

**CLONING AND BIOCHEMICAL CHARACTERIZATION OF  
CG16936, A PUTATIVE GLUTATHIONE TRANSFERASE IN  
*DROSOPHILA MELANOGASTER***

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**FACULTY OF SCIENCE  
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**DISSERTATION SUBMITTED IN FULFILMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF MASTER OF  
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## ABSTRACT

CG16936, which was classified as DmGSTE12 was studied in this project. This study cloned and expressed CG16936 and the resulting recombinant CG16936 has been reactive towards 1,2-dichloro-4-nitrobenze (DCNB) (0.4324  $\mu\text{mol}/\text{min}/\text{mg}$ ) but of no activities towards, 1-chloro-2,4-nitrobenzene, *trans*-2-hexenal, *p*-nitrobenzyl chloride (NBC), ethacrynic acid (EA), *trans*-4-phenyl-butene-2-one (PBO), hexa-2,4-dienal, *trans,trans*-hepta-2,4-dienal, 1,2-epoxy-3-*p*-nitrophenoxy propane (EPNP), bromosulfophthalein (BSP) and *trans*-oct-2-enal. In previous work, it was shown that CG16936 was expressed in response to odorant treatment to fruit flies. Our work investigated the direct contribution of the recombinant CG16936 towards conjugation of odorants Glutathione (GSH). No evidence of conjugated product yielded when the recombinant protein acted upon GSH and *trans*-2-hexenal on thin layer chromatography. The absence of enzymatic activity hence suggested the inability of CG16936 to conjugate *t*-2-hexenal to GSH. Upon exposure to benzaldehyde, expression of CG16936 was demonstrated unchanged. The evidences suggested that CG16936 could have assumed other physiological function other than detoxification of odorant and its direct involvement in oxidative stress.

## ABSTRAK

Kajian ini adalah CG16936, yang telah diklasifikasikan sebagai DmGSTE12. Kajian ini telah mengklon dan mengekspreskan CG16936. Keputusan menunjukkan rekombinan CG16936 adalah reaktif terhadap 1,2-dichloro-4-nitrobenze (DCNB) dengan aktiviti spesifik sebanyak 0.4324  $\mu\text{mol/min/mg}$ , namun tiada sebarang aktiviti ditunjukkan terhadap 1-chloro-2,4-nitrobenzene (CDNB), *trans*-2-hexenal, *p*-nitrobenzyl chloride (NBC), ethacrynic acid (EA), *trans*-4-phenyl-butene-2-one (PBO), hexa-2,4-dienal, *trans,trans*-hepta-2,4-dienal, 1,2-epoxy-3-*p*-nitrophenoxy propane (EPNP), bromosulphophthalein (BSP) dan *trans*-oct-2-enal. Dalam satu kajian yang terdahulu, didapati *Drosophila melanogaster* telah mengekspres *CG16936* sebagai gerakbalas terhadap rawatan pengahbau. Dalam kajian ini, kami juga mengkaji sumbangan secara langsung rekombinan CG16936 terhadap konjugasi dengan pengahbau Glutation (GSH). Namun, apabila protein rekombinan bertindak balas dengan GSH dan *t*-2-hexenal, tiada sebarang bukti yang menunjukkan produk konjugasi di atas lapisan kromatografi nipis. Ketiadaan aktiviti oleh CG16936 terhadap *t*-2-hexenal mencadangkan ketidakupayaanya untuk menghasilkan produk konjugasi dengan substrat, *t*-2-hexenal dan GSH. Selain itu, pendedahan *Drosophila melanogaster* terhadap benzaldehyde tidak mendorong atau mengekspres protein CG16936. Bukti yang diperolehi mencadangkan CG16936 mungkin mempunyai fungsi fisiologi selain dari penyahtoksik pengahbau dan penglibatan secara langsung dalam tekanan oksidatif.

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Finally, I dedicate this dissertation to my late grandmother, Rasamah whom I lost a few months back. She was the one most eager to see me graduate; hence this is for you Amayee. I hope I made you proud.

<b>List of Content</b>	<b>Pages</b>
Abstract	i
Abstrak	ii
Acknowledgement	iii
List of Content	iv
List of Figures	viii
List of Tables	x
List of Abbreviations	xi
Introduction	1
Chapter 1: Literature review	2
1.1 GSTs and its roles	2
1.2 Gluthathione	10
1.3 Structure of GSTs	11
1.4 Classifications and nomenclature	16
1.5 Gene regulations	19
1.6 GST purification	22
1.7 Olfactory GSTs	23
1.8 CG16936	25
1.9 <i>Drosophila melanogaster</i>	27
1.10 Objectives	28
Chapter 2: Materials and method	29
2.0 Materials	29
2.0.1 Chemicals	29
2.0.2 Buffers	30
2.0.3 Kits	30
2.0.4 Columns/ matrice for purification	31

2.0.5 Equipments	31
2.0.6 Insects	32
2.1 Methodology	33
2.1.1 Total RNA extraction from <i>Drosophila melanogaster</i>	33
2.1.2 cDNA synthesis	33
2.1.3 Polymerase chain reaction (PCR)	34
2.1.4 Agarose gel electrophoresis of DNA	35
2.1.5 DNA extraction from agarose gel	35
2.1.6 Digestion with restriction enzymes	36
2.1.7 Ligation of digested PCR product and pET-30a(+)	37
2.1.8 Transformation with <i>E.coli</i> BL21(DE3) pLysS	37
2.1.9 Ligation of PCR product with pGEM-T vector.	38
2.1.10 Transformation with NovaBlue Singles™ Competent Cells	38
2.1.11 Plasmid extraction.	39
2.1.12 Digestion of ligated pGEM-T plasmid with restriction enzymes	39
2.1.13 Cell culturing and lysis	40
2.1.14 Purification of recombinant CG16936	41
2.1.15 SDS-Polyacrylamide Gel (SDS-PAGE)	42
2.1.16 Bradford assay	43
2.1.17 Assay for GSTs	43
2.1.18 Thin layer chromatography	47
2.1.19 <i>Drosophila</i> exposure with/without odorant	48
Chapter 3 Results	49
3.1 RNA extraction	49
3.2 Polymerase chain reaction	50

3.2.1 PCR gel image	51
3.3 Cloning of the PCR product	52
3.4 Plasmid purification	53
3.4.1 Sequencing results	53
3.4.2 Plasmid digestion	55
3.4.3 PCR using plasmid as template	56
3.5 Purification of recombinant CG16936	57
3.5.1 GSTrap™ HP and S-Hexylglutathione	57
3.5.2 Sep-Pak C18	59
3.5.3 Superdex 75	60
3.5.4 HiPrep 16/60 Sephacryl S-200	61
3.5.5 HiTrap ANX FF	62
3.5.6 HiTrap DEAE FF	63
3.5.7 HisTrap™ FF Crude	64
3.5.8 S-2,4-(dinitrophenyl) GSH (DNP-SG)	65
3.5.9 Bromosulfophthalein GSH (BSP-SG)	66
3.5.10 Affi-Gel Blue Gel column	67
3.6 Substrate specificities	68
3.7 Thin layer chromatography	69
3.8 Exposure to odorant	70
Chapter 4 Discussion	71
4.1 Cloning and expression of CG16936	71
4.2 Purification of recombinant CG16936	76
4.3 Substrate specificities	85
4.4 Investigations of CG16936 as an olfactory GST	88
4.4.1 Exposure of Drosophila with odorant	88



4.4.2 Conjugation of CG16936 with odorant substrate	90
Chapter 5 Conclusion	91
References	92
Appendices	105

<b>List of figures</b>	<b>Page</b>
Figure 1.1: Activities of Cytochrome P450s, Microsomal epoxide hydrolase and Glutathione S-transferase with three different xenobiotics	2
Figure 1.2: 4-HNE is metabolized through its conjugation by reaction catalyzed by GST	5
Figure 1.3: Example functions catalyzed in the GST superfamily showing reactions in which GSH is consumed by conjugation to the product.	9
Figure 1.4: Structure of glutathione	10
Figure 1.5: GST monomer (left) and dimer (right) showing N-terminal thioredoxin-like domain in blue/cyan and the C-terminal all $\alpha$ -helical domains in red. GSH is shown in dark grey to delineate the active site.	12
Figure 1.6: Active sites of S/C and Y-type GSTs highlighting the extensive hydrogen bonding network which anchors the glutathione tripeptide	14
Figure 1.7: Conjugation GST with the common substrates in the presence of GSH	15
Figure 1.8: <i>Drosophila melanogaster</i> specimens	27
Figure 3.1: 1% Agarose gel electrophoresis image of PCR	51
Figure 3.2: Map of pET-30a(+) vector	52
Figure 3.3: Gel image of purified plasmids from 6 random colonies	53
Figure 3.4: Blast search tool results of the recombinant CG16936	54
Figure 3.5: Expansion of Sequence ID: AE013599.4, featuring GST E12 isoform A and C	54
Figure 3.6: Gel image showing the digestion of pET 30	55
Figure 3.7: Gel image of PCR performed using extracted plasmid as template	56
Figure 3.8: SDS-PAGE of purification of CG16936 using GSTrap™ HP	58
Figure 3.9: SDS-PAGE of purification of CG16936 using S-Hexyl glutathione	58
Figure 3.10: SDS-PAGE of purification of CG16936 using Sep-Pak C18	59

## List of figures (Cont.)

Figure 3.11: SDS-PAGE of purification of CG16936 using Superdex 75	60
Figure 3.12: SDS-PAGE of purification of CG16936 using Sephacryl S-200	61
Figure 3.13: SDS-PAGE of purification of CG16936 using Anion Exchanger (HiTrap ANX FF)	62
Figure 3.14: SDS-PAGE of purification of CG16936 using HiTrap DEAE FF	63
Figure 3.15: SDS-PAGE of purification of CG16936 using HisTrap™ FF Crude	64
Figure 3.16: SDS-PAGE of purification of CG16936 using DNP-SG.	65
Figure 3.17: SDS-PAGE of purification of CG16936 using BSP-SG.	66
Figure 3.18: SDS-PAGE of purification of CG16936 using Affi-Gel Blue Gel column	67
Figure 3.19: Progress of conjugating products for 20 minutes with DCNB of the recombinant CG16936	69
Figure 3.20: T.L.C analysis of recombinant CG16936 conjugating activity against DCNB and <i>t</i> -2-hexenal	69
Figure 3.21: Progress of conjugating products for 20 minutes with DCNB comparing exposed and non-exposed <i>Drosophila</i>	70
Figure 4.1: Patterns of alternative splicing. Constitutive sequences present in all final mRNAs are gray boxes. Alternative RNA segments that may or may not be included in the mRNA are hatched boxes	73

<b>List of Tables</b>	<b>Page</b>
Table 2.1: Forward primer and reverse primer used for the amplification <i>CG16936</i>	34
Table 2.2: Summary of columns and buffers used.	41
Table 2.3: SDS-PAGE gel formulations	43
Table 3.1: Gene sequence of CG16936 aligned with forward and reverse primers	50
Table 3.2: Substrates specificity of recombinant CG16936	68
Table 4.1: Summary of different properties and chromatography techniques	76
Table 4.2: Summary of the recombinant CG16936 purification	84

## List of abbreviations and symbols

GST	Glutathione transferase
GSH	Glutathione
CDNB	1,chloro-2,4-dinitrobenzene
DCNB	1,2-dichloro-4-nitrobenzene.
PCR	Polymerase chain reaction
BLAST	Basic local alignment search tool
SDS-PAGE	Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
kb	kilobase
bp	Base pair
LB	Luria Bertani
<i>E.Coli</i>	<i>Escherichia coli</i>
IPTG	Isopropyl $\beta$ -D-1- thiogalactopyranoside
rpm	Revolutions per minute
FPLC	Fast protein liquid chromatography
t.l.c	Thin layer chromatography
°C	Degree Celsius
kDa	kilo Dalton

# INTRODUCTION

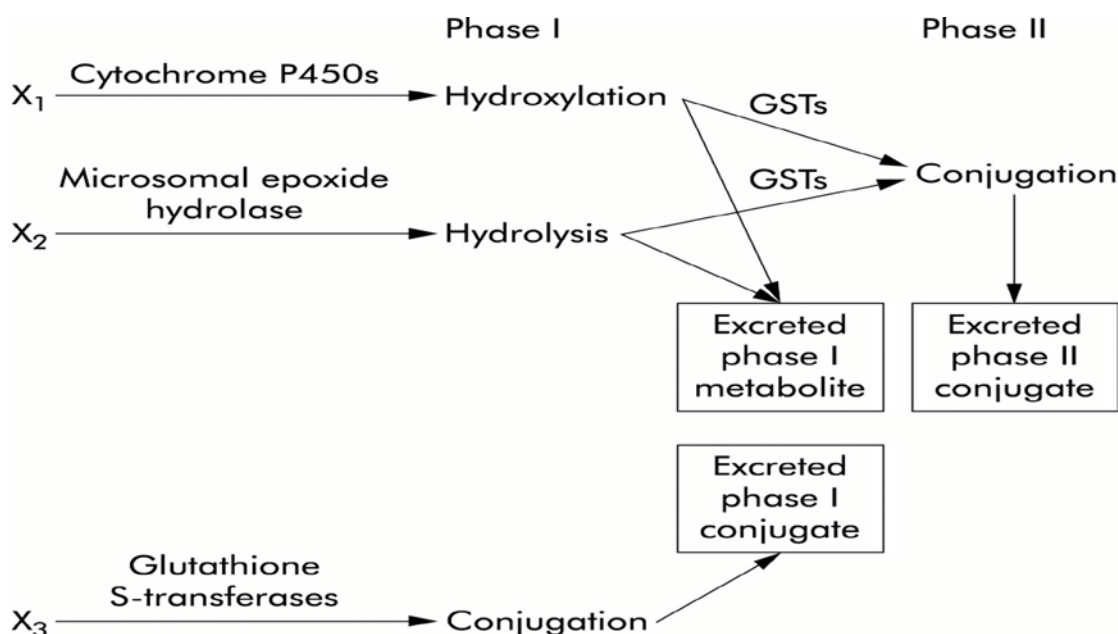
Living organisms mainly insects such as *Drosophila* are exposed to many different compounds such as insecticides, pesticides, toxicants and carcinogens mainly through pests control programs and harmful toxic compounds that are produced by plant, fungal toxins (e.g. plant phenols and aflatoxins) and reactive oxygen species. It is important that these organisms have ability to survive the threat posed by endogenous compound or by exogenous compounds in order to adapt to the environment (Hayes and McLellan, 1999). Most of these toxic compounds are able to bind and alter the structure of nucleic acids, lipids and proteins (Chasseaud, 1979). All living things pose a group of enzymes that are known as detoxification enzyme in order to neutralise the effect of these toxic compounds (Habig and Jakoby, 1980).

Glutathione S-transferase, recently classified as glutathione transferases (GSTs; EC 2.5.1.18) comprises a wide range of enzymes that is found plentiful in almost all living things such as bacteria, mammals, plants, insects, helminthes and yeast (Sheehan *et al.*, 2001). GSTs in mammals are well studied because it has been found to be associated with its resistance with drugs and in cancer epidemiology (Tew, 1994; Hayes and Pulford, 1995). This group of enzymes are said to be involved in the phase II detoxification. It catalyses the conjugation of glutathione to the hydrophilic centre of the toxic compounds such as insecticides, drugs and endogenous toxic substrates that is present in the organism (Ding *et al.*, 2003; Enayati *et al.*, 2005)

# CHAPTER 1: LITERATURE REVIEW

## 1.1 GSTs and its roles.

The hydrophobic compounds and xenobiotics are detoxified in 3 different phases. The phase 1 detoxification pathway starts with oxidation by cytochrome P450 monooxygenase (Guengerich, 1991). This family of microsomal proteins are in charge of the oxidation, among other wide range of reactions by them (Guengerich, 1990). It is then continued with phase II detoxification where GSTs and a variety of other enzymes are involved in a conjugation reaction (Mannervik and Danielson, 1988). Both phase I and II pathways are important because it involves the processes of converting the lipophilic, non-polar metabolites into a more water-soluble and less toxic metabolites which makes it to be easily excreted out of the cell.



**Figure 1.1:** Activities of Cytochrome P450s, Microsomal epoxide hydrolase and Glutathione S-transferase with three different xenobiotics (Sandford and Silverman, 2012)

The most important part of detoxification by GSTs are their ability to conjugate glutathione with a wide range of hydrophobic compound (Armstrong, 1997) which plays a crucial role in cell survival. For this purpose GSTs are present in almost all organisms and has been classified into at least 10 different classes which is done based on its structure (tertiary and quaternary), sequence similarity, immunological properties and substrate specificity (Sheehan *et al.*, 2001). Each isozyme has unique catalytic activity although it shows to have a broad range of substrate specificities.

Basically, the capacity of GSTs to conjugate the thiol group of the cysteine from the reduced GSH to a wide range of electrophiles and the affinity to bind to various hydrophobic compounds. As a result, the solubility of the compounds are increased, hence helps in the excretion process (Lumjuan *et al.*, 2007)

Reduced glutathione are used as a cofactor instead of a conjugate by some GSTs to catalyze a dehydrochlorination reaction (Clark and Shamaan, 1984). GSTs are also found to be involved in the transportation of drugs, hormones and other metabolites through its ability to bind a hydrophobic compound that are not its substrate. This also helps with the storage and sequestration process (Hayes and Pulford, 1995).

Besides that, the activation of a stress response mechanism can also be associated with the changes in GST levels (Wilhelm *et al.*, 1997). The cellular glutathione levels are said to be altered as a step to protect the cells against oxidative stress and electrophiles (Hayes and Pulford, 1995). In addition to that, when cells are going through oxidative stress, the GSTs are said to exhibit the glutathione peroxidase activity which can be associated with various physiological roles such as signal transduction, apoptosis, differentiation and cell proliferation (Ketterer *et al.*, 2001).

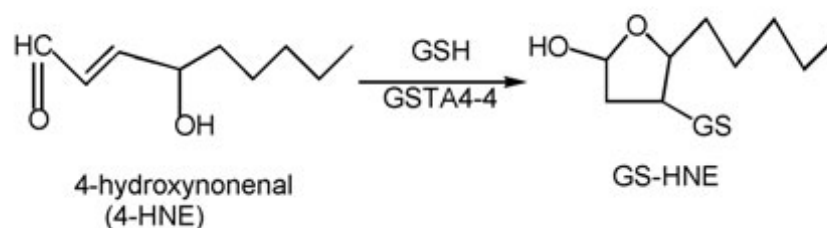


Furthermore, many proteins which have a GST like domains in their structure are associated with other roles such as in protein assembly and folding (Blocki *et al.*, 1992; Koonin *et al.*, 1994). The resistance of various anticancer agents and chemical carcinogens has been associated with the over expression of GSTs in mammalian tumour cells (Hayes and Pulford, 1995).

Wide range of GSTs has been found to be involved in various physiological roles. For example, the ability of the Omega class GST to regulate the  $\text{Ca}^{2+}$  channel activity of the ryanodine receptor which helps to protect cells from apoptosis that is induced by  $\text{Ca}^{2+}$  (Dulhunty *et al.*, 2001). The Bcl-2 family member, the Bax protein has known to regulate the programmed cell death by promoting apoptosis. The Theta class GST has been identified as a Bax-interacting protein because of its role in the regulation of the apoptotic cell death (Kampranis *et al.*, 2000; Fesik, 2001). On the other hand, GSTP1-1 has been found to be expressed at high level in tumours which is important in the diagnosis of cancer and its therapy (Sato, 1989). Besides that, this has been found to be helpful to monitor the development of drug resistance in patients who are being treated with chemotherapy (Hamada *et al.*, 1994).

Not only the roles of GSTs differ among different classes, in fact it differs among different isozyme of the same class. The GSTA4-4 is said to be involved in defending against the product of cell membranes that generates harmful compounds. High activity was observed with 4-hydroxynonenal (4-HNE), which is a product of lipid peroxidation unlike the other Alpha class GSTs (Bruns *et al.*, 1999) whereas the GSTA3-3 is responsible to catalyze the double bond of isomerisation of 5-androstene-3,17-dione (precursor to testosterone) and 5-pregnene-3,20-dione (precursor to

progesterone), in steroid hormones biosynthesis. It has also been found to have high activity with steroid substrates as well (Johansson and Mannervik, 2001).



**Figure 1.2:** 4-HNE is metabolized through its conjugation by reaction catalyzed by GST (Srivastava *et al.*, 1998).

Alterations of the intracellular signal transduction are among other interactions of GSTs. The c-Jun N-terminal kinase (JNK) signal transduction pathway is regulated by GST and GST activities are affected by the JNK interaction. Besides that, different isoforms of GST reacts with different types of regulatory mechanism in JNK pathway while others are found to interact with protein kinases (Udomsinprasert *et al.*, 2004). GSTs can also serve as maleylacetoacetate isomerases and thiol transferases (Board *et al.*, 1997, 2000).

In plants, most glutathione transferases are associated with the detoxification and conjugation of herbicides. It is classified as secondary metabolism enzymes and was discovered based on its ability to detoxify the photosystem II inhibitor atrazine in maize (Dixon *et al.*, 2010). Its activity has been reported in many major crops and weeds (Hatton *et al.*, 1996). Besides the roles in the detoxification of herbicides, plant GSTs has also said to be involved in responses to abiotic and biotic stress, developmental changes and hormones based on the changes in its expressions (Dixon *et al.*, 2002; Frova, 2006). Many studies have reported that members of GSTs are being

expressed selectively in response to environmental stress and cell division (Marrs, 1996; Moons, 2005).

The involvement of GSTs has also been observed in the synthesis of sulphur-containing secondary metabolites such as volatiles and glucosinolates, and the conjugation, transport and storage of reactive oxylipins, phenolics and flavonoids (Dixon *et al.*, 2010).

There also has been increasing evidence showing that GSTs are involved in various external stress responses. The expressions of GST genes in plants are stimulated by these external stress responses such as hydrogen peroxide, heat, toxic chemicals and dehydration (Marrs, 1996; Fujita and Hossain, 2003). The stress-related hormones, for example the ethylene (Zhou and Goldsbrough, 1993), abscisic acid (Dixon *et al.*, 1998) and salicylic acid (Wagner *et al.*, 2002) are influenced by GST expressions. The catalysis of anthocyanins production, are examples of other roles played by the plant GST (Marrs, 1996; Sheehan *et al.*, 2001). Many transgenic plants have demonstrated the roles of GSTs in stress responses. For example, salt resistance of transgenic tomato seedlings is promoted by the over expressions of GST (Roxas, 1997). Besides that, the capability of plants to endure high or low temperature and high salt concentration are said to be enhanced by the over expressions of GSTs in rice (Takesawa *et al.*, 2002) and tobacco (Roxas, 1997).

In insects, the GSTs are mainly responsible for the insecticides resistance (for example DDT), which targets the nervous and results the death of larvae during life stages transition (Enayati *et al.*, 2005; Low *et al.*, 2007). The most important part of DDT detoxification would be the dehydrochlorination which is catalysed by GSTs.

Although no conjugation was observed of DDT with glutathione, the glutathione has been found to be an important cofactor in this reaction. The active site of the GST generates the thiolate anion which acts as a general base and a hydrogen atom is extracted from DDT which then eliminates the chlorine and generates DDE (Clark and Shamaan, 1984). However, lindane which is an organochlorine insecticide is detoxified by the conjugation to glutathione.

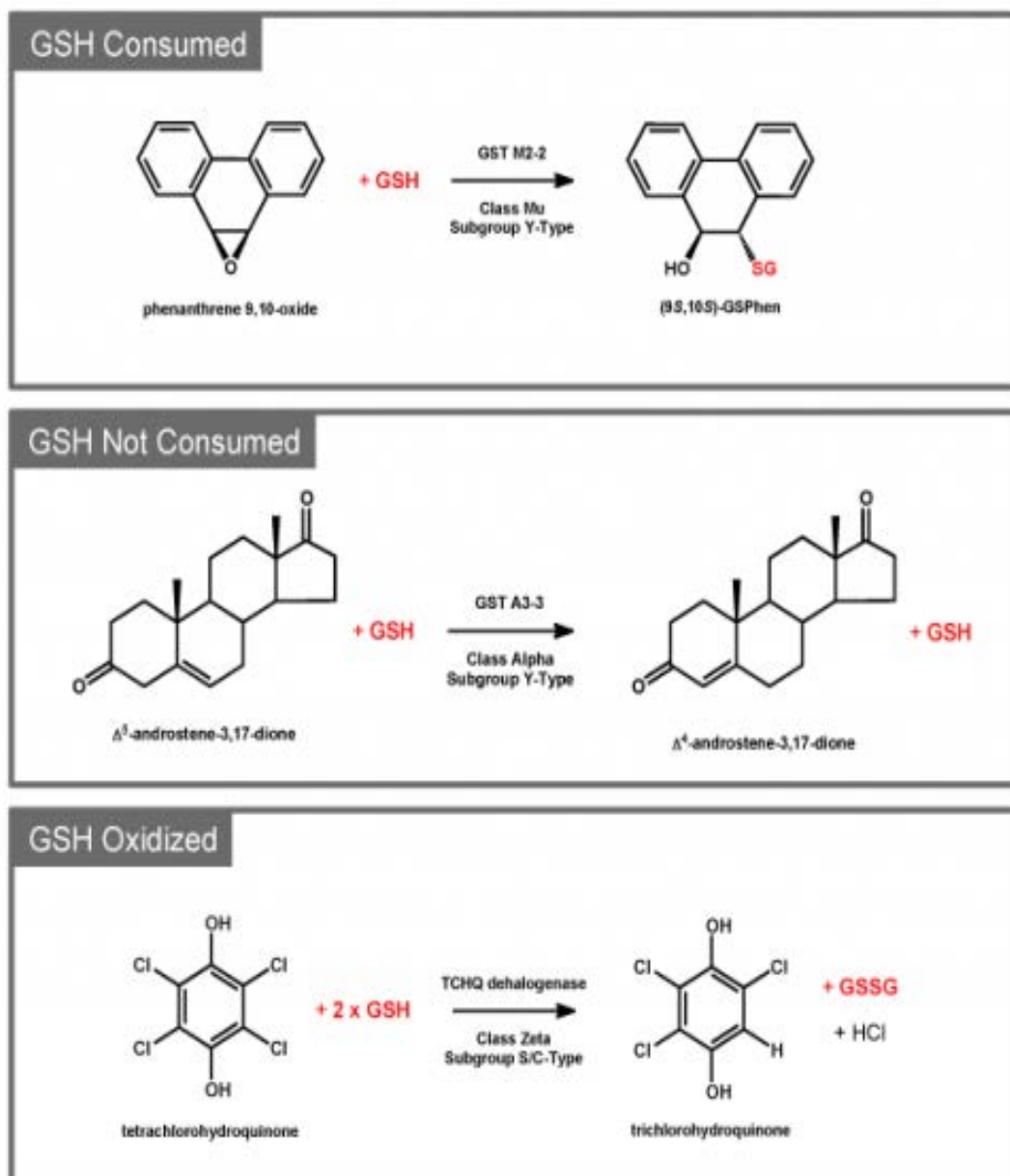
Besides organochlorines, GSTs are also found to be involved in the resistance of organophosphate (Hayes and Wolf, 1988). Two distinct pathways are responsible in its detoxification that results from the conjugation of glutathione to organophosphate insecticides. The first path is the O-dealkylation where the glutathione conjugates with the alkyl portion of the insecticides, for example the tetrachlorvinphos in resistant houseflies (Oppenoorth *et al.*, 1979) while the second path is known as the O-dearylation where the leaving group reacts with glutathione. For example is the methyl parathion and parathion detoxification that happens in the diamondback moth *Plutella xylostella* (Chiang and Sun, 1993).

GSTs have also been found to interact with pyrethroids. Although metabolism of pyrethroid insecticides by GSTs has not been detected, there is evidence that GSTs may contribute to pyrethroid resistance in several ways. A study on the defence mechanism of *Tenebrio Molitor* against pyrethroids revealed that the molecule of GST binds pyrethroid insecticides in a sequestering mechanism adding to the defence of the organism either as a passive way of detoxification or as a facilitating one (Kostaropoulos *et al.*, 2001).

The second contribution would be through GST's protective role in oxidative stress, with this oxidative stress being a by-product of pyrethroid toxicity (Vontas *et al.*, 2001). Another recent study also reported that the Omega class GST from the lepidopteran silkworm moth, *Bombyx mori*, may also be contributing to resistance by binding pyrethroid as well as having a role in oxidative stress response (Yamamoto *et al.*, 2009).

Some GSTs are also said to be involved in lipid peroxidation. The Sigma class GST, namely DmGSTS1-1 were found to have high activity with 4-hydroxynonenal (4-HNE) but has no activity with towards the universal GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). The 4-HNE has shown to be cytotoxic and found to be associated in the etiology of various degenerative diseases at higher levels. It is an electrophilic aldehyde that is derived from the lipid peroxidation and to have role in cell signalling (Singh *et al.*, 2001).

This enzyme has been suggested to have protective role related to oxidative stress because of its high abundance in the adult *Drosophila*, where its localization in tissues that are highly aerobic, for example the flight muscle and its sensitivity to oxidative damage. This kind of function is somewhat same to the Alpha class GSTs from mammals (Singh *et al.*, 2001).

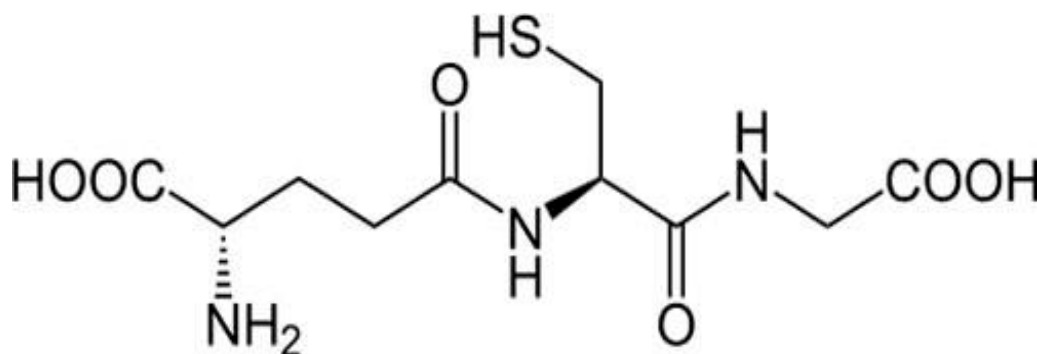


**Figure 1.3:** Example functions catalyzed in the GST superfamily showing reactions in which GSH is consumed by conjugation to the product (top). GSH is not consumed during steroid isomerisation (middle), and GSH is oxidized to glutathione disulfide (GSSG) during xenobiotic dehalogenation (bottom)  
(Adapted from Armstrong, 2010)

## 1.2 Glutathione

Glutathione (GSH) are found most abundantly in aerobic organisms, especially in the liver. It is a water soluble tripeptide that is comprised of three amino acids, namely cysteine, glycine and glutamic acid. It has a potent reducing agent, the thiol group and plays a wide range of functions. It is an important endogenous anti oxidant and play a key role in the maintenance of redox balance in the cell and in the detoxification of xenobiotics (Main *et al.*, 2012). The enzymatic conjugation of the GSH conversion is helped by the Glutathione S-transferases.

It appears in the form of either oxidized (GSSG) or in reduced (GSH) state (Anderson, 1998). The oxidized glutathione can be reduced with the help of glutathione reductase, using the NADPH as an electron donor. The ratio of reduced glutathione to oxidized glutathione is important as it can be used to measure the cellular toxicity (Pastore *et al.*, 2003).



**Figure 1.4:** Structure of glutathione

(Adapted from: <http://chemistry.about.com/od/factsstructures/ig/Chemical-Structures---G/Glutathione.htm>)

### 1.3 Structure of GSTs

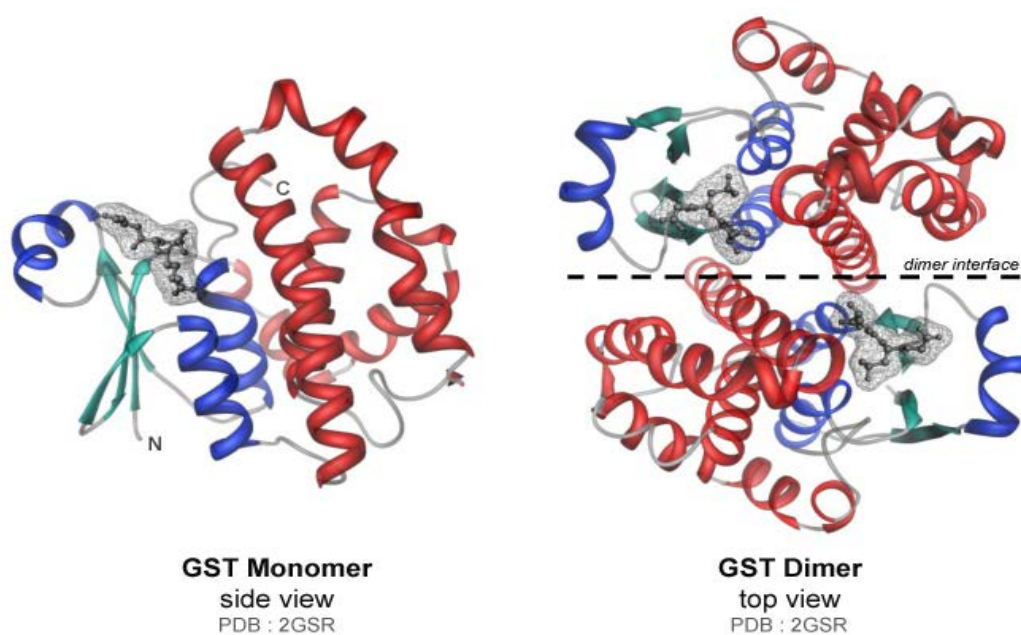
The discovery of a catalytic activity for the addition of glutathione (GSH) to 1, 2-dichloro-4-nitrobenzene in cytosolic extracts of liver in 1961 initiated the increasing interest in the genetics and enzymology of the glutathione transferases culminating in the elucidation of the three-dimensional structures of several cytosolic isozymes. The structural information is important for our understanding of the catalytic mechanisms of the GSTs, evolution of the protein fold and molecular basis for their involvement in detoxification endogenous and xenobiotic electrophiles. The mammalian cytosolic GSTs (The alpha-, Mu- and pi-class) were among the first to be structurally characterized because of their relevance to toxicology, cancer and drug metabolism.

The GST enzymes are large family of enzymes where the loci encoding the gene have been found on at least seven chromosomes (Strange *et al.*, 2001). Cytosolic GSTs (soluble) consist of two subunits forming either identical (homodimers) or non-identical (heterodimers) and are approximately 23-28 kDa in size. This hybridizing is strictly with subunits from the same class. Large numbers of enzymes are formed by the formation of homo- and hetero-dimers from just a limited number of genes. This random dimer formation is also found to happen post translationally as well (Hayes and Pulford, 1995).

The structural interactions of the subunits are very important especially for the stability and dimer assembly, which is why it is class-specific. These findings explained by the availability of crystal structure from the main classes. The novel GST classes were defined by the unusual architecture at the interface between subunit (Board *et al.*, 2000; Rossjohn *et al.*, 1998; Ji *et al.*, 1995).



Each GST subunit contains a specific glutathione binding site, which known as the G-site and the non specific electrophilic ligand binding site (H-site) which is found to be adjacent to each other. Each monomer has polypeptide chains that folds into 2 domains and are joined together by a variable linker region. The polypeptides fold in a very similar way between classes although the amino acid sequence identity is low. Each class, however, still possess a very unique property among them, especially at the C-terminus (Dirr *et al.*, 1994; Wilce and Parker, 1994).



**Figure 1.5:** GST monomer (left) and dimer (right) showing N-terminal thioresoxin-like domain in blue/cyan and the C-terminal all  $\alpha$ -helical domains in red. GSH is shown in dark grey to delineate the active site.

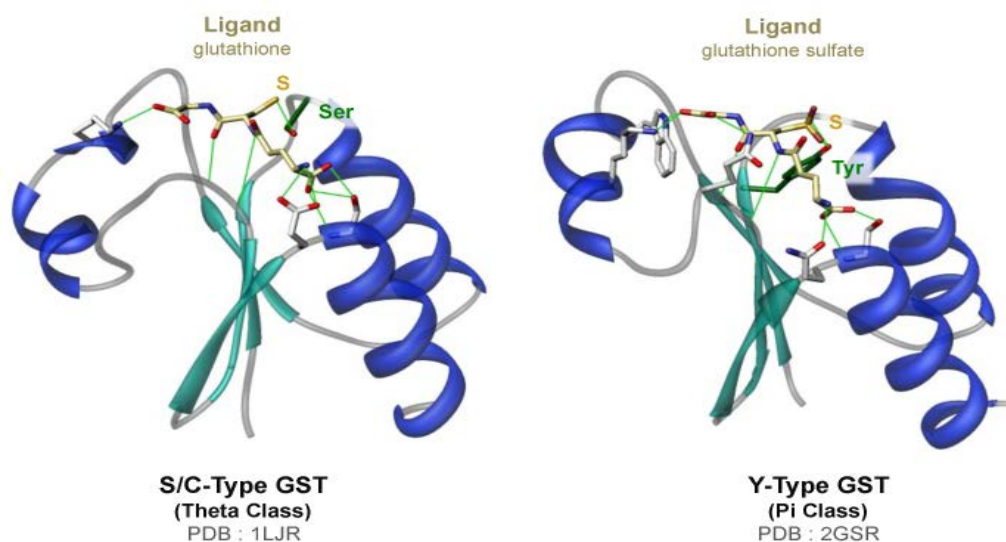
(Adapted from Armstrong, 2010)

The N-terminal domain or the domain 1 (Approximately 1–80 residues) consists of four beta sheets and three flanking alpha helices ( $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$ - $\alpha$ ) and said to be almost one third of the protein. The Helix  $\alpha$ 2 is a mobile surface exposed by helix whereas the cores of the secondary structure elements are formed by helices  $\alpha$ 1 and  $\alpha$ 3 (Wilce and Parker, 1994). Its conformation is somewhat similar to the thioredoxin fold that is found in many proteins that bind GSH or cysteine. Majority of residues that are involved in the binding of glutathione binding is from this domain. The primary structure at the N-terminus involves an important part of the active site and tends to be more conserved than others, within the classes (Armstrong, 1997).

A fundamentally conserved interaction that are found in all classes of cytosolic GSTs and in mitochondrial GSTs is a *cis*-proline residue (found at the N-terminal towards the end of strand  $\beta$ 3) that forms hydrogen-bond interactions with the backbone amine group of the GSH-cysteinyl moiety. The region containing helix  $\alpha$ 2 shows greatest variability among the various classes of GSTs. This secondary structure element contains residues that interact with the glycine-residue of GSH. Recent studies of the structural similarity and sequence analysis of cytosolic GSTs revealed two major subgroups (Atkinson and Babbitt, 2009). These subgroups can be classified as Y-GSTs, which utilize a tyrosine residue to activate GSH, and the S/C-GSTs that utilize serine (or in the beta- and omega-class GST, cysteine) in their interaction with GSH.

In both the Y- and S/C-GSTs (and mitochondrial GSTs), the catalytic GSH-activating residue occurs in a “catalytic loop” following the first  $\beta$ -strand in the thioredoxin-like domain. Human GSTs such as the alpha, mu, pi and sigma-class isozymes fall into the Y-GSTs whereas all the other classes of GSTs fall into the S/C-GST subfamily (Oakley, 2011). Importance of this hydroxyl group has been

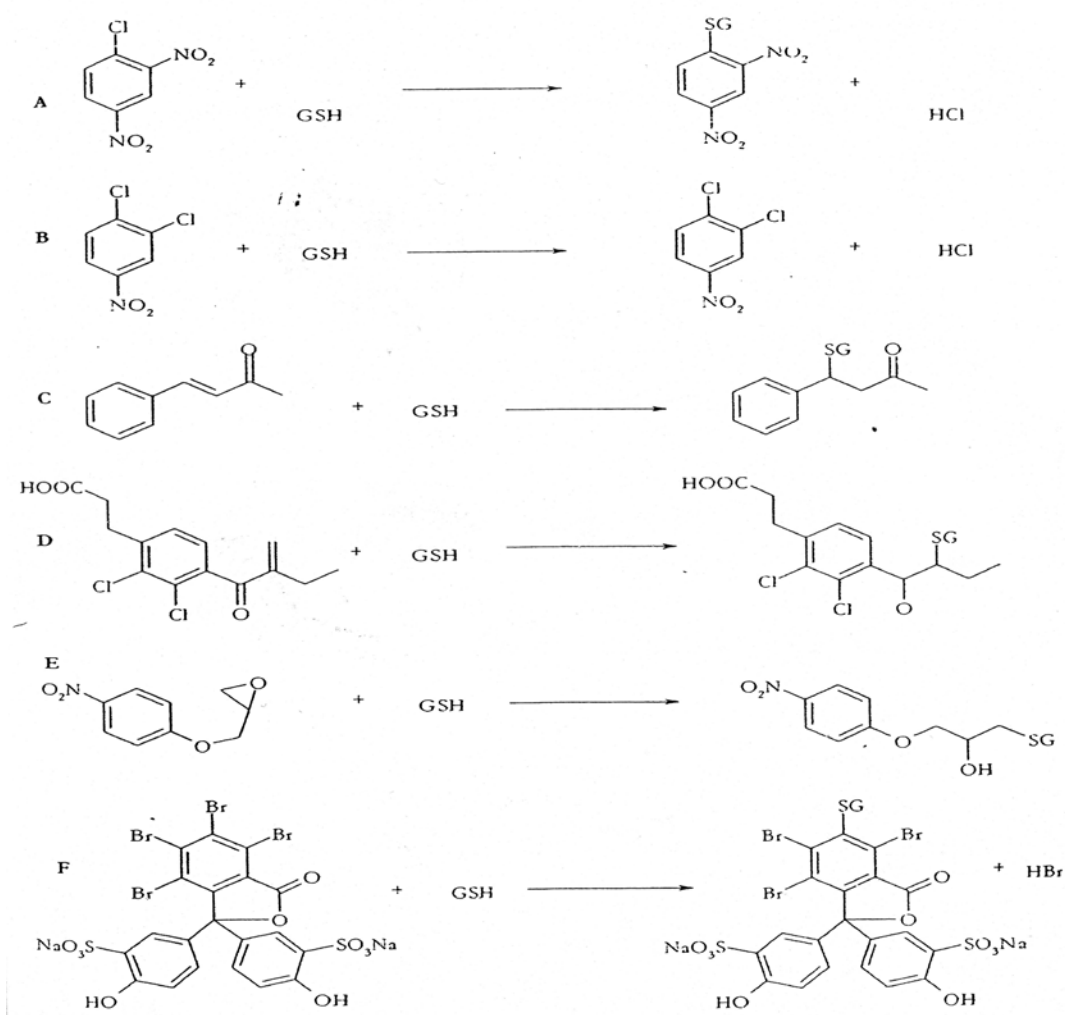
demonstrated in several mutagenesis studies (Kong *et al.*, 1992). Similar studies have also confirmed the importance of serine in S/C-type GSTs for example in human theta-class (Tan *et al.*, 1996).



**Figure 1.6:** Active sites of S/C and Y-type GSTs highlighting the extensive hydrogen bonding network which anchors the glutathione tripeptide.  
(Adapted from: <http://enzymefunction.org/about/bridging-projects/gst-superfamily>)

The larger C-terminal domain or domain 2 (which is two-thirds of the GST) consists of a variable number of alpha helices (Enayati *et al.*, 2005). The location of the binding site for hydrophobic co substrate (H-site) was first revealed in the crystal structure of the human pi-class, hGSTP1-1 (Reinemer *et al.*, 1992). The hydrophobic H-site is variable and is adjacent to the G-site and is responsible to interact with the electrophilic substrates. It is largely formed in a cleft between the N- and the C-terminal domains, both of which may contribute residues to its formation. The H-site varies greatly in shape and chemical character between classes (Oakley, 2011). Their quaternary structure has been found to be essential for their activity and their wide range of substrate specificities is due to the high level of diversity in the quaternary region where each monomeric active site functions independently (Mannervik and Danielson, 1988).

A number of model substrates are widely used to characterize the substrate specificity of GSTs. Some of which are 1-chloro-2, 4-dinitrobenzene (CDNB) and 1, 2-dichloro-4-nitrobenzene (DCNB) although the activity of most insect GST enzymes is higher with CDNB compared to DCNB. Other common substrates used include *trans*-4-phenyl-3-butene-2-one (PBO), Ethacrynic acid (EA), 1, 2-Epoxy-3-nitrophenoxyp propane (EPNP) and Sulfobromophthalein (BSP).



**Figure 1.7:** Conjugation GST with the common substrates in the presence of GSH. (A)CDNB, (B) DCNB, (C) PBO, (D) EA, (E) EPNP and (F) BSP (Adapted from Alias, 2007).

#### 1.4 Classifications and nomenclature

The GST enzyme super family are a wide group of enzymes that has been subdivided into a number of classes and subclasses based on a few criterias such as amino acid or nucleotide sequence similarity, immunological, kinetic and tertiary or quaternary structural properties (Sheehan *et al.*, 2001).

Three main groups of GSTs have been identified to date according to their location within the cell. The first group will be the microsomal GSTs (also known as membrane –associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Jakobsson *et al.*, 1999; Sheehan *et al.*, 2001). The microsomal GSTs are trimeric, membrane bound protein. Structurally, the microsomal GST is very different from the cytosolic GST. However, both group of GSTs catalyse similar reactions (Gakuta and Toshiro, 2000; Prabhu *et al.*, 2001). Three microsomal GST genes were found in mosquito *Anopheles gambiae* while a single microsomal GST gene was found in fruit fly *Drosophila melanogaster* (Toba and Aigaki, 2000; Ranson *et al.*, 2002).

The second group of GSTs will be the mitochondrial GSTs. This group of GSTs has been classified as kappa class and is found in the mammalian mitochondria and peroxisomes (Morel *et al.*, 2004; Lander *et al.*, 2004). This group of GST are structurally very different from the microsomal and cytosolic GSTs (Robinson *et al.*, 2004). Most mitochondrial GSTs identified are from rat and human but none was detected in the insect species to date.

The third group of GSTs are known as the cytosolic GSTs which occur in the cytoplasm of the cell. It is also referred to as soluble GST and is the only group that has been implicated in insecticide resistance (Hemingway *et al.*, 2004; Enayati *et al.*, 2005). There are 4 general class of GST that appears in most of the animals including insect species which are Theta, Omega, Sigma and Zeta (Hayes and McLellan, 1999; Pearson, 2005), two of which (Theta and zeta) appears in all living organism including plants (Dixon *et al.*, 2010) and Bacteria (Skopelitou *et al.*, 2012).

The mammalian cytosolic GST have been assigned to 7 classes including Alpha, Mu, Omega, Pi, Sigma, Theta and Zeta in which Alpha, Mu and Pi appears in all vertebrates such as fish (Mannervik *et al.*, 2005).

Bacteria are also characterized by multiple GST genes of wide sequence just like other eukaryotic organisms. Two groups of GSTs have been identified in bacteria. First is the cytosolic GSR which comprise of four different classes that is Beta, Chi, Theta and Zeta (Sheehan *et al.*, 2001; Wikteliuss and Stenberg, 2007; Rossjohn *et al.*, 1998; Vuilleumier, 1997). Next is the bacterial fosfomycin-resistance proteins FosA and FosB, which is only found in bacteria but not much is known about this protein (Armstrong, 2000).

The plant GSTs are found to differ quite significantly in sequence compared to the mammalian GSTs (Dixon *et al.*, 2002). Based on recent GSTs classifications for plants, four main types of GSTs have been identified to occur in most plants. They are Phi, Zeta, Tau and Theta. In addition to these classes, there are 2 distinct groups that belong to the GST superfamily based on sequence similarity. These groups are found in *Arabidopsis*. One is the DHARs (Dehydroascorbate Reductase) which were recently

found in other plants as well (Jakobsson *et al.*, 1999) and a new lambda class GSTs (Droog, 1997; Wongsantichon and Ketterman, 2005). These group enzymes differ from other plant GSTs by being monomeric and plant specific. Finally, some genes encoding the microsomal GSTs have been identified in plants (Dixon *et al.*, 2002).

Initially, the insect cytosolic GSTs were assigned to numbers according to their order of elution during purification and isoelectric points (Clark *et al.*, 1985) and then came up with two distinct classes of GSTs, Class I and Class II (Fournier *et al.*, 1992). Class I GSTs were encoded by multigene while Class II GSTs were encoded by a single gene (Beall *et al.*, 1992; Ding *et al.*, 2003). However, due to the increasing numbers of insect GSTs, it was reassigned using Greek letters.

There are 6 insect GSTs that have been identified to date. The four classes (Theta, Omega, Sigma and Zeta) and additional two classes, Delta and Epsilon that is insect specific (Saisawang *et al.*, 2011). The Delta and Epsilon are the largest classes of GSTs, occupying almost half the GSTs in insects. However, it is also noticed that some insects such as Hymenoptera (ants and bees) do not have any Epsilon class (Oakeshott *et al.*, 2010) while Delta class GSTs are said to be found in non-insect Species such as arthropods and crustaceans (Lee *et al.*, 2008; Zhao *et al.*, 2010). Both Delta and Epsilon classes are important as it is found to be actively involved in insecticide detoxification in insects.

A recent study on the proteome of *Drosophila melanogaster* cytosolic GSTs identified 36 cytosolic GST genes that expresses 41 protein products. The Omega class are found to have four genes, one of which alternatively splices to yield five proteins in total. Zeta class has 2 genes, one of which encodes three spliced products to produce 4

proteins while the Theta class has four genes that encode five proteins (Saisawang *et al.*, 2011).

The Delta and Epsilon, each has more than 10 members each with over 25 genes when combined. The Delta class has 11 members, DmGSTD1- DmGSTD10 plus a recently identified member, DmGSTD11. While the Epsilon class are found to have 14 members, DmGSTE1-DmGSTE10 plus additional of 4 recently characterized members: CG5224, CG16936, CG11784 and CG4688 which are denoted as DmGSTE11-DmGSTE14 respectively (Saisawang *et al.*, 2011).

### **1.5 Gene regulations**

GSTs are generally induced by environmental signals or in a tissue- or development-specific manner in response to various inducers and are differentially regulated in organisms such as the mammals. For example the human kidney, liver and testis are full of Alpha class GSTs whereas the Pi class are plentiful in the brain, lung and skin. This does not only apply to the human but is also expected in insects. The dietary effect of various compounds such as insecticides on GST expressions were described in two review articles (Clark, 1989; Yu, 1996).

Variations in the level of GST activity in different insect tissues have also been reported in several species. For example, in *Drosophila melanogaster*, the Delta (DmGSTD1) is dominant at the larval fat body while the Epsilon class (DmGSTE1) is dominant at the tubule (Yang *et al.*, 2007). A study by Li *et al.*, (2009) also revealed that eight of eleven GST genes were expressed in all tissue examined, but there were some noticeable variations in expression levels among different tissues of the aquatic midge *Chironomus tentans* specifically, the GSTd2 was expressed in a limited number



of tissues including midgut and fat bodies, whereas GSTu1 was virtually undetectable in salivary glands and hemolymph.

Besides that, it is also known that the level of GST activity fluctuates throughout the life stages of insects. For example, in *Aedes aegypti*, overall GST activity with CDNB (1-chloro-2,4-dinitrobenzene) and DCNB (1,2-dichloronitrobenzene) increase throughout larval stage, reaching its peak in pupa stage, but later on decreases in adult insects (Hazelton and Lang, 1983). Another example is the Sigma class GST from the spruce budworm (*Choristoneura fumiferana*) was expressed at a very low level in feeding larvae but highly expressed in a diapausing larva (Feng *et al.*, 1999). Studies on the development expression patterns on the same species revealed the 2 GSTs, the CfGSTs4 and the CfGSTd5 were found to be present in all tissues and not specifically related to a particular tissue (Zheng *et al.*, 2007). The CfGSTs4, similar to CfGSTd3 were found to be expressed in all tissue at the 6<sup>th</sup> instar larval development of the insect whereas the CfGSTd5, similar to CfGSTd2 were expressed in a higher level in the fat body when insects were about to molt (Huang *et al.*, 2009)

Many studies have reported that most regulations takes place at transcriptional level. The promoter regions of GSTs are said to have many transcriptional elements that may possibly involved in its induction. However, the importance of these elements in the aspects of protein functional studies is still unknown. For example, the Delta class GST in a DDT-resistant *Aedes aegypti* has been found to be highly expressed due to a mutation in a trans-acting repressor element (Ranson *et al.*, 2002)

Studies on the variation in activity by a particular GST can provide further details on the function of different GSTs. However, the mechanism that involved in the controlling the GST expression and the protein regulation in the cell are still unclear. What is known is that GSTs expressions are induced by a series of chemical stresses that happens within the cell as an adaptive response mechanism. Wide range of compounds has been found to be capable of inducing individual isozymes which activates the expression in an isoenzyme-specific manner (Mannervik *et al.*, 2005).

A study on the potency of inducers of NAD(P)H:(quinone-acceptor) oxidoreductase is shown to be parallel with their efficiency as substrates for glutathione transferases. Besides, it is also reported that virtually all glutathione transferase substrates are inducers, and their potencies in the nitrobenzene series correlate linearly with the Hammett sigma or sigma- values of the aromatic substituents, precisely as previously reported for their efficiencies as glutathione transferase substrates (Spencer *et al.*, 1991).

## 1.6 GST purification.

There are various ways to purify GSTs. One of the simplest method will be through affinity chromatography. It is easily purified by using glutathione immobilized to a matrix such as sepharose. The most commonly used columns for the purification of GSTs and GST-tagged proteins will be GSTrap and GSH-agarose that are commercially available. However, the problem using these columns is that not all GST isoforms bind to it. For example, some epsilon class GSTs has no binding affinity towards GSTraps.

The glutathione conjugate of bromosulfophthalein (BSP) was used as a ligand to trap GSTs. However, this too trapped limited isoforms of GSTs such as from the Sigma and Epsilon class, leaving behind classes such as Zeta and Omega (Alias and Clark, 2007).

A recent publication by Pal *et al.* (2012) revealed the use of S-substituted glutathiones as affinity ligands. Among the ligands that were newly introduced are S-(2,4-(dinitrophenyl) glutathione (DNP-SG) that binds wide range of isoforms and also non-GST proteins. Others were S-(2-chloro-4-nitrophenyl) glutathione (CNP-SG) and S-(4-nitrobenzyl) glutathione (NB-SG). All matrices were observed to bind different isoforms of GST.

## 1.7 Olfactory GST

Insects are always exposed to cytotoxic xenobiotics, such as plant derived products, insecticides and herbicides. In order to protect the insects from these harmful compounds, their olfactory systems are designed to trap and sample the volatile hydrophobic compounds. These molecules that enter the olfactory systems are odorants and pheromones from other organisms. Most odorants are small, volatile, lipophilic molecules that enter the mucus flow and reach the odorant receptors which are located on the cilia of the olfactory sensory neurons (Green *et al.*, 2005).

Although there is not much detail known to what happens to the xenobiotics that enters the olfactory sensilla but it is known that the entry of harmful xenobiotics is somehow limited by a group of biotransformation enzymes. The presence of the odorant binding proteins in the sensillum could be responsible for restricting the entry of the toxic compounds. This is just a possibility as there is no evidence to prove the restrictions of odorant binding proteins to the entry of these volatile compounds.

However, the concentration of these harmful substances is believed to be limited by detoxification via a series of enzymatic processes, which helps to reduce the damage caused to the cells. Xenobiotic metabolism is accomplished through the action of the biotransformation enzymes (Rogers *et al.*, 1999). Three groups of such enzymes have been identified in the olfactory system of mammals to date. They are cytochrome P-450, Glutathione transferases and UDP-glucuronosyltransferases (Nef *et al.*, 1989; Ben-Arie *et al.*, 1993).

A few studies has suggested that the olfactory enzymes are responsible of catalyzing the transformation of odorants, which at the same time helps to terminate the olfactory signal as well based on the various odorant types that is modified by them and its presence in the olfactory system (Lazard *et al.*, 1991; Ben-Arie *et al.*, 1993).

*Drosophila melanogaster* has a relatively simple olfactory system, consisting of -1000 receptor neurons (as compared to 100 million in humans) and it is located in the third segment of the antenna (Stocker *et al.*, 1990). The studies on the functional analysis of an olfactory receptor in *Drosophila melanogaster* have identified almost 59 olfactory receptor genes which encode seven trans membrane-domain proteins (Stortkuhl and Raffael, 2001). Recent studies on the *Drosophila* olfactory coding system also proved that it could detect the odor of acid (Ai *et al.*, 2010).

Many studies have been done to prove the presence of GSTs in the mammalian olfactory system such as rats (Ben-arie *et al.*, 1993), cattle (Longo *et al.*, 1991) and humans (Aceto *et al.*, 1989). These olfactory GSTs were found to catalyse glutathione conjugation of several classes that includes unsaturated aldehyde and ketones, as well as epoxides. It is also believed to mediate the covalent modifications of odorants, the agonists of olfactory neurons, thereby affecting their neutralization and clearance (Ben-arie *et al.*, 1993).

Olfactory specific GST was also detected in the Sphinx Moth *Manduca sexta* and was named as GST-msolf1. Studies showed that the endogenous GSTs of male and female antennae is able to modify *trans*-2-hexenal, a plant derived green leaf aldehyde which is known to stimulate the olfactory system of *Manduca sexta* and hence concluded that the GST-msolf1 play dual role of protecting the olfactory system from

harmful xenobiotics and inactivating aldehyde odorants, especially the components of *Manduca sexta* sex pheromone (Rogers *et al.*, 1999).

## 1.8 CG16936

The main aim of this research is to investigate the CG16936, a putative Glutathione s-transferase. It is believed to be an Epsilon class GST. A similarity percentage of 59% to 64% was matched to the *Drosophila* GSTs from the Epsilon class (Alias and Clark, 2007). In general, GSTs that share greater than 60% identity are assigned within a class, whereas those with less than 30% identity are assigned to separate class (Sheehan *et al.*, 2001).

The gene that codes this enzyme is about 672 base pairs in length which encodes a protein of 223 amino acids. Most GSTs are intronless. In *Drosophila melanogaster*, 20 out of 37 genes are intronless (10 Epsilon and 10 Delta GSTs) (Sawicki *et al.*, 2003). However, the CG16936 contains intron which means that it has to be reverse-transcribed into cDNA before the Polymerase chain reaction (PCR) is performed.

A recent study on the *Drosophila* systems approach to xenobiotic metabolism revealed that the CG16936 is found most abundant in the tubule of the adult and larvae (Yang *et al.*, 2007). Several lines of evidence have also suggested that the tubule may be the dominant tissue for xenobiotic mechanism in adult *Drosophila*.

A previous study on chemical challenges, GSTs from adult *Drosophila melanogaster* was partially purified and chemicals such as Phenobarbital and Paraquat were pre treated to all GSTs. There were increases in the relative amounts of all

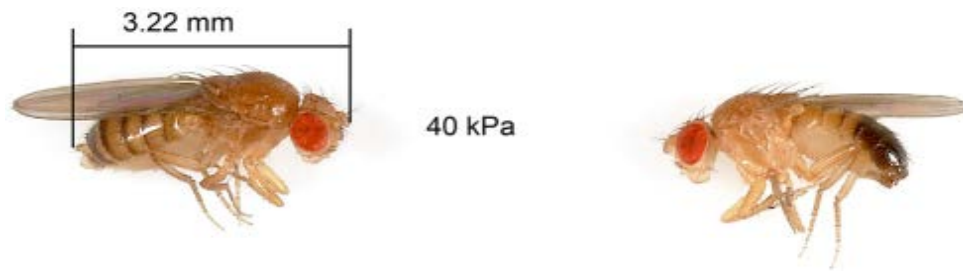
isoforms detected except for CG16936 (Alias and Clark, 2007). This probably shows that this enzyme may not be really be involved in insecticide detoxification.

The CG16936 have been previously reported to have a possible role in the olfactory system of *Drosophila melanogaster* (Anholt and Mackay, 2001). Besides that, a recent study on the molecular evolution of Glutathione S-transferase in genus *Drosophila* also revealed that some uncharacterized genes such as the CG1681, CG1702, and CG16936 are found to be among the slowest evolving compared to others such as CG4623, CG4688, and CG11784 that were quickly evolving among the GST family (Low *et al.*, 2007).

A study on the proteome of *Drosophila melanogaster*, CG16936 were purified and revealed 4 different isoforms of this GST with the same molecular weight (25.4kDa) but different pI value ranging from 5.0 to 5.3 (Pal *et al.*, 2012) respectively although theoretically it was said to have a pI value of 5.9 (Alias and Clark, 2007).

## 1.9 *Drosophila melanogaster*

*Drosophila melanogaster* is a fruit fly about 3 mm long and are easily found around unripe and spoiled fruits. Among members of the animal kingdom, it is an important eukaryotic model. Its known genetics and availability of methods for molecular interactions makes it well suited for research purposes. Many classical genetics information can be obtained to study the physiological functions or even to study the gene of interest by using the *Drosophila* model. Besides that, it is easily bred, cheap and easy to maintain in the laboratory.



**Figure 1.8:** *Drosophila melanogaster* specimens (left is female and right is male)

(Adapted from: <http://www.geochembio.com/biology/organisms/fruitfly/>)

### **Classifications:**

Domain: Eukarya

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Family: Drosophilidae

Genus: *Drosophila*

Species: *melanogaster*



### **1.10 Objectives**

1. To clone and express recombinant CG16936.
2. To purify and determine the substrate specificity of recombinant CG16936.
3. To evaluate the response of CG16936 expression *in vivo* when treated with odorant.
4. To investigate the direct contribution of recombinant CG16936 towards conjugation of odorants to GSH.

## CHAPTER 2: MATERIALS AND METHOD

### 2.0 Materials

#### 2.0.1 Chemicals

0.5M Tris-HCl pH 6.8 (Bio Rad), 1,2-Dichloro-4-nitrobenzene (DCNB) (Sigma), 1,2-Epoxy-3-nitrophenoxypropane (EPNP) (Sigma), 1.5M Tris-HCl pH 8.8 (Bio Rad), 1-Chloro-2,4-dinitrobenzene (CDNB)(Sigma), 1kb DNA Ladder (Promega), 2-mercaptoethanol (Merck), 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (Sigma), Acetic acid (System), Acetone (Sigma), Agarose LE analytical grade (Promega), Ammonium Persulphate (Bio Rad), Ammonium Sulphate (System), Ampicilin Sodium (Duchefa Biochemier), BamHI enzyme (Fermentas), BenchMark™ Protein Ladder (Invitrogen), Benzaldehyde (Friendemann Schmidt), Bioneer Accu Power™ PCR premix, Bromosulphophthalein (BSP) (Sigma), Bromophenol Blue (BDH), Bovine Serum Albumin (Sigma), Butan-1-ol (System), Chloroform (System), Competent cells (*E.coli*BL21(DE3)pLysS) (Novagen), Coomassie Brilliant Blue G-250 (Sigma), Diethyl pyrocarbonate water (Fermentas), Ethacrynic acid (EA) (Sigma), Ethanol (System), Ethidium bromide (Sigma), FastDigest EcoRI enzyme (Fermentas), FastDigest NdeI enzyme (Fermentas), Glutathione reduced (Sigma), Glycerol (System), Hexa-2,4-dienal (Sigma), HindIII enzyme (Fermentas), Imidazole (Bioworld), Isopropanol (Fisher Scientific), Isopropyl  $\beta$ -D-thiogalactopyranoside (Gold Bio.Com), Kanamycin Sulphate (CALBIOCHEM), Luria Bertani Agar (Pronadisa), Luria Bertani Broth (Promega), Lysozyme (Sigma), 30% (w/v) Acrylamide/bis-acrylamide (29:1) (Bio Rad), Methanol (System), Ninhydrin (Sigma), NovaBlue Singles™ Competent Cells (Novagen), Nuclease-free water (Fermentas), Ortho-Phosphoric acid (System), pET-30a(+) plasmid DNA(Novagen), *p*-Nitrophenyl Chloride (NBC) (Sigma), Potassium Chloride (System), PureZOL™ RNA isolation reagent (Bio Rad), SOC medium (Invitrogen), Sodium Chloride (System), Sodium dihydrogen phosphate (System), Sodium Dodecyl

Sulphate (Sigma), Tetramethylethylenediamine (Bio Rad), *trans*-2-Hexenal (Sigma), *trans,trans*-Hepta-2,4-dienal (Sigma), *trans*-4-Phenyl-3-butene-2-one (PBO) (Sigma) and Tris base (Promega).

### 2.0.2 Buffers:

- 10x Buffer BamHI (Fermentas)
- TBE buffer (0.09M Tris-Borate and 2mM EDTA, pH 8.0)
- Buffer A ( 0.1M Sodium Phosphate buffer pH 6.8)
- Buffer B ( 0.1M Tris buffer, pH 9.0)
- Buffer C ( 0.1M Sodium Phosphate buffer, pH 7.5)
- SDS reducing buffer [0.5 M Tris-HCl pH 6.8, glycerol, 10% (w/v) SDS and 0.5% (w/v) Bromophenol Blue and  $\beta$ -mercaptoethanol(prior to use)]
- 10X Tris/Glycine/SDS running buffer (Biorad)

### 2.0.3 Kits

- First Strand cDNA Synthesis Kit (Fermentas)
- LaboPass™ Gel Extraction Kit
- pGEM-T Easy Vector Ligation Kit (Promega)
- T4 DNA Ligase Kit (Bioline)
- innuPREP Plasmid Rapid Kit (Analytik Jena)

#### **2.0.4 Columns/ Matrice for protein purification**

- GSTrap™ HP (GE Healthcare)
- Hi-Trap Desalting(G-25) (GE Healthcare)
- HisTrap™ FF Crude (GE Healthcare)
- HiPrep 16/60 Sephacryl S-200 (GE Healthcare)
- HiTrap ANX FF (GE Healthcare)
- HiTrap DEAE FF (GE Healthcare)
- S-Hexylglutathione (Sigma)
- Superdex 75 (Sigma)
- Affi-Gel Blue Gel (Biorad)
- S-2,4-(dinitrophenyl) GSH (DNP-SG) (Gift from Prof. Alan Clark, Department of Biological Sciences, Victoria University of Wellington, New Zealand)
- Bromosulfophthalein GSH (BSP-SG) (Gift from Prof. Alan Clark, Department of Biological Sciences, Victoria University of Wellington, New Zealand)
- Sep-PakC18 cartridge (Waters)

#### **2.0.5 Equipments**

The equipments that have been used in this study include:

- Amersham Bioscience AKTA FPLC™
- Pellet Pestle Motor Grinder (Sigma-Aldrich)
- Biorad Mycycler thermal cycler (Polymerase chain reaction machine)
- Agarose gel electrophoresis cell (Biorad)
- Alpha Innotech UV transilluminator
- Centrifuge machine (Eppendorf)
- Microwave
- Vortex mixer

- Water bath
- PCR air clean cupboard
- Shaking incubator
- pH meter
- Mini-protean tetra cell (Biorad)
- Jasco V630 spectrophotometer (Alpha Analytical)
- Laminar hood
- Sonicator
- T.L.C Silica Gel 60 F<sub>254</sub> (Merck)
- Centrifugal concentrator (Vivaspin 20, Sartorius Stedim)

#### **2.0.6 Insects**

*Drosophila melanogaster* that is used in this study were obtained from the Department of Genetics, University of Malaya and bred at the lab using medium containing oats, sugar and agar (Full recipe at Appendix D).

## 2.1 METHODOLOGY

### 2.1.1 Total RNA extraction from *Drosophila melanogaster*.

One ml of PureZOL reagent was added per 50-100 mg of fresh *Drosophila melanogaster* and was homogenised in a 2 ml round-bottom micro centrifuge tubes using the pellet pestle motor grinder (Sigma-Aldrich). Homogenised sample was incubated at room temperature for 5 minutes. Homogenate was then split into 1 ml aliquots in 2 ml round-bottom micro centrifuge tubes. A total of 0.2 ml of chloroform was added per 1 ml of PureZOL reagent used. Sample was then mixed vigorously for 15 seconds and incubated at room temperature (28°C) for 2 to 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous upper phase was transferred to a new 2 ml micro centrifuge tubes and 0.5 ml of isopropanol was added per 1 ml of PureZOL reagent used. Tube was mixed by inversion. Sample was then incubated at room temperature (28°C) for 10 minutes and centrifuged at 12,000 x g for 15 minutes at 2-8°C (At this point, RNA can be seen as a pellet at the bottom of the tube). Supernatant was carefully removed and pellet was washed once with 75% (v/v) ethanol, adding 1 ml per 1 ml of PureZOL reagent used. Sample was vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C. Supernatant was removed and RNA pellet was air-dried. Pellet was resuspended in 50-100 µl DEPC H<sub>2</sub>O. Total RNA was then stored in -70°C until further analysis.

### 2.1.2 cDNA synthesis

Ten µl RNA (~50ng) was heated at 65°C for 10 minutes and was quenched on ice. First strand cDNA Synthesis Kit (Fermentas) were used. The following components were added into a sterile, nuclease-free 0.2 ml tube on ice as per recommended in the manufacturer's manual: 6 µl Template RNA (50 ng), 1 µl oligo (dT)<sub>18</sub> Primer (0.5 µg/µl), 4 µl DEPC-treated water, 4 µl 5 X reaction buffer, 1 µl RiboLock RNase Inhibitor

(20 units/ $\mu$ l), 2  $\mu$ l 10 mM dNTP mix and 2  $\mu$ l M-MuLV Reverse Transcriptase (20 units/ $\mu$ l) which sums up to a total volume of 20  $\mu$ l. The reaction mixture was mixed gently and spun down (quick spin) to make sure all components were at the bottom of the tube. It is then incubated for 60 minutes at 37°C for the cDNA synthesis. Reaction was terminated by heating at 70°C for 5 minutes. Reverse transcription reaction product was directly used in PCR application or stored at -70°C until further use.

### 2.1.3 Polymerase chain reaction (PCR)

PCR were carried out to amplify *CG16936*. With a total volume of 5  $\mu$ l of cDNA template (50ng), 0.5  $\mu$ l 10 mM reverse primer, 0.5  $\mu$ l of 10 mM forward primer and 14  $\mu$ l of distilled water were added to Bioneer Accu Power<sup>TM</sup> PCR Premix in a 0.2 ml micro centrifuge tube. PCR was performed with hot start of 95°C for 3 minutes then followed by 35 cycles of amplification with denaturation at 95°C for 30 seconds, annealing at temperature 62°C which was determined according to the melting temperature of primers for 30 seconds and elongation for 1 minute at 72°C per 1 kb. After 35 cycles, it was set to hold at 4°C for keeping the samples. Primers used were as per Table 2.1 below.

Forward primer/ BamHI:	5' CGC <u>GGA TCC</u> ATG TCA AAG CCA GCT CTG TA 3'	Tm: 64°C
Reverse primer/ Hind III:	5' CCC <u><b>AAG CTT</b></u> CTA CTT GCC ACG GTT TTC TG 3'	Tm: 63°C

**Table 2.1:** Forward primer and reverse primer used for the amplification *CG16936*

Forward primer was designed with inclusion of the BamHI restriction site (underline) followed by initiation codon ATG whereas the reverse primer was designed with HindIII (underline with bold case) for *CG16936*. PCR product was then loaded into a 1% (w/v) agarose gel.

#### **2.1.4 Agarose Gel electrophoresis of DNA.**

A total of 0.4 g of agarose powder was dissolved in 40 ml of 0.5 x TBE buffer to prepare 1% (w/v) gel. The agarose solution was then micro waved to completely dissolve the agarose powder into the buffer. It was then left to cool down to 55°C to 60°C before pouring into the gel tray. Proper comb (1.5 mm) was inserted to form the well and gel was allowed to harden for 30 minutes. Comb was removed and gel was placed in the gel tank. 0.5 x TBE buffer was poured until gel was completely covered with the buffer and allowed to soak for 30 minutes. Two µl of loading dye (Blue/Orange Loading Dye, 6 x) was mixed with 10 µl sample (PCR product) and pipetted into the well. One kb DNA Ladder (Promega) was used as marker. The loading dye/buffer gives colour and density to the sample to facilitated loading into the wells. The dye was negatively charged in neutral buffers and thus moves in the same direction as the DNA during electrophoresis, allowing us to monitor the progress of the gel. Electrophoresis was performed at 60 V for approximately 1 hour and 30 minutes or until the marker reaches bottom of the tank. Gel was taken out of the gel tank and placed in a tupperware containing ethidium bromide (0.5 mg/ml) and stained for 10 minutes with gentle shaking. Gel was then placed in distilled water and destained for 20 minutes. The DNA bands were visualized by ultraviolet radiation (302 nm wavelength) and the image saved.

#### **2.1.5 DNA extraction from agarose gel.**

DNA fragment was exercised from the agarose gel with a sharp scalpel. Labopass Gel extraction kit was used. Gel slice was transferred into a 1.5 ml reaction tube and 650 µl of gel solubilizer are added. It was incubated for 10 minutes at 50°C until the agarose gel slice was completely dissolved. Fifty µl binding optimizer was added and suspension was mixed by vortexing. Sample was applied onto spin filter



located in a 2.0 ml receiver tube. Cap was closed and centrifuged at 10,000 x g for 1 minute.

Filtrate was discarded and receiver tube was re-used. A total of 700 µl washing solution [70% (v/v) ethanol] was added and centrifuged at 10,000 x g for 1 minute. Filtrate was discarded and receiver tube was reused. Spin filter was placed back into the 2.0 ml receiver tube. It was then centrifuged at max speed for 2 minutes to remove all traces of ethanol. Receiver tube was discarded and spin filter was placed into a 1.5 ml elution tube. Ten to twenty µl elution buffer was added into the spin filter and incubated at room temperature for 2 minutes. It was centrifuged at 8,000 x g for 1 minute. Half of the extracted DNA was used to be digested with restriction enzymes while other half was used to ligate with pGEM-T vector.

#### **2.1.6 Digestion with restriction enzymes.**

Two different restriction enzymes were used based on the map of the cloning vector used. HindIII and BamHI were chosen because it includes 6x Histidine tagging to the gene of interest which will assist with purification process. The following components were added into a 0.2 ml micro centrifuge tube accordingly: 18 µl nuclease-free water, 2 µl 10 x Buffer BamHI, 10 µl PCR reaction mixture (~0.5 µg), 1 µl BamHI enzyme and 2 µl HindIII enzyme. Tube was mixed gently and spun down for a few seconds. Tube was then incubated at 37°C for 16 hours. Digested PCR product was purified using the gel purification method.

For the digestion of pET30a, the following components were added in a 0.2 ml micro centrifuge tube accordingly: 16 µl nuclease-free water, 2 µl 10x Buffer BamHI, 1 µl pET-30a(+) plasmid DNA, 1 µl BamHI enzyme and 2 µl HindIII enzyme. Tube was mixed gently and spun down for a few seconds. Tube was then incubated at 37°C for 16 hours. Digested plasmid was purified using the gel purification method.

### **2.1.7 Ligation of digested PCR product and pET-30a(+).**

Ligation was done using the T4 DNA Ligase kit. Digested PCR product, digested pET-30a(+) plasmid DNA, T4 DNA Ligase Kit was thawed and placed on ice. (Solutions were mixed during the thawing process to avoid localized concentrations of salt). A ligation-reaction mixture was prepared according to the following scheme: 2 µl 10 x T4 Ligase Reaction Buffer, 2 µl 10 x ATP Solution, 2 µl digested vector, 6 µl digested PCR product, 1 µl T4 DNA Ligase and 7 µl of water (ddH<sub>2</sub>O). The ligation-reaction mixture was mixed gently (by pipetting the mixture up and down) and was then incubated for 16-24 hours (for maximum yield) at 12-14°C. Ligation-reaction mixture was stored at -20°C until further use. Cloned product was confirmed using the agarose gel electrophoresis method.

### **2.1.8 Transformation with *E.coli* BL21 (DE3) pLysS**

Competent cells are thawed on ice. Super Optimal Broth with Catabolite repression (SOC) medium was also thawed and warmed to room temperature (28°C). 1-2 µl ligation-reaction mixture was added to the competent cells tube (50 µl), mixed gently (gentle flicking) and incubated on ice for 30 minutes. The cells were then heated in a 42°C water bath for 45 seconds without shaking, followed by incubation on ice for another 5 minutes. A total of 250 µl room temperature SOC medium was added to the tube containing the transformation mixture and was incubated at 37°C for 1 hour with shaking at 250 rpm. Hundred µl of the transformation mixture was plated onto LB (Luria Bertani) agar containing Kanamycin (30µg/ml) [(pET-30a(+)) has the kanamycin resistant gene)]. Plate was incubated at room temperature until the transformation mixture has absorbed into the agar. Plate was then inverted and incubated at 37°C overnight.

### **2.1.9 Ligation of PCR product with pGEM-T vector.**

Ligation of purified PCR product with pGEM-T was done using the pGEM-T Vector Ligation Kit. The following were added to a 0.2 ml micro centrifuge tube according to the protocol provided by the manufacturer: 5 µl 2 x Rapid Ligation Buffer, 1 µl pGEM®-T vector, 3 µl purified PCR product product, and 1 µl T4 DNA Ligase. Reaction was mixed by pipetting and incubated overnight at 4°C to produce maximum amount of transformants.

### **2.1.10 Transformation with NovaBlue Singles™ Competent Cells**

Competent cells are thawed on ice and mixed gently to ensure that cells are evenly suspended. 5 µl of the ligation mixture was added directly to the cells. Tube was flicked gently to mix. Tube was placed on ice for 5 minutes. Tube was heated for exactly 30 seconds in a 42°C water bath, without shaking. It was then placed on ice for 2 minutes. 250 µl of room temperature SOC medium was added to tube. Tube was shaken at 37°C (250 rpm) for 30 minutes prior to plating. 100 µl of the transformation mixture was plated onto X-gal plate and covered with aluminium foil to prevent light exposure. Plate was incubated at room temperature until the transformation mixture has absorbed into the agar. Plate was then inverted and incubated at 37°C overnight. Colonies in white were cells containing plasmid with insert while the colonies in blue are cells containing plasmid without insert. One bacteria colony was inoculated into a 5-ml LB broth containing kanamycin (30 µg/ml) and placed in a shaking incubator (250rpm) at 37°C and left overnight for the bacteria cells to multiply.

### **2.1.11 Plasmid extraction.**

InnuPREP Plasmid Rapid Kit was used to extract the plasmid. Three-5 ml of the overnight *E.coli* culture from section **2.1.10** was transferred into a 1.5 ml reaction tube. It was centrifuged for 1 minute at maximum speed to pellet the cells and supernatant was removed completely. Bacterial cell pellet was resuspended in 200 µl re-suspension buffer completely by vortexing or by pipetting up and down. Two hundred µl lysis buffer was added and mixed carefully by inverting the tube 5-10 times. 300 µl of neutralization buffer was added and mixed gently, but thoroughly, by inverting the tube 4-6 times. Sample was applied onto a Pre-filter located in a 2.0 ml receiver tube. It was centrifuged at 10,000 x g for 2 minutes. Clarified filtrate was applied onto a spin filter located in a 2.0 ml receiver tube and centrifuged at 10,000 x g for 1 minute. Filtrate was discarded and the 2.0 ml receiver tube was re-used. 500 µl washing solution A was added to the spin filter and centrifuged at 10,000 x g for 1 minute. Filtrate was discarded and 2.0 ml receiver tube was re-used. 700 µl washing solution B was added to the spin filter and centrifuged 10,000 x g for 1 minute. Filtrate was discarded and the tube was centrifuged at maximum speed for 2 minutes to remove all traces of ethanol. The 2.0 ml receiver tube was discarded. Spin filter was placed into 1.5 ml elution tube and 30 µl of elution buffer was added to the centre of the spin filter. Tube was incubated at room temperature for 1 minute and centrifuged at 6,000 x g for 1 minute. Extracted plasmid DNA was kept at -20°C for further analysis.

### **2.1.12 Digestion of ligated pGEM-T plasmid with restriction enzymes.**

Digestion was carried out as specified in the manufacturer's manual. Plasmid was double digested with FastDigest NdeI and EcoRI with a suitable common buffer, which gave maximal activity to both enzymes. Mixture was mixed gently and spun down. It was then incubated at 37°C in a heat block for 5 minutes. The NdeI and EcoRI

were chosen because these two restriction sites were present in both pGEM-T and pET30. After digestion, the reaction mixture was loaded into an 1% (w/v) agarose gel. The lower band at approximately 700 bp which was the PCR product, was gel purified and used to ligate into the expression vector, pET30 and transformed in the *E.coli BL21* for protein expression as mentioned in section **2.1.7** and **2.1.8**.

#### **2.1.13 Cell culturing and lysis.**

One gram of LB broth was dissolved in 50 ml of distilled water in a conical flask and autoclaved. The flask was cooled to 37°C and kanamycin was added to a final concentration of 30 µg/ml. One bacteria colony was inoculated into the flask and placed in a shaking incubator (250rpm) at 37°C and left overnight for the bacteria cells to multiply. The next day, 10 ml of the fresh bacterial culture was added into a new 500 ml LB broth containing kanamycin (30 µg/ml) and placed in shaking incubator (250 rpm) at 37°C for 5 hours. IPTG was added to the final concentration of 1 mM to the flask and continued shaking at 37°C for an additional of 3 hours.

Bacterial culture was then centrifuged at 6,000 rpm for 15 minutes at 4°C to pellet the cells. Cell pellet was then resuspended with 10 ml of lysis buffer [(25 mM sodium phosphate, 1.0 mM EDTA, lysozyme (0.25 mg/ml)] and left to shake for 15 minutes. Crude lysate was centrifuged at 10,000 x g for 30 minutes at 4°C to remove cellular debris. Supernatant containing the protein of interest was transferred to a clean tube without disturbing the pellet and stored on ice.

### 2.1.14 Purification of recombinant CG16936

Crude lysate of the bacterial lysis was subjected to affinity chromatography using several types of matrices. Affinity chromatography was carried out using Amersham Bioscience AKTA FPLC™ connected to a fraction collector. Each column was equilibrated with about 30 ml of binding buffer to ensure proper equilibration. 5 ml of crude lysate was injected; allowing the sample to flow through the column followed by washing of the column with another 20 ml of binding buffer to ensure all the unbound protein was washed out completely. For columns such as bromosulphophthalein (BSP) and HisTrap™ FF Crude, additional washing with 1 M sodium chloride or potassium chloride was required to remove the non-specific protein binding. Once washed, the protein was eluted out using elution buffer as stated below (Table 2.2).

As for Superdex 75 and Sephacryl S-200, no elution buffer was required as the protein was eluted out according to its size. Every peak obtained was collected and enzyme activity was determined. Table 2.2 is a summary of the columns and buffers that was used for purification.

**Table 2.2:** Summary of columns and buffers used.

No.	Column	Binding Buffer	Elution Buffer	Washing Buffer
1.	GSTrap™ HP	25 mM Sodium Phosphate buffer, pH7.4	10 mM GSH, pH7.4	Nil
2.	S-Hexylglutathione	25 mM Sodium Phosphate buffer, pH7.4	10 mM GSH, pH7.4	Nil
3.	DNP-SG	25 mM Sodium Phosphate buffer, pH7.4	10 mM GSH, pH7.4/ 20 mM GSH pH9.6	Nil
4	BSP-SG , Hi-Trap Desalting(G-25)	25 mM Sodium Phosphate buffer, pH7.4	2 mM BSP, pH 7.4/ 20 mM GSH pH7.4	1 M NaCl 1 M KCl
5	HisTrap™ FF Crude	25 mM Sodium Phosphate buffer + 30 mM imidazole, pH7.4	300 mM imidazole, pH7.4	1 M NaCl
6	Superdex 75	25 mM Sodium Phosphate buffer, pH7.4	Nil	Nil
7	Sephacryl S-200	25 mM Sodium Phosphate Buffer, pH7.4	Nil	Nil

**Table 2.2**, continued.

8.	HiTrap ANX FF	25 mM Sodium Phosphate buffer, pH7.4	1M NaCl, pH7.4	Nil
9.	DEAE	25 mM Sodium Phosphate buffer, pH7.4	1M NaCl, pH7.4	Nil
10	Affi-Gel Blue Gel	25 mM Sodium Phosphate buffer, pH7.4	150 mM NaCl, pH7.4	Nil
11	Sep-Pak C18	2% (v/v) Methanol + 0.1% (v/v) Trifluoroacetic acid	20% (v/v) Methanol	Nil

### 2.1.15 SDS-Polyacrylamide gel (PAGE)

Polyacrylamide gel casting was performed using the Bio-Rad MINI PROTEAN II System (Bio-Rad Laboratories, USA) following the manufacturer's instructions. SDS-PAGE gel formulations are as described in Table 2.3 below. The resolving gel (lower part) was prepared and allowed to polymerize for 30 minutes to an hour and overlaid with distilled water. The overlaid distilled water was poured away and replaced with the stacking gel. A comb was placed on top of the stacking gel to form well. After polymerization, comb was removed and the stacking gel was washed with distilled water to remove the unpolymerized acrylamide solution.

**Table 2.3:** SDS-PAGE gel formulations

<b>Solution</b>	<b>4% Stacking Gel (ml)</b>	<b>12% Resolving gel (ml)</b>
30% (w/v) Acrylamide/bis-acrylamide (29:1)	3.3	4.0
1.5 M Tris-HCl pH 8.8	-	2.5
0.5 M Tris-HCl pH 6.8	6.3	-
Distilled water	15	3.4
10% (w/v) SDS	0.25	0.1
TEMED	0.005	0.005
10% (w/v) Ammonium Persulphate	0.125	0.05

The electrophoresis apparatus were assembled following the instructions for the Bio-Rad Mini-PROTEAN® II System. Sample was then diluted with SDS reducing buffer (at least 1:2) and heated at 95°C for 4 minutes. Samples and protein standard

markers were loaded into the wells. Electrophoresis was performed in descending directions, with running buffer 1X Tris-glycine buffer (25 mM Tris-HCl pH 8.6, 192 mM Glycine, and 0.1% (w/v) SDS) with a constant voltage of 120 volts until the bromophenol marker reaches the bottom edge of the gel tank. Once done, the electrophoresis apparatus was disassembled and gel was stained in Colloidal Coomassie staining solution [(5% (w/v) Coomassie Brilliant Blue, 85% (v/v) Ortho-phosphoric acid and Ammonium Sulphate] and left overnight. Gel was then washed with 20% (v/v) methanol and viewed under visible white light.

#### **2.1.16 Bradford assay**

A total of 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% (v/v) ethanol and 100 ml of 85% (w/v) phosphoric acid was added to the mixture. The solution was then diluted to 1 liter when the dye has completely dissolved, and was filtered through Whatman #1 paper just before use. Filtered Bradford solution was wrapped in aluminium foil and stored in dark as it is light sensitive. Standards (BSA) ranging from 20-100 µg was prepared in 100 µl volume.

Hundred µl of sample was added to 5 ml Bradford reagent and mixed well using vortex. Mixture was incubated for 5 minutes in the dark. Absorbance of the mixture was measured at 595 nm (Bradford, 1976).

#### **2.1.17 Assay for GSTs**

1-Chloro-2,4-dinitrobenzene (CDNB)

All the below were added in a cuvette accordingly: 2.85 ml Buffer A (0.1M Sodium Phosphate, pH 6.8), 0.05 ml 60 mM GSH (freshly prepared), 0.05 ml sample. The sample was replaced with potassium phosphate buffer for negative control. Finally,



0.05 ml of 60 mM CDNB was added (which makes the total volume 3 ml) and mixed well. Progress on conjugating products with CDNB was monitored over time by the change of absorbance at 340 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 3 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is 9600  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Habig *et al.*, 1974).

#### 1,2-Dichloro-4-nitrobenzene (DCNB)

All the following were added in a cuvette accordingly: 2.80 ml Buffer B (0.1 M Tris buffer, pH 9.0), 0.05 ml 240 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer B for negative control. Finally, 0.05 ml 24 mM DCNB was added (total volume 3 ml) and mixed well. Progress on conjugating products with DCNB was monitored over time by the change of absorbance at 344 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 20 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is 8400  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Motoyama and Dauterman, 1974).

#### *p*-Nitrophenyl Chloride (NBC)

All the following were added in a quart cuvette accordingly: 2.60 ml Buffer A (0.1 M Sodium Phosphate, pH 6.8), 0.25 ml 60 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer A for the negative control. Finally, 0.05 ml 60 mM NBC was added (total volume 3 ml) and mixed well. Progress on conjugating products with NBC was monitored over time by the change of absorbance at 310 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 10 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is 1900  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Habig *et al.*, 1974).

1,2-Epoxy-3-nitrophenoxypropane (EPNP).

All the following were added in a quart cuvette accordingly: 2.60 ml Buffer A (0.1 M Sodium Phosphate, pH 6.8), 0.25 ml 60 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer A for negative control. Finally, 0.05 ml 30 mM EPNP was added (total volume 3 ml) and mixed well. Progress on conjugating products with EPNP was monitored over time by the change of absorbance at 360 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 10 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is 500  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Habig *et al.*, 1974).

Sulfobromophthalein (BSP)

All the following were added in a quart cuvette accordingly: 2.60 ml Buffer C (0.1 M Sodium Phosphate, pH 7.5), 0.25 ml 60 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer C for negative control. Finally, 0.05 ml 2 mM BSP was added (total volume 3 ml) and mixed well. Progress on conjugating products with BSP was monitored over time by the change of absorbance at 330 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 10 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is 4500  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Habig *et al.*, 1974).

Ethacrynic acid (EA)

All the following were added in a quart cuvette accordingly: 2.8 ml Buffer A (0.1 M Sodium Phosphate, pH 6.8), 0.05 ml 15 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer A for negative control. Finally, 0.05 ml 12 mM EA is added (total volume 3 ml) and mixed well. Progress on conjugating products

with EA was monitored over time by the change of absorbance at 270 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 10 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is 5000  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Habig *et al.*, 1974).

#### *trans*-4-Phenyl-3-butene-2-one (PBO)

All the following were added in a quartz cuvette accordingly: 2.8 ml Buffer A (0.1M Sodium Phosphate, pH 6.8), 0.05 ml 15 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer A for negative control. Finally, 0.05 ml 3 mM PBO was added (total volume 3 ml) and mixed well. Progress on conjugating products with PBO was monitored over time by the change of absorbance at 290 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 10 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is -24800  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Habig *et al.*, 1974).

#### Hexa-2,4-dienal

All the following were added in a quartz cuvette accordingly: 2.8 ml Buffer A (0.1M Sodium Phosphate, pH 6.8), 0.05 ml 150 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer A for negative control. Finally, 0.05 ml 3 mM hexa-2,4-dienal was added (total volume 3 ml) and mixed well. Progress on conjugating products with hexa-2,4-dienal was monitored over time by the change of absorbance at 280 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 10 minutes at 25°C. Molar absorption coefficient  $\xi_m$ , is -34200  $\text{l.mol}^{-1}.\text{cm}^{-1}$  (Brophy *et al.*, 1989).

*trans,trans*-Hepta-2,4-dienal.

All the following were added in a quart cuvette accordingly: 2.8 ml Buffer A, 0.05 ml 150 mM GSH (freshly prepared) and 0.10 ml sample. The sample was replaced with buffer A for negative control. Finally, 0.05 ml 3 mM *trans,trans*-Hepta-2,4-dienal is added (total volume 3 ml) and mixed well. Progress on conjugating products with *trans,trans*-Hepta-2,4-dienal was monitored over time by the change of absorbance at 280 nm using the Jasco V630 spectrophotometer. This standard GST assay was measured for 10 minutes at 25°C.

Molar absorption coefficient  $\xi_m$ , is  $-30300 \text{ l.mol}^{-1}.\text{cm}^{-1}$  (Brophy *et al.*, 1989).

#### **2.1.18 Thin Layer Chromatography.**

A 0.2 mm thick t.l.c silica gel 60 F<sub>254</sub> plate was cut into 10 cm X 10 cm. A light pencil line was drawn at about 1 cm from the end of the chromatographic plate. Each sample was spotted within a distance of 0.5 cm each. A separate capillary was used for every sample. Each spot was made as small as possible, no more than 2-3 mm in diameter. A developing chamber was prepared using a large beaker as a chamber. The developing solvent, a mixture of butan-1-ol/acetic acid/water (12:3:5, v/v/v) was poured into the beaker at a depth of approximately 1cm was covered with aluminium foil to prevent evaporation of the solvent and to allow saturation in the chamber for about 15 minutes. The prepared t.l.c plate was then placed into the developing chamber and once again covered with aluminium foil. The plate was removed once the solvent has risen to near the top of the plate (about 1 cm from the top). Plate was kept in the hood until the majority of solvent has evaporated from the plate. Once dried, the plate was stained with ninhydrin (0.25 %, w/v in acetone). The stained spots were outlined with a marker.

#### **2.1.19 *Drosophila* exposure with/without odorant.**

Six bottles containing fresh medium containing oats, sugar and agar (full recipe in appendix D) was prepared. Approximately 200 mg of adult *Drosophila* was transferred into each bottle. A Q-tip dipped in distilled water is inserted into 3 bottles each to act as negative control while a Q-tip dipped into odorant (benzaldehyde) was inserted into the remaining 3 bottles to be used as the positive control. The *Drosophila* was exposed for 5 days continuously replacing the Q-tips with freshly dipped odorant/water every day. After 5 days, Q-tips were removed and *Drosophila* was kept in -20°C for further analysis.

## CHAPTER 3: RESULTS

### 3.1 RNA extraction

The CG16936 is an epsilon class GST that have not been characterized previously. The gene that codes this protein contains intron. Thus, RNA instead of DNA was extracted from *Drosophila melanogaster*. The concentration of RNA extracted was quantified using the absorbance readings of the Spectrophotometer. Absorbance was read at 260 nm and 280 nm. A crude way to calculate the concentration from the absorption at 260 nm was used:

Beer Lambert's law:  $A = e C l$

Where A is the measured absorption at 260 nm

e is the RNA extinction coefficient (0.040)

C the RNA concentration

and l is the path length (1 cm)

Purity of nucleotide was evaluated by using the ratio of

$\text{Absorbance}_{260\text{nm}} / \text{Absorbance}_{280\text{nm}}$  as a range of 1.7 to 2 is considered pure RNA

(Glasel, 1995).

### 3.2 Polymerase chain reaction (PCR)

PCR reaction was performed according to the conditions mentioned earlier. Gene sequence for CG16936 was obtained from Flybase, a database of *Drosophila* Genes and Genomes (<http://flybase.org>). Primers were designed and used to amplify the gene. Gene sequence and primer sequences used are as below:

**Table 3.1:** Gene sequence of CG16936 aligned with forward (red) and reverse (green) primers, with the restriction sites underlined.

5'CGCGGATCCATGTCAAAGCCAGCTCTGTA 3'

5'CGC**GGATCC**ATGTCAAAGCCAGCTCTGTATTATGCCACCCTAAGTCCCCCATCGCGCGC  
CGTCCTCCTCACGGCTAAGGCGATCGGACTCGACCTTGAACCTACGGTAGGTTCCCACACCG  
TGCCACTTAACTCAATTAACAGAGTTTTCGCCCCGAATGCAGGCCAATTAACCTGCTGAAGG  
GAGAGCATCTGACTCCGGAATTCTCAAGCTGAACCCCCAGCACACCATCCCGACCCTGA  
TCGACGGCGAGGCCACTATCATTGACTCGCACGCCATCTGCGCCTACCTGGTGGAGAAGT  
ATGGCCAGAAGGAGCAGCAGCTCTATCCGAAGGAATTGGTGCAGCGCGCCAACGTGGAT  
GCTCGGCTCCATCTGGACTCCGGCCACCTCTTCGCGCGCCTGCGCTTCTTTACGAGCCCAT  
CCTGTATTATGGATCGACGGACTGCTCCATCGACAAGATCGCATAATCCAGAAGTGCTGG  
GAGATCCTAGAGGGATTCTCAAGGATCAGCCGTATTTGTGTGGTTCTGATCTAACCATCG  
CAGACTTTTTCGCGCGTGGCCACCGTAACCTCGGTGAACGACACCGCTCCCATCGATGAAT  
TTAAGTTTCCCAAGATGCACGCCTGGCTGAAGCGTCTGGCAGAGCTACCCTACTACCAGG  
AGGTAAACGGCGACGGCGCTGACGAGCTTAAGAGCATCTTCAAGGCCAAGCTGG

5'CAGAAAACCTGGCAAG**TAG**AAGTTGCG-3'

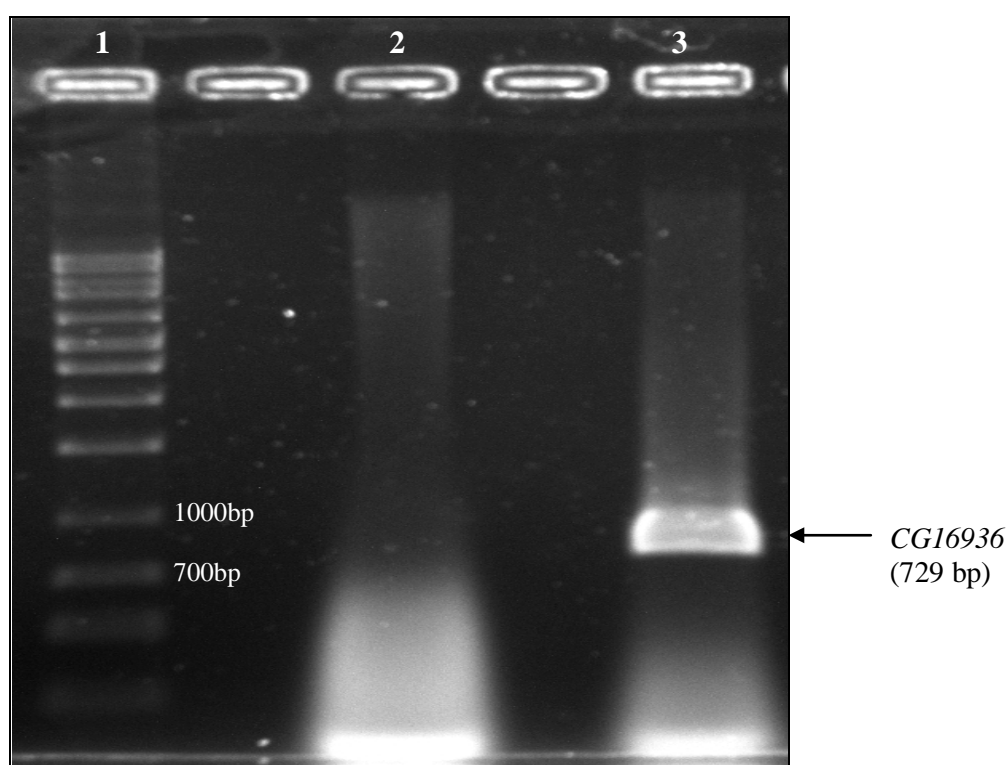
3'GTCTTTTGGCACCGTTCATCTTCGAACCC-5'

Both the start codon (ATG) and stop codon (TAG) is in bold case in the beginning and ending of the gene sequence, with restriction enzyme highlighted in yellow. The forward primer (red) includes the restriction site for HindIII (underlined)

and aligned at the beginning of the sequence while the reverse primer (green) includes the restriction site for BamHI (underlined) and aligned at the ending.

### 3.2.1 PCR gel image.

Figure 3.1 shows the gel image of amplified *CGI6936*. Gel was stained with ethidium bromide. A single band was observed in between 700 bp and 1000 bp (lane 3) whereas no band was observed on lane 2 which is the negative control where everything added was similar except primers.



**Figure 3.1:** 1% (w/v) Agarose gel electrophoresis image of PCR

Lane 1: 1 kb DNA Ladder

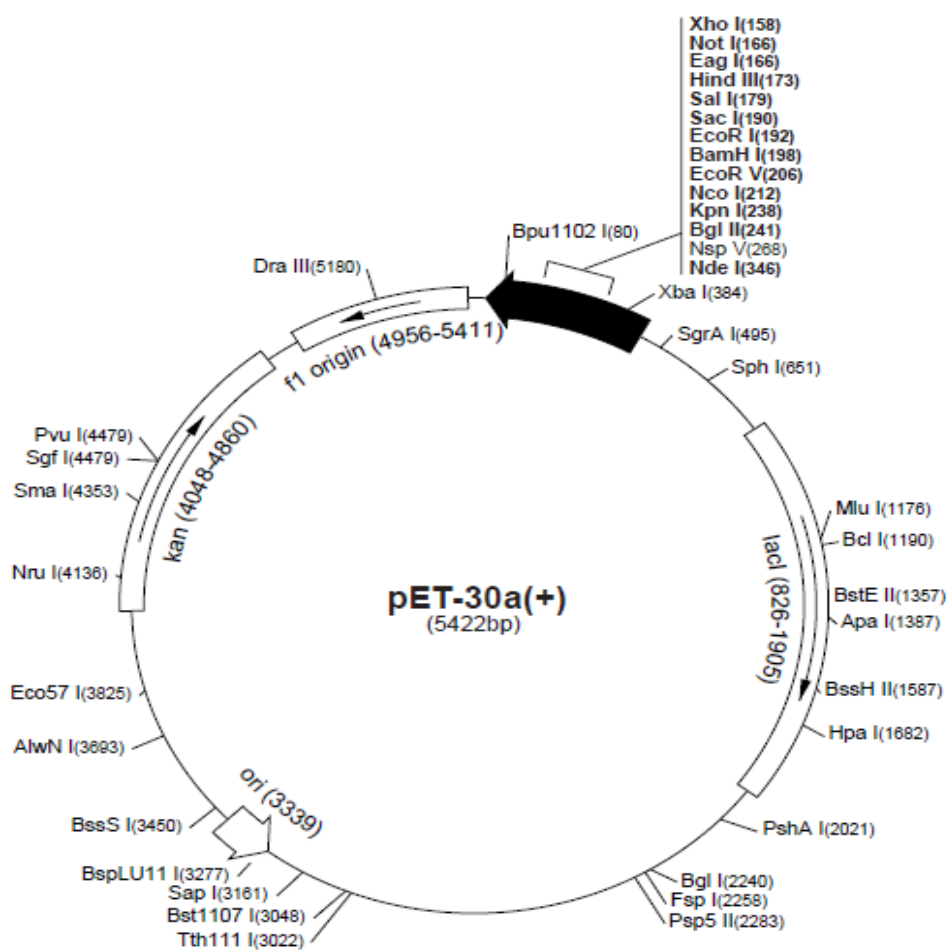
Lane 2: Negative control

Lane 3: PCR product of *CGI6936*



### 3.3 Cloning of the PCR product.

PCR product is the extracted from the agarose gel and digested with BamHI and HindIII. (List of cutters and non-cutters (restriction enzymes) for the CG16936 gene sequence was obtained from [www.restrictionmapper.org](http://www.restrictionmapper.org)). It was then matched with the cutters and non-cutters of pET-30a(+).) The cloning vector used was pET-30a(+) and digested with the same restriction enzymes. The restriction sites for BamHI and HindIII are located at the multiple cloning site of the pET-30a(+) plasmid DNA as shown in Figure 3.2.

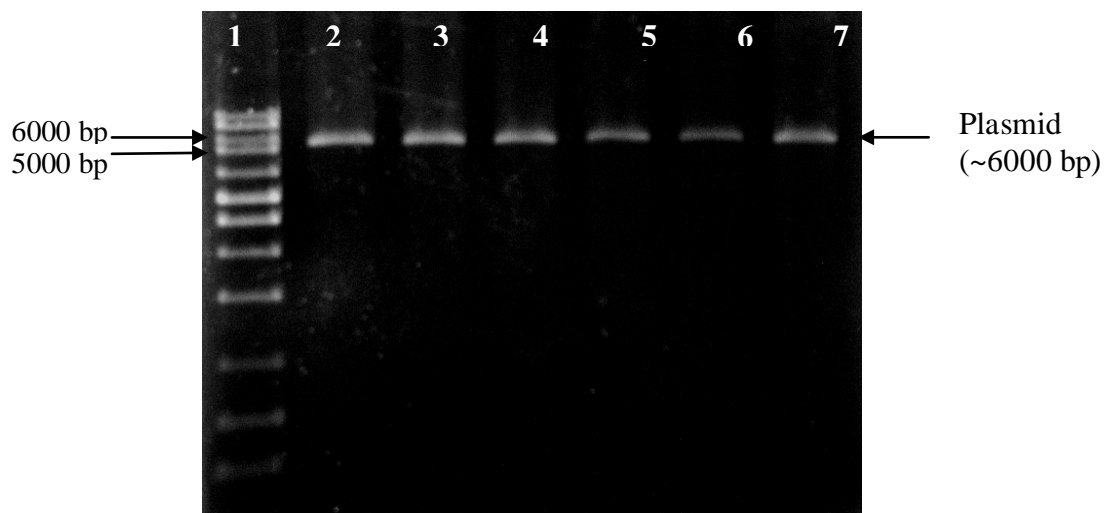


**Figure 3.2:** Map of pET-30a(+) vector

(Adapted from <http://www.synthesisgene.com/vector/pET-30a>)

### 3.4 Plasmid purification.

Six random colonies were picked from the transformation plate and cultured in LB broth containing kanamycin. Plasmid was purified from all 6 and was loaded into 1% (w/v) agarose gel, stained with ethidium bromide. Figure 3.3 shows the gel image of purified plasmids from 6 random colonies where a single band is observed in each lane in the range of between 5000 to 6000 bp. The size of a ligated plasmid is expected at the range of 6000 bp.



**Figure 3.3:** Gel image of purified plasmids from 6 random colonies.

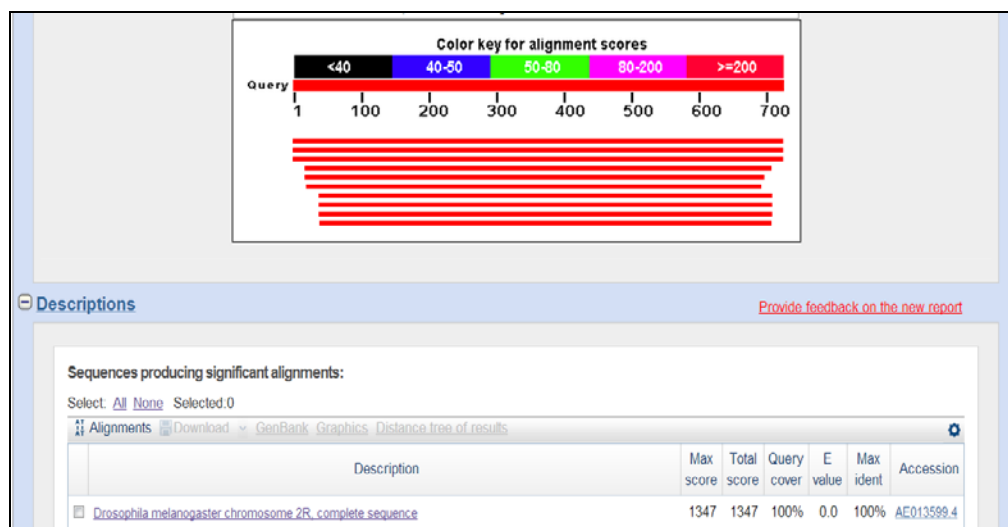
Lane 1: 1kb DNA Ladder

Lane 2-7: Purified plasmid from 6 random colonies.

#### 3.4.1 Sequencing results.

Purified plasmid containing *CG16936* were sent for sequencing to First Base Laboratories for identification. Results obtained were analyzed using Basic Local Alignment Search Tool (BLAST). Figure 3.4 shows the BLAST search tool results that revealed 100% similarity with the *Drosophila melanogaster* chromosome 2R, complete sequence which denotes the glutathione S-transferase, E12 isoform A and isoform C

(Figure 3.5). CG16936 has recently been classified as Glutathione transferase E12 (Saisawang *et al.*, 2011).



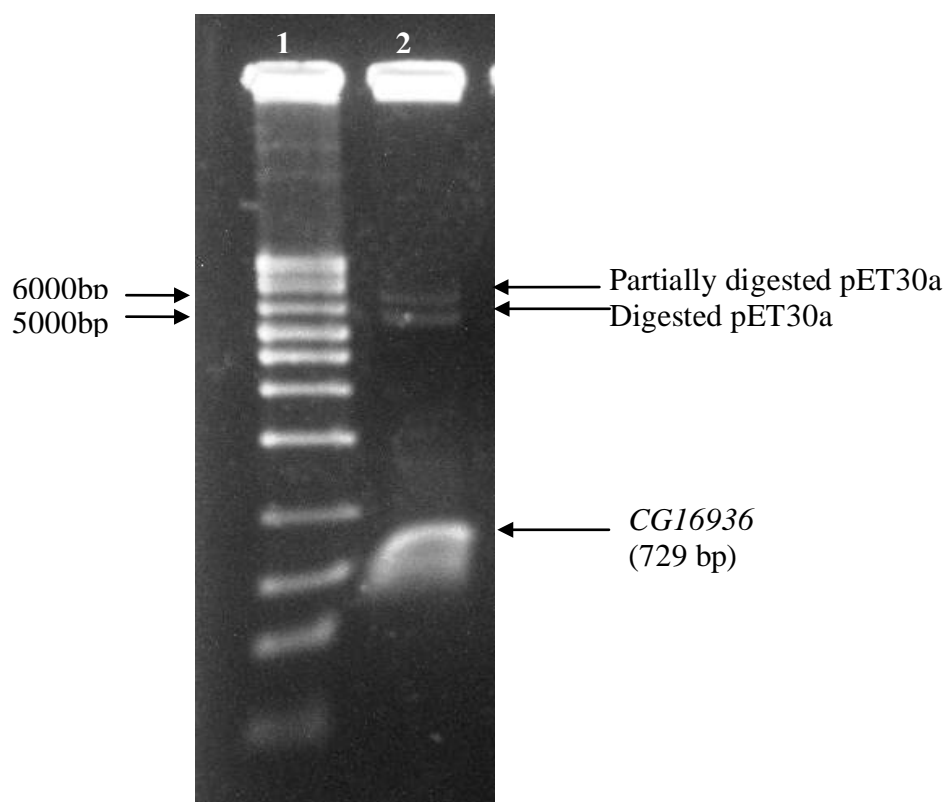
**Figure 3.4:** BLAST search tool results of the recombinant *CG16936* (Obtained from <http://blast.ncbi.nlm.nih.gov/>)



**Figure 3.5:** Expansion of Sequence ID: AE013599.4, featuring GST E12 isoform A and C

### 3.4.2 Plasmid digestion.

The purified plasmid was digested with BamHI and HindIII enzymes. Digested product was loaded into 1% (w/v) agarose gel to confirm the ligation product. Figure 3.6 shows the gel image of the digested product. There were 3 bands in total, 2 bands at the range of 5000 bp to 6000 bp which was the ligated plasmid (top) and digested plasmid (bottom). The third band was noticed in between 700 and 1000 bp which is the digested PCR product. The position of the band in that range proves that the ligated product was *CG16936*.



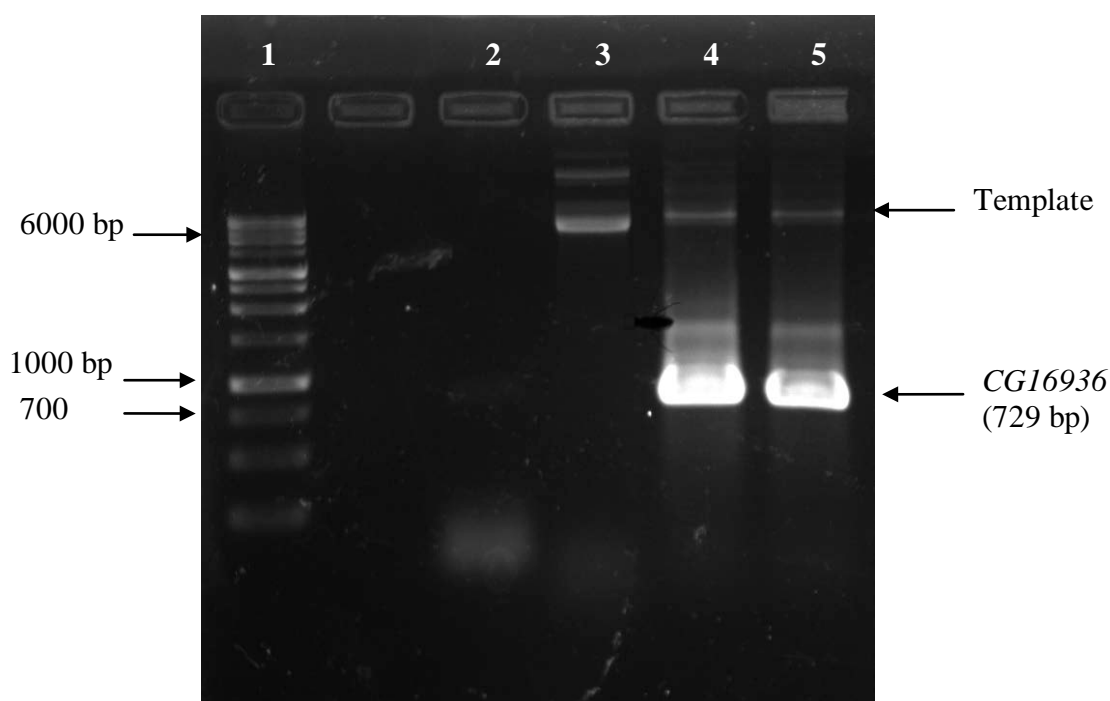
**Figure 3.6:** Gel image showing the digestion of the ligated pET 30.

Lane 1: 1kb DNA Ladder

Lane 2: Digested pET30a Plasmid.

### 3.4.3 PCR using Plasmid as template.

PCR was performed to further confirm that the ligation product was correct. The same plasmid that was extracted before was used as a template to perform PCR to reconfirm that the ligated product was *CG16936*. It was then loaded on a 1% (w/v) agarose gel and stained with ethidium bromide. Figure 3.7 shows band on lane 4 and 5 between the range of 700-1000 bp proved that the ligated product was *CG16936*.



**Figure 3.7:** Gel image of PCR performed using extracted plasmid as template.

Lane 1: 1kb DNA Ladder

Lane 2: PCR without template.

Lane 3: PCR with plasmid but no primers.

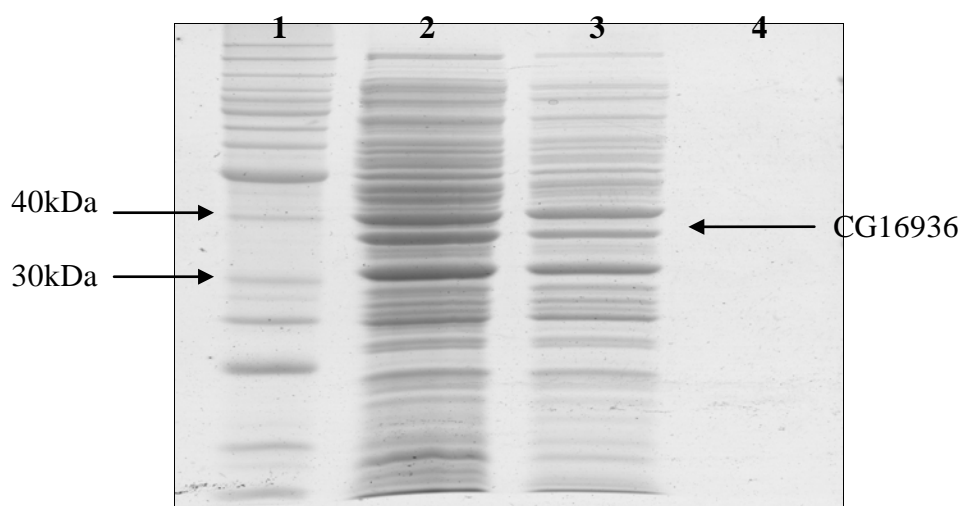
Lane 4 and 5: PCR with primers and plasmid as template (replicates)

### **3.5 Purification of recombinant CG16936.**

The recombinant CG16936 protein was tried purified using multiple matrices. A total 12 columns were tried in order to get a purified GST. Chromatography was carried out using an Amersham Bioscience AKTA FPLC<sup>TM</sup>. Eluted protein was concentrated using centrifugal concentrator (Vivaspin 20) and purity of the protein was analyzed on 12% (w/v) SDS-PAGE stained with colloidal Coomassie Brilliant Blue G250.

#### **3.5.1 GSTrap<sup>TM</sup> HP and S-hexyl glutathione**

CG16936 could not bind to both GSTrap<sup>TM</sup> HP and S-hexyl glutathione. This was confirmed by checking the activities of elution and the flow through. No activity was detected with the elution but flow through had high activity with DCNB. It was then concentrated and the purity level was checked on SDS-PAGE. Gel was stained with Colloidal Coomassie Brilliant Blue G250. Figure 3.8 and 3.9 shows the SDS gel image of purification using GSTrap<sup>TM</sup> HP and S-Hexyl glutathione respectively. No band was observed in lane 4 which was loaded with the elution sample in both figures.



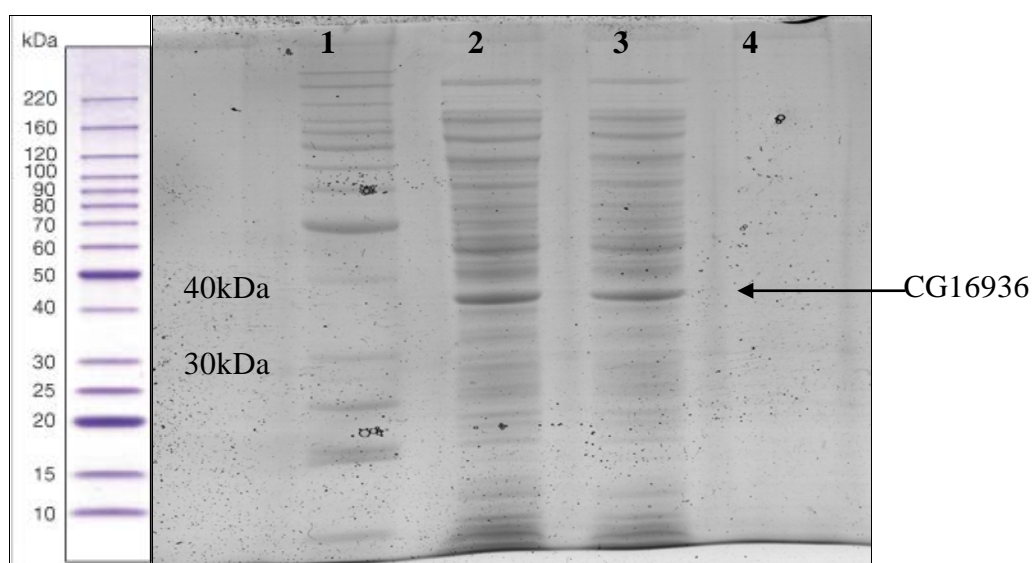
**Figure 3.8:** SDS-PAGE of purification of CG16936 using GSTrap™ HP.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude lysate

Lane 3: Flow through fraction

Lane 4: Elution with 10 mM GSH, pH 7.4



**Figure 3.9:** SDS-PAGE of purification of CG16936 using S-Hexyl glutathione.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

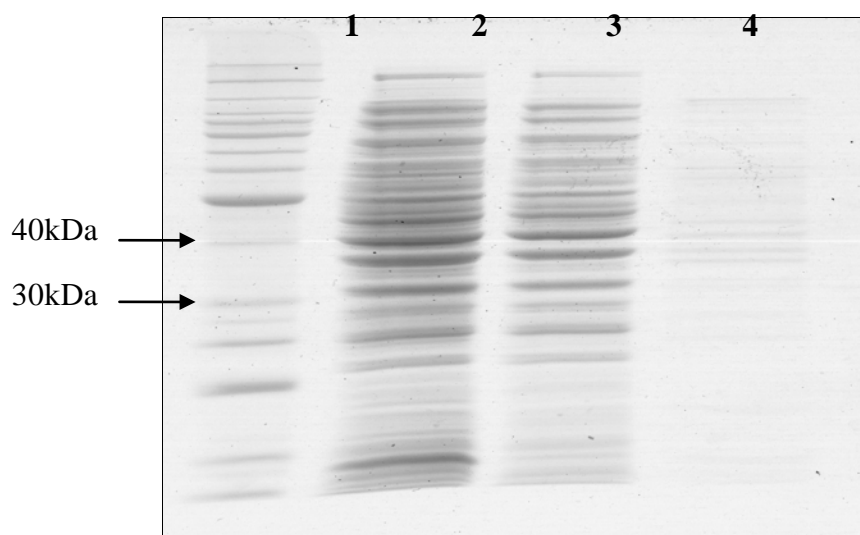
Lane 2: Crude lysate

Lane 3: Flow through fraction

Lane 4: Elution with 10 mM GSH, pH 7.4

### 3.5.2 Sep-Pak C18

The Sep-Pak C18 was used next. Both the flow through fraction and elution had no activity when tested with DCNB. It was concentrated using the centrifugal concentrator and loaded on the third lane of SDS-PAGE. Gel was stained with colloidal Coomassie Brilliant Blue G250. Figure 3.10 shows the SDS-PAGE of purification of CG16936 using Sep-Pak C18. As observed, the third lane has multiple faint bands with no distinct band of CG16936.



**Figure 3.10:** SDS-PAGE of purification of CG16936 using Sep-Pak C18.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude lysate

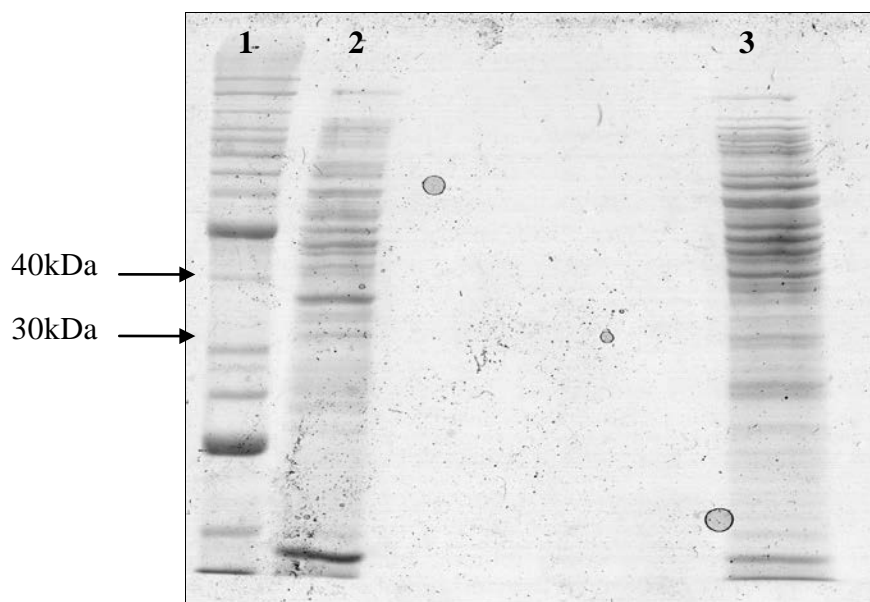
Lane 3: Flow through fraction

Lane 4: Concentrated elution



### 3.5.3 Superdex 75

A total of three peaks were obtained from Superdex 75. However, only the third peak gave a mild activity when tested with DCNB. It was concentrated using the centrifugal concentrator and loaded on the third lane of SDS-PAGE. Gel was stained with colloidal Coomassie Brilliant Blue G250. Figure 3.11 shows the SDS-PAGE of purification of CG16936 using Superdex 75. As it can be seen, the third lane has multiple bands with no distinct band of CG16936.



**Figure 3.11:** SDS-PAGE of purification of CG16936 using Superdex 75

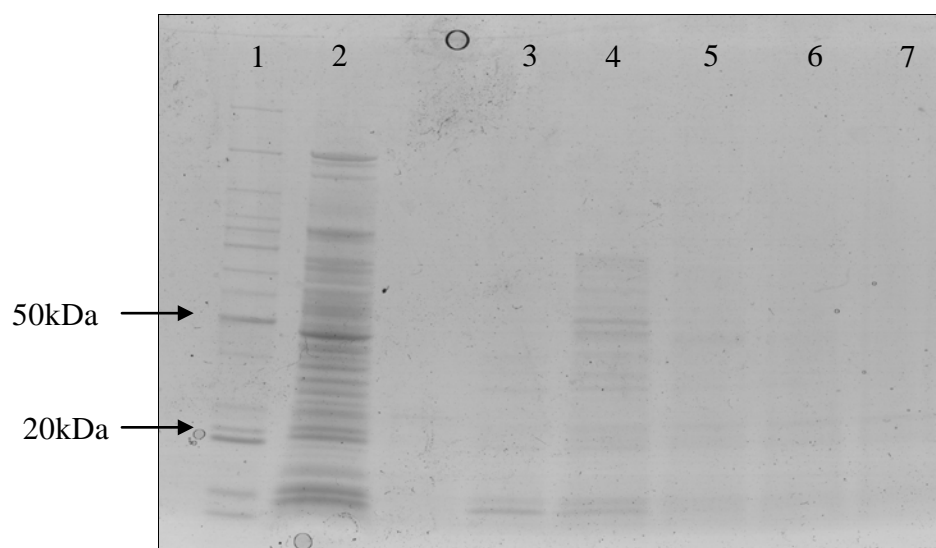
Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude lysate

Lane 3: Third peak

### 3.5.4 HiPrep 16/60 Sephacryl S-200.

Figure 3.12 shows the SDS-PAGE purification of CG16936 using Sephacryl S-200. A total 5 peaks were obtained from the size exclusion chromatography column (Sephacryl S-200). Only the second peak (Lane 4) and third peak (Lane 5) gave activity with DCNB. All of the peaks were concentrated and loaded into the gel. Gel was stained with colloidal Coomassie Brilliant Blue G250. Based on figure 3.12, it was noticed that the column did not help much with the purification as multiple faint bands were observed.



**Figure 3.12:** SDS-PAGE of purification of CG16936 using HiPrep 16/60 Sephacryl S-200.

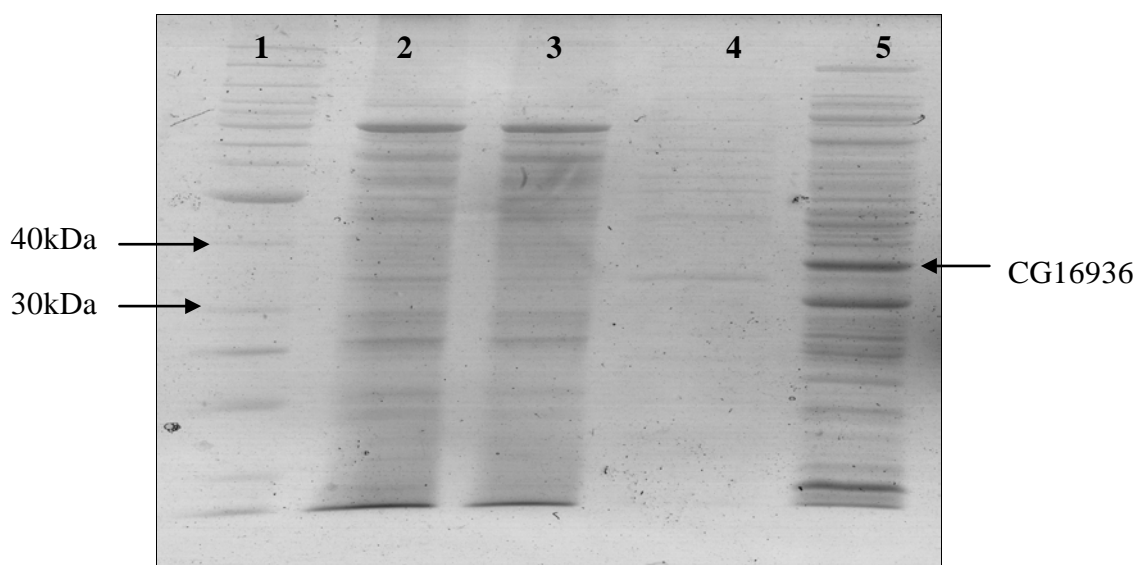
Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude bacterial lysate

Lane 3-7: Peaks 1 to 5 respectively

### 3.5.5 Anion exchanger (HiTrap ANX FF)

Figure 3.13 shows the SDS-PAGE of purification of CG16936 using HiTrap ANX FF column. Gradient elution was performed with 0.5 M NaCl from 0% to 100% for 20ml. Elution from the first 10ml had low activity but the second 10ml had high activity with DCNB. No activity detected in the flow through fraction. Elution was concentrated and loaded on SDS-PAGE, stained with colloidal Coomassie Brilliant Blue G250. Lane 4 and 5 shows the concentrated elution from this column with multiple light bands and two intense bands (lane 5 only) in between 30 kDa and 40 kDa.



**Figure 3.13:** SDS-PAGE of purification of CG16936 using HiTrap ANX FF

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude bacterial lysate

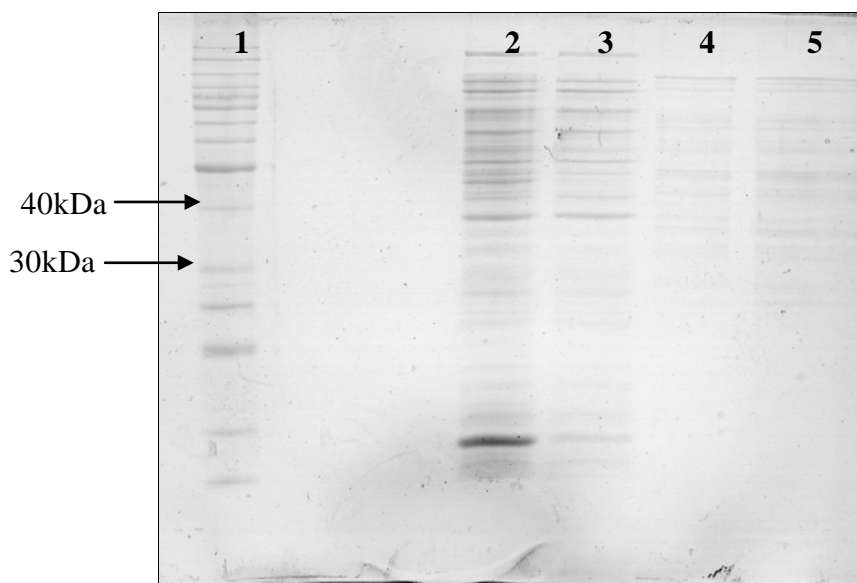
Lane 3: Flow through fraction from ANX

Lane 4: First 10 ml of 0.5 M NaCl gradient

Lane 5: Second 10 ml of 0.5 M NaCl gradient

### 3.5.6 Diethylaminoethanol-sepharose (HiTrap DEAE FF)

Figure 3.14 shows the SDS-PAGE of purification of CG16936 using HiTrap DEAE FF. Gradient elution was performed with 1.0 M sodium chloride from 0% B to 100% for 20 ml. Two peaks were obtained. Elution of the first peak had activity with DCNB but no activity was detected in the second peak and the flow through fraction. It was concentrated and loaded on SDS-PAGE, stained with colloidal Coomassie Brilliant Blue G250. Lane 4 and 5 shows the concentrated elution from this column with multiple bands however **no** intense band of the protein of interest were observed.



**Figure 3.14:** SDS-PAGE of purification of CG16936 using HiTrap DEAE FF.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude bacterial lysate

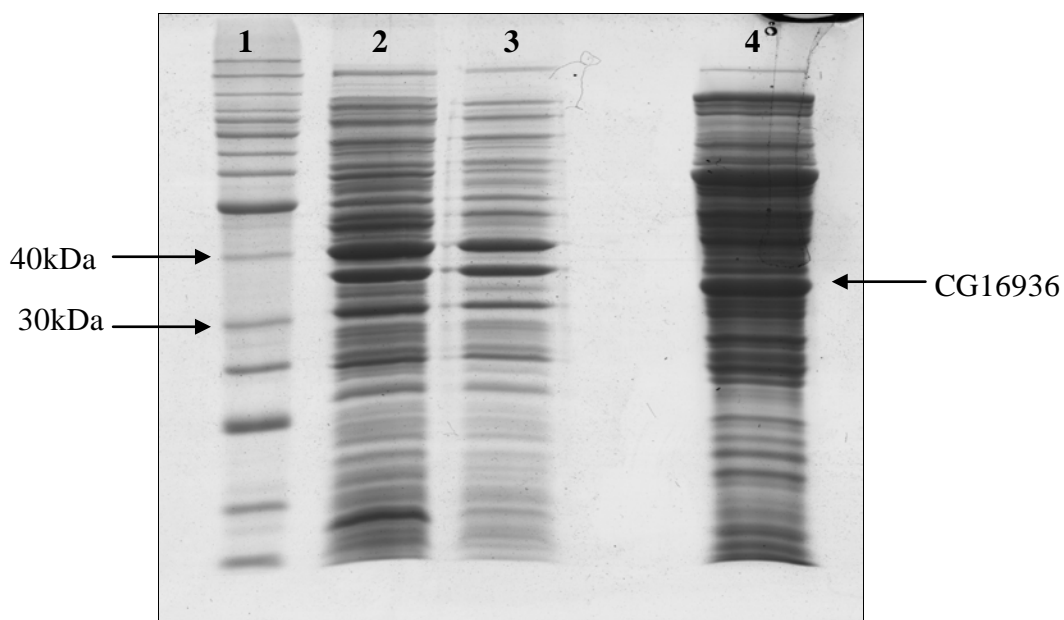
Lane 3: Flow through fraction from DEAE

Lane 4: First peak of elution from 1.0 M NaCl, pH 7.4 gradient

Lane 5: Second peak of elution from 1.0 M NaCl, pH 7.4 gradient

### 3.5.7 HisTrap™ FF Crude

Figure 3.15 shows the SDS-PAGE of purification of CG16936 using HisTrap™ FF Crude. Protein was eluted out with 300 mM imidazole, pH 7.4. Elution had high activity with DCNB and no activity was detected with flow through fraction. Fraction with activity concentrated and loaded on SDS-PAGE, stained with colloidal Coomassie Brilliant Blue G250. Lane 5 shows the concentrated elution from this column with multiple bands, with almost the same intensity in the range of 25 kDa and 50 kDa, including the protein of interest.



**Figure 3.15:** SDS-PAGE of purification of CG16936 using HisTrap™ FF Crude.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

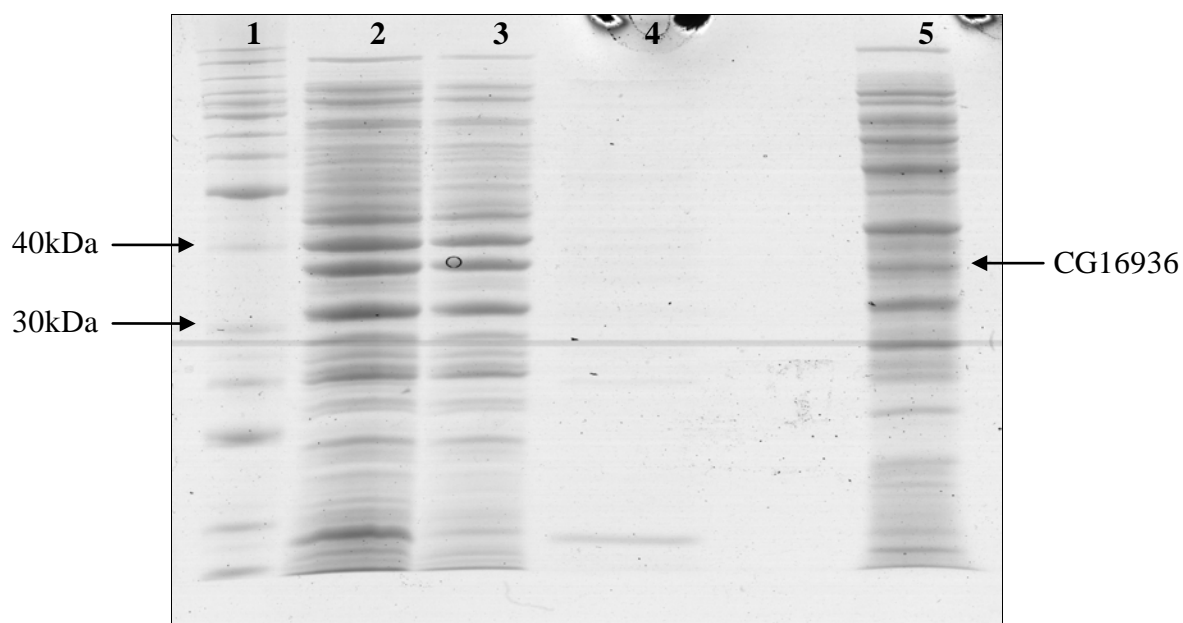
Lane 2: Crude bacterial lysate

Lane 3: Flow through fraction from HisTrap™ FF Crude

Lane 4: Elution with 300 mM imidazole, pH 7.4

### 3.5.8 S-2, 4-(dinitrophenyl)-GSH (DNP-SG)

Figure 3.16 shows the SDS-PAGE of purification of CG16936 using S-2, 4-(dinitrophenyl)-GSH matrix. The column was washed with 1.0 M of sodium chloride, pH 7.4 to remove any non-specific protein bindings and eluted with 20 mM GSH, pH 9.6. Elution had activity, while no activity was detected in flow through fraction. Elution was then concentrated and loaded on SDS-PAGE, stained with colloidal Coomassie Brilliant Blue G250. Lane 5 shows the concentrated elution from this column with multiple bands, with the same intensity.



**Figure 3.16:** SDS-PAGE of purification of CG16936 using DNP-SG.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude bacterial lysate

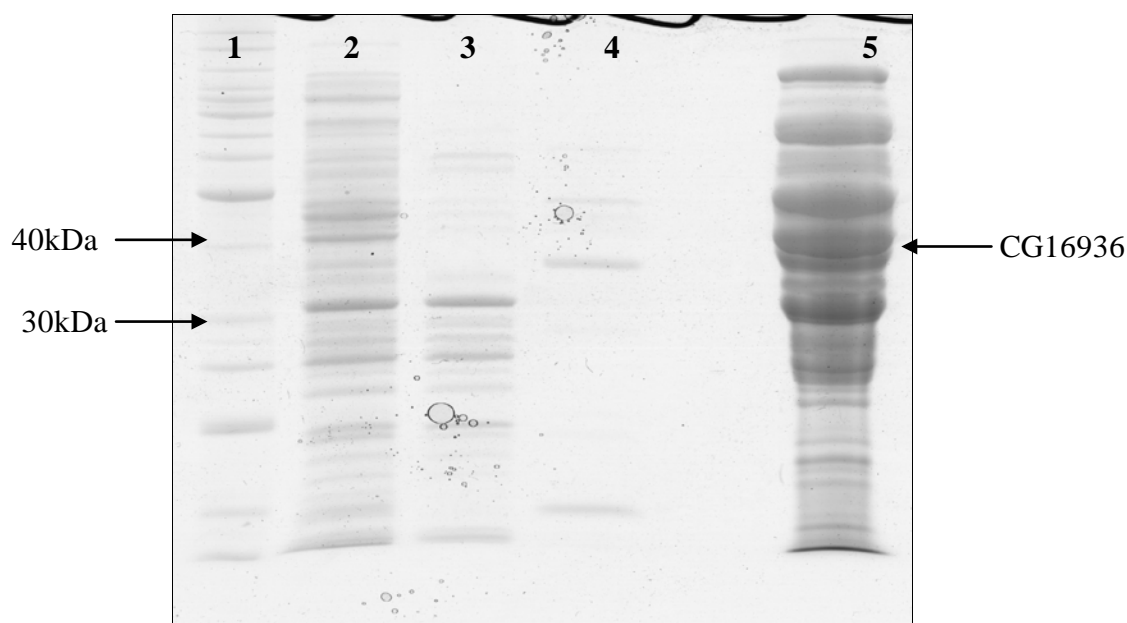
Lane 3: Flow through fraction from DNP-SG

Lane 4: Wash with 1M NaCl, pH 7.4

Lane 5: Elution with 20 mM GSH, pH 9.6

### 3.5.9 Bromosulphophthalein-GSH (BSP-SG)

Figure 3.17 shows the SDS-PAGE image of purification of CG16936 using Bromosulphophthalein-GSH matrix. The column was washed with 0.5 M of potassium chloride, pH 7.4 to remove any non-specific protein binding. Then it was eluted with 2 mM BSP in phosphate buffer, pH 7.4. Activity with DCNB was detected with the elution but no activity in the flow though. Elution was concentrated and loaded on SDS-PAGE, stained with colloidal Coomassie Brilliant Blue G250. Lane 5 shows the concentrated elution with multiple bands of non specific proteins, some of which are similarly intensified with the target protein, all above 25 kDa.



**Figure 3.17:** SDS-PAGE of purification of CG16936 using BSP-SG.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)

Lane 2: Crude bacterial lysate

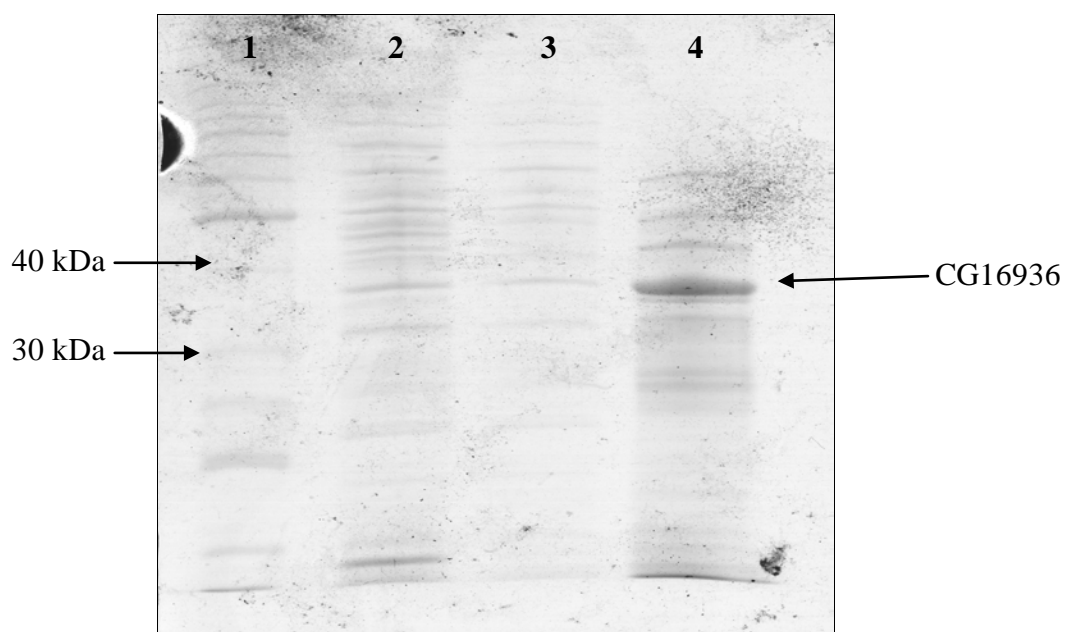
Lane 3: Flow through fraction from BSP-SG

Lane 4: Wash with 0.5 M KCl, pH 7.4

Lane 5: Elution with 2 mM BSP, pH 7.4

### 3.5.10 Affi-Gel Blue Gel column.

The final matrix to be tried was the Affi-Gel Blue Gel matrix to purify CG16936. The target protein was eluted with 150 mM NaCl in phosphate buffer, pH 7.4. Elution with this column gave the highest activity with DCNB, almost 10 fold more than the negative control. No activity was detected in the flow through fraction. Elution was concentrated and loaded on SDS-PAGE, stained with colloidal Coomassie Brilliant Blue G250. Based on figure 3.18, the partially purified CG16936 can be seen as a more intensified band compared to the other bands, at the molecular weight in between 30 kDa and 40 kDa (lane 4).



**Figure 3.18:** SDS-PAGE of purification of CG16936 using Affi-Gel Blue Gel column.

Lane 1: BenchMark™ Protein Ladder (Invitrogen)  
Lane 2: Crude bacterial lysate  
Lane 3: Flow through fraction from Affi-Gel Blue Gel  
Lane 4: Elution with 150 mM NaCl, pH 7.4



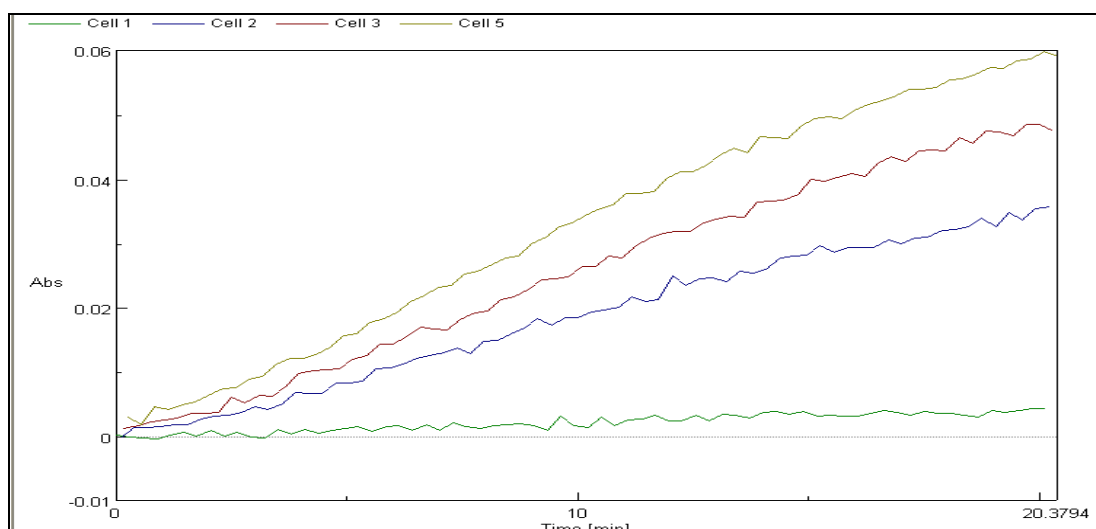
### 3.6 Substrates specificity.

**Table 3.2:** Substrates specificity of the recombinant CG16936 protein (average of 3 independent replicates).

Substrates	Specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ )
1-Chloro-2,4-dinitrobenzene (CDNB)	ND
1,2-Dichloro-4-nitrobenzene (DCNB)	0.4324 $\pm$ 0.0016
<i>trans</i> -2-Hexenal	ND
2,4-Hexadienal	ND
<i>trans</i> -2-Octenal	ND
<i>trans</i> -4-Phenyl-butene-2-one	ND
<i>trans,trans</i> -Hepta-2,4-dienal	ND
Ethacrynic acid	ND
<i>p</i> -Nitrobenzyl chloride	ND
1,2-Epoxy-3- <i>p</i> -nitrophenoxy propane	ND
Bromosulfophthalein (BSP)	ND

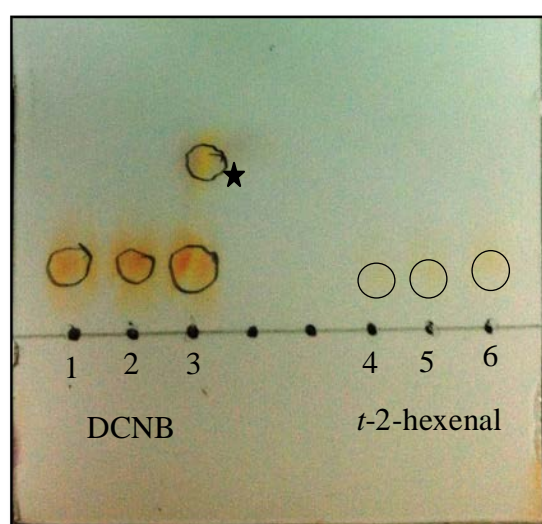
\*ND denotes not detected.

Table 3.2 lists out the different substrates used to check the substrates specificity of CG16936. Based on table 3.2, DCNB was the only substrate to have activity with the enzyme with a specific activity of 0.4324  $\mu\text{mol}/\text{min}/\text{mg}$  however no activity was detected with other substrates including CDBN. Figure 3.19 (next page) shows the progress of conjugating products for 20 minutes with DCNB of the recombinant CG16936. Based on the figure 3.19, it shows the high activity of the enzyme with DCNB compared to the negative control. No activity was detected with *trans*-2-hexenal and *t*-4-phenyl-3-butene-2-one (PBO) that are known as olfactory GST substrates.



**Figure 3.19:** Progress of conjugating products for 20 minutes with DCNB of the recombinant CG16936.

### 3.7 Thin layer chromatography



Lane 1: DCNB  
 Lane 2: GSH+ DCNB  
 Lane 3: GSH + DCNB + CG16936  
 Lane 4: *t*-2-hexenal  
 Lane 5: GSH + *t*-2-hexenal  
 Lane 6: GSH + *t*-2-hexenal + CG16936

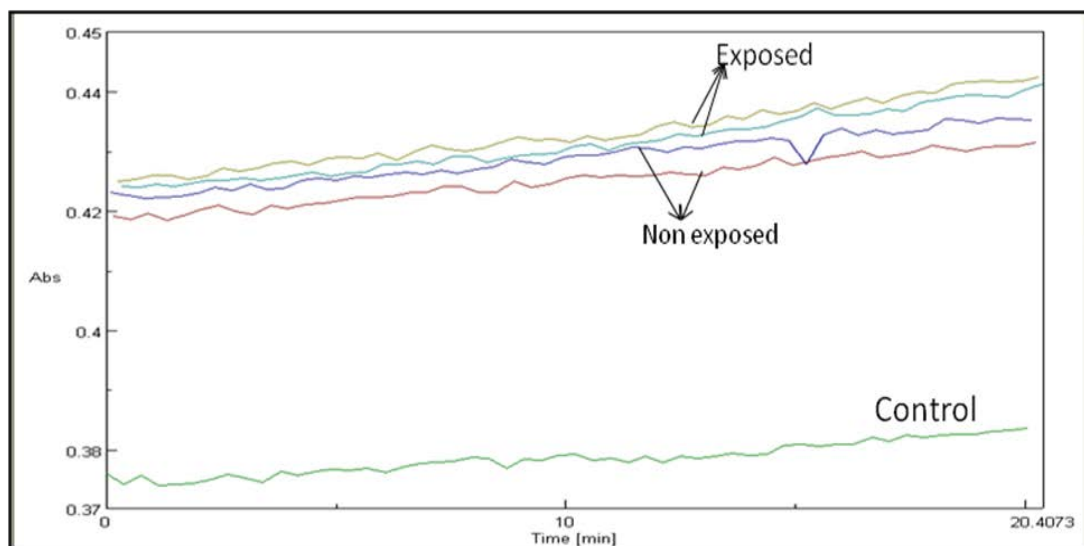
**Figure 3.20:** T.L.C analysis of recombinant CG16936 conjugating activity against DCNB and *t*-2-hexenal.

★ Denotes the presence of GSH-DCNB conjugate

Figure 3.20 shows the conjugated reaction product of GSH-DCNB that is indicated by an extra spot at lane 3 and is marked with a star. However no extra spot was observed at any other lane including lane 6 that contains the recombinant CG16936 with *t*-2-hexenal which reveals the inability of CG16936 to conjugate *t*-2-hexenal to GSH.

### 3.8 Exposure to odorant.

#### 3.8.1 Comparison between the activity of exposed and non-exposed *Drosophila melanogaster*.



**Figure 3.21:** Progress of conjugating products for 20 minutes with DCNB comparing exposed and non-exposed *Drosophila*.

*Drosophila* was exposed with Benzaldehyde and the GSTs were purified using DNP-SG matrix. Activities of the purified GSTs between the exposed and non-exposed were compared to see any significant increase in the expression of CG16936. Figure 3.21 shows that activities from the exposed and non exposed *Drosophila* with DCNB showed almost the same results which means there were no increase in the GST expressed on both conditions.

## CHAPTER 4 DISCUSSION

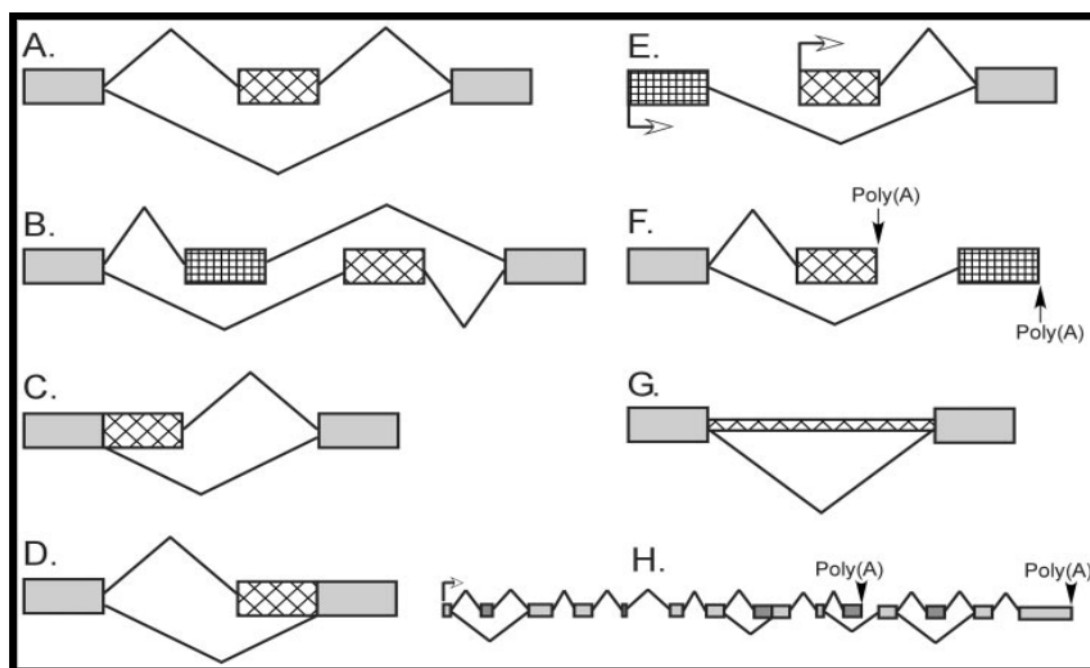
### 4.1 Cloning and expression of CG16936

Gene Sequence of CG16936 was obtained from FlyBase database (flybase.org). CG16936 is a protein coding gene that is 672 base pairs in length which encodes 223 amino acids. The gene that encodes this protein contains intron in between the sequence. Generally, a gene that contains intron will not be able to express the desired protein because introns are non-coding sequence. Within a cell, introns are removed at transcription level by undergoing a process called alternative splicing and the sequences at both ends are joined together in the final mature RNA which is also known as messenger RNA (mRNA). The new mRNA will be translated by ribosome to the desired protein. So, in this project RNA instead of DNA were isolated from *Drosophila melanogaster*. Bearing in mind that RNAs are short-lived and easily degraded, it was isolated from freshly killed insects and were immediately reversed-transcribed to a much stable cDNA using the first strand cDNA synthesis kit. This cDNA was used as a template for the amplification of *CG16936* as per the conditions mentioned earlier in Chapter 2.1.3. Many trials and errors were done by modifying the annealing temperature and cycling time before concluding the 62°C was the best annealing temperature and increasing the cycling time from the usual 32 to 35 cycles would be sufficient to amplify enough *CG16936* for the cloning process.

After amplification, PCR product was analysed on 1% (w/v) agarose gel. The band was in the range of 700 bp to 1000 bp (Figure 3.1), but the expected range was supposed to be in between 500 bp to 700 bp. To confirm the gene sequence was *CG16936*, the PCR band was gel purified and sequenced.

The sequencing results were analysed using Basic Local Alignment Search Tool (BLAST) to identify the gene sequence. Result matched 100% to the *Drosophila melanogaster* chromosome 2R, complete sequence (Figure 3.4). The result was clicked to expand, and the gene sequence was aligned to Glutathione s-transferase E12 isoform A and C (Figure 3.5). A recent paper on the studies of the proteome of *Drosophila melanogaster* has classified CG16936, as Glutathione transferase, E12 (DmGST E12) (Saisawang *et al.*, 2011). Another paper on the purifications of GSTs from *Drosophila melanogaster* identified 4 isoforms of CG16936 with the same molecular weight but differing pI values ranging from 5.0 to 5.4 (Pal *et al.*, 2012) although theoretically, it was said to have a pI value of 5.9 (Alias & Clark, 2007). It is known that alternative splicing could actually help producing multiple protein isoforms with just a single gene (Black, 2003).

Many studies have proved the existence of alternative splicing in insect genes. For example, Ranson *et al.*, (1998) reported the involvement of alternative RNA splicing in generating multiple functional GST transcripts in the *Anopheles gambiae*. Another study on the proteome of *Drosophila melanogaster* identified 36 cytosolic GST genes that expressed 41 protein products through alternative splicing (Saisawang *et al.*, 2011). In addition to that, out of 28 genes that encodes cytosolic GSTs in *Anopheles gambiae*, two were alternatively spliced which resulted to a total of 32 transcripts (Ding *et al.*, 2003). Based on these facts, it can be assumed that CG16936 that was expressed in this project was longer in sequence because it could be a result of a newly spliced gene. Hence, the difference in the size of the amplified and the theoretical gene sequence.



**Figure 4.1:** Patterns of alternative splicing. Constitutive sequences present in all final mRNAs are grey boxes. Alternative RNA segments that may or may not be included in the mRNA are hatched boxes (Black, 2003).

Once the gene identity was confirmed, the purified *CG16936* were used to ligate to the pGEM-T using the pGEM-T easy ligation kit. Upon ligation, it was transformed into the NovaBlue Singles™ Competent Cells and transformation mixture was plated onto the X-gal plate. Usage of this plate allowed us to choose the correct colony that contains the gene of interest through blue white screening method. The blue colonies are bacteria cells without *CG16936*-plasmid, while the white colonies are the ones carrying *CG16936*-plasmid. White colonies were picked and cultured in LB broth containing ampicillin. Plasmid were purified from the bacteria culture and digested with restriction enzymes as per mentioned in Chapter 2. It was then ligated to an expression vector, pET30a(+).

Restriction enzymes were chosen by comparing the non-cutters and cutters of the gene sequence of CG16936 and pET-30a(+) vector. The pET-30a(+) system were used to express the protein because many studies proved to have successfully cloned GSTs with this vector and had a well expressed protein. For example, the cloning of a pi-class GST from freshwater mussels *Cristaria plicata* (Hu *et al.*, 2012) and GSTs from *Drosophila melanogaster* (Sawicki *et al.*, 2003). The pET30a(+) are one of the best vectors known for its good expression level because it uses the T7 promoter system that can be induced by adding IPTG. Besides that it also carries the His.Tag sequence at the N-terminal. Choosing the appropriate restriction enzymes will help to attach the histidine tagging to the protein of interest which could help with protein purification later.

Upon digesting the pET30a(+) plasmid, it was loaded on a 1% (w/v) agarose gel to check the digestion level. The band that contained the digested plasmid was carefully excised and gel purified. This was done for two reasons. Firstly was to remove the undigested plasmids. Secondly, was to remove the short fragments resulted from the digested plasmid to prevent religation and circularization of the plasmid. Next, the purified digested plasmid was ligated with the digested *CG16936* using T4 DNA ligase kit as per instruction.

Ligated plasmids were transformed into *E.coli* BL21 (DE3) pLysS host. This strain was used because it is the best competent cells that provide varying levels of expression control with T7 promoter-driven, vectors such as the pET vector. It was used in the cloning and expression of delta class GSTs in *Drosophila melanogaster* (Sawicki *et al.*, 2003) and Epsilon class GSTs in *Anopheles Cracens* (Wongtrakul *et al.*, 2009). Transformed *E.coli* was spread on agar plate containing Kanamycin. The plate was

incubated for 16 hours at 37°C. The cells that didn't uptake the ligated plasmid will not be able to grow while the cells that contained the pET30-CG16936 will be able to develop resistance towards kanamycin as the pET30a(+) has a kanamycin resistant gene. This was an additional step to be selective towards the colonies that contained the plasmid.

Six random colonies were picked and cultured in LB broth containing Kanamycin sulphate. The colony that doesn't have the plasmid will not be able to grow. Plasmids were purified from these bacterial cultures using plasmid purification kit. Upon purification, it was loaded into 1% (w/v) agarose gel to check for purity and size. It showed a single band at approximately 6000 bp (figure 3.3). The size of pET30a and *CG16936* is about 5400 and 700 bp respectively. The size of the ligated plasmid was expected at 6000 bp approximately. Once the size has been confirmed, the purified plasmid was then double digested and the mixture was loaded into 1% (w/v) agarose gel to check if the insert was in correct size.

The gel image showed 3 bands in total (Figure 3.6). Two bands were at the range of 5000 bp and 6000 bp which is the undigested plasmid (top) and digested plasmid (bottom). A single band was observed in between 700 bp and 1000 bp, which was believed to be *CG16936*.

Finally, to confirm that the insert was *CG16936*, the same plasmid was used as a template to perform PCR using the Forward and Reverse primers specific for *CG16936*. The PCR product was loaded on 1% (w/v) agarose gel once again to check if there is any gene amplified and there was single band in between 700 bp and 1000 bp (Figure 3.7). All those procedures confirmed the ligation of *CG16936* with pET30a. The



transformed bacteria were cultured at bigger volume (1000 ml) for purification purpose. It was noticed that the protein was expressed at a very low level. No over expressions were observed on SDS PAGE, even after induction with various concentrations of IPTG up to 1 mM as per recommended by pET system manual. Besides that, different induction time and temperature were tried as well but none of it gave over expression of the protein. So, large amount of bacterial culture were used to get sufficient amount of protein.

## 4.2 Purification of recombinant CG16936.

The chromatography techniques are used to separate biomolecules according to its differences in their specific properties. Generally, proteins are separated based on four basic properties that are size, charge, hydrophobicity and ligand specificity. All four properties have different techniques and summarized in Table 4.1.

**Table 4.1:** Summary of different properties and chromatography techniques.

Property	Technique
Size	Separation based on protein size (gel filtration)
Charge	Separation based on the ionic properties (Ion exchange chromatography)
Hydrophobicity	Based on the hydrophobicity of the protein (Hydrophobic interaction chromatography, Reversed phase chromatography)
Ligand specificity	Based on the affinity towards ligand (Affinity chromatography)

The purification of CG16936 took the most time. A total of 11 different matrices were tried before choosing the column that could give the best result. The first column to be tried was GSTrap™ HP. This column was chosen for an obvious reason that it should be able to trap most GSTs. However, the CG16936 had no affinity towards this

column. The protein came out in the flow through fraction and was confirmed by the high activity in the flow through and no activity with the elution. To double confirm this fact, the elution was concentrated (using a protein concentrator, vivaspin) and analysed on SDS-PAGE. As expected, the lane that was loaded with the elution was clear with no bands (Figure 3.8). A few studies have also confirmed that some isoforms of the epsilon class GSTs are unable to bind to GSTrap™ HP (Saisawang *et al.*, 2011).

S-hexyl glutathione was tried next. It is a ligand that is used for the affinity chromatography of Glutathione transferases and glutathione peroxidases. This column has shown to trap several GSTs that were unable to be trapped by GSTrap™ HP (Alias & Clark, 2007). However, this column was also unable to retain the CG16936. This was confirmed by checking the enzyme activity of both the flow through fraction and elution which revealed that only the flow through had activity with DCNB. No band was observed on the SDS gel image as well (Figure 3.9, chapter 3). Purification was then repeated with the same column but this time the crude was allowed to flow through the Sephadex G-25 to remove any endogenous inhibitors that may inhibit the binding of CG16936 to this column (Pal *et al.*, 2012). The G-25 is a desalting column that has the potential to bind any inhibitory pigments with sufficient affinity to separate them from the protein of target. The fraction that had activity with DCNB was then chromatographed with S-hexylglutathione. However, it gave the same results as GSTrap™ HP with activity in the flow through and no band was observed on SDS-PAGE. This confirmed that CG16936 has no affinity towards this matrix as well. It was reported previously that GSTs that are active towards DCNB could not bind at all or were less firmly bound to the GSH-affinity gel than the ones that are active with CDNB (Stenersen *et al.*, 1987).

Sep-Pak C18 is a cartridge column with strong hydrophobicity that can be used to absorb analytes of even weak hydrophobicity from aqueous solutions. This column is mostly used to separate proteins and peptides based on their hydrophobicity. The binding strength of the protein to the column depends on the hydrophobicity of the protein itself. The more hydrophobic the protein is, the stronger it binds to the column and higher concentration of salt is needed to elute. Purification with this column did not give the desired result as well where multiple bands were observed on SDS-PAGE (Figure 3.10) and no activity was detected in the elution. The crude supernatant may have contained much different kinds of protein with hydrophobic properties that will also bind to this column. The usage of this column must be accompanied with another column that uses different purification technique such as ion exchange or ligand specificities to help reduce the binding of non specific protein binding and get higher protein purity.

Superdex 75 and Sephacryl S-200 are gel filtration columns that is used to separate protein, peptides and other macromolecules according to their molecular size as they pass through a porous structure. Molecules with diameter greater than the largest pores within the resin material will not enter the particle but passes through the column and be eluted first. Whereas, the smaller molecules, will enter the resin particle and will be eluted later in order of decreasing molecular weight.

Superdex 75 separates molecules in range of 3000 to 70000 dalton. Purification with superdex 75 gave 3 peaks, none of which gave activity with DCNB. When analyzed on SDS-PAGE, it showed multiple bands with the same intensity. This is probably because proteins from bacterial lysate may have molecular weight almost similar to CG16936. When being eluted out, these proteins come out together with

CG16936 as one peak which gives multiple bands on SDS PAGE (Figure 3.11). For this reason, a column with a broader range of molecular weight was used instead. Sephacryl S-200 was chosen as its range was between 5000 and 250000 dalton. Having in mind that a column with a broader range of molecular weight should be able to separate the proteins better, Sephacryl S-200 was chosen. A total of 5 peaks were obtained from the purification using Sephacryl S-200. The second and third peak gave activity with DCNB. The elutions (from the second and third peak) were then analyzed on SDS-PAGE but multiple faint bands were observed as well (Figure 3.12).

HisTrap™ FF Crude columns are packed with Ni Sepharose High Performance and is designed for the purification of histidine tagged proteins by immobilized metal ion chromatography (IMAC). This column was chosen next as CG16936 has polyhistidine tagging attached. First the enzyme was eluted with an increasing gradient of imidazole concentration (0 to 500 mM imidazole) to determine the most optimum concentration of imidazole needed to elute the target protein. The concentration was set at 300mM and elution gave significant activity with DCNB. However, the SDS gel showed multiple bands, with a number of intense bands (Figure 3.15). The column was tried washing with different concentrations of sodium chloride before elution to remove the non specific bindings. This did not help as low concentration of sodium chloride gave no difference while higher concentration of washing stripped off the target protein as well. The disadvantage of using this HisTrap is that this column has high affinity towards poly-histidine. Proteins with polyhistidines (as short as 3 histidines in a row), in the amino acid sequence of a protein could easily bind to this column. The washing step did not help much with the removal of non specific binding as multiple bands were still observed on SDS gel.

Chromatography method that separates ions and polar molecules based on the charge is known as ion exchange chromatography. There are two types of ion exchangers, cation exchanger where the positively charged molecules will be attracted to a negatively charged resin while anion exchanger attracts the negatively charged molecules to a positively charged resin. Binding of the protein to the ion exchanger depends on the pI value of the protein itself and the pH it is exposed to. The CG16936 has a pI value of 5.9 (Alias and Clark, 2007). Buffer with a higher pH value than the pI value of the protein will make the protein to be negatively charged. Hence, the protein will be attracted to a positively charged resin (Anion exchanger). For that purpose a weak anion exchanger (ANX resin, Diethyl aminopropyl) with a running buffer of pH7.4 was used. After loading the sample, a fair amount of running buffer, approximately 20 to 30 ml were allowed to pass through the column before elution. To determine the optimum concentration of elution buffer, a gradient of increasing concentration was performed (0% to 100% 0.5 M NaCl, pH7.4). Two peaks were obtained. Both fractions were checked for activity with DCNB and revealed a significant activity in the second peak and a much lower activity in the first peak. Elution was concentrated using centrifugal concentrator and the purity level was analysed on SDS PAGE. The fraction with high activity had multiple bands with 2 intensified bands in between the range of 30 kDa and 50 kDa (Figure 3.13). CG16936 was expected to be one of the bands.

DEAE (Diethyl aminoethyl resin) is another type of weak anion exchanger was employed next. The same elution method was used. However, the final concentration of the eluting buffer was increased from 0.5 M NaCl pH 7.4 to 1.0 M NaCl pH 7.4. Almost similar results were obtained as ANX column. Two peaks were obtained. Elution of the first peak had activity with DCNB but no activity was detected in the second peak and

the flow through fraction. Multiple bands were observed on SDS-PAGE (Figure 3.14). Purification was repeated with a slight modification where the pH of the running and eluting buffer was increased to 8.0. CG16936 was eluted out using a step wise elution of 30%, 40% and 50% of 1.0 M NaCl, pH8.0. The percentage was chosen based on the peaks obtained from the gradient elution. Once again, multiple bands were observed on SDS-PAGE. This may be due to the fact that a lot of other bacterial proteins will also be negatively charged besides CG16936, hence the binding and multiple bands. Usage of this column followed by another column with a different technique should be able to give a better purity.

A recent study on the purification of GST proteome of *Drosophila melanogaster* introduced the use of S-substituted glutathiones as affinity ligands (Pal *et al.*, 2012). Two of the matrices were tried. First was the glutathione conjugate of bromosulphophthalein (BSP-SG). Supernatant was injected into the column and about 20-30 ml of running buffer was allowed to flow through the column to make sure the unbound was completely removed. The column was then washed with 0.5 M NaCl pH 7.4 to clear the non-specific protein binding and 2 mM BSP pH 7.4 was used to elute out the protein of interest. Both the wash and elute fraction gave activity with DCNB with the elute giving slightly higher activity. However, this gave multiple bands on SDS PAGE (Figure 3.17). The same method was repeated but this time replacing the wash buffer with 1.0 M KCl, pH7.4. This change managed to remove most of the non specific binding including our protein of interest. As a result, high activity was detected in the wash fraction instead of the elution and a few faint bands on the SDS gel, in the elute lane.

Assuming that 1 M potassium chloride was too strong, the concentration was reduced to 0.5 M KCl and used to wash. However, that too didn't help much as multiple intensified bands were seen on SDS-PAGE. Another problem faced with this matrix was the activity. BSP can act as substrate or an inhibitor for GSTs (Prapanthadara *et al.*, 2000). For CG16936, it portrayed as an inhibitor hence the elution has to be filtered by using an additional column during elution. A desalting column (G-25) was used to trap the BSP, allowing only the target protein to be eluted out. However, sometimes not all BSP could be trapped resulting it to be eluted out together with CG16936 and it inhibits the activity when enzyme activity assay was performed. Having that in mind, this column was not preferred.

The DNP-SG (S-2,4-(dinitrophenyl)-GSH is a product conjugate that can be used as a ligand as well. It has shown to trap many isoforms of GST including Epsilon class GSTs (Pal *et al.*, 2012). Purification method used for this column was the same as BSP except the eluting buffer which was replaced with 20 mM GSH, pH 9.6. Almost similar results were obtained with multiple bands were observed on SDS-PAGE (Figure 3.16). Different washing techniques with 1M sodium chloride and 1 M potassium chloride also did not help with the purification in obtaining a purified CG16936.

The final matrix to be used was Affi-Gel Blue Gel. This matrix purifies a large range of proteins because the blue dye functions as an ionic, hydrophobic, aromatic or sterically active binding site in various applications. Upon applying the sample, the column was left to run for about 20 to 30 ml of running buffer. Target protein was first eluted out using a gradient of increasing concentration of eluting buffer (0% to 100% 1 M NaCl, pH 7.4) to determine the optimum concentration of elution buffer. Two peaks were obtained from the elute where the first peak gave almost 10 fold activity more than

the negative control. No activity was detected with the void fraction and the second peak. The fraction that gave activity was concentrated and loaded on SDS-PAGE. Multiple faint bands and a single intensified band were observed in between the range of 30 kDa and 40 kDa, which is our protein of interest, CG16936 (Figure 3.18).

To get a better result, stepwise elution was performed using 10%, 20% and 30% of elution buffer. These percentages were chosen based on the concentration at which the peak came out earlier when the gradient elution was performed. However, this elution didn't help much because multiple bands were observed on SDS-PAGE. A similar method was repeated by increasing the buffer pH to 8.0 (Both running and elution buffers). This too didn't help much with the purification. Once again, the purification was repeated by eluting the protein with a constant concentration of 150 mM NaCl, pH 7.4. The lane had multiple faint bands and a single intensified band in between the range of 30 kDa and 40 kDa, similar to the one obtained previously. Purification with this column gave the best result in terms of the enzyme activity and the purity based on the bands observed on SDS PAGE.

Purification was also tried by combining two or more columns. For example, the combination of BSP and DEAE, BSP and HisTrap, vice versa etc. The problem with combining columns was that it still doesn't entirely purify and help to exhibit a single band on SDS PAGE. Eluting from one column and loading into another column costs protein loss and protein degradation which leads to the loss in activity as well. Taking into consideration that the expression of CG16936 was low, the loss of protein suffered during purification by combining two columns and the end result of low activity and multiple bands, thus we decided to stick to single column purification.



Back to analysing all single column purifications that have been done, a few factors were considered. The most ideal purification will be the one that gives the highest purity on SDS PAGE, highest enzyme activity and saves time. Based on these factors, the affi-gel blue gel matrix was chose. Among all the columns, this matrix gave the highest activity in the elute with almost 10 fold more activity compared to the negative control and a single intense band in the range of 30-40 kDda among multiple light bands. Purification with this column was very straight forward with no washing required, which helped us to save time. The high activity also suggested that almost 90% of the protein were trapped and eluted with not much hassle. Considering all the factors above, all the subsequent purifications were done with this column.

Theoretically, the molecular weight of CG16936 is 25.4 kDa (Pal *et al.*, 2012). However, the protein size on SDS PAGE showed a much bigger size, in between 30kDa to 40kDa. This may due to the fact that the size of the gene sequence that was amplified was much higher (729 base pairs) than the expected 672 base pairs. This means the protein expressed will be bigger in size too (243 instead of 224 amino acids in length).

Table 4.2 is a summary of the recombinant CG16936 purification. After comparing the results of all columns based on the activity of the elution and bands obtained on SDS PAGE, we chose to use Affi-Gel Blue Gel for all the subsequent purifications as it gave the best result in terms of activity and purity.

**Table 4.2:** Summary of the recombinant CG16936 purification.

Column	Activity with DCNB	SDS-PAGE	Reference figure
GSTrap™ HP	Activity in the flow through	No band in the elute lane	Figure 3.8
S-hexyl-GSH	Activity in the flow through	No band in the elute lane	Figure 3.9
Sep-Pak C18	No activity	Multiple faint bands	Figure 3.10
Superdex 75	No activity in all peaks	Multiple faint bands	Figure 3.11
Sephacryl S-200	Activity in the second and third peak	Multiple faint bands	Figure 3.12
ANX	Activity in the second peak	Two intensified band (30-40 kDa) among multiple faint bands	Figure 3.13
DEAE	Activity in the first peak	Multiple faint bands	Figure 3.14
HisTrap™ FF Crude	High activity in the elution	Multiple intensified bands	Figure 3.15
DNP-SG	Activity in the elution	Multiple intensified bands	Figure 3.16
BSP-SG	Activity in the elution	Multiple intensified bands	Figure 3.17
Affi-Gel Blue gel	High activity in the elution	A single intensified band (30-40 kDa) among multiple faint bands	Figure 3.18

### 4.3 Substrate Specificities

After purifications with Affi-gel blue gel column, the protein concentration was determined with Bradford assay using Bovine Serum Albumin (BSA) as standard. GST spectrophotometric activity assay was performed as described in Chapter 2. GSTs can catalyse many different substrates with at least 100 known to date (Hayes and Pulford, 1995). A number of model substrates are widely used to characterize the substrate specificity of GSTs. A total of 11 different GST substrates were checked for activity. All activity was performed using partially purified CG16936. No activity was detected in the crude lysate probably due to the presence of inhibition factor.

Based on the result on Table 3.2, it shows that the only substrate that reacted with CG16936 was DCNB with a specific activity of 0.4324  $\mu\text{mol}/\text{min}/\text{mg}$ . This result is consistent with the previously reported activity of CG16936 with DCNB (Alias and

Clark, 2007). A few other studies have also shown that DCNB is a more preferred substrate compared to CDNB by most Epsilon class GST, especially CG16936 (Pal *et al.*, 2012). The result shows that the CG16936 was highly specific towards its substrate as it only reacted with DCNB of all the other 11 substrates. This includes the absence of activity with CDNB which is the universal substrate for most GSTs from animals to insects. Another notable result was the absence of activity with *trans*-2-hexenal which is an odorant substrate for olfactory GSTs.

Every substrate that was used was common GST substrates, some of which has its own functions. Activities with this particular substrate will help to understand the role of the enzyme that reacts with it. For example, *trans*-2-hexenal and *trans*-4-Phenyl-butene-2-one (PBO) are said to be olfactory GST substrates. Studies done by Ben-Arie *et al.*, (1993) on the olfactory GSTs of rat has listed out a few odorants that were olfactory GST substrates. The absence of activity with these two substrates suggested that CG16936 has no olfactory related detoxification functions.

Next, is the use of *trans*-2-octenal. *Trans*-2-octenal is an aldehyde resulted from the end product of lipid peroxidation. GSTs that react to these substrates can be associated to their function in oxidative stress. For example, the Sigma class GST in *Drosophila melanogaster* (Singh *et al.*, 2001). The DmGSTS1-1 is said to be inactive towards the commonly used substrate CDNB but has high activity for 4-hydroxynonenal (4-HNE) an aldehyde derived from lipid peroxidation. The localization of this enzyme in flight muscle suggests that it may have a protective role against oxidative stress. Almost four different types of aldehydes (*trans*-2-hexenal, *trans*-2-octenal, 2,4-hexadienal, *trans,trans*-hepta-2,4-dienal) were checked for activity with

CG16936. Absences of activity with these substrates suggested that probably this enzyme may not be involved in oxidative stress as well.

The high activity with DCNB implies its participation in detoxification of pesticide. However, work done by Alias and Clark (2007) indicated that CG16936 expression remained unchanged in adult fruit flies when treated with pesticides such as Phenobarbital and paraquat which contradicts its participation in insecticides detoxifications.

Most Epsilon class GSTs are associated with insecticide resistance. Although in general it is said to detoxify insecticides, some Epsilon GSTs has portrayed to be responsible to detoxify certain substrate only. For example, the GSTE2 are particularly associated with DDT detoxification (Lumjuan *et al.*, 2005) however the GSTE3 has no activity with DDT but showed to have high activity with 4-HNE which suggest its involvement in oxidative stress (Wongkratul *et al.*, 2009).

The ability to utilize 4-HNE was reported in GSTE1 by Sawicki *et al.* (2003) and activity with HNE (Saisawang *et al.*, 2011). However, GSTE1 showed no response to H<sub>2</sub>O<sub>2</sub> exposure in the strain of *Anopheles gambiae* (Ding *et al.*, 2005). Enzymes associated to oxidative stress will have increased expression level when exposed to hydrogen peroxide but the GSTE1 showed no response although it has proven activity with 4-HNE. This was similar to CG16936, where exposure to chemicals such as phenobarbital did not induce the enzyme but the enzyme still has high activity with DCNB. Based on all the evidences mentioned above, the role of this enzyme in *Drosophila melanogaster* was inconclusive.

## 4.4 Investigations of CG16936 as an olfactory GST

### 4.4.1 Exposure of *Drosophila* to odorant

Six bottles of *Drosophila melanogaster* were prepared. Three bottles of the flies were exposed to odorant as per described in chapter 2. Benzaldehyde was chosen as preferred odorant because a previous study on the olfactory system of *Drosophila* reported that exposure to this odorant has expressed the *CG16936* (Anholt and Mackay, 2001). Upon the exposure, the insect was homogenized and purified using the DNP-SG matrix and eluted with 20 mM GSH, pH 9.6. This column was chosen because it has proven to capture the multiple isoforms of CG16936 (Pal *et al.*, 2012).

The elute was then checked for activity with DCNB. DCNB was chosen as the preferred substrate to check the activity of the purified GST because not many GSTs react with DCNB. Since the entire *Drosophila* cells were homogenized, it contains many other GSTs besides CG16936. Usage of DNP-SG will trap many different isoforms of GSTs as well. To minimize the reaction of other GSTs, a substrate that is not most preferred by most GSTs were used. Besides that, DCNB is the only substrate that CG16936 reacts with high activity. The activities of the purified GSTs from the exposed and non-exposed were compared and not much difference was observed in the activities of both samples (refer to Figure 3.21). The activity level was almost similar which implies that the exposure to the odorant did not give any effects in terms of the expression of CG16936 in *Drosophila melanogaster*.

Olfactory GSTs are said to be responsible in protecting the olfactory system of insects from harmful xenobiotics by inactivating aldehyde odorants (Rogers *et al.*, 1999). The exposure to odorants such as volatile hydrophobic compounds will express more of these enzymes in order to detoxify these compounds. The expression level can

be associated with the enzyme activity. High enzyme activity can be interpreted as high expression. However, no such observations were noticed. Due to the no response in the expression after being exposed to odorant, we didn't further proceed to 2D gel analysis. 2D gel analysis would have been useful to visualise the expression level of CG16936 by comparing the spot intensity of exposed and non exposed samples as it can be interpreted that more intensified spot can be considered as high expression level.

Although the *CG16936* was shown to be expressed in response to exposure to odorant; benzaldehyde, there was no evidence of the gene being translated into CG16936 protein and that it participated in olfactory detoxifications. A gene expression does not necessarily mean that the expressed gene was translated into functional protein. Gene expression is found to be a random process and not all genes that are expressed will be translated into protein. The transcription and translation processes suggested to be random, which leads to cell to cell variations in mRNA and protein levels (Raj and Oudenaarden, 2008).

Another factor to be considered is the location of the enzyme. A study on the *Drosophila* system revealed that the CG16936 was found most abundant in the tubule of the adult and larvae (Yang *et al.*, 2007) whereas most olfactory GSTs are found in the head, or to be more specific in the olfactory system of the organism. For example, olfactory GSTs from the sphinx moth were isolated from the antennae (Rogers *et al.*, 1999) and in the rat were found in the vomeronasal organ (Green *et al.*, 2005) and the olfactory epithelium (Ben-Arie *et al.*, 1993). Having this in mind, the location of CG16936 does not exactly portray its role as an olfactory GST but more convinced to be involved in basic detoxifications of pesticides.

#### 4.4.2 Conjugation of CG16936 with odorant substrate.

A spectrophotometric assay was performed and an aliquot of the reaction was loaded on the t.l.c plate to check the ability of CG16936 to conjugate with odorant substrates. GST reactions were carried out with and without the addition of the recombinant CG16936. The plate was stained with ninhydrin to identify the spots which appeared in orange colour. Based on figure 3.20, it shows the conjugated reaction product with DCNB, indicated by an extra spot at lane 3 and marked with a star. DCNB was used as a control substrate because of its proven activity with CG16936. However no extra spot was observed at any other lane including lane 6 that contains the recombinant CG16936 and *t*-2-hexenal which reveals that there was no conjugation. This was expected as no activity was detected with this substrate as well. *Trans*-2-hexenal, a plant derived green leaf aldehyde that has leafy odour, known to be the best substrate for most olfactory GSTs such as the rat olfactory and the sphinx moth (Rogers *et al.*, 1999).

Odorants are found to be the most efficient substrates for olfactory enzymes. The ability to conjugate with an odorant substrate is an important characteristic of olfactory GSTs. Conjugation between olfactory GSTs and *t*-2-hexenal on t.l.c plate were observed in the studies of characterization of mouse vomeronasal organ (Green *et al.*, 2005). The absence of conjugation of the recombinant CG16936 with this substrate indicates the inability of CG16936 to detoxify odorants and doubts its ability to act as an olfactory GST in *Drosophila melanogaster*.

## CHAPTER 5 CONCLUSION

CG16936 has been recently classified as Glutathione transferase E12. Our recombinant CG16936 has shown to have high activity towards DCNB with a specific activity of 0.4324 $\mu$ mol/min/mg. No activities were detected with the other 11 GST substrates including CDNB, the universal substrate and *t*-2-hexenal, an odorant substrate specific for olfactory GSTs. The absence of activity and inability of the recombinant CG16936 to conjugate with *t*-2-hexenal suggested that this enzyme was unable to detoxify odorant substrates. Besides that, exposure of *Drosophila* to benzaldehyde did not induce or over express the CG16936 protein. Thus, our findings suggested that in contrary, the CG16936 may have not specifically be responsible to detoxification of odorant. Its activity towards DCNB implies its participation in detoxification of pesticides and other general conjugate substrates.

Future work suggestions would be to investigate more on the olfactory GSTs of *Drosophila melanogaster*. This can be done by exposing the flies with various other odorants, besides benzaldehyde to see the gene and protein expressions. Besides that, maybe the head can be isolated and checked for olfactory GSTs upon exposure. For some reasons, the *CG16936* was found to be expressed when exposed to benzaldehyde. Function of that can be investigated as well. Due to time constrain, we did not proceed to other methods of purification and reported it as partially purified. Future work can be done trying other purification methods and a pure CG16936 can be used to do kinetic studies as well as for characterization of this enzyme which can help us to understand the function CG16936 better.



## REFERENCES

- Ai, M., Min., S., Grosjaen, Y., Leblanc, C., Bell.,R., Benton, R. & Suh, G.S.B. (2010). Acid sensing by the *Drosophila* Olfactory System. *Nature*, 468(7324), 691-695.
- Aceto, A., Dillio, C., Angelucci S., Longo, V., Gervasi, P.G. & Federici, G. (1989). Glutathione transferases in human nasal mucosa. *Archives of Toxicology*, 63(6), 427–431.
- Alias, Z. & Clark, A.G. (2007). Studies on the glutathione S-transferases proteome of adult *Drosophila melanogaster*. Responsiveness to chemical challenge. *Proteomics*, 7, 3618 – 3628.
- Anderson, M.E. (1998). Glutathione: an overview of biosynthesis and modulation. *Chemico- Biological Interaction*, 111–2, 1–14.
- Anholt, R.R.H. & Mackay, T.F.C. (2001). The Genetic architecture of odor-guided behaviour in *Drosophila melanogaster*. *Behaviour Genetics*, 31, 17-27.
- Armstrong, R.N. (1997). Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chemical Research Toxicology*, 10, 2–18.
- Armstrong, R.N. (2000). Mechanistic Diversity in a Metalloenzyme Superfamily. *Biochemistry*, 39, 13625 – 13632.
- Armstrong, R. (2010). *Glutathione Transferases (GST) superfamily*. Retrieved from <http://enzymefunction.org/about/bridging-projects/gst-superfamily>.
- Atkinson, H.J. & Babbitt, P.C. (2009). Glutathione transferase are structural and functional outliers in the thioredoxin fold. *Biochemistry*, 48, 11108-11116.
- Ben-Arie, N., Khen, M. & Lancet, D. (1993). Glutathione S transferase in rat olfactory epithelium: Purification, molecular properties and odorant biotransformation. *Biochemical Journal*, 292, 379–384.
- Beall, C., Fyrberg, C., Song, S. & Fyrberg, E. (1992). Isolation of a *Drosophila* gene encoding glutathione S-transferase. *Biochemical Genetics*, 30, 515–527.

- Black, D.L. (2003). Mechanisms of alternative pre messenger RNA splicing. *Annual reviews of Biochemistry*, 72(1), 291-336.
- BLAST: Basic Local Alignment Search Tool*. Retrieved 11, November, 2011 from <http://blast.ncbi.nlm.nih.gov/Blast.cgi>
- Blocki, F.A., Schlievert, P.M. & Wackett, L.P. (1992). Rat liver protein linking chemical and immunological detoxification systems. *Nature (London)*, 360, 269-270.
- Board, P.G., Baker, R.T., Chelvanayagam, G. & Jermini, L.S. (1997). Zeta, a novel class of glutathione transferases in a range of species from plants to humans. *Biochemical Journal*, 328, 929-935.
- Board, P.G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermini, L.S., Schulte, G.K., Danley, D.E., Hoth, L.R., Griffor, M.C., Kamath, A.V., Rosner, M.H., Chrnyk, B.A., Perregaux, D.E., Gabel, C.A., Geoghegan, K.F. & Pandit, J. (2000). Identification, characterization, and crystal structure of the Omega class glutathione transferases. *Journal of Biological Chemistry*, 275, 24798-24806.
- Bradford, M.M. (1976). A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.
- Brophy, D.M., Southan, C. & Barrett, J. (1989). Glutathione transferase in the tapeworm *Moniezia expansa*. *Biochemical Journal*, 262(3), 939-946.
- Bruns, C.M., Hubatsch, I., Ridderstroëm, M., Mannervik, B. & Tainer, J.A. (1999). Human Glutathione Transferase A4-4 Crystal Structures and Mutagenesis Reveal the Basis of High Catalytic Efficiency with Toxic Lipid Peroxidation Products. *Journal of Molecular Biology*, 288, 427-439.
- Chasseaud, L.F. (1979). The role of glutathione S-transferase in metabolism of chemical carcinogens and other electrophilic agents. *Advances in Cancer Research*, 26, 175-274.
- Chiang, F.M & Sun, C.N. (1993). Glutathione transferase isozymes of diamondback moth larvae and their role in the degradation of some organophosphorus insecticides. *Pesticide Biochemistry and Physiology*, 45, 7-14.

- Clark, A.G. (1989). The comparative enzymology of the glutathione S-transferases from non-vertebrate organisms. *Comparative Biochemistry and Physiology B*, 92, 419–446.
- Clark, A.G. & Shamaan, N.A. (1984). Evidence that DDT-dehydrochlorinase from the housefly is a glutathione S-transferase. *Pesticide Biochemistry and Physiology*, 22, 249-261.
- Clark, A.G., Dick, G.L., Martindale, S.M. & Smith, J.N. (1985). Glutathione S-transferases from the New Zealand grass grub, *Costelytra zealandica*, their isolation and characterization and the effect on their activity of endogenous factors. *Insect Biochemistry*, 15, 35–44.
- Ding, Y., Ortell, F., Rossiter, L.C., Hemingway, J. & Ranson, H. (2003). The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BioMed Central Genomics*, 4, 35-50.
- Ding, Y., Hawkes, N., Meredith, J., Eggleston, P., Hemingway, J. & Ranson, H. (2005). Characterization of the promoters of Epsilon glutathione transferases in the mosquito *Anopheles gambiae* and their response to oxidative stress. *Biochemical Journal*, 387, 879-888.
- Dirr, H.W., Reinemer, P. & Huber, R. (1994). X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. *European Journal of Biochemistry*, 220, 645-661.
- Dixon, D.P., Cummins, I., Cole, D.J. & Edwards, R. (1998). Glutathione-mediated detoxification systems in plants. *Current Opinion in Plant Biology*, 1, 258–266.
- Dixon, D.P., Laphorn, A. & Edwards, R. (2002). Plant glutathione transferases. *Genome Biology*, 3(3), 1-10.
- Dixon, D.P., Skipsey, M., & Edwards, R. (2010). Roles for glutathione transferases. *Phytochemistry*, 71, 338-350.
- Drosophila melanogaster*, fruit fly. Retrieved 7, July, 2012 from <http://www.geochembio.com/biology/organisms/fruitfly/>
- Droog, F. (1997). Plant glutathione S-transferases, a tale of Theta and Tau. *Journal of Plant Growth Regulation*, 16(2), 95-107.

- Dulhunty, A., Gage, P., Curtis, S., Chelvanayagam, G. and Board, P. (2001). The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. *Journal Biological Chemistry*, 276, 3319–3323.
- Enayati, A.A., Ranson, H. & Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology*, 14, 3–8.
- Feng, Q.L., Davey, K.G., Pang A.S.D., Primavera M., Ladd, T.R., Zheng, S.C., Sohi, S.S., Retnakaran, A. & Palli, S.R. (1999). Glutathione S-Transferases from the spruce budworm, *Choristoneura fumiferana*: Identification, characterization, localization, cDNA cloning and expression. *Insect Biochemistry and Molecular Biology*, 29, 779-793.
- Fesik, S.W. (2001). Insights into programmed cell death through structural biology. *Cell* (Cambridge, Mass.) 103, 273–282.
- FlyBase: A Database for Drosophila Genes and Genomes*. Retrieved 10, January, 2010 from <http://flybase.org>.
- Frova, C. (2006). Glutathione transferases in the genomics era: New insights and perspectives. *Biomolecular Engineering*, 23(4), 149-169.
- Fujita, M. & Hossain, M.Z. (2003). Modulation of Pumpkin Glutathione S-Transferases By Aldehydes and Related Compounds. *Plant and Cell Physiology*, 44(5), 481-490.
- Fournier, D., Bride, J.M., Poire, M., Berge, J.B. and Plapp, F.W. (1992). Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. *Journal of Biological Chemistry*, 267, 1840–1845.
- Gakuta, T. & Toshiro, A. (2000). Disruption of the microsomal glutathione S-transferase-like gene reduces life span of *Drosophila melanogaster*. *Gene*, 253, 179–187.
- Glutathione Chemical Structure*. Retrieved from 6, July, 2012 from <http://chemistry.about.com/od/factsstructures/ig/Chemical-Structures---G/Glutathione.htm>.
- Glasel, J.A. (1995). Validity of Nucleic Acid Purities Monitored by A260/A280 Absorbance Ratios, *Biotechniques* 18:62-63.

- Green, N., Weech, M. & Walters, E. (2005). Localization and characterization of glutathione-S-transferase isozymes, alpha, mu, and pi within the mouse vomeronasal organ. *Neuroscience Letters*, 375, 198-202
- Guengerich, F.P. (1991). Oxidation of toxic and carcinogenic chemicals by human cytochrome p-450 enzymes. *Chemical Research in Toxicology*, 4, 391–407.
- Guengerich, F. P. (1990). Enzymatic oxidation of xenobiotic chemicals. *Critical Review in Biochemistry and Molecular Biology*, 25, 97-153.
- Habig, H., Pabst, M.J. & Jacoby, W.B. (1974). Glutathione S-transferases: The first step in mercapturic acid formation. *Journal of Biological Chemistry*, 249, 7130-7139.
- Habig, W.H. & Jakoby, W.B. (1980). An enzymatic basis of detoxification. Vol.2 pp. 63-94. Academic press, New York.
- Hatton, P.J., Dixon, D., Cole, D.J. & Edwards, R. (1996). Glutathione transferase activities and herbicide selectivity in maize and associated weed species. *Pesticide Science*, 46, 267–275.
- Hamada. S., Kamada. M., Furumoto. H., Hirao. T. & Aono, T. (1994). Expression of glutathione S-transferase  $\pi$  in human ovarian cancer as an indicator of resistance to chemotherapy. *Gynecologic Oncology*, 52, 313-319.
- Hayes, J.D. & Wolf, C.R. (1988). Role of glutathione-S-transferase in drug resistance. In *Glutathione Conjugation*, Cap. X, Sies, H. and Ketterer, B. eds, pp. 315-355. Academic Press, London.
- Hayes, J.D. & Pulford, D. J. (1995). The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Critical Review in Biochemistry and Molecular Biology*, 30, 445–600.
- Hayes, J.D. & McLellan, L.I. (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radical Research*, 31, 273-300.
- Hazelton, G.A. & Lang C.A. (1983). Glutathione S-transferases activity in a yellow fever mosquito (*Aedes Aegypti*) during growth and aging. *Biochemical Journal*, 210, 281-287.

- Hemingway, J., Hawkes, N.J., Mccarroll, L. & Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Molecular and population biology of mosquitoes*, 34(7), 653-665.
- Hu, B., Deng, L., Wen, C., Yang, X., Pei, P., Xie, Y. And Luo, S. (2012). Cloning, identification and functional characterization of pi-class glutathione s transferase from the freshwater mussels *Cristaria plicata*. *Fish & Shellfish Immunology* , 32, 51-60.
- Huang, Y., Krell, P.J., Ladd, T., Feng, Q., & Zheng, S. (2009). Cloning, Characterization And Expression Of Two Glutathione S-Transferase cDNAs In The Spruce Budworm, *Choristoneura Fumiferana*. *Archives Of Insect Biochemistry And Physiology*, 70, 44–56.
- Ji, X.H., Rosenvinge, E.C., Johnson, W.W., Tomarev, S.I., Piatigorsky, J., Armstrong, R.N. & Gilliland, G.L. (1995). 3-Dimensional structure, catalytic properties and evolution of a sigma-class glutathione transferase from squid, a progenitor of the lens S-crystallins of cephalopods. *Biochemistry*, 34, 5317-5328.
- Johansson, A.S., Mannervik, B. (2001). Human glutathione transferase A3–3, a highly efficient catalyst of double-bond isomerization in the biosynthetic pathway of steroid hormones. *Journal of Biological Chemistry*, 276, 33061–33065.
- Jakobsson, P.J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A. & Persson, B. (1999). Common structural features of MAPEG- a widespread superfamily of a membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Science*, 8, 689-692.
- Kampranis, S.C., Damianova, R., Atallah, M., Toby, G., Kondi, G., Tsiachlis, P. N. & Makris, A.M. (2000). A novel plant glutathione S -transferase/peroxidase suppresses Bax lethality in yeast. *Journal of Biological Chemistry*, 275, 29207–29216.
- Ketterer, B. (2001). A bird's eye view of the glutathione transferase field. *Chemico-Biological Interaction*, 138, 27–42
- Kong, K.H., Nishida, M., Inoue, H. & Takashashi, K. (1992). Tyrosine-7 is an essential residue for the catalytic activity of human class pi glutathione s-transferase-chemical modification and site directed mutagenesis studies. *Biochemical and Biophysical Research Communications*, 182, 1122-1129.

- Koonin, E.V., Mushegian, A.R., Tatusov, R.L., Altschul, S.F., Bryant, S.H., Bork, P. & Valencia, A. (1994). Eukaryotic translation elongation factor 1 contains a glutathione transferase domain. Study of a diverse, ancient protein superfamily using motif search and structural modeling. *Protein Science*, 3, 2045–2054.
- Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E., & Papadopolou-Mourkidou, E. (2001). Glutathione S-transferases in the defense against pyrethroids in insects. *Pesticide Biochemistry and Physiology*, 31, 313–319.
- Lander, J.E., Parsons, J.F., Rife, C.L., Gilliland, G.L. & Armstrong, R.N. (2004). Parallel evolutionary pathways for glutathione transferases: structure and mechanisms of the mitochondrial class Kappa enzyme rGSTK1-1. *Biochemistry*, 43, 352–261.
- Lazard, D., Tal, N., Rubenstein, M., Khen, M., Lancet, D. & Zupko, K. (1990). Identification and biochemical analysis of novel olfactory-specific cytochrome P-450IIA and UDP-glucuronosyl transferase. *Biochemistry*, 29, 7433–7440.
- Lee, K.W., Raisuddin, S., Rhee, J.S., Hwang, D.S., Yu, I.T., Lee, Y.M., Park, H.G. & Lee, J. S. (2008). Expression of glutathione S-transferase (GST) genes in the marine copepod 16 *Tigriopus japonicus* exposed to trace metals. *Aquatic Toxicology*, 89, 158-166
- Li, X., Zhang, X., Zhang, J., Zhang, Xin, Starkey, S.R. & Zhu, K.Y. (2009). Identification and characterization of eleven glutathione S-transferase gene from aquatic midge *Chironomus tentans*. *Insect Biochemistry and Molecular Biology*, 39, 745-754
- Lumjuan, N., McCarroll, L., Prapanthadara, L., Hemingway, J. & Ranson, H. (2005). Elevated activity of an Epsilon class Glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, 35, 861-871.
- Lumjuan, N., Stevenson, B.J., Prapanthadara, L., Somboon, P., Brophy, P.M., Loftus, B.J., Severson, D.W. and Ranson, H. (2007). The *Aedes aegypti* glutathione transferase family. *Insect Biochemistry and Molecular Biology*, 37, 1026-1035.
- Longo, V., Mazzaccaro, A., Naldi, F. & Gervasi, P.G. (1991). Drug-metabolizing enzymes in liver, olfactory and respiratory epithelium of cattle. *Journal of Biochemical Toxicology*, 6, 123–128.

- Low, W.L., Ng, H.L., Morton, C.G, Parker, M.W., Batterham, P. & Robin, C. (2007). Molecular evolution of Glutathione S-transferase in genus *Drosophila*. *Genetics*, 177, 1363–1375.
- Main, P.A., Angley, M.T., O'Doherty, C.E., Thomas, P. & Fenech, M. (2012). The potential role of the antioxidant and detoxification properties of glutathione in autism spectrum disorders: a systematic review and meta-analysis. *Nutrition & Metabolism*, 9(1), 35.
- Mannervik, B. & Danielson, U. (1988). Glutathione transferases—structure and catalytic activity. *Critical Review in Biochemistry and Molecular Biology*, 23, 283–337.
- Mannervik, B., Board, B.G., Hayes, J.D., Listowsky, I. & Pearson, W.R. (2005). Nomenclature for mammalian soluble Glutathione Transferases. *Methods in Enzymology*, 401, 1-8.
- Marrs, K.A. (1996). The functions and regulation of glutathione S-transferases in plants. *Annual Review in Plant Physiology and Plant Molecular Biology*, 47, 127–158.
- Map of pET-30a(+) vector. Retrieved 3, January, 2010, from <http://www.synthesisgene.com/vector/pET-30a>.
- Maps sites for restriction enzymes in DNA sequence. Retrieved 10, January, 2010, from <http://www.restriction mapper.org>.
- Moons, A. (2005). Regulatory and functional interactions of plant growth regulators and plant glutathione S-transferases (GSTs). *Plant Hormones*, 72, 155–202.
- Morel, F., Rauch, C., Petit, E., Piton, A., Theret, N., Coles, B. & Guillouzo, A. (2004). Gene and protein characterization of the human glutathione S-transferase kappa and evidence for a peroxisomal localization. *Journal of Biological Chemistry*, 279, 16246–16253.
- Motoyama, N. & Dauterman, W.C. (1977). Purification and properties of housefly glutathione s-transferase. *Insect Biochemistry*, 7(4), 361-369.
- Nef, P., Heldman, J., Lazard, D., Margalit, T., Jaye, M., Hanukoglu, I. & Lancet, D. (1989). Olfactory-specific cytochrome P-450. *Journal of Biological Chemistry*, 264, 6780–6785.



- Oakley, A. (2011). Glutathione transferases: A structural perspective. *Drug metabolism Reviews*, 43(2), 138-151.
- Oakeshott, J. G., Johnson, R. M., Berenbaum, M. R., Ranson, H., Cristino, A. S. and Claudianos, C. (2010). Metabolic enzymes associated with xenobiotic and chemosensory responses in 24 *Nasonia vitripennis*. *Insect Molecular Biology*, 19(1), 147-163.
- Oppenoorth, F.J., Van der Pas, L.J.T. & Houx, N.W.H. (1979). Glutathione S-transferase and hydrolytic activity in a tetrachlorvinphos-resistant strain of housefly and their influence on resistance. *Pesticide Biochemistry and Physiology*, 11, 176-188.
- Pal, R., Blakemore, M., Ding, M. & Clark, A.G. (2012). A Study of the Glutathione Transferase Proteome of *Drosophila melanogaster*: Use of S- Substituted Glutathiones as Affinity Ligands, Affinity Chromatography, Sameh Magdeldin (Ed.), ISBN: 978-953-51-0325-7.
- Pastore, A., Piemonte, F., Locatelli, M., Russo, A.L., Gaeta, L.M., Tozzi, M. & Federici, G. (2003). Determination of blood total, reduced, and oxidized glutathione in paediatric subjects. *Clinical Chemistry*, 47(1), 1467-1469.
- Prapanthadara, L., Promtet, N., Koottathep, S., Somboon, P., & Ketterman, A.J., (2000). Isoenzymes of glutathione S-transferase from the mosquito *Anopheles dirus* species B: the purification, partial characterization and interaction with various insecticides. *Insect Biochemistry and Molecular Biology* 30, 395-403.
- Pearson, W.R. (2005). Phylogenies of glutathione transferase families. *Methods in Enzymology*, 401, 186–204.
- Prabhu, K.S., Reddy, P.V., Gmpricht, G., Hildenbrandt, G.R., Scholz, R.W., Sordillo, L.M. & Reddy, C.C. (2001). Microsomal glutathione S-transferase A1–1 with glutathione peroxidase activity from sheep liver: molecular cloning, expression and characterization. *Biochemical Journal*, 360, 345–354.
- Raj, A. & Oudenaarden, A.V. (2008). Nature, nurture, or chance: Stochastic gene expression and its consequences. *Cell*, 135(2), 216-226.
- Ranson H., Collins F. & Hemingway J. (1998). The role of alternative mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione S-transferase family. *Proceedings of National Academy of Sciences U.S.A.*, 95,14284–14289

- Ranson, H., Claudianos, C., Ortell, F., Abgrall, C., Hemingway, J., Sharakhova, M.V., Unger, M.F., Collins, F.H. & Feyereisen, R. (2002). Evolution of supergene families associated with insecticide resistance. *Science*, 298, 179–181.
- Reinemer, P., Dirr, H.W., Ladenstein, R., Huber, R., Lo Bello, M. & Federici, G. (1992). Three dimensional structure of class pi glutathione S-transferase from human placenta in complex with S-hexylglutathione at 2.8 Å resolution. *Journal Molecular Biology*, 227, 214-226.
- Robinson, A., Huttley, G.A., Booth, H.S. & Board, P.G. (2004). Modelling and bioinformatics studies of the human Kappa-class glutathione transferase predict a novel third glutathione transferase family with similarity to prokaryotic 2-hydroxychromene 2-carboxylate isomerases. *Biochemical Journal*, 379, 541–552.
- Rogers, M.E., Jani, M.K. & Vogt, R.G. (1999). An olfactory Specific Glutathione S-transferase in the Spinx moth *Manduca sexta*. *The Journal of Experimental Biology*, 202, 1625-1637.
- Rossjohn, J., Polekhina, G., Feil, S. C., Allocati, N., Masulli, M., Dillio, C. & Parker, M.W. (1998). A mixed disulfide bond in bacterial glutathione transferase : functional and evolutionary implications. *Structure*, 6, 721-734.
- Roxas, V.P., Smith, R.K., Allen, E.R. & Allen, R.D., (1997). Overexpression of glutathione S-transferase/ glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nature Biotechnology*, 15, 988-991.
- Saisawang, C., Wonsantichon, J. & Ketterman, A.J. (2011). A preliminary characterization of the cytosolic glutathione transferase proteome from *Drosophila melanogaster*. *Biochemical Journal*, 442(1), 181-190.
- Sandford, A.J. & Silverman, E.K (2012). Chronic obstructive pulmonary disease. *Thorax*, 57, 736-741.
- Sato, K. (1989). Glutathione transferases as markers of preneoplasia and neoplasia. *Advances in Cancer Research*, 52, 205–255.
- Sawicki, R., Singh, S.P., Mondal A.K, Benes, H. & Zimniak, P. (2003). Cloning, expression and biochemical characterization of one Epsilon-class (GST-3) and ten Delta-class (GST-1) glutathione S-transferase from *Drosophila melanogaster*, and identification of additional nine members of the Epsilon class. *Biochemical Journal*, 370, 661-669.

- Sheehan, D., Meade, G., Foley, V.M. & Dowd, C.A. (2001). Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochemical Journal*, 360, 1–16.
- Singh, S.P., Coronella, J.A., Benes, H., Cochrane, B.J. & Zimniak, P. (2001). Catalytic function of *Drosophila melanogaster* glutathione S-transferase DmGSTS1-1 in conjugation of lipid peroxidation end products. *European Journal Biochemistry*, 268, 2912-2923.
- Skopelitou, K., Dhavala, P., Papageorgiou, A.C. & Labrou, N.E. (2012). A Glutathione Transferase from *Agrobacterium tumefaciens* Reveals a Novel Class of Bacterial GST Superfamily. *PLoS ONE* 7(4): e34263. doi:10.1371/journal.pone.0034263.
- Spencer, S.R., Xue, L., Klenz, E.M. & Talalay, P. (1991). The potency of inducers of NAD(P)H: (quinine-acceptor) oxidoreductase parallels their efficiency as substrates for glutathione transferases. *Biochemistry Journal*, 273, 711-717.
- Stenersen, J., Kobro, S., Bjerke, M., & Arend, U. (1987). Glutathione transferases in aquatic and terrestrial animals from nine phyla. *Comparative Biochemistry and Physiology C*, 86(1), 73–82.
- Strange, R.C., Spiteri, M.A., Ramachandran, S. & Fryer, A.A. (2001). Glutathione-S-transferase family of enzymes. *Mutation Research*, 482, 21–26.
- Stocker, R.F., Lienhard, M.C., Borst, A. & Fischbach, K.F. (1990). Neuronal architecture of the antennal lobe in *Drosophila melanogaster*. *Cell Tissue Research*, 262(1), 9–34.
- Stortkuhl, K. & Raffael, K. (2001). Functional analysis of an olfactory receptor in *Drosophila melanogaster*. *Neurobiology*, 98(16), 9381-9385.
- Srivastava, S., Chandra, A., Wang, L.F., Seifert, W.E.J., DaGue, B.B., Ansari, N.H., Srivastava, S.K. & Bhatnagar, A. (1998). Metabolism of the lipid peroxidation product, 4-hydroxy-trans-2-nonenal, in isolated perfused rat heart. *Journal of Biological Chemistry*, 273, 10893–900.
- Takesawa, T., Ito, M., Kanzaki, H., Kameya, N. & Ikuo, N., (2002). Over-expression of glutathione S-transferase in transgenic rice enhances germination and growth at low temperature. *Molecular Breeding*, 9, 93-101.

- Tan, K.L., Chelvanayagam, G., Parker, M.W. & Board, P.G. (1996). Mutagenesis of the active site of the human theta class glutathione transferase GSTT2-2: catalysis with different substrate involves different residues. *Biochemical Journal*, 319, 315-321.
- Tew, K.D. (1994). Glutathione-associated enzymes in anticancer drug resistance. *Cancer Research*, 54, 4313–4320.
- Toba, G. & Aigaki, T. (2000). Disruption of the microsomal glutathione S-transferase-like gene reduces life span of *Drosophila melanogaster*. *Gene*, 253, 179–187.
- Udomsinprasert, R., Bogoyevitch, M.A. & Kettermann A. J. (2004). Reciprocal regulation of glutathione S-transferase spliceforms and the Drosophila c-Jun N-terminal kinase pathway components. *Biochemical Journal*, 383, 483-490
- Vontas, J.G., Small, G.J. & Hemingway, J. (2001). Glutathione S-transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochemical Journal*, 357, 65–72.
- Vuilleumier, S. (1997). Bacterial glutathione S-transferases: what are they good for?. *Journal of Bacteriology*, 179, 1431–1441.
- Wagner, U., Edwards, R., Dixon D.P. & Mauch, F., (2002). Probing the diversity of the *Arabidopsis* glutathione S-Transferase gene family. *Plant Molecular Biology*, 49, 515-532.
- Wilce, M.C.J. & Parker, M.W. (1994). Structure and function of Glutathione S-transferase. *Biochimica et Biophysica Acta*, 1205, 1-18.
- Wilhelm, D., Bender, K., Knebel, A. & Angel, P. (1997). The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents. *Molecular and Cellular Biology*, 17, 4792–4800.
- Wikteliuss, E. & Stenberg, G. (2007). Novel class of glutathione transferases from cyanobacteria exhibit high catalytic activities towards naturally occurring isothiocyanates. *Biochemical Journal*, 406, 115–123.
- Wongsantichon, J. & Kettermann, A. (2005). Alternative splicing of glutathione transferases. *Methods in Enzymology*, 401, 100-116.

- Wongtrakul, J., Wongsantichon, J., Vararattanavech, A., Leelapat, P., Prapanthadara, L. & Ketterman, A.J. (2009). Molecular cloning and expression of several new *Anopheles cracens* Epsilon GSTs. *Protein & Peptide*, 16, 75-81.
- Yamamoto, K., Nagaoka, S., Banno, Y. & Aso, Y. (2009). Biochemical properties of an omega-class glutathione s-transferase of the silkworm, *Bombyx mori*. *Comparative Biochemistry and Physiology C*, 149, 461-467.
- Yang, J., McCart, C., Woods, D. J., Terhzaz, S., Greenwood, K.G., Constant, R.H. & Dow, J.A.T. (2007). A *Drosophila* systems approach to xenobiotic metabolism. *Physiological Genomics*, 30, 223-231.
- Yu, S.J. (1996). Insect glutathione S-transferases. *Zoological Studies*, 35, 9-19.
- Zhou, J. & Goldsbrough, P.B. (1993). An *Arabidopsis* gene with homology to glutathione S-transferase is regulated by ethylene. *Plant Mol Biol*, 22, 517-523.
- Zhao, D., Chen, L., Qin, C., Zhang, H., Wu, P. & Zhang, F. (2010). A delta-class glutathione transferase from the Chinese mitten crab *Eriochei sinensis*: cDNA cloning, characterization, and mRNA expression. *Fish & Shellfish Immunology*, 29, 698-703.
- Zheng, S.C., Deng, H.M., Ladd, T.R., Tomkins, B., Krell, P.J. & Feng, Q. (2007). Cloning and characterization of two glutathione S-transferase cDNAs in the spruce budworm, *Choristoneura fumiferana*. *Archive of Insect Biochemistry and Physiology*, 66, 146-157.

## APPENDICES

### **Appendix A: Buffer Solution Preparation**

#### **Buffer A- Sodium Phosphate Buffer (0.1 M, pH 7.4)**

12 g of  $\text{NaH}_2\text{PO}_4$  was dissolved in approximately 900 ml of  $\text{dH}_2\text{O}$ . The pH of the solution was adjusted 7.35 at 20°C and the volume was made up to 1000 ml.

#### **Buffer B- Tris Buffer (0.1 M, pH 9.0)**

12.11 g Tris base was dissolved in approximately 900 ml of  $\text{dH}_2\text{O}$  and the pH was adjusted 9.0 at 20°C. The volume was then made up to 1000 ml.

#### **Buffer C- Sodium Phosphate Buffer (0.1 M, pH 7.5)**

12 g of  $\text{NaH}_2\text{PO}_4$  was dissolved in approximately 900 ml  $\text{dH}_2\text{O}$  and the pH was adjusted to 7.5 at 20°C. The volume was then made up to 1000 ml.

### **Running buffer for FPLC**

#### **Sodium Phosphate buffer (25 mM, pH 7.4)**

3 g  $\text{NaH}_2\text{PO}_4$  was dissolved in approximately 900 ml of  $\text{dH}_2\text{O}$ . The pH of the solution was adjusted to 7.35 at 20°C and the volume was made up to 1000 ml.

### **Elution buffers for FPLC**

#### **Glutathione (10 mM, pH 7.4)**

0.307 g of reduced glutathione was dissolved in 100 ml of 25 mM phosphate buffer and pH was adjusted to 7.4 at 20°C.

**Imidazole (300 mM, pH 7.4)**

2.0424 g of imidazole was dissolved in 100 ml 25 mM phosphate buffer and pH was adjusted to 7.4.

**Bromosulfophthalein (2 mM, pH 7.4)**

0.167 g of BSP was dissolved in 100 ml 25 mM phosphate buffer and pH was adjusted to 7.4.

**Sodium chloride (1 M, pH 7.4)**

5.844 g of sodium chloride was added to 100 ml of 25 mM Phosphate buffer and pH was adjusted to 7.4

**Potassium Chloride (1 M, pH 7.4)**

7.456 g of potassium chloride was added to 100 ml of 25 mM phosphate buffer and pH was adjusted to 7.4.

**Appendix B: Preparations for cell culturing.****Luria Bertani broth**

20 g of LB broth was dissolved in 1000 ml dH<sub>2</sub>O. The solution was then autoclaved at 120°C for 20 minutes.

**Kanamycin stock solution (30 mg/ml)**

1.5 g kanamycin sulphate is dissolved in 50 ml deionized water. Solution was filter sterilized and stored in -20°C.

**Ampicilin stock solution (100 mg/ml)**

5 g ampicilin sodium salt is dissolved in 50 ml deionized water. Solution was filter sterilized and stored in -20°C.

**X-gal stock solution (40 mg/ml)**

400 mg of 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside were dissolved 10 ml dimethylformamide. Solution is protected from light by wrapping in aluminium foil. Solution is stored in -20°C.

**IPTG stock solution (100 mM)**

238.3 mg isopropyl  $\beta$ -D-thiogalactopyranoside dissolved in 10 ml deionized water. Solution is filter-sterilized and store in -20°C.

**LB Agar plates preparation**

35 g of LB Agar was dissolved in 1000 ml dH<sub>2</sub>O. The solution was then autoclaved at 120°C for 20 minutes.

The autoclaved LB agar was allowed to cool to 55°C. 1 ml kanamycin stock solution was added and mixed. For blue/white screening, 2 ml X-gal stock solution and 0.5 ml IPTG stock solution were added. Agar was poured into plates, allowed to set, inverted and stored at 4°C under sterile conditions



## **Appendix C: Solutions for SDS Polyacrylamide Gel Electrophoresis**

### **SDS (10% (w/v))**

10 g SDS was dissolved in 50 ml miliQ dH<sub>2</sub>O with gentle shaking. Then the volume was made up to 100 ml.

### **SDS Sample Buffer**

The buffer consisted of 62.5 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol. To prepare a buffer solution of 2 ml, 0.25 ml of 0.5 M Tris-HCl, pH 6.8, 0.4 ml glycerol, 0.4 ml 10% (w/v) SDS, 0.1 ml β-mercaptoethanol, 0.1 ml of 0.5% (w/v) bromophenol blue and 0.75 ml miliQ H<sub>2</sub>O were mixed. To prepare sample in sample buffer, the sample was diluted at least 1:4 ratio. Then, the sample was heated at 95°C for 4 minutes.

### **SDS gel staining.**

Colloidal Coomassie Brilliant Blue G-250.

One g of Coomassie Brilliant Blue G-250 was added in 20 ml of dH<sub>2</sub>O was sonicated until it dissolves. 11.8 ml of 85% (w/v) phosphoric acid is added to the CBB solution. 100 g of ammonium sulphate was dissolved in approximately 600 ml of dH<sub>2</sub>O. Then the CBB solution was added little by little and made up to 1 litre.

**Appendix D: Medium for *Drosophila* breeding.**

Ingredients	Percentage (%)
Agar	1.0
Sucrose	6
Brown sugar	3
Oatmeal	10
Yeast	1.5
Acid mix	1.5

Acid Mix: Propionic acid and phosphoric acid.

All the ingredients were added in 250 ml water and boiled together. Once done, it was poured into an appropriate bottle and let it cool overnight before transferring *Drosophila* to it.

**Appendix E: Solutions for t.l.c****Developing solvent**

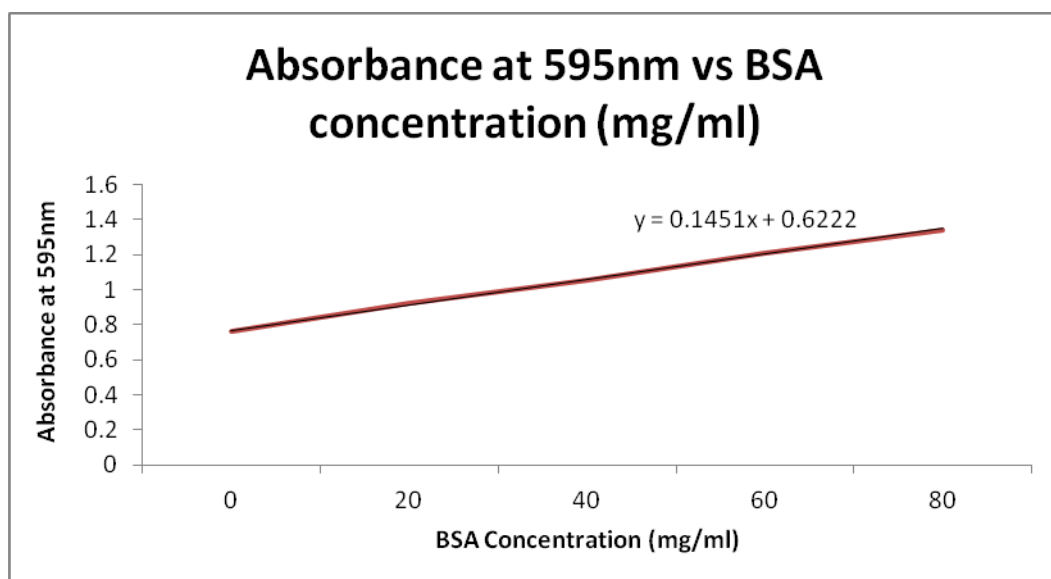
12 ml of Butan-1-ol with mixed together with 3 ml of acetic acid and 5ml water and poured into a beaker and covered with aluminium foil.

**Staining solution**

A total of 0.25 g ninhydrin was dissolved in 100 ml acetone and mixed well.

## Appendix F: Bradford assay

### BSA graph



Graph: Absorbance at 595 nm vs. BSA concentration (mg/ml)

Bradford method was used to determine the concentration of the partially purified CG16936. Absorbance was recorded at different concentrations of BSA (Table below) and graph was plotted. Based on the equation derived from the graph, protein concentration was determined and specific activity was calculated for the substrate specificities.

Table: Absorbance at 595 nm of different concentrations of BSA.

BSA concentration (mg/ml)	Absorbance at 595 nm
0	0.7595
20	0.9224
40	1.0571
60	1.2055

## **Appendix G: Substrates preparations**

### **1-Chloro-2,4-dinitrobenzene (CDNB)**

0.2430 g of CDNB was dissolved in 20 ml of ethanol. It was mixed well and stored at room temperature, in a universal bottle covered with aluminium foil to protect it from light.

### **1,2-Dichloro-4-nitrobenzene (DCNB)**

0.092 g of DCNB was dissolved in 20 ml of ethanol. It was mixed well and stored at room temperature, in a universal bottle covered with aluminium foil to protect it from light.

### ***p*-Nitrophenyl chloride (NBC)**

0.2058 g of NBC was dissolved in 20 ml of ethanol. It was mixed well and stored in a universal bottle, at room temperature.

### **1,2-Epoxy-3-nitrophenoxypropane (EPNP)**

0.1171 g of EPNP was dissolved in 20 ml ethanol. It was mixed well and stored in a universal bottle, at room temperature.

### **Sulfobromophthalein (BSP)**

0.0334 g of BSP was dissolved in 20 ml ethanol. It was mixed well and stored in a universal bottle, at room temperature.

**Ethacynic acid (EA)**

0.0727 g was dissolved in 20 ml ethanol. It was mixed well and stored in a universal bottle, at room temperature.

**Trans-4-phenyl-3-butene-2-one (PBO)**

0.0876 g was dissolved in 20 ml ethanol. It was mixed well and stored in a universal bottle, at room temperature.

**Trans-2-hexenal**

2.326  $\mu$ l of trans-2-hexenal stock in 20 ml ethanol. It was mixed well and stored in a universal bottle, at room temperature.

**Trans-Oct-2-enal**

11.19  $\mu$ l of trans-octenal stock in 50 ml Buffer A. It was mixed well and stored in a volumetric flask covered with aluminium, at room temperature.

**2,4-Heptadienal**

94.94  $\mu$ l of 2,4-heptadienal stock in 250 ml Buffer A. It was mixed well and stored in a volumetric flask covered with aluminium, at room temperature.

**Hexa-2,4-dienal**

82.42  $\mu$ l of hexa-2,4-dienal stock in 250 ml buffer A. It was mixed well and stored in a volumetric flask covered with aluminium, at room temperature.