In vitro Characterization of Glutathione Transferases from *Sarcoptes scabiei*

Eva U. Molin
Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health
Uppsala

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Cover: Crystal structure of *Sarcoptes scabiei* Delta GST I (SsGSTD1-1).
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Abstract

The mite *Sarcoptes scabiei* causes sarcoptic mange, or scabies, a disease that affects both animals and humans worldwide. The ectoparasite not only causes suffering in the host but also financial losses in e.g. pig herds. Infections can be cured with acaricides but treatment failures have been reported. Very little is known about drug detoxification in *S. scabiei*, and hence more information on underlying enzymatic mechanisms could prolong the lifespan of acaricides used in treatment of both animals and humans.

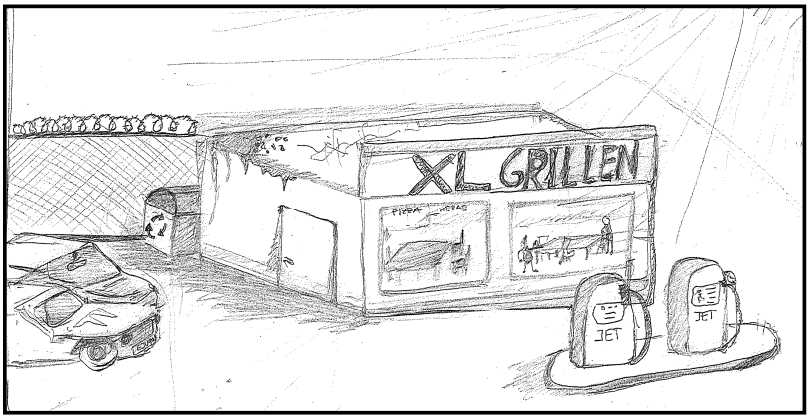
The aim of this thesis was to characterize glutathione transferases (GSTs) from *S. scabiei*, investigate their possible involvement in resistance development and their potential as antigens in serology. GSTs are multifunctional enzymes with fundamental roles in the cellular detoxification, and these enzymes have been linked to drug resistance in various organisms. GSTs have also been suggested to confer acaricide resistance in *S. scabiei*.

The first Delta GST occurring outside insects was identified, and phylogenetically classified. The crystal structure of this GST was also determined, which was the first Acari (mites and ticks) GST structure. The 3D-structures of two other Delta GSTs were predicted using homology modeling. Possible binding modes between the three Delta GSTs and various acaricides and the synergist diethyl maleate (DEM) were investigated using docking studies. Recombinant versions of the Delta GSTs and three Mu GSTs were biochemically characterized under steady-state conditions, and inhibition analyses with various acaricides and DEM were conducted. Additionally, the potential of the GSTs as antigens in serology was investigated.

The results showed that all *S. scabiei* GSTs, except one, were catalytically active and significantly inhibited by various acaricides. However none of the GSTs were good antigens for serology. The docking studies showed that *S. scabiei* GSTs could metabolize or bind various acaricides, hence strengthening the suggestion that GSTs might confer resistance to acaricides in *S. scabiei*.

Keywords: *Sarcoptes scabiei*, GST, glutathione, resistance, acaricide, ivermectin, lindane, permethrin, inhibition, crystal structure

Author's address:
Eva U. Molin, SLU, Department of Biomedical Sciences and Veterinary Public Health & SVA, Department of Virology, Immunobiology and Parasitology
P.O. SE-751 89 Uppsala, Sweden
E-mail: Eva.Molin@sva.s



Drawing: Mats Ander

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I EU Pettersson, EL Ljunggren, DA Morrison & JG Mattsson (2005). Functional analysis and localisation of a delta-class glutathione S-transferase from *Sarcoptes scabiei*. *International Journal for Parasitology* 35, 39-48.

II EU Molin & JG Mattsson (2008). Effect of acaricides on the activity of glutathione transferases from the parasitic mite *Sarcoptes scabiei*. *Parasitology* 135, 115-123.

III EU Molin, R Friemann, M Ingelman, F Söderbom & JG Mattsson (2009). Structure-function studies of *Sarcoptes scabiei* glutathione transferases: Modelling of various acaricides and the synergist diethyl maleate to three Delta GSTs. Manuscript.

The contribution of XX to the papers included in this thesis was as follows:

I Conceived and designed the experiments: ELL & JGM.
Performed the experiments: DAM (phylogeny), ELL & EUP.
Analyzed the data: ELL, EUP, DAM & JGM.
Wrote the paper: ELL, EUP, DAM & JGM.

II Conceived and designed the experiments: EUM & JGM.
Performed the experiments: EUM.
Analyzed the data: EUM & JGM.
Wrote the paper: EUM & JGM.

III First authors: EUM & RF
Conceived and designed the experiments: EUM, FS, JGM, MI & RF.
Performed the experiments: EUM, MI (crystallization) & RF (crystal structure determination, homology modelling and docking studies).
Analyzed the data: EUM, JGM, MI & RF.
Wrote the paper: EUM, FS, JGM, MI & RF.

Abbreviations

ABC	ATP-binding cassette transporter
CDNB	1-chloro-2,4-dinitrobenzene
CYP450	cytochrome P450
DDT	dichlorodiphenyltrichloroethane
DEM	diethyl maleate
GABA	γ-aminobutyric acid receptor
GSH	reduced glutathione
GST	glutathione transferase
LGIC	ligand-gated ion channel
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
mbp	maltose binding protein gene
MRP	multidrug resistance-associated protein
PDB	Protein Data Bank
PgP	P-glycoprotein
qRT-PCR	quantitative real-time PCR
SMIPP	scabies mite inactivated protein paralogues
Vssc	voltage-gated sodium channel

1 Introduction

The World Wide Web search motor Google™ was created to bring order to the information chaos we are encountering with the use of internet. It can also be used to analyze what are the hot topics of the world today, hence how "famous" they are. When I started writing this thesis I got curious about how famous is *Sarcoptes scabiei* (Table 1).

Table 1. Google™ searches performed 22nd of June 2009.

Search word	Number of hits
Sarcoptes scabiei	76 700
sarcoptic mange	119 000
scabies	1 210 000
bird flu	9 310 000
swine flu	11 700 000
malaria	12 700 000
cancer	182 000 000

It is obvious that *S. scabiei* and its infection, called sarcoptic mange in animals and scabies in humans, are not very famous or particularly hot. Infections caused by *S. scabiei* are a worldwide problem among both animals and humans. The infection is very troublesome and often makes it possible for secondary, sometimes lethal, infections to establish (McCarthy *et al.*, 2004). According to the World Health Organization (WHO), about 300 million human cases of scabies are reported each year and, even though the infection occurs among any socioeconomic group, it is more prevalent in overcrowded and poor areas with insufficient water-supplies (WHO, 2009). Hence, *S. scabiei* is a problem for the poor and needy. Could this be a reason for the low number of hits? Probably yes, together with the fact that

S. scabiei infections alone seldom cause deaths as e.g. malaria infections do. However, it causes widespread misery for the host.

As mentioned above, an *S. scabiei* infection often makes it possible for other, often bacterial, infections to establish. This together with the severe itching connected with sarcoptic mange, makes it important to treat infected hosts as quickly as possible. The infection is treatable; however during recent years there have been reports of treatments failures that cannot be explained by wrong doses or failure to treat the surroundings to stop reinfections to occur. Hence, we might be facing a resistance[1] problem. To fight this it is important to know the underlying mechanisms.

The aim of this thesis is primarily to characterize glutathione transferases (GSTs) from *S. scabiei* and investigate their possible involvement in drug detoxification and resistance development, and also to investigate if the GSTs could be used as antigens for serological diagnosis of *S. scabiei* infections. More information on the enzymatic mechanisms of drug metabolism in *S. scabiei* is essential to understand the resistance development, and new findings could prolong the lifespan of acaricides used in treatment of both animals and humans. Additionally, I want to make people aware of the problem with sarcoptic mange and scabies and hence make *S. scabiei* more famous.

[1] What is drug resistance? When is an individual resistant to a drug? When is a population resistant to a drug? For some organisms, e.g. parasitic roundworms (nematodes), there are specific methods to detect and supervise resistance and for those organisms a specified threshold for resistant or sensitive has been established (Demeler *et al.*, 2009; Höglund *et al.*, 2009). For mites this is not available, and hence for *S. scabiei* it is not as simple to define resistance. In this thesis I will use the term resistance all the way trough. However, one should bear have in mind that for *S. scabiei* not much research has been done on this topic.

2 Background

The parasitic disease sarcoptic mange (infection in animals) or scabies (infection in humans) is caused by the itch mite *Sarcoptes scabiei* that affects both animals and humans worldwide. The infection is immunopathological and arises because of the burrowing activities of the mites in the upper layer of the skin (epidermis) of its host. Host cells are disrupted both mechanically and due to the secretion of cytolytic components. These cytolytic components and mite derived antigenic substances, faeces or eggs cause the immunopathogenic reactions (McCarthy *et al.*, 2004; Burgess, 1994). The primary clinical sign is an intense itching, and later on cellular changes in the skin might appear (Burgess, 1994). Depending on the immunopathological status of the host, the symptoms as well as the intensity, spread and course of event can vary greatly (Donabedian & Khazan, 1992). Some host animals (e.g. red foxes) are very susceptible and infections, often leading to high mortality rates (McCarthy *et al.*, 2004; Mörner, 1992). In the pig industry, between 50 and 95% of the herds were estimated to be infected by *S. scabiei* approximately ten years ago (Cargill *et al.*, 1997), hence various preventive measures were taken in order to limit the damages caused, both from an animal welfare and an economical perspective (Firkins *et al.*, 2001).

Most studies of *S. scabiei* are performed on mites from human hosts and much less has been done on *S. scabiei* from animal hosts. Hence, the background of this work will include many references to various aspects of human scabies together with references on sarcoptic mange, focusing on red foxes (*Vulpes vulpes*), dogs and pigs. For many years there has been a discussion about whether *S. scabiei* is one species or should be divided in multiple species, specific for each host organism. Even though it seems like there might be strains of mites more or less adapted to different hosts, there is so far no genetic evidence of multiple species or subspecies (Morrison, 2005). Either way, the bias in background information should not affect this

thesis. However, the species-question is an important factor to keep in mind when discussing the results.

In the Background I will first give a general background to the parasite. Some of the topics are closely linked to this thesis while others, e.g. clinical features, diagnosis and epidemiology of *S. scabiei* are not the focus of this thesis. However, to give an overall background of the parasite I will go through these topics as well. After the parasite has been presented the background of treatments, drug resistance, and GSTs will follow.

2.1 *Sarcoptes scabiei*

2.1.1 Classification and Morphology

The obligate parasite *S. scabiei* is a microscopic burrowing mite that belongs to the phylum Arthropoda, the class Arachnida and the subclass Acari (Fig. 1). The female is about 400 μm long and 300 μm wide while the male is approximately half her size. The body has an oval, tortoise-like shape with eight short legs that hardly project beyond the body brim. The most important characters are the numerous ridges and scales on the back of the mite, which are not seen on many other mange mites on mammals. Most of the mite is creamy white except for the legs and the mouthparts that are brown (Urquhart *et al.*, 2006; Burgess, 1994; Arlian, 1989).

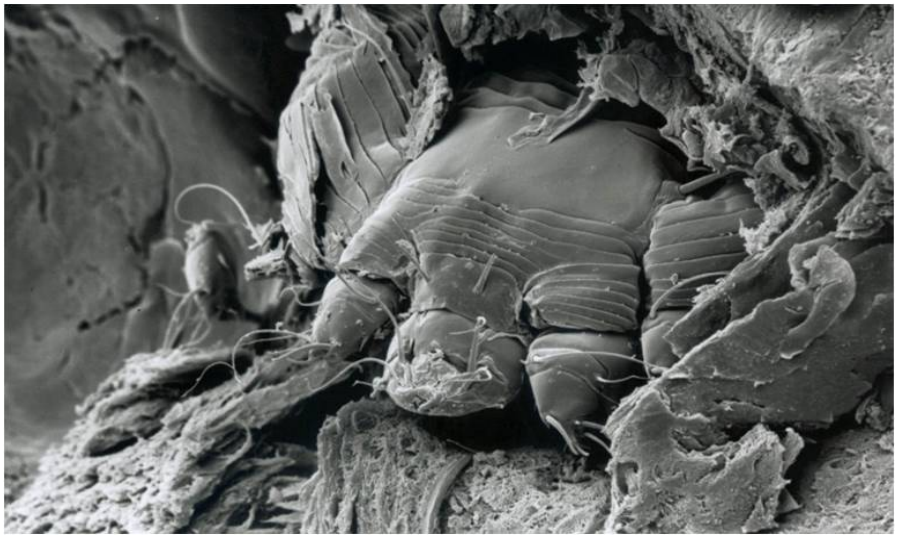


Figure 1. Electron microscopy picture of an *S. scabiei* mite in the skin of a red fox. Photo: Set Bornstein and Tapio Nikkilä.

2.1.2 Life Cycle

The life cycle of *S. scabiei* is direct and all life stages occur in the host (Fig. 2). *S. scabiei* is a burrowing mite, which means that it digs tunnels in the epidermis of its host. The fertilized females lay their eggs in these tunnels and the eggs hatch in 3-5 days. A six-legged larva is hatched, digs new tunnels and creates small "moulting pockets" where it develops to protonymph, tritonymph, and later on to an adult mite. The entire life cycle is completed in 10-21 days (Urquhart *et al.*, 2006; Green, 1989). Most life cycle studies have been performed on *S. scabiei* derived from human hosts and some on *S. scabiei* from canine hosts. However, it is presumed that the life cycle is the same in other hosts as well (Urquhart *et al.*, 2006; Arlian, 1989).

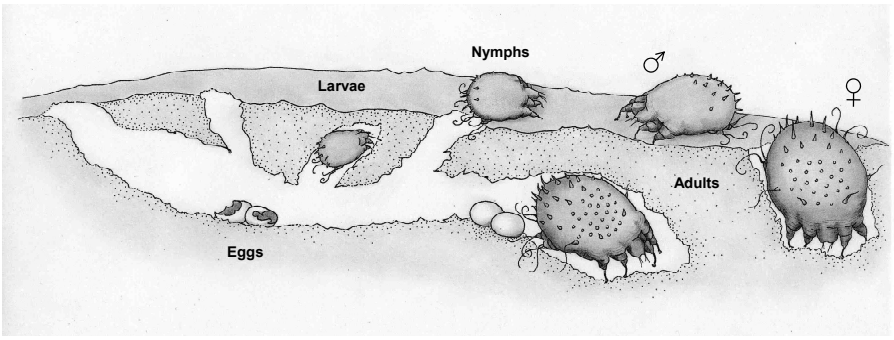


Figure 2. The life cycle of *S. scabiei*. The picture shows different life stages of *S. scabiei* in the epidermis of a host. For a more detailed description of the life cycle, see the text (section 2.1.2). Drawing: Katarina Näslund.

2.1.3 Transmission, Survival and Infectivity

It has been shown that all life stages frequently leave the tunnels and mooch around on the skin surface of the host (Arlian & Vyszenski-Moher, 1988). This suggests direct contact, including sexual contact, as the most important way of transmission (Walton & Currie, 2007). Additionally, *S. scabiei* has shown host-seeking behavior; hence a less important, but still a source of transmission, are fomites (e.g. bedding, clothing, towels, i.e. all sorts of objects) (Arlian, 1989).

The survival of *S. scabiei* off its host is very much affected by the ambient temperature and relative humidity (Arlian *et al.*, 1989; Arlian *et al.*, 1988a). Lower temperatures and higher relative humidity generally increase the survival time. In general, all life stages survive 1-9 days at 15-25 °C and 25-97% relative humidity. The ability of the mites to remain infective off the

host is important for the transmission and it has been shown, at room conditions that *S. scabiei* derived from pigs can infect new hosts for 24 hours and *S. scabiei* derived from canines remained infective for 36 hours after the death of its host. In general, experiments indicate that mites keep their infectivity for at least one half to two thirds of their survival time off their host. At -25 °C female mites survived for approximately one hour but were not infective and would not penetrate the skin of its host (Arlian, 1989).

Due to the requirements for *S. scabiei* to stay alive and infective off its host there is, so far, no *in vitro* culture system for *S. scabiei*. Today, the only way to culture *S. scabiei* in the laboratory is to use live animals as hosts. This system is used in various laboratories (Pasay *et al.*, 2009), but because this is ethically questionable, and only working with mite from naturally infected animals and humans limits the ability to obtain sufficient numbers of mites, it is desirable to find a way around this problem. One solution, for some applications, is to develop a molecular *in vitro* system and work with recombinant proteins expressed by e.g. bacterial cells.

2.1.4 Clinical Features and Diagnosis

Clinical signs of a primary *S. scabiei* infection in humans arise 4 to 6 weeks after infection (Walton & Currie, 2007). First an intense itching takes place and later on cellular changes in the skin might appear (Burgess, 1994). Depending on the immunological status of the host, the symptoms can vary a lot as well as the intensity, spread and course of event. For example, in humans with a normally functioning immune system the infection is limited to 10-100 parasites in total, but in individuals with reduced immune response you can find local areas with as many as 1400 mites/cm^2 or more (Donabedian & Khazan, 1992). When humans are infected with *S. scabiei* mites from animals the incubation period is shorter and the symptoms decline faster (Bornstein, 1995).

Clinical features in animals vary depending on which host organism is infected. Compared to humans the first visible symptoms are harder to identify in many animals because of the thick fur; however an intense itching is usually observed early (Bornstein, 1995).

A severe kind of *S. scabiei* infections may occur in several host species, e.g. humans, dogs, red foxes (Fig. 3) and pigs, called crusted or Norwegian scabies (Walton & Currie, 2007; Bornstein, 1995; Paterson *et al.*, 1995). This disease is characterized by a high number of mites and eggs, and the skin gets crusted in a loose, scaly and flaky or thick and adherent manner. Often these infections last much longer than regular *S. scabiei* infections (Walton & Currie, 2007). Crusted scabies is most often seen in individuals

with some kind of immunodeficiency but it occasionally occur in immunocompetent individuals as well (Roberts *et al.*, 2005).

Figure 3. Red fox with severe *S. scabiei* infection. Photo: Bengt Ekberg.

The primary confirmation of an *S. scabiei* infection is by a skin scraping, where an oil-covered scalpel blade is scraped across the skin of the infected individuals, where the scraped material stick to the blade. After this, collected material is analyzed using a microscope (Hengge *et al.*, 2006). This technique has a relatively low sensitivity; hence repeated scrapings might be necessary. Alternatively, the burrow ink test can be used. In this test the tunnels are identified through staining with a specific ink (Woodley & Saurat, 1981). Another approach is to take a blood sample and perform an enzyme-linked immunosorbent assay (ELISA) to detect *S. scabiei* specific antibodies (Bornstein *et al.*, 2006; Hollanders *et al.*, 1997). However, this serology on antibody detection is limited by the lack of *in vitro* culture systems, lack of reliable recombinant diagnostic antigens and cross-reactivity to house dust mite (*Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphys maynei*) antigens (van der Heijden *et al.*, 2000).

2.1.5 Epidemiology

If an infection is maintained in a population without the need of external inputs it is called endemic. Human *S. scabiei* infections are endemic in many tropical and subtropical areas worldwide (Walton & Currie, 2007).

In more developed parts of the world the infections occur more sporadically, and often as institutional outbreaks (de Beer *et al.*, 2006; Estes & Estes, 1993). Epidemiological analyses have shown that the occurrence of scabies does not depend on sex, race, age or socioeconomic status; however the general main risk factor to obtain scabies infection is poverty and overcrowded living conditions (Walton *et al.*, 2004b; Gibbs, 1996). This also accounts for red foxes where high population densities are known to raise the sarcoptic mange prevalence (Davidson *et al.*, 2008).

In Sweden, *S. scabiei* almost exterminated the population of red foxes (*Vulpes vulpes*) during the 1980's. Some foxes survived though, and since then the fox population has increased again. Nowadays it seems like the foxes can overcome a mange infection. It has been demonstrated that animals and humans with a *S. scabiei* reinfection have a lower mite burden and that some previously infected individuals can eliminate a reinfection (Arlian *et al.*, 1996). Hence, the decrease of *S. scabiei* infections in red foxes in Sweden might be a consequence of the selection during the eighties and the development of some kind of herd immunity (Arlian *et al.*, 1996). This has also been proposed for the red fox population in Norway, where they have showed that the sarcoptic mange prevalence decreased even though the population density raised (Davidson *et al.*, 2008). The mange epidemic among red foxes in Sweden is well described (Bornstein, 1995). The infection has also spread to other wild and domestic animals. For instance, it infected the previously naïve domestic dog population in Sweden, as it did in Norway (Bornstein, 1995). As mentioned earlier, *S. scabiei* also affects pigs where the infection, apart from animal welfare aspects, causes severe economic losses in the pig industry (Firkins *et al.*, 2001; Arends *et al.*, 1999).

In general, the Swedish pig population is very healthy compared to populations in other countries and the herds are associated with extended control programs (Wallgren *et al.*, 2004). In the 1990's the worldwide prevalence of sarcoptic mange varied from 20 to 90% within the pig populations and 70 to 90% of the herds were reported as infected (Wallgren & Bornstein, 1997). However, the introduction of macrocyclic lactones on the market facilitated eradication programs and the sarcoptic mange infections could be combatted. As a consequence of that, the Swedish pig herds are today declared free from sarcoptic mange (Wallgren *et al.*, 2004).

It has been proposed that there is a cyclic pattern of *S. scabiei* infections. However these data may not be truly representative, as the data often are based on different diagnostic and recording methods and come from countries with different social and physical environments (Walton & Currie, 2007; Orkin, 1971). However, in resource-rich communities, scabies seems to occur in cyclic epidemics in e.g. nursing homes and other institutional-living situations (Scheinfeld, 2004).

The estimated worldwide prevalence of *S. scabiei* infections in humans is 300 million cases, with large variations between different countries (WHO, 2009). In e.g. UK, practitioners recorded approximately 1200 new cases of scabies per year in the 1990s; and in remote Aboriginal areas of Northern Australia the prevalence of scabies is around 50% in children and 25% in adults (Wong *et al.*, 2002; Downs *et al.*, 1999). In Sweden, there are no up-to-date prevalence data available for scabies. But for sarcoptic mange in domestic dogs there are case reports from one of the biggest Swedish insurance companies for animals (AGRIA). These numbers show that 200 to 500 cases of *S. scabiei* infections have been reported per year from 1995 to 2007 (oral communication[2]). Additionally, this shows that since 2001 the number of cases seems to have declined.

More detailed information on the epidemiology of *S. scabiei* is unfortunately hard to find. This could be explained by the problems with diagnosing *S. scabiei* infections and the fact that the infections often are mistaken for other skin diseases (Hengge *et al.*, 2006).

2.1.6 Host Interaction and Immunology

An *S. scabiei* infection is immunopathological and arises because of the burrowing of the mite in the epidermis of its host. Host cells are lysed both mechanically and due to the secretion of cytolytic components. These cytolytic components and mite derived antigens are believed to cause the immunopathogenic or hypersensitivity reactions (Hengge *et al.*, 2006; McCarthy *et al.*, 2004; Burgess, 1994). In an experiment using rabbits as an experimental model it was shown that host protective immunity was acquired when the rabbits were exposed to an *S. scabiei* infection (Arlian, 1989). This immunity develops during the period from infection and first symptom, in approximately 4 weeks, and longer (more than 100 days) exposure to the parasite seems to raise the immunity (Walton *et al.*, 2004b; Estes & Estes, 1993). It has also been shown that infections with *S. scabiei* from dogs induce immunity in rabbits (Arlian *et al.*, 1996; Arlian *et al.*,

[2] Agneta Egenvall, Ruminant Medicine and Veterinary Epidemiology, Unit for Epidemiology, Swedish University of Agricultural sciences, Uppsala, Sweden.

1994). The predominant lymphocytes are T lymphocytes (Falk & Matre, 1982) and a humoral immune response is also evoked, because serum antibodies, primarily IgE and IgG, directed at *S. scabiei* antigens have been detected (Walton *et al.*, 2004b; Arlian, 1989).

Although scabies mite inactivated protease paralogues (SMIPPs), paramyosin and glutathione transferases have been suggested as important antigens (Fischer *et al.*, 2009; Dougall *et al.*, 2005; Mattsson *et al.*, 2001) the information on *S. scabiei* specific diagnostic antigens is limited.

2.1.7 Host Specificity

Before the introduction of molecular techniques morphological analysis was the major tool to differ *S. scabiei* mites from each other. These analyses show no or little differences between mites from different hosts and do not indicate that there are host-specific species of *S. scabiei* (Arlian, 1989). On the other hand, experimental cross-infection studies were unsuccessful at transferring mites from one host to another (Arlian *et al.*, 1988b; Arlian *et al.*, 1984). These results show that transmission of *S. scabiei* between different host species is unusual. However, mites from dogs have established infections on New Zealand white laboratory rabbits (Arlian *et al.*, 1984) and mites from red foxes have transmitted the infection to dogs. Also, mites from various animals, e.g. dogs, pigs and foxes, infect humans; hence *S. scabiei* is classified as a zoonosis (although, these infections are generally self-limiting) (Bornstein, 1995; Beck, 1965).

With the introduction of molecular techniques many research groups have tried to find an answer to the question if *S. scabiei* is host-specific or not. However, the results are contradictory (Alasaad *et al.*, 2009). Different phylogenetic analyses have been performed, and e.g. Walton *et al.* (2004a), analyzed mitochondrial sequences from mites derived from animals and humans from different continents. They concluded that genotypes were different for human mites compared to animal mites, hence animal-human transmission is rare and not important to consider when e.g. establishing control programs. However, these findings have been questioned by subsequent phylogenetic analyses performed on the same data set. These results show that genotypes are shared between hosts, no matter what continent they came from. Hence, today there is no evidence of host specificity for *S. scabiei* (Morrison, 2005).

Today a designation of different "variants" of *S. scabiei* is used, e.g. *S. scabiei* var. *hominis* and *S. scabiei* var. *canis*; however I'm not certain that it is a good way to classify *S. scabiei* at this time. As mentioned above, there is no evidence of host specificity for *S. scabiei* and I think the "variant"

designation confuses more than it clarifies. Hence, I will not use these designations in this thesis.

2.2 Treatment of *S. scabiei* Infections

S. scabiei infections are cured with chemotherapeutic drugs called acaricides or scabicides. In this thesis I will use the term acaricides. Mainly these drugs are applied topically (on the skin) but oral treatments and injections are also available. There are various acaricides available on the market, and choice is mainly based on host species, age, state of health, potential toxicity, cost and availability (Walton *et al.*, 2004b).

A few years ago lindane (organochlorine) was the topical acaricide mainly used to treat *S. scabiei* infections in humans. However, because of its potential neurotoxicity it has been abandoned in Australia and the European Union (Walton & Currie, 2007; Hengge *et al.*, 2006). However, lindane is still used in many parts of the world because of its efficacy and cost-effectiveness (Hengge *et al.*, 2006). In Australia and the European Union permethrin (pyrethroid) is instead the most commonly used topical acaricide nowadays. Worldwide, oral treatments or injections with ivermectin (macrocyclic lactone) are also widely used. In many areas, especially where *S. scabiei* is endemic, prophylactic treatments are more cost-effective than treatment after diagnosis. These treatments are usually performed with either permethrin or ivermectin. However, when treating crusted scabies it is common to use a combination of any topical acaricide together with oral ivermectin (Meinking *et al.*, 1995). Other acaricides used, but not as commonly as permethrin and ivermectin, are e.g. sulphur compounds, benzyl benzoate and crotamiton (Walton *et al.*, 2004b). New drugs are also starting to occupy the market. These include essential oils in which terpenoids are most likely to be the active substance (Walton *et al.*, 2004c). When comparing the cost of the acaricides, permethrin is clearly more expensive than the others, which is a problem in poorer parts of the world (Mounsey *et al.*, 2008; Walton *et al.*, 2004b).

In Sweden, there are various drugs available for pigs, and the active substances are ivermectin, foxim (organophosphorous compound) and doramectin (macricyclic lactone). The recommended treatment practice is prophylactic treatment of sows 1-4 weeks prior to farrowing and often a subsequent treatment is performed (Eliasson-Selling, 2000; Wallgren & Bornstein, 1997). For dogs there are three topical formulations available and the active substances in these are permethrin, foxim and selamectin

(macrocyclic lactone) (FASS, 2009). The acaricides used for veterinary purposes in Sweden are summarized in Table 2.

Table 2. *Drugs used for sarcoptic mange treatment in dogs and pigs in Sweden (FASS, 2009).*

Trade name	Active substance	Host species	Formulation
Bimectin® vet.	Ivermectin	Pig, cattle, reindeer	Injection
Dectomax Suis vet.	Doramectin	Pig	Injection
Exspot® vet.	Permethrin	Dog	Spot-on
Ivomec® vet.	Ivermectin	Pig	Oral in feed
Ivomec® vet.	Ivermectin	Pig, cattle, reindeer	Injection
Noromectin vet.	Ivermectin	Pig, cattle, reindeer	Injection
Sebacil® vet.	Foxim	Dog, pig, sheep	Cutaneous solution
Sebacil® pour on vet.	Foxim	Pig	Pour-on
Stronghold	Selamectin	Dog, cat	Spot-on
Virbamec® vet	Ivermectin	Pig, cattle, reindeer	Injection

2.2.1 Acaricide Resistance and *S. scabiei*

The intensive use of e.g. pyrethroids and ivermectin has led to resistance problems in arthropods and nematodes worldwide (Gilleard, 2006; Hengge *et al.*, 2006). Problems arising with resistance are a threat to the general health of both animals and humans. Hence, it is important to have reliable techniques to monitor if and when resistance to a drug develops and also to develop new drugs (Walton *et al.*, 2000). Whether we have resistance to acaricides among *S. scabiei* mites is not yet firmly decided. Earlier, treatment failures have been shown to arise because of e.g. incorrect application of the acaricide and failure to treat all contacts leading to reinfections (Wong *et al.*, 2002). However, recently there have been reports of treatment failures in human patients where these factors could be ruled out (Currie *et al.*, 2004; Walton *et al.*, 2000; Purvis & Tyring, 1991; Roth, 1991; Hernandez-Perez, 1983), and hence it seems like resistance is establishing. Both clinical treatment failures and *in vitro* resistance have been documented for ivermectin (Mounsey *et al.*, 2009a; Holt *et al.*, 2007; Currie *et al.*, 2004). For example, a recent acaricide sensitivity assay using ivermectin showed that the *in vitro* survival times of *S. scabiei* mites derived from human patients have doubled over 10 years (Mounsey *et al.*, 2009a). Treatment failures with lindane have also been reported (Purvis & Tyring, 1991; Roth, 1991). Additionally, *in vitro* efficacy studies in Northern Australia using 5% permethrin showed that when permethrin was introduced on the market 100% of *S. scabiei* mites were dead after 1-hour exposure, but approximately

ten years later 35% of the mites were still alive after 3 hours and 4% were still alive after 18-22 hours of exposure (Walton *et al.*, 2000; Fraser, 1994).

As mentioned earlier, prophylactic treatments are often used in some areas. This, together with the fact that *S. scabiei* has a short and direct life cycle, could favor resistance development (Mounsey *et al.*, 2008). So far, there are no reports of resistance problems for animals. However, for quite a long time the recommended treatment for infected dogs (two treatments 10-14 days apart) has not been sufficient, and more than two treatments have been necessary (oral communication[3]). Additionally, prophylactic treatments are the treatment of choice in e.g. Swedish pig herds (Wallgren & Bornstein, 1997) and could easily speed up possible resistance to acaricides.

2.3 Resistance Mechanisms to Acaricides

In this thesis we have investigated how recombinant versions of *S. scabiei* GSTs interact with 11 different acaricides: the organophosphorous compounds chlorpyriphos, coumaphos, diazinon and ethion; the pyrethroids deltamethrin, flumethirn and permethrin; the formamidine amitraz; the macrocyclic lactone ivermectin; and the organochlorines dichlorodiphenyltrichloroethane (DDT) and lindane. Because resistance has been reported to ivermectin, lindane and permethrin for *S. scabiei*. I will concentrate on these groups of substances and just briefly go through the other substances.

Generally, drug resistance occurs through three different strategies: 1) changes in the drug target, resulting in structural differences altering the interaction of the acaricide with the target, hence reducing the sensitivity of the drug; 2) elevated removal of the drug by membrane bound efflux pumps; or 3) changes in metabolic enzymes, through binding or metabolization of the drug before it reaches its target site (Ffrench-Constant, 2007; Li *et al.*, 2007; Walton *et al.*, 2004b; Feyereisen, 1995).

2.3.1 Macrocyclic Lactones

The resistance mechanisms of the macrocyclic lactone ivermectin are proposed to be complex, multifactorial and differ between closely related organisms. The target for ivermectin is the ligand-gated ion channels (LGICs). These channels mediate neurotransmission in muscles and neurons; and LGICs have also been suggested as the target for ivermectin in *S. scabiei* (Mounsey *et al.*, 2007). Mutations in LGIC genes have been linked to

[3] Set Bornstein, National Veterinary Institute, Uppsala, Sweden.

ivermectin resistance among both nematodes and arthropods (Dong, 2007; McCavera *et al.*, 2007). Also, ivermectin is a substrate for the ATP-binding cassette (ABC) transporters, e.g. P-glycoprotein (PgP), and allelic changes, together with increased transcription of the genes for this efflux pump, have been linked to resistance in many species, including parasites (Eng & Prichard, 2005). Additionally, analysis of the macrocyclic lactone abamectin indicates that metabolic mechanisms, so far mainly cytochrome P450s (CYP450s), might be involved in resistance development in arthropods (Clark *et al.*, 1995). A newly performed study showed an elevated expression of metabolic enzymes, e.g. GSTs, and PgP in ivermectin exposed *S. scabiei* mites compared to unexposed mites (Mounsey *et al.*, 2009b).

2.3.2 Organochlorines

The organochlorine lindane is a so called cyclodiene and the target of these substances is the γ-aminobutyric acid (GABA) receptor, an important receptor in the nervous system. Experiments have shown that a single amino acid change in this receptor confers resistance in arthropods (Ffrench-Constant, 2007; Feyereisen, 1995). Also, metabolic enzymes, e.g. GSTs, have been linked to lindane resistance (Syvanen *et al.*, 1996). Another organochlorine included in this thesis is the well-known bridged diphenyl DDT. The target site for this substance is the voltage-gated sodium channel (Vssc). This channel is essential for normal transmission of nerve impulses, and it has been shown that changes in these channels, together with elevated metabolization of the drug by various GSTs, confer resistance in insects (Wang *et al.*, 2008; Davies *et al.*, 2007).

2.3.3 Pyrethroids

Target alteration and enzymatic degradation are the main mechanisms involved in resistance to pyrethroids (e.g. permethrin, deltamethrin and flumethrin) (Walton *et al.*, 2004b). The target for permethrin is the Vssc, and it has been shown that only a few mutations in this gene confer resistance (Pasay *et al.*, 2008; Davies *et al.*, 2007; Ffrench-Constant, 2007). These point-mutations are known as knockdown resistance or *kdr* type resistance, and are present among many different arthropods (Rosario-Cruz *et al.*, 2009). Additionally, metabolic enzymes, e.g. CYP450s, esterases and GSTs, have been linked to permethrin resistance in arthropods. For example, GSTs are proposed to be involved in resistance development to pyrethroids in the German cockroach (*Blattelidae* species) (Enayati & Motevalli Haghi, 2007). Additionally, in the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*, an arthropod closely related to *S. scabiei*, both a target

alteration in *Vssc* and metabolic degradation by esterases have been shown to confer resistance to permethrin (Jamroz *et al.*, 2000). Alterations in the *Vssc* gene and metabolic enzymes, e.g. GST, esterase and CYP450, have lately been proposed to confer permethrin resistance in *S. scabiei* (Pasay *et al.*, 2009; Pasay *et al.*, 2008)

2.3.4 Organophosphorous Compounds and Formamidines

The organophosphorous compounds (e.g. foxim, diazinon, ethion, chlorpyriphos and coumaphos) have the enzyme acetylcholinesterase as their target. Mutations in this enzyme have been identified as the main reason for resistance (Ffrench-Constant, 2007; Feyereisen, 1995). Also, metabolic enzymes, e.g. GSTs, have been linked to organophosphorous resistance (Huang *et al.*, 1998). Resistance to the formamidine amitraz has been reported for the cattle tick *R. microplus,* but the resistance mechanism is still unknown (Jonsson & Hope, 2007).

2.3.5 Drug metabolism

For all three acaricides to which resistance has been reported for *S. scabiei*, metabolic enzymes have an important role. Because this is the focus of this thesis I will give a deeper introduction of drug metabolism in eukaryotic cells.

All eukaryotic cells have a molecular system that takes care of foreign or unwanted molecules, so-called xenobiotics. The system makes the xenobiotics more water soluble and hence less toxic to the cell, and transport the processed xenobiotics out of the cell. This system consists of various enzymes, e.g. CYP450 and GSTs. Earlier this system was divided into "Phase I": including oxidation, reduction, and hydrolyses of xenobiotics, "Phase II": conjugation reactions, and "Phase III": transport of the processed xenobiotic out of the cell. However, further research revealed that it was more complicated than this. Today, it is proposed that the drug metabolism system should consist of five groups of reactions (Josephy & Mannervik, 2006):

1. *Oxidations*. Mainly catalyzed by CYP450s but also e.g. peroxidases, and alcohol dehydrogenase. Included in old "Phase I".
2. *Reductions*. Relevant substrates are e.g. aldehydes and compounds containing nitro groups. Included in old "Phase I".
3. *Reactions between electrophilic groups of xenobiotic substrates and the most important cellular nucleophiles, water and glutathione*. Include e.g. the

hydrolysis of esters, amides, epoxides, and many reactions catalyzed by GSTs. Included in old "Phase I" and "Phase II".

4. *Reactions of nucleophilic functional groups in xenobiotics with endogenous agents*. Includes the processes such as glucuronidation, sulfation and amino acid conjugation. Included in old "Phase I".
5. *Membrane transport processes*. Include the ATP-dependent export of glutathione conjugates and glucuronides across cell membranes, catalyzed by transporters as the multidrug resistance-associated protein (MRP). Included in old "Phase III".

Because this thesis deals with GSTs, my work concerns group 3: reactions between electrophilic groups of xenobiotic substrates and the most important cellular nucleophiles, water and glutathione. GSTs are a big family of enzymes that have been linked to resistance to many different insecticides and acaricides (Li *et al.*, 2007). In the next section I will give you a deeper background of these enzymes.

2.4 Glutathione Transferase

GSTs are a large family of soluble or microsomal multifunctional enzymes with important roles in drug metabolism in eukaryotic cells. Not all of them are transferases but they are still collectively called GSTs because the first findings of these enzymes concerned transfer reactions (Josephy & Mannervik, 2006). First, GSTs were linked to cancer development in humans. For example, a mutated *GSTM1* gene renders higher risk of developing proximal colon cancer (Hadfield *et al.*, 2001; Rebbeck *et al.*, 1999). It has also been shown that an increased GST activity can confer resistance to cancer treatments (McIlwain *et al.*, 2006; Uchida, 2000). GSTs' main reaction is to catalyze the conjugation between the tripeptide glutathione (GSH) with electrophiles (reagents attracted to electrons) to form more water soluble, non-toxic compounds, for further transportation out of the cell. In addition, GSTs can have non-catalytic functions, e.g. binding of non-substrate ligands (Frova, 2006). In this thesis I will concentrate on the GST conjugation reactions but also briefly on their ligand binding properties.

2.4.1 Glutathione

The tripeptide GSH (Fig. 4) is a thiol, a compound that contains a functional group composed of a sulfur-hydrogen bond ("-SH"), occurring in all aerobic organisms (Edalat, 2002; Reed, 1990).

The concentration of GSH in humans is approximately between 0.3 and 10 mM, depending on cell (Samiec *et al.*, 2000; Smith *et al.*, 1996). GSH has many important functions in the cell. For example, GSH defends the cell against oxidative and electrophilic chemical compounds. The functional thiol group makes GSH a good nucleophile for reactions with the electrophilic chemical compounds. Most organic electrophilic compounds can form conjugates with GSH under suitable conditions, unless e.g. steric hindrance occurs (Josephy & Mannervik, 2006).

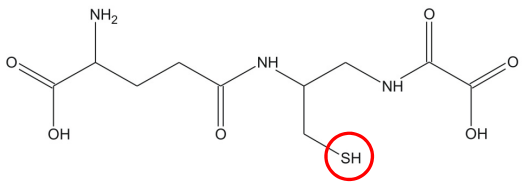


Figure 4. Chemical structure of the tripeptide GSH. The enzymatically important thiol group is indicated with a red circle.

There are many GSH dependent enzymes that are part of the cellular protection against xenobiotic substances. Some examples are selenium dependent glutathione peroxidases and glyoxalase I and II (Arthur, 2000; Thornalley, 1993). Another GSH-dependent enzyme, and the one important for this thesis, is GST. As mentioned above GSTs have many properties, among which catalyzing the conjugation of GSH to various electrophilic compounds is one of the most investigated (Edalat, 2002).

2.4.2 Classification, Nomenclature and Evolution of GST

Earlier the GSTs were called "glutathione S-transferases", but this naming is misleading because the sulfur atom alone (from GSH) is not transferred. However the abbreviation "GST" is still kept even though the recommended enzyme name is "glutathione transferase" (Josephy & Mannervik, 2006).

Human GSTs were the first GSTs investigated, and the nomenclature system used has been applied on GSTs from other species as well (Chelvanayagam *et al.*, 2001). The soluble GSTs are today divided into many different classes based mainly on sequence similarities, and these classes are designated by the names of the Greek letters Alpha, Mu, Pi, and so on, abbreviated in Latin capitals: A, M, P etc. Within each class the enzymes are numbered using Arabic numbers. For example, the gene for the Mu class 1

is written *GSTM1* or *gstm1* (italicized capital letters for human genes and italicized lower case letters for other species). The functional enzyme can be either homo- or heterodimeric, and homodimeric enzymes are named e.g. GSTM1-1 while the heterodimeric enzyme is named e.g. GSTA1-2, which means that the enzyme constitutes of polypeptides expressed from varying exons. When needed, a prefix is added to the enzyme name to distinguish GSTs from different species, e.g. mGSTA1-1 and rGSTA1-1 from mouse and rat, respectively (Josephy & Mannervik, 2006). For human GSTs, enzymes belonging to the same class may have more than 90% sequence identity, and a variable lower limit is 50% sequence identity to distinguish enzymes from different groups. This classification means that the human GSTs cluster on different chromosomes, which lends strong support to this classification system. Additionally, the numbers of exons and introns show distinctive differences between classes (Josephy & Mannervik, 2006).

Today seven classes of cytosolic GSTs are recognized in mammals: Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega (Frova, 2006; Josephy & Mannervik, 2006). Plants have six classes: Lambda, Phi, Tau, dehydroascorbatereductase (DHAR), Theta and Zeta (Frova, 2006). Six classes have been recognized in insects: Delta, Epsilon, Sigma, Theta, Zeta and Omega (Enayati *et al.*, 2005). In bacteria the picture is less clear, but Theta and bacteria specific Beta GSTs are present together with other enzymes that possibly are GSTs (Frova, 2006).

The microsomal GSTs are membrane-bound proteins with distinctive structural differences compared to the soluble ones, and form their own family called membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Josephy & Mannervik, 2006; Enayati *et al.*, 2005).

The proposed evolution of the GST family is based on sequence comparisons. From this, Theta GSTs are considered the first GST to have evolved, with their introduction predating the prokaryotic-eukaryotic split (Frova, 2006). After this, the mammalian and plant specific GSTs have evolved from a Theta-class gene duplication (Pemble & Taylor, 1992). The overall structures of GSTs describe a high folding similarity, not only among GSTs, but also with many other protein families. Hence, the evolution of GSTs is considered in a larger context together with the evolution of e.g. the thioredoxin family. Uniquely for GSTs, their functions are multiple and quite diversified, but they still share many functional links with members of the thioredoxin family (Frova, 2006). To further investigate the evolution of GSTs it has been shown that it is changes in the active site that are important to look at (Frova, 2006; Josephy & Mannervik, 2006).

In most organisms, including e.g. insects, it has been shown that local gene duplications have resulted in expansions of the GST family. Because minor amino acid changes have shown dramatic effects on enzyme activity, gene duplication can be a mechanism to specify reactions by GSTs to face particular ecological niches (Ranson *et al.*, 2002).

2.4.3 *S. scabiei* GSTs

Through expressed sequence tag (EST) analysis various genes coding for GSTs have been identified in the *S. scabiei* genome (Dougall *et al.*, 2005; Pettersson *et al.*, 2005; Fischer *et al.*, 2003; Ljunggren *et al.*, 2003). Sequences for six genes from mites derived from humans are deposited in GenBank. The corresponding genes from mites derived from red foxes have also been identified (one has been deposited in GenBank), and no consistent sequence differences have been shown. Three of the *S. scabiei* GSTs have been classified as Delta GSTs and three as Mu GSTs, and all of them are homodimeric enzymes (based on *in vitro* analyses of recombinant versions of the enzymes). A summary of the *S. scabiei* GSTs and their nomenclature is presented in Table 3.

2.4.4 GST Gene Organization, Regulation and Expression

GSTs are a very variable family of enzymes and different organisms often have many genes encoding unique GSTs. To raise the variability some species, e.g. *Musca domestica* (house fly), the *Anopheles* species (mosquito malaria vectors) and humans, also use alternative splicing. This feature includes the variable use of exons, i.e. exons can be excluded or included in the finally processed mRNA (Fig. 5). Using this technique the organism can produce many more GST variants than there are actual genes (Josephy & Mannervik, 2006; Enayati *et al.*, 2005).

The level of GST activity can vary a lot between different life stages and also between different tissues of e.g. insects. This is probably due to differences in gene regulation, and it has been proposed that *gst* expression is regulated by many different inducers or environmental signals (Clark, 1989). For example, very high GST activities have been identified in the midguts of insects, which is an important site for the detoxification of xenobiotics (Enayati *et al.*, 2005).

Table 3. *Nomenclature for* S. scabiei *GSTs. This nomenclature was agreed on in September 2009 by the Swedish and Australian research groups working on* S. scabiei *and GSTs.*

Gene	Protein	GenBank nucleotide accession number	Previous designations
ssgstd1	SsGSTD1-1	AY649788[a]	Delta-class GST (Paper I)
		AY825935[b]	ssGST delta I (Paper II)
			GST-delta 2 (Mounsey *et al.*, 2009b)
ssgstd2	SsGSTD2-2	AY825936[b]	GST-delta 3 (Mounsey *et al.*, 2009b)
ssgstd3	SsGSTD3-3	AY825937[b]	GST-delta 1 (Mounsey *et al.*, 2009b)
ssgstm1	SsGSTM1-1	AY825933[b]	*S. scabiei* GST (Fischer *et al.*, 2003)
			SsGST01 (Dougall *et al.*, 2005)
			ssGST mu II (Paper II)
			GST-mu 1 (Mounsey *et al.*, 2009b)
ssgstm2	SsGSTM2-2	AF462190[b]	ssGST mu I (Paper II)
			GST-mu 2 (Mounsey *et al.*, 2009b)
ssgstm3	SsGSTM3-3	AY825934[b]	GST-mu 3 (Mounsey *et al.*, 2009b)

[a] Sequence from *S. scabiei* derived from red foxes

[b] Sequence from *S. scabiei* derived from humans

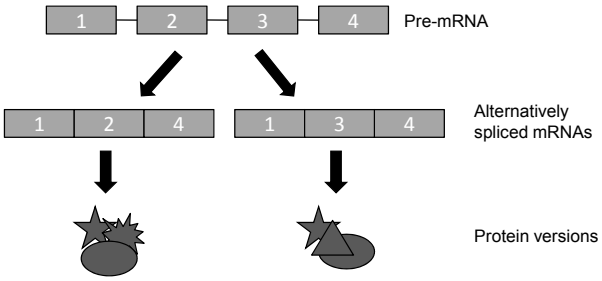


Figure 5. Schematic picture of alternative splicing. Each numbered box corresponds to an exon.

2.4.5 Structure of Soluble GSTs

Normally, functional GSTs are dimeric enzymes where each subunit is approximately 25 kDa. To know more about their catalytic properties the three-dimensional structures for many GSTs have been solved by X-ray crystallography. Even though the amino acid sequence identity is low

between some of them, the over-all three-dimensional structures are very similar, but with significant differences in the active site region (Josephy & Mannervik, 2006). For example, six Delta GSTs from insects have had their structures determined: a Delta GST from the Australian sheep blowfly *Lucilia cuprina* complexed with glutathione (Wilce *et al.*, 1995), and five from the mosquito malaria vectors *Anopheles dirus* (Ad) and *Anopheles gambiae* (Ag): AdGST1-3 (complexed with glutathione, RCSB Protein Data Bank (PDB) ID: 1JLV), AdGST1-4 (apo form, PDB ID: 1JLW) (Oakley *et al.*, 2001), AdGSTD1-5 (complexed with glutathione sulfonic acid, PDB ID: 1R5A) (Udomsinprasert *et al.*, 2005), AdGSTD1-6 (complexed with glutathione sulfonic acid, PDB ID: 1V2A) and AgGSTd1-6 (complexed with s-hexylglutathione, PDB ID: 1PN9) (Chen *et al.*, 2003). So far, no structure for GSTs from the Acari subclass (mites and ticks) has been determined.

Each subunit of GST consists of two domains; the N-terminal and the C-terminal (Fig. 6). The N-terminal includes approximately the 80 first amino acids and is essentially an α/β structure. The C-terminal domain is an α-structure and includes most of the amino acids interacting with the electrophilic substrates. Few amino acids are conserved in the GST family, but those are all important for proper folding of the enzyme (Josephy & Mannervik, 2006).

2.4.6 Active Site of GST

The GSTs have a high specificity for the tripeptide GSH, and this is associated with the N-terminal domain. This region also includes a proline residue that is highly conserved among GSTs and thioredoxin proteins. In GSTs, this proline renders a sharp turn of the polypeptide backbone, which allows the formation of two hydrogen bonds between the chain of the protein and the GSH. The "G-site", which is the binding pocket for GSH, has multiple polar bonds that contribute to the binding of the charged GSH (Josephy & Mannervik, 2006). As mentioned in section 2.5.3, Delta and Mu GSTs are found in *S. scabiei*. In Delta GSTs a serine residue is important for the catalysis, by hydrogen bonding to the GSH, and in Mu GSTs this residue is a tyrosine (Josephy & Mannervik, 2006; Udomsinprasert *et al.*, 2005). None of these residues in the G-site seems to be responsible for ionization of the thiol group of GSH. Instead this reaction may be promoted by the positive end of the dipole formed by the α1-helix (Josephy & Mannervik, 2006).

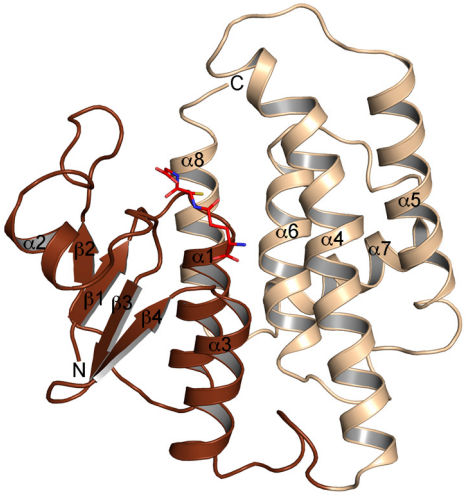


Figure 6. An *A. dirus* species B Delta GST (AdGSTD1-3) subunit with the various α-helices and β-sheets numbered. The N-terminal domain is shown in brown and the C-terminal in beige. The tripeptide GSH (red, blue and yellow) is shown in the structure to highlight the active site position.

The "H-site" is the hydrophobic binding site for binding of the various electrophilic substrates. This site varies a lot more than the G-site between different GSTs. Here the β1-strand and the α1- and α4-helices and part of the C-terminal domain are mainly active. They end up close to each other in the folded enzyme and form a pocket close to the G-site. This pocket is quite flexible and may allow an induced fit of the substrate. Many experiments propose that differences in the H-site are the main reason for substrate specificity differences, and hence changes in the H-site are proposed to be the main feature to establish new evolutionary mechanisms among GSTs (Josephy & Mannervik, 2006).

2.4.7 Acaricide Resistance and GST

In section 2.3.5 I briefly introduced the drug metabolism of eukaryotic cells. In this section I will go deeper into how the GST mechanisms are associated with resistance to acaricides.

Many GSTs have been shown to metabolize xenobiotics or non-covalently bind xenobiotics without further metabolization. The non-covalent binding is a so-called ligandin mechanism, where the enzyme binds e.g. a xenobiotic without further metabolization, acting as a binding and transport protein (Edalat, 2002). In this thesis I will mainly concentrate on the metabolic features of GSTs, but the ligandin function is mentioned because this feature could be important in the emerging resistance to

acaricides in *S. scabiei*. As before, I will concentrate on macrocyclic lactones, organochlorines and pyrethroids.

Most studies of organochlorine resistance in insects are performed with DDT in insects, e.g. *Anopheles* and *Drosophila* species. These studies have shown that various GSTs, e.g. Delta GSTs, dehydrochlorinate DDT and that the resistant strains over express these GSTs, and hence no gene amplification is present (Verhaeghen *et al.*, 2009; Lumjuan *et al.*, 2005; Ortelli *et al.*, 2003; Ranson *et al.*, 2001). Additionally, site-specific mutations of GSTs have been linked to DDT resistance in insects (Low *et al.*, 2007). GSTs have also been shown to metabolize lindane (Wei *et al.*, 2001; Syvanen *et al.*, 1996). For example, in *M. domestica* GST metabolization of lindane has been linked to resistance, and here over expression of the gene also seems to be an important factor (Syvanen *et al.*, 1996). For pyrethroids, GSTs are mainly involved in ligandin activities or taking care of the detoxification of peroxidation products induced by pyrethroids (Li *et al.*, 2007), but in the *Anopheles* species they are also suggested to have metabolic activities conferring resistance (Verhaeghen *et al.*, 2009). In the brown planthopper *Nilaparvata lugens* it has been shown that most GSTs involved in pyrethroid resistance had peroxidase activity (Vontas *et al.*, 2002). Resistant strains of this insect had more copies of the gene compared to susceptible strains. Hence pyrethroid resistance was conferred by gene amplification (Li *et al.*, 2007). To date, there are no reports of mite GSTs conferring resistance to the macrocyclic lactone ivermectin.

2.4.8 Acaricide Resistance and *S. scabiei* GSTs

GSTs have been suggested to confer resistance to ivermectin and permethrin in *S. scabiei*. Quantitative real-time PCR (qRT-PCR) has been performed to investigate whether the expression of GSTs is changed in ivermectin exposed compared to unexposed mites (Mounsey *et al.*, 2009b) and in permethrin resistant compared to sensitive *S. scabiei* mites (oral communication[4]). The qRT-PCR results for permethrin could be challenged because the experiment was performed with mites from different hosts (resistant strain from dog and sensitive strain from pigs). Hence host-parasite interactions might affect the results, and this has to be further investigated. However, the qRT-PCR results indicate that SsGSTD3-3 and SsGSTM1-1 are significantly upregulated in ivermectin exposed mites compared to unexposed mites while both of these as well as SsGSTD1-1 are upregulated in permethrin resistant mites compared to sensitive mites.

[4] Kate Mounsey, Queensland Institute of Medical Research, Brisbane, Australia and Menzies School of Health Research, Darwin, Australia.

Additionally, results published by Pasay *et al*. (2009) show that treating permethrin resistant *S. scabiei* mites with a mixture of permethrin and diethyl maleate (DEM) raised the sensitivity for the drug. DEM is a common GST inhibitor and synergist. Synergists block metabolic pathways, hence restoring susceptibility to a drug. It has also been shown that the total GST activity in permethrin resistant mites is higher compared to GST activity in sensitive mites. This elevated GST activity was significantly inhibited by the addition of DEM (Pasay *et al*., 2009). However, again, the results need further testing because the experiment was performed with mites from different hosts. Hence host-parasite interactions might affect the results.

3 Aims of the Thesis

The overall aim of this thesis was to characterize GSTs from *S. scabiei* and to investigate their possible involvement in acaricide detoxification, and also to investigate if the GSTs could be used as antigens for serological diagnosis of *S. scabiei* infections. More information on the enzymatic mechanisms in *S. scabiei* is essential to understand the resistance development, and new findings could prolong the lifespan of acaricides used in treatment of both animals and humans.

More specifically, the aims of the separate studies were to:

- Classify and characterize a new *S. scabiei* GST. Further, to establish an *in vitro* system for biochemical and antigenic characterization of *S. scabiei* GSTs and use this system to characterize the new *S. scabiei* GST (I)

- Biochemically characterize one Delta and two Mu *S. scabiei* GSTs and investigate their possible interactions with acaricides (II)

- Solve the crystal structure of one *S. scabiei* Delta GST and use this structure together with homology modeling and docking studies to characterize possible interactions between all *S. scabiei* Delta GSTs and various acaricides and DEM (III)

4 Comments on Materials and Methods

Detailed descriptions of the materials and methods used are given separately in each paper (I-III).

4.1 The *S. scabiei* DNA (I-III)

All molecular work in this thesis was performed on DNA or RNA extracted from *S. scabiei* mites derived from wild Swedish red foxes (*Vulpes vulpes*). Isolation of mites was performed as previously described by Bornstein and Zakrisson (1994). Briefly, pieces of skin from red foxes with natural sarcoptic mange infections were put in Petri dishes and left at room temperature under an electric light source. Because of the warmth from the light source, live mites, of all life stages, migrated to the inside of the lids of the Petri dishes, and after 2-4 hours the migrated mites were derived and killed by freezing (-70 °C). Before DNA or RNA extraction an aliquot of mites (including all life stages) were thawed and manually homogenized in a ceramic mortar filled with liquid nitrogen.

4.2 Phylogeny (I)

In paper I phylogenetic analysis was performed to investigate which class the newly identified *S. scabiei* GST belonged to. The main objective in doing this was to find a representative group of GSTs from other organisms to compare with. Hence, most amino acid sequences from model species that had had their GSTs evaluated (the insects *Anopheles gambiae* and *Drosophila melanogaster*, the vertebrates *Homo sapiens* and *Mus musculus*, and the nematode *Caenorhabditis elegans*) were chosen. Also, known Acari GSTs and a selection of platyhelminth and crustacean sequences were included.

For this thesis an additional phylogenetic analysis of the Mu GSTs was performed. This analysis was performed because SsGSTM3-3 differs notably from SsGSTM1-1 and SsGSTM2-2, both in the sequence identity and in the catalytically behavior. This analysis included manually derived alignments of peptide sequences for the N- and C-terminal domains from the Pfam database (http://pfam.sanger.ac.uk) for all of the Metazoa (protein families PF02798 and PF00043, respectively); 791 accessions were included in the analysis and 183 amino acids were aligned.

The final alignments were edited by hand for consistency using MacClade v4.05 (http://www.macclade.org) and the alignments were analyzed by neighbor-joining based on distances derived from the Jones-Taylor-Thornton amino acid substitution model, using the Phylip v3.6a3 package (http://evolution.genetics.washington.edu/phylip.html). For the first analysis, the robustness of the branches was evaluated using 1000 bootstrap replicates, and the stability of the topology was assessed by comparing it to the maximum-likelihood tree based on the same substitution model.

4.3 Genomic Organization (II)

It is well known that *gsts* often cluster in the genome in various organisms (Soranzo *et al.*, 2004; Ortelli *et al.*, 2003; Morel *et al.*, 2002), and hence analyses to identify possible gene clusters in the *S. scabiei* genome were performed. As a first and simple test specific primers, directed upstream in the 5'-end and downstream in the 3'-end, for each *S. scabiei gst* were produced. Then a regular PCR was run, and the identified fragments were sequenced and analyzed.

4.4 Recombinant Protein Preparation (I-III)

4.4.1 Subcloning

All open reading frames (ORFs) for the *S. scabiei* GSTs, used in biochemical analysis, were subcloned into the expression vector pET-14B (Novagen, Madison, WI). The resulting plasmids had the *ssgst* linked to a histidine tag (His_6-tag) in the 5'-end. In paper I, *ssgstd1* was also subcloned into a modified version of the expression vector pMAL-c2 (New England Biolabs, Waltham, MA) to compare the enzymatic activity of the enzyme, expressed from different plasmids. A His_6-tag followed by a stop codon was introduced between the *Pst*I and *Hind*III sites of pMAL-c2. The resulting plasmid had

the *ssgstd1* ORF linked to the *Escherichia coli* gene coding for a maltose binding protein (*mbp*) in the 5'-end and a His_6-tag coding sequence in the 3'-end.

SsGSTD1-1 used for crystallization, immunization of rabbits and also biochemical analyses in paper I, had its ORF subcloned into another modified version of the expression vector pMAL-c2. The *mbp* gene was removed and a His_6-tag followed by a stop codon was introduced as described above. The *ssgstd1* ORF had an additional AAA-codon (lysine) inserted directly after the start codon, to enhance the expression of the recombinant protein (Stenstrom *et al.*, 2001). The resulting plasmid had *ssgstd1* linked to a His_6-tag in the 3'-end. A test to subclone the other *S. scabiei gsts* into this modified pMAL-c2 vector was performed as well, but for unknown reasons the cloning was unsuccessful. Hence, the pET-14b system was used for the biochemical analysis.

4.4.2 Protein Expression and Purification

All recombinant *S. scabiei* GSTs, except for rSsGSTD3-3 and rSsGSTM3-3, were satisfactorily expressed in *E. coli* strain BL21(DE3). For rSsGSTD3-3 and rSsGSTM3-3, expression in BL21(DE3) did not result in any detectable expression, hence *E. coli* strain Rosetta(DE3) had to be used instead. All recombinant *S. scabiei* GSTs were satisfactorily expressed either in LB- or minimal medium supplemented with various heavy metal ions.

Purification of the recombinant enzymes was performed using pre-packed affinity- or ion exchange columns (Amersham Biosciences, Uppsala, Sweden). All recombinant *S. scabiei* GSTs, except for rSsGSTD3-3 and rSsGSTM3-3, were purified in one step using their GST-activity. Here GSTrap FF columns were used, where the matrix contains GSH which the enzymes bind to. The reason for not using GSTrap for rSsGSTD3-3 and rSsGSTM3-3 was because these two enzymes did not bind sufficiently to the GSTrap matrix. Hence, rSsGSTD3-3 and rSsGSTM3-3 were purified in two steps using their His_6-tags. As a first step, HiTrap Chelating columns were used. The matrix of this column includes e.g. Ni^{2+}-ions which the His_6-tag of the recombinant protein binds to in a chelating manner. This affinity purification step was followed by ion exchange purification with HiTrap Q columns to get sufficient purity of the recombinant protein. A summary of expression and purification strategies is shown in Table 4. Additionally, Table 4 shows which paper each *S. scabiei* GST was included in. SsGSTM3-3 was not included in any of the papers, but analyses have been done and the results will be presented in Results and discussion.

Table 4. *Summary of expression and purification strategies for recombinant* S. scabiei *GSTs and which paper each* S. scabiei *GST is included in.*

Protein	Plasmid name	Bacterial cells	Column	Paper
rSsGSTD1-1[a]	pPU98	BL21(DE3)	GSTrap	I
rSsGSTD1-1[b]	pPU99	BL21(DE3)	GSTrap	I- III
rSsGSTD1-1[c]	pPU100	BL21(DE3)	GSTrap	I and III
rSsGSTD2-2	pPU111	BL21(DE3)	GSTrap[d]	III
rSsGSTD3-3	pPU114	Rosetta(DE3)	1) HiTrap Chelating 2) HiTrap Q[e]	III
rSsGSTM1-1	pPU109	BL21(DE3)	GSTrap	II
rSsGSTM2-2	pPU108	BL21(DE3)	GSTrap	II
rSsGSTM3-3	pPU116	Rosetta(DE3)	1) HiTrap Chelating 2) HiTrap Q[f]	-

[a] rSsGSTD1-1 used in biochemical analysis.

[b] rSsGSTD1-1 used for biochemical analysis.

[c] rSsGSTD1-1 used for crystallization, immunization of rabbits and biochemical analyses.

[d] 5 mM DTT was added to harvested cells before sonication and 0,1 mM DTT was added to each fraction of eluted protein after purification.

[e] Binding buffer: 20 mM TrisHCl pH 7.5, elution buffer: 20 mM TrisHCl pH 7.5 with 0.5 mM NaCl.

[f] Binding buffer: 20 mM TrisHCl pH 10.0, elution buffer: 20 mM TrisHCl pH 10.0 with 0.5 NaCl.

4.5 Western Blot Analyses and Immunolocalization (I)

GSTs have also been suggested as possible diagnostic antigens for *S. scabiei* infections (Dougall *et al.*, 2005). Hence all six recombinant *S. scabiei* GSTs were analyzed for their interactions with various sera from naturally infected animals. The rSsGSTD1-1 was used to detect IgG antibodies (ab's) in sera from dogs and pigs, and also IgE ab's in dog sera. The remaining recombinant *S. scabiei* GSTs were used to detect IgG and IgE ab's in sera from red foxes. The results for rSsGSTD1-1 are presented in paper I, while the remaining results are presented in Results and discussion.

To investigate where in the parasite SsGSTD1-1, SsGSTM1-1 and SsGSTM2-2 were located; immunolocalization analyses were performed, as described in paper I. The first step was to produce polyclonal ab's specifically directed at each one of the recombinant versions of *S. scabiei* GSTs. For SsGSTD1-1 the whole purified recombinant enzyme was used for

immunization of rabbits, while only short peptides of SsGSTM1-1 and SsGSTM2-2 were used. The two Mu enzymes have a high amino acid identity, and hence the peptide immunization strategy was chosen to be able to immunize with short peptides of the enzymes that clearly differed.

4.6 Structure Determination of SsGSTD1-1 (III)

Knowledge about the three-dimensional structure of an enzyme can give valuable information for understanding how the enzyme works. In paper III X-ray crystallography was used to determine the structure of SsGSTD1-1. Briefly, this experimental method uses diffraction patterns resulting from the interaction of X-rays with the electrons in the molecule to determine the relative position of its atoms (Fig. 7). Because the X-ray scattering from one single molecule is too weak to detect, crystals that consist of millions of ordered copies of the same molecule are used.

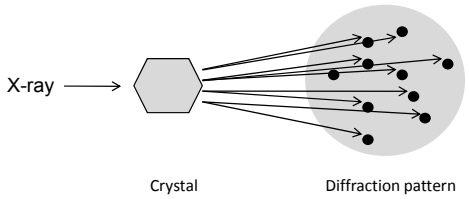


Figure 7. Schematic description of X-ray crystallography.

To obtain crystals through the hanging drop vapor-diffusion technique, a highly pure sample of rSsGSTD1-1 was used. A solution of the purified enzyme was mixed with a crystallization solution of pH 7.0 including polyethylene glycol (PEG) and HEPES. Two constructs of SsGSTD1-1 were tested, one with a His_6-tag in the N-terminal part and one with a His_6-tag in the C-terminal part of the enzyme. The construct with a C-terminal His_6-tag quickly produced ordered crystals, while the other construct did not give rise to any usable crystals. However, further modification would probably have provided good crystals for this construct as well, but this was not done. Before diffraction data were derived at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France, crystals were incubated in protective cryo solution supplemented with 25% (v/v) glycerol and flash-frozen in liquid nitrogen.

As mentioned in the Background (section 2.4.5), GSTs from different classes and different organisms share more or less the same three-dimensional

structure. Earlier, many GSTs have had their three-dimensional structure solved; hence the molecular replacement method was used to determine the structure of SsGSTD1-1. This method tries to determine both the orientation and the position of the experimental data compared to the already solved structure. For the experiment in paper III the structure for a Delta GST from the mosquito *Anopheles dirus* species B (AdGST1-3, PDB ID: 1JLV) was used, because it had the highest sequence identity compared to SsGSTD1-1 of those Delta GSTs that had had their three-dimensional structures deposited in PDB.

4.7 Homology Modeling and Docking Studies (III)

For SsGSTD2-2 and SsGSTD3-3, homology modeling was used to predict their three-dimensional structures. First a ClustalW2 sequence alignment together with SsGSTD1-1 was performed, and SsGSTD1-1 was then used as a template for the modeling of SsGSTD2-2 and SsGSTD3-3.

To investigate possible binding modes between the *S. scabiei* Delta GSTs and various acaricides and DEM, docking studies were performed. This is a computational technique that explores possible binding modes between e.g. an enzyme and a substrate. The docking studies in paper III were performed with the crystal structure of SsGSTD1-1 and the homology models of SsGSTD2-2 and SsGSTD3-3. For the acaricides DDT, ethion, lindane and permethrin and the synergist DEM a search space centered on and encompassing the active site was used. For the acaricide ivermectin, which is a much bigger molecule and which was assumed not to being able to enter the active site, the search space was enlarged to include the whole enzyme. The molecular structures of the acaricides and DEM are shown in Figure 1 in paper III.

4.8 Kinetic Analysis (I-III)

4.8.1 Steady State Analysis (I-III)

To determine the apparent kinetic parameters (K_M, k_{cat} and k_{cat}/K_M) for *S. scabiei* GSTs in the presence of varying concentrations of GSH and the model substrate 1-chloro-2,4-dinitrobenzene (CDNB), a spectrophotometric assay under steady-state conditions was used. To calculate the apparent K_M value the data were plotted as a non-linear regression plot according to the Michaelis-Menten equation. Under steady-state conditions the k_{cat} value was calculated as the ratio between the

maximum velocity and total enzyme concentration. Graphs and calculations were performed using GraphPad Prism v4. Each assay was run in triplicate, and the results were expressed as the calculated mean with standard error of mean. The data were analyzed by comparing 95% confidence intervals, with intervals that did not overlap zero indicating significant differences in apparent kinetic parameters.

K_M is the so-called Michaelis constant and its units are mol l^{-1} or M. Roughly, this constant indicates how tightly the enzyme binds a substrate; a substrate bound weakly will have a higher K_M value and a substrate bound tightly will have a lower K_M value. The k_{cat} parameter is the so-called turnover number and its unit is s^{-1}. This parameter is a rate constant that represents the number of μmoles of substrate converted per μmole of enzyme per second. The k_{cat}/K_M parameter is known as the catalytic efficiency of the enzyme and it represents how efficiently free enzyme reacts with free substrate.

The model substrate CDNB is a well-known substrate widely used in GST research worldwide. The main objective with the steady-state analyses in this thesis was to detect differences between the recombinant *S. scabiei* GST enzymes, and not to investigate what reactions the enzymes catalyze in the parasite; hence CDNB was a good substrate to use. It is also a good substrate to start with when characterizing new GSTs. The reaction is easy to prepare and easy to follow using a regular spectrophotometer, measuring at a wavelength of 340 nm, at which the product *S*-2,4-dinitrophenylglutathione (DNP-SG) has maximal absorbance (Josephy & Mannervik, 2006) (Fig. 8).

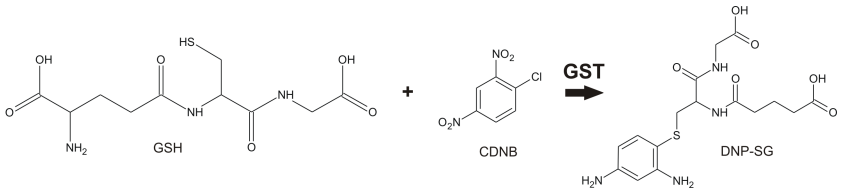


Figure 8. Chemical reaction between GSH and CDNB catalyzed by GST.

4.8.2 Inhibition Analysis with Acaricides and DEM (II-III)

In paper II and III it was investigated whether various acaricides did inhibit the reaction between *S. scabiei* GSTs and CDNB. In total 11 different acaricides were tested: four organophosphorous compounds (the organothiophosphates diazinon, ethion, chlorpyriphos and coumaphos), three pyrethroids (the pyrethroid esters deltamethrin, flumethrin and

permethrin), one formamidine (amitraz), one macrocyclic lactone (ivermectin), and two organochlorines (lindane and the bridged diphenyl DDT).

The standard GST assay described above were used, but with fixed GSH, CDNB and acaricide concentrations. All acaricides were diluted in methanol and because I did not know the stability of the diluted acaricides I prepared new dilutions before each set of measurements. Additionally, all controls included the same concentration of methanol as the samples with acaricide. The general acaricide concentration used was 0.1 mM; however in paper II 0.2 mM of the acaricides was also analyzed, but this concentration were excluded in further analysis because there were dilution problems for some acaricides at this concentration. The use of 0.1 mM acaricide was chosen because this is a well established *in vitro* setup of similar experiments performed by other research groups (da Silva Vaz *et al.*, 2004; Prapanthadara *et al.*, 1998). Each assay was run in triplicate, and the results were expressed as the calculated mean with standard error of mean. The data were analyzed by comparing 95% confidence intervals, with intervals that did not overlap zero indicating significant change of activity.

Permethrin, ivermectin and lindane were chosen because they have been or are widely used acaricides to treat *S. scabiei* infections, and resistance problems have been reported for all three. Because this thesis is focusing on resistance in *S. scabiei*, these three acaricides are the most important included in our work.

DDT is banned from use in most parts of the world today. However it has been used a lot for many years worldwide, and as mentioned in the Background, the resistance in e.g. insects is wide-spread, and many studies, including structural studies, have been performed where mutations in GSTs have been linked to DDT resistance. The rest of the acaricides were chosen to have various acaricides from different acaricide groups for comparison.

One active substance used in sarcoptic mange treatment in Sweden is foxim (organophosphorous compound). Foxim was not included in this analysis; however, four other organophosporous compounds were analyzed instead (see above). The active substances abamectin and doramectin (both macrocyclic lactones), which are also used to treat sarcoptic mange infections in Sweden, are also not included in our analysis. However, these molecules are closely related to ivermectin, which was included.

As mentioned in the Background (section 2.4.8) the synergist DEM is a well-known GST inhibitor. Because this synergist has been suggested to reduce resistance to permethrin in *S. scabiei* (Pasay *et al.*, 2009) I analyzed how DEM inhibits the different recombinant *S. scabiei* GSTs. The

experiment was setup to resemble the experiment performed by Pasay *et al.* (2009). The results for the recombinant *S. scabiei* Delta GSTs are included in paper III, while the results for the recombinant *S. scabiei* Mu GSTs will be presented in Results and Discussion.

5 Results and Discussion

5.1 The GST Family in *S. scabiei* (I-III)

Using phylogenetic analysis the first Delta GST outside insects was identified (Paper I). Additionally, two other Delta GSTs and three Mu GSTs have been identified (Dougall *et al.*, 2005; Fischer *et al.*, 2003). Hence, the *S. scabiei* genome includes at least six different genes coding for GSTs. However, to be certain about the total number of *gsts* in the *S. scabiei* genome, whole-genome sequencing would be necessary.

It has been shown that *gsts* from the same class have a similar distribution of exons and introns (Josephy & Mannervik, 2006). Analyses if the S. scabiei *gst* nucleotide sequences showed that all *S. scabiei* Delta genes had introns while only one of the Mu genes had an intron. In more detail, *ssgstd1* and *ssgstd2* had two introns and *ssgstd3* and *ssgstm3* had one intron each.

It has also been shown that *gsts* from the same class often cluster in the genome (Soranzo *et al.*, 2004; Ortelli *et al.*, 2003; Morel *et al.*, 2002). A simple PCR-test to see if any gene clustering of the *S. scabiei gsts* could be detected was performed. The results showed that *ssgstm1* and *ssgstm2* cluster somewhere in the *S. scabiei* genome with a 500 bp long intergenic region (Fig. 2, paper II). Because both of these genes also lack introns the clustering indicates that evolutionary gene duplication might have occurred, so that the *ssgstm1* and *ssgstm2* originate from a single gene (Soranzo *et al.*, 2004; Kumar & Reddy, 2001). This gene duplication has probably occurred to tailor the diversification and adapt to a new ecological niche. Through a small number of substitutions, important differences in enzymatic activity have been achieved (Enayati *et al.*, 2005). No other clusterings were detected using this simple PCR technique; hence more work has to be done to find out how the *gst* genes are localized in the *S. scabiei* genome, e.g. southern blot

analyses or, once again, whole-genome sequencing would give useful knowledge about this.

Each subunit for SsGSTD1-1, SsGSTD2-2, SsGSTM1-1 and SsGSTM2-2 had predicted molecular weights of approximately 26 kDa, while SsGSTD3-3 had a predicted weight of 25 kDa and SsGSTM3-3 a predicted weight of 31 kDa. The amino acid sequence identities between classes were below 20% while the identities within a class varied from 36% to 77% (Table 5).

Table 5. *Amino acid sequence identity (%) for* S. scabiei *GSTs*.

	SsGSTD1-1	SsGSTD2-2	SsGSTD3-3	SsGSTM1-1	SsGSTM2-2
SsGSTD1-1	100	-	-	-	-
SsGSTD2-2	68	100	-	-	-
SsGSTD3-3	40	39	100	-	-
SsGSTM1-1	19	16	14	100	-
SsGSTM2-2	18	17	14	77	100
SsGSTM3-3	15	13	13	38	36

When comparing the sequences of the three *S. scabiei* Mu GSTs, SsGSTM3-3 stands out. It has a quite low amino acid sequence identity compared to the other two Mu GSTs (Table 5), and has an extra tail of 29 amino acids. Also, as is evident later on in this chapter, this enzyme does not have the same biochemical properties as the other two Mu GSTs. An extra phylogenetic analysis to determine if this *S. scabiei* GST actually belongs to the Mu class was performed. In this analysis SsGSTM3-3 did cluster with the included Mu GSTs; however it was in the fringe of the group (Fig. 9). In the nucleotide sequence of *ssgstm3* there was a repeated sequence with 5 thymines, starting at position 705 in the ORF, which was where the extra tail started (Fig. 10). This repetition of thymines could cause a shift in the transcription of the gene or translation of the mRNA; hence different ORFs might be produced. This could result in a dysfunctional version of the enzyme, or it might be different functional versions of the enzyme present in the mite. These different versions of the enzyme could have different biochemical properties, to perform different tasks in the mite. To find an answer to this, one would have to capture the GSTs actually expressed in the parasite and analyze these native versions of the enzymes.

Figure 9. Phylogenetic tree for the Mu GSTs from Metazoa, showing the locations of the three *S. scabiei* gene sequences (boldface). Aligned peptide sequences for the N-terminal and C-terminal domains were manually collacted from the Pfam database for all of the Metazoa (protein families PF02798 and PF00043, respectively). This alignment (791 accessions, 183 aligned amino acids) was analysed by neighbor-joining based on distances derived from the Jones-Taylor-Thornton substitution model, using the Phylip v3.6a3 package. The labels are the accessions names from the UniProt database, along with the GST annotation ("-" if unannotated). Only the relevant part of the full phylogenetic tree is shown, and the Alpha, Pi and Sigma classes have been collapsed to single nodes. The scale bar represents the inferred genetic distance. Accession number Q8I9R9 to SsGSTM1-1, Q2YFF0 corresponds to SsGSTM2-2 and Q2YFE9 to SsGSTM3-3.

```
           551                                                  600
SsGSTM1-1  CACCAGAAAT TTTCACCAAA TTTCCAAACC TAAATAGCTA CATTACT...
SsGSTM2-2  CACCGGAAGT TTTTGCAAAA TTTCAAAACC TAGGCAACTA TGTTAAT...
SsGSTM3-3  ATCCAGATCT CTTTGGGCAA AATCAACAAT GGAGAAATTT GATTGATTTT

           601                                                  650
SsGSTM1-1  ......CGCA TTGAATCGAT GCCGAAAATC TCTGCCTACA TCAAACAACA
SsGSTM2-2  ......CGAT TCGAATCGAT GCCAAAAATA TCGGCCTATC TTAAACAACA
SsGSTM3-3  CTACATCGAA TCGAATCGTT TCCAACCATA AAAGAATA.. TCAATATTCT

           651                                                  700
SsGSTM1-1  AGAGCCTCAA TTATTCAAC. ....GGTCCA ATGGCGAAAT GGAATA...C
SsGSTM2-2  ACAACCTCAA TTCTTCAAC. ....GGTTTG ATGGCGAAAT GGAATA...C
SsGSTM3-3  GAGGATTATA TTCGTCATCC TAGTGGCCTA TTGATCGCCT GGTACGAGGC

           701                                                  750
SsGSTM1-1  AAAATATTA. .......... .......... .......... ..........
SsGSTM2-2  AAAATATTA. .......... .......... .......... ..........
SsGSTM3-3  GAAATTTTTC TCCACATTCA ATCGATCGTT AGGTGATCAA CCAAGCGAAC

           751                                        791
SsGSTM1-1  .......... .......... .......... .......... .
SsGSTM2-2  .......... .......... .......... .......... .
SsGSTM3-3  AATTACGAAA AGAATTCATT AGGTCAGAGA TGGTTAGCAA T
```

Figure 10. Nucleotide sequence alignment of *S. scabiei* Mu *gst* partial ORFs (from position 551 to the end of each ORF). Repeated thymine sequence that might cause a transcription or translation shift is underlined in red.

When comparing the *S. scabiei* GST amino acid sequences from mites derived from red foxes to mites derived from humans differences for two of the enzymes were detected. The amino acid sequences for SsGSTD2-2 differed at two positions while the sequences for SsGSTM2-2 differed at one position (Fig. 11). Sequence analysis performed in Australia has also identified amino acid differences at the same positions in SsGSTD2-2 between mites derived from humans, pigs and dogs (oral communication[5], data not shown). What the amino acid differences really mean is hard to say because too little is known about the *S. scabiei* genome and variations there in. However, the positions are not directly involved in catalytically important motifs of the enzyme (Fig. 13). The amino acid differences identified might indicate species or geographical specificities, but it could also be regular differences at an individual level.

[5] Kate Mounsey, Queensland Institute of Medical Research, Brisbane, Australia and Menzies School of Health Research, Darwin, Australia.

```
A)
                1                                                    50
SsGSTD2-2_h  MGSIRPIIYW MAESPPCRTL YAVTKLLGID CEWKVLDLSQ KEHMKPDFLT
SsGSTD2-2_r  MGSIRPIIYW MAESPPCRTL YAVTKLLGID CEWKVLDLSQ KEHMKSDFLT

                51                                                   100
SsGSTD2-2_h  INPFHCVPTM VESDGFKLWE SRVICKYLIE SRNIETALYP KDLKKRAIID
SsGSTD2-2_r  INPFHCVPTM VESDGFKLWE SRVICKYLIE SRNIETALYP KDLKKRAIID

                101                                                  150
SsGSTD2-2_h  RCLHFDLGTL YRALADVVYD AFYVGKPNLA KLPRLEEVLQ MMEDNLAKTN
SsGSTD2-2_r  RCLHFDLGTL YRALADVVYD AFYVGKPNLA KLPRLEEVLQ MMEDNLAKTN

                151                                                  200
SsGSTD2-2_h  SNYLAQTDEP TLADISTYFS LSILEIVSEF DLAKYFKLFS WKQRMNEFIK
SsGSTD2-2_r  SNYLAQSDEP TLADISTYFS LSILEIVSEF DLAKYFKLFS WKQRMNEFIK

                201                          228
SsGSTD2-2_h  SIDDGTFATG QANIIAFAKK MMDQHKA.
SsGSTD2-2_r  SIDDGTFATG QANIIAFAKK MMDQHKA.


B)
                1                                                    50
SsGSTM2-2_h  MSSKPTLGYW NIRGLAQPIR MMLSYAGVDF VDKRYNYGPA PDFDRSEWLN
SsGSTM2-2_r  MSSKPTLGYW NIRGLAQPIR MMLSYAGVDF VDKRYNYGPA PDFDRSEWLN

                51                                                   100
SsGSTM2-2_h  EKFNLGLDFP NLPYYIDGDV KLTQSLAILR YLARKHKLDG HNEQEWLRIA
SsGSTM2-2_r  EKFILGLDFP NLPYYIDGDV KLTQSLAILR YLARKHKLDG HNEQEWLRIA

                101                                                  150
SsGSTM2-2_h  LCEQQIVDLY MAMGRISYDP NFEKLKPDYL EKLPDNLKLF SEFLGDHPFV
SsGSTM2-2_r  LCEQQIVDLY MAMGRISYDP NFEKLKPDYL EKLPDNLKLF SEFLGDHPFV

                151                                                  200
SsGSTM2-2_h  AGTNLSYVDF FVYEYLIRLK AMTPEVFAKF QNLGNYVNRF ESMPKISAYL
SsGSTM2-2_r  AGTNLSYVDF FVYEYLIRLK AMTPEVFAKF QNLGNYVNRF ESMPKISAYL

                201                 219
SsGSTM2-2_h  KQQQPQFFNG LMAKWNMKY
SsGSTM2-2_r  KQQQPQFFNG LMAKWNTKY
```

Figure 11. Amino acid sequence alignment of A) SsGSTD2-2 from mits derived from humans (h) and mites derived from red foxes (r), and B) SsGSTM2-2 from mites derived from humans (h) and mites derived from red foxes (r). Identified amino acid differences are underlined in red.

5.2 Expression, Purification and Steady State Analysis (I-III)

5.2.1 Protein Expression and Purification

All recombinant *S. scabiei* GSTs, except for rSsGSTD3-3 and rSsGSTM3-3, were successfully expressed in BL21(DE3) *E. coli* strain. For rSsGSTD3-3 and rSsGSTM3-3, Rosetta(DE3) *E. coli* strain had to be used instead. Rosetta(DE3) is a BL21 derivate created to enhance expression of proteins

containing codons rarely used in *E. coli*. These codons are AGG, AGA, AUA, CUA,CCC and GGA (Tegel *et al.*, 2009), and so the number of these codons in the *S. scabiei gsts* was counted. The *ssgstm1* had a total of 10 rarely used codons, *ssgstd1*, *ssgstd2* and *ssgstm2* had 11, *ssgstd3* had 15 and *ssgstm3* had 22. Hence, *ssgstd3* and *ssgstm3* had a higher number of rare codons compared to the other genes, which could explain the need to use the Rosetta(DE3) strain for successful expression of these genes. This has also been shown for expression of human recombinant proteins in *E. coli* BL21(DE3) compared to Rosetta(DE3) (Tegel *et al.*, 2009).

Purification of the recombinant enzymes was performed through affinity or ion exchange purification. The rSsGSTD1-1, rSsGSTD2-2, rSsGSTM1-1 and rSsGSTM2-2 were all successfully purified using their GSH binding activity, while this was not possible for rSsGSTD3-3 and rSsGSTM3-3. For these two enzymes the His_6-tag had to be used to obtain successful purification. To enhance the purification yield different suggestions from the GSTrap manufacturer were tested, but this did not improve the purification results using the GSH binding activity. These findings indicate that rSsGSTD3-3 and rSsGSTM3-3 did not bind GSH sufficiently to allow purification using their GSH binding activity, or that the proteins were misfolded when expressed in *E. coli*.

5.2.2 Steady State Analysis

In the steady-state analysis all recombinant enzymes, except for rSsGSTM3-3, were catalytically active with the model substrate CDNB. Comparing them class-wise, the Delta GSTs had more or less the same parameters with some notable differences (Table 6). For example, rSsGSTD2-2 and rSsGSTD3-3 had significantly higher turnover numbers (k_{cat}) for CDNB than rSsGSTD1-1, and rSsGSTD3-3 had a significantly higher catalytic efficiency (k_{cat}/K_M) for GSH compared to rSsGSTD1-1 (Table 6). Interestingly, rSsGSTD3-3 did not bind GSH any more weakly than the other enzymes, because the K_m values were in the same range for all enzymes. A weaker binding of GSH was expected for rSsGSTD3-3 because it was not possible to purify this enzyme using its GSH-binding properties. Why it was not possible to use the GST-activity to purify rSsGSTD3-3 is still an open question. Because it was possible to purify rSsGSTD3-3 satisfactorily using the His_6-tag, no further attempts were performed.

Regarding the recombinant Mu enzymes, rSsGSTM1-1 and rSsGSTM2-2 were catalytically active with CDNB but rSsGSTM3-3 showed no activity (Table 7). The rSsGSTM2-2 bound CDNB more weakly (K_M) than rSsGSTM1-1 and rSsGSTM1-1 had a higher turnover number (k_{cat}) for

GSH than rSsGSTM2-2. Also the catalytic efficiencies (k_{cat}/K_M) for both CDNB and GSH differed between the two Mu enzymes (Table 7).

Table 6. *Kinetic parameters for steady-state catalysis of CDNB and GSH by recombinant* S. scabiei *Delta GSTs*.

Kinetic parameters	rSsGSTD1-1	rSsGSTD2-2	rSsGSTD3-3
K_m (mM) CDNB	0.3 ± 0.0	0.4 ± 0.1	1.2 ± 0.4
K_m (mM) GSH	0.5 ± 0.1	0.7 ± 0.2	0.3 ± 0.0
k_{cat} (S^{-1}) CDNB	1.9 ± 0.1[a]	14.0 ± 1.3[b]	20.3 ± 2.7[b]
k_{cat} (S^{-1}) GSH	2.0 ± 0.1[a]	4.6 ± 0.3[a]	7.0 ± 0.3[a]
k_{cat}/K_m (S^{-1} mM^{-1}) CDNB	6.9 ± 0.8	39.5 ± 9.3	16.0 ± 5.3
k_{cat}/K_m (S^{-1} mM^{-1}) GSH	4.3 ± 0.5[c]	6.5 ± 1.5	21.0 ± 3.4[b]

Values are from three independent experiments, showing the mean ± standard error. Significant differences were based on 95% confidence intervals.

[a] Significant difference compared to the other two enzymes.

[b] Significant difference compared to rSsGSTD1-1.

[c] Significant difference compared to rSsGSTD3-3.

Table 7. *Kinetic parameters for steady-state catalysis of CDNB and GSH by recombinant S. scabiei Mu GSTs*.

Kinetic parameters	rSsGSTM1-1	rSsGSTM2-2	rSsGSTM3-3
K_m (mM) CDNB	0.3 ± 0.0[a]	4.2 ± 0.6[a]	nd
K_m (mM) GSH	0.3 ± 0.0	0.4 ± 0.0	nd
k_{cat} (S^{-1}) CDNB	10.3 ± 0.6	6.2 ± 0.4	nd
k_{cat} (S^{-1}) GSH	9.4 ± 0.3[a]	3.8 ± 0.1[a]	nd
k_{cat}/K_m (S^{-1} mM^{-1}) CDNB	38.3 ± 6.1[a]	1.5 ± 0.2[a]	nd
k_{cat}/K_m (S^{-1} mM^{-1}) GSH	30.6 ± 3.4[a]	9.5 ± 1.1[a]	nd

Values are from three independent experiments, showing the mean ± standard error. Significant differences were based on 95% confidence intervals.

nd=not detectable

[a] Significant difference compared to the other rSsGSTM.

Comparing the kinetic parameters of *S. scabiei* recombinant GSTs with parameters for recombinant GSTs from other organisms was difficult, because the recombinant enzymes were most often produced in different expression systems, which might affect the results (Paper I). However, I compared the kinetic parameters for rSsGSTD1-1, rSsGSTD2-2 and rSsGSTD3-3 with the parameters for the Delta GSTs that have been crystallized, and had their structures deposited in PDB, and saw that most of the parameters are in the same range (Udomsinprasert *et al.*, 2005; Chen *et*

al., 2003; Oakley *et al.*, 2001). However, the catalytic efficiency (k_{cat}/K_M) for the recombinant *S. scabiei* Delta GSTs generally seems to be a little bit lower. This could be due to the differing expression systems used or biological differences between GSTs from different organisms.

As mentioned earlier, rSsGSTM3-3 does not share the characteristics of the other recombinant *S. scabiei* Mu GSTs. It has obvious sequence differences and also completely different biochemical properties. The steady-state results indicate that SsGSTM3-3 either performs different tasks in the parasite compared to the other *S. scabiei* GSTs, or a dysfunctional version of the enzyme not active in the parasite was detected. To find out if it is a functional enzyme with other catalytically properties, its interaction with other GST substrates has to be evaluated.

5.3 Localization of *S. scabiei* GSTs in the Mite

To investigate where in the mite SsGSTD1-1, SsGSTM1-1 and SsGSTM2-2 localized, rabbits were immunized to produce specific polyclonal antibodies. When the analysis for SsGSTD1-1 was performed none of the other Delta GSTs had been identified, and hence a cross-reactivity test could not be performed. The results show that the SsGSTD1-1 localized in the integument of the epidermis, in cavities surrounding vital organs; with minor staining was also observed in the intestine. No staining was observed in the host tissue (Fig. 5, paper I). Later on, when the other two Delta GSTs were cloned and expressed, the antibodies were tested for cross-reacticvity, and unfortunately the polyclonal antibodies towards rSsGSTD1-1 also detected rSsGSTD2-2 and rSsGSTD3-3 (data not shown). Hence, the localization results probably concerned all three *S. scabiei* Delta GSTs, and could not differentiate the location between the *S. scabiei* Delta GSTs in the mite. More work has to be done to produce more specific antibodies toward the various Delta GSTs to investigate their localization in the mite.

For SsGSTM1-1 and SsGSTM2-2 small peptides of the enzymes were used to immunize rabbits, because it was assumed that there would be cross-reaction between antibodies produced if using the whole enzymes. The antibodies produced did not cross-react, nor did they cross-react with SsGSTM3-3. However, no good immunolocalization results were obtained using these antibodies, because the background signal was too high (data not shown).

5.4 *S. scabiei* GSTs as Diagnostic Antigens

GSTs in general have been suggested as good antigens for various organisms (Dougall *et al.*, 2005). All recombinant *S. scabiei* GSTs were analyzed to investigate if they were antigens that could be used to serologically detect *S. scabiei* infections. Tur results showed that none of the recombinant *S. scabiei* GSTs is good diagnostic antigens. IgG antibodies from sera from red foxes reacted with rSsGSTM1-1 however, but only with sera from individuals that had severe mange. This has also been seen in experiments with sera from human patients, where only antibodies from patients with crusted scabies reacted with SsGSTM1-1 (Dougall *et al.*, 2005).

5.5 3D-structures of *S. scabiei* Delta GSTs (III)

To learn more about the catalytic properties of the *S. scabiei* Delta GSTs rSsGSTD1-1 was crystallized to determine its three-dimensional structure, and homology modeling was performed to predict the structures for SsGSTD2-2 and SsGSTD3-3.

5.5.1 Overall structure and the active site of SsGSTD1-1

The crystal structure of SsGSTD1-1 was determined using the molecular replacement technique, where the coordinates of *A. dirus* species B GSTD1-3 (AdGST1-3) were used as the template. The structure was refined with good stereochemistry to a final R_{factor} of 20.0% (R_{free} 25.1%) to 2.5 Å resolution. This is the first GST structure determined for the subclass Acari (mites and ticks). SsGSTD1-1 is a homodimer adopting the GST canonical fold, with each subunit consisting of two distinct domains (Fig. 12). The N-terminal domain (residues 1-85) has a topology similar to that of the thioredoxin fold, consisting of four β-sheets with three flanking α-helices. A conserved *cis*-proline residue (Pro58 in the loop between helix 2 and 3), although not playing a direct role in catalysis, is thought to maintain the protein in a catalytically favorable conformation (Fig. 13) (Allocati *et al.*, 1999). The larger α-helical C-terminal domain (residues 86-227) contains five α-helices, and shows greater variability across the GST classes than the N-terminal domain (Board *et al.*, 2000; Reinemer *et al.*, 1996; Ji *et al.*, 1995; Sinning *et al.*, 1993; Reinemer *et al.*, 1992) (Fig. 13).

The active site is located in a deep cleft formed by the interface of the two domains (Fig. 16A). The active site can be further divided into the more conserved GSH-binding site (G-site) and a variable hydrophobic substrate-binding site (H-site). The G-site consists of the N-terminal domain residues Ser14, Gln43, His55, Cys56, Val57, Pro58, Glu70, Ser71 and

Arg72 (Fig. 13 and 15A). As in other Delta GSTs, the conserved S14 seems to be important for catalysis. The large H-site is comprised of residues Met11, Ala15, Pro16, Leu38, Phe39, Tyr111, Leu114, Tyr119, Ile121, Leu122, Phe123, Met173, Leu211 and Phe214 (Fig. 13 and 15B).

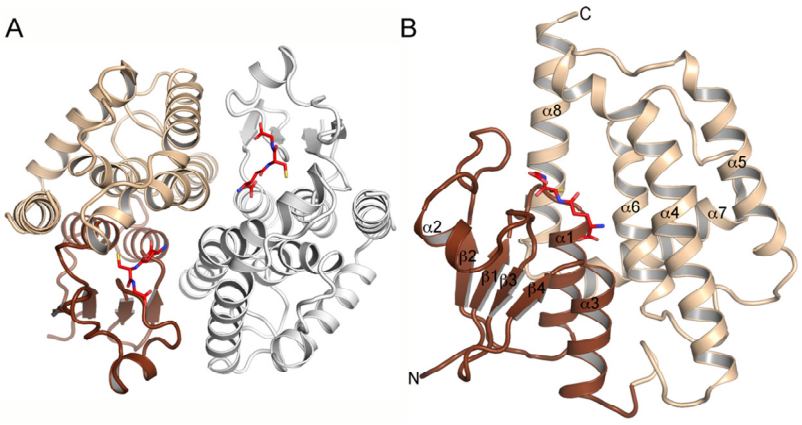


Figure 12. Overall structure of SsGSTD1-1. A) The structure of the homodimeric SsGSTD1-1. Where the N- and C-terminal domains of the monomer are colored on brown and beige, respectively. The second monomer is represented in grey. GSH (red) are shown in stick representation. GSH was modeled in the G-site based on the structure of AdGST1-3. B) Structure of one monomer with its numbered secondary structure elements. The figure was produced using PyMOL (http://www.pymol.org).

The electron density map showed the presence of a modified cysteine residue (Cys56) (Fig. 14). The density indicated that there are two or three additional atoms covalently attached to the cysteine. However, the side chain of the modified cysteine is too far away (4 Å) from the GSH for an interaction to occur. Unfortunately, the resolution of the structure was not enough to further analyze the electron density surrounding Cys56. To investigate whether Cys56 is a conserved amino acid among Delta GSTs from various arthropods, an amino acid sequence alignment with a total of 40 arthropod amino acid sequences was performed. The results showed that 50% of the aligned sequences had a cysteine, 40% a threonine, 2% a serine and 2% an asparagine at this position. Hence, more than 90% of the aligned sequences had an amino acid with a polar side chain. This information suggests that Cys56 is a conserved and probably catalytically important amino acid, but more has to be done to elucidate what action it has.

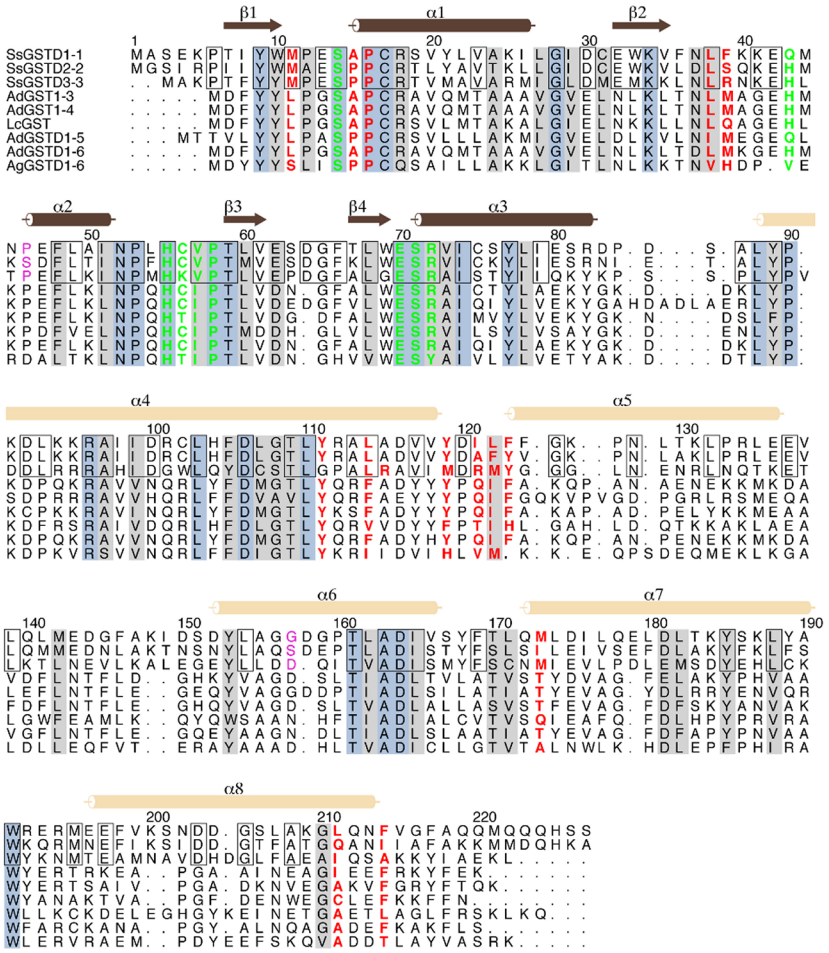


Figure 13. Structure-based sequence alignment of Delta GSTs. The sequences of known 3D Delta GST structures are aligned. The top three sequences are from S. scabiei and are aligned according to sequence, while the bottom sequences are aligned according to a structural comparison with SsGSTD1-1. The secondary structure elements for SsGSTD1-1 are given above the sequences, where the N- and C-terminal domains are shown in brown and beige, respectively. The sequence numbering is given above the alignment and refers to SsGSTD1-1. Amino acids conserved in all sequences are shown against a light blue background while amino acids against grey background have a conservation value of 7 (Livingstone & Barton, 1993). Amino acids conserved in S. scabiei are boxed. Amino acids lining the G- and H-sites are colored in green and red, respectively. Amino acid differences identified between SsGSTD2-2 when comparing sequences from mites derived from dogs and mites derived from humans are highlighted with pink letters (positions 46 and 157). The figure was made using ALSCRIPT (Barton, 1993).

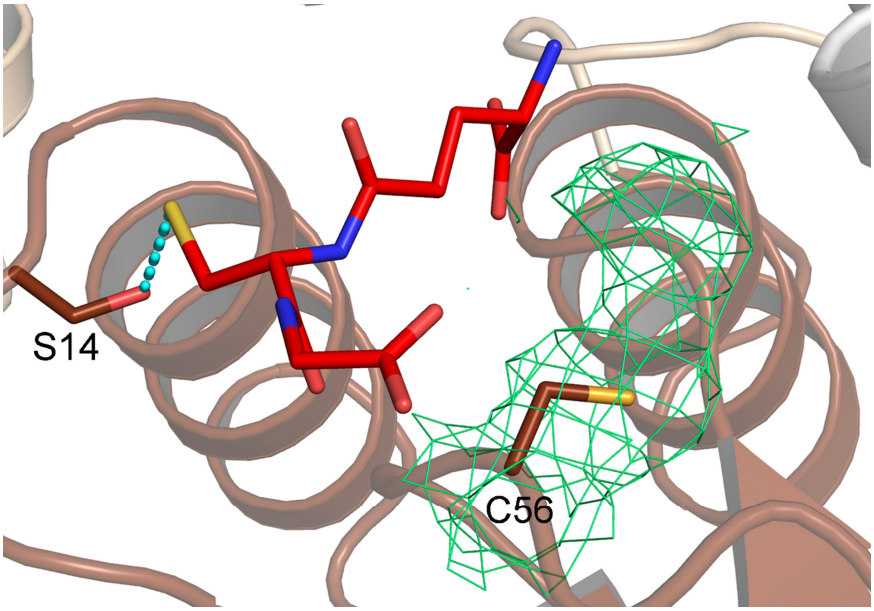


Figure 14. Electron density map of the modified Cys56. The modified Cys56 is shown in green. The modeled GSH (red), the catalytic Ser14 and Cys56 are shown in stick representation. The figure was produced using PyMOL (http://www.pymol.org).

5.5.2 Homology modeling of SsGSTD2-2 and SsGSTD3-3

Homology models of SsGSTD2-2 and SsGSTD3-3 were successfully built using SsGSTD1-1 as the template. The results demonstrated a strong structural conservation within the *S. scabiei* Delta GSTs. Structural changes in one of the hydrophobic dimer interactions was observed, indicating that the interaction area might be locally different in SsGSTD2-2 and SsGSTD3-3 compared to SsGSTD1-1.

5.5.3 Active site and electrostatic surface potential comparisons

The results showed that the G-site is highly conserved among the *S. scabiei* Delta GSTs, which is generally seen among GSTs (Josephy & Mannervik, 2006). Interestingly, SsGSTD2-2 has a cysteine at position 56 (numbering according to SsGSTD1-1), same as in SsGSTD1-1, while SsGSTD3-3 has a lysine. Hence, SsGSTD3-3 is a Delta GST lacking an amino acid with a polar side chain at this position. What catalytic importance this difference has, we analyses cannot tell. The shapes of the H-sites are diverse among the *S. scabiei* Delta GSTs (Fig. 15C), with SsGSTD1-1 and SsGSTD2-2 having the smallest and largest entrance to the active site, respectively (Fig. 16A-C).

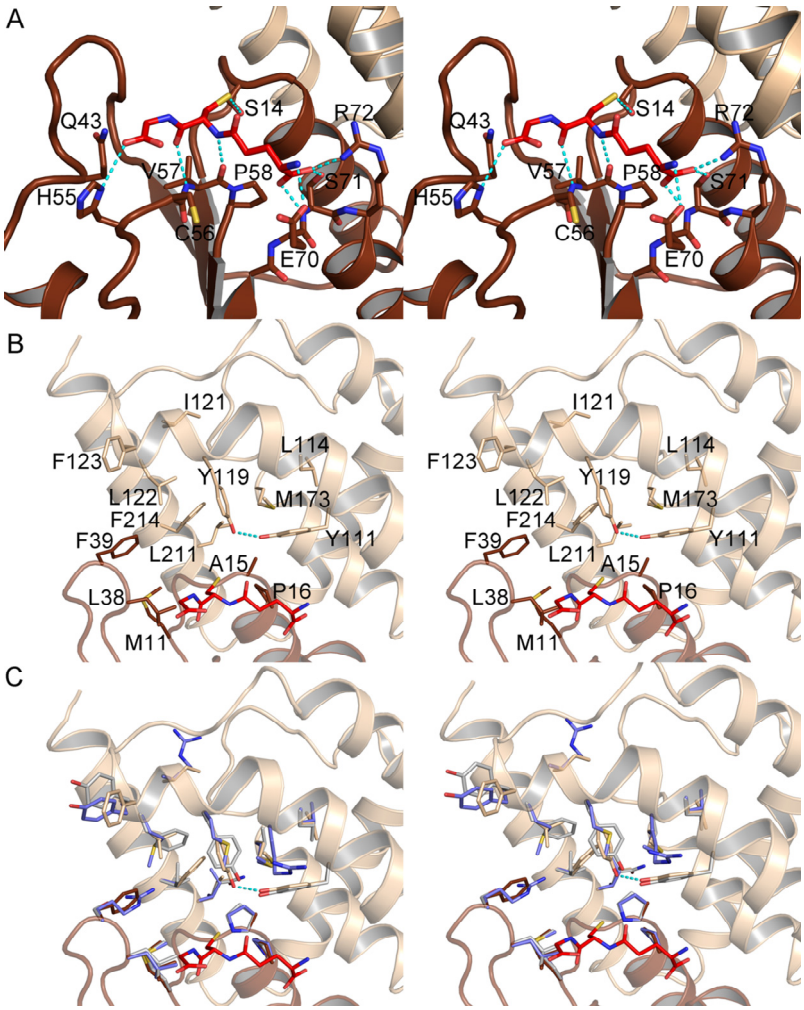


Figure 15. The active site of *S. scabiei* Delta GSTs. Stereo representation[6] of the SsGSTD1-1s' G-site (A), H-site (B), and the overlap of the active sites of SsGSTD1-1 and the homology models of SsGSTD2-2 (grey) and SsGSTD3-3 (blue) (C). GSH (red) is shown in stick representation and was modeled in the G-site based on the structure of AdGST1-3. Possible hydrogen-bonded contacts are shown by dotted lines. The figure was produced using PyMOL (http://www.pymol.org).

[6] How to view the stereo representations (http://edge-3D.com/how_to.htm): 1) Hold the stereo-pair at a comfortable viewing distance, wearing your glasses if you normally wear them. 2) The stereo-pair should be approximately in the center of your visual field and evenly illuminated. 3) Look "through" the paper, i.e. look to the distance. 4) A third image will appear between the left and the right pictures. 5) Without converging the eyes, slowly focus on the third, center image. 6) A 3D picture will emerge.

The molecular surface potentials for the crystal structure of SsGSTD1-1 and the two homology models for SsGSTD2-2 and SsGSTD3-3 were analysed. Interestingly, the electrostatic properties were quite dissimilar. The surface of SsGSTD1-1 is overall more negatively charged than SsGSTD2-2 and SsGSTD3-3 (Fig. 16A-C). The electrostatic surface potential also differs in and around the active site, with SsGSTD1-1 and SsGSTD3-3 being the most negatively and positively charged, respectively (Fig. 16D-F). These findings might indicate that either the enzymes are located in different compartments of *S. scabiei* (Stella *et al.*, 2007) or that they perform different tasks in the mite cells. For example, the differing electrostatic properties might indicate that the enzymes have differing ligandin actions in the cell, interacting with different charged ligands (Habig *et al.*, 1974). These actions might e.g. be important for transportation of various molecules out of the cell via MRPs (Josephy & Mannervik, 2006). Furthermore, differences in electrostatic potential could potentially be used to produce specific GST inhibitors (Mahajan *et al.*, 2006).

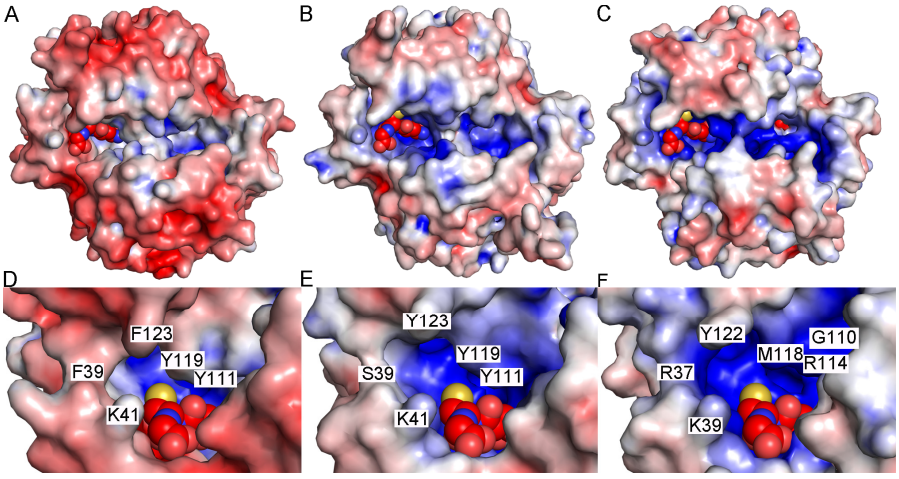


Figure 16. Electrostatic surface potential of *S. scabiei* GSTs. Surface representation of the electrostatic surface potential (blue, electropositive, and red, electronegative) of A) the structure of SsGSTD1-1, B) the homology model of SsGSTD2-2 and C) the homology model of SsGSTD3-3. D-F) shows the electrostatic surface potentials around the active sites of SsGSTD1-1, SsGSTD2-2 and SsGSTD3-3, respectively. Amino acids at the rims of the active sites are labeled. GSH (red) is shown as spheres. The surfaces were calculated using the APBS plugin (Baker *et al.*, 2001) of PyMol (http://www.pymol.org).

5.6 Enzyme-Acaricide and Enzyme-DEM Interactions (II-III)

Inhibition analyses were performed to investigate whether various acaricides (amitraz, chlorpyriphos, coumaphos, DDT, deltamethrin, diazinon, ethion, flumethrin, ivermectin, lindane and permethrin) and the synergist DEM inhibited the activity of all *S. scabiei* GSTs active with the model substrate CDNB. Further, docking studies were performed to investigate potential binding modes of ethion, DDT, ivermectin, lindane and permethrin and DEM with the *S. scabiei* Delta GSTs.

Because resistance has been reported to ivermectin, lindane and permethrin for *S. scabiei,* I will concentrate this section on these compounds. Results for the other acaricides (amitraz, chlorpyriphos, coumaphos, DDT, deltamethrin, diazinon, ethion and flumethrin) are presented in paper II (Mu GSTs) and III (Delta GSTs).

5.6.1 Delta GSTs

GSTs have never been shown to metabolize ivermectin; however, this acaricide significantly inhibited the CDNB activity of rSsGSTD2-2 (12%) (Table 8). Interestingly, ivermectin significantly enhanced the CDNB activity of rSsGSTD3-3, while for rSsGSTD1-1 there was no significant change. Because GSTs have not been associated with ivermectin metabolization, and ivermectin is a relatively large molecule that was assumed not to be able to enter the active site, large-scale docking studies including the whole enzyme and not only the active site were performed. These analyses revealed that ivermectin has many different binding opportunities on all three *S. scabiei* Delta GSTs (data not shown). Interestingly, SsGSTD2-2 can bind ivermectin in a way that allows part of the ivermectin molecule to enter the active site. This was not seen for the other *S. scabiei* Delta GSTs. This could explain the differences detected in the inhibition analysis, where only rSsGSTD2-2 was significantly inhibited. This change in CDNB activity could have two possible scenarios: 1) ivermectin blocks the active site entrance, and hence less CDNB can enter the active site; or 2) ivermectin is metabolized. Concerning the enhancement of CDNB activity for rSsGSTD3-3, this is probably explained by ivermectin binding the enzyme in a way that changes the enzyme structure, hence improving the interactions between CDNB and GSH. Both the SsGSTD3-3 explanation and scenario 2 for SsGSTD2-2 indicate that the enzymes might perform ligandin functions, which stops further actions of ivermectin without metabolizing it.

Table 8. *Inhibition (%) of 0.1 mM ivermectin, lindane or permethrin on the enzyme activity with CDNB for rSsGSTD1-1, rSsGSTD2-2, rSsGSTD3-3, rSsGSTM1-1 and rSsGSTM2-2 compared to control reactions without any acaricide present.*

Acaricide	rSsGSTD1-1	rSsGSTD2-2	rSsGSTD3-3	rSsGSTM1-1	rSsGSTM2-2
Ivermectin	2 ± 3.9	12 ± 0.4[a]	-33 ± 5.3[a]	16 ± 9.7	-3 ± 6.5
Lindane	3 ± 3.2	23 ± 1.0[a]	46 ± 0.7[a]	9 ± 0.9	-3 ± 4.1
Permethrin	9 ± 0.6[a]	21 ± 1.9[a]	24 ± 12.0	37 ± 2.4[a]	7 ± 4.9
Control	0 ± 0.0	0 ± 1.0	0 ± 2.0	0 ± 2.6	0 ± 0.6

Values are from three independent experiments, showing the mean ± standard error. Significant differences were based on 95% confidence interval.

[a] Significant inhibition.

The largest inhibition of CDNB-activity observed for any of the recombinant *S. scabiei* Delta GST-acaricide combinations was for rSsGSTD3-3 together with lindane. The rSsGSTD3-3 lost almost half of its activity in the presence of this acaricide (Table 8). This was in sharp contrast to rSsGSTD1-1, which did not show any reduction of activity (3% ± 3.2) while rSsGSTD2-2 lost approximately 20% of its CDNB-activity. The docking studies yielded one possible binding mode of lindane for each *S. scabiei* Delta GST. These results revealed that lindane binds deeper into the H-site in SsGSTD2-2 compared to SsGSTD1-1 (Fig. 17). In SsGSTD1-1, Phe39 hinders this deeper binding. However, in both SsGSTD1-1 and SsGSTD2-2 lindane binds as it was in close proximity (about 2.5 Å) to the GSH for a conjugation or dehydrochloronation to occur. In SsGSTD3-3 Arg38 prevents lindane binding as in SsGSTD1-1 or SsGSTD2-2, and instead pushes lindane into the more hydrophobic interior of the H-site of SsGSTD3-3 (Fig. 17). The lindane binding in SsGSTD3-3 is located approximately 6 Å away from the GSH thiol group, suggesting that lindane is probably not metabolized by SsGSTD3-3. Hence, the inhibition of the CDNB-activity of rSsGSTD3-3 detected in the inhibition analyses was probably due to bound lindane stopping CDNB-GSH conjugation, without further metabolization of lindane. For SsGSTD1-1 a conjugation probably did not occur, because no affect on the CDNB-activity in the inhibition analysis was detected; however, this could be a result of varying affinities resulting in CDNB outcompeting lindane. With a different setup of the experiment, conjugation might be detected. For SsGSTD2-2 a conjugation between lindane and GSH are possible and the inhibition analysis showed a significant inhibition by lindane on the CDNB-activity for SsGSTD2-2. Lindane metabolization by GSTs has been shown in other organisms, and strengthens the results for SsGSTD2-2 (Wei *et al.*, 2001; Syvanen *et al.*, 1996).

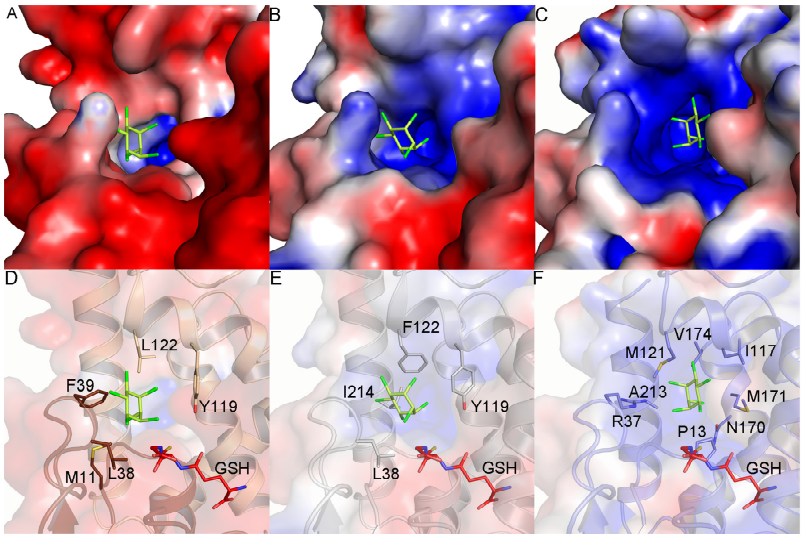


Figure 17. Docking results of lindane. The active sites of the structure of SsGSTD1-1 (A and D) and the homology models of SsGSTD2-2 (B and E) and SsGSTD3-3 (C and F) shown as surface representation of the electrostatic surface potential (blue, electropositive, and red, electronegative) and stick representation of side-chain residues that are interacting with the lindane molecule. For the sake of clarity, hydrogen atoms are not displayed. The surfaces were calculated using the APBS plugin (Baker *et al.*, 2001) of PyMOL (http://www.pymol.org).

The acaricide permethrin significantly inhibited SsGSTD1-1 (9%) and SsGSTD2-2 (21%), and the mean inhibition of SsGSTD3-3 was in the same range as for SsGSTD2-2, but the results were not statistically significant (Table 8). In contrast to the lindane docking analysis, the docking studies for permethrin yielded multiple possible binding modes of this acaricide in the active site of each *S. scabiei* Delta GST (Fig. 18). The results showed that the amino acid at position 214 (numbering according to SsGSTD1-1) could have an important role for the specificity for permethrin in *S. scabiei* Delta GSTs. This amino acid is a phenylalanine, isoleucine and alanine in SsGSTD1-1, SsGSTD2-2 and SsGSTD3-3, respectively. Due to a smaller side-chain residue at this position, the permethrin ligand can penetrate deeper into the H-site of both SsGSTD2-2 and SsGSTD3-3. Also, due to the smaller Ser39 (Phe in SsGSTD1-1) permethrin has a tighter binding to SsGSTD2-2 compared to SsGSTD1-1. Overall, the docking studies showed that all three *S. scabiei* Delta GSTs could bind permethrin for a conjugation to occur. For example, the GSH thiolate in SsGSTD2-2 is positioned at a distance of approximately 3 Å from the linker region between the

phenoxybenzyl- and dichlorovinyl group on permethrin, suggesting that SsGSTD2-2 could metabolize permethrin. Earlier research has shown that GSTs can bind permethrin but it has not been proven that a conjugation occurs (Jirajaroenrat *et al.*, 2001; Kostaropoulos *et al.*, 2001). However, both the inhibition analyses and docking studies indicate that all three *S. scabiei* Delta GSTs could be able to bind or metabolize, permethrin.

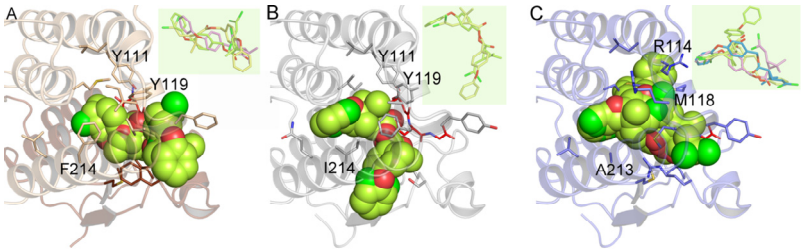


Figure 18. Docking results of permethrin. Representation of possible binding sites of permethrin in SsGSTD1-1 (A), SsGSTD2-2 and SsGSTD3-3 (C). The top low energy clusters are overlapped in the active site and shown in CPK model representation. For clarity, the conformations of permethrin are also shown in stick representation in the green box areas. Amino acids lining the H-site and the modeled GSH (red) are shown in sticks. Imposrtant H-site amino acids are labeled. For the sake of clarity, hydrogen atoms are not displayed. The figure was produced using PyMOL (http://www.pymol.org).

The synergist DEM is a well-known GST inhibitor which often is used to deplete cells of GSH. This is achieved by GSTs effectively catalyzing the conjugation of DEM to GSH. Enzyme activity for all three recombinant *S. scabiei* Delta GSTs was significantly inhibited by the addition of DEM (Table 9). Interestingly, rSsGSTD1-1 and rSsGSTD2-2 lost almost all of their CDNB-activity, while rSsGSTD3-3 kept approximately 35% of its activity. As for lindane, the docking studies yielded one possible binding mode of DEM of each *S. scabiei* Delta GST. The results showed no obvious differences between the enzymes, explaining the inhibition results (Fig. 11, paper III).

Table 9. *Inhibition (%) of DEM on the enzyme activity with CDNB for rSsGSTD1-1, rSsGSTD2-2, rSsGSTD3-3, rSsGSTM1-1 and rSsGSTM2-2 compared to control reactions without DEM present.*

Synergist	rSsGSTD1-1	rSsGSTD2-2	rSsGSTD3-3	rSsGSTM1-1	rSsGSTM2-2
DEM	98 ± 0.1	96 ± 0.8	68 ± 3.5	56 ± 7.0	65 ± 4.3
Control	0 ± 1.3	0 ± 1.4	0 ± 8.2	0 ± 1.8	0 ± 4.0

All values are from three independent experiments, showing the mean ± standard error. The data were analyzed by comparing 95% confidence intervals and all assays showed significant inhibition.

5.6.2 Mu GSTs

The rSsGSTM1-1 activity with CDNB was significantly inhibited by permethrin (37%), while ivermectin and lindane gave slight reductions of activity compared to the controls, but the results were not statistically significant (Table 8). Enzyme activity for both rSsGSTM1-1 and rSsGSTM2-2 was significantly inhibited by DEM, where both enzymes lost approximately 60% of their activity (Table 9). None of the *S. scabiei* Mu GSTs has been crystallized; hence the findings cannot be supported with structure-function studies as for the *S. scabiei* Delta GSTs. However, a brief tertiary structure comparison of SsGSTM1-1 and SsGSTM2-2 (Fig. 1, paper II) showed that approximately one-fifth of the amino acids differing between SsGSTM1-1 and SsGSTM2-2 were located in the N-terminal domain of the enzyme, and the rest in the C-terminal domain. Most of the differing amino acids appeared on the outer surface of the enzymes, and clustered around the cleft surrounding the active site opening. These results suggest that the differing amino acids might account for the different interactions between the Mu GSTs and the various acaricides and DEM, even though the amino acid differences were located outside of the active site. It has also previously been shown that single amino acid differences outside the active site can affect the GST activity (Ortelli *et al.*, 2003; Ketterman *et al.*, 2001). Still, not much is known about SsGSTM3-3, except that it differs a lot from the other two *S. scabiei* Mu GSTs. Hence, further structure-function studies of this enzyme are necessary to investigate its possible involvement in drug detoxification.

Because the results indicate that *S. scabiei* Mu GSTs might metabolize or bind various acaricides I think it would be interesting to crystallize the Mu GSTs to find out more about their interactions with, especially, permethrin and ivermectin.

6 Concluding Remarks

The overall aim of this thesis was to characterize GSTs from *S. scabiei*, investigate their possible involvement in acaricide detoxification and their potential as diagnostic antigens. Have I done that? Let me sum up!

The *S. scabiei* genome includes at least six different genes coding for GST enzymes. Three of them express enzymes belonging to the Delta class (SsGSTD1-1, SsGSTD2-2 and SsGSTD3-3), and three of them express enzymes belonging to the Mu class (SsGSTM1-1, SsGSTM2-2 and SsGSTM3-3). All recombinant versions of these enzymes, except rSsGSTM3-3, were catalytically active with the model substrate CDNB; and subsequent inhibition analyses showed that the catalytically active enzymes interacted with various acaricides. The crystal structure for SsGSTD1-1 was determined, and the 3D-structures for SsGSTD2-2 and SsGSTD3-3 were predicted by homology modeling. In the docking studies different binding modes for ivermectin, lindane and permethrin and the three Delta enzymes were detected, which indicated that *S. scabiei* Delta GSTs could bind or metabolize the acaricides (Table 10), hence stopping the acaricides to perform further tasks. These results gave valuable information concerning the inhibition results, and deepened our knowledge about possible involvement of GSTs in detoxification of resistance in *S. scabiei*.

None of the recombinant enzymes are good diagnostic antigens for serology; however SsGSTM1-1 seems to encounter the immune response in red foxes when a severe infection has been established. Unfortunately, the immunohistochemistry analyses failed, but the obvious differences seen in electrostatic surface representation could indicate that the enzymes are localized in different compartments of the mite. Another suggestion for the differences in electrostatic surface representation is that they perform different catalytically tasks in the mite.

Table 10. *Summary of possible interactions between the* S. scabiei *Delta GSTs and ivermectin, lindane and permethrin, shown by inhibition analyses and docking studies.*

Acaricide	SsGSTD1-1	SsGSTD2-2	SsGSTD3-3
Ivermectin	B	B (+ M)	B
Lindane	?	B + M	B
Permethrin	B + M	B + M	B + M

B=binds the acaricide

M=metabolizes the acaricide

Bringing the results together with the qRT-PCR experiments performed with ivermectin (Mounsey *et al.*, 2009b) and permethrin (oral communication[7]), and the synergist experiments performed by Pasay *et al.* (2009), gives a deeper knowledge of resistance development in *S. scabiei*. The qRT-PCR experiments showed that SsGSTD3-3 and SsGSTM1-1 are upregulated in ivermectin exposed mites, while those two as well as SsGSTD1-1 are upregulated in a permethrin resistant strain of *S. scabiei*. Hence, it has been proposed that these GSTs are involved in resistance development to ivermectin and permethrin in *S. scabiei*. The characterization of these enzymes presented in this thesis also strengthens these findings, and in my opinion these three GSTs are the most important *S. scabiei* GSTs to focus on today. The results also indicate that SsGSTD2-2 could metabolize or bind all three acaricides, but this enzyme was not upregulated in the qRT-PCR analysis for ivermectin and permethrin. However, it could be important for the resistance to lindane. More analyses have to be done to investigate this.

Because SsGSTM3-3 was not catalytically active with CDNB this enzyme was not further characterized, but more knowledge is needed about SsGSTM3-3 to include or exclude this enzyme from the group of interesting *S. scabiei* GSTs. Additionally, one have to keep in mind that we do not know if there are additional GSTs in the *S. scabiei* genome. Maybe there is another GST that is the important one in resistance development?

The lifespans of many of the currently used acaricides are threatened due to resistance development. Hence, one prospect of this thesis was to collect information on how to overcome this problem. As mentioned earlier, treatment of mites from a permethrin resistant *S. scabiei* strain with a combination of permethrin and DEM increased the sensitivity to the drug (Pasay *et al.*, 2009). The results presented in this thesis also showed that all *S. scabiei* GSTs were strongly inhibited by DEM. Hence, one suggestion for

[7] Kate Mounsey, Queensland Institute of Medical Research, Brisbane, Australia and Menzies School of Health Research, Darwin, Australia.

the future could be to add DEM when treating *S. scabiei* infections. Probably other synergists should be included as well because resistance in *S. scabiei* probably is conferred by other metabolic enzymes as well, e.g. CYP450 and esterases (Pasay *et al.*, 2009).

In the first section of the background I stated that it is important to keep the species-question in mind when discussing our findings. Especially, this is important when transforming the *in vitro* results to real life. Will findings from experiments performed on GSTs from mites derived from red foxes also apply for interactions between *S. scabiei* and other hosts? As mentioned earlier this is our belief today, because there is no conclusive evidence of host specificity for *S. scabiei*. However, thorough analyses of the *S. scabiei* genome from different hosts from different geographical areas together with deeper studies on specific *S. scabiei*-host interactions have to be performed, to interpret our *in vitro* results in relation to the *in vivo* situation.

Finally, how famous is *S. scabiei* and its infections? Has something happened since I started writing this thesis (Table 11)?

Table 11. *Google™ searches performed 22nd of June and 7th of October 2009.*

Search word	Number of hits 22nd of June	Number of hits 7th of October
Sarcoptes scabiei	76 700	76 700
sarcoptic mange	119 000	121 000
scabies	1 210 000	1 460 000
bird flu	9 310 000	11 200 000
swine flu	11 700 000	38 800 000
malaria	12 700 000	12 700 000
cancer	182 000 000	173 000 000

Still it is obvious that *S. scabiei* and its infections are not very famous. The infections have, however, a slightly higher number of hits the 7th of October compared to 22nd of June (Table 11). The only big change, seen for the included search words, is for "swine flu" which has increased its celebrity with approximately 30 million hits, which is not surprising because of the ongoing pandemy. Hopefully, this thesis will make at least a small dent on the number of hits that can be found, but, more importantly, make people aware of the problem with sarcoptic mange and scabies and hence make *S. scabiei* more famous.

7 Future Research

Because the results presented in this thesis have shown that it is possible to start to unravel the complex issue of acaricide resistance in *S. scabiei* using a combination of various modeling tools and biochemical studies, further research of the underlying mechanisms of resistance in *S. scabiei* probably have been or will be initiated. Additionally, we have settled a small part of this complex issue, and there is still a considerable need for further studies within this field of research. Below, some suggestions for future research in relation to the findings presented in this thesis are noted:

- Determination of the crystal structure of all *S. scabiei* GSTs, to investigate their interactions with various acaricides. Also, it would be of importance to crystallize the *S. scabiei* GSTs in complex with various acaricides, to determine the binding modes.

- Enzymatic analyses followed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses to detect GSH conjugates of the acaricides.

- Biochemical analyses of the *S. scabiei* GSTs with various substrates to investigate which reactions the enzymes catalyze in the mite.

- Comparative studies, using e.g. qRT-PCR, for field and/or experimental mite isolates, from mites derived from the same host, to identify differences in response to various acaricides, in particular ivermectin and permethrin.

- Whole-genome sequencing of the *S. scabiei* genome, to investigate how many GSTs are present in the genome and how they are localized.

- Thorough sequence analyses of *S. scabiei* comparing sequences from mites derived from the same host, mites derived from different hosts, and also comparing both of these groups from different geographical locations. This will tell us if *S. scabiei* is host specific or not, or if there are geographical differences between mite populations.

- Research to establish an *in vitro* culture system without the use of live animals. This would be a good tool for further studies in all *S. scabiei* research fields.

- Worldwide collaboration to speed up and improve *S. scabiei* research, not only for the resistance research field but also for improving e.g. diagnostic tools.

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