EFFECT OF SYNTHETIC PYRETHROID LAMBDA-CYHALOTHRIN ON *HELICOVERPA ARMIGERA* GLUTATHIONE S-TRANSFERASES

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ABSTRACT

EFFECT OF SYNTHETIC PYRETHROID INSECTECIDE LAMBDA-CYHALOTHRIN ON *HELICOVERPA ARMIGERA* GLUTATHIONE S-TRANSFERASES

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Helicoverpa armigera is a polyphagous pest. Due to excessive use of insecticides, the field populations of *H. armigera* have become resistant to synthetic pyrethroids by one or combination of three mechanisms; reduced penetration through the cuticle, decreased nerve sensitivity and enhanced metabolism by the detoxification enzymes especially glutathione S-transferases.

In this study, gut sections of *H. armigera* were obtained from Adana and Antalya field populations and susceptible populations from Israel. Each gut section was homogenized separately in 1.0 ml, 40 mM and pH 7.5 phosphate buffers. GST activity was determined using CDNB as substrate. Product formation linearly increased up to 29.5µg proteins in 20mM, pH 7.5 phosphate buffers. Maximum reaction rate was reached at 30°C. The Vmax and Km values for GST towards CDNB and GSH were calculated with Lineweaver-Burk and Eadie-Scatchard plots as CDNB Vmax; 6.54µmol/min/mg, 6.35µmol/min/mg , Km; 0.29mM, 0.28mM ,respectively and as GSH Vmax; 6.42µmol/min/mg,

6.65µmol/min/mg, Km; 0.22mM, 0.23mM, respectively. Cytosolic GST activity of each individual from Adana, Antalya and susceptible populations were determined under optimized conditions.

The mean of GST activity in Adana population (n=50) and Antalya population (n=50) were found 7.824µmol/min/mg and 9.518µmol/min/mg, respectively. The mean of GST activity in susceptible population (n=50) was determined as 3.272µmol/min/mg. According to these results, GST activities of Adana and Antalya field populations' showed statistically significant increase ($p<0.05$) than susceptible *H. armigera* populations with ANOVA method. In addition, Antalya population showed statistically increase ($p<0.05$) GST activity than Adana.

Key words: *Helicoverpa armigera*, Glutathione S-transferases, CDNB, Synthetic Pyrethroids, Specific activity.

ÖZ

SENTETİK PRETROİDLİ İNSEKTİSİT LAMBDA-CYHALOTHRİN'İN *HELİCOVERPA ARMİGERA*'NIN GLUTATYON S-TRANSFERAZLARI ÜZERİNDEKİ ETKİSİ

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Helicoverpa armigera polifag bir zararlıdır ve tarla populasyonları yaygın olarak kullanılan sentetik piretroid'lere karşı direnç kazanmaktadır. Direnç kütikuladan insektesitlerin geçişinin, insektesite karşı sinirsel duyarlılığın azaltılması ve insektisiti metabolize eden detoksifikasyon enzimlerinin, özellikle glutatyon S-transferazların aktive edilmesi mekanizmalardan birinin yada birkaçının birlikte etkili olmasıyla oluşmaktadır.

Bu çalışmada, İsrail'den getirtilen herhangi bir insektisite maruz kalmamış hassas, Adana ve Antalya'dan toplanan *H. armigera* örnekleriden elde edilen mide bölümleri GST kaynağı olarak kullanıldı. Her mide bölgesi ayrı ayrı 1,0 ml, 40 mM, pH 7,5 fosfat tamponu içerisinde homojenize edildikten sonra, GST aktiviteleri CDNB substratı varlığında ölçüldü. Ürün oluşumu, 20 mM ve pH'sı 7.5 fosfat tamponunda, ortamdaki 29.5µg protein'e kadar doğrusal olarak arttı. En yüksek reaksiyon hızı 30°C'de saptandı. GST enzimi'nin, substratı olan CDNB ve kofaktörü GSH için Km ve Vmax değerleri hem Lineweaver-Burke

hemde Eadie-Scatchard grafikleriyle hesaplandı. CDNB için Vmax; 6.54µmol/dk/mg, 6.35µmol/dk/mg, Km; 0,29mM, 028mM olarak hesaplandı. GSH için Vmax; 6.42µmol/dk/mg, 6.65µmol/dk/mg, Km; 0,22mM, 023mM olarak hesaplandı. Sitozolik GST enzim aktivitesi Adana, Antalya ve Hassas populasyonun her bireyi için ayrı ayrı optimum şartlarda ölçüldü. Adana populasyonu (n=50) ortalama GST aktivitesi 7.824µmol/dk/mg, Antalya populasyonu (n=50) 9.518µmol/dk/mg olarak bulundu. Hassas populasyonun (n=50) GST aktivitesi 3,272µmol/dk/mg olarak belirlendi. Bu sonuçlara göre, Adana ve Antalya tarla populasyonları, hassas populasyonla ANOVA metoduna göre karşılaştırıldığında istatistiksel olarak önemli ($p<0.05$) bir artış göstermiştir. Ayrıca, Antalya örneklerinin GST aktivitelerinin Adana örneklerinden istatistiksel olarak önemli bir artış gösterdiğide ($p<0.05$) belirlendi.

Anahtar kelimeler: Helicoverpa armigera, Glutatyon S-transferazlar, CDNB, Sentetik piretroid'ler , Spesifik aktivite.

To my lovely wife…

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER I

INTRODUCTION

At the production of the economical important crops such as groundnut, tomatoes and cotton one of the most common problem is destruction of these crops by insect invasion. In order to prevent this, farmers commonly use insecticides. However, using these insecticides also cause resistance development at the insects through those insecticides.

Helicoverpa armigera is a major pest of the cotton, maize, sorghum, pigeon pea, chickpea, soybean, groundnut, sunflower, and a range of vegetables. It occurs in Africa, Asia, southern Europe and Australia. Its polyphagous nature, their wide geographic range and their ability to adapt to diverse cropping systems have contributed to this pest status. *H.armigera* has developed resistance all of the insecticides that have been deployed against it in any quantity. Although pyrethroids are known as not any side effect on human health, common and excessive usage of these insecticides has caused resistance development in the field populations of the *H.armigera*. For lowering the rate of the resistance development synthetic pyrethroids like λ-Cyhalothrin has been commonly used against *H.armigera*, recently. It has been thought that one of the factors that cause to develop this resistance in *H.armigera* is the function of the detoxification enzymes like glutathione S-transferases.

1.1. Taxonomy of *Helicoverpa armigera*

Family: Noctuidae

Sub-family: Heliothinae

Species: *Helicoverpa armigera*

Common Name(s): Corn Ear Worm, Tomato Grub or Worm, Cotton Bollworm, Bollworm, Common Bollworm, Bean Pod Borer, Lucerne Budworm, Flower Caterpillar, Climbing Cutworm and Heliothis Worm or Grub.

Synonyms and changes in combination: The moth currently called *H. armigera* has most commonly been referred to in the past as: *Bombyx obsoleta F.*, *H. obsoleta F.* and *Noctua barbara*. It has also been reported as *Heliothis armigera* and *Heliothis armiger* (Zalucki *et al.*, 1986).

1.1.1. Life Cycle of Helicoverpa armigera

In more temperate regions of *H. armigera*'s range, it completes 2½ generations per year with pupae over-wintering in the soil (**Figure 1.1**). In more tropical areas, this species may continue to be active throughout the year with multiple overlapping generations. During its lifetime, a single female may oviposit from 500 to 3,000 eggs, averaging close to 1,000. As many as 1,500 eggs may be laid by a female over a 14 day period, with peak lying at about 7 days. Eggs hatch after only three days at 25 ºC (9 days at 17ºC). The eggs hatch in 3 to 7 days in warm weather, and larvae are mature after 2 to 3 weeks. Fully developed larvae move to the soil where they form an earthen cell 2-10 cm below the surface. When the chamber is dug, an exit tunnel is also dug, to ensure the emerging adults can escape **(Figure 1.2)**. The pupal period generally lasts from 8-21 days depending on temperature. Diapausing pupae can over-winter in the soil in more temperate areas for long periods (> 175 days). In tropical areas, diapauses

can be induced by drought. Adults, that are mainly nocturnal in their mating and egg laying activities, can live as long as 2-3 weeks (Jallow and Zalucki, 1998).

H. armigera is highly migratory, and can fly long distances (Fitt, 1989). It tends to over-winter as pupae in the soil of late-planted summer crops and is therefore considered to remain in the local cropping area. Moth emergence from these over-wintering pupae often begins between September and October in the southern spring and may take several generations to build to high numbers. Crop damage by *H. armigera* is therefore most common during the later parts of summer. The ecology of this species is responsible for it being a predominantly late summer pest. The localized activity of *H. armigera* within cropping regions is also thought to contribute to its ability to readily build resistance to insecticides. The larvae can, however, be difficult to kill with insecticides, even if susceptible. Once they shelter in plant structures or plant tissue, they are difficult with insecticides (Deuter *et al.*, 2000).

In general, *Helicoverpa* species have been preferentially feeding on buds, flowers and fruits. However, larvae may not always be able to feed on the plant structure on which they hatched, or be able to continue feeding once they have started, if plants respond to feeding by mobilizing secondary metabolites. The preference for fruiting structures and the tendency to move from one fruit to another, often without consuming each fruit completely, is the main reason why extensive damage often results to crops even when the number of large larvae is relatively low (Zalucki *et al.*, 1986).

In Turkey, *H. armigera* is usually found at the Marmara, Aegean, Mediterranean, Black Sea, Central Anatolia and Southeastern Anatolia region. Especially in summer, the larvae of them mature between 10-15 days at Çukurova. Fully developed larvae move to the soil where they form an earthen cell 1-6 cm below the surface. The pupal period generally lasts from 9-12 days in summer. It completes 5 generations per year, third of which are on cotton. The plants, first generation of *H. armigera* develop on and last generation of it fed on,

which form earthen cell, are important host plants for *H. armigera* ecology. (Zirai Mücadele Teknik Talimatı, 1995)

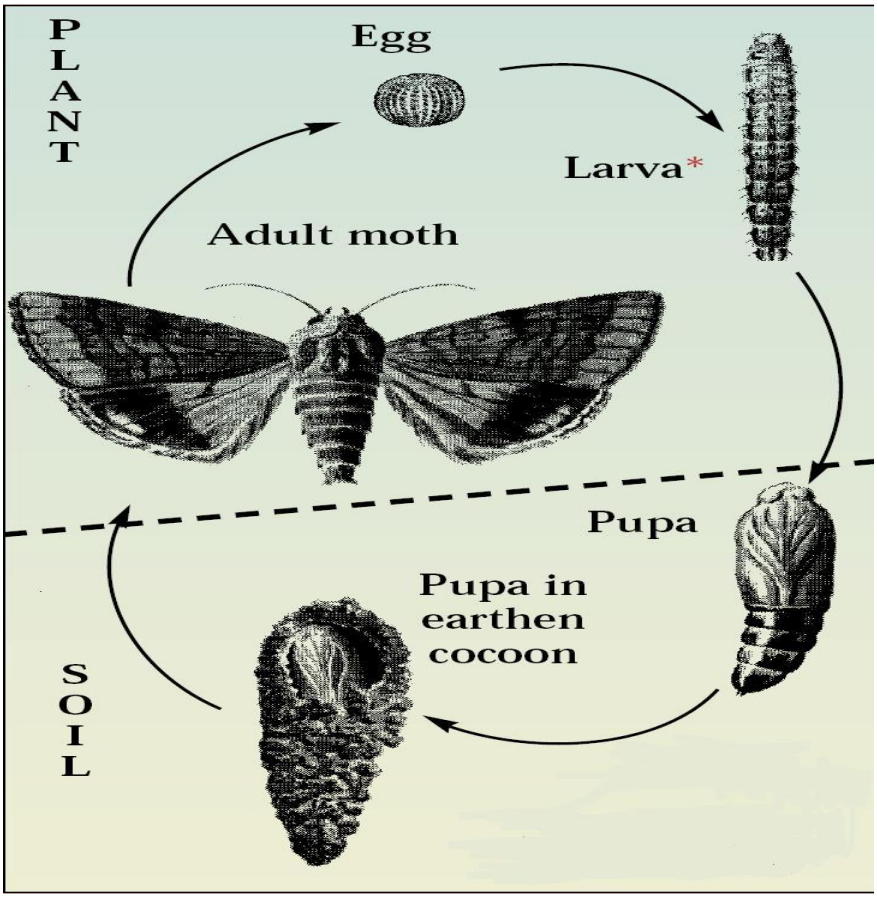

Figure 1.1. Life Cycle of *Helicoverpa armigera* (I.M.P.G., 2000)

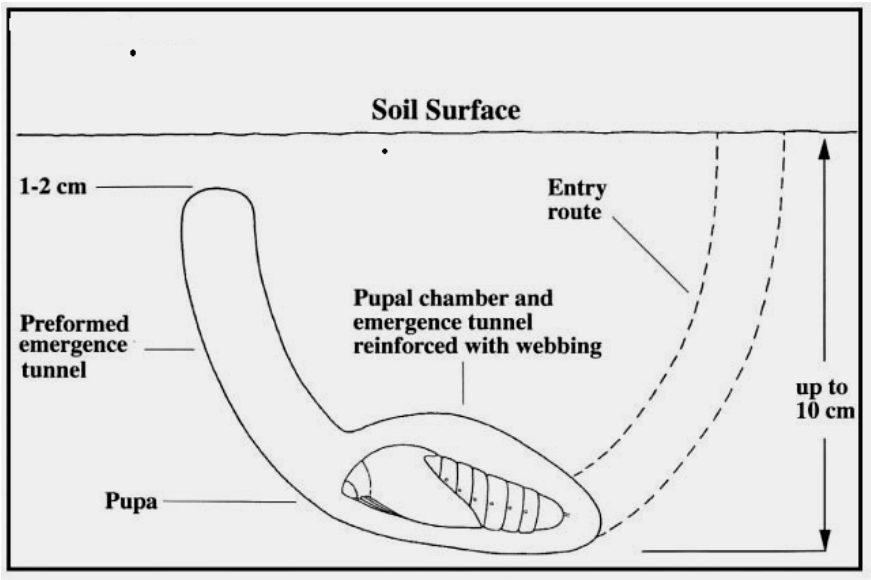

Figure 1.2. *Helicoverpa armigera* Pupa in Soil (Cotton CRC information and identification guide)

1.1.2. Morphology of *Helicoverpa armigera*

1.1.2.1. Morphology of *Helicoverpa armigera* Egg

Eggs are dome-like with a ribbed surface. The eggs are small (approximately 0.5 mm in diameter; about half the size of a pinhead) and sub-spherical (dome shaped with a slightly flattened bottom) in shape. Eggs are usually laid singly, making detection difficult near buds, flowers, fruits, or on leafy plant parts. They are initially pale green, sometimes with black dots, and they later change to cream and then brown (CPC, 2002; Deuter *et al.*, 2000; DEFRA, 2001; Zalucki *et al.*, 1986).

1.1.2.2. Morphology of *Helicoverpa armigera* Larva

Early instars are predominantly green, and appear spotted because of dark spiracles and tubercle bases. Larvae pass through four, five, or sometimes even

six instars, and ultimately reach 30 to 40 mm in length, and they usually display striped patterns and may vary in color from light green to brown to black and have distinct hairs when held up to the light **(Figure 1.3)**. There is a good deal of color variation in the larvae. For example, larvae may have white, instead of black spots. Superimposed on the dorsal bands are numerous lighter longitudinal lines, which are wrinkled or wavy. There are often dark, raised spots on the back, at the base of fine hairs. In *H. armigera*, there is a dark triangular area on the back of the first abdominal segment of the third, fourth and fifth instars of the larvae. Larvae have a posture when disturbed characteristic of a number of species in this family: it lifts its head and curls it under the front of the body. If even more disturbed, it lets go and drops, rolling into a spiral (CPC, 2002; Deuter *et al.*, 2000; DEFRA, 2001; Zalucki *et al.*, 1986).

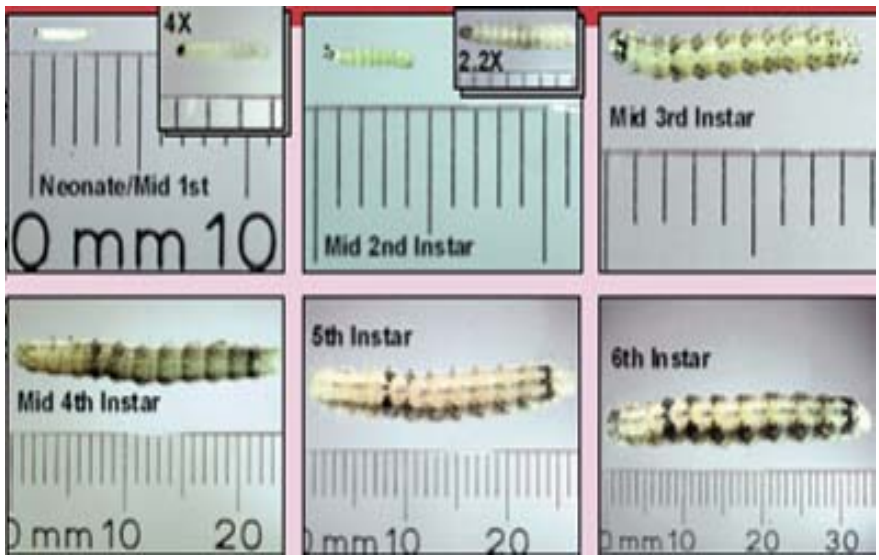

Figure 1.3. *Helicoverpa armigera* Larval Stages (I.M.P.G., 2000)

1.1.2.3. Morphology of *Helicoverpa armigera* Pupa

Pupae are 14-20 mm long, pale brown in color with a tinge of green, turning darker brown as the adult develops within. Pupae can be separated reliably by measuring the distance between the outer edges of the cremaster spines at the junction with the cremaster (> 0-22 mm, *H. armigera*; <0-20 mm, *H. punctigera*) (CPC, 2002; Deuter *et al.*, 2000; DEFRA, 2001; Zalucki *et al.*, 1986).

1.1.2.4. Morphology of *Helicoverpa armigera* Adult

Stout-bodied moth of typical noctuid appearance, with 3.5-4 cm wing-span; broad across the thorax and then tapering, 14-18 mm long; color variable, but male usually greenish-grey and female orange-brown. Forewings have a line of seven to eight blackish spots on the margin and a broad, irregular, transverse brown band. Hind wings are pale-straw color with a broad dark-brown border that contains a paler patch; they have yellowish margins and strongly marked veins and a dark, comma-shaped marking in the middle. Antennae are covered with fine hairs (Garcia-Tejero, 1957; Hardwick, 1965; Cayrol, 1972; Delatte, 1973).

1.1.3. Destruction Types of *Helicoverpa armigera*

1.1.3.1. Destruction Types of *Helicoverpa armigera* on Cotton

Bore holes are visible at the base of flower buds, the latter being hollowed out. Bracteoles are spread out and curled downwards. Leaves and shoots may also be consumed by larvae. Larger larvae bore into maturing green bolls; young bolls fall after larval damage. Adults lay fewer eggs on smooth-leaved varieties.

1.1.3.2. Destruction Types of *Helicoverpa armigera* on Tomatoes

Young fruits are invaded and fall; larger larvae may bore into older fruits. Secondary infections by other organisms lead to rotting. Eggs are laid on the silks,

larvae invade the cobs and developing grain is consumed. Secondary bacterial infections are common.

1.1.3.3. Destruction Types of *Helicoverpa armigera* on Sorghum

Larvae feed on the developing grain, hiding inside the head during the daytime.

1.1.3.4. Destruction Types of *Helicoverpa armigera* on Chickpea

Foliage, sometimes entire small plants are consumed; larger larvae bore into pods and consume developing seed.

1.1.3.5. Destruction Types of *Helicoverpa armigera* on Pigeon pea

Flower buds and flowers bored by small larvae may drop; larger larvae bore into locules of pods and consume developing seed. Short duration and determinate varieties are subject to greater damage.

1.1.3.6. Destruction Types of *Helicoverpa armigera* on Groundnut

Leaves, sometimes flowers attacked by larvae; severe infestations cause defoliation.

1.1.4. Negative Effect of *Helicoverpa armigera* on Australian Economy

Since its larvae of *H. armigera* is the nearly omnivorous that cause the damage; this is a result of them feeding mostly on flower buds, flowers, developing seed, fruits and leaves so it is one of the most important economic pests of most crops. But in particular, they may devastate corn, cotton, maize, grain legumes and oilseeds. For example, it costs Australian agriculture $AUS225 million per annum. To deal with this species, growers estimated the cost of insect control on cotton at roughly $30/ha in 1966 and this increased rapidly to more than $800/ha in 1998. It was estimated in 1991 that in the preceding decade, Helicoverpa species alone had accounted for a yield reduction of 7% in

Queensland cotton crops despite expenditure of about AUS$7.5 million on control (Sequeira, 2001).

1.2. Pyrethroid Insecticides

Pyrethroids are a partially refined extract of the chrysanthemum flower. Pyrethrum is prepared by first drying, then crushing the flowers of Chrysanthemum cinerariaefolium into a powder. The extract prepared by solvent extraction is pyrethrin. The discovery of photo stable synthetic pyrethroids in 1977 nurtured a profound increase in agricultural use of this insecticide (Elliott, 1977; Sattelle and Yamamoto, 1988). These formulations were most attractive given their effectiveness, absence of persistent residues following decomposition in the environment, and their relatively low mammalian toxicity (Sattelle and Yamamoto, 1988). The difference in toxicity between mammals and insects lies, not only in quantitatively more sodium channel sensitivity in insects, (Narahgashi *et al.*, 1998) but also by augmented metabolic detoxification in mammals by enzymatic action (Casida, 1997).

When given orally, pyrethroids are hydrolyzed by hepatic microsomal enzyme, pyrethroid carboxyl esterase, which eliminates the pesticide and its metabolites almost completely from the body in 2 to 4 days (Ruzo, 1978). This rapid hydrolysis in mammalian liver prevents the Central Nerve System (CNS) effects that kill insects. Although natural pyrethrins are useful, they have been surpassed in terms of effectiveness and diversity of applications by synthetic analogues (Picollo, 1998).

1.2.1. Lambda-cyhalothrin

Lambda–cyhalothrin is a pyrethroid insecticide registered by the U.S. Environmental Protection Agency (EPA) in 1988 (EPA, 1988). Pyrethroids are synthetic chemicals that are structurally similar to the natural insecticides pyrethrins. Scientists developed pyrethroid insecticides to have enhanced

biological activity and desired physical and chemical properties relative to pyrethrins. Pyrethroids affect the nervous system of an organism. They act by disrupting the gating mechanism of sodium channels that are involved in the generation and conduction of nerve impulses. They also disrupt the sodium channel activation gate by keeping it in the open position. Delayed closing of the gate results in prolonged excitation of nerve fibers (WHO, 1990). Lambda–cyhalothrin is similar to the pyrethroid cyhalothrin. Cyhalothrin is a mixture of four isomers, and two of these isomers compose lambda–cyhalothrin (WHO, 1990; Fed. Regist, 1998). Due to their similarity, researchers sometimes use toxicity tests conducted with evaluate the toxicity of lambda–cyhalothrin (Fed. Regist, 1998). It is a colorless to beige solid that has a mild odor. It has low water solubility (5x 10^{-3} mg/L) and is nonvolatile with vapor pressure of 1.5 x 10^{-9} mmHg at 20 °C (WHO, 1990; Tomlin, 1997). Lambda–cyhalothrin causes rapid paralysis and death to an insect when ingested or exposed externally (A world Compendium, 1997). Temperature influences insect paralysis and the toxicity of it (Toth. *et al.*, 1990).

In laboratory studies, lambda–cyhalothrin hydrolyzed in water (pH 9) with a half life of approximately 7 days. No hydrolysis occurred in water at lower pH values (pH 5 and 7) (WHO, 1990). The half-life of it on plant surfaces is 5 days (GLEAMS, 1993). The low water solubility and high binding affinity of it indicates a low potential to contaminate ground water (Vogue *et al.*, 1994).

Lambda-cyhalothrin is highly to moderately toxic when ingested. The acute oral lethal dose 50 (LD50) in rats is 79 mg/kg for males and 56 mg/kg for females. In mice, the acute oral LD50 is 19.9 mg/kg. It is moderately toxic when applied to the skin. The acute dermal LD50 in rats is 632 mg/kg for males and 696 mg/kg for females. In skin irritation studies, lambda-cyhalothrin causes no skin irritation in rabbits. The EPA classifies it as very low in toxicity for skin effects. It also causes mild eye irritation in rabbits. The U.S. EPA categorizes it as moderately toxic for eye effects. In studies guinea pigs, it did not cause skin

sensitization (WHO, 1990; Tomlin, 1998). In a 4-hour inhalation study with a lambda-cyhalothrin product, the lethal concentration 50 (LC50) ranged from 0.315 to 0.175 mg/L, indicating moderate toxicity (EPA, 1988).

1.3. Resistance to Pyrethroids

This was observed in the field soon after widespread use of the photo stable synthetic pyrethroids. A major contributory factor is that pyrethroids exhibit cross-resistance to DDT, which is thought to act at the same binding site on the voltage-gated sodium channel. The two main resistance mechanisms observed in the field are increased detoxification and target-site insensivity. These mechanisms can occur singly or together. For example, target-site insensitivity was thought to be the main resistance mechanism when resistance to pyrethroids was first reported in Australian *H. armigera*, but metabolic resistance is now implicated as the major mechanism. In Indian *H. armigera*, site-insensitivity is considered to be an additional mechanism (Kranthi *et al*., 2001).

1.3.1. Increased Metabolism

This is manifested in two main forms: overproduction of esterases (that can sequester and/or hydrolyze pyrethroids) and increased mixed-function oxidize activity. Since these are general mechanisms, it not surprising that cross resistance to other classes (*e.g*. organophosphates and carbamates) has been observed in the field. However, metabolic degradation can be minimized in general by the use of synergists and in particular by incorporation of appropriate structural features within the pyrethroid molecule. Potential for this approach has been demonstrated in the case of *H. armigera* (Forrester *et al.,* 1993a). The occurrence of negative cross-resistance for some classes of insecticides towards insects resistant to pyrethroids offers a possibility not observed frequently for the other main classes of insecticide. For example, chlorfenapyr is a proinsecticide that is activated within insects by the action of mixed function oxidizes (Brooke *et al.*, 2001).

Another example is indoxacarb, likewise activated but by esterase. These insecticides, therefore, are more effective against pyrethroid-resistant insects than against the susceptible strains. The practical value of this phenomenon has been demonstrated in the control of *H. armigera* (Gunning and Devonshire, 2001).

1.3.2. Site Insensitivity

This form of resistance occurs as a consequence of mutations in the amino acid sequence of the voltage-gated sodium channel. Changes have been shown to occur at (or close by) positions 1014 (for kdr) and 918 (super-kdr) (Williamson *et al.*, 1996). The level of resistance due to the former (kdr mutation) alone is generally similar for all pyrethroid molecules (20–50 fold). In contrast, it is much higher (up to 500 fold) for the super-kdr mutation (methionine to threonine in houseflies). The importance of a methionine residue at position 918 for sensitivity to pyrethroids has recently been demonstrated by Vais *et al.,* (2000). They observed a significant increase in the sensitivity of mammalian sodium channels to pyrethroids when the isoleucine residue at the position equivalent to 918 in houseflies was replaced with methionine. In contrast to the kdr mutation, the levels of resistance observed for the super kdr mutations are closely linked to the structure of the pyrethroid molecule (Farnham and Khambay, 1995a, 1995b; Beddie *et al.*, 1996). Thus, there is scope for the identification and development of pyrethroids that retain high efficacy against the pest but exhibit relatively low levels of resistance. The kdr and super kdr mutations can occur singly or in combination. For example, in houseflies and Plutella xylostella (diamond-back moth), both mutations are found but in Aphis gossypii only the super kdr mutation is found. Recent studies have indicated different combinations of these mutations can confer variable levels of resistance (Vais *et al.*, 2001).

1.3.3. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides around the world

1.3.3.1. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in Australia

Before the introduction of pyrethroids in 1977 in Australia, *H. armigera* had developed severe resistance to DDT in the Ord River Valley (Wilson, 1974), New South Wales (Goodyer *et al.*, 1975; Goodyer and Greenup, 1980) and Queensland (Kay, 1977). Resistance to endosulfan (Kay, 1977; Kay *et al.*, 1983; Gunning and Easton, 1994), Ops (Goodyer and Greenup, 1980; Kay *et al.*, 1983) and carbamates (Gunning *et al.*, 1992) was also known to be present. Resistance to pyrethroids first appeared in 1983 (Gunning *et al.,* 1984), and immediately a resistance management strategy was implemented, which restricted the use of pyrethroids to a 42-day window during January February (from 1990 they were restricted to a 35-day window) (Forrester, 1990; Forrester *et al.*, 1993a). Endosulfan use was also limited. An effective weekly monitoring scheme based on survival of fourth-instar larvae of *H. armigera* after treatment with a diagnostic dose of fenvalerate was initiated and much data accumulated on the effects of selection and survival of resistant individuals. Later monitoring also determined the likely presence of a metabolic resistance based on enhanced monooxygenase activity by treating larvae with both fenvalerate and the metabolic inhibitor piperonyl butoxide (PBO). Based on these results, PBO could be added to the last of the three (maximum) sprays in the pyrethroid window. This strategy undoubtedly held pyrethroid resistance in check for a number of years although there appeared to be a steady rise in the proportion of the population that was resistant to pyrethroids (Forrester *et al.*, 1993a). *H. armigera* in unsprayed refugia readily became contaminated with resistant individuals (Gunning and Easton, 1989; Forrester *et al.*, 1993a), and similar levels of resistance were found in other crops, such as maize (Glenn *et al.*, 1994). This gradual loss of pyrethroid efficacy together with the development of an immunodiagnostic to distinguish the eggs of

H. armigera from those of *H. punctigera*, the use of *Bacillus thuringiensis* (Bt) and other insecticides and the advent of Bt-transgenic cotton led to a complete reorganization of the strategy and a relaxation on the use of the now less useful pyrethroids. The situation is continuing to deteriorate, with resistance to pyrethroids increasing steadily.

1.3.3.2. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in New Zealand

A programme to monitor resistance to fenvalerate in *H. armigera* was initiated in 1991 in tomato, maize and lucerne crops in New Zealand. A significant trend of declining mortality from 1992 to 1994 was seen and this suggests an increase in the frequency of resistance to the pyrethroids (Cameron *et al.*, 1995; Suckling, 1996). Management strategies have been devised to counter this problem (Suckling, 1996).

1.3.3.3. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in Thailand

Wangboonkong (1981) first reported inadequate control of *H. armigera* in Thailand soon after the introduction of pyrethroids, but it was not known whether resistance was the cause. Significant resistance to pyrethroids was found in populations of *H. armigera* from the Tak Fa area of Nakonsawan in Thailand in 1985 (Ahmad and McCaffery, 1988). These insects were also resistant to DDT and carbaryl. Pyrethroid resistance was again noted in Thai populations of the insect by Ernst and Dittrich (1992).

1.3.3.4. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in Indonesia

After the introduction of pyrethroids in the 1980s, resistance to was found in populations of *H. armigera* collected from the cotton-growing areas of South

Sulawesi, Indonesia, in 1987 and early 1988 (McCaffery *et al.*, 1991a). These populations were also resistant to endosulfan and DDT.

1.3.3.5. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in China

Almost all groups of conventional insecticides have been used to control *H. armigera* in China. DDT resistance was first detected in *H. armigera* in Henan province (Anon, 1974), and subsequently in Jiangsu and Hebie provinces (Zhu *et al.*, 1982), together with resistance to carbaryl. Pyrethroids such as fenvalerate and deltamethrin have been widely used since 1983 with others such as cyhalothrin, cypermethrin, esfenvalerate, fenpropathrin and cyfluthrin being used from the mid- to late-1980s.There were no substantial changes in susceptibility until around 1989, but in the following years resistance to pyrethroids was widely detected in a number of areas including Jiangsu, Henan and Shandong provinces (Tan *et al.*, 1987; Shen *et al.*, 1991, 1992, 1993; Wu *et al.*, 1996, 1997b). The development of this resistance led to calls for a resistance management strategy to restrict pyrethroid use, to promote greater emphasis on the use of alternations with other insecticides and to promote the use of biological control (Shen *et al.*, 1992). Although levels of resistance to pyrethroids are still high, recent lower populations have alleviated the problem to some degree.

1.3.3.6. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in India

Pyrethroid insecticides were first used in India in 1980 for the control of a number of pests, including *H. armigera*. In 1987 resistance to pyrethroids was first noted in India in Andhra Pradesh (Dhingra *et al.*, 1988; McCaffery *et al.*, 1988, 1989; Phokela *et al.*, 1989a, 1989b) in populations that were also resistant to DDT and slightly resistant to endosulfan (McCaffery *et al.*, 1989). Numerous other studies confirmed the high incidence of pyrethroid resistance, especially in the cotton- and pulse-growing regions of central and southern India, and also

confirmed its gradual spread to other regions of the country (see, for example, Phokela *et al.*, 1990; Mehrohtra and Phokela, 1992; Armes *et al.*, 1992, 1996; Sekhar *et al.*,1996; Jadhav and Armes, 1996). Pyrethroid resistance has recently been found in the Punjab close to populations over the border in Pakistan, leading Armes *et al.*, (1996) to the conclusion that pyrethroid resistance is ubiquitous in *H. armigera* in the Indian subcontinent. Resistance to pyrethroids is frequently accompanied by resistance to endosulfan, to OPs such as quinalphos and monocrotophos, and to the oxime carbamate methomyl (Armes *et al.*, 1992, 1996).

1.3.3.7. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in Pakistan

As a result of pyrethroid use since the early 1980s, moderate to high levels of resistance to pyrethroids were found in populations of *H. armigera* collected from various regions of Pakistan from 1991 onwards (Ahmad *et al.*, 1995). These insects were also resistant to the OP monocrotophos, showed moderate resistance to endosulfan and had low-level resistance to the OPs chlorpyrifos and profenofos and the carbamate thiodicarb. Interestingly, in a subsequent study these authors showed variations in resistance to pyrethroids depending on their structure. Although resistance varied from location to location, the general trend was for moderate to high resistance to chemicals like cypermethrin, a low-to-moderate resistance to compounds like deltamethrin and comparatively low resistance to others like lambda-cyhalothrin (Ahmad *et al.*, 1997). With the loss of efficacy of the pyrethroids farmers have begun to use other non-pyrethroid compounds, with the result that levels of pyrethroid resistance were lower in 1997 than in previous years (Ahmad, 1998).

1.3.3.8. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticides in Israel

Since 1987 a strictly observed insecticide resistance management strategy has been in place in cotton fields in Israel. This is designed to maintain susceptibility to a range of insecticides, including pyrethroids, in *H. armigera* and other cotton pests. Monitoring studies show that, despite slight fluctuations during the season, susceptibility to cypermethrin did not alter during the period 1987 1991 (Horowitz *et al.*, 1993); control continued to be achieved despite a very marked decline in the number of sprays applied (Horowitz *et al.*, 1995).

1.3.3.9. Resistance of *Helicoverpa armigera* to Pyrethroid Insecticide in Turkey

Resistance to synthetic pyrethroids was found in populations of *H. armigera* in 1984, after their initial use around 1980 (Anon, 1986). Similar findings were reported by Ernst and Dittrich (1992). Uğurlu (2001) found that there was resistance development at field populations of *H. armigera* through synthetic pyrethroids lambda-cyhalothrin 20-41 folds and tralomethrin 15-24 folds compared to susceptible population.

1.4. Glutathione S-Transferases Enzyme Family

The Glutathione S-Transferases (GSTs) (EC.2.5.1.18) are enzymes that participate in cellular detoxification of endogenous as well as foreign electrophilic compounds.

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

GSTs as a part of detoxification systems are evolved to protect cells against reactive oxygen metabolites by conjugating the reactive molecules to the nucleophile scavenging tripeptide glutathione (GSH, γ-glu-cys-gly) (Coles and Ketterer, 1990). GSTs serve as transporters of potentially harmful substances out of the cell with different electrophilic species (Jemth and Mannervik., et *al.,* 1999). Following conjugation, these generally harmless GSH adducts, or their mercapturic metabolites, are secreted into the bile or urine.

GSH, first described in 1888 as philothion, is the most ubiquitous and abundant non-protein thiol in mammal cells and serves as a necessary nucleophile in a number of detoxification reactions (Tew, 1994). In addition to its role in intracellular detoxification, it participates in interconversions of arachidonic acid pathway metabolites (prostaglandins and leukotrienes) (Flatgaard *et al*., 1993) and contributes to regulation of protein and DNA synthesis (Rass, 1988) (**Figure 1.4**).

Maintenance of a homeostatic GSH content is achieved by both de novo synthesis and salvage synthesis and a number of interrelated pathways are also involved (**Figure 1.5).**

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

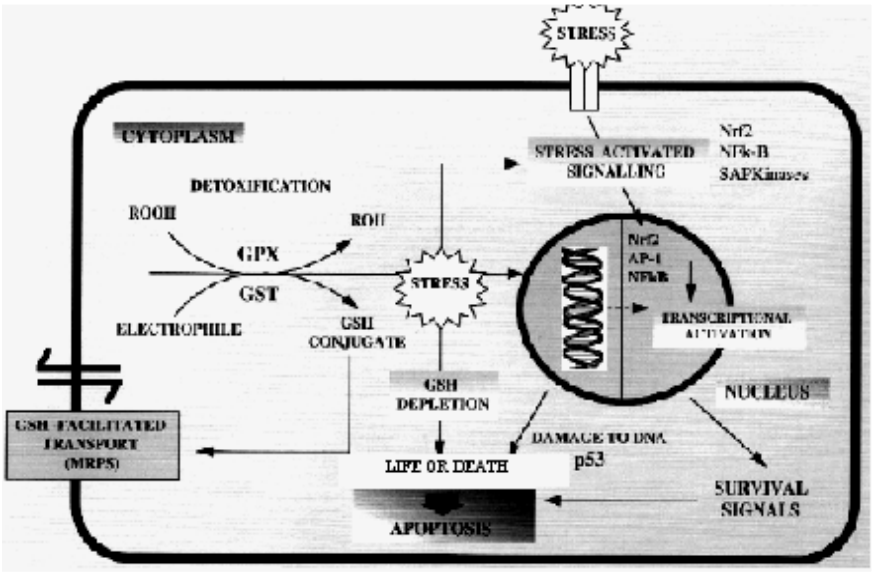

Figure 1.4. Mechanism for Cellular Protection by Glutathione (McLellan, 1999).

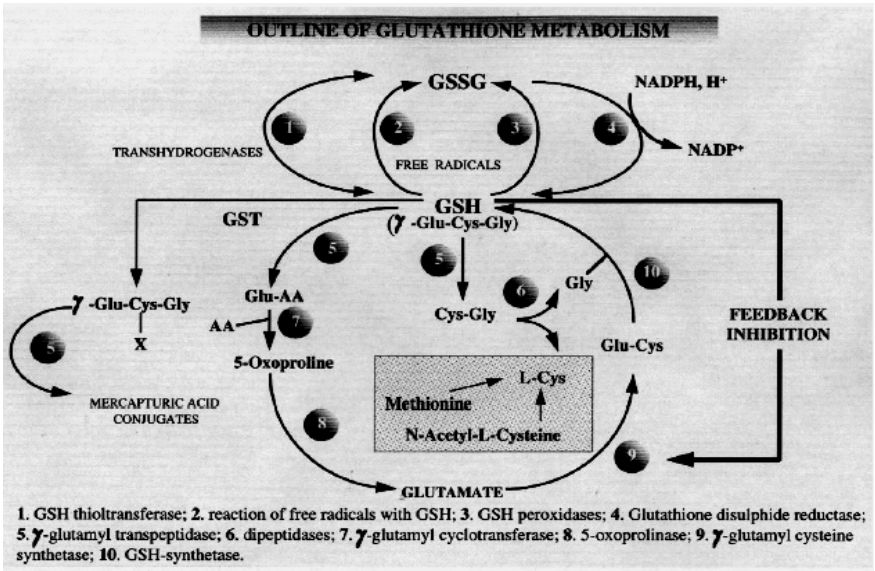

Figure 1.5. Glutathione metabolism (McLellan, 1999).

In those species so far investigated, soluble forms of GSTs are homo or heterodimers of different subunits with distinct substrate specificities (Mannervik and Jensson, 1982) having molecular weight from 20,000 to 25,000. Sequences and the known three-dimensional structures suggest that these proteins share a common ancestry, though the precise details of their evolution remain obscure. They are expressed at high levels in mammalian liver constituting up to 4% of the total soluble proteins (Eaton *et al.,*1999) and at least six distinct classes of soluble GSTs have been identified thus far: alpha (α), mu (μ), pi (π), sigma (σ), theta (θ), and zeta (δ). This classification is in accordance with the substrate specificity, chemical affinity, structure, amino acid sequence and kinetic behavior of the enzyme (Landi, 2000). The amino acid sequence identity within class is greater than 70%, whereas the interclass identity is usually less than 30% (Rossjohn *et al.,* 1998).

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

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

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

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

1.4.1. Nomenclature and Classification of Glutathione-S-Transferases

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

On the basis of sequence similarity, plant GSTs can be divided into four classes: phi, zeta, tau and theta (Edwards, 2000). The phi and tau classes are unique to plants and are relatively well characterized, being encoded by large gene families in all plant species studied to date (Dixon, 2000). In contrast, the theta and zeta GSTs are less well represented in plants and the presence of homologues in animals and fungi suggests common essential functions in all eukaryotes (Hayes and McLellan, 1999). Although the enzyme has been isolated from numerous animals and plant sources the most thoroughly studied group of isozymes are those purified from rat liver cytosol, in spite of the presence of the membrane bound forms of GSTs (Morgenstein and De Pierre, 1982) known as

kappa class GST (Pemble *et al.*, 1996), most of the studies were done with the soluble forms.

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

Several nomenclatures have been proposed for rat GST subunits over the years. The most widely used one was proposed by Jackoby et al., using Arabic numeral nomenclature. This system is of value because it is unambiguous and allows simple displaying of subunit combinations. But this system has disadvantage that it is not clear to show which gene family each subunit belongs. The most important advantage of this system is its usefulness to group GSTs by subfamily and immediate identification of subunits that will dimerize. A class-based subunit nomenclature has been proposed. In this system, subunits are grouped by gene family and then numbered according to their order of discovery; this system for defining GST was originally devised for the human transferases (Mannervik, 1992), but it is generally applicable. In this nomenclature system, single capital letter abbreviations are used to signify the alpha (A), the mu (M), the pi (P), the sigma (S), and the theta (T) classes, and Arabic numerals are employed for numbering each of the separate gene products; for example, the class alpha subunits are called A1, A2, A3, etc. The dimeric GST isoenzymes are represented by the single letter suffix (signifying class) followed by hyphenated Arabic numerals (signifying each of the two subunits). Hence the class alpha heterodimer formed between Ya1 (A1) and Yc1 (A3), are GST Ya1Yc1, is designated GSTA1-3 (Hayes and Pulford, 1995).

1.4.2. Functions and Structure of GSTs

1.4.2.1. Catalytic Activities of GSTs

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

GSH is synthesized within the cytosol and depleted in it by conjugation reactions and by the reaction of H_2O_2 and biologically generated radicals across the cell membrane. Oxidation of GSH results in the formation of glutathione disulphide (GSSG), but this is rapidly returned to the reduced state by glutathione reductase, thus maintaining the GSH: GSSG ratio at around 99:1 (**Figure 1.3**). The third group of GSH can participate in two main types of reaction involving either a one- or a two-electron transfer. These reactions allow GSH to perform key roles within a normal cell, including conservation of the redox status of a cell and participation in certain detoxification reactions (Kearns, 1998).

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

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

1.4.2.2. Structure of the GSTs

For all cytosolic GST classes representative crystal structures are available with the exception of the kappa class. Including those structures of the members of mammalian GSTs are from classes α [hGSTA1-A from human liver (Sinning and Armstrong, 1993); μ [rGSTM1-1 from rat liver (Ji, 1992); hGSTM2-2 from human muscle (Rahgunathan, 1994)]; π [pGSTP1-1 from pig lung (Reinemer, 1991); hGOTP1-1 from human placenta (Reinemer, 1992.)]; δ [s GSTS1-1 from squid digestive gland (Ji, 1995) and θ [from *Lucilia cuprina* (Wilce, 1995), from *Anabidopsis thaliana* (Reinemer, 1996), human (Rossjohn *et al.*, 1998) and a GST from *Schistosoma japanicum* (SjGST) (Lin, 1994), providing the structural basis for investigations of the enzyme active site. A comparison of some structures is provided in **Figure 1.6**.

Although there is low level of sequence identity across the classes, all the structures follow a similar folding, with each subunit consisting of two domains of different structure (**Figure 1.7**). Domain I, the N-terminal domain functions to provide the binding site for glutathione, called as G-site, and a Domain II contains essentially all of the H-site known as xenobiotic substrate binding domain. In addition, it has been also shown that the glutathione binding domain (G-site) is highly conserved (not identical) in all classes.

The N- terminal domain consists of 4β sheets with 3 flanking α-helices (**Figure 1.7**). This domain (approx. residues 1-80) adopts a topology similar to that of thioredoxin fold (Wilce, 1995). The fold consists of distinct N-terminal and C- terminal motifs which have β α β and β β α arrangement respectively and which are linked by α-helix (α-2 in **Figure 1.7**).

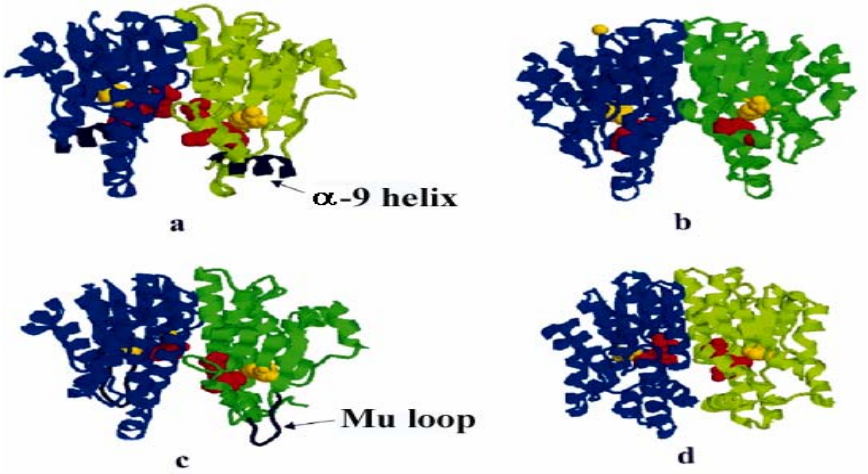

Figure 1.6. Mammalian GST structures (Sheehan, 2001)

Subunits are distinguished by colour (green and blue), and structures are represented to emphasize the relative arrangements around the active site of the right-hand subunit and the inter-subunit cleft. Catalytically essential tyrosine or serine residues are represented in space-filling mode and highlighted in yellow, while the ligand with which the enzyme was co-crystallized is shown in red, identifying the location of the active site. Class-specific features of the Alpha and Mu structures are shown in black. Protein database codes are given in parentheses: (a) human Alpha class (1GUH; [Sinning, 1993]); (b) human Pi class (1GSS; [Reinemer, 1992]); (c) rat Mu class (6GST; [Xiao, 1996]); (d) human Theta class (1LJR; [Rossjohn, 1998]). β α β begins with an N terminal β – strand (β -1), followed by an α- helix (α -1) and then a second β strand (β -2) which is parallel to β -1. A loop region leads into a second α-helix (α -2), which connects with the C terminal motif. This motif consists of two sequential β-strands (β-3 and β-4), which are antiparallel and which are followed by a third α helix (α-3) at the c terminus of the fold. The four sheets are in the same plane, with two helices (α-1 and α-3) below this plane and α-2 above it, facing the solvent. The loop

connecting α-2 and β-3 shows characteristic proline residue which is in the less favored cis conformation and is highly conserved in all species.

GSTs and known as the cis-pro loop. Although it does not play direct role in catalysis, this loop appears to be important in maintaining the protein in a catalytically competent structure. (Allocati *et al.*, 1999) In GSTs, domain I is highly conserved and provides most of the GSH binding site. It is connected to Domain by a short linker sequence (**Figure 1.8**).

Domain II (approx. residues 87-210) begins at the C terminus of the linker sequence and consists of five α helices in the case of pi and mu classes. (Ji, 1992; Reinemer, 1991) and six α helices in the case of the α class (Sinning *et al*., 1993). The number of helices in domain II varies widely between classes. The C terminal domain is less similar between the three mammalian classes than the N-terminal domain (**Figure 1.6** and **1.8**). (Dirr, 1994; Wilce, 1995). It contributes most of the residues that interact with the hydrophobic second substrate, as well as contributing a highly conserved aspartic acid residue (occurring in helix α -4) to the GSH binding site. Differences in the C terminal domain may be responsible for the differences in substrate specificity between the three classes (Wilce, 1995).

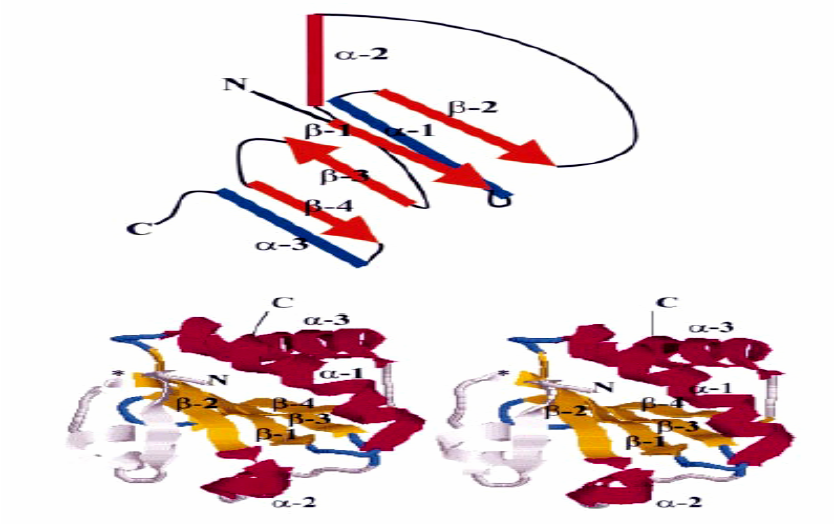

Figure 1.7. The thioredoxin fold (Sheehan, 2001)

A schematic diagram representing the thioredoxin fold is shown at **Figure 1.7** a RasMol depiction of the thioredoxin dimer (Katti, 1990). In the diagram, α-helices are shown as cylinders, while β-sheets are shown as orange arrows. The four β-sheets are essentially co-planar, with one helix (α-2) shown in red above this plane and the other two α-helices (α-1 and α-3) shown in blue below the plane. The cis-Pro loop links α-2 to β-3. In GSTs, domain 2 is connected to the C-terminus by a short linker peptide. In thioredoxin itself, β-sheets are coloured yellow, while α-helices are magenta. The thioredoxin fold has an extra β-sheet and α-helix at the N-terminus (residues 1±21) ending at the point denoted by * where the fold proper begins. These additional N-terminal features are coloured grey.

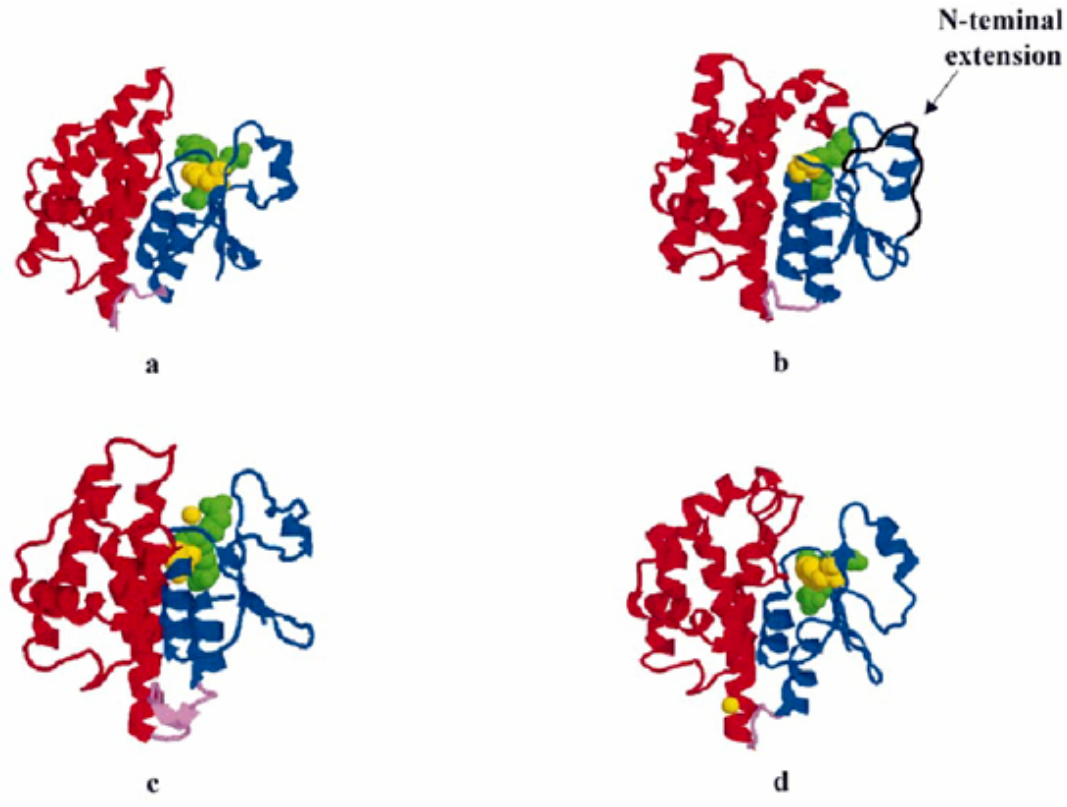

Figure 1.8. Domain structure of GST subunits (Sheehan 2001).

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

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

example during conjugation between CDNB and GSH (Widersten *et al.,* 1992; Kolm *et al.,* 1992).

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

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

This particular interaction is not observed in the class sigma and theta enzymes due to the absence of Phe residue and the hydrophobic socket between the helices 4 and 5 (Armstrong, 1997). The most conserved region of structure in all of the cytosolic enzymes is a core ββα motif that is responsible for recognition of the γ- glutamyl portion of the peptide (**Figure 1.9)**. The cis prolyl residue that precedes this region helps to conserve the overall fold of the domain. The only other highly conserved interaction is between the carbonyl and NH groups of the cysteinyl residue and the main chain of the protein just preceding the conserved cis-prolyl residue. The similarity in this region of structure for the two most divergent classes of cytosolic enzyme, theta and alpha, is obvious and much more highly conserved (**Figure 1.9)** than are the interactions between the proteins and sulphur of GSH (**Figure 1.10)**. The most fundamental difference among the GSH binding sites of the various enzyme classes involves the interaction of protein with the sulfur of the peptide (**Figure 1.10)**.

The theta class enzymes thought to be the evolutionary precursor of the alpha, mu, pi, and sigma class proteins, utilizes the hydroxyl group of a serine residue located near the N-terminus of the polypeptide to activate the sulphydryl

group of bound GSH. Whereas, the class alpha, mu, pi, and sigma enzymes have hydroxyl group of tyrosyl residue in a slightly different position to act as a hydrogen bond donor to the sulphur which lowers the pKa of the thiol in the E.GSH complex so that it is ionized at physiological pH. In class alpha additional residue except from seryl or tyrosyl group gathers additional stabilization from its positive charge of Arg15 (Armstrong, 1997).

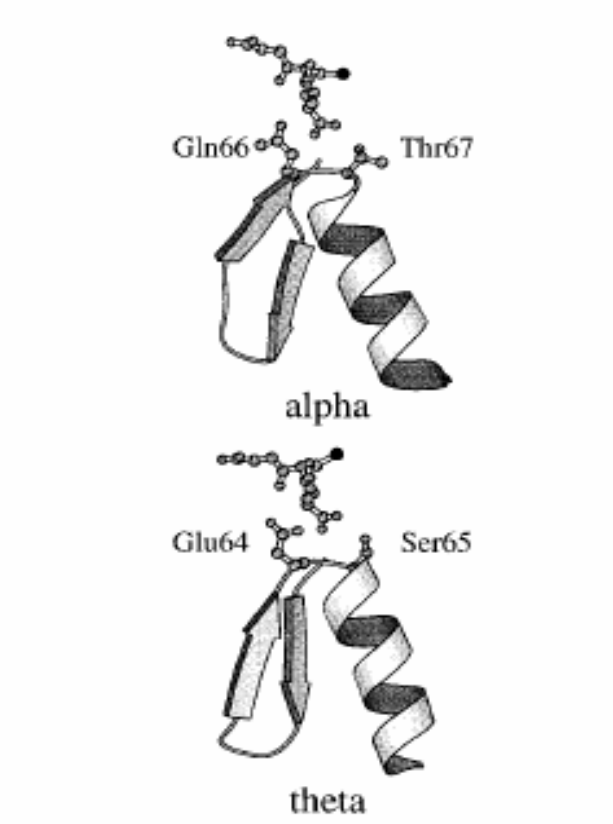

Figure 1.9. Representation of the highly conserved core ββα motif which is responsible for the recognition of the γ-glutamyl residue of GSH. Glutathione and the side chains located at the turn between the β4-strand and α3-helix involved in hydrogen-bonding interactions with the α-amino and α-carboxyl moieties of the γ-glutamyl residue are illustrated in ball-and-stick. The two motifs represented are from what are considered to be the most divergent classes of cytosolic GSH transferases, the theta and alpha classes (Armstrong, 1997)

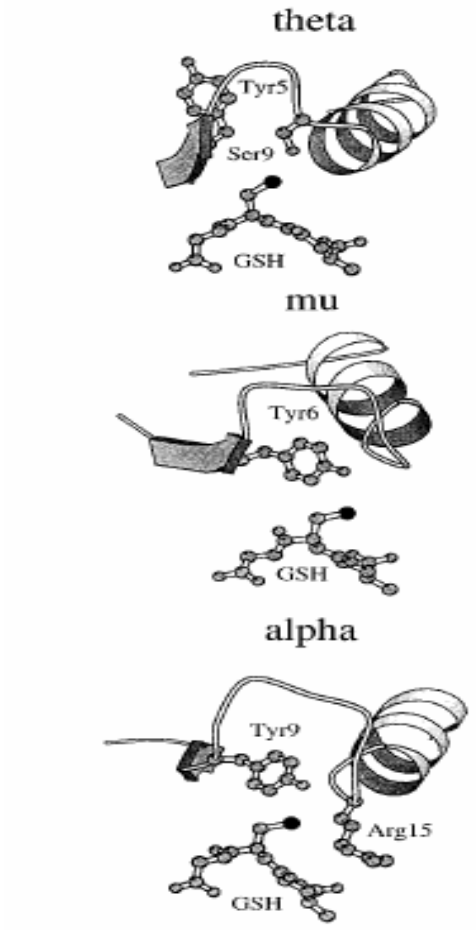

Figure 1.10. Evolution of first-sphere interactions between the active sites of class theta, mu, and alpha enzymes and the sulfur of GSH. It is possible that the tyrosine residue often, but not always, found near the N-terminus of the class theta enzymes was recruited in the evolution of the protein to the other classes. The most recently evolved enzyme, class alpha, has an additional residue (Arg15) conscripted into the first sphere of the sulfur of GSH (Armstrong, 1997).

Three membrane bound glutathione transferases are known, one of which appears to be involved in xenobiotic mechanism. Microsomal GSH transferase I is an integral membrane protein that has been characterized from both rats and humans where it is found in large amounts in liver and is distributed in both

microsomal and outer mitochondrial membrane. This protein has no relationship with any of the known cytosolic enzymes with respect to sequence. However, it shows some similarity to two other membrane bound GSH transferases, leukotrienes C_4 synthase and micosomal GSH transferase II, in which the three proteins are about the same size, share a small amount of sequence identity and are all membrane bound. There is no so much information about the xenobiotic mechanisms of microsomal GSTs.

A proposed membrane topology for the microsomal enzyme is illustrated in **Figure 1.11** (Armstrong, 1997). Each subunit consists of five membrane spanning regions, at least two of which are likely to be α-helices perpendicular to the membrane surface. The N-terminus of the enzyme appears to be located on the luminal side of the endoplasmic reticulum while the C- terminus and the active site face the cytosol. The overall topology is most simply described as an N terminal in, C-terminal out orientation with five membrane spanning regions. Although it is unclear what the GSH-binding motif looks like, the enzyme lowers the pKa of the thiol of bound GSH as in the case of all cytosolic GSTs.

1.4.3. GSTs Catalyzed Reactions and Substrates

1.4.3.1. Glutathione Conjugation and Detoxification

All GSTs have the ability to conjugate GSH with compounds containing an electrophilic center. The electrophilic functional group for conjugation reactions can be provided by a carbon, nitrogen, or a sulfur atom. Such groups are present in arene oxides, aliphatic and aromatic halides, α,β- unsaturated carbonyls, organic nitrate esters, organic thiocyanates, olefins, organic peroxides, quinines and sulfate esters (Mannervik, 1985; Mannervik and Danielson, 1988). The range of compounds that contain electrophilic center is extremely large and includes the parent chemical or metabolite of the carcinogens (**Table 1.1**) benzo[a]pyrene, 5-

methylcrysene, aflatoxin B1, 7,12-dimethybenz[a]anthracene, and 4-nitroquinoline-N-oxide (**Figure 1.10**).

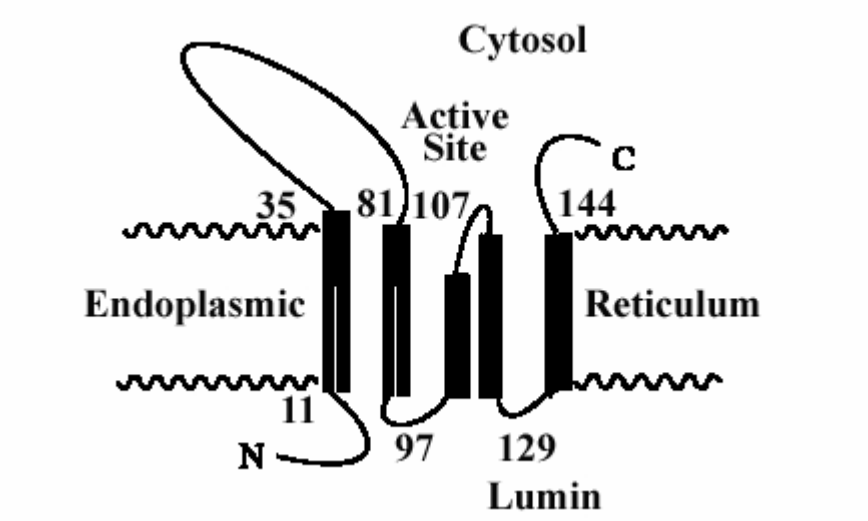

Figure 1.11. A proposed membrane topology for the microsomal GSH transferase subunit derived from sites of proteolysis, chemical modification and mutagenesis (Anderson, 1994a). Experimental evidence and hydropathy analysis indicate that there are at least three membrane spanning regions: residues 11-35, 81-97, and 129. Two of the membrane spanning regions are thought to be α-helices (Hebert, 1995).

Table 1.1. Examples of GSTs Substrates from Different Compound Categories (Hayes and Pulford, 1995)

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

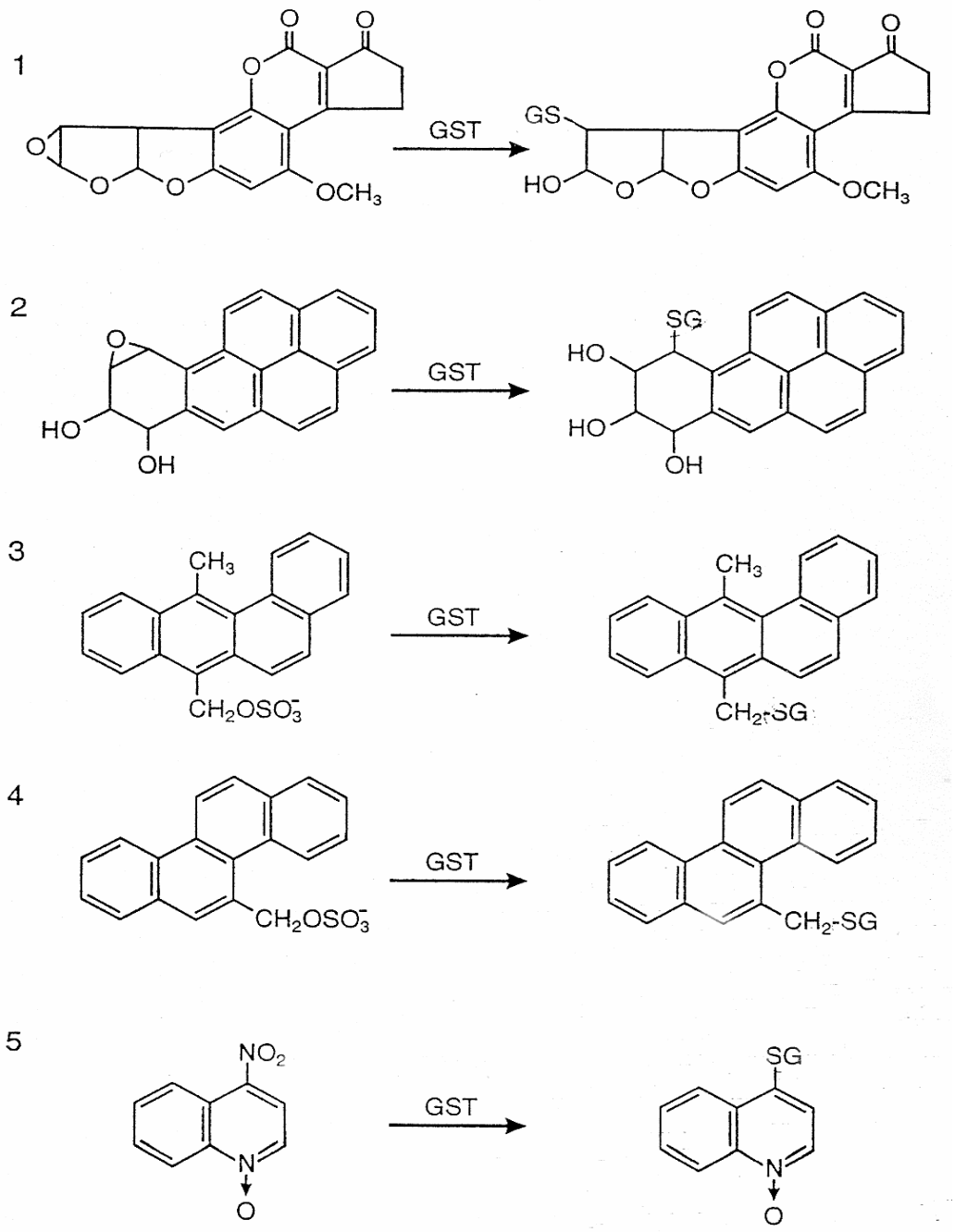

Figure 1.12. Detoxification of carcinogens by GSTs. The following reactions are catalyzed by GST: (1) aflatoxin B_1-8,9-epoxide; (2) benzo[a]pyrene-7,8-diol-9,10-oxide;(3) 7-hydroxymethylbenz[a]anthracene sulfate; (4) 5-hydroxymethyl-chrysene sulfate; (5) 4-nitroquinoline *N*-oxide (Hayes and Pulford, 1995)

GST also detoxifies the pesticides, alachlor, atrazine, dichloro-diphenyltrichloroethane (DDT), lindane, and methyl parathion (**Figure 1.13**), the oxidative-damage products acrolein, base propenals, cholesterol α-oxide, fatty acid hydroperoxides, and 4-hydroxynonenal (**Figure 1.14**), the anticancer drugs chlorambucil, melphalan, thiotepa, 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU), and cyclophosphamide, as well as the antibiotic fosfomycin (**Figure 1.15**).

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

The conjugation reaction between GSH and xenobiotics represents the first step in the synthesis of mercapturic acids, an important group of excretion products that were first identified more than 100 years ago in the urine of animals treated with bromobenzene. In that reaction, after the conjugation with GSH, the glutathione conjugate is converted to the corresponding cysteine conjugate following sequential removal of glutamate and glycine. Cysteine conjugate then is either metabolized to a mercapturate by acetylation or cleaved to a mercaptan by β-lyase (C-S lyase). In addition to the mercapturic acid pathway, methylation of the thiol to form the methylthio-containing metabolite and the glucuronidation of the mercaptan to form the thioglucuronide represent important metabolic steps for the biotransformation of the cysteine conjugate. (Boyland and Chasseaud, 1969; Pickett and Lu, 1989) (**Figure 1.16)**.

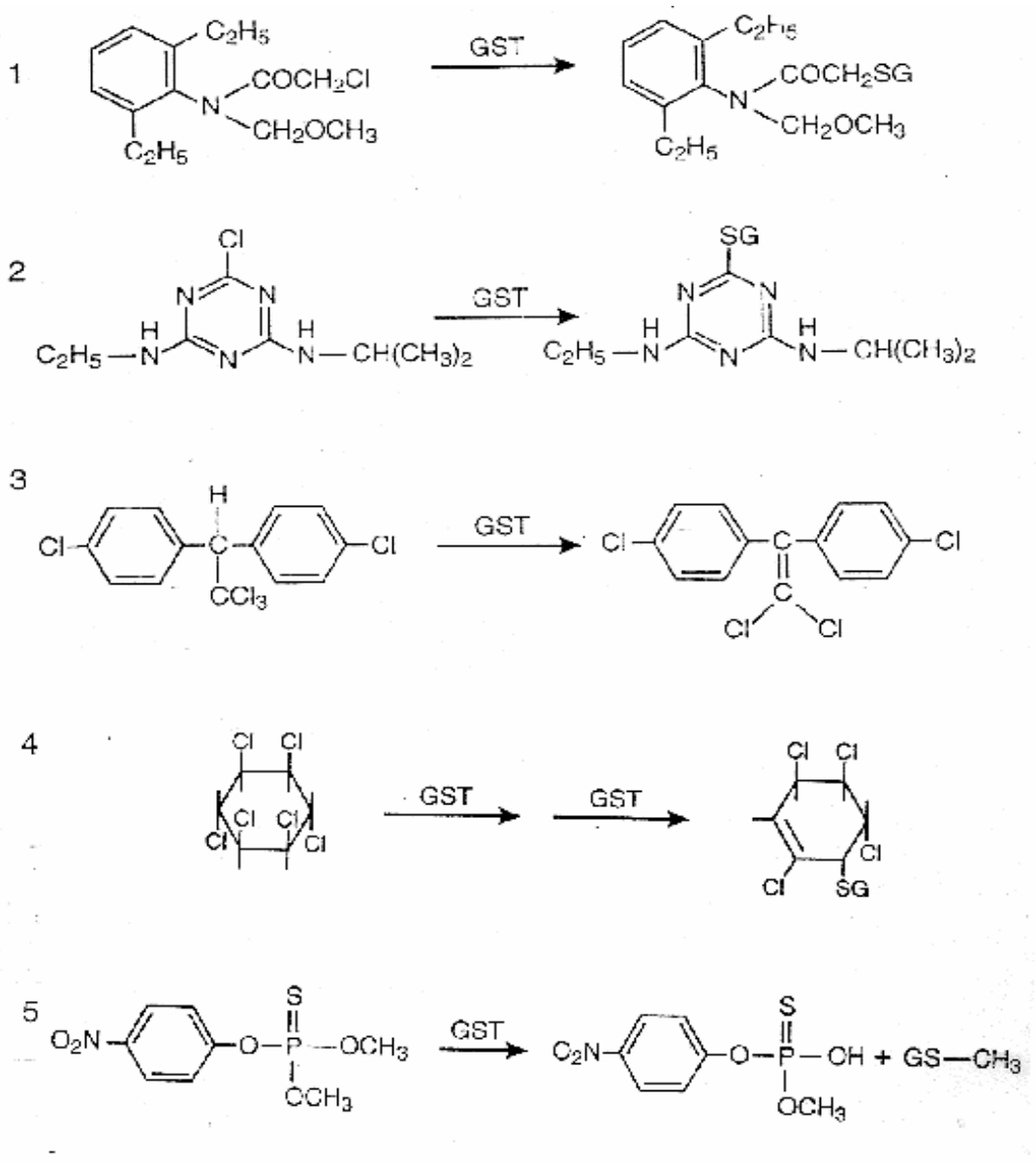

Figure 1.13. Metabolism of pesticides by GSTs: (1) alachlor; (2) atrazine; (3) DDT; (4) lindane; (5) methyl parathion (Hayes and Pulford, 1995).

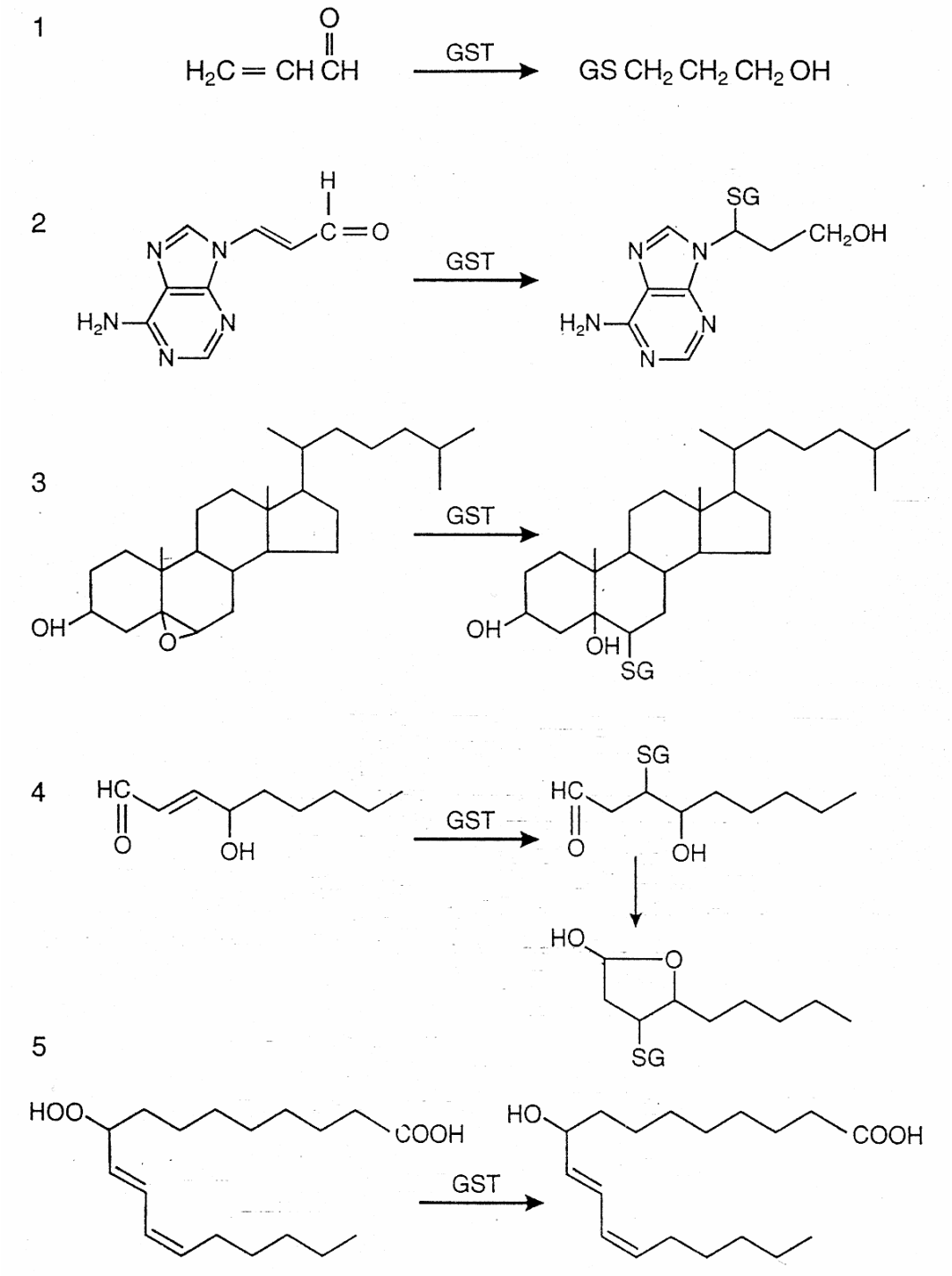

Figure 1.14. Examples of GST substrates that are produced by oxidative stress: (1) acrolein; (2) adenine propenal; (3) cholesterol-5, 6-oxide; (4) 4-hydroxynon-2-enal (5) 9-hydroperoxy-linoleic acid (Hayes and Pulford, 1995).

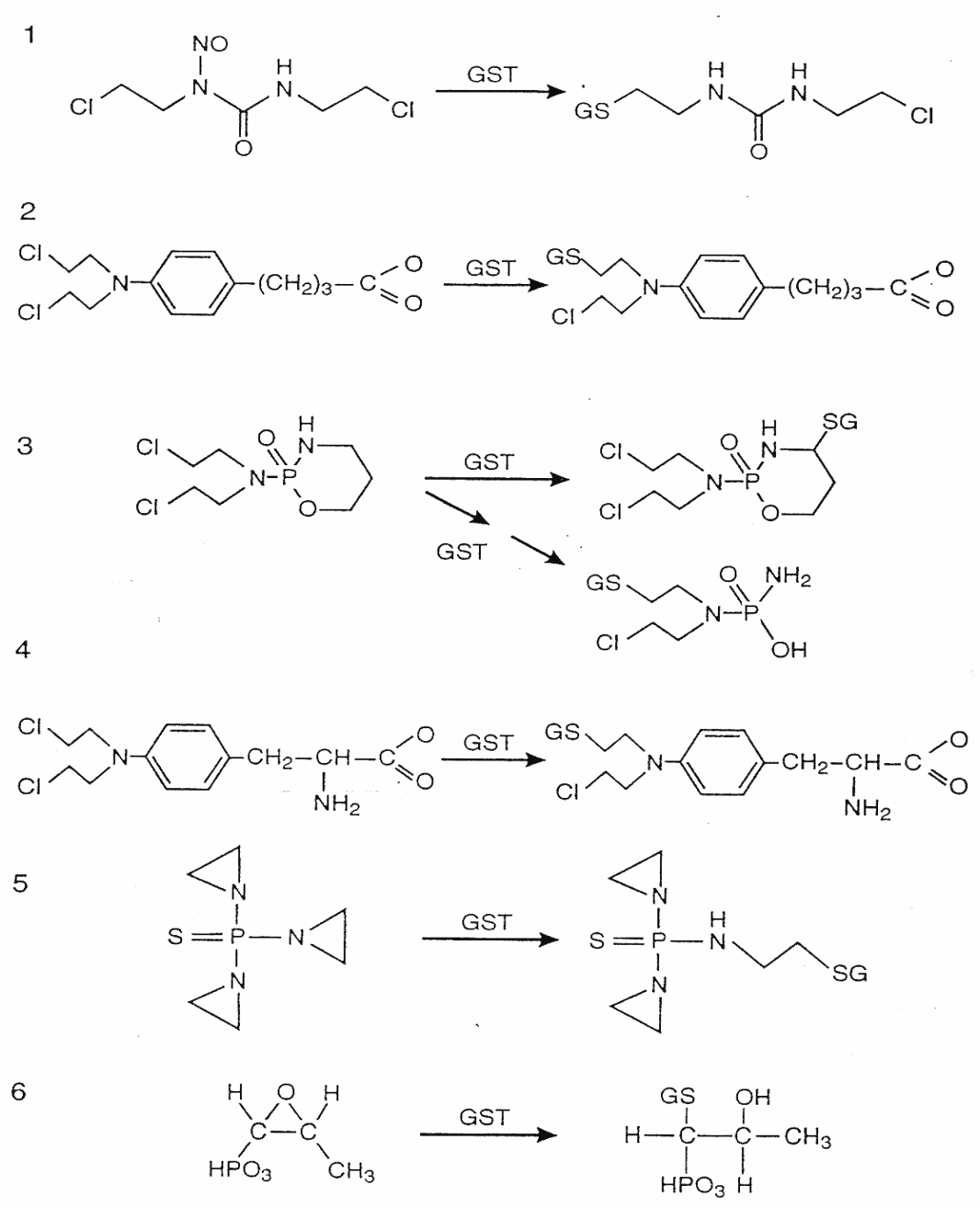

Figure 1.15. Examples of chemotherapeutic agents that are GST substrates: (1) BCNU; (2) chlorambucil; (3) cyclophosphamide; (4) melphalan; (5) thiotepa; (6) fosfomycin (Hayes and Pulford, 1995).

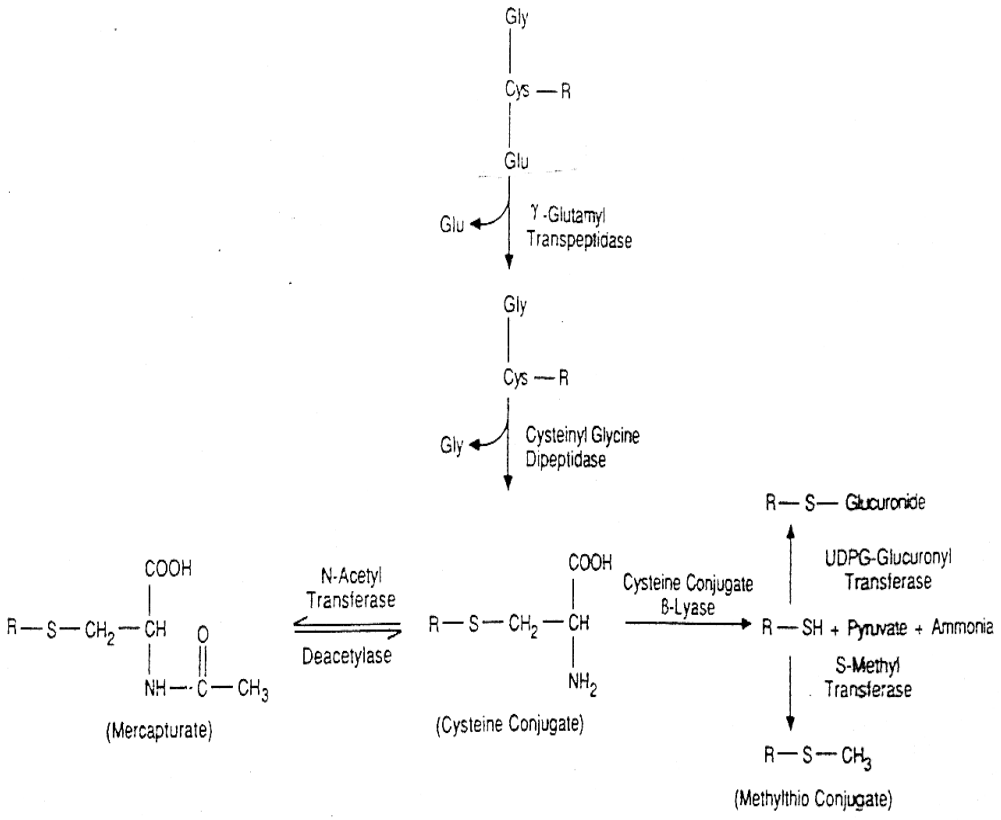

Figure 1.16. Metabolism of glutathione conjugates (Pickett and Lu, 1989)

1.4.3.2. Glutathione Conjugation and Toxification

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

A potentially more serious situation can arise with a small number GST substrates that yield a GSH conjugate, or a metabolite of the conjugate, that is

more reactive than the parental compound: These two groups of compounds have been referred to as directly acting toxic GSH conjugates and indirectly acting toxic GSH conjugates.

Directly acting toxic GSH conjugates are formed from a number of alkyldihalides (Rannug, 1978) as in the case of dihaloethanes and dihalomethanes toxification. The conjugates from dihaloethanes may rearrange spontaneously prior to interaction with DNA, but those formed from dihalomethanes, probably do not rearrange (**Figure 1.17**). GST-catalyzed reactions between 1,2 dihaloalkenes and GSH may yield S-(2-haloalkyl) glutathiones, which can form episulfonium ions intermediates are potent electrophiles and act as alkylating agents (Van Bladeren, 1979). In the case of dihalomethanes the s-methylglutathione conjugate appears to be the ultimate mutagen.

Indirectly acting toxic GSH conjugates are formed from various halogenated alkenes and alkynes. They include hexachlorobutadiene, tetrachloroethane, trichlorotrifluoropropane, and dichloroacetylene (**Figure 1.18**), all of which are nephrotoxic and also possibly nephrocarcinogenic (Dekant, 1994). These compounds are all preferentially metabolized by membrane bound GST, rather than by the soluble GST (Andersson *et al.,*1994b).

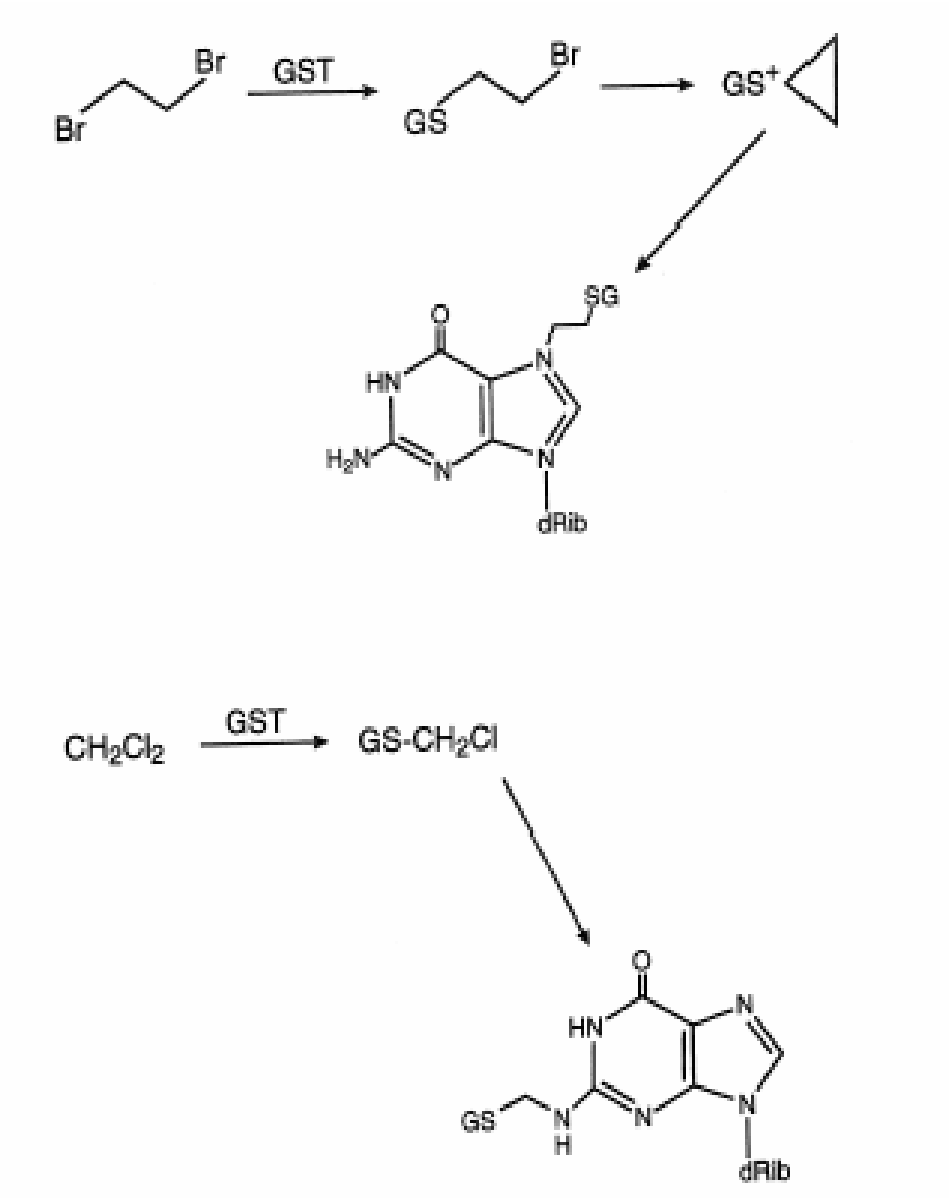

Figure 1.17. Reaction between GS conjugates formed between (1) ethylene dibromide and DNA and (2) methylene chloride and DNA.

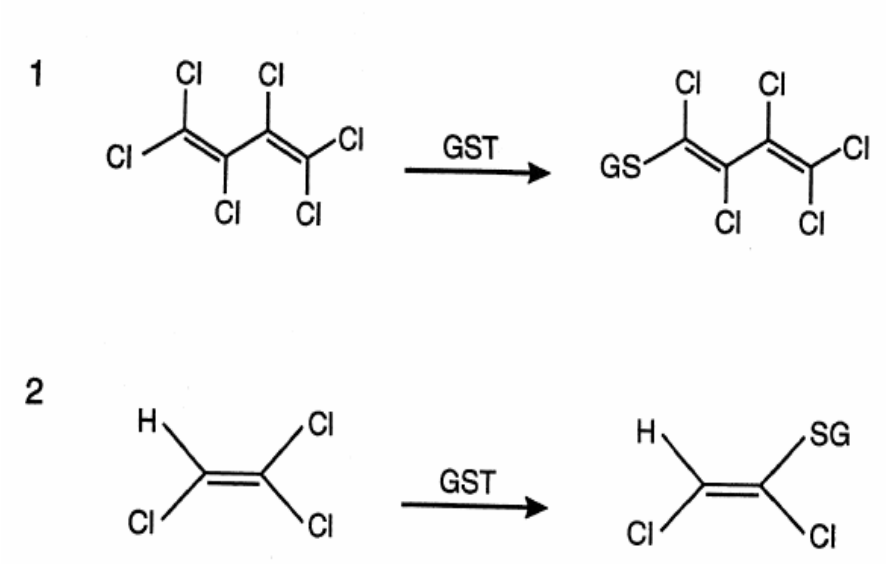

Figure 1.18. Examples of compounds that form indirectly acting toxic GSH conjugates: (1) hexachloro-1,3-butadiene; (2) trichloroethene. (Hayes and Pulford 1995).

1.4.3.3. Peroxidase Activity of GSTs

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

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

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

to give the overall reaction,

iii. ROOH + 2GSH ⟶ ROH + GSSG + H2O

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

1.4.3.4. Isomerase Activity of GST

In addition to their role as detoxication enzymes, the GSTs have been suggested to be involved in different facets of biological signalling such as synthesis of various prostaglandins, in the 5-lipoxygenase pathway, and in the interactions with protein kinases of signal transduction systems (Johansson, 2002). GST A3-3 is the most recent example of GSTs with a connection to biological signalling (Johansson, 2001). GST A3-3 efficiently catalyzes double-bond isomerizations of Δ^5- androstene 3,17 dione (Δ^5-AD) and of Δ^5-pregnene-3,20-dione, intermediates in the biosynthesis of the steriod hormones progesterone and testosterone (Johansson, 2001) (**Figure 1.19**). There are other isomerization reactions catalyzed by GSTs. The cis-trans conversion of retinoic acid is a GSH independent reactions catalyzed by GSTP1-1 (Chen *et al*., 1988).

Zeta class GSTs are involved in the catabolic pathway of tyrosine and phenylalanine by catalyzing the GSH-dependent cis-trans isomerization of maleyacetone to fumaryacetone and malay aceto acetic acid to fumarylaceto acetic acid (**Figure 1.20**). An even smaller number of GST isoenzymes possess ketosteroid isomerase activity and catalyze the conversion of Δ 5-3 ketosteroids to Δ 4- 3-ketosteroids. The physiological significance of these isomerization of maleylacetoacetic acid occurs in the pathway of tyrosine degredation in mamallian liver (Keen, 1978) (**Figure 1.20**).

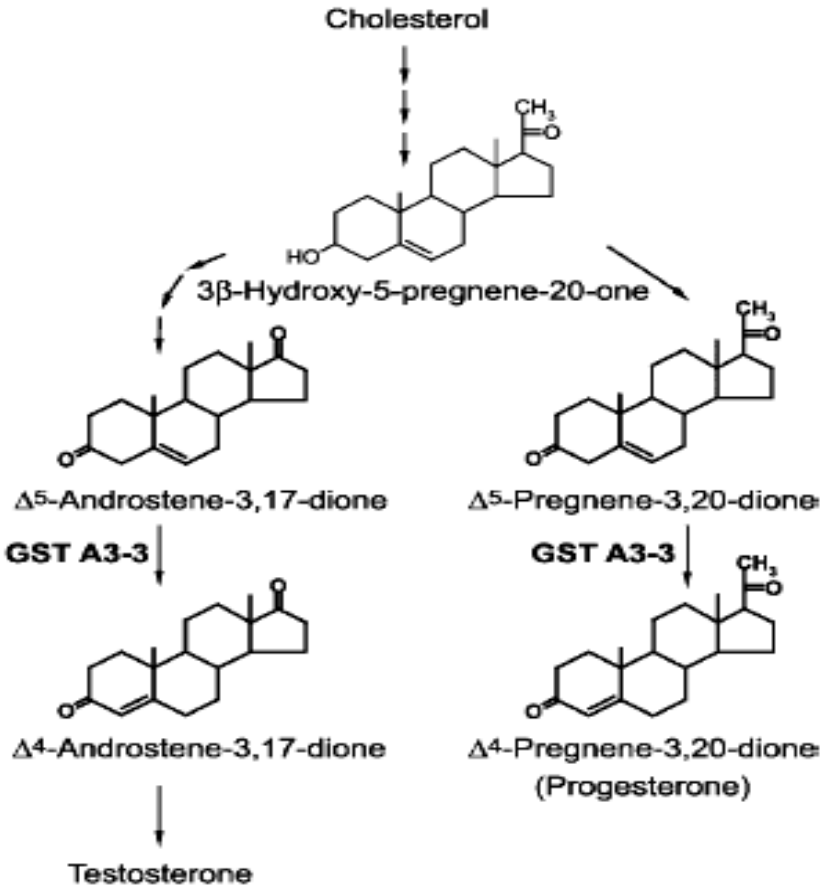

Figure 1.19. Two biosynthetic pathways leading to the steroid hormones testosterone and progesterone. The obligatory Δ^5- Δ^4 isomerization of the 3-ketosteroids is catalyzed efficiently by human glutathione transferase A3-3 present in steroidogenic tissues (Johansson, 2001).

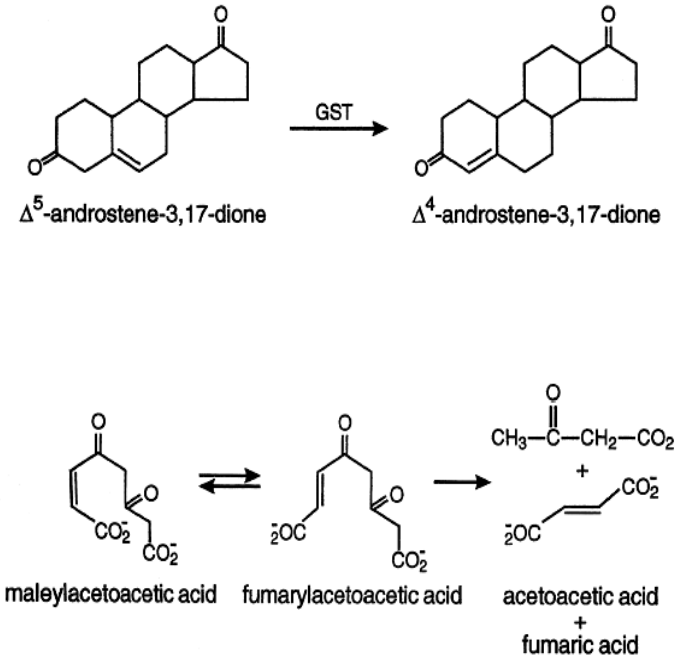

Figure 1.20. Isomerization of Δ5-androstene-3,17-dione and maleylacetoacetic acid, both of which are catalyzed by GST (Hayes and Pulford, 1995).

1.5. Scope of The Work

Although glutathione S-transferases (GST) are not the only detoxification enzymes that are responsible for the development of resistance through synthetic pyrethroids in *H. armigera*, it is thought that they have a role in the formation of this resistance. In order to understand and explain their role a lot of research has been done throughout the world. However, there is not so much work related with the role of the GSTs on *H. armigera* resistance through pyrethroids in Turkey.

The aim of this study is to investigate the role of GSTs in synthetic pyrethroid resistance observed at *H. armigera* in Turkey. Thus, first the conditions of the activity measurement of GST enzymes for susceptible population of *H.*

armigera were optimized. After that, GST activity of field and susceptible populations were determined under optimized conditions and compared statistically. Furthermore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of selected cytosols from these three populations was performed to analyze the possible changes of GST isozymes between Adana, Antalya field populations and susceptible population of *H. armigera*.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), bovine serum albumin (BSA), dipotassium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), SDS-PAGE molecular weight markers, ammonium persulphate (APS), N,N'-methylene-bisacrylamide (Bis), hydroxymethyl aminomethane (Tris), N,N,N',N'-tetramethylenediamine (TEMED), acrylamide, silver nitrate, glycerol, sodium thiosulfate, dithiothreitol (DTT), formaldehyde, were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. 2-mercaptoethanol, ether, ultra pure methanol, potassium dihydrogen phosphate, potassium sodium tartrate tetrahydrate and Ciocalteu's Folin phenol reagent were from Merck, Darmstadt, Germany. Copper (II) sulphate 5-hydrate extra pure and sodium carbonate were from Riedel de-Haen. All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2. Methods

2.2.1 Preparation of S9 fractions from *Helicoverpa armigera* midguts

Samples of *H. armigera* larvae were obtained from cotton fields in Adana and Antalya province in 2002–2003. The susceptible strain of *H. armigera* was obtained from Volcani Center, Agricultural Research Organization, in Israel.

Larvae were fed on artificial diet, allowed to pupate. Emerging adults were allowed to breed and the resulting first and second generation of sixth instar larvae used for the experiments.

In order to remove midguts of *H. armigera*, larvae were paralyzed by keeping on ice. 2-days-old sixth instars larvae were cut along their length by razor blade on ice midguts were removed. Midguts were immediately cleaned in 1.15 M KCl in order to remove fats or other unwanted substances like Malpighi tubes. Then they were individually dried on filter paper and their weights were recorded. Then, they were placed into eppendorf tube and stored in deep freezer at -80^{o}C until they were homogenized.

Each midgut was homogenized (5 times for 30 seconds with 60 seconds intervals) in 1ml of 40mM potassium phosphate buffer, pH 7.5, containing 1mM EDTA, with ultraturrax homogenizer. The homogenate was centrifuged at 10,000 x g for 30 min in a Sigma Hettich centrifuge. The supernatant (S9 fraction) was used as the enzyme source.

2.2.2 Protein Determinations

The protein concentrations in the prepared crude extracts were determined by the method of Lowry *et al.,* (1951) with crystalline bovine serum albumin (BSA) as a standard. Aliquots of 0.1 ml of 1:10 diluted midgut S9 fractions were taken into test tubes and were completed to a final volume of 0.25 ml with distilled water. Then, alkaline copper reagent was prepared by mixing 2% copper sulfate, 2% sodium potassium tartarate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 1.25 ml of the alkaline copper reagent was added to each tube, mixed by vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.125 ml of 1 N Folin Phenol reagent was added to each test tube, mixed immediately within 8 seconds by vortex and incubated 30 minutes at room temperature. The intensity of color developed in each tube was measured at 660 nm.

The protein concentrations in the crude extracts were calculated from a standard calibration curve that was constructed from the corresponding $O.D_{660nm}$ values of BSA standards (0 to 200 µg). The protein concentrations in the prepared S9 fractions were found to be in the range of 1.5-3 mg/ml.

2.2.3. Determination of GST Activities of S9 Fractions with CDNB

Glutathione S-transferase activity measurements were done by modified Habig *et al.*, method of using 1-chloro-2,4-dinitrobenzene(CDNB) as a substrate and in the presence of cofactor GSH. GST activities against the substrates CDNB were determined spectrophotometrically by monitoring the formation of the conjugation product under the conditions given in **Table 2.1** (Gillham, 1971; Habig *et al*., 1974; Habig and Jakoby, 1981). GSTs enzyme activity assays were conducted with CDNB at room temperature.

Table 2.1. Glutathione S-transferase enzyme activity assay medium

Constituents	Stock Concentration	Added Volume	Final Concentration
Potassium phosphate buffer, pH 7.5	40 mM	500 µl	20 mM
GSH	50 mM	20 µl	1.0 mM
CDNB	20 mM	50 µl	1.0 mM
Enzyme Source (S9 fraction) 1.5-3.0 mg/ml	500µg/ml in 40mM Potassium phosphate buffer, pH 7.5	50 µl	25 µg
dH_2O	-	380µl	-

2.2.4. SDS-Polyacrylamide Gel Electrophoresis

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

- Bovine Albumin (M_r 66000)
- Egg Albumin (M_r 45000)
- Glyceraldehyde-3-phosphate Dehydrogenase (M_r 36000)
- Carbonic Anhydrase (M_r 29000)
- Trypsinogen (M_r 24000)
- Trypsin Inhibitor (M_r 20000)

2.2.4.1. Preparation of Reagents

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

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

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

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

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

60.0 g acrylamide were dissolved in about 150 ml distilled water and then 1.6 gm BIS (Bis-acrylamide) were added and solution was completed to 200 ml with distilled water. Finally, the solution was filtered through course filter paper.

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

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

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

(D) 10 % SDS Solution

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

(E) Catalyst (10 % Ammonium Persulfate "APS")

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

(F) Tracking Dye (0.05 % Bromophenol Blue)

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

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

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

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

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

2.5 ml	1 M Tris-HCl, pH 6.8
4.0 ml	Glycerol
2.0 ml	2-mercaptoethanol
0.4 ml	Tracking Dye
0.8 gr	SDS

Completed to 10.0 ml with distilled water

2.2.4.2 Electrophoresis Procedure

Vertical slab gel electrophoresis was carried out to run two gels simultaneously. The assembly of the glass plate cassettes (10.5 X 10 cm) and the process of gel casting were done according to instruction manual provided with the apparatus. Once the cassettes were properly assembled and mounted, the preparation of the separating and stacking gels was started.

The 12 % and 5 % stacking gel polymerizing solutions were prepared just before use by mixing the given volumes of stock solutions in the written order as given in **Table 2.2**. The separating gel solution was first prepared with the TEMED added just before casting the gel into the glass assembly from the edge of one of the spacers until the desired height of the solution (about 7 cm) was obtained. Then, the liquid gel was overlaid with distilled water, without disturbing the gel surface, to obtain an even interface between the separating gel and the stacking gel. The gel was then allowed to polymerize at room temperature for a minimum of 30 minutes. After polymerization, the layer of water was removed completely using filter paper without hurting the gel surface. The stacking gel was then poured on the top of the resolving gel and the comb was inserted into the

layer of the stacking gel without trapping air bubbles under the teeth of the comb. The gel was then allowed to polymerize for a minimum of 30 minutes. After the gel was polymerized, the comb was removed carefully and the wells were washed with distilled water and filled with electrode buffer. At this point, the gel cassettes were removed from the casting stand, mounted and clamped onto the running frame with the notched glass plate of each cassette facing inside. When running only one gel, the blank plastic plate, provided with the system, was mounted in the place of the second cassette in the casting stand and in the running frame. Aliquots from the protein samples to be analyzed and from the standards mixture were diluted 3:1 with the 4X sample buffer (3 parts sample and 1 part sample buffer), to have the samples in 62.5 mM Tris-HCl buffer, pH 6.8, 2 % SDS, 5% 2-mercaptoethanol, 10 % glycerol and 0.001% bromophenol blue. Then the samples and standards were placed in a boiling water bath for 2 minutes. Afterwards, protein samples (7.2µg protein) and molecular weight standards (8 – 13.7 µl) were loaded into different wells.

Table 2.2. Formulations for SDS-PAGE separating and stacking gels

	Separating Gel		**Stacking Gel**
Monomer Concentration	**12 %**	**15 %**	**5%**
Acrylamide/bis	6.0 ml	7.5 ml	0.81 ml
Distilled water	5.0 ml	3.5 ml	2.86 ml
1.5 M Tris-HCl, pH 8.8	3.75 ml	3.75 ml	----
0.5 M Tris-HCl, pH 6.8	----	----	1.25 ml
10% (w/v) SDS	150 µl	150 µl	50 µl
10 % APS	93, µl	93 µl	25 µl
TEMED	7.5 µl	7.5 µl	5 µl
Total monomer	15 ml	15 ml	5 ml

After loading the samples, the running buffer (135 ml) was added to the compartment formed by the running frame and the cassettes (the upper buffer compartment) and the system was checked for leakage. The running buffer (250ml) was then also added to the outer tank (the lower buffer compartment). Thereafter, the running frame was inserted into the outer tank, the safety cover was replaced and the leads were plugged into the EC250-90 electrophoresis power supply. The power supply was adjusted to give a constant current of 15 mA when the samples were in the stacking gel and 30 mA when the samples passed to the separating gel. Under these conditions the voltage was about 50V at the beginning and elevated up to 100V at the end of the run that took a total of about 4 hours. The power supply was switched off, when the dye front is just 0.3 cm from the lower end of the glass plates, the running frame was taken out and the buffer was removed from the upper buffer compartment. Afterwards, the clamps were detached and the cassettes were removed from the running frame. To gain access to the gels in the cassette, the glass plates were pried apart using a spatula taking care not to chip the edges of the glass plates. The left-top corner of each gel was cut to indicate the order of wells. The gels, usually adhered to one of the glass plates, were taken carefully using gloves and placed in the previously prepared appropriate solutions to stain the samples which have been resolved on the gels, or to prepare the gels for subsequent blotting.

2.2.4.3 Silver Staining of the SDS-PAGE Gel

The silver staining of the SDS-PAGE gels was carried out with a revised method of Blum *et al.,* (1987) as explained in **Table 2.3**.

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

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

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

Table 2.3. Procedure for rapid Method of Silver Staining of Proteins in Polyacrylamide Gels

Steps	Solution[a]	Time of Treatment
1) Fix	40 % Methanol; 0.5 ml 37 % HCOH /liter water	Incubate for 10 min.
2) Wash	With dH_2O	2 X 5 min
3) Pretreat	$Na_2S_2O_3.5H_2O$ (0.2 g/liter)	1 min
4) Rinse	With dH_2O	2 X 20 sec
5) Impregnate	$AgNO_3$ 0.1 %	10 min
6) Rinse	Once with H_2O, once with developer solution.	2 X 20 sec
7) Develop	Na_2CO_3 3% (wt g/v) 0.5 ml 37 % HCOH /liter[b] 0.0004% (wt g/v) $Na_2S_2O_3.5H_2O$	Agitate slowly until bands appear.
8) Stop	Add 2.5 ml of 2.3 M citric acid/ 50 ml developer solution	10 min
9) Wash	With dH_2O	10 min
10)Drying Solution	10% Ethanol, 4% Glycerol, dH_2O	10 min

[a] The solutions should be prepared freshly
[b] Add formaldehyde immediately before use

2.2.5. Statistical Analysis

Differences in measured GST activities between susceptible, Adana and Antalya populations of *H. armigera* were assessed by student t-test and ANOVA

method with the help of MINITAB 13.0 statistics software. Data were expressed as mean ± Standard Error of Mean. The relationship between field populations of Adana and Antalya samples and susceptible samples activity were analyzed and a probability of 0.05 ($p<0.05$) was set as the level of statistical significance.

CHAPTER III

RESULTS

3.1. Helicoverpa armigera GST Activity

Glutathione S-transferase (GSTs) activities in the S9 fractions, prepared from *H. armigera* midguts, were determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate by monitoring the thioether formation at 340 nm as described by Habig and co-workers (Habig *et al*., 1974). The optimum conditions for the maximum enzyme activity were established and average activity of susceptible strain (n=50) was determined as 3.272 µmol/min/mg protein of S9 fraction.

3.2. Characterization of *Helicoverpa armigera* GST Activity

3.2.1. Effect of Enzyme Amount on *Helicoverpa armigera* GST Activity

Effect of enzyme amount on the GST activity was measured by changing the final protein concentration in the 1.0 ml reaction mixture between 14.6 µg and 146.5µg protein. It was found that the activity was proportional with enzyme amount up to 29.5µg protein in 1.0 ml reaction mixture. In order to obtain sufficient quantity of product for spectrophotometric determinations, 25µg protein was routinely used throughout in this study. Effect of protein amount on glutathione S-transferases enzyme activity was shown in **Figure 3.1**.

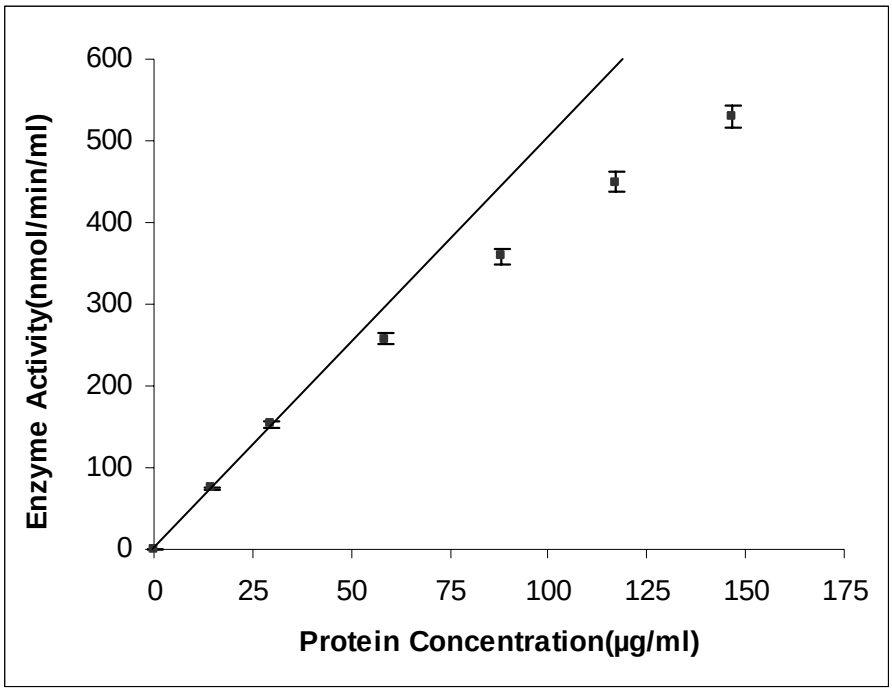

Figure 3.1. Effect of Enzyme Amount on *Helicoverpa armigera* GST Activity (Values were expressed as mean ± S.E.M)

The reaction mixture was prepared as mentioned at **Table 2.1** and the reaction was started by the addition of *H. armigera* S9 fraction in a final volume of 1.0 ml. The reaction was carried out at room temperature (25 °C) for three minutes. Each point was the average of duplicate determinations.

3.2.2. Effect of pH on *Helicoverpa armigera* GST Activity

Effect of pH on GST activity was measured by changing the pH of 20 mM phosphate buffer between 6.5 and 8.0 in the 1.0 ml reaction mixture. It was found that the activity increased from pH 6.5 through pH 7.5, then decreased linear until pH 8.0. In order to obtain maximum activity for spectrophotometric determinations, 20 mM phosphate buffer pH 7.5 was routinely used throughout this study. **Figure 3.2** shows the effect of pH on GST activity.

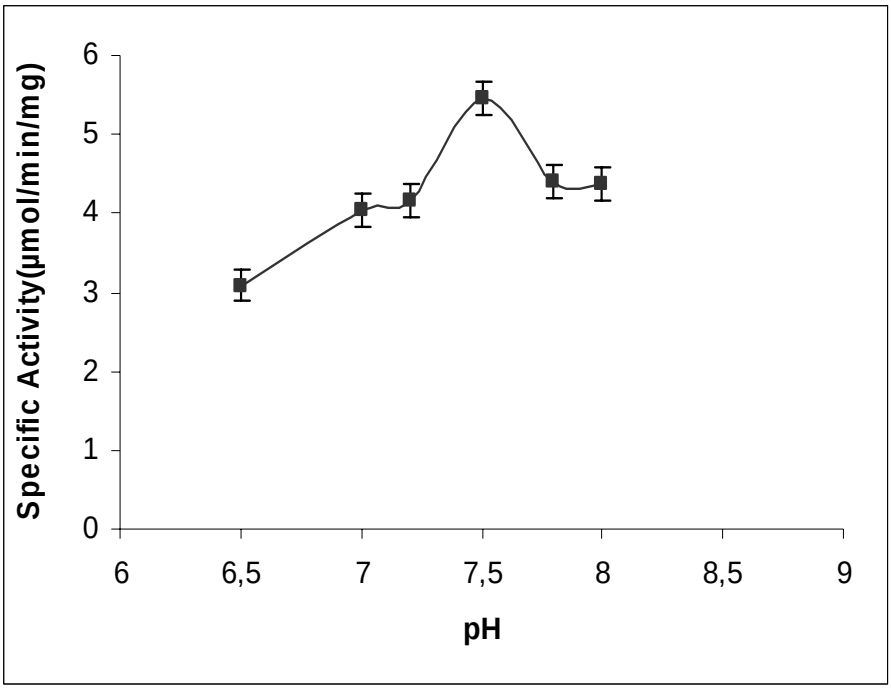

Figure 3.2. Effect of pH on GST Activity (Values were expressed as mean ± S.E.M)

3.2.3. Effect of Phosphate Buffer Concentration on *Helicoverpa armigera* GST activity

Effect of phosphate buffer concentration on GST activity was measured by changing pH 7.5 phosphate buffer concentrations in the 1.0 ml reaction mixture between 5mM and 50mM. It was found that the maximum activity was observed at 20mM phosphate buffer. Therefore, 20mM was routinely used throughout this study. Effect of phosphate buffer concentration on GST activity was shown in **Figure 3.3**.

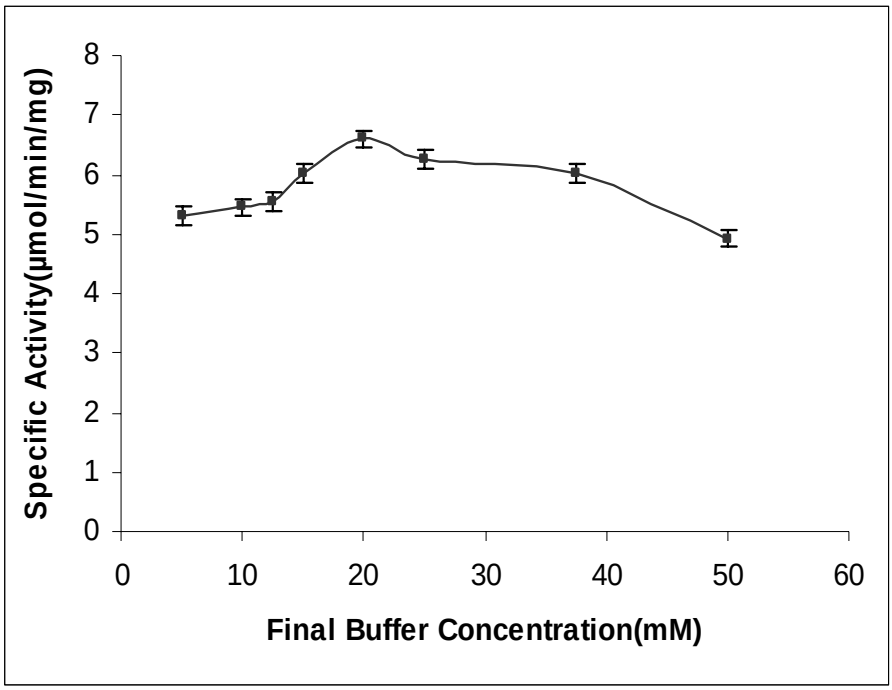

Figure 3.3. Effect of Phosphate Buffer on GST Activity (Values were expressed as mean ± S.E.M)

3.2.4. Effect of Temperature on *Helicoverpa armigera* GST Activity

The effect of temperature on GST activity was detected by incubating the reaction mixture at 7 different temperatures 17, 25, 30, 32, 35, 37 and 40 °C. According to the measurement it was seen that GST enzyme activity increased up to 30°C. Thus, kinetic studies were done at room temperature. Effect of the temperature on *H. armigera* GST was shown in **Figure 3.4**.

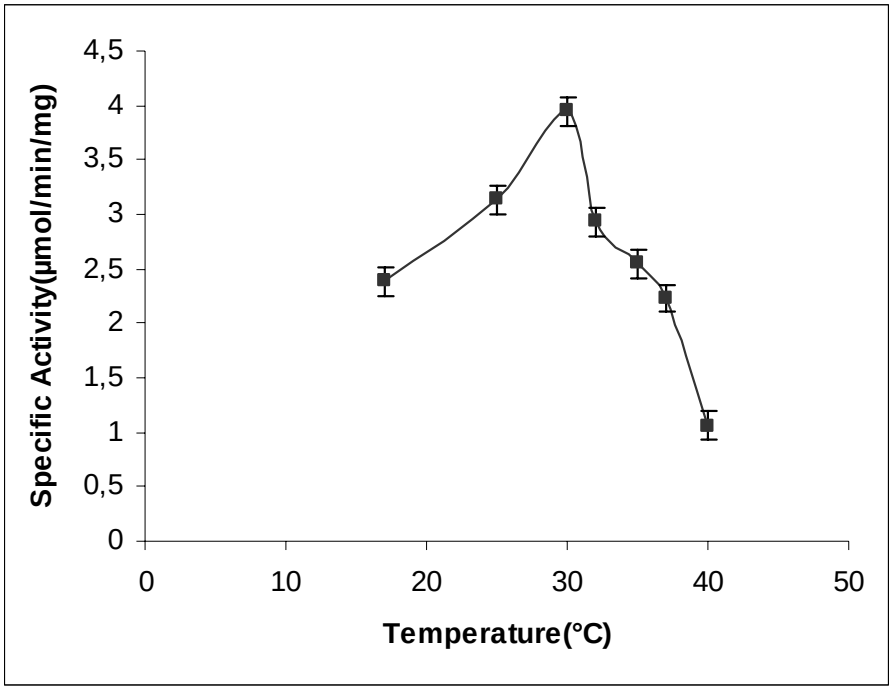

Figure 3.4. Effect of Temperature on GST activity (Values were expressed as mean ± S.E.M)

3.2.5. Effect of Substrate (CDNB) Concentration on *Helicoverpa armigera* GST Activity

The effect of substrate 1-chloro-2,4-dinitrobenzene (CDNB) concentration on GST activity was measured by changing CDNB concentrations in the 1.0 ml reaction mixture between 0.1mM and 2.5mM. The effect of substrate concentration is shown **Figure 3.5**. It was found that GST activity was reached saturation at around 1mM CDNB. Therefore, 1mM CDNB concentration was routinely used throughout this study. In addition, *H. armigera* GST Km and Vmax were calculated for CDNB by constructing Lineweaver–Burk **(Figure 3.6)** and Eadie-Scatchard plot **(Figure 3.7)**. With the help of Lineweaver–Burk plot GST Km (CDNB) and Vmax (CDNB) were calculated as 0.29mM and 6.54 µmol/min/mg, respectively. However, at the Eadie-Scatchard plot GST Km (CDNB) and Vmax (CDNB) were calculated as 0,28mM and 6.35 µmol/min/mg, respectively.

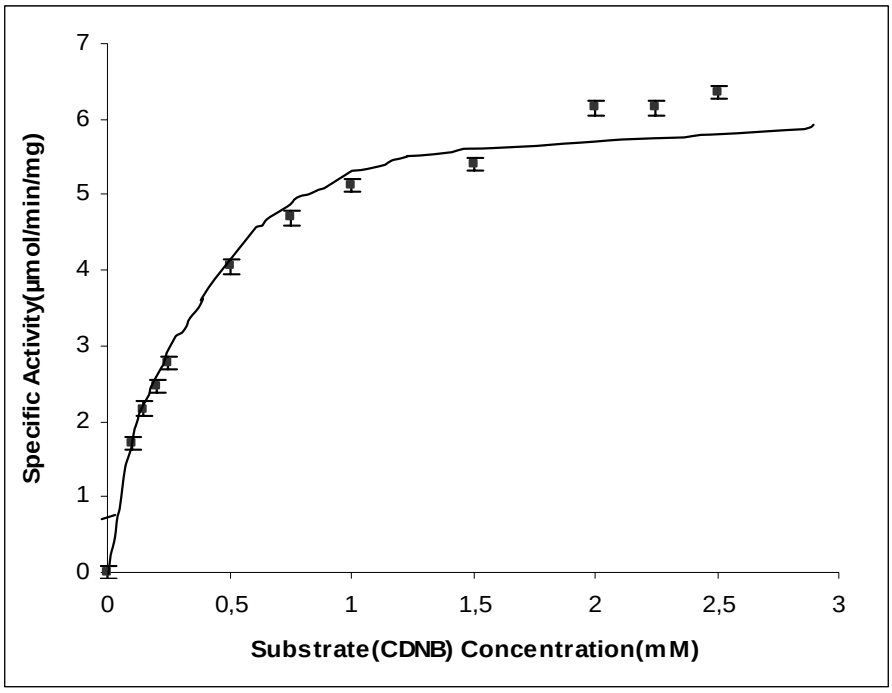

Figure 3.5. Effect of CDNB Concentration on *H. armigera* GST Activity (Values were expressed as mean ± S.E.M)

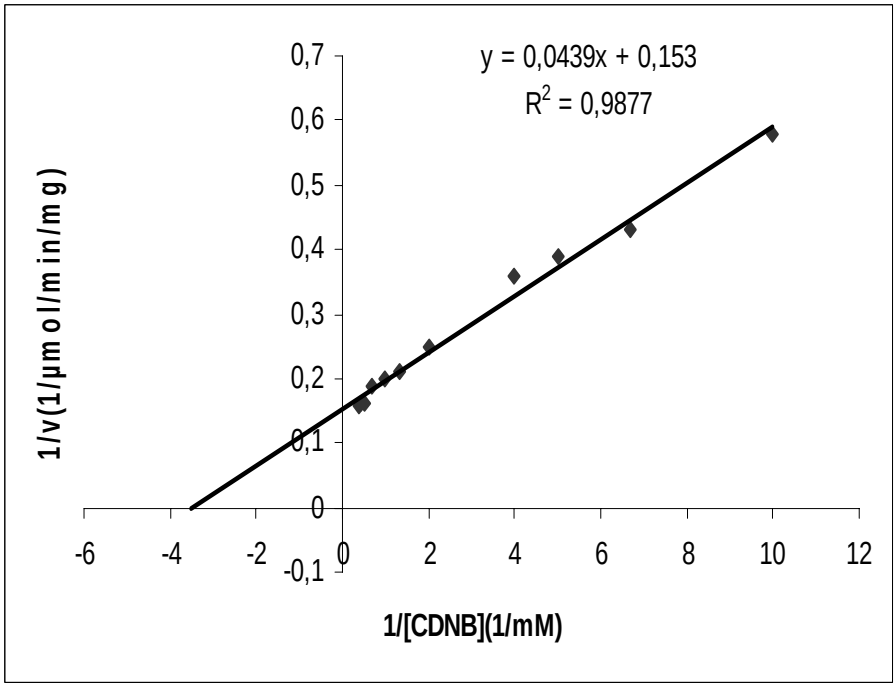

Figure3.6. Lineweaver-Burk Plot of GST Activity against Substrate (CDNB)

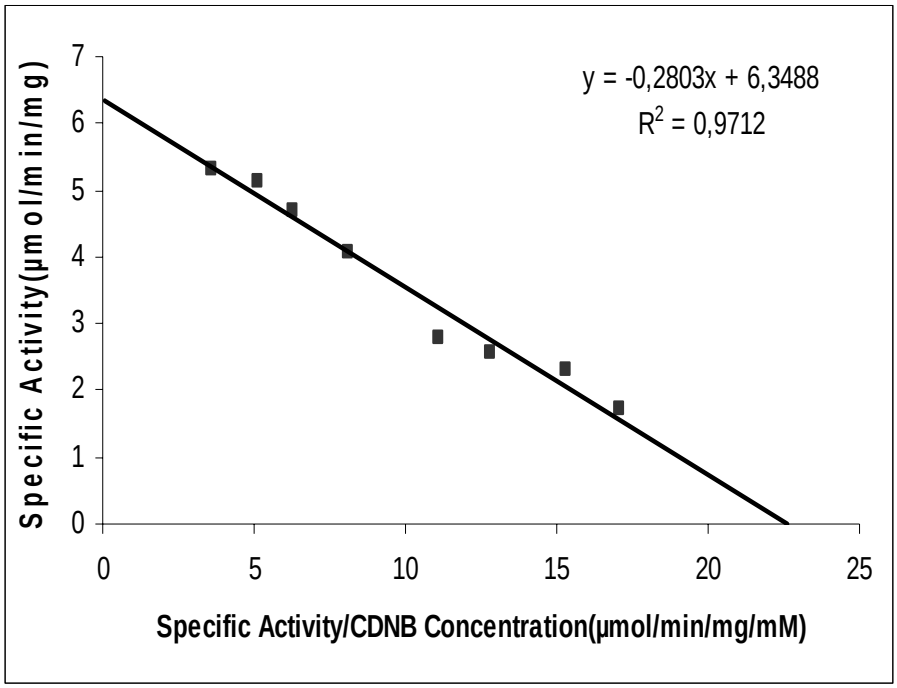

Figure 3.7. Eadie-Scatchard Plot of GST Activity against Substrate (CDNB)

3.2.6. Effect of Cofactor Reduced Glutathione (GSH) Concentration on *Helicoverpa armigera* GST Activity

The effect of reduced glutathione (GSH) concentration on GST activity was measured by changing GSH concentrations in the 1.0 ml reaction mixture between 0.1mM and 1.25mM. The effect of GSH concentration is shown in **Figure 3.8**. It was found that GST activity was reached saturation at around 1 mM GSH. Therefore, 1mM GSH concentration was routinely used throughout this study. In addition, *H. armigera* GST Km and Vmax were calculated for GSH by constructing Lineweaver –Burk **(Figure 3.9)** and Eadie-Scatchard plot **(Figure 3.10)**. According to the Lineweaver–Burk GST Km (GSH) and Vmax (GSH) were found as 0.22mM and 6.42 µmol/min/mg, respectively. However, at the Eadie-Scatchard plot GST Km (GSH) and Vmax (GSH) were calculated as 0.23mM and 6.65 µmol/min/mg, respectively.

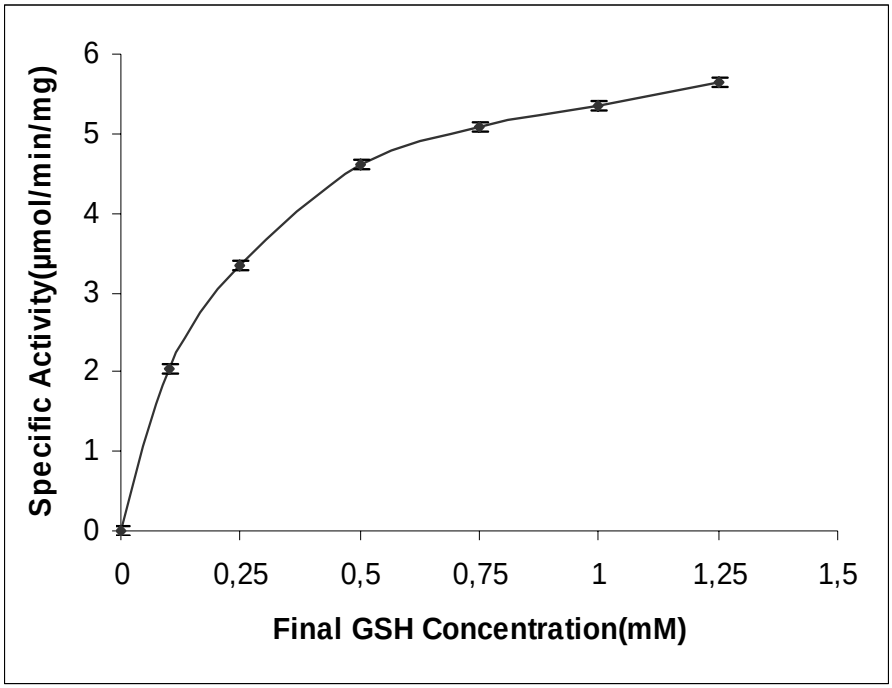

Figure 3.8. Effect of GSH Concentration on GST Activity (Values were expressed as mean ± S.E.M)

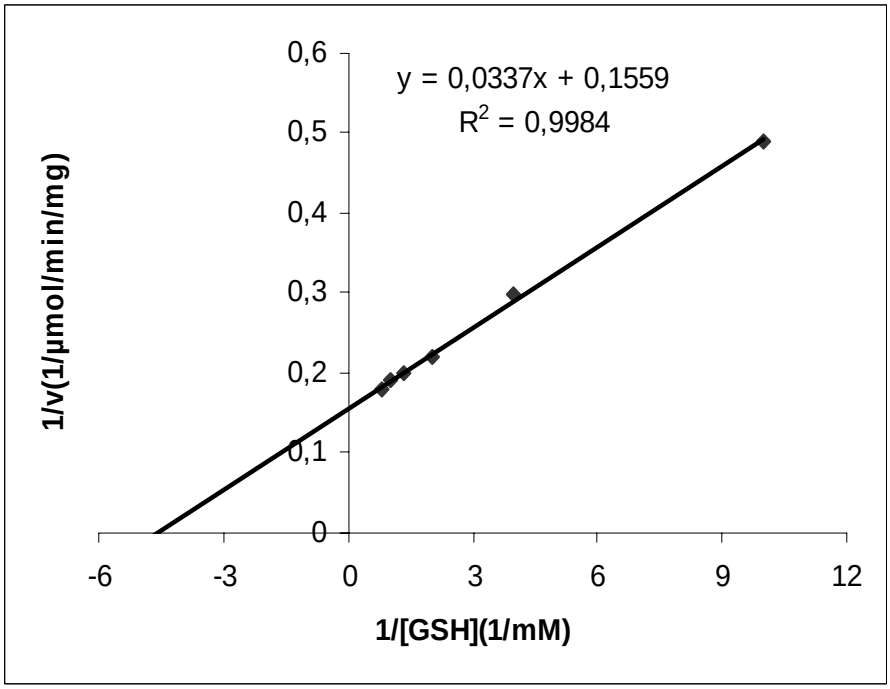

Figure 3.9. Lineweaver-Burk Plot of GST Activity against Cofactor (GSH)

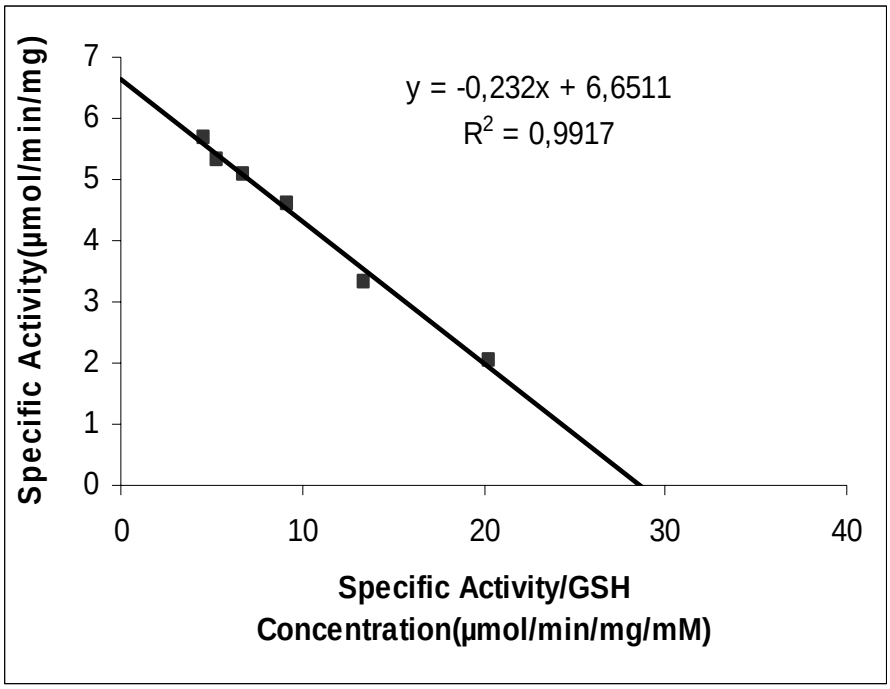

Figure 3.10. Eadie-Scatchard Plot of GST Activity against Cofactor (GSH)

3.2.7. Reaction Time Course of *Helicoverpa armigera* GST

Figure 3.11 shows the reaction time course plot of GST. It was found that absorbance linearly increased during first 60 seconds then it slightly deviated from linearity between 60-180 seconds. Therefore, activity calculations of the field population and susceptible samples were done according to their absorbance changes at the initial 60 seconds.

The reaction 1ml mixture was prepared with pH 7.5, 20mM phosphate buffer containing 1mM CDNB, 1mM GSH and 29.3 µg/ml GST enzyme. The measurement was done at room temperature.

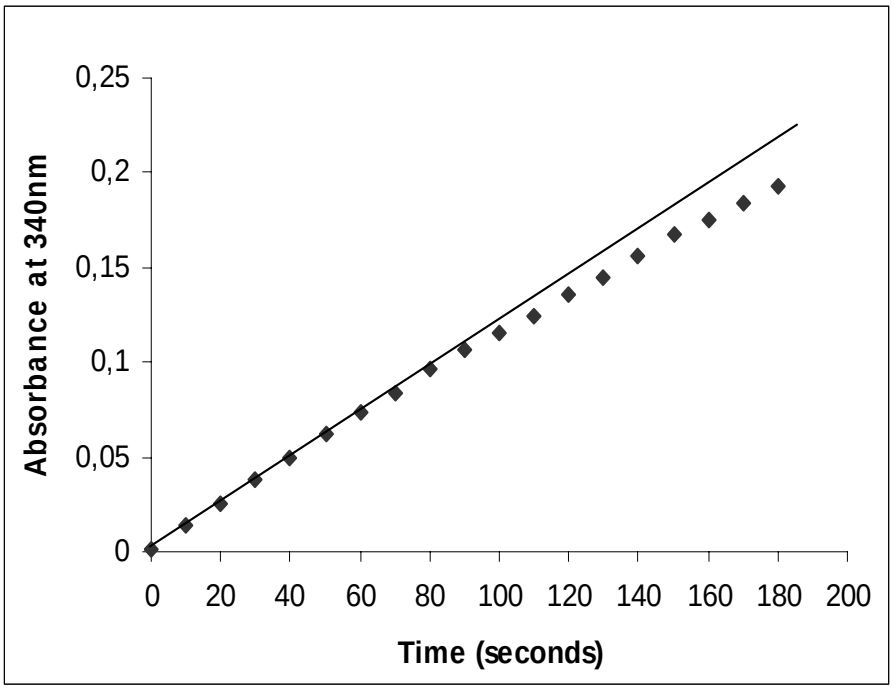

Figure 3.11. GST Reaction Time Course Plot

3.3. GST Enzyme Activities of Susceptible Samples of *Helicoverpa armigera*

Under optimized reaction conditions, 50 susceptible samples enzyme activities were measured. At the probability plot (**Figure 3.12**) the data are plotted against a theoretical normal distribution in such a way that the points should form an approximate straight line. For susceptible 50 samples mean, standard deviation and standard error of mean were calculated with the help of Sample t test. According to sample t-test, average activity was determined as 3.272µmol/min/mg. Also, standard deviation and standard error of mean were found as 1.058 and 0.15, respectively.

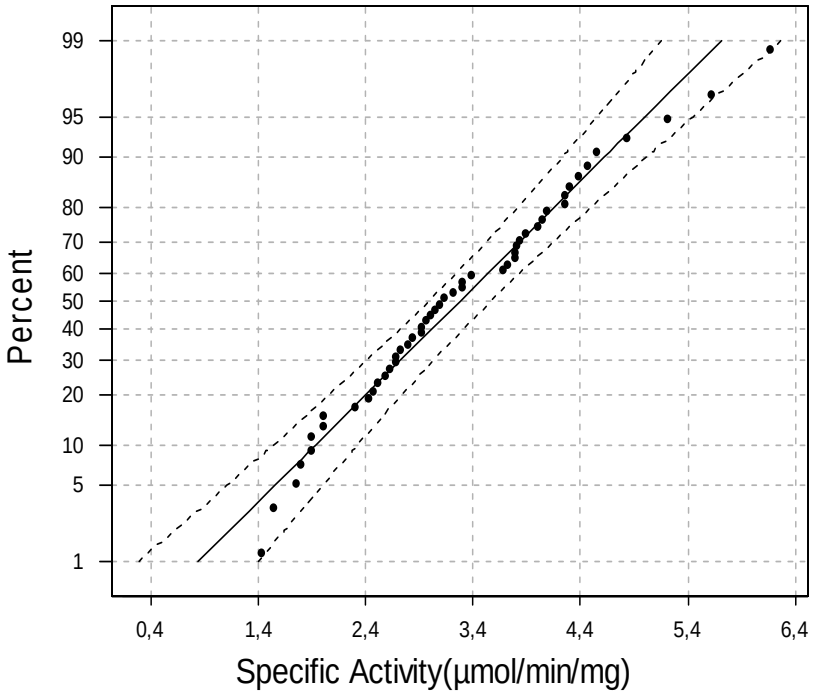

Figure 3.12. Normal Probability Plot of Susceptible Samples GST Activities

3.4. GST Enzyme Activities of Adana Field Samples of *Helicoverpa armigera*

50 Adana field samples GST enzyme activities were measured at pre-determined conditions. **Figure 3.13** shows the normal probability plot of these measured activities. As Adana samples GST activities mean, standard deviation and standard error of mean were calculated with the help of Sample t test. According to sample t-test, Average activity was determined as 7.824µmol/min/mg. Also, standard deviation and standard error of mean were found as 2.962 and 0.419, respectively.

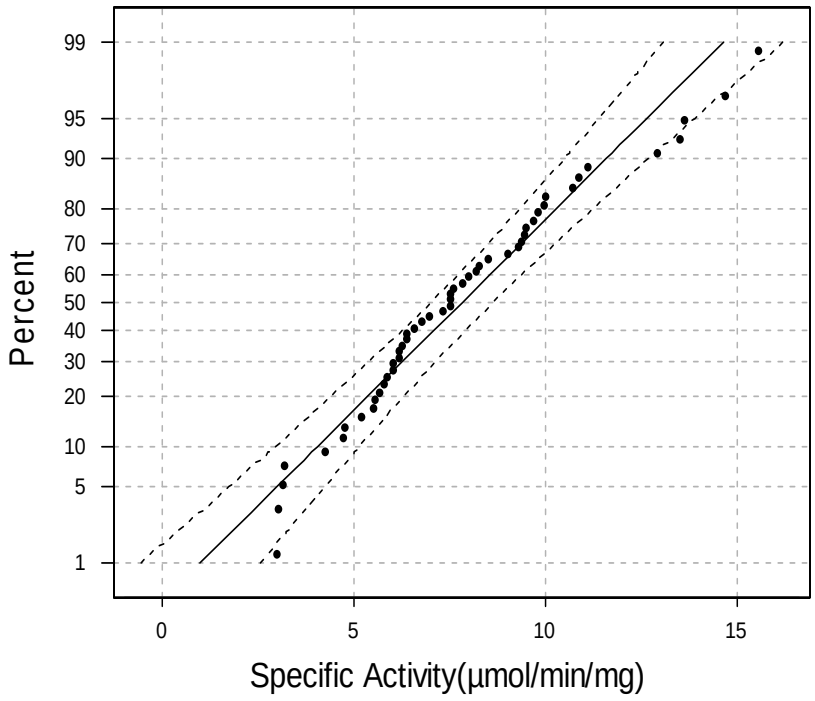

Figure 3.13. Normal Probability Plot of GST Activities of Adana Samples

3.5. GST Enzyme Activities of Antalya Field Samples of *Helicoverpa armigera*

50 Antalya field samples GST enzyme activities were also measured at optimum reaction conditions. These activities mean, standard deviation and standard error of mean were calculated with the help of Sample t test. According to sample t-test, Average activity was determined as 9.518µmol/min/mg. Also, Standard deviation and Standard error of mean were found as 3.67 and 0.519, respectively. The distribution of these activities was also mentioned in **Figure3.14**.

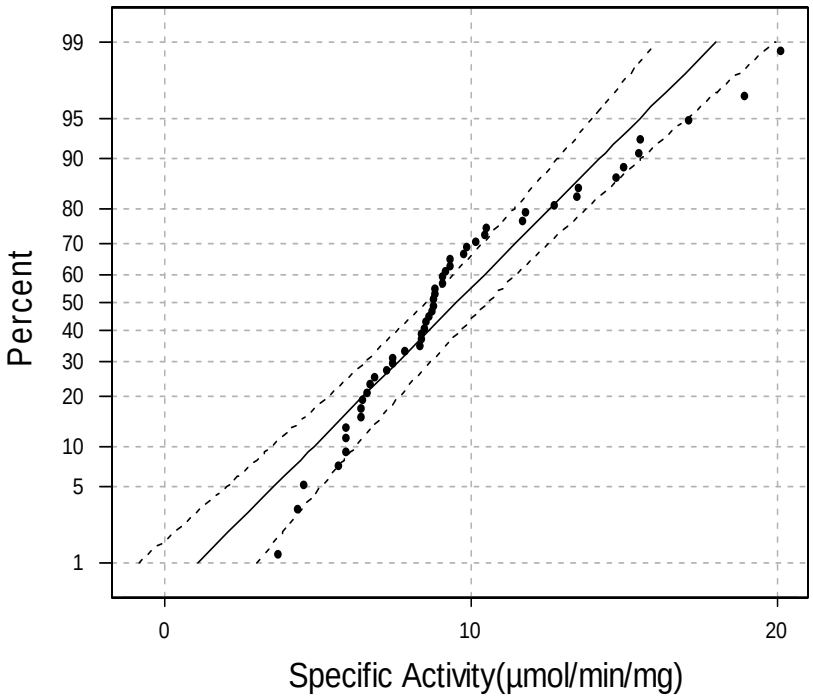

Figure 3.14. Normal Probability Plot of GST Activities of Antalya Samples

In order to analyze to the similarity or difference between the field and susceptible populations and also field populations each other their p values were calculated comparatively with ANOVA method. According to these results that Adana and Antalya populations GST activities are completely different from susceptible population ($p<0.05$) and also each other ($p<0.05$).

As a comparison of the activities of these populations mean values of the each population were used to construct **Figure 3.15**. It can be seen from that figure field populations of the *H. armigera* show higher GST activity than susceptible population. Antalya samples also showed the higher GST activity than Adana samples.

Table 3.1. Summary of Sample t- test Results of *H. armigera* Populations

Variable	Sample Amount(N)	Mean (µmol/min/mg)	Standard Deviation	Standard Error of Mean
Susceptible	50	3.272	1.058	0.15
Adana field	50	7.824	2.962	0.419
Antalya field	50	9.518	3.670	0.519

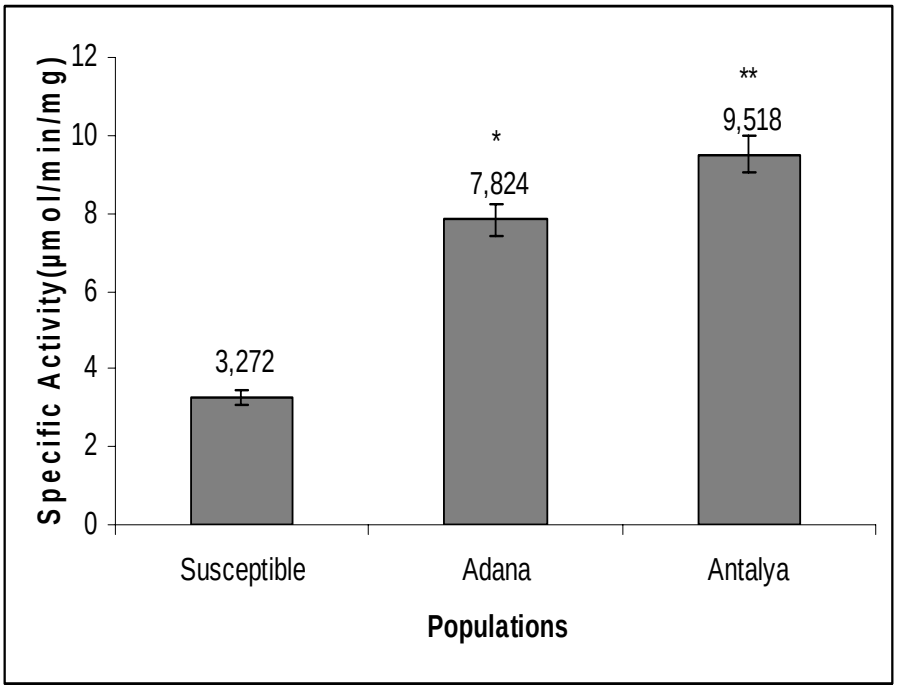

Figure 3.15. Comparison of Average GST activities of *Helicoverpa armigera* populations **(**Values were expressed as mean ± Standard Error of Mean)
(*) significantly different than susceptible, $p<0.05$
(**) significantly different than Adana, $p<0.05$

3.6. SDS-PAGE of the S9 Fractions from *Helicoverpa armigera* Populations

After GST activity measurments were done, selected *H. armigera* samples' S9 fractions were analyzed in order to analyze whether GST isozymes induction could have been occured or not in field populations. **Figure 3.16** show the photograph of SDS-PAGE gel after silver staining. In the **Figure 3.16** lanes contained molecular weight markers and S9 fractions prepared from individuals representing the each populations. According to the this SDS-PAGE result that some of the GST isozymes could be induced in the field population compared to susceptible ones.

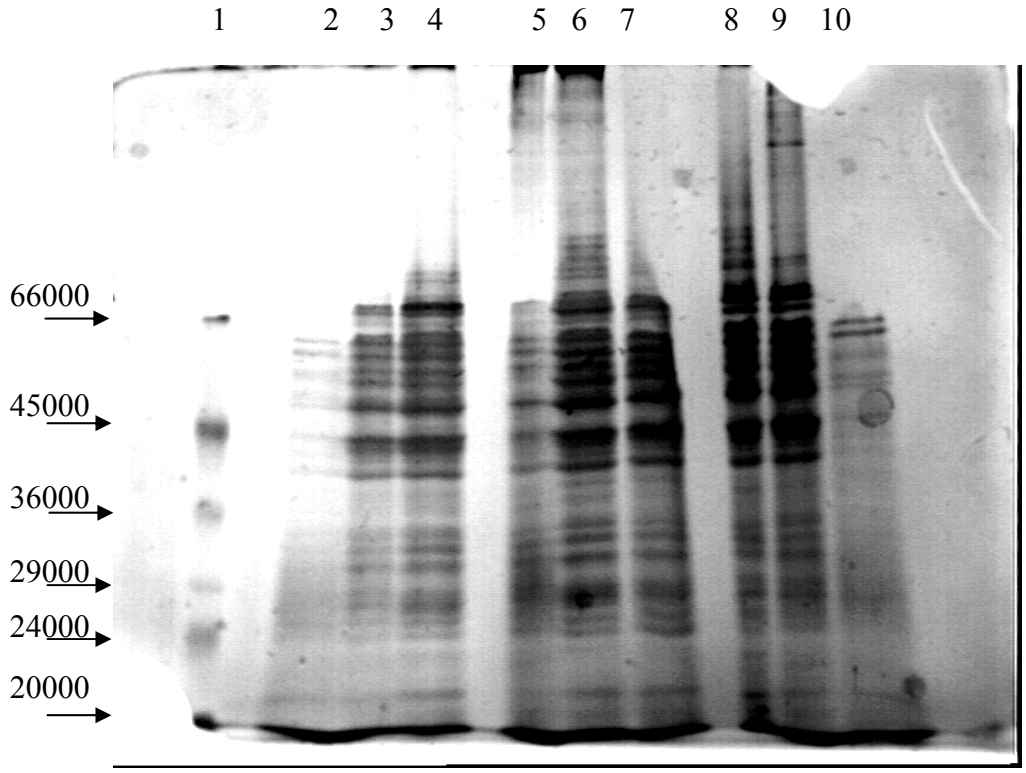

Figure 3.16. SDS-PAGE (12 %) stained with silver, of the S9 fractions from each popultaions and molecular weight markers (Each well contains 7.2 µg protein)

Lane 1	SDS-PAGE Standards
Lane 2	Cytosolic Fraction of Adana (Low Activity)
Lane 3	Cytosolic Fraction of Adana (Median Activity)
Lane 4	Cytosolic Fraction of Adana (High Activity)
Lane 5	Cytosolic Fraction of Antalya (Low Activity)
Lane 6	Cytosolic Fraction of Antalya (Median Activity)
Lane 7	Cytosolic Fraction of Antalya (High Activity)
Lane 8	Cytosolic Fraction of Susceptible (Low Activity)
Lane 9	Cytosolic Fraction of Susceptible (Median Activity)
Lane 10	Cytosolic Fraction of Susceptible (High Activity)

CHAPTER IV

DISCUSSION

Glutathione S-transferases (GSTs) (E.C.2.5.1.18) are a group of multifunctional detoxification enzymes catalyzing the conjugation of reduced glutathione (GSH) with electrophilic substrates (Chasseaud, 1979). The conjugates are then eliminated from the cell via the glutathione S-conjugate export pump and subsequently transformed in animals to give excretable mercapturic acids (Dykstra and Deuterman, 1978 and Ishikawa, 1992). Thus, glutathione-dependent conjugation has been regarded as an important detoxification mechanism in insects as well as in mammals.

Due to heavy selection pressure over the past 40 years, *Helicoverpa armigera* has developed resistance to organochlorine, carbamate, organophosphate and pyrethroid insecticides in many countries (McCaffery, 1998). All the major mechanisms of resistance such as delayed penetration, nerve insensitivity and metabolic detoxification have been implicated as the cause of pyrethroid resistance in *H. armigera* (Ahmad *et al.,* 1989; Gunning *et al.,* 1991; Forrester *et al.,* 1993b; Wu *et al.,* 1995, 1997a; McCaffery, 1998; Ahmad and McCaffery, 1999; Tan and McCaffery, 1999; Martin *et al.,* 2002).

GSTs are important in phase I metabolism of organophosphorous and organochlorine compounds and play a significant role in resistance to these insecticides in insects (Motoyoma and Dauterman, 1980; Oppenoorth, 1979). They are also important in phase II metabolism of reactive metabolites formed by microsomal oxidation (Menzie, 1978). Recently, GSTs were found to be involved in pyrethroid tolerance through sequestration (Kostaropoulos *et al.,* 2001) and pyrethroid resistance through antioxidant defense in insects (Vontas *et al.,* 2001).

Insect glutathione S-transferases have been shown to be active toward numerous electrophilic xenobiotics including halogenated compounds(e.g., 1-chloro-2-4-dinitrobenzene), nitro compounds (e.g., *p*-nitrophenyl acetate), α,β-unsaturated carbonyl compounds (e.g.,*trans*-4-phenyl-3-buten-2-one), isothiocyanates (e.g., allyl isothiocyanate), organothiocyanates (e.g., benzyl thiocyanate) oxides (e.g., styrene oxide), organophosphates (e.g., diazinon), and organic hydroperoxides (e.g., cumene hydroperoxide). However, very little is known about substrate specificity of individual GST isozymes in insects. This information is crucial for understanding the molecular mechanisms of detoxification in insects. Furthermore, it is important to understand GST gene regulation in pest species. Limited work indicates the involvement of de novo protein synthesis (Yu, 1996).

Glutathione S-transferases enzyme family has been implicated as one of the major mechanisms for neutralizing the toxic effects of insecticides in insects (Grant *et al.,* 1991, Feurnier *et al.,* 1992, Ku *et al.,* 1994, Syvanen *et al.,* 1994 and 1996, Ranson *et al.,* 1997, Huang *et al.,* 1998). Recently, the management of *H. armigera*, the American bollworm, has become increasingly difficult due to development of resistance to various groups of insecticides, particularly pyrethroids (Armes *et al.,* 1992).

In recent years, synthetic pyrethroids such as tralomethrin and lambda-cyhalothrin have been commonly used in Turkey. As they are very effective at beginning and require low dosage applying, they are quickly favored by farmers. Since there are so many pests for cotton in Turkey, usually much higher amount of insecticides has been applied from air to large cotton farming areas to control these pests. This would have caused developing resistance to these insecticides very quickly. Thus, the resistance development caused by these insecticides should be carefully under observation.

In this study, we tried to understand the effect of the lambda-cyhalothrin on *H. armigera* GSTs and to enlighten the mechanism of the developed resistance so help to produce effective resistance management strategies against to this insect. In order to do that we first optimized the GST activity measurement conditions for *H. armigera*. Then, at optimum assay conditions susceptible samples, Adana and Antalya field samples of *H.armigera* GST activities were measured. Finally, selected cytosolic fractions from each population were applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze GST isozymes between field and susceptible samples and also between field samples.

The Km and Vmax values of *H. armigera* GSTs were calculated both its substrate 1-chloro-2,4-dinitrobenzene (CDNB) and cofactor reduced glutathione (GSH) by constructing Lineweaver-Burk and Eadie-Scatchard plots. Although Km and Vmax values of GST for CDNB were calculated as 0.29 mM and 6.54 µmol/min/mg in Lineweaver-Burk plot, in the Eadie-Scatchard plot Km and Vmax values of GST for CDNB were calculated as 0.28 mM and 6.35 µmol/min/mg, respectively. According to the Lineweaver–Burk plot, Km and Vmax values of GST for GSH were found as 0.22 mM and 6.42 µmol/min/mg, respectively. However, at the Eadie-Scatchard plot Km and Vmax values of GST for GSH were calculated as 0.23 mM and 6.65 µmol/min/mg, respectively. It could be easily seen that substrate Km and Vmax values from Lineweaver–Burk and Eadie-Scatchard plots were quite close to each other. It is concluded that both plot results confirm each other very well. According to these results, as Vmax of GST can not be exceeding theoretically, both substrate and cofactor Vmax values were nearly same. Because of Km is a constant value for a given enzyme toward that substrate, Km values of GSH and CDNB were different from each other. In addition, we can also easily conclude that affinity of the reduced glutathione (0.225mM) has higher affinity toward *H. armigera* GSTs than CDNB (0.285mM).

In order to see the distribution of all GST activities in each population well, we plotted all data of the each population separately against a theoretical normal distribution in such a way that the points should form an approximate straight line. Departures from this straight line indicate departures from normality. Thus, data, which were deviated so much from normal distribution, were easily seen and omitted. As we carried out the statistical analysis without omitting deviated data, there were no differences between the results.

We found that, GST activity in Adana and Antalya field populations were significantly higher ($p<0.05$) compared to susceptible samples as seen **Table 3.1** and **Figure 3.15**. GST activity increased 138% in Adana samples and 185% in Antalya samples compared to susceptible population results. This marked increase in GST activity suggests that there will be an increased in metabolism of GSTs for detoxifying the synthetic pyrethroid lambda-cyhalothrin or other toxicants in Antalya and Adana field samples. These results are in consistency with the study of Martin *et al.,* 2002.

We also found that GST activity of the Antalya samples were significantly higher ($p=0.013$) compared to the Adana samples. According to our results, GST activities of Antalya samples showed statistically significant ($p<0.05$) increase 20% more than Adana samples. This marked increased in GST activity suggest that *H.armigera* samples in Antalya could have exposed much more synthetic pyrethroid lambda-cyhalothrin than Adana samples. There were other reasons like amount and type of other insecticides used in the sample collection area in Adana and Antalya (cross-resistance) or qualitative and quantitative differences of GST isozymes in these *H. armigera* samples.

We analyzed individual S9 fractions on SDS-PAGE. The SDS-PAGE was run on 12 % gels, stained with silver nitrate short staining method and is given at **Figure 3.16**. GST isozymes appear usually in between 24 kDa to 30 kDa range. Couple of bands whose molecular weights are 24 kDa, 26 kDa and 29 kDa were seen on the stained gels. Although, the western-blot analysis was not done for

these protein bands to see whether they bands were exactly GST isozyme bands or not, there were observed some meaningful changes in the band intensity related to the levels of GSTs. After western blot analysis, we can evaluate this SDS-PAGE result more correctly. According to the literature, highly polyphagous insects like *H. armigera* and fall armyworm have evolved multiple glutathione S-transferases which may help detoxify the diverse toxics found in their host plants. For instance, Yu (1989) purified four isozymes with three subunits molecular weights ranging from 27.5 kD to 32 kD in larval midguts of *H. armigera*. In contrast, Chien and Dauterman (1991) isolated only one GST isozyme with homodimer with two equal size subunits having molecular weights 23.9 kD. Renuka *et al.,* (2003) were purified GST that only one isozyme having molecular weight of 30 kD.

CHAPTER V

CONCLUSION

GST activity of *H. armigera* was characterized in S9 fractions prepared from midguts. The optimum conditions for maximum GST activity were determined as product formation linearly increased up to 29.5µg protein in 20mM potassium phosphate buffer, pH 7.5. Maximum reaction rate was reached at 30°C and GSTs were saturated with its substrate (CDNB) and its cofactor (GSH) around 1mM concentration. The Km and Vmax values of were calculated for CDNB 0.285 mM and for GSH 0.225 mM.

Both Antalya and Adana field samples of *H. armigera* showed 185% and %138 higher ($p<0.05$) GST activity, respectively than the susceptible samples.

Antalya field samples of *H. armigera* showed the higher GST activity than Adana samples. The increase was found statistically significant ($p<0.05$).

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APPENDIX A

Table A.1. Kinetics of glutathione S-tranferase isozymes from fall armyworm larvae midgut (Yu, 2002)

Isozyme	Km(mM)[a]	Vmax(μmol/min/mg)[a]
MG GST-1	0.91	2.35
MG GST-2	2.26	3.00
MG GST-3	1.11	3.33
MG GST-4	0.65	6.67
MG GST-5	3.33	10.0
MG GST-6	1.00	1.42

[a] CDNB as substrate.