Studies on Human and Drosophila melanogaster Glutathione Transferases of Biomedical and Biotechnological Interest

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To the martyrs of APS attack,
Peshawar
List of Publications

This thesis is based on the following papers, which are referred to in the text by Paper I, Paper II, Paper III and Paper IV:


IV. A.M.A. Mazari, B. Mannervik, Drosophila GSTs display outstanding catalytic efficiencies with the environmental pollutants 2,4,6-trinitrotoluene and 2,4-dinitrotoluene, Biochem Biophys Rep 5 (2016) 141-145.
Additional publications

Abstract

Glutathione transferases (GSTs, EC.2.5.1.18) are multifunctional enzymes that are universally distributed in all cellular life forms. They play important roles in metabolism and detoxification of endogenously produced toxic compounds and xenobiotics. GSTs have gained considerable interest over the years for biomedical and biotechnological applications due to their involvement in the conjugation of glutathione (GSH) to a vast array of chemical species. Additionally, the emergence of non-detoxifying functions of GSTs has further increased their biological significance. The present work encompasses four scientific studies aimed at investigating human as well as fruit fly Drosophila melanogaster GSTs.

Paper I presents the immobilization of GSTs on nanoporous alumina membranes. Kinetic analyses with 1-chloro-2,4-dinitrobenzene followed by specificity screening with alternative substrates showed a good correlation between the data obtained from immobilized enzymes and the enzymes in solution. Furthermore, immobilization showed no adverse effects on the stability of the enzymes. Paper II presents inhibition studies of human hematopoietic prostaglandin D$_2$ synthase (HPGDS), a promising therapeutic target for anti-allergic and anti-inflammatory drugs. Our screening results with an FDA-approved drug library revealed a number of effective inhibitors of HPGDS with IC$_{50}$ values in the low micromolar range. Paper III concerns the toxicity of organic isothiocyanates (ITCs) that showed high catalytic activities with GSTE7 in vitro. The in vivo results showed that phenethyl isothiocyanate (PEITC) and allyl isothiocyanate in millimolar dietary concentrations conferred toxicity to the adult fruit flies leading to death or shortened life-span. The transgenic female flies overexpressing GSTE7 showed increased tolerance against PEITC toxicity compared to the wild-type. However, the effect was opposite in male flies overexpressing GSTE7 after one week exposure. Notably, the transgene enhanced the oviposition activity of flies with and without ITCs exposure. Paper IV highlights Drosophila GSTs as efficient catalysts of the environmental pollutant and explosive 2,4,6-trinitrotoluene and the related 2,4-dinitrotoluene degradation. This result suggests the potential of GST transgenes in plants for biotransformation and phytoremediation of these persistent environmental pollutants.
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Abbreviations

GSH     Glutathione
GSTs    Glutathione transferases
DCNB    1,2-Dichloro-4-nitrobenzene
CDNB    1-Chloro-2,4-dinitrobenzene
DMSO    Dimethyl sulfoxide
ITCs    Isothiocyanates
AITC    Allyl isothiocyanate
PEITC   Phenethyl isothiocyanate
DHAR    Dehydroascorbate reductases
TCHQD   Tetrachlorohydroquinone dehalogenase
IPTG    Isopropyl-beta-D-thiogalactopyranoside
DDT     1,1,1-Trichloro-2,2-bis-(p-chlorophenyl) ethane
MAPEG   Membrane-associated proteins in eicosanoid and glutathione metabolism
JNK     Jun N-terminal kinase
ASK1    Apoptosis stimulating kinase 1
MAPK    Mitogen-activated protein kinase
APTEX   3-Aminopropyltriethoxysilane
CDI     N,N´-Carbonyldiimidazole
TEA     Triethylamine
HPGDS   Hematopoietic prostaglandin D2 synthase
PGD2    Prostaglandin D2
PGH2    Prostaglandin H2
TRPA1   Transient receptor potential A1
DNT     2,4-Dinitrotoluene
TNT     2,4,6-Trinitrotoluene
1. Introduction

The introduction is aimed at presenting a detailed overview of phase II detoxication enzyme glutathione transferases, their classification, functional roles and their importance in biomedical and in biotechnological applications.

1.1. The detoxification enzyme system

All living organisms are frequently exposed to a wide array of harmful environmental chemicals, referred to as xenobiotics. These non-nutritional foreign chemical species are potentially toxic to the organisms because they pose electrophilic centers that can readily modify nucleophilic groups in proteins and nucleic acids, causing toxic and mutagenic effects. The majority of the xenobiotics are naturally occurring compounds of animal, microbial and plant origins. Furthermore, a range of novel man-made chemicals such as pharmaceuticals, insecticides, pesticides and the by-products of urbanization that contaminate the ground water and soil pose a serious challenge to the environment and to the organisms [1]. To minimize the cellular damage from the exposure to these xenobiotics, organisms have evolved a complex three-phase detoxification system by which they metabolize these xenobiotics into less harmful peptide derivatives and facilitate their excretion from the body [2].

- Phase I (functionalization/activation): The phase I detoxification enzyme system is mainly composed of cytochrome P450 monooxygenases (P450s) and is generally considered as the first line of enzymatic defense against xenobiotics. This superfamily of enzymes metabolize a wide range of xenobiotics by using NADPH as a cofactor and molecular oxygen to introduce a reactive substituent such as a hydroxyl group that will prepare the compound for phase II conjugation reactions. Sometimes this biotransformation results into more toxic molecules than the parent xenobiotics as a consequence of this reaction [3]. If these
activated molecules of phase I response are not further metabolized by phase II detoxication enzymes, they may lead to cellular damage of proteins, nucleic acids and other tissues [4].

- **Phase II (conjugation):** The phase II detoxication enzyme system mostly consists of conjugating enzymes, which are generally responsible for the deactivation of the xenobiotics and the phase I activated metabolites by conjugation with endogenous hydrophilic molecules and facilitate their excretion from the organism. A variety of conjugation reactions occur in the body ranging from coupling to glutathione (GSH), to sulfation, to glucuronic acid, and to amino acids. Glutathione transferases catalyze the inactivation of electrophilic compounds with the tripeptide glutathione. This superfamily of enzymes are of particular importance as being one of the major contributors in the defense mechanism against xenobiotics and are the subject of this thesis [4-6].

- The Phase III detoxication system consists ATP-binding cassette super family of transporters (ABC) including proteins named as multidrug-resistance-associated protein and P-glycoprotein that recognize the glutathione conjugates as substrates and actively pump these toxins out of the cell, thereby decreasing the intracellular concentrations of xenobiotics [2,7].

### 1.2. Glutathione transferases

Glutathione transferases (GSTs) belong to a superfamily of multifunctional enzymes and have been studied for more than five decades now. The enzymes were first reported in 1961 by two independent research groups because of their involvement in the catalysis of glutathione conjugation with various arylhalides in soluble rat liver extracts [8,9]. The discovery of this enzyme family was further aided by improved purification methods allowing the separation of isoenzymes [10,11]. At the beginning, it was suggested that these enzymes should be named after the substrate they catalyzed. However, soon it was realized that these isoenzymes have overlapping substrate specificities [10,12] and this nomenclature was deemed inappropriate, so later the name glutathione transferases was assigned [5]. GSTs are widely distributed in nature and
have been identified in many groups of prokaryotes and eukaryotes including insects, plants and mammals. The primary role of GSTs as principal phase II detoxication enzymes is to catalyze the conjugation of the tri-peptide glutathione GSH (γ-glutamyl-cysteinyl-glycine) with hydrophobic cosubstrates bearing an electrophilic center and to render them into more soluble and less harmful peptide derivatives. This way, the GSTs protect the living cells from the toxic effects of electrophilic compounds of both endogenous and exogenous origins. Apart from their catalytic functions, GSTs are also involved in metabolic processes such as prostaglandin biosynthesis, steroid hormone biosynthesis, peroxide degradation, dehydroascorbate reduction, cellular signaling and other functions [1,13].

1.2.1. Classification of GSTs

Three distinct superfamilies of GSTs have been recognized so far: the cytosolic, the mitochondrial or kappa and the microsomal. The kappa class GSTs are structurally different from microsomal and cytosolic GSTs [14] and are found in peroxisomes and the mitochondria of mammalian cells. However, the kappa GSTs have not yet been identified in insects [15,16]. The microsomal GSTs, also known as membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) are homotrimeric proteins which are found in sub-cellular membranes in a wide spectrum of organisms. In humans, six microsomal GST genes have been identified [17], while in fruit fly Drosophila melanogaster only one microsomal GST gene exist [18].

1.2.2. Cytosolic GSTs

Among all the GST classes, the cytosolic GSTs (also known as soluble or canonical GSTs) are the most numerous class of enzymes ubiquitously found in almost all cellular life forms including mammals, insects, plants, bacteria and fungi [19-23] (Table 1). Based on amino acid sequence similarity and other structural properties, the cytosolic GSTs have been subdivided into various classes [21,24]. In mammals, seven different classes of cytosolic GSTs have been recognized so far and are named by Greek letters: Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta [19]. In insects six different classes of cytosolic GSTs exist, namely Theta, Omega, Zeta and Sigma classes of GSTs, which also appear to
exist in almost all eukaryotes plus additional two classes: Delta and Epsilon. Both of these cytosolic GST classes are generally found in arthropods (such as insects, crabs and mites). However, the first three classes, Alpha, Mu and Pi are only present in vertebrates such as mammals [1] and fish [25]. In plants, six different classes of cytosolic GSTs exist, which are named as Lambda, Phi, Tau, Theta, Zeta, DHAR (dehydroascorbate reductases), and TCHQD (tetrachlorohydroquinone dehalogenase) [26]. The bacterial specific classes of cytosolic GSTs identified are Beta and Chi, while the fungal GST classes are Alpha, Mu and Gamma.
Table 1. Classes and biological functions of cytosolic GSTs (adopted and modified from) [27].

<table>
<thead>
<tr>
<th>Organism</th>
<th>Class</th>
<th>Function</th>
<th>Active site residue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammals</strong></td>
<td>Alpha</td>
<td>Isomerase activities, drug metabolism, peroxidase activity, detoxication</td>
<td>Tyrosine</td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td>Drug metabolism</td>
<td>Tyrosine</td>
</tr>
<tr>
<td></td>
<td>Pi</td>
<td>Drug metabolism</td>
<td>Tyrosine</td>
</tr>
<tr>
<td></td>
<td>Theta</td>
<td>Prevention of hepatocarcinogenesis, metabolism of industrial compounds</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Zeta</td>
<td>Phe/Tyr catabolism, isomerase activity, dehalogenation, peroxidation</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Omega</td>
<td>Oxidative stress</td>
<td>Cysteine</td>
</tr>
<tr>
<td></td>
<td>Sigma</td>
<td>Prostaglandin D₂ synthesis</td>
<td>Tyrosine</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td>Beta</td>
<td>Catabolism of organic compounds</td>
<td>Cysteine</td>
</tr>
<tr>
<td></td>
<td>Chi</td>
<td>Cysteine</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Phi</td>
<td>Detoxication function, against oxidative stress, signaling function, non-catalytic binding of flavonoids, participation in intermediary metabolism</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Tau</td>
<td>Unknown</td>
<td>Serine</td>
</tr>
<tr>
<td><strong>Plants</strong></td>
<td>Tau</td>
<td>Unknown</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Theta</td>
<td>Prevention of hepatocarcinogenesis, metabolism of industrial compounds</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Zeta</td>
<td>Prevention of hepatocarcinogenesis, metabolism of industrial compounds</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Lambda</td>
<td>Oxidative stress</td>
<td>Cysteine</td>
</tr>
<tr>
<td></td>
<td>DHAR</td>
<td>Unknown</td>
<td>Cysteine</td>
</tr>
<tr>
<td></td>
<td>TCHQD</td>
<td>Possibly Serine</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td>Alpha</td>
<td>Detoxication of environmental xenobiotics, signaling</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Mu</td>
<td>Unknown</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Gamma</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Insects</strong></td>
<td>Delta</td>
<td>Detoxication of insecticides, peroxidase activity, oxidative stress</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Epsilon</td>
<td>Detoxication of insecticides, peroxidase activity, oxidative stress</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Theta</td>
<td>Acts against by-products of oxidative stress, May have a role in muscle function</td>
<td>Tyrosine</td>
</tr>
<tr>
<td></td>
<td>Sigma</td>
<td>Tyrosine degradation pathway</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Zeta</td>
<td>Unknown</td>
<td>Serine</td>
</tr>
<tr>
<td></td>
<td>Omega</td>
<td>Unclear (probably act against oxidative stress)</td>
<td>Cysteine</td>
</tr>
</tbody>
</table>
1.2.2.1. Insect specific classes of GSTs

There are two classes of GSTs, the Delta and Epsilon class, which appear to exist uniquely in insects and other arthropods with few exceptions; honey bee and jewel wasp do not have any Epsilon class. Both of these classes are the most numerous classes of GSTs, present in the mosquito *Anopheles gambiae* and the fruit fly *Drosophila melanogaster*. There are 25 GST genes in *Drosophila melanogaster* in the Delta and Epsilon classes combined [25]. The expanded number of both these classes of GSTs in insects and their functional role of being the major contributors in detoxication reactions, for both endogenous and xenobiotic compounds, suggests that these enzymes play an important role in the adaptation of the insects to their specific environments [26].

1.2.2.2. Structure of GSTs

Most of the cytosolic GSTs are composed of two subunits either homodimers, of a single gene product or heterodimers encoded by different genes with a subunit molecular mass of approximately 25 kDa. Each subunit of the dimeric enzyme is composed of two distinct functional domains, the thioredoxin like N-terminal domain and the C-terminal domain. In each subunit, there are two substrate-binding sites: a hydrophilic GSH binding site termed as G-site and an adjacent hydrophobic H-site. The G-site is typically formed by N-terminal domain (residues 1-80) but in some cases residues from other subunit can contribute [28], which is quite conserved in all soluble GSTs for GSH binding (Fig. 1A), whereas the H-site is largely composed of C-terminal domain residues for binding structurally diverse electrophilic substrates [21,24]. Although each subunit of the dimeric enzyme has a kinetically independent active site [29], cooperativity have also been reported [30], however the quaternary structure of the enzyme is compulsory for the catalytic activity.

GSTs act by catalyzing the nucleophilic attack of GSH on electrophilic substrates by locating the substrate into close proximity with GSH in the active site of the enzyme, and by activating the thiol group of GSH, which acts as a base [31]. In most of the mammalian classes of GSTs (Alpha, Mu, Pi and Sigma), the active site residue tyrosine is responsible for the GSH activation for catalyzes, whereas in Theta, Zeta, Delta and Epsilon classes of GSTs this role is performed by a serine residue. In Omega GSTs the catalytic residue is a cysteine. The hydroxyl group
of tyrosine/serine acts as a hydrogen bond donor to the sulfhydryl group of GSH, and stabilizes the highly reactive thiolate anion which is responsible for the nucleophilic attack on electrophilic substrates [13]. Generally, all cytosolic GSTs contain a very similar conserved tertiary structure [6]. Currently, more than three hundred structures of GSTs have been solved and deposited in Protein Data Bank (PDB), out of which, approximately 5% of the total solved structures belongs to insect GSTs [20]. More recently, DmGSTE6 and DmGSTE7 crystal structures have been determined and the coordinates are available in PDB with the entry codes 4pnf and 4png, respectively [32]. Fig. 1. below shows a representative crystal structure of DmGSTE6 monomer and dimer.

**Figure 1.** A) DmGSTE6 monomer (chain A) showing the location of the secondary structure elements as well as selected residues. The GSH ligand is rendered as sticks. The labels A and B denote α-helices and β strands, respectively. B) DmGSTE6 dimer (Chains A and B). Chain A is colored as in monomer. Chain B is in pastels. The non-crystallographic two-fold rotation axis relating the two subunits is shown as a black line. The GSH ligand is rendered as sticks.
1.3. Biological functions of GSTs

GSTs are best known as major contributors in cellular detoxication against a variety of cytotoxic and genotoxic compounds [1]. Studies have revealed that up-regulation of GST expression in tumor cells have a significant role in the development of resistance against anticancer drugs in animals [33]. In plants, GSTs are primarily responsible for herbicide detoxication [34]. In insects, elevated GST activities have been associated with resistance to all major classes of insecticides [35]. For example, in mosquitoes strains of *Anopheles gambiae* and *Anopheles dirus*, the enhanced rates of DDT dehydrochlorination by GSTs confers resistance to DDT [36,37]. Furthermore, studies have shown that GSTs play important roles in the metabolism of endogenously produced lipid peroxidation products and in defense mechanisms against oxidative stress. For example, human Alpha class GSTA4-4 catalyzes the conjugation of GSH to the toxic 4-hydroxynonenal, which is a product of lipid peroxidation [38].

1.3.1. GSTs in steroid hormone biosynthesis

Among the cytosolic GSTs, there are enzymes that are involved in the biosynthesis of steroid hormones. Human GSTA3-3 from the Alpha class has been demonstrated to have steroid double-bond isomerase activity with Δ^5^androstene-3,17-dione and Δ^5^pregnene-3,20-dione, which are close precursors of testosterone and progesterone, respectively [39]. This reaction is also catalyzed by endogenous 3β-hydroxysteroid dehydrogenases. However, the isomerase activity of GSTA3-3 is 200 times more efficient than that of 3β-hydroxysteroid dehydrogenases. Additionally, the selective tissue expression of GSTA3-3 in steroidogenic tissues further points to its role in steroid hormone biosynthesis.

1.3.2. GSTs in prostaglandin biosynthesis

The human Sigma class GSTS1-1 is known as human hematopoietic prostaglandin D\(_2\) synthase (HPGDS) because of its involvement in catalyzing the isomerization reaction of prostaglandin H\(_2\) (PGH\(_2\)) to prostaglandin D\(_2\) (PGD\(_2\)), a mediator of allergic and inflammatory responses [40]. There is compelling evidence that the overproduction of PGD\(_2\) in
human mast cells and T helper type 2 (Th2) cells mediates various allergic and inflammatory responses by activating two G-protein-coupled receptors, the D prostanoid receptor 1 (DP1) and the CRTH2 receptor (chemoattractant receptor-homologous molecule expressed on (Th2) cells [41]. Therefore, HPGDS is believed to be a promising therapeutic target for designing anti-allergic and anti-inflammatory drugs. In the present study a diverse library of FDA-approved compounds was screened for the identification of novel and potent inhibitors of HPGDS. The crystal structure of rat HPGDS revealed that the enzyme has features in common with Mu and Pi classes of enzyme (for example the active site residue tyrosine). However, the enzyme had a unique hydrophobic substrate binding site (H-site) for accommodating its large physiological substrate PGH₂. The proposed catalytic mechanism of HPGDS-catalyzed isomerization of PGH₂ to PGD₂ is initiated by the hydroxyl group of Y8, which acts as a hydrogen bond donor to the thiol group of GSH, resulting in the formation of a reactive thiolate anion, which engages in nucleophilic attack on the oxygen atom of PGH₂. The nucleophilic attack results in the formation of a GSH-PGH₂ adduct. A subsequent attack by thiolate anion in solution on C11-hydrogen results in the cleavage of the O-S bond and formation of the C11 carbonyl. Finally, the hydrogen atom transferred to the C9-hydroxyl anion from GSH completes the product formation [40] (Fig. 2.).

![Figure 2. A) HPGDS-catalyzed isomerization of PGH₂ to PGD₂. B) The proposed mechanism of HPGDS-catalyzed isomerization of PGH₂ to PGD₂.](image)
1.3.3. GSTs as ligandins

In addition to their well-established catalytic functions, numerous GSTs from the Alpha, Mu and Pi classes have been reported to bind a range of non-substrate ligands such as heme, bilirubin, steroids and various xenobiotics [42,43]. It has also been shown that the interaction between the GST and the non-substrate ligand often but not always occurs at the dimer interface and interestingly, there is an interaction between the active site of the dimer and the non-substrate ligand-binding site [44].

1.3.4. GSTs in the regulation of cell signaling

Kinase-mediated cellular signaling pathways play important roles in the regulation of stress responses, cell survival, proliferation and apoptosis [45]. Recently, several mammalian GST isoforms have been reported to have a role in cellular signaling pathways. For example human GSTP1-1 negatively regulates the activity of Jun N-terminal kinase (JNK) by inhibiting its activity via specific protein-protein interactions [46], whereas GSTM1-1 has also been shown to have a regulatory function in apoptosis stimulating kinase 1 (ASK1) [47]. More recently, several GST isoforms from Drosophila melanogaster Delta, Omega, Sigma and Zeta classes, have been reported to have regulatory roles in the modulation of the p38b mitogen-activated protein kinase (MAPK) signaling pathway [48,49].

1.3.5. Insect GSTs and resistance to plant allelochemicals

The ability of the insects to metabolize and thereby degrade xenobiotics and other detrimental chemicals is of considerable importance for their survival in a chemically unfriendly environment. Recent advances in insect genetics have been instrumental for our understanding the mechanisms involved in the insecticides resistance. However, less is known about the insect resistance to plant allelochemicals that are present in their natural environment. One of the major defenses that insects have evolved in the co-evolutionary `arms race´ with plants, is the biotransformation of plant toxins [50]. There are examples of metabolic resistance, where overproduction of detoxication enzymes conferred resistance against plant chemicals in both herbivorous and detritivorous insects such as mosquito larvae feeding on the plant debris.
GSTs are very important detoxication enzymes, which are involved in the biotransformation of various xenobiotics including plant toxins. Hence their role in resistance to plant allelochemicals have been studied in various crop-feeding insects such as moths and butterflies. Glucosinolates present in plants are considered as important weapons for their defense against the attack from various predators, such as insects and microorganisms. For example, the glucosinolates of Brassicaceae host plants render the oviposition of specialist insects like the Pieridae butterflies, the caterpillars feeding on these plants resist ITCs toxicity [51]. Another example is Arabidopsis thaliana, which contains a series of indolic and aliphatic glucosinolates as their primary defense mechanism against herbivorous aphids feeding on these plants [52].

The reaction between ITCs and the ubiquitous tri-peptide GSH is spontaneous and results in the formation of dithiocarbamates [5]. However, many GSTs have been reported to catalyze this reaction very efficiently [53]. Given the roles above that GSTs play, it can be assumed that the insect GSTs provide protection against ITC toxicity, but this notion has not yet been tested experimentally. In the present study, we have created a transgenic Drosophila melanogaster overexpressing the enzyme GSTE7, which is shown to be highly active with ITC substrates in vitro, and investigated the effect of allyl isothiocyanate (AITC) and phenethyl isothiocyanate (PEITC) on the transgenic flies.

1.3.5.1. Drosophila, an attractive model organism for longevity experiments

The fruit fly Drosophila melanogaster has been used as a model organism in biological research for over a century now [54]. Although, the genome size of fruit fly is approximately 5% of a typical mammalian genome, but it still has great resemblances with the human genome. In fact, 75% of the known disease-related genes in humans have their orthologues in fruit fly, which makes Drosophila research highly relevant for human biology and medicine [55,56]. The key features that make Drosophila an attractive model organism for longevity assays include its inexpensive culturing, short lifespan, plentiful progeny and most importantly, the ease of manipulating its genetics. Another aspect of Drosophila research that has gained a considerable importance in the modern world is the use of Drosophila to test the mutagenic properties of environmental toxicants and as a model to understand how organisms
respond to an ever changing environment [57]. Today, we are living in an environment that is experiencing rapid fluctuations in climatic conditions due to global warming. It will be advantageous for us, if not crucial, to understand how these climatic variations and the by-products of industrialization will affect the growth, reproduction and survival of the key species including agricultural pests, pollinators and wild food stocks. The ease and availability of genetic tools make *Drosophila melanogaster* an excellent model organism for investigating these processes across multiple generations of individuals within a time span that may allow us to identify solutions to problems before they become unresolved.

1.4. Biomedical and biotechnological applications of GSTs

GSTs are of immense importance in the fields of pharmacology and toxicology due to their involvement in the metabolism of broad spectrum of chemical structures, including chemotherapeutic agents, carcinogens, insecticides and endogenous metabolites, and therefore, provide targets for anti-cancer drug therapies. The elevated levels of GSTs in mammalian tumor cells have been associated with resistance to various anti-cancer agents [1]. HPGDS is another important therapeutic target for designing anti-allergic and anti-inflammatory drugs, since the enzyme is involved in the biosynthesis of PGD2, a mediator of allergic and inflammation responses [41]. Furthermore, GSTs can play an important role in diagnostics. For example, GSTP1-1 has been shown to be overexpressed in various tumors, suggesting its possible role as a useful immunohistological marker for diagnosing cancer [58].

GSTs have been widely used in conventional molecular biology as GST fusion tags for recombinant protein overexpression and pull-down assays; because of their enhanced solubility, stability and ease of purification [59,60,61]. In addition to the above mentioned biomedical and conventional uses, GSTs have gained considerable interest over the years for their use in biotechnological applications. In agriculture, GSTs are exploited to produce transgenic plants for increased resistance to herbicides. For example the transgenic *Arabidopsis thaliana* showed increased tolerance towards fluorodifen herbicide when introduced with a catalytically efficient GST transgene [62]. There are certain crops which are susceptible to various herbicides due to lack of GSTs
with herbicide detoxifying activities such as wheat and tobacco [63]. Additionally, immobilized GSTs can be used as herbicide biosensors [64]. Studies have revealed that resistance to insecticides is often related to elevated GST activities [35].
2. Aims of the study

The aims for each paper are detailed below.

**Paper I:** The multifunctional enzyme GSTs catalyze the conjugation of tri-peptide GSH with a broad range of electrophilic substrates and the characterization of individual GST is often labor intensive. In this paper we aimed at developing a method for the screening of large sets of substrates by immobilizing GSTs on nanoporous membranes.

**Paper II:** Human hematopoietic prostaglandin D₂ synthase (HPGDS) is involved in the biosynthesis of prostaglandin D₂ (PGD₂), a mediator of allergy and inflammation responses. In this paper we aimed at identifying new inhibitors of human hematopoietic prostaglandin D₂ synthase from a FDA-approved drug library.

**Paper III:** ITCs are known plant toxins and many GSTs catalyze the inactivation of these compounds. In this study we aimed to investigate the *in vivo* effects of these electrophilic molecules on the transgenic fruit flies overexpressing GSTE7.

**Paper IV:** To identify efficient GSTs for catalysis of environmental pollutant and the explosive TNT and the related DNT for potential transgenic applications in plants for phytoremediation.
3. Methodological Considerations

The more important methods employed in the present work are summarized in this section, however the readers are referred to the publications in the thesis for detailed description of methods.

3.1. Covalent immobilization of the enzymes (Paper I)

The schematic for covalent immobilization of GSTs on nanoporous alumina membranes can be explained under following steps. In the first step the alumina membranes were silanized with approximately 500 times excess of APTES and TEA in the presence of dry acetonitrile by constant shaking at room temperature for 1h. Subsequently, the membranes were extensively washed with MQ water, ethanol and acetone followed by drying at 110 °C. In the second step, the silanized membranes were activated with the excess of CDI in the presence of TEA for 1h at room temperature with
constant shaking. Thereafter, the membranes were extensively washed as per step one and dried in vacuum desiccator containing dry silica gel as a water absorbent and connected to a water pump for evaporation of air and solvent for 30 minutes.

In the third step, the enzymes subjected to immobilization were incubated with the activated membranes in 20 mM sodium borate buffer, pH 9 for 20 h in an incubator shaker. The unbound enzymes were later washed away by excess of 25 mM sodium phosphate buffer pH, 6.5. The membranes with immobilized enzymes are now ready to use or can be stored at 4 °C for further usage.

3.2. Flow system design

The design of the reactor and the flow system is schematically presented below in Fig. 4.

**Figure 4:** Flow system design. The membranes with immobilized enzymes were placed between two silicone gaskets in a 13-mm Swinnex polypropene holder and was fitted between an Amersham P-50 pump and a Shimadzu spectrophotometer UV-1601 equipped with a 70-µl quartz flow cuvette. The system can be used in a circulating mode as well as in a single pass mode. In circulating mode, the fluid inlet connected to the pump takes the reaction mixture from the reservoir and passes it through the enzyme reactor followed by the detector back into the reservoir. In single pass mode, the reaction mixture is collected separately or led to waste after detector (Paper I, Fig. 1).
3.3. Enzyme activity assays (Papers I, II, III, IV)

The catalytic activities of the purified enzymes with the electrophilic substrates except DNT and TNT were determined spectrophotometrically. The reactions were performed at 30 °C in 0.1 M sodium phosphate buffer, pH 6.5. Isothiocyanate stock solutions were prepared in acetonitrile (2% final concentration in the assay), while CDNB, FDNB and DNT stocks were prepared in ethanol (5% final concentration in the assay). The TNT stock was kindly provided as 0.5 mM aqueous solution by Dr. Rune Berglind, Swedish Defence Research Agency (FOI). The enzymatic activities with 1 mM DNT and 0.2 mM TNT accompanying 1 mM GSH were determined in 0.1 M sodium phosphate buffer, pH 6.5, at 30 °C for 30 min. The nitrite formation was quantified colorimetrically by using Griess assay as described by French et al [65].

3.4. Enzyme inhibition assays (Paper II)

HPGDS catalyzed conjugation of GSH with CDNB was used as a biochemical assay for inhibition studies by using conditions as described earlier. Preliminary screening of the US Drug Collection for GST inhibition was performed spectrophotometrically in 96-well plate reader (Spectramax PLUS 384 Molecular Devices). The US Drug Collection compounds were available in a stock concentration of 10 mM in DMSO. The final DMSO concentration in the reaction mixture of 300 µl was 3.3% (v/v), which gave negligible inhibition of enzyme activity.

IC$_{50}$ values of the most potent inhibitors were determined on a UV-2501 PC (Shimadzu) spectrophotometer by using a series of inhibitor concentrations where the solubility of the drug allowed and the background reactions were corrected by same solvent concentrations. For the determination of $K_I$ values and inhibition type of most potent compounds, one of the substrates (CDNB) was kept at near-saturating concentrations, while the concentration of the other substrate (GSH) and the inhibitor were varied.
3.5. Construction of transgenic flies and overexpression of GSTE7 (Paper III)

The gene encoding GSTE7 was custom synthesized by DNA 2.0 (Menlo Park, CA, USA) and was provided in pJ201 cloning vector. The coding sequence of GSTE7 from the pJ201 plasmid was PCR amplified and was inserted into pUAST attB vector [62] by Not I and Xba I. Embryos from y¹ sc¹ v¹ P{nos-phiC3\textbackslash int.NLS}X; P\{Cary-P\}attP2 flies bearing the attP2 landing site on chromosome 3L (68A4) were injected with the plasmid by Rainbow Transgenic Flies, Inc., Camarillo, CA, USA. The transgene was translocated into chromosome 3 by way of \( \Phi \) C31-mediated integration system. The resulting transgenic flies were then crossed with a fruit fly strain containing the Actin-Gal4 driver for ubiquitous expression. The schematic outlining of the binary Gal4-UAS system is presented below in Fig. 5.

![Schematic representation of the binary Gal4-UAS system](image)

**Figure 5:** Schematic representation of the binary Gal4-UAS system used for the induction of ubiquitous overexpression of GSTE7 (Paper III, Fig. 1).
3.6. *In situ* hybridization

For construction of the *in situ* hybridization probe, the coding sequence of GSTE7 from pJ201 plasmid was PCR amplified with the forward CTCGGGATCCATGCCCAAATTGAT and reverse primers ATCGAAGCTTATTCGATGCGAAAGTG and was inserted into pGEM-T Easy (Promega) by TA-cloning. Thereafter, the plasmid was linearized by using Apa I restriction enzyme and a digoxigenin-labeled RNA probe transcribed with SP6 RNA polymerase and the whole-mount *in situ* hybridization of fruit fly embryos was performed as described previously with a GSTE7 probe [63].

3.7. Survival and egg-laying assays

For survival and egg-laying assays, approximately 100 Actin-Gal4/CyO female virgin flies were crossed with 40 *W*111*8* (control) and approximately the same number of Actin-Gal4/CyO female virgin flies were crossed with 40 UAS-GST*E7* homozygous males. The crosses were kept in food bottles and the flies were flipped to fresh food bottles after 2-3 days of mating. The offspring from the crosses were allowed to mate for 3-5 days after hatching in the food bottles. A day prior to setting up the survival and egg-laying assays, fresh fly food with and without isothiocyanates was prepared as described in Paper III. The non-Cy, 3-5 days old male and female flies from the crosses were then separated and divided into 10 ml fresh food vials at the first day of the assay. For survival assays, the fly food containing PEITC was changed 3 times per week. For egg-laying assays, the food (with or without isothiocyanate) was changed every day and the number of eggs deposited in the vials were counted each day for seven consecutive days.

For any food avoidance behavior induced by PEITC addition, a coloring agent xylene cyanol was used in fly food (with or without PEITC) to monitor the uptake of food by means of flies abdomen turning blue. To setup this assay male and female flies were separated and put into food vials (5 flies/vial). After three hours in food vials, the flies of both sexes had a blue-colored abdomen from both control food, and the food supplemented with PEITC.
4. Results and discussion

GSTs are amongst the most important families of detoxifying enzymes existing in nature. They play a pivotal role in detoxication across multiple kingdoms and phyla. The broad substrate specificity of GSTs including drugs and xenobiotics have afforded them considerable importance in biomedical and biotechnological research. Important findings of the present work are summarized and discussed in this section.

4.1. Stability, kinetics and substrate specificity of immobilized GSTs (Paper I)

Because the enzymes are biological materials, their enzymatic activity decreases with the passage of time. Therefore, the maintenance of enzyme activity is crucial for enzyme immobilization. An earlier example of GST immobilization was reported by Paddeu and co-workers, who immobilized a non-specified GST on a silica matrix using 3-glycidoxypropyltrimethoxysilane [66]. GSTs are generally known as relatively stable enzymes. The stability comparison of immobilized GSTE7 and the corresponding enzymes in solution showed a good correlation (Fig. 6.). After six days of repeated use at room temperature and storage in the refrigerator, the immobilized GSTE7 retained 70 % of its initial activity, which is comparable to the other enzymes in solution. The half-life of the enzyme under stated conditions was approximately 10 days. The tolerance to immobilization conditions was also encouraging, since the enzymes retain more than 90 % of their initial activities after 24 h of incubation at room temperature in 25 mM sodium borate buffer, pH 9. These findings suggested that the chemical modifications thus had no adverse effects on the stability of the enzymes and the system could be used for the screening purposes for several hours without any necessary corrections.
The $K_m$ values obtained for the immobilized enzymes were relatively higher than the enzymes in solution, while the $k_{cat}$ values for immobilized enzymes were somewhat lower compared to the corresponding enzymes in solution (Paper1, Table 1). These results can be justified because there is a general agreement that the kinetic parameters of the immobilized enzymes can be much different from the free enzymes in solution due to certain mass transport limitations, steric effects, and micro-environmental changes \[67\]. However, our substrate specificity screening data, when normalized against the data obtained for CDNB, showed a good correlation between immobilized enzymes and the enzymes in solution (Paper 1, Fig. 4). From this, a general conclusion can be made that the substrate selectivity pattern for the immobilized enzymes is similar to those in solution. Hence, these results are encouraging for the use of immobilized GSTs for screening purposes. An obvious advantage of immobilized enzymes is that the same set of enzyme can be used repeatedly, which is ofcourse economical and also diminishes random errors from enzyme delivery.
4.2. Inhibition studies of HPGDS, a promising therapeutic target (Paper II)

PGD$_2$ is an eicosanoid produced by mast cells and Th2 cells after IgE-dependent activation [68]. It triggers a range of physiological and pathophysiological responses associated with allergy and inflammation, such as airways eosinophilia, hypersensitivity and mucus hypersecretion. These effects are mediated by interaction of PGD$_2$ with two G-protein-coupled receptors, DP1 and CRTH2 or DP2, associated with inflammatory conditions [41]. PGD$_2$ is isomerized from its precursor PGH$_2$ both in the central nervous system and in peripheral tissues by two structurally distinct enzymes, lipocalin prostaglandin D synthase (LPGDS) and hematopoietic prostaglandin D synthase (HPGDS), also known as GSTS1-1 [69,70]. HPGDS is highly expressed in mast cells [71], thus represents a promising therapeutic target for modulating the proinflammatory effects of PGD$_2$. Recently, a very comprehensive review has been published that disclosed a range of potent and selective inhibitors of HPGDS and described their potential therapeutic applications [72].

In the present investigation, we have been interested in finding out potent and novel inhibitors of HPGDS from a FDA-approved US Drug Collection. The obvious advantage of finding inhibitors for HPGDS among FDA-approved compounds suggested for other indications would be a shortcut to their clinical applications. Our screening results identified 23 compounds as effective inhibitors of HPGDS activity with $IC_{50}$ values in the low micromolar range (Paper II, Table 2). Among the selected best 23 inhibitors, erythrosine sodium, suramin, tannic acid and sanguinarine sulfate were identified as the most potent inhibitors with $IC_{50}$ values of 0.2, 0.3, 0.4, and 0.6 µM, respectively. Ethacrynic acid was the least potent inhibitor in this list with an $IC_{50}$ value of 44 µM, already a known inhibitor for GSTP1-1 and other GSTs [73]. Furthermore, the kinetic inhibition analysis with the best inhibitors revealed erythrosine sodium as a nonlinear competitive inhibitor. However, suramin, tannic acid and sanguinarine sulfate were characterized as linear competitive inhibitors with $K_i$ values 0.1, 0.2, and 1.0 µM, respectively. The $K_i$ value for erythrosine sodium could not be determined due to the nonlinear behavior (Paper II, Fig. 2A and B). Investigations based on catalytic activity measurements and inhibition studies indicated that the soluble GSTs subunits are kinetically independent [29,74]. However, cooperative properties of GSTs with some substrates and inhibitors
have also been uncovered [30]. A possible explanation for nonlinear behavior with erythrosine could be cooperativity between the two sub-units of the enzyme. Additionally, in the list of our most potent inhibitors, two compounds were of particular interest namely, montelukast sodium (a leukotriene receptor antagonist) and tranilast (an interleukin-6 inhibitor) with IC$_{50}$ values of 2.0 µM and 13.7 µM, respectively. Both of these drugs are already in the market for their clinical applications against symptoms related to allergy and asthma [75,76]. In contrast to tranilast, which has been reported as a mild inhibitor of HPGDS [77], herein for the first time, we report montelukast sodium as an inhibitor of HPGDS. These findings suggests that tranilast and montelukast sodium exert some of their therapeutic effects by suppressing PGD$_2$ formation via inhibiting HPGDS activity.

Bioactivities of our most potent inhibitors further warrant their therapeutic potential as these compounds are already in medical applications. Suramin is a polysulfonated naphthalurea, with broad clinical implications including antiprotozoal, trypanocidal and antiviral effects. It is prescribed for the symptoms related to sleep sickness caused by trypanosomes [78], onchocerciasis [79], and prostate cancer [80]. Tannic acid is a food additive. It has been reported that tannic acid has a potential role as an anti-cancer agent for cholangiocarcinoma [81]. More recently, Karakurt and Adali [82] have reported the inhibitory effects of tannic acid on proliferation, migration and invasion of prostate cancer. Interestingly, they have shown that tannic acid also modulates phase I and phase II detoxication enzymes. Erythrosine sodium is a color additive with applications in food industry and in pharmaceuticals for coating purposes. Sanguinarine has diverse biological activities including antimicrobial [83], anti-plaque [84], and anti-inflammatory activities [85]. Our results suggest that these compounds may have additional pharmacological effects by preventing PGD$_2$ formation.

4.3. Toxic effects of organic isothiocyanates on fruit flies overexpressing GSTE7 (Paper III)

Organic isothiocyanates are allelochemicals occurring in plants as unreactive glucosinolates. They are released upon hydrolysis by the action of endogenous myrosinases [86]. These reactive molecules are toxic and constitute a direct defense mechanism against herbivorous insects and other enemies. Several studies have been reported regarding the direct effects of ITCs on insects [87,88].
The toxicity of ITCs towards insects was shown to be comparable to that of synthetic insecticides [89]. *Drosophila melanogaster* contains 36 GST genes in six different classes, that encode 41 different proteins [90]. Among all the GSTs from *Drosophila*, the members of the Epsilon class may be uniquely interesting because of their involvement in defense against toxicants. Gene expression changes in *Drosophila* associated with aging or oxidative stress include up regulation of GSTs, [91,92] and the GSTE7 was amongst the limited number of GSTs overexpressed in long-lived flies [93]. Therefore, this enzyme was subjected to activity studies with ITCs featuring both aliphatic and aromatic chemical substituents and transgenic studies.

The *in vitro* enzymatic activity results showed that *Drosophila melanogaster* GSTE7 efficiently catalyzes the inactivation of organic isothiocyanates (Paper III, Table 2). To investigate the *in vivo* effects of ITCs, we constructed a transgenic fruit fly overexpressing GSTE7 by taking advantage of a binary Gal4-UAS system. There are numerous genetic tools available for manipulating the *Drosophila* genome. One of the powerful tools to study individual gene functions in the fruit fly is the use of transposable elements (*P*-element) mediated germ-line modifications [94]. *P*-element mediated alterations in *Drosophila melanogaster* have been used for inserting as well as knocking out genes. However, these gene insertions occur randomly that can strongly effect the gene expression and complicate the analysis of transgenes [95]. In the present investigation, we have used a site-specific ΦC31 mediated integration system that does not need any cofactor for mediating the recombination between attB and endogenous landing site attP [96].

Among the tested ITCs substrates, phenethyl isothiocyanate (PEITC) and allyl isothiocyanate (AITC) featuring both aromatic and aliphatic substituents were selected for *in vivo* studies. A concentration of 0.25 mM PEITC in standard fly food was toxic to the flies and considerably shortened their lifespan, whereas, higher PEITC concentrations had lethal effects on both wild-type and transgenic flies. The results obtained from survival assays with 0.25 mM PEITC, demonstrated that the transgenic overexpression of GSTE7 could protect female flies from the initial acute toxic effects of PEITC, whereas no positive long-term effect on survival was observed (Paper III, Fig. 2A). By contrast, the effect of the transgene on male flies were opposite to that of females. A higher mortality rate was noticed in male flies overexpressing GSTE7 after the first few days of exposure (Paper III, Fig. 2B). The reason for this toxic influence observed in male flies is not yet clear, but a possible explanation could be the excessive ITC conjugation that might have led to GSH
depletion and toxicity. Since GSH is involved in many important reactions in the cell and the excessive conjugation may shift the tissue redox balance, which is known to influence longevity [97].

Furthermore, a concentration of 4 mM AITC in standard fly food appeared to be fatally toxic to both the wild-type and the transgenic flies, and the flies were dead after a few hours of exposure. By contrast, 1 mM AITC concentration showed no obvious effects on the survival of flies. Additionally, we investigated the effects of ITCs on the oviposition activity of transgenic flies by counting the number of eggs laid by 3-5 days old flies for seven consecutive days in the presence or absence of 0.15 mM PEITC and 1 mM AITC in standard fly food. The wild-type flies were used as control in the assay (Paper III, Fig. 3). The results demonstrated that the transgene has a significant influence on the rate of oviposition and surprisingly, this effect was pronounced both in the presence and absence of ITCs. The oviposition rate was approximately 5- to 2-fold higher in the flies overexpressing GSTE7 compared to wild-type flies.

ITCs are strong electrophiles produced in plants and exert toxicity in biological tissues. In addition to that, ITCs act as repellents for certain insects [51]. Studies have revealed that reactive electrophiles classified as noxious chemicals such as AITC (in wasabi) that activates transient receptor potential A1 (TRPA1), which in turn acts on the gustatory receptors leading to the food avoidance [98]. To investigate any such possibility, we have used xylene cyanol in both, the isothiocyanate-containing food and the standard fly food. The blue abdomen of the flies confirmed the utilization of food with no obvious differences in color. So the harmful effects of ITCs in our experiments cannot be primarily explained by any food avoidance in these experiments, and the GSTE7 transgene thus has provided protection. Furthermore, the gene expression profile of GSTE7 in FlyAtlas Anatomical Expression Data [99] shows its high expression in the digestive tract of both larvae and adult flies. This tissue expression suggests that it could be a normal function of GSTE7 to metabolize the toxic electrophiles such as ITCs arising from food and liquid uptake.
4.4. Drosophila GSTs are efficient catalysts of DNT and TNT degradation (Paper IV)

The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) and its byproduct (DNT) are major worldwide military pollutants. The existence of these highly persistent compounds at military ranges and at former manufacturing sites, presents serious health and environmental challenges. Due to chemically resistant structures, these compounds are highly recalcitrant to biodegradation. Studies based on laboratory evaluations demonstrated that most of the nitro-substituted explosives are toxic to almost all classes of organisms from algae to bacteria to plants to mammals and to invertebrates [100].

In the present investigation, we have heterologously expressed and purified a set of GSTs comprising human Alpha, Mu, Pi, and Sigma classes as well as two Epsilon class GSTs from Drosophila melanogaster and subjected to activity studies with the environmental pollutants, DNT and TNT. The catalytic activities with TNT revealed Drosophila GSTE6 and GSTE7 as the most efficient enzymes with specific activities of 62.7 ± 2.6 nmol•min⁻¹•mg⁻¹ and 20.0± 2.0 nmol•min⁻¹•mg⁻¹, respectively. The catalytic activities of GSTE6 and GSTE7 with DNT substrate were more of a similar magnitude with specific activities of 20.5 ± 1.4 nmol•min⁻¹•mg⁻¹ and 14.3 ± 1.4 nmol•min⁻¹•mg⁻¹, respectively. The human GSTs showed moderate activities with both substrates. Among the different human GSTs, GSTA2-2 was the most active enzyme with DNT (2.6 ± 0.4 nmol•min⁻¹•mg⁻¹), whereas < 0.01nmol•min⁻¹•mg⁻¹ activity was detected for this enzyme with TNT. The kinetic parameters of the most active enzymes (Paper IV, Table. 2) showed that GSTE6 has almost 3-fold higher catalytic efficiency ($k_{cat}/K_m$) with TNT than that of GSTE7, while the $k_{cat}$ values were not significantly different. On the contrary, both the $k_{cat}$ and $K_m$ values with DNT substrate were approximately two-fold higher for DmGSTE6 than for DmGSTE7. As a result, the catalytic efficiencies of both the enzymes with DNT substrate were identical.

Cytosolic GSTs belong to a multigene family of enzymes known to conjugate GSH to electrophilic molecules. In insects, GSTs are involved in the detoxification of insecticide xenobiotics [35]. Since GSTs have evolved the ability to catalyze the conjugation of GSH with a vast array of different chemical species, suggesting that GSTs might play a central role in the detoxification of TNT and its precursors.
A limited number of plant GSTs from poplar and *Arabidopsis*, Tau class have been reported to have catalytic activities with TNT [101,102]. Additionally, two GSTs from poplar have been reported to be up regulated in response to TNT [103,104,105]. However, the observed catalytic activities of these Tau class GSTs appears not to be efficient enough to play a significant role in the biotransformation of TNT. A comparative catalytic activity analysis between GSTE6 and the previously reported poplar GSTU16 and GSTU45, showed that the *Drosophila* GSTE6 is >1000 times more active than poplar GSTs (Paper IV, Table 2). Additionally, GSTE6 showed approximately, 50 to 100 times higher catalytic activities with TNT substrate compared to *Arabidopsis* GSTU25, GST24, respectively. In conclusion, our results suggest that these catalytically efficient enzymes underline the potential of geneting engineering in plants for biotransformation and phytoremediation of TNT and its by-product DNT.
5. Conclusions

The major findings of this thesis are listed and described below:

**Paper I:** From this work, a general conclusion can be made that the substrate selectivity pattern for the immobilized enzymes is similar to those in solution. Hence, these results are encouraging for the use of immobilized GSTs for screening purposes. An obvious advantage of immobilized enzymes is that the same set of enzyme can be used repeatedly, which is of course economical and also diminishes random errors from enzyme delivery.

**Paper II:** From our screening results, we conclude that the potent and novel inhibitors of HPGDS have potential to be used in the treatment of conditions involved with inappropriate PGD$_2$ synthesis such as allergic asthma. However, we suggest that further characterization of these compounds should be done by using natural substrate PGH$_2$ in the inhibition assays. Additionally, we conclude that these drugs could possibly serve as lead compounds for the synthesis of new inhibitors for human HPGDS.

**Paper III:** Herein, we conclude that overexpression of *Drosophila melanogaster* GSTE7 protects female flies from initial acute toxic effects of PEITC, however there was no long term protection. Additionally, the transgene enhances the oviposition activity of the flies and surprisingly, this effect was pronounced both in the presence and absence of ITCs.

**Paper IV:** We conclude that GSTE6 and GSTE7 are efficient catalysts of TNT and DNT degradation, hence underline the potential of geneting engineering in plants for biotransformation and phytoremediation of these toxic environmental pollutants.
6. Populärvetenskaplig sammanfattning på svenska

Glutationtransferaser (GST, EC.2.5.1.18) är multifunktionella enzymer som förekommer i alla cellulära livsformer. De spelar en viktig roll i metabolism och avgiftning av såväl endogent producerade giftiga föreningar som xenobiotika. GST har rönt stor uppmärksamhet genom sin potential för biomedicinska och biotekniska tillämpningar på grund av den katalytiska rollen i konjugering av glutation (GSH) med ett brett spektrum av kemiska substanser. Upptäckten av dessa enzymers icke-avgiftande funktioner har ytterligare ökat vår insikt om deras biologiska signifikans. Föreliggande avhandling omfattar fyra vetenskapliga studier som syftar till att undersöka GST i människa och i bananflugan Drosophila melanogaster.

Artikel I redogör för immobilisering av tre enzymer på nanoporösa aluminium-membran: två enzymer tillhörande klass Epsilon, GSTE6 och GSTE7 från bananfluga, samtidet humana GSTS1-1, känt som hematopoetisk prostaglandin D2-syntas (HPGDS). Kinetiska analyser med 1-klor-2,4-dinitrobensen följt av specificitet-screening med alternativa substrat visade en god korrelation mellan resultat erhållna från immobiliserade enzymer och resultat från enzymer i lösning. Vidare uppvisade immobiliseringen ingen negativ effekt på stabiliteten hos dessa enzymer.


Artikel III gäller toxiciteten av organiska isotiocyanater (ITC) med vilka GSTE7tidigare har uppvisat hög katalytisk aktivitet in vitro. Resultaten in vivo visar att fenetylisotiocyanat (PEITC) och allylisotiocyanat i millimolära koncentrationer i vuxna bananflugors kost leder till dödsfall eller förkortad livslängd. Transgena hon-flugor som överuttryckte GSTE7 visade ökad tolerans mot PEITCs toxicitet jämfört med vildtypen. En veckas exponering hade dock motsatt effekt.
på han-flugor som överuttryckte GSTE7. Värt att notera är att transgenen förbättrade äggläggningsaktiviteten hos flugor både med och utan exponering för ITC.

Artikel IV belyser Drosophila-GST som effektiva katalysatorer av avgiftning av den explosiva miljöföroreningen 2,4,6-trinitrotoluen och den besläktade 2,4-dinitrotoluen. Detta resultat demonstrerar potentialen hos GST-transgener i växter för fytoremediering och biotransformation av dessa persistenta miljögifter.
7. Acknowledgements

It is a matter of great pleasure to express my sincere gratitude to my supervisor Prof. Bengt Mannervik for accepting me as a PhD student and for believing in me. I will never forget your unlimited support, excellent guidance, inspiring attitude, valuable suggestions and encouragement. You are not only a great scientist, a great mentor, but also a very kind person. Thanks alot Bengt.

I would also like to thank Anne-Charlotte for the kind invitations to Gräsö and Stockholm and for your hospitality.

I like to thank Prof. Kerstin Iverfeldt for being my co-supervisor.

I am profoundly grateful to Prof. Mattias Mannervik for giving me lab space to work with fruit flies and for the discussions that we had in our meetings and also Prof. Gunnar Johansson for his valuable discussions on enzyme kinetics. I would like to thank my co-authors Olle Dahlberg and Marcus Kjellander for their contribution in this work. A big thank to my co-author Usama Hegazy for all his help and guidance, especially when I started to work with the library screening.

Special thanks to our former and current group members, for providing wonderful research environment, valuable scientific discussions in our group meetings and for good loughs. Special thanks to Birgitta and Helena for your support and nice company. I really appreciate your help in correcting the reports of my students written in Swedish. Yaman, you are really a true friend and a nice colleague. I cannot forget the fun we had in our corridor parties at lappis. I would also like to thank our project students Aram, Thanh and André for being part of our group and for valuable contribution in our research. I wish you all a very successful scientific future.

I am also grateful to all my fellow PhD students, Researchers, Teachers and the Staff at the Department of Neurochemistry, Stockholm University for creating such a friendly and an excellent research environment.
Finally, I would like to thank Julika (ku-ku) and my family for their never ending love, support and encouragement, which enabled me to complete this work. Special thanks to Ahmad bhai, Ismail bhai, Sheedu khan, Dr. Safdar Mazari, Saleem Khan and Saeed Ahmad.

At last but not least, I am thankful to my dearest friends from Västerås and from Uppsala for their supportive attitude and very nice company. Specially, Chaudhary Zahid Shafqat, Ammad Shafqat, Deemi bhai, Nadeem bhai, Ayyaz Khalil (urf mara), Daniyal (urf teeku), AD, Dr, Raza, Hussain Mussarat, Obaid Aftab, Taj Muhammad, Usman Arif, Furqan Shani and Gulati. In case if I have forgotten someone; a big thank to you as well 😊
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