the pH values of the collected fractions as well as the GSTs activity against MS were measured. The fractions with the highest activity against MS (eluted in the gradient at pH 7.2 – 7.3) were pooled and dialysed against 0.5 liter of 10 mM potassium phosphate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol for 8 hours at 4°C. The dialysed sample was then concentrated against sucrose for 2 hours in the cold room. The protein content and the activity of the concentrated sample against MS were determined immediately after dialysis and the remaining sample was stored at -80°C.

The Polybuffer exchanger (PBE 96) was regenerated in the column, without repacking, by washing with 1.0 M NaCl (about 5 bed volumes) to remove the bound substances. Strongly bound proteins can be removed by washing with 0.1 M HCl. If HCl is used, the gel must be re-equilibrated to a

higher pH as soon as possible after washing. The gel is then ready for re-equilibration with start buffer at the desired pH. The resin is stored, in the dark, at 4°C in 24 % ethanol as an antimicrobial agent.

2.2.6 SDS-Polyacrylamide Gel Electrophoresis:

Polyacrylamide slab gel electrophoresis, in the presence of the anionic detergent sodium dodecyl sulfate (SDS), was performed on 4 % stacking gel and 12 % or 15 % separating gel in a discontinuous buffer system as described by Laemmli (1970). The six proteins given below were used as molecular weight standards.

- Bovine Albumin (M_r 66000)
- Egg Albumin (M_r 45000)
- Carbonic Anhydrase (M_r 29000)
- Trypsinogen (M_r 24000)
- Trypsin Inhibitor (M_r 20100)

2.2.6.1 Preparation of Reagents:

(A) Stock Separating Gel Buffer: (1.5 M Tris-HCl, pH 8.8)

36.3 gm Tris base were dissolved in about 100 ml distilled water and pH 8.8 was adjusted with 1 M HCl. Finally completed to 200 ml.

(B) Stock Stacking Gel Buffer :(0.5 M Tris-HCl, pH 6.8)

12.1 gm Tris base were dissolved in about 100 ml distilled water and pH 6.8 was adjusted with 1 M HCl. Finally completed to 200 ml.

(C) Stock Gel Solution: (Acrylamide-BIS, 30 % A, 2.67 % C)

60.0 gm acrylamide were dissolved in about 175 ml distilled water and then 1.6 gm BIS (Bis-acrylamide) were added and solution was completed to 200 ml with distilled water. Finally, the solution was filtered through course filter paper.

Note: % A represents acrylamide monomer percent concentration and % C indicates the crosslinking monomer concentration, which were calculated as below:

% A = [(gm acrylamide/total volume)] X 100

% C = [gm BIS/(gm acrylamide + gm BIS)] X 100

(D) 10 % SDS Solution:

10 gm SDS were dissolved in water with gentle stirring and completed to a final volume of 100 ml.

(E) Catalyst: (10 % Ammonium Persulfate “APS”)

Prepared freshly by dissolving 100 mg ammonium persulfate (APS) in a final volume of 1 ml distilled water.

(F) Tracking Dye: (0.05 % Bromophenol Blue)

Tracking dye solution was prepared by dissolving 5 mg solid bromophenol blue in a final volume of 10 ml.

(G) 5 X Electrode (Running) Buffer: (25 mM Tris, 192 mM Glycine, pH 8.3)

Stock running buffer solution was prepared by dissolving and completing 15 gr Tris base, 72 gr glycine to 1 liter distilled water. The pH of the buffer was not adjusted with acid or with base. This buffer was diluted 1:5 and 1 gr solid SDS was added to 1 liter of buffer before use.

(H) 4 X Sample Dilution Buffer: (SDS Reducing Buffer)

0.25 M Tris-HCl buffer, pH 6.8 containing 8 % SDS, 40 % glycerol, 20 % 2-mercaptoethanol, 0.004 % bromophenol blue. It was prepared by mixing the following volumes of given solutions :

2.5 ml	1 M Tris-HCl, pH 6.8
4.0 ml	Glycerol
2.0 ml	2-mercaptoethanol
0.4 ml	Tracking Dye
0.8 gm	SDS
Distilled water to 10.0 ml	

2.2.6.2 Electrophoresis Procedure:

Vertical slab gel electrophoresis was carried out using the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A.) that can be used to run two gels simultaneously. The assembly of the glass plate cassettes (8.3 X 7.4 cm) and the process of gel casting were done according to instruction manual provided with the apparatus. Once the cassettes were properly assembled and mounted, the preparation of the separating and stacking gels was started.

The 12 % or 15 % separating gel and 4 % stacking gel polymerizing solutions were prepared just before use by mixing the given volumes of stock solutions in the written order as given in **Table 7**. The separating gel solution was first prepared with the TEMED added just before casting the gel into the glass assembly from the edge of one of the spacers until the desired height of the solution (about 5 cm) was obtained. Then, the liquid gel was overlaid with distilled water (about 0.5 ml), without disturbing the gel surface, to obtain an even interface between the separating gel and the stacking gel. The gel was then allowed to polymerize at room temperature for a minimum of 30 minutes. After polymerization, the layer of water was removed completely using filter paper without hurting the gel surface. The stacking gel was then poured on the top of the resolving gel and the comb was inserted into the layer of the stacking

gel without trapping air bubbles under the teeth of the comb. The gel was then allowed to polymerize for a minimum of 30 minutes. After the gel was polymerized, the comb was removed carefully and the wells were washed with distilled water and filled with electrode buffer. At this point, the gel cassettes were removed from the casting stand, mounted and clamped onto the running frame with the notched glass plate of each cassette facing inside. When running only one gel, the blank plastic plate, provided with the system, was mounted in the place of the second cassette in the casting stand and in the running frame.

Aliquots from the protein samples to be analyzed and from the standards mixture were diluted 3:1 with the 4X sample buffer (3 parts sample and 1 part sample buffer), to have the samples in 62.5 mM Tris-HCl buffer, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue. Then the samples and standards were placed in a boiling water bath for 5 minutes. Afterwards, protein samples and molecular weight standards (5 – 25 µl) were loaded into different wells using a 25 µl Hamilton syringe with a tipped needle.

Table 7. Formulations for SDS-PAGE separating and stacking gels

	Separating Gel		Stacking Gel
Monomer Concentration	**12 %**	**15 %**	**4 %**
Acrylamide/bis	12.0 ml	15.0 ml	1.3 ml
Distteled water	10.0 ml	7.0 ml	6.1 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	----
0.5 M Tris-HCl, pH 6.8	----	----	2.5 ml
10% (w/v) SDS	300 µl	300 µl	100 µl
10 % APS	185 µl	185 µl	50 µl
TEMED	15 µl	15 µl	10 µl
Total monomer	30 ml	30 ml	10 ml

After loading the samples, the running buffer (135 ml) was added to the compartment formed by the running frame and the cassettes (the upper buffer compartment) and the system was checked for leakage. The running buffer (250ml) was then also added to the outer tank (the lower buffer compartment). Thereafter, the running frame was inserted into the outer tank, the safety cover was replaced and the leads were plugged into the EC250-90 electrophoresis power supply. The power supply was adjusted to give a constant current of 15 mA when the samples were in the stacking gel and 30 mA when the samples passed to the separating gel. Under these conditions the voltage was about 50 V at the beginning and elevated up to 150 V at the end of the run that took a total of about 1.5 hours. The power supply was switched off, when the dye front is just 0.5 cm from the lower end of the glass plates, the running frame was taken out and the buffer was removed from the upper buffer compartment. Afterwards, the clamps were detached and the cassettes were removed from the running

frame. To gain access to the gels in the cassette, the glass plates were pried apart using a spatula taking care not to chip the edges of the glass plates. The left-top corner of each gel was cut to indicate the order of wells. The gels, usually adhered to one of the glass plates, were taken carefully using gloves and placed in the previously prepared appropriate solutions to stain the samples which have been resolved on the gels, or to prepare the gels for subsequent blotting.

2.2.6.3 Silver Staining of the SDS-PAGE Gel:

The silver staining of the SDS-PAGE gels was carried out with a revised method of Blum and co-workers (Blum *et al.,* 1987) as explained in **Table 8**.

The relative mobility (R_f) of each protein was determined by dividing its migration distance from the top of the separating gel to the center of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel.

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

Table 8. Procedure for rapid Method of Silver Staining of Proteins in Polyacrylamide Gels.

Steps	Solution[a]	Time of Treatment
1) Fix	40 % Methanol; 0.5 ml 37 % HCOH /liter water	Incubate for 10 min.
2) Wash	water	2 X 5 min
3) Pretreat	$Na_2S_2O_3.5H_2O$ (0.2 g/liter)	1 min
4) Rinse	H_2O	2 X 20 sec
5) Impregnate	$AgNO_3$ 0.1 %	10 min
6) Rinse	Once with H_2O, once with developer solution.	2 X 20 sec
7) Develop	Na_2CO_3 3% (wtg/v) 0.5 ml 37 % HCOH /liter*** 0.0004% (wtg/v) Na2S2O3.5H_2O	Agitate slowly until bands appears.
8) Stop	Add 2.5 ml of 2.3 M citric acid/ 50 ml developer solution	10 min
9) Wash	water	10 min
10)Drying Soln.	10% Ethanol 4% Glycerol water	10 min

a The solutions should be prepared freshly *** add formaldehyde immediately before use

The R_f values (abscissa) were plotted against the known molecular weights (logarithmic scale ordinate) and standard line was drawn and its slope was used in the calculation of the molecular weight of proteins.

2.2.7 Western Blotting:

2.2.7.1 Electroblotting of the Gels from SDS-PAGE:

Electroblotting was carried out using EC140 Mini Blot Module of the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A.), and Polyvinylidene difluoride (PVDF) was used as a blotting membrane. The gels obtained from the SDS-PAGE were electroblotted directly.

Prior to electroblotting, the gels taken from SDS-PAGE were placed for 30 min, with shaking, in the Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol) (Towbin *et al.*, 1979).

While the gels were incubated in the transfer buffer, the other system components and the transfer membrane were prepared. All of the electroblotting procedure was carried out wearing gloves. The PVDF transfer membrane, with the dimensions of the gel to be transferred, was soaked in 100 % methanol for 30 seconds with shaking, to overcome the hydrophobicity of the membrane. Then, the wet membrane is washed several times with distilled water and then with transfer buffer until it was equilibrated (it should submerged into the solution not floating over it), the point at which the membrane is ready to bind the proteins. The membrane should not be allowed to dry, otherwise proteins will not bind to it. The membrane does dry during the procedure, the wetting procedure should be repeated again. Afterwards, two pieces of filter paper, the Scotch Brite sponge pads, and the transfer membrane were soaked in the transfer buffer for 15 min with continuous shaking.The blotting stack was assembled on the top of stainless steel grid cathode located in the trough of the frame stand of the Mini Blot Module, to which a small amount of transfer buffer was added. The configuration of the assembly was as follows:

Top	Cover with Palladium Wire Anode
	Sponge Pad
	Sponge Pad
	Filter Paper
	PVDF Transfer Membrane
	Gel
	Filter Paper
	Sponge Pad
Bottom	Frame stand with Stainless steel Grid Cathode

After the above assembly was prepared, the cover of the electroblotting module was pressed onto the blotting stack and fixed with the clamps after turning assembled blotting module upright and then filled with the transfer buffer (about 100 ml). Thereafter, the fully assembled module was inserted into the outer tank and the safety cover with leads was replaced. The red lead was connected to the anode (+) and the black lead to the cathode (-), were the proteins will be transferred as anions to the direction of anode. The transfer process was performed at room temperature for 50 minutes using a constant voltage of 15–20 V. when the blotting was finished, the PVDF membrane was immediately removed and placed in the proper solutions, previously prepared, either for total protein staining or immunostaining.

2.2.7.2 Immunostaining of the PVDF Membranes

Immunostaining was carried out according to the instruction manual provided with the Amplified Alkaline Phosphatase (AP) Western Blotting Kit (Bio-Rad) that was used in the immunostaing of the electroblotted PVDF membranes. All of the incubations were performed in a minimum of 5 ml of solutions in each step with continuous shaking at room temperature.

The electroblotted PVDF membrane was incubated in the blocking solution (5 % non-fat dry milk in TTBS buffer) for 1 to 2 hours. Afterwards, the membrane was incubated with the antibodies diluted in the blocking solution for 1 to 2 hours, in the polyclonal anti-GSTT2-2 (1/5,000 diluted) or the polyclonal anti-GST mu, pi and alpha (1 / 7,500 diluted) were used. The membrane was then washed five times, each for 5 min with TTBS and incubated with the secondary antibody (biotinylated goat anti-rabbit) with the 1/7,500 dilution in TTBS for 1 to 2 hours. During the secondary antibody incubation period, the streptavidin - biotinylated AP complex was prepared by the addition of streptavidin to biotinylated AP (both 1/7,500 diluted in TTBS) and allowed to stand at least 1 hour and not more than 3 hours at room temperature.

After the incubation with secondary antibody, the membrane was washed again with TTBS (five times, each 5 min) and then incubated for 1-2 hours in the previously prepared streptavidin - biotinylated AP complex. Afterwards, the membrane was washed three times, 5 min each, again with TTBS and the AP color developing solution (BCIP/NBT) was added. The specific protein bands started to appear after 10 - 30 min. Finally, the membranes were carefully dried and the images were obtained using a scanner connected to the computer.

CHAPTER III

RESULTS

3.1 The GSTs Activities of Bovine Liver Cytosolic Fractions Against MS, CDNB and 4-NBC:

Glutathione S-transferases (GSTs) activities in the cytosolic fractions prepared from bovine liver were determined spectrophotometrically using 1-menapthylsulfate (MS), 1-chloro-2,4-dinitrobenzene (CDNB) and 4-nitrobenzyl-chloride (pNBC) as substrates by monitoring the thioether formation at 298 nm, 340 nm and 310 nm, respectively, as described by Habig and co-workers (Habig *et al*., 1974).

The average value of GST enzyme activity of bovine liver cytosols was calculated as 21.2 ∓ 1.45 (Mean ∓ S.E., n = 29) unit/ml towards 1-MS and 6744.3 ∓ 885 (Mean ∓ S.E., n = 29) unit/ml towards CDNB.

3.2 Purification of GSTT2-2:

The purification of the GST class **Theta** GSTT2-2 from bovine liver was carried out basically according to the purfication protocols used by Hiratsuka et al., (1997) with some modifications. This method included the sequential Anion exchange DEAE cellulose column, *S*-hexylglutathione agarose affinity, dye-ligand (Matrex Gel Orange A) and chromatofocusing column chromatography.

3.2.1. DEAE Cellulose Anion Exchange Column Chromatography :

As described under "Methods", cytosolic fractions of bovine liver having total of 600 mg protein and 25,6 unit/mg of GST enzyme activity against 1-MS, 180 unit/mg of GST enzyme activity against CDNB and 6.5 unit/mg of GST enzyme activity towards pNBC were applied to the column (1.5 X 15.0 cm).

As it is shown in **Table 10** with this step almost 67.% of cytosolic proteins were eliminated. The flow through fractions having highest activity towards 1-MS were combined, pooled and applied immediately to the *S*-hexylglutathione agarose affinity column. The combined fraction had a total of 100 mg protein with 38.7 unit/mg of GSTs activity towards 1-MS and 410 unit/mg of GSTs activity towards CDNB and 18,6 unit/mg of GSTs activity towards pNBC. According to this result DE52 column provided about 1.51 purification fold with about 50.3 % recovery of the enzyme activity.

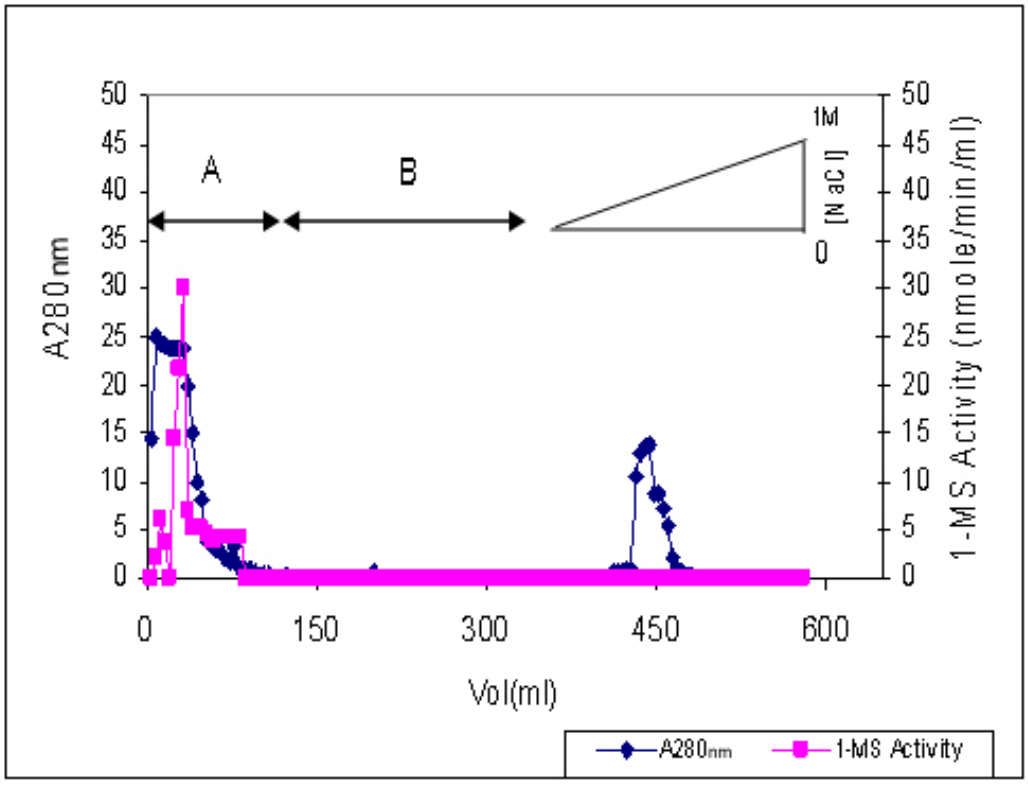


Figure 26 : Purification profile for bovine Liver GSTT2-2 on DEAE Cellulose anion exchange column chromatography. The freshly prepared cytosolic fraction having 30 mg/ml protein and 25.6 unit/mg of GST activity against 1-MS, 410 unit/mg of GST activity against CDNB and 195 unit/mg of GST activity towards pNBC were applied to the column as explained under 'Methods'. A: Sample Application, B: Washing C: Gradient with 100ml washing buffer and 100 ml washing buffer containing 1M NaCl.

3.2.2 Affinity Column Chromatography on *S*-Hexylglutathione Agarose:

As described under "Methods", the combined fraction, from the previous step, was applied to the *S*-hexylglutathione agarose affinity column (1.0 cm X 5.0 cm). Afterwards, the column was washed with the equilibration buffer until no absorption of effluent at 280nm was detected, and then the column was further washed with the same buffer containing 0.2 M NaCl. Afterwards, the bound proteins were eluted with 20 ml of 50 mM Tris-HCl buffer, pH 9.5 containing 0.2 M NaCl and 10 mM GSH.

The unbound fractions with the highest activity against 1-MS were combined in one fraction for further purification (**Figure 27**).

The combined fraction had a total of 20.25 mg protein with 27 unit/mg of GST enzyme activity towards 1-MS and 10.3 unit/mg of GSTs against pNBC. Although it was very small when compared with that of eluted fractions CDNB activity was also determined in this combined fractions as a value of 72 unit/mg According to these results, the *S*-hexylglutathione agarose affinity column provided about 1.05 purification fold and 16 % recovery of the enzyme activity.

The enzyme activity in the bound fractions towards CDNB was determined as 4791 unit/mg.

The unbound combined fraction from the affinity column, with most of the GSTs activity against 1-MS, was applied immediately to the dye binding orange A column for further purification.

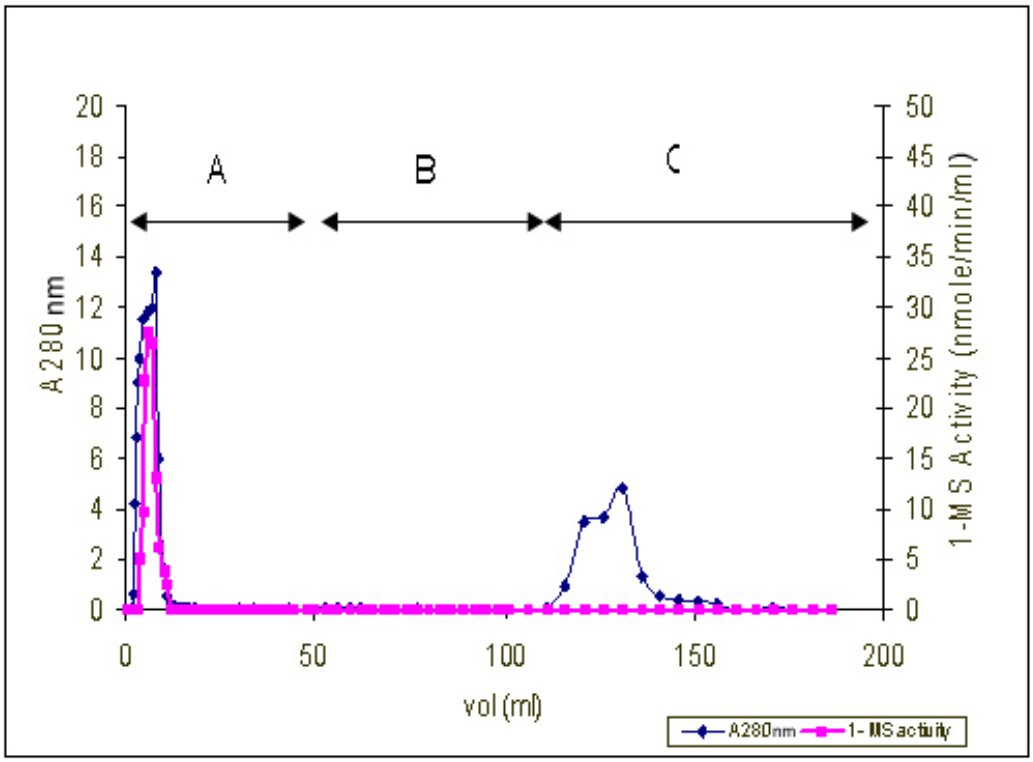


Figure 27 : Purification profile for bovine Liver GSTT2-2 on S-Hexylglutathione Affinity column chromatography. A: Sample application and washing B: Washing with equilibration buffer containing 0.2 M NaCl, C: Elution of the bound GSTs with 50mM Tris-HCl, pH: 9.5, containing 0.2 M NaCl, 10mM GSH.

3.2.3 Dye-Ligand Column Chromatography on Matrex Gel Orange A:

As described under "Methods" the fractions from previous step of affinity chromatography having the highest activity against 1-MS were applied to the column (1.0 cm X 15 cm). Afterwards, the column was washed with the equilibration buffer until no absorption of effluent at 280 nm was detected. The bound proteins were eluted from the column with a linear NaCl gradient consisting of 100 ml of the equilibration buffer and 100 ml of the same buffer containing 1.0 M NaCl.

As shown in **Figure 28**, all of the GSTs with activity against 1-MS bound to the column and the fractions with the highest activity against 1-MS were eluted in the salt gradient at about 0.2 M NaCl. The fractions having highest activity towards 1-MS were pooled in one fraction and collected for further purification. The combined fraction of this step had a total protein of 1.68 mg with 98 unit/mg of enzyme activity towards 1-MS and 55 unit/mg of pNBC respectively.

The pooled fractions from the Orange A purification steps were combined together and dialysed against 1 liter of 25 mM ethanolamine-acetic acid buffer, pH 9.6, containing 2 mM 2-mercaptoethanol for 8 hours at 4°C. Afterwards, the sample was applied to the chromatofocusing column.

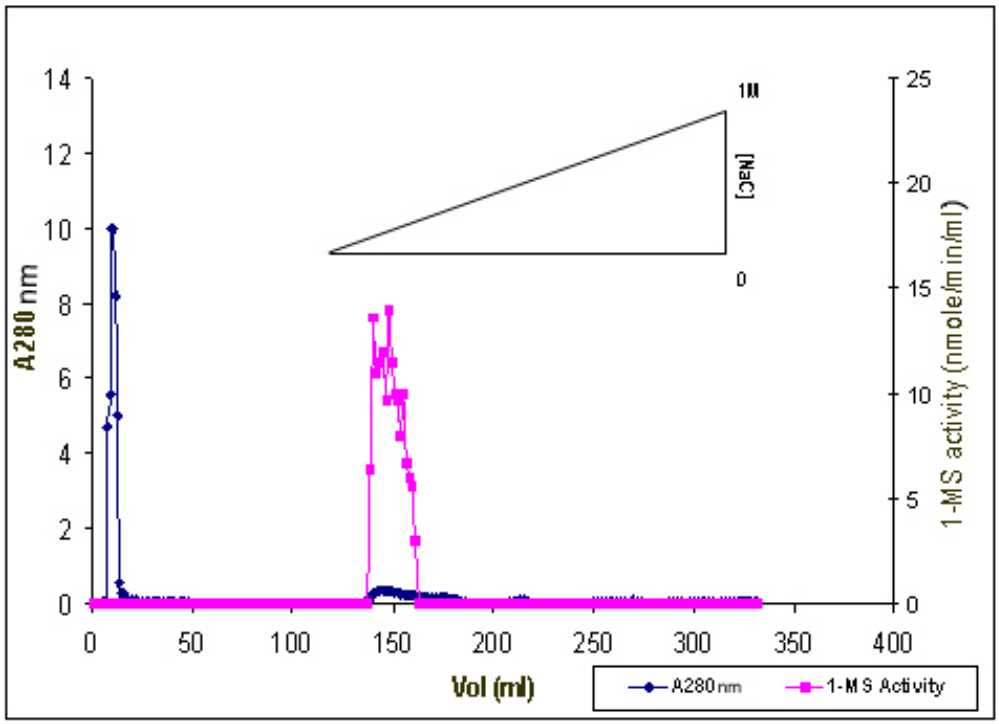


Figure 28: The Purification Profile of bovine liver cytosolic GSTs (GSTT2-2) on Matrex Gel Orange A dye Binding column (1.0 cm X 15 cm).

3.2.4. Chromatofocusing Column Chromatography:

As described under "Methods", the dialyzed combined fraction from previous Matrex Gel Orange A chromatography step was applied to a Polybuffer exchanger PBE 94 chromatofocusing column (1.0 cm X 17 cm). The combined fraction containing a total of 1.68 mg protein with 98 unit/mg of GST enzyme activity towards 1-MS was loaded to the column at a flow rate of 10 ml/hour. Afterwards, the column was washed with the equilibration buffer until no absorption of effluent at 280 nm was detected.

The pH gradient was developed with about 120 ml of a 10 % (v/v) Polybuffer 96 – acetic acid , pH 6.0, containing 2 mM 2-mercaptoethanol. Fractions of 1.0 ml each were collected and the pH values of the collected fractions as well as the GSTs activity against 1-MS were measured. The fractions with the highest activity against 1-MS were eluted in the gradient at pH 7.2 – 7.3 (**Figure 29)**. The combined fraction had a total of 1.8 mg protein with 77 unit/mg of GSTs activity towards 1- MS .

All of the purification process results are summarized in **Table 9** for the purification of GSTT2-2 from bovine liver cytosolic fraction

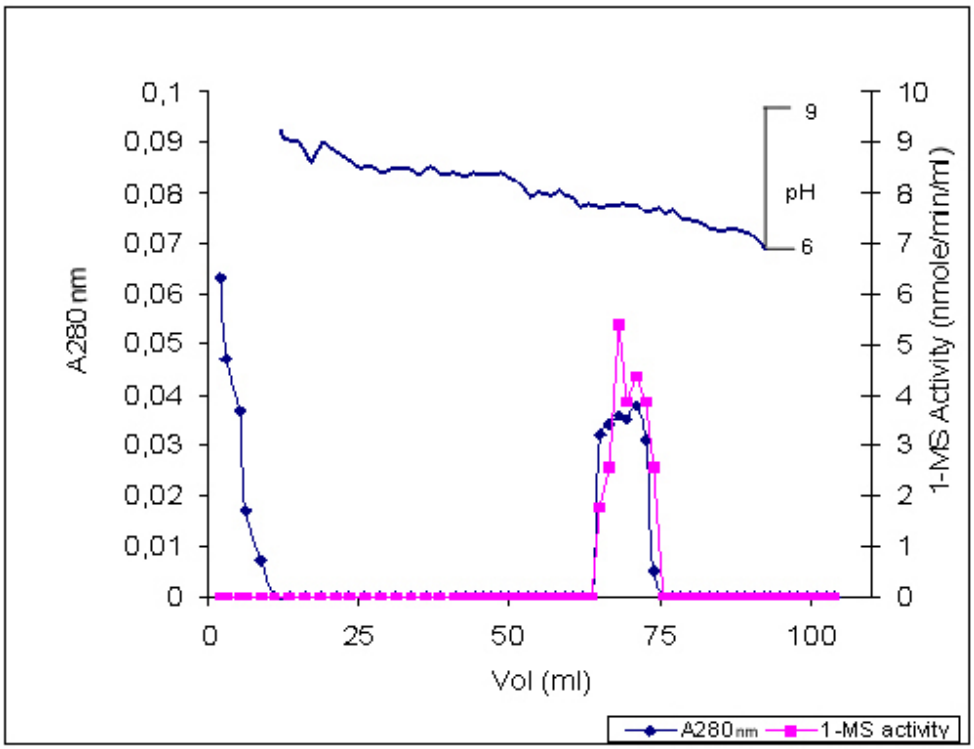


Figure 29: The Purification Profile of bovine liver cytosolic GSTs (GSTT2-2) on the chromatofocusing column (1 cm X 17 cm).

Table 9 : Purification table for GSTT2-2 from bovine liver

FRACTIONS	Vol (ml)	Protein (mg/ml)	Total Protein (mg)	Protein Recovery (%)	Total Activity nmole/min	Activity Recovery (%)	SA (nmole/min/mg)	Enrichment
Bovine Liver Cytosol	20	30	600	100	769	100	25.6	'1'
DE52 Column	10	10	100	33.3	387	50.3	38.7	1.5
Affinity Column	4.5	4.5	20,25	15	122	15.86	27	1.05
Orange A Column	4	0.42	1.68	1.4	41	5.3	98	3.8
Chromatofocusing Column	6	0.3	1.8	1.5	23	3	77	3

Table 10: Enzyme activities with different substrates during purification steps.

	1-MS (unit/mg)	**CDNB (unit/mg)**	**4-NBC (unit/mg)**
Cytosol	25,6	180	6.5
DE 52 Column Eluate	38,7	410	18.6
Affinity Column Eluate	27	72	10.2
Affinity Bound Eluate	-	4791	-
Orange A Eluate	98	-	55
Chromatofocusing Eluate	77	-	ND

3.3 Immunological Characterization of the Purified GSTT2-2:

3.3.1 SDS-PAGE and Western Blotting:

The fraction eluted from the purification columns and the purified GST (GSTT2-2) were analyzed by SDS-PAGE in order to resolve the subunit composition of GSTT2-2 from bovine liver cytosol.

Figure 30 shows the photograph of one typical gel from SDS-PAGE after silver staining. The lanes contained molecular weight markers and fractions from the different purification steps. The SDS-PAGE revealed that the GSTT2-2 is a homodimer with a subunit molecular weight of about 25,200 Da. This was also confirmed by the Western Blotting, where the gel obtained from the SDS-PAGE was electroblotted onto PVDF membrane and then immunostained with polyclonal GSTT2-2 antibodies using the amplified alkaline phosphatase immunblotting kit (**Figure 31**).

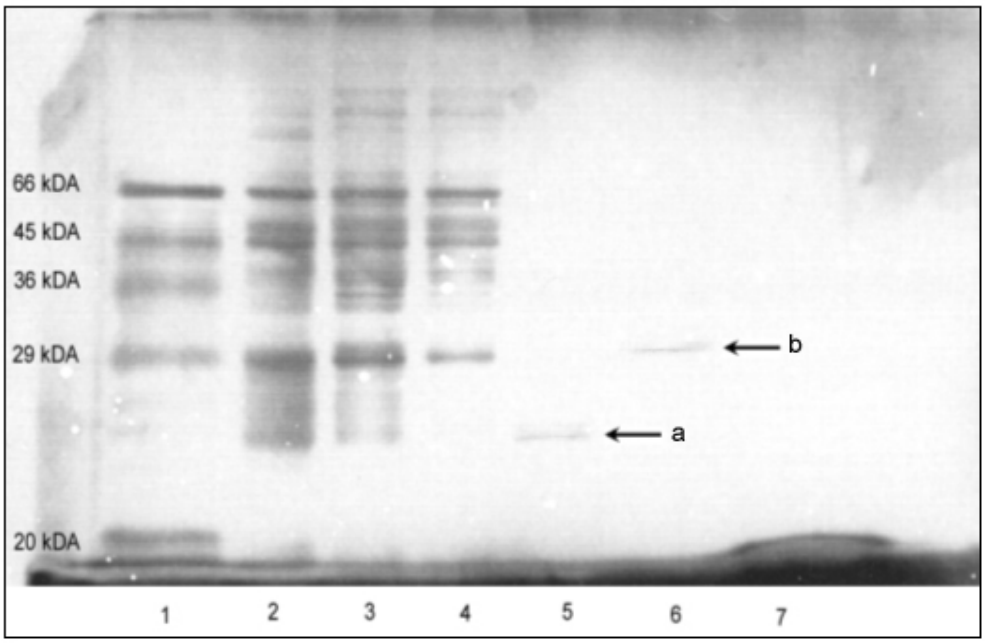


Figure 30: SDS-PAGE stained with silver (12 %), of the purification fractions and molecular weight markers (Each wel contains 10 µg of protein except for Affinity bound fraction which has 3 µg of protein).

Lane 1	SDS-PAGE Standards
Lane 2	Cytosolic Fraction
Lane 3	DE 52 Column Pooled fraction
Lane 4	S- Hexylglutathione Affinity Column Pooled Fraction
Lane 5	S- Hexylglutathione Affinity Bound Fraction (Arrow a)
Lane 6	Orange A Column Pooled Fraction (Arrow b)
Lane 7	Chromatofocusing Column Pooled Fraction

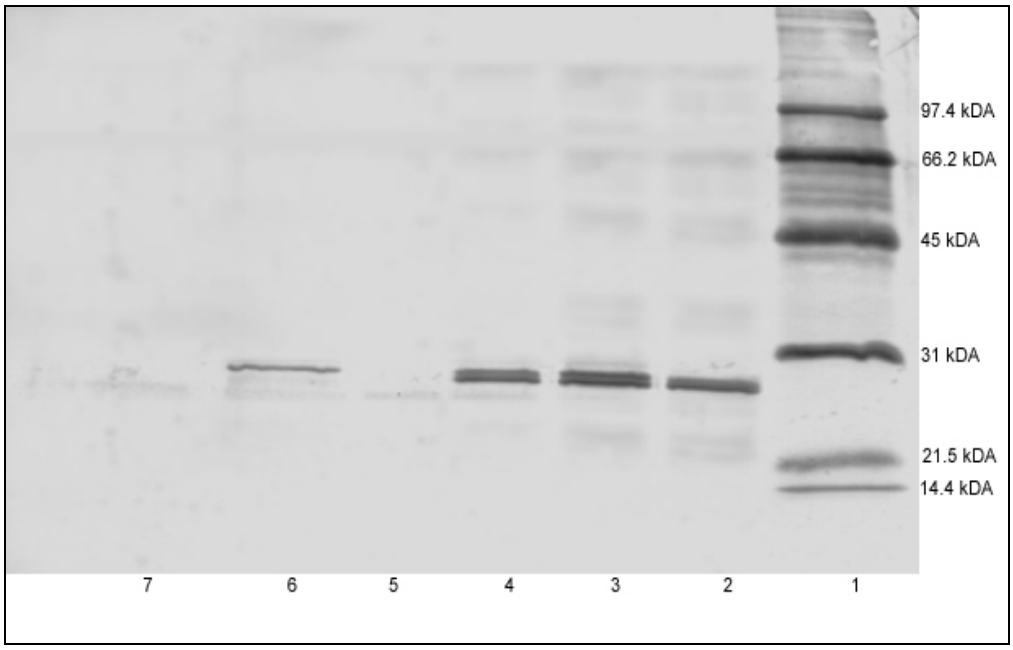


Figure 31: Western blotting of the purification fraction (each well containing 20 µg of protein) (PVDF membrane immunostained with polyclonal anti-GSTT2-2 antibody).

Lane 1	Biotinylated SDS_PAGE Standard
Lane 2	Cytosolic Fraction
Lane 3	DE 52 Column Pooled fraction
Lane 4	S- Hexylglutathione Affinity Column Pooled Fraction
Lane 5	S- Hexylglutathione Affinity Bound Fraction
Lane 6	Orange A Column Pooled Fraction
Lane 7	Chromatofocusing Column Pooled Fraction

To confirm the accuracy of this study of GST enzyme purification, as a positive control we have blotted the fractions from purification steps with other GST antibodies other than theta.

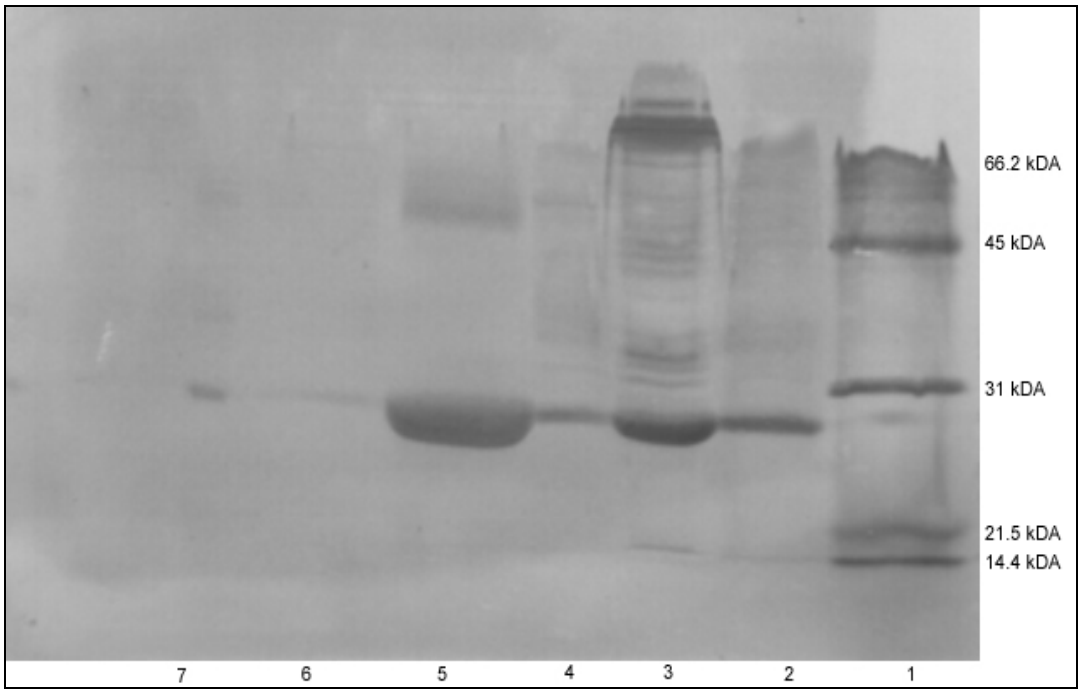


Figure 32: Western blotting of the purification fraction (each well containing 20 µg of protein) (PVDF membrane immunostained with polyclonal anti-GST alpha)

Lane 1	Biotinylated SDS_PAGE Standard
Lane 2	Cytosolic Fraction
Lane 3	DE 52 Column Pooled fraction
Lane 4	S- Hexylglutathione Affinity Column Pooled Fraction
Lane 5	S- Hexylglutathione Affinity Bound Fraction
Lane 6	Orange A Column Pooled Fraction
Lane 7	Chromatofocusing Column Pooled Fraction

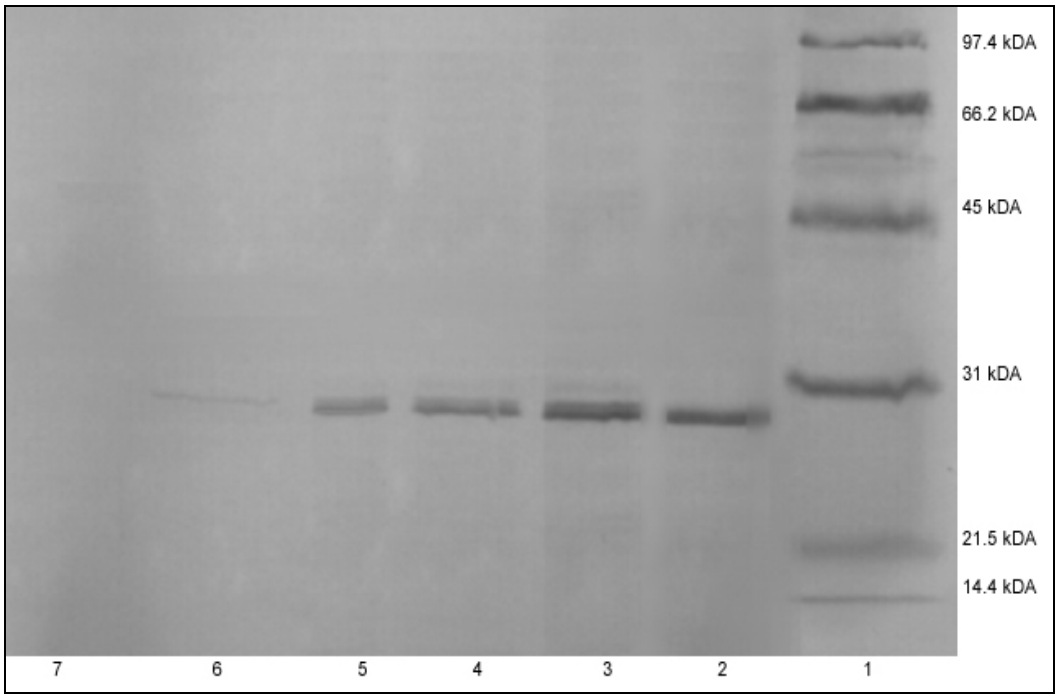


Figure 33: Western blotting of the purification fraction (each well containing 20 µg of protein) (PVDF membrane immunostained with polyclonal anti-GSTpi antibody).

Lane 1	Biotinylated SDS_PAGE Standard
Lane 2	Cytosolic Fraction
Lane 3	DE 52 Column Pooled fraction
Lane 4	S- Hexylglutathione Affinity Column Pooled Fraction
Lane 5	S- Hexylglutathione Affinity Bound Fraction
Lane 6	Orange A Column Pooled Fraction
Lane 7	Chromatofocusing Column Pooled Fraction

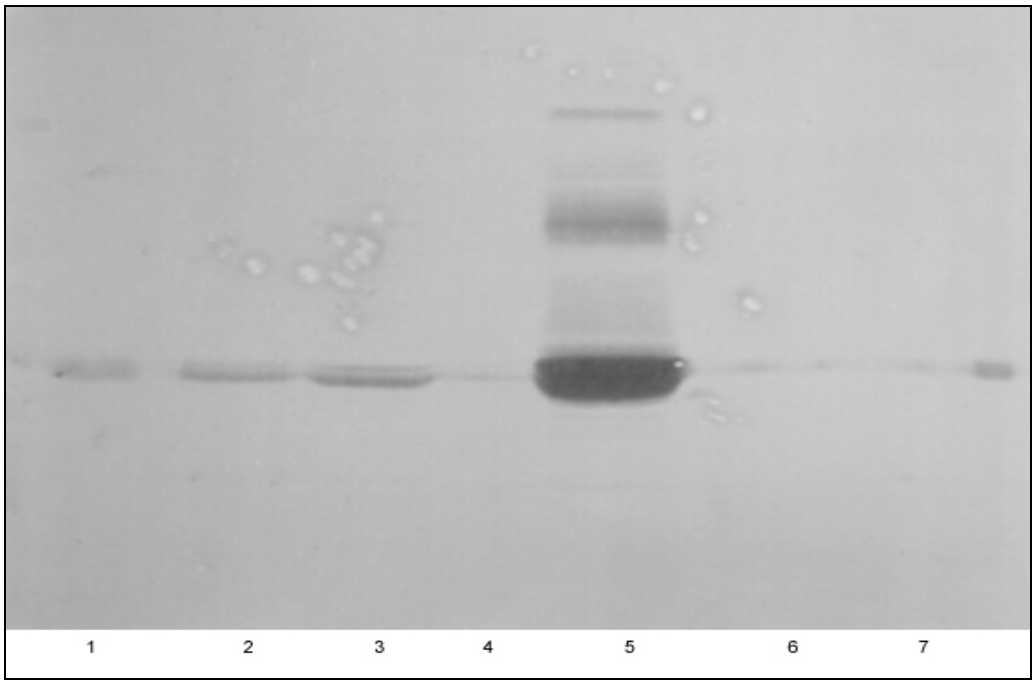


Figure 34: Western blotting of the purification fraction (each well containing 20 µg of protein) (PVDF membrane immunostained with polyclonal anti-GSTMu antibody).

Lane 1	Biotinylated SDS_PAGE Standard
Lane 2	Cytosolic Fraction
Lane 3	DE 52 Column Pooled fraction
Lane 4	S- Hexylglutathione Affinity Column Pooled Fraction
Lane 5	S- Hexylglutathione Affinity Bound Fraction
Lane 6	Orange A Column Pooled Fraction
Lane 7	Chromatofocusing Column Pooled Fraction

CHAPTER IV

DISCUSSION

It has been suggested that the Glutathione-S-transferases (EC2.5.1.18) are a group of multifunctional proteins involved in the detoxification of a broad spectrum of xenobiotics (Jackoby and Habig 1980). The conjugation of toxic electrophiles with GSH not only decreases their ability to modify macromolecules but allows their elimination from the cell via the glutathione-S-conjugate efflux pumps (Ishikawa 1988).

It has been also concluded that, besides their catalytic functions, in which reduced glutathione participates as a nucleophile, the glutathione-S-transferases act as ligand binding proteins, thus lowering the intracellular concentration of a wide spectrum of hydrophobic nonsubstrate chemicals such as heme, billirubin and steroids (Mannervik 1988, Coles and Ketterer 1990).

GSTs also play a key role in preventing a variety of reactive metabolites from their attacks on cellular biomacromolecules which may induce necrosis and tumorigenesis of tissues. Either excessive formation or relatively low GST mediated scavenging of the reactive metabolites may result in their covalent binding to DNA, causing mutation or death of cells.

The first genetic studies of the GSTs in humans demonstrated the existence of three genetically distinct groups of cytosolic GST isoenzymes.

Further studies by Mannervik et al., (Mannervik 1985) demonstrated that these groups were found in other mammals and represented three evolutionary classes termed alpha, mu and pi. Subsequent studies have identified an additional class of cytosolic GSTs termed Theta (Board 1997). The theta class GSTs are characterized by the presence of an active-site serine residue, which contrasts with an active-site tyrosine residue that is conserved throughout the alpha, mu and pi class GSTs (Board 1997). Therefore mammalian GSTs were subdivided into six species independent gene classes according to their sequence homologies and enzymatic, physicochemical and immunological properties. The classes are termed alpha, mu, pi, sigma, zeta and theta. All mammalian cytosolic GSTs occur as homo or heterodimers of the kinetically independent subunits (Kunze 1997). After that in 1996, Pemble *et al*., has described, a soluble, mitochondrial specific GST termed kappa, although little is known about the structure, function or evolution of this class.

A membrane bound microsomal GST has been well characterized and seems to be structurally and genetically distinct from the cytosolic enzymes (Board 1997). Three membrane bound glutathione transferases are known, one of which appears to be involved in xenobiotic metabolisms. Microsomal GSH transferase I is an integral membrane protein that has been characterized from both rats and humans where it is found in large amounts in liver and is distributed in both microsomal and outer mitochondrial membranes.

Expression level of GST and factors effecting them have significant biological and clinical implications. Overexpression of some GSTs display resistance to certain anti-cancer drugs. (Hayes and Pulford 1995). On the other hand some genetic deficiencies in these enzyme class are risk factors for cataract and several forms of cancer such as lung and liver carcinoma in rats. (Sherrat 1997).

Recently, a polymorphism for the human GSTT2 gene has been described in Australians and Europeans, but this needs to be investigated further (Coggan, 1998).

Unlike the class alpha, mu, pi GSTs, the class Theta enzymes are not abundant, stable or readily purified by affinity chromatography (Meyer 1991). Eventhough, the rat and mouse GST T1-1 enzymes have been studied by several research groups, little progress has been made in characterizing the human transferase at the protein level. Meyer has purified GSTT1-1 from human liver sufficient for amino acid sequencing, however, it was insufficient to allow either its catalytic properties to be established or antibodies to be raised against it (Meyer 1991).

The theta class GST family contains a desperate group of enzymes from a diverse range of organisms, including bacteria, plants and insects. The mammalian theta class GST family have been only recently identified in humans, rats and mice. The inability of this class of enzymes to bind to GSH affinity matrices which is routinely used for GST purification and their low activity with the common CDNB, have meant that they have been the least studied of the established mammalian GST classes (Board, Coggan and Wilce 1995).

The factors for hGSTT2-2 and presence of a buried sulfate binding pocket at one end of the H site make the mammalian theta class enzymes specific for a much narrower range of substrates and suggest that they could play very specific detoxification role in contrast to the other GST classes (Rossjohn 1998).

To date, two theta class GSTs hGSTT1-1 and hGSTT2-2, have been identified, cloned and sequenced from human tissues. These enzymes share 55% sequence identity and exhibit distinct substrate specificity (Pemble 1994, Tan 1996, Hussey 1992, Tan 1996).

Recently, a number of non-mammalian GST crystal structures have been determined; these include two theta enzymes from insect and plant (Wilce 1995; Reinemer 1996). These enzymes share same sequence similarity to the mammalian theta class enzymes and they both adopt the canonical GST fold.

The two known theta class structures have implicated a serine residue in promoting the thiolate anion form of GSH and this observation has been supported by site directed mutagenesis (Board 1995, Tan 1996).

In comparison to GSTs of known three dimensional structure, hGSTT2-2 is novel in that it possesses 40 residue C-terminal extension and a unique sulfatase activity. Furthermore, hGSTT2-2 displays less than 15% interclass sequence identity and the structure is therefore expected to reveal additional unique features beyond that seen in published GST structures.

For the crystal structure study of mammalian theta class Rossjohn and coworkers have found that a serine residue appear to activate substrate, GSH, rather than a tyrosine residue as found in the other mammalian GST classes. Two surprising features of the structures were found: the active site is buried, and the enzyme appears to have a purpose-built sulfate binding site. Such a site has not been observed in any other GST structure up to date.

In this study, the GSTT2-2 was purified from bovine liver by applying the cytosol sequentially to the anion exchange, S-hexylglutathione affinity, matrix gel Orange A and choromatofocusing column.

In this study the liver samples were obtained from slaughter house. The GST activities in those samples were determined by using 1-MS, CDNB and 4-NBC as substrate.

Throughout this study the most important problem was very low activity towards 1-MS to follow the subsequent purification steps. Additionally, obtaining activity from cytosolic fractions were not sufficient to continue further because of loosing activity due to instability of the enzyme throughout the study. The problem of loosing activity was also mentioned in the literature (Hussey and Hayes 1992).

In that study, it was concluded that after storage at 4ºC for 24 hour the activity of hepatic enzyme towards 1-MS was typically decreased to about 20 % of that recovered in freshly prepared liver cytosol. And Again it was concluded in that study that due to that dramatic loss of activity the sample was applied immediately, without prior dialysis, to the subsequent columns.

At the beginning of this study the sample that was used to purify GSTT2-2 was sheep liver. During studies with sheep liver cytosol the average enzyme activity towards 1-MS were found as 4.26 ∓1.95 unit/ml and that of CDNB was 27948 ∓ 745 unit/ml. Starting the purification of enzyme from sheep liver having that much activity resulted not any activity determination even at the end of the first step of purification. In other words for each application of the sheep liver cytosol to the column chromatography, we had not determine any activity towards 1-MS in any fractions at the end of the DEAE cellulose anion exchange column chromatography.

However, the high activity obtaining problem reached to the point where decision making in changing the sample was unavoidable. For this reason the different samples such as rat, bovine and sheep were searched and compared with each other based on the activity towards 1-MS. During this comparison study the activity levels of the samples were ranked and it was found out that, rat liver samples had the highest activity towards 1-MS as a value of 75.85 ∓ 2.62 unit/ml and the bovine liver comes next with the enzyme activity of 12.54 unit/ml which was more than ten times higher the value obtained from the sheep of 4.26 ∓1.95 unit/ml. Since the study with rat liver for this enzyme had completed and due to the subject problem, after two years of study on sheep liver it was decided to replace the study sample with the bovine depending on these results (**Table 11**).

After changing the study sample from sheep liver to bovine we managed to obtain high activity sample to begin the purification but another problem of loosing activity, in other words, problem in stability still existed.

Table 11: Comparison of the 1-MS activity of sheep, bovine, rat liver cytosols.

	SHEEP	BOVINE	RAT
1-MS Activity (Unit/ml)	4.26∓.95 (Mean ∓ S.E., n = 10)	12.54	75,85 ∓ 2.62 (Mean ∓ S.E., n = 4)

At the beginning of this study the cytosols were prepared by using 20mMTris-HCl buffer containing 0.15 M KCl, 2mM EDTA, 25 μM PMSF. The average enzyme activity with this conditions were calculated as 21.04 ∓ 1.41 unit/ml and 6786.7 ∓ 927 unit/ml for 1-MS and for CDNB respectively. Due to the stability problem that was mentioned above, the homogenization buffer conditions were changed with that of column of 10 mM phosphate buffer containing 0.15 M KCl, 2mM EDTA, 25 μM PMSF. The average enzyme activity of the liver samples homogenized with these condtions were found as 20.1 ∓ 1.5 unit/ml for 1-MS (**Table 12**). Although the average enzyme activity of the liver samples homogenized with these conditions resulted a little decrease, we concluded that this change would keep the enzyme activity stable during the long period of purification steps.

Table 12: Comparison of 1-MS activity with different homogenization conditions.

Homogenization performed by 20mMTris-HCl pH:7.0 containing 0.15M KCl, 2mM EDTA, 25 μM PMSF	
1-MS activity (Unit/ml)	21.04 ∓ 41 (Mean∓S.E.; n=10)
Homogenization performed by 10mMphosphate buffer pH: 7.0 containing 0.15M KCl, 2mM EDTA, 25 μM PMSF	
1-MS activity (Unit/ml)	20.1 ∓ 5 (Mean∓S.E.; n=19)

To overcome the stability problem we faced throughout this study, after changing the homogenization buffer conditions, we also tested the effect of ionic strength on enzyme stability by adding different NaCl concentration to the enzyme assay mixture. The enzyme activity which was calculated as 21.5 unit/ml for 1-MS without adding salt to the assay condition,was increased to 24.1 unit/ml with 5mM NaCl, to 25.1 unit/ml with 10 mM NaCl and to 28.2 unit/ml with 20 mM NaCl concentration indicating that, increase in ionic strength increases the enzyme activity at a maximum value 24% with the addition of 20 mM NaCl concentration to the assay mixture.

All the activity determination until now were performed at 25 ºC. The next parameter that we checked to overcome the stability problem was the temperature effect. While the enzyme activity were determined as 16.9 unit/ml at 25 ºC, the enzyme activity of the same sample was determined as 29.7 unit/ml at 37 ºC towards 1-MS meaning 76% increase in enzyme activity. This result were supported by testing this effect with another new liver sample. The enzyme activity of this sample at 25 ºC was determined as 18.5 unit/ml and increase in temperature to 37 ºC caused increase in the enzyme activity of this sample to the value of 26.5 unit/ml, in other words, around 43% increase in enzyme activity observed. With this results it was concluded to perform all the enzyme assays at 37 ºC .

In this study the method of Hiratsuka *et.al.*,(1997) was followed with some modifications. The first modification was the use of Matrix Gel Orange A instead of Blue Sepharose 6B. Although the functions of both columns were the same Matrix Gel Orange A was preferred because it was previously used for human breast tumor GSTT1-1 purification in our laboratory.

In the original protocol; since the homogenization buffer was different from that of columns the purification study was started by dialyzing the homogenate towards the column buffers. At this stage after several trials made, the second modification was decided by changing the homogenization buffer with that of columns by the addition of some preservatives such as DTT, resulted in the elimination of the dialysis step taking at least 24 hours in cold room. This elimination was helpful for preserving the activity throughout the study . Because we had continuos stability problem especially staying the enzyme in the cold room. The cytosol having GST activity towards 1-MS was determined as 33.6 unit/ml for a freshly prepared cytosol and the enzyme activity of the same cytosol after staying 3 days in the cold room (4 ºC) was determined as 1.15 unit/ml meaning that around 96% decrease in enzyme activity in 3 days period of time in the cold room. With an otherfreshly prepared liver sample having cytosolic GST activity towards 1-MS as

21.8 unit/ml and again after 8 days in the cold room that cytosol lost all of its activity. Not only the cytosolic fractions but also the liver pieces had lost their activity in deep freezer (-80ºC). The liver sample homogenized and the activity was determined in fresh cytosol as 16 unit/ml. A piece of the same sample again homogenized after 5 months later and the activity were determined as 5.9 unit/ml meaning that almost 63 % of cytosolic enzyme activity towards 1- MS were lost in deep freeze (-80ºC) as in the form of liver pieces.

. Cytosolic fractions having highest activity towards 1-MS was applied to anion exchange column and the flow through fraction from the column having high 1-MS activity were collected and immediately applied to S-hexylglutathione agarose column. The first DEAE cellulose chromatography column step provided the sample free of 60 % of the cytosolic proteins.

Except for the theta class all the soluble GSTs were removed after the S-hexylglutathione affinity column, the next chromatography step was matrix gel orange A, up to this step the activities were checked by using CDNB and 4NBC including 1-MS, since all the soluble GSTs other than theta should bind to the affinity matrices from now on there were no need to check the activity with CDNB.

The flow-through fractions from S-hexylglutathione affinity column, having highest activity, towards 1-MS were collected and immediately applied to the next column.

The next purification step was matrix gel Orange A. Since the purpose of using this column were to purify especially the theta class GSTs as explained before the T2-2 and T1-1 were bound to the column and eluted together. Although both T2-2 and T1-1 activity in this column were determined, two separate peaks could not be obtained.

But in that study Hiratsuka and co-workers with rat liver they have found that T2-2 and T1-1 come as a two separate peaks and T2-2 comes first and T1-1 next with an overlapping tails of these two isoenzyme peaks.

The problem of low level of protein containing GSTT2-2 eluted from Matrex Gel Orange A caused the further purification step efficiency loss. Instead of proceeding the purification at this stage, the fractions having high activity and protein content belonging to Matrex Gel Orange A columns of different purification steps of bovine liver samples might polled, concentrated and applied to the last chromatofocusing column. However due to the instability of this enzyme, even sometimes we could not obtain the active fractions after Matrex Gel Orange A. For this reason this application was not preferred because the purification steps took long period of time and the active fractions from previous purification sets of Matrex Gel Orange A might lost activity during the storage at –80^{o} C. The root cause of the problem was not being the cytosolic fractions only, it had found that the eluted active fractions from Matrex Gel Orange A stored in ice box, in cold room (+4^{o} C) had lost total activity towards 1-MS at the end of 24 hours period of time.

. The fractions eluted from Orange A were subjected to PBE 94 chromatofocusing column. The highest activity peak towads 1-MS was eluted in PBE 94 chromatofocusing column between pH: 7.2-7.3. This step provided 3 fold enrichment with 3 % recovery against 1-MS. And the activity against 4-NBC even detected until previous orange A step were totally not detected in the pooled fraction from this column.

In the literature, the most recent studies related with GST purification and characterization were performed by using different sources such as mollusks (Zhou Wan 2003), fungus (Cerniglia 2001) and fish (Melgar 2002, Angelucci 2000, Melgar Riol 2001). In those studies, GSTs were purified by using S-hexylglutathione affinity and then HPLC column chromatography steps.

Since those enzyme source on which the experiments had carried out, were not previously studied ones and again those studies were not targeted to isolate a specific GST isozymes those recent studies could not guide and clarify the reasons of problems especially stability problems faced during this study.

Additionally the recent studies related with GSTT2-2 were aimed to characterize the kinetic behavior of the enzyme after purifying it. In the previous study related with the enzyme kinetic from rat GSTT2-2 by using recombinant enzyme concluded that the previous report of instability and loss of activity in purified enzyme from liver extracts could not observed in that study of using recombinant rGSTT2-2 as an enzyme source. It was concluded that the recombinant enzyme had retained most of its activity in 20mM pipes buffer pH6.5 ,1M NaCl even stored at 4ºC for more tha a year (Jemth and Mannervik 1996). And depending on this result again in most recnt study related with GSTT2-2 recombinat enzyme was used (Jemth and Mannervik 1999, 2000).

The isolated isoenzyme determined from the fraction of Orange A was migrated as a single band in SDS-PAGE with the estimated subunit Mr of around 28,200 using molecular marker proteins on the same gel which was similar to the calculated value (27,000) obtained by Pemble *et. al.,* (1994) and also competible with the results of Sherrat *et. al.,* (1997) (around 28.000 Da for T1-1, that of Hiratsuka *et. al.,* (1997) and that of Abu Hijleh (1999). This result was not obtained with the fractions from chromatofocusing due to the low protein content.

The purified fractions of GSTT2-2 from bovine liver were further examined by immunoblot analysis after SDS-PAGE using anti GSTT2-2, anti GST alpha, anti GST mu, and anti GST pi polyclonal antibodies. The immunoblot analysis suggest that the purified GSTT2-2 could be detected from all of the column fractions except for the affinity bound. Because only the Theta class GSTs could not bind to the affinity matrices.

In flow through fractions of affinity column, the immunostaining had not been observed with anti GST alpha, anti GST mu, and anti GST pi polyclonal antibodies but immunostaining was observed with these antibodies in column fractions including affinity bound one indicating that all GST isozymes other than theta had bound to the affinity matrices as it was expected.

CHAPTER V

CONCLUSION

The enzyme purified with 3% recovery with the fold of 3 at the end of four chromatographic steps.

The column fractions were applied to the 12% SDS-PAGE. The silver stained SDS-PAGE had resulted by giving a single band with the affinity bound fraction and almost single band with the eluted fraction of Matrex Gel Orange A at around Mr of 28,200 Da.

The fractions from column fractions including cytosol were stained with anti GST antibodies. With the result of antibody staining, it had concluded that all the theta isozymes had eluted from the affinity column because the affinity bound fraction lane did not result in antibody staining with antiGSTT2-2. On the other hand, immunostaining the column fractions with the GST antibodies of alpha, mu and pi resulted in the staining with affinity bound fractions meaning that all the GSTs other than theta were bound to the affinity column.

Although the protein content level after the Matrex Gel Orange A decreased drastically, immunostaining with antiGSTT2-2 with this fraction also gave the positive result with this antibody.

Staining with Pi class antibody the affinity flow through fractions had also gave positive result and put us to conclude the presence of leakage in S-

Hexyglutathione Affinity Column and this result were also confirmed by obtaining the CDNB activity from flow through fractions of this column, even though this activity level was very low when compared with that of bound fractions.

Since the polyclonal anti GSTT2-2 antibody was used in this study especially in the chromatofocusing and Matrex Gel Orange A the positive stain with this antibody might also result of GSTT1-1 this result should also be confirmed by using monoclonal antiGSTT1-1 or antiGSTT2-2 antibodies.

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