Effect of acaricides on the activity of glutathione transferases from the parasitic mite *Sarcoptes scabiei*

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SUMMARY

Glutathione transferases (GSTs) are a family of multifunctional enzymes with fundamental roles in cellular detoxication. Here we report the molecular characterization of 3 recombinant GSTs belonging to the mu- and delta-class from the parasitic mite *Sarcoptes scabiei*. Kinetic constants were determined, and the effect of acaricides, including organothiophosphates, pyrethroid esters, a formamidine, a macrocyclic lactone, an organochlorine as well as a bridged diphenyl acaricide, on the activity of the GSTs were tested using 1-chloro-2,4-dinitrobenzene (CDNB) as model substrate. Our results showed that enzymes from the same class and with high amino acid sequence identity have significantly different kinetic properties. For instance, one mu-class GST lost more than 50% of its activity in the presence of one of the organothiophosphates while the activity of the second mu-class GST was only slightly reduced under identical conditions. Tertiary structure modulations indicated that structural differences were the crucial factor for the different kinetic patterns observed. Genome analysis showed that the two mu-class GSTs are organized in tandem in the *S. scabiei* genome. Taken together these results show that GSTs might be involved in the metabolism of acaricides in *S. scabiei*.

Key words: *Sarcoptes scabiei*, glutathione transferase, enzyme activity, acaricide, kinetic analysis, genome organization.

INTRODUCTION

The disease sarcoptic mange or scabies is caused by the parasitic itch mite *Sarcoptes scabiei* (Acari: Sarcoptidae) and affects both animals and humans worldwide. The infection is immunopathological and arises because of the burrowing of the mite in the epidermis of its host. In the burrows the mites reproduce and develop through their different stages, larvae and nymph, to adult mites (Burgess, 1994; McCarthy *et al*. 2004). The primary symptom is an extreme itching and, depending on the immunological status of the host, the symptoms spread and the course of events can vary considerably (Donabedian and Khazan, 1992). Some animal species (e.g. foxes) are more susceptible than others and infections often lead to extensive mortality (Mörner, 1992; McCarthy *et al*. 2004). In the international pig industry, between 50% and 95% of the herds are estimated to be infected by *S. scabiei* (McCarthy *et al*. 2004), and consequently various preventive measures are taken to limit the damage, both from an animal welfare as well as an economic perspective (Firkins *et al*. 2001).

Glutathione transferases (GSTs) are a large family of hetero- or homo-dimeric multifunctional enzymes (Sharp *et al*. 1991; Enayati *et al*. 2005) which catalyses nucleophilic attack by reduced glutathione (GSH) on non-polar electrophilic substrates (Enayati *et al*. 2005). Consequently, the enzymes are important members of the enzymatic system that deals with foreign toxic compounds (xenobiotics) in all living organisms (Sheehan *et al*. 2001). The role of GSTs in detoxication, as well as their extensive ligand binding properties, has linked the GSTs to resistance against various active ingredients used in treatments towards insects (Feyereisen, 1995; Hemingway, 2000; Enayati *et al*. 2005). Many of these active substances, e.g. organochlorines and pyrethroid esters, together with the more parasite-specific macrocyclic lactones, are used extensively for treatment of scabies, and the number of reports on resistance concerning these substances is now increasing (Currie *et al*. 2004; Walton *et al*. 2004; Heukelbach and Feldmeier, 2006).

Because of the linkage of GSTs to drug resistance and the limited information available about resistance mechanisms in ticks and parasitic mites of veterinary importance (Foil *et al*. 2004), we have conducted experiments to further study GSTs from *S. scabiei*. To date 6 different *S. scabiei* GSTs are identified and 3 of them belong to the mu-class and 3 of them to the delta-class (Fischer *et al*. 2003; Dougall *et al*. 2005; Pettersson *et al*. 2005). Here we have analysed 1 of the delta-class GSTs (Pettersson *et al*. 2005) and 2 of the mu-class GSTs (Fischer *et al*. 2003; Dougall *et al*. 2005). We used basic

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Table 1. Oligonucleotides used in genome organisation analysis

Primer name	Nucleotide sequence	Descript	Direction
OP459	5′-ATCATTCTGATGGGCTGCGCAAGGC-3′	mu I 5′ end	reverse
OP460	5′-TCAATAAAATTCGGATTGATTGACC-3′	mu II 5′ end	reverse
OP461	5′-ACACTACGACATGGTGCACTTTCTGGC-3′	delta 5′ end	reverse
OP462	5′-TCGATTGAAAGCGATGACACC-3′	mu I 3′ end	forward
OP463	5′-CTTATCCGTGTCAAAATTTTTGCA-3′	mu II 3′ end	forward
OP464	5′-ATGGTTCGCTTGCTAAAGGGCTTC-3′	delta 3′ end	forward
OP469[a]	5′-TCAGGGACAAATGAATACC-3′	mu II 3′ end	forward
OP470[a]	5′-TATCGGCATCATTCGAAGCT-3′	mu I 5′ end	reverse
OP471	5′-CTGCGCAAGGCCACGTATATTCCAAT-3′	mu I 5′ end	reverse

[a] Sequencing primer.

steady-state analysis to define the kinetic characteristics of the 3 enzymes and investigate the interaction between the recombinant forms of the enzymes and various commercial acaricides. Additionally, the relative organization of the 3 GST genes in the *S. scabiei* genome were analysed by PCR and sequence analysis.

MATERIALS AND METHODS

Sarcoptes scabiei *DNA, expression vector and* Escherichia coli *strains*

An aliquot of *S. scabiei* mites was isolated from the skin of red foxes as described previously (Bornstein and Zakrisson, 1993), and genomic DNA was extracted as described by Walton *et al.* (1997). The genomic DNA (*S. scabiei* var. *vulpes*) was then amplified using Genomiphi DNA amplification kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions, and used for amplification of *S. scabiei* GST mu open reading frames (ORFs). For the initial cloning step the PCR-script Amp Electroporation-Competent Cell Cloning Kit (Stratagene, La Jolla, CA) was used according to the manufacturer's instructions. The T7-promotor driven vector pET-14b (Novagen, Madison, WI) was used as the expression vector. *Escherichia coli* strain XL-1 Blue MRF' (Stratagene) was used for cloning and preparation of expression plasmids, and *E. coli* strain BL21(DE3) (Stratagene) was used for high-level expression.

Chemicals

In this study we evaluated 4 organothiophosphate acaricides (diazinon, ethion, chlorpyriphos and coumaphos), 3 pyrethroid ester acaricides (deltamethrin, flumethrin and permethrin), 1 formamidine acaricide (amitraz), 1 macrocyclic lactone endectoside (ivermectin), 1 organochlorine acaricide (lindane) and finally 1 bridged diphenyl acaricide (DDT). All acaricides were purchased from Sigma Aldrich (St Louis, MO) and diluted in methanol. Chlorpyriphos, coumaphos, deltamethrin, amitraz, ivermectin, lindane and DDT were prepared as 10 mM stock solutions, while diazinon, ethion, flumethrin and permethrin were prepared as 100 mM stock solutions.

Genomic organization

Oligonucleotides complementary towards the 5′- and 3′-parts of the *S. scabiei* GST delta (GenBank Accession number AY649788), mu I (GenBank Accession number AY825933) and mu II (GenBank Accession number AF462190) genes (Table 1) were used to amplify DNA fragments and determine the relative organization of GST delta, mu I and mu II in the *S. scabiei* genome. The reaction mixture contained 1× GeneAmp PCR buffer (Tris-HCl, pH 8·3, 50 mM KCl, 10 mM, 1·5 mM $MgCl_2$, 0·001% (w/v) gelatin) (Applied Biosystems, Foster City, CA), 20 pmol of each primer (multiplex PCR with a mix of OP459, OP460, OP461, OP462, OP463 and OP464 or a standard PCR with OP463 and OP471), 200 μM of each deoxynucleotide, 1 μl of genomic DNA (amplified using Genomiphi DNA amplification kit) and 1 U of AmpliTaq DNA polymerase (Applied Biosystems). After initial incubation at 94 °C for 1 min the DNA was amplified for 30 cycles. Each cycle consisted of denaturation at 94 °C for 15 s, annealing at 54 °C for 15 s and extension at 72 °C for 3 min, and ended with a final extension at 72 °C for 6 min. Amplicons were purified using the Geneclean II kit (QBIOGene, Irvine, CA) according to the manufacturer's instruction, and eluted with a mixture of 10 μl of TE-buffer, pH 8·0, and 10 μl of distilled water.

Amplification of mu-class GST cDNA open reading frames

The ORFs of the two GST mu genes (mu I: GenBank Accession number AY825933 and mu II: GenBank Accession number AF462190) were both amplified from genomic DNA extract by PCR in 40 μl reactions using the forward primer OP455 (5′-CCG CTC GAG ATG TCT TCG AAA CCA ACT

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CTT-3′) and the reverse primer OP456 (5′-CGG GAT CCT TAA TAT TTT GTA TTC CAT TTC GCC-3′), both of which were constructed based on the mu I GST sequence. To insert the ORFs into the multicloning site of the pET-14b vector, primers were designed with linkers (underlined) containing restriction sites for *Xho*I and *Bam*HI, respectively. The reaction mixtures contained 1× *Pfu* reaction buffer (20 mM Tris-HCl, pH 8·8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0·1% Triton X-100, 0·1 mg/ml BSA) (Stratagene), 20 pmol of each primer, 200 μM of each deoxynucleotide, 1 μl of genomic DNA (amplified using Genomiphi DNA amplification kit) and 1 U of *Pfu Turbo* DNA polymerase (Stratagene). After 2 initial 5-min incubations at 93 °C and 45 °C, respectively, the DNA was amplified for 25 cycles. Each cycle consisted of denaturation at 93 °C for 1 min, annealing at 45 °C for 2 min and 30 s, and extension at 72 °C for 2 min; and the PCR ended with a final extension at 72 °C for 10 min. Amplicons were purified as described above.

DNA sequencing

DNA was sequenced by the dideoxynucleotide chain termination method using ABI PRISM Big Dye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems). Each 20 μl reaction contained 2 μl of Big Dye solution, 5 pmol of primer (control sequencing of plasmids with T3, T7, T7pro or T7ter; genomic organization analysis with OP469 or OP470), 3 μl of 5× Big Dye sequencing buffer and 150–300 ng purified plasmid. Sequence reactions were carried out with the following programme: (96 °C for 10 s, 45 °C for 5 s and 60 °C for 4 min) × 25. The reaction products were purified and concentrated through ethanol and sodium acetate precipitation, and the resulting pellets were resuspended in 11 μl of formamide, before product separation was performed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Bioinformatics

The sequences were processed using the Vector NTI program suite 9 (Informax Inc., Oxford, UK), and predictions of transcription factor binding sites were performed using Transcriptional Factor Search (http://www.cbrc.jp/research/db/TFSEARCH.html). Putative amino acid sequences and protein sizes were calculated using the Translate tool available through the ExPASy server (http://www.expasy.org).

Tertiary structure comparison

Coordinates for the human mu-class GST M2-2 complexed with glutathione (PDB ID: 1XW5) were downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/Welcome.do), and used in remodelling analysis to compare the predicted tertiary structure differences between the two *S. scabiei* mu-class GSTs. Alignment of the amino acid sequences for the two *S. scabiei* mu-class GSTs was performed, and differences between the two sequences were highlighted in the amino acid sequence of the human mu-class GST. Thereafter, this was visualized in the 3-dimensional model of the human mu GST using the 3D Molecule Viewer in Vector NTI.

Subcloning of the S. scabiei *GST mu ORFs*

The mu I amplicon produced with OP455/OP456 was digested and cloned into the *Xho*I-*Bam*HI sites of pET-14b. After identification of positive clones by colony PCR, the resulting plasmid was purified, control sequenced and designated pPU108. The second OP455/OP456 amplicon (mu II) was digested and cloned into the same sites of pET-14b. After purification and control sequencing the resulting plasmid was named pPU109. For expression of the GST delta gene (GeneBank Accession number: AY649788) a previously described construct, pPU99, was used (Pettersson *et al*. 2005).

Protein expression and purification

For high-level expression pPU99 and pPU109 were freshly transformed into *E. coli* strain BL21(DE3), and expressed in minimal medium at 18–20 °C as earlier described (Pettersson *et al*. 2005). The plasmid pPU108 was also freshly transformed into BL21(DE3) cells, and a single colony was inoculated in 20 ml of LB medium with ampicillin (50 μg/ml) and incubated on a shaker at 37 °C overnight. On day 2, 20 ml of the overnight culture was inoculated in 1 litre of LB medium with ampicillin (50 μg/ml). The new culture was grown on a shaker at 37 °C until OD_{600} reached ∼0·5, and then the recombinant gene expression were induced by addition of isopropylthiogalactoside (IPTG) to a final concentration of 0·5 mM. Induced cells were incubated on a shaker at 37 °C for 2 h.

All cell cultures were resuspended in 1×PBS, pH 7·3. To prevent protein degradation, the buffer was supplemented with Complete Protease Inhibitor (Roche Molecular, Basel, Switzerland) according to the manufacturer's instructions. Harvested cells were lysed by sonication, and cell debris was collected by centrifugation (9000 ***g***, 4 °C, 30 min). The resulting supernatant was filtered through a 0·45-μm filter, and the recombinant ssGSTs were purified by affinity chromatography (1 ml GSTrap FF, GE Healthcare) according to the manufacturer's instructions using a peristaltic pump P-1 (GE Healthcare).

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Protein expressions and purification steps were checked by 12% SDS-PAGE and stained in 0·1% Coomassie Brilliant Blue solution. Final protein concentrations were estimated by the Bradford method (Bradford, 1976).

Determination of kinetic parameters

To determine the apparent K_m, k_{cat} and k_{cat}/K_m values for the recombinant *S. scabiei* GSTs, the enzyme activity towards the substrate 1-chloro-2,4-dinitrobenzene (CDNB) was determined by a spectrophotometric assay under steady-state conditions, as described by Habig *et al*. (1974) and Widersten *et al*. (1996). In the assays, a CDNB range from 0·05 to 6 mM was used with a fixed reduced glutathione (GSH) concentration of 5 mM, and a GSH range from 0·25 to 10 mM with a fixed CDNB concentration of 6 mM. The GST activities were measured spectrophotometrically in an Ultrospec 1000 UV/Visible spectrophotometer (GE Healthcare) and 1 cm spectrophotometer cells. For the substrate CDNB a molar extinction coefficient ($\Delta\varepsilon$) of 9·6 $mM^{-1}\,cm^{-1}$ was used (Habig *et al*. 1974). All reactions were performed at 21 °C, and the initial rates were recorded as the change in absorbance at 340 nm during 2 min. Before every measurement the spectrophotometer was calibrated with a control reaction (no enzyme present) to compensate for non-enzymatic activity. 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 $[E]_{tot}$. Graphs and calculations were performed using GraphPad Prism v4 (GraphPad Software, Inc., San Diego, CA).

Determination of enzyme activity in the presence of acaricides

The study was done using the standard GST assay conditions described above, but with fixed substrate and ligand concentrations. In the absence and presence of various acaricides we used a CDNB concentration of 1 mM with a GSH concentration of 5 mM. All controls contained the same concentration of methanol as the samples with acaricide (2% or 4% depending on the volume of acaricide added). The acaricides diazinon, ethion, chlorpyriphos, ivermectin, lindane and DDT were all added to the reactions at concentrations of 0·1 and 0·2 mM. Flumethrin, permethrin and deltamethrin were all added to the reactions at a concentration of 0·1 mM due to solubility problems at 0·2 mM. The acaricide amitraz was added to the reactions with ssGST delta and ssGST mu I at concentrations of 0·1 mM and 0·2 mM, but only added at a concentration of 0·1 mM to the reactions with ssGST mu II due to measuring problems at 0·2 mM. Each assay was run in triplicate and the results were expressed as the calculated mean with standard error of mean. The data were analysed by comparing 95% confidence intervals, with intervals that did not overlap zero indicating significant reduction of activity.

RESULTS

Sequence analysis and subcloning

With the primers OP455 and OP456 both versions of the GST mu genes (mu I: GenBank Accession number AY825933 and mu II: GenBank Accession number AF462190) were amplified from the *S. scabiei* genome. Our sequence analysis showed that none of the analysed mu genes had any intron. Alignments of the amino acid sequences of the 3 *S. scabiei* GSTs showed that the identity between the 2 mu-class GSTs was 77·2%, and the identity between the ssGST delta and ssGST mu I and ssGST mu II was 17·9% and 18·8%, respectively.

For further characterization of the mu-class GSTs, we subcloned the ORFs into the expression vector pET-14b (pPU108 and pPU109) that had the GSTs fused to an N-terminal His_6-tag under the control of the T7 promoter. Both his-tagged mu-class GSTs from pPU108 and pPU109 had estimated molecular weights of 28 kDa. The delta-class GST from pPU99 was also expressed from a pET-14b background and also had an estimated molecular weight of 28 kDa (Pettersson *et al*. 2005).

Expression and purification of S. scabiei *GSTs*

All 3 constructs were transformed into BL21(DE3) for expression of the recombinant ssGST proteins. Induction of the expression by addition of IPTG resulted in production of high yields of recombinant protein of expected molecular weight from all constructs, as indicated by SDS-PAGE analysis (data not shown).

After preparation of bacterial lysates, all recombinant ssGSTs were affinity-purified on glutathione columns by means of the GST activity. The recombinant mu I GST from pPU108 was named ssGST mu I and the recombinant mu II GST from pPU109 was named ssGST mu II. The recombinant delta GST was named ssGST delta.

Kinetic analysis

All 3 recombinant enzymes showed enzymatic activity under steady-state conditions, using CDNB as substrate, and from the Michaelis-Menten plots we were able to determine the various kinetic parameters (Table 2). The enzymatic data suggested that the enzymes had unique kinetic properties regardless of

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Table 2. The kinetic parameters for steady-state catalysis of CDNB and GSH by ssGST delta, ssGST mu I and ssGST mu II as described in the Materials and Methods section

(Values are from 3 independent experiments, showing the mean ± standard error.)

Kinetic parameters	ssGST delta	ssGST mu I	ssGST mu II
K_m (mM) CDNB	0·3 ± 0·0[a]	4·2 ± 0·6	0·3 ± 0·0
K_m (mM) GSH	0·5 ± 0·1	0·4 ± 0·0	0·3 ± 0·0
k_{cat} (s^{-1}) CDNB	1·9 ± 0·1[a]	6·2 ± 0·4	10·3 ± 0·6
k_{cat} (s^{-1}) GSH	2·0 ± 0·1	3·8 ± 0·1	9·4 ± 0·3
k_{cat}/K_m (s^{-1} mM^{-1}) CDNB	6·9 ± 0·8[a]	1·5 ± 0·2	38·3 ± 6·1
k_{cat}/K_m (s^{-1} mM^{-1}) GSH	4·3 ± 0·5	9·5 ± 1·1	30·6 ± 3·4

[a] Result has previously been reported (Pettersson *et al*. 2005).

Table 3. Effect of 11 different acaricides on the enzyme activity of ssGST delta, ssGST mu I and ssGST mu II

(Procedures as described in the Materials and Methods section. All values are from 3 independent experiments, showing the mean ± standard error.)

			Reduction of activity (%)		
Acaricide group	Acaricide	mM	ssGST delta	ssGST mu I	ssGST mu II
Organothiophosphates	Diazinon	0·1	3·0 ± 3·2	−4·0 ± 5·3	7·9 ± 3·0
		0·2	10·0 ± 1·5[a]	1·0 ± 4·3	8·0 ± 0·4[a]
	Ethion	0·1	10·0 ± 3·5	3·0 ± 4·6	27·0 ± 1·1[a]
		0·2	26·0 ± 1·7[a]	ND[b]	ND[b]
	Chlorpyriphos	0·1	9·0 ± 0·9[a]	5·0 ± 3·8	25·0 ± 0·9[a]
		0·2	20·0 ± 1·0[a]	26·0 ± 3·1[a]	29·0 ± 0·4[a]
	Coumaphos	0·1	12·0 ± 3·7	3·0 ± 5·4	53·0 ± 1·8[a]
Pyrethroid esters	Flumethrin	0·1	19·0 ± 4·5[a]	18·0 ± 3·8[a]	50·0 ± 1·6[a]
	Permethrin	0·1	9·0 ± 0·6[a]	7·0 ± 4·9	37·0 ± 2·4[a]
	Deltamethrin	0·1	13·0 ± 2·9[a]	14·0 ± 3·7[a]	39·0 ± 4·0[a]
Formamidine	Amitraz	0·1	1·0 ± 1·4	2·0 ± 4·2	32·0 ± 3·8[a]
		0·2	2·0 ± 1·1	0·0 ± 4·8	ND[b]
Macrocyclic lactone	Ivermectin	0·1	2·0 ± 3·9	−3·0 ± 6·5	15·0 ± 9·7
		0·2	5·0 ± 2·1	13·0 ± 4·8	12·0 ± 3·1
Organochlorine	Lindane	0·1	3·0 ± 3·2	−3·0 ± 4·1	8·6 ± 0·9
		0·2	4·0 ± 1·0	3·0 ± 1·2	12·0 ± 0·7[a]
Bridged diphenyl	DDT	0·1	9·0 ± 2·8	6·0 ± 1·7	26·0 ± 1·3[a]
		0·2	21·0 ± 4·3[a]	20·0 ± 2·1[a]	24·0 ± 0·7[a]
	Control	[c]	0·0 ± 0·0	0·0 ± 0·6	0·0 ± 2·6
		[d]	0·0 ± 0·6	0·0 ± 0·7	0·0 ± 1·1

[a] Significant reduction of enzyme activity based on 95% confidence interval.
[b] Not detectable.
[c] Same amount of methanol as for 0·1 mM of acaricide.
[d] Same amount of methanol as for 0·2 mM of acaricide.

the class to which they belonged. For example, ssGST mu II had the highest catalytic efficiency (k_{cat}/K_m) for both CDNB and GSH whereas ssGST delta and ssGST mu II had identical apparent affinities (K_m) towards CDNB.

The greatest change in activity we observed, for any of the enzyme-acaricide combinations, was for ssGST mu II together with coumaphos, one of the organothiophosphate acaricides (Table 3). The ssGST mu II lost more than half of its activity in the presence of 0·1 mM of the acaricide. This was in sharp contrast with ssGST mu I, which only showed limited reduction of activity (3·0%) in the presence of 0·1 mM coumaphos. Two other organothiophosphates, diazinon and ethion, both significantly reduced the activity of ssGST mu II as well as ssGST delta. Only 1 of the tested organothiophosphates (chlorpyriphos) significantly reduced the activity of all 3 enzymes. Among the remaining substances tested, only flumethrin, deltamethrin and DDT gave a significant reduction of enzymatic activity for all 3 ssGST enzymes. Moreover, only the ssGST mu II enzyme activity was significantly reduced by amitraz and lindane; and the macrocylic lactone ivermectin

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gave a slight reduction in activity compared to the controls but the results were not statistically significant.

Tertiary structure comparison of mu-class GSTs

The fact that the two mu-class GSTs had close to 80% sequence identity, but had different kinetic characteristics and were affected differently by the various acaricides, suggested that there might be important structural differences. Through a sequence alignment of ssGST mu I and ssGST mu II we first identified which amino acid residues had weak or no biochemical similarities. Approximately one-fifth of these residues were located in the N-terminal domain (aa 1–80) and the rest in the C-terminal domain. To highlight these differences we used a 3-dimensional structure of the human mu-class GST M2-2 in complex with GSH (Fig. 1). By using this model it was clear that most of the structural differences were located on the outer surface of the proteins. Interestingly, the model also predicted that the differences between the *S. scabiei* mu-class GSTs were clustered around the catalytically important cleft that is between the two enzyme monomers. However, none of the structural differences seem to be located in the active site, e.g. in the actual GSH binding site. This suggests that these differences at the mouth of the cleft might account for how the two GST enzymes interact with various types of acaricides.

Genomic organization of the GST genes

The relative organization of the 3 GST genes in the *S. scabiei* genome was targeted by PCR using different gene-specific primers (Table 1). The primers were located in the 5′- and 3′-ends of the genes and directed outward from the genes. Only 1 of the tested combinations of primers, OP463 and OP459, resulted in any amplification (data not shown). The approximately 700 bp product linked the two mu GST genes to each other, which could also be confirmed by DNA sequencing (Fig. 2). To fully resolve the whole intergenic region we generated an additional PCR fragment with OP471 and OP463. The 500-nt-long intergenic region had an AT content of about 70%, with several homopolymeric stretches, in contrast to the coding sequences that had an AT content below 62%. Analysis of the intergenic region with the Transcriptional Factor Search tool identified several high-scoring potential heat shock transcription factor binding sites. However, this result might be biased by the high AT content.

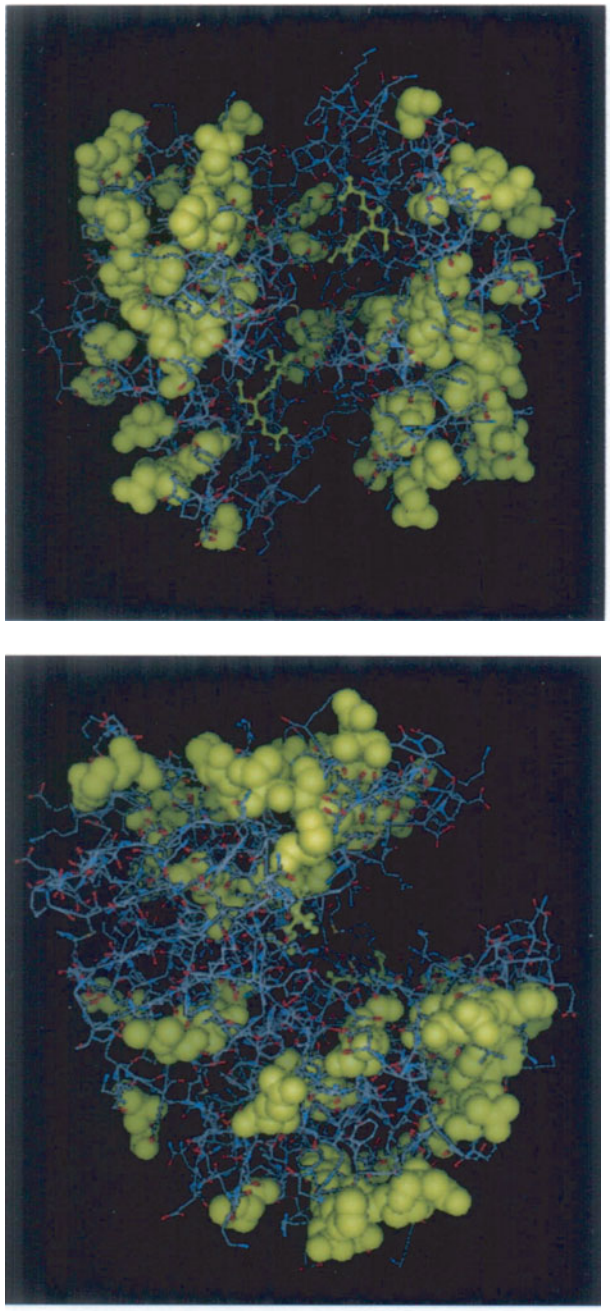

Fig. 1. Two different angles of the 3D-model of a homodimer of a human mu-class GST complexed with glutathione (PDB ID: 1XW5 and complexed glutathione shown as yellow sticks). Predicted structure differences between ssGST mu I and ssGST mu II are shown as yellow space filled spheres in each monomer in both pictures.

DISCUSSION

In this paper we report the functional characterization of 3 recombinant *Sarcoptes scabiei* glutathione transferases (GSTs) belonging to the mu- and delta-classes. Our results indicated that GSTs from the same class and with high amino acid sequence identity have significantly different kinetic properties. Tertiary structure modulations indicated that structural differences are the vital factor for these different properties. Our results also showed that all three of the *S. scabiei* GSTs analysed interact with different kinds of commercial acaricides, and thereby

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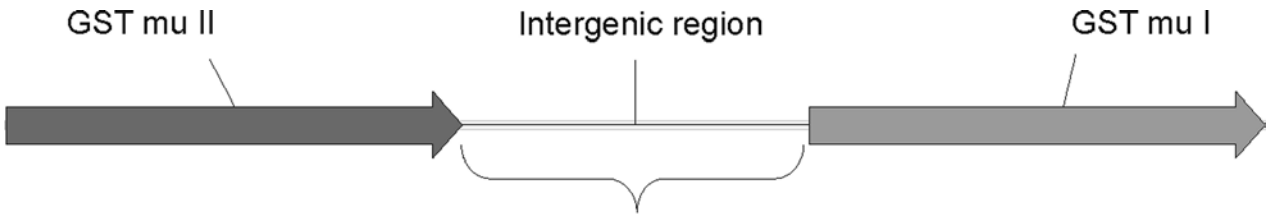


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1   taaaaattca atgtgaattg attgcaatga ctacaataag atcaatcgaa
51  atcgaataaa aacgatatga tattccataa tttgagtgat tatcattttc
101 tattcaattt atacagacga aaaaaaaatt tcagggacaa atgaatacca
151 gagaattgtt gtgcaaaaaa aaaaaacaga cccgaatctc catcaatgct
201 actactatca atgaaacaaa attttctttt ctaaaaccaa tttaattcat
251 tgcttctact ctatgacgaa atagcttcga atgatgccga taaccgagag
301 tcatcaattg tgattagatt gagggtaagt gatggagaga tgttgatggc
351 ggtgaatgat aataattata ttgaggcgtt tttcttctaa ttttcatttt
401 cgttttcgga tataaataaa acattttcga atcgaaaaaa aaatctctcc
451 attgaataga atcgtttcaa tcatcgaaat cgaaaaaatt cgaaacggaa
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Fig. 2. Schematic picture of the relative gene organization of GST mu I and GST mu II in the *Sarcoptes scabiei* genome. The nucleotide sequence below shows the 500 nt long intergenic region.

might be involved in the mite's defence towards these substances. Finally, in genome organization analysis it was shown that the two mu-class GSTs cluster in the *S. scabiei* genome.

All three recombinant enzymes showed enzymatic activity towards the model substrate CDNB, and the analysis demonstrated that there are no class-specific kinetic characteristics; instead structural differences seem to be the important factor. Since the two mu-class GSTs have high amino acid sequence identity (77·2%) but differ in their kinetic behaviour, we performed amino acid alignment and tertiary structure predictions to determine what might influence the enzymatic differences. The alignment analysis showed that approximately four-fifth of the amino acid differences appeared in the C-terminal domain and one-fifth in the N-terminal domain. These results correspond with the fact that the N-terminal domain is more conserved among the GSTs generally (He *et al*. 1999). The C-terminal domain includes many of the residues that interact with the electrophilic substrates (Enayati *et al*. 2005). For example, our results showed that ssGST mu I had a significantly lower apparent affinity towards the substrate CDNB than ssGST mu II (K_m = $4{\cdot}2 \pm 0{\cdot}6$ mM and $0{\cdot}3 \pm 0{\cdot}0$ mM, respectively), and also a significantly lower catalytic efficiency ($k_{cat}/K_m = 1{\cdot}5 \pm 0{\cdot}2\ s^{-1}\ mM^{-1}$ and $38{\cdot}3 \pm 6{\cdot}1\ s^{-1}\ mM^{-1}$, respectively). The N-terminal domain, on the other hand, includes the so-called G-site that is involved in the GSH binding (Enayati *et al*. 2005). In the tertiary structure predictions, where we studied the amino acids that differed between GST mu I and GST mu II in a 3D model of a human mu-class GST, we were able to verify that none of the differing amino acids in the N-terminal domain were located in the actual GSH-binding site. However, previous studies have shown that single amino acid differences outside the active sites can affect the enzyme activity (Ketterman *et al*. 2001; Ortelli *et al*. 2003). To summarize, most of the predicted tertiary differences between GST mu I and GST mu II were found on the exterior surface of the enzymes close to the cleft, which is between the two monomers. Catalytically important sites are located inside this cleft, and even minor conformational differences in the structure on the exterior surface of the cleft most certainly influence the kinetic properties of the enzyme (Ketterman *et al*. 2001). Because our analyses are performed using recombinant enzymes the exact values for the native enzymes might be slightly different.

Our kinetic experiments where we added acaricides to the reaction between GSH and CDNB, is the first attempt to characterise the effect of acaricides on *S. scabiei* enzymes. This study shows that all three GSTs might interact with various active

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substances used in acaricides. Interestingly, pyrethroid esters reduce the ssGST mu II activity at least 2-fold compared to their reduction of ssGST mu I activity as well as ssGST delta activity. Additionally, ssGST mu II lost more than 50% of its activity in the presence of coumaphos while the activity of the ssGST mu I was only slightly reduced under identical conditions. Moreover, the organochlorine lindane only reduced ssGST mu II activity. This indicates that each enzyme has its own profile of interaction with the various substances, which is in keeping with other GSTs and organisms (Jirajaroenrat *et al*. 2001; da Silva Vaz Jr. *et al*. 2004*b*). Many studies have shown that GSTs are associated with resistance against several of the active substances used in pest management of various insects. The GST-contributed resistance is achieved through different mechanisms: gene amplification or most often increased transcriptional rate of one or more GST enzyme (da Silva Vaz Jr. *et al*. 2004*a*; Enayati *et al*. 2005). Thereafter, the enzymes either convert the acaricides to more water-soluble substances through conjugation of the acaricide to GSH (Enayati *et al*. 2005), or passively lower the toxicity through a sequestering mechanism (Kostaropoulos *et al*. 2001). Moreover, there are examples of structural mutations of GSTs that render a reaction where GSH is used more as a co-factor than as a conjugate (Hemingway, 2000). Many of the active substances used in insecticides are also used in acaricides. However, very little is known about the mechanisms concerning resistance in ticks and mites (da Silva Vaz Jr. *et al*. 2004*a*, *b*). *S. scabiei* resistance has been reported both for the organochlorine lindane and the pyrethroid ester permethrin (Walton *et al*. 2004; Heukelbach and Feldmeier, 2006), and both of these acaricides strongly reduce the activity of ssGST mu II with CDNB as substrate. With the current results we cannot identify whether the function of the GSTs are inhibited by the acaricides, or if the acaricides are consumed in the assays. Hence, further analyses are needed to investigate this. Interestingly, GST mu II is identified as a major antigen in human patients with crusted scabies (Dougall *et al*. 2005).

In the genome organization analysis we showed that the two mu-class GSTs clustered in the *S. scabiei* genome. This result corresponds with previous findings, where GST-clusters have been identified for different GST subfamilies in various organisms (Morel *et al*. 2002; Ortelli *et al*. 2003; Soranzo *et al*. 2004). The clustering of the two mu-class GST genes and the lack of introns in both these genes indicate that a gene duplication might have occurred during evolution, so that the two mu-class genes originate from a single gene (Kumar and Reddy, 2001; Soranzo *et al*. 2004). This gene duplication has probably occurred to tailor the diversification and adapt to a new ecological niche; through a small number of amino acid substitutions, important differences in enzymatic activity have been achieved (Enayati *et al*. 2005).

GSTs have been described as having a 'promiscuous substrate specificity' (Angelucci *et al*. 2005) that works in favour for the organism but also provides one of the important obstacles for any treatment regime. Nevertheless, the functional and structural differences that are present among GSTs, between parasite and host, need to be better understood if we want to be successful in future parasite control. The present work clearly shows that *S. scabiei* has a large repertoire of GST enzymes to aid of its survival.

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