Functional analysis and localisation of a delta-class glutathione *S*-transferase from *Sarcoptes scabiei*☆

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Abstract

The mite *Sarcoptes scabiei* causes sarcoptic mange, or scabies, a disease that affects both animals and humans worldwide. Our interest in *S. scabiei* led us to further characterise a glutathione *S*-transferase. This multifunctional enzyme is a target for vaccine and drug development in several parasitic diseases. The *S. scabiei* glutathione *S*-transferase open reading frame reported here is 684 nucleotides long and yields a protein with a predicted molecular mass of 26 kDa. Through phylogenetic analysis the enzyme was classified as a delta-class glutathione *S*-transferase, and our paper is the first to report that delta-class glutathione *S*-transferases occur in organisms other than insects. The recombinant *S. scabiei* glutathione *S*-transferase was expressed in *Escherichia coli* via three different constructs and purified for biochemical analysis. The *S. scabiei* glutathione *S*-transferase was active towards the substrate 1-chloro-2,4-dinitrobenzene, though the positioning of fusion partners influenced the kinetic activity of the enzyme. Polyclonal antibodies raised against *S. scabiei* glutathione *S*-transferase specifically localised the enzyme to the integument of the epidermis and cavities surrounding internal organs in adult parasites. However, some minor staining of parasite intestines was observed. No staining was seen in host tissues, nor could we detect any antibody response against *S. scabiei* glutathione *S*-transferase in sera from naturally *S. scabiei* infected dogs or pigs. Additionally, the polyclonal sera raised against recombinant *S. scabiei* glutathione *S*-transferase readily detected a protein from mites, corresponding to the predicted size of native glutathione *S*-transferase.

1. Introduction

The microscopic mite *Sarcoptes scabiei* (Arthropoda: Arachnida: Acari) causes the disease sarcoptic mange or scabies that affects both animals and humans worldwide. The infection is immunopathological and arises because of the mite and the tunnels it digs in the skin of its host. Host cells are lysed both mechanically and due to the secretion of cytolytic components. These cytolytic components and antigens from mite bodies, faeces or eggs are believed to cause the immunopathogenic reactions (Burgess, 1994). Sarcoptic mange is usually associated with an intense itching, and later on cellular changes in the skin appear (Burgess, 1994). Depending on the immunological status of the host, the symptoms as well as the intensity, spread and course of events can vary a lot (Donabedian and Khazan, 1992). Some animal species (e.g. foxes) are very susceptible and infections often lead to high mortality rates (Mörner, 1992). In the pig industry, various preventive measures are taken in order to limit the damage caused by mange, both from an animal welfare and an economical perspective (Firkins et al., 2001).

Glutathione *S*-transferase (GST) is a cytolytic enzyme found in all eukaryotic organisms (Sharp et al., 1991). The enzyme is multifunctional, catalysing the conjugation between different forms of the tripeptide glutathione,

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e.g. reduced glutathione (GSH) (Krause et al., 2001; Ouaissi et al., 2002), and many structurally different substrates (Ouaissi et al., 2002; Yang et al., 2003). GST participates in a number of different reactions that are important in the defence of the cells against oxidative attacks by oxygen and free radicals (Sharp et al., 1991). The enzyme often has immunomodulatory properties, and GST secreted from parasites (i.e. protozoa and helminths) could play a part in evasion of the immune response of the host (Ouaissi et al., 2002). Consequently, GSTs have been identified as potentially good vaccine candidates against different parasite infections (Ouaissi et al., 2002). However, data on the immunomodulatory properties of GST from parasitic arthropods are so far very limited.

We have previously identified an expressed sequence tag (EST) from *S. scabiei* with sequence similarity to other arthropod GSTs (Ljunggren et al., 2003). In a smaller EST-study of *S. scabiei* isolated from humans, Fischer et al. (2003) also identified an EST with similarity to GST. A preliminary comparison suggests that these two GSTs are structurally different. Given the importance of GSTs in other parasite infections and the limited amount of molecular data available for *S. scabiei*, we have conducted experiments to further study the *S. scabiei* GST identified in our previous EST-study. To do this we have looked at the phylogenetic classification as well as the kinetic properties of recombinant forms of the *S. scabiei* GST. We have also assessed the localisation of the enzyme in and around the parasite in skin biopsies. Finally, the potential immune response from *S. scabiei* infected animals toward the protein has been examined.

2. Material and methods

2.1. Sarcoptes scabiei cDNA, expression vectors and Escherichia coli strains

A λ-phage lysate with the cDNA clone corresponding to EST SAS0751 (Ljunggren et al., 2003), GenBank accession number BM522085, was used for amplification of the *S. scabiei* GST cDNA. Two types of expression systems were used, one using the tac-promotor driven vector pPU16, a modified pMAL-c2 vector (Mattsson et al., 2001), and one using the T7-promotor driven vector pET-14b (Novagen, Madison, WI). For the construction of a new *S. scabiei*-specific expression system the tac-promotor driven vector pMAL-c2x (New England Bio-labs, Waltham, MA) was used as backbone. *Escherichia coli* strain XL-1 Blue MRF′ cells (Stratagene, Hercules, CA), was used for cloning and preparation of expression plasmids. *Escherichia coli* strain BL21(DE3) was used for high-level expression.

2.2. Template preparation and DNA sequencing

The complete insert corresponding to EST SAS0751 was amplified by PCR in 40 μl reactions using the vector specific primers T3 (5′-AAT TAA CCC TCA CTA AAG GG-3′) and T7 (5′-GTA ATA CGA CTC ACT ATA GGG C-3′). The reaction mixtures contained $1 \times Pfu$ 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^{-1} BSA; Stratagene, La Jolla, CA], 0.2% Tween, 0.25 μM of each primer, 200 μM of each deoxynucleotide, 1 μl of template and 1 U of *Pfu Turbo* DNA polymerase (Stratagene). The amplification was done in a PTC 200 machine (MJ Research, Waltham, MA). After two initial 5 min incubations at 93 and 50 °C, respectively, the cDNA was amplified for 30 cycles. Each cycle consisted of 1 min of denaturation at 93 °C, 2 min and 30 s of annealing at 50 °C and 2 min of extension at 72 °C. The PCR ended with a final extension for 10 min at 72 °C. The amplicon was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and was eluted with 50 μl distilled water.

DNA was sequenced by the dideoxynucleotide chain termination method using ABI PRISM Big Dye Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Each 20 μl reaction contained 2 μl Big Dye solution, 5 pmol of primer [T3, T7, OP244 (5′-GCG ATC ATC GAT CGT TGT-3′, OP245 (5′-TAC AAT GTT CCC AGA TCG-3′) or OP260 (5′-ATG ATG GTT CGC TTG CTA AAG G-3′)], 6 μl dilution buffer [200 mM Tris–HCl (pH 9.0) and 10 mM $MgCl_2$], dH_2O and 10–20 ng purified PCR-product. Sequence reactions were carried out with the following program: (96 °C for 10 s, 45 °C for 5 s and 60 °C for 4 min) $\times 25$. 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).

2.3. Bioinformatics

The sequences were processed using the Vector NTI program suite 8 (Informax Inc., Oxford, UK). Putative amino acid sequences, isoelectric points (pI) and protein sizes were calculated using the Compute pI/Mw program available through the ExPASy server (http://www.expasy.org). The GST class of the final sequence was identified by database searches and phylogenetic analysis. The protein sequences of a representative collection of the known cytosolic GST classes (i.e. excluding the microsomal MAPEG and mitochondrial kappa classes) were obtained from the DDBJ/EMBL/GenBank database, including most of the sequences from several model species that have had their GST complement thoroughly evaluated (the insects *Anopheles gambiae* and *Drosophila melanogaster*, the vertebrates *Homo sapiens* and *Mus musculus*, the plant

Arabidopsis thaliana, and the nematode *Caenorhabditis elegans*), along with all of the known Acari complete sequences (including the *S. scabiei* GST identified by Fischer et al., 2003) and a selection of platyhelminth and microfungal sequences. These peptide sequences were aligned using ClustalW v1.83 (ftp://ftp.ebi.ac.uk/pub/software/mac/clustalw/) with the default parameter settings. Since ClustalW can perform poorly when sequence identity is < 30% (Raghava et al., 2003; Thompson et al., 1999) and the sequence identity between GST classes is always below this value, the sequences were first aligned within classes, and then the multiple alignment was progressively built up using these alignments as profiles. The final alignment was edited by hand for consistency using MacClade v4.05 (http://www.macclade.org/). The alignment was analysed by neighbour-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). 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.

2.4. Amplification of the S. scabiei GST cDNA open reading frame

The open reading frame (ORF) of the *S. scabiei* GST cDNA (ssGST) was amplified by PCR from the EST SAS0751 clone with the forward primer OP248 (5′-GGA ATT CCA TAT GAT GGC TTC AGA GAA ACC AAC-3′) and the reverse primer OP249 (5′-CGG GAT CCT TAA GAG CTA TGT TGT TGT TGC-3′). To simply insert the ORF into the multicloning-site of the pET-14b vector, the primers were designed with linkers (underlined) containing restriction sites for *Nde*I and *Bam*HI, respectively. For the sub-cloning of the ssGST ORF into pPU16, the cDNA was amplified with the forward primer OP250 (5′-CGG GAT CCA TGG CTT CAG AGA AAC CAA-3′) and the reverse primer OP251 (5′-AAC TGC AGA GAG CTA TGT TGT TGT TGC-3′). To facilitate the cloning the linkers were designed with restriction sites for *Bam*HI and *Pst*I, respectively. For the sub-cloning into pMAL-c2x, the ssGST ORF was amplified with the forward primer OP258 (5′-GGA ATT CCA TAT GAA ATC AGA GAA ACC AAC AAT CTA TTG G-3′) and the reverse primer OP259 (5′-CCC CCG GGA GAG CTA TGT TGT TGT TGC-3′). The linker sequences carried recognition sites for *Nde*I and *Ava*I, respectively. While maintaining the start codon, the +2 codon GCT (alanine) was changed to an AAA-codon (lysine) in OP258 to enhance the expression of the recombinant protein (Stenström et al., 2001). All oligonucleotides used for cloning had additional nucleotides preceding the restriction sites to achieve efficient digestion by the restriction enzymes. All PCR conditions were as described above.

2.5. Subcloning of the S. scabiei GST cDNA ORF

The OP248/OP249 amplicon was digested and cloned into the *Nde*I–*Bam*HI sites of pET-14b. After identification of positive clones by colony PCR, the resulting plasmid was purified, control sequenced and designated pPU99. The OP250/251 amplicon was digested and cloned into the *Bam*HI–*Pst*I sites of pPU16. After purification and control sequencing the resulting plasmid was called pPU98.

To prepare the pMAL-c2x vector for further manipulation we firstly removed the maltose binding protein gene (MalE) and substituted it with the OP258/OP259 ssGST ORF, digested with *Nde*I and *Ava*I. Then, a short stretch of nucleotides coding for a hexahistidine-tag ending with a stop codon was inserted down-stream of the multicloning-site. After plasmid purification and control sequencing the resulting plasmid was designated pPU100.

2.6. Protein expression, purification and immunisation

For high-level expression, pPU98, pPU99 and pPU100 were freshly transformed into *E. coli* strain BL21(DE3). A single colony was inoculated into 20 ml of LB medium with ampicillin (50 µg ml^{-1}) and incubated on a shaker at 37 °C overnight. On day 2, 10 ml of the overnight culture was inoculated in 1 l fresh minimal medium (MM/CA; Pryor and Leiting, 1997) supplemented with 50 µg ml^{-1} carbenicillin. The MM/CA cultures were grown on a shaker at 37 °C until the OD$_{600}$ reached ∼0.9. Then the cells were cooled for 5 min in a water bath to a temperature of 20 °C. The recombinant gene expression was induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 0.5 mM. Induced cells were transferred to a shaker and incubated at 18 °C overnight. After incubation, cells were collected by centrifugation (4000 × g, 4 °C, 10 min). About half of the cells were resuspended in HiTrap Start buffer [1 × phosphate buffer (pH 7.4), 10 mM imidazole] and the rest in PBS (pH 7.3). To prevent protein degradation both buffers were supplemented with Complete Protease Inhibitor (Roche Molecular, Basel, Switzerland) according to the manufacturer's instructions.

Harvested cells from the high-level expression were lysed by sonication. Cell debris was collected by centrifugation (9000 × g, 4 °C, 30 min) and the resulting supernatant was filtered through a 0.45 µm filter. The recombinant ssGSTs were purified by affinity chromatography (1 ml HiTrap chelating HP columns or 1 ml GSTrap FF columns, both Amersham Biosciences, Uppsala, Sweden) and ion exchange chromatography (1 ml HiTrap Q HP columns, Amersham Biosciences) according to manufacturer's instructions on an ÄKTA-FPLC system (Amersham Biosciences). For ion exchange purification, 20 mM Tris–HCl (pH 7.0) was used as starting buffer, and elution was performed with a gradient of 0–0.5 M NaCl-solution in 10 or 20 column volumes. Buffer changes were performed on NAP columns according to the manufacturer's instructions

(Amersham Biosciences). A cell lysate was prepared from *E. coli* transformed with pPU16 followed by purification on a HiTrap chelating column. The resulting protein extract was used as a control in the GST enzyme assays.

All protein expressions and purification steps were checked by 10 or 15% SDS-PAGE and stained in 0.1% Coomassie Brilliant Blue solution. Final protein concentrations were estimated by the Bradford method (Bradford, 1976). Estimation of the protein purity was done using the image processing and analysis software ImageJ, available through the National Institute of Health (NIH, http://rsb. info.nih.gov/ij/). Recombinant GST expressed from pPU100 and purified with GSTrap was used to immunise two rabbits according to European Guidelines for Animal Health under ISO 9001:2000 standards (MedProbe, Oslo, Norway). For production details see http://www.medprobe. com. The ssGST from pPU100 was chosen for immunisation since it lacks the MBP and had the highest enzyme activity.

2.7. Western blot analyses

Protein fractions prepared by GSTrap purification were separated on 15% SDS-PAGE, and then transferred onto a nitrocellulose membrane at 300 mA for 45 min with a mini-Trans-Blot transfer cell (BioRad, Hercules, CA). The membrane was blocked in TBS [Tris buffered saline: 100 mM Tris–HCl (pH 7.5), 0.9% NaCl] with 5% non-fat dried milk for 1 h at RT or overnight at 4 °C. For the Western blot analysis, a number of sera were analysed: six were from dogs diagnosed with scabies, three were from healthy dogs, five were from pigs diagnosed with scabies and four were from healthy pigs. Two polyclonal sera raised against the recombinant version of *S. scabiei* GST were included in the analyses as well as a serum specific towards *Schistosoma japonicum* GST (sjGST-ab) available from Amersham Biosciences. The membranes were cut into strips and incubated for 90 min at RT. All clinical sera were diluted 1:100 and immune sera were diluted 1:1000. The strips were washed with T-TBS [0.1% Tween in TBS] and then incubated with various secondary antibodies (a mouse anti-dog and rabbit anti-mouse IgG mixture for the dog sera, diluted 1:1000, a rabbit anti-pig IgG for the pig sera, also diluted 1:1000, a rabbit anti-goat IgG for the sjGST-ab, diluted 1:200 and a pig anti-rabbit IgG for the polyclonal sera, diluted 1:1000) labelled with horseradish peroxidase. After a 1 h incubation at RT, the strips were washed with T-TBS and the bound secondary antibodies were visualised by the ECL fluorescent detection reagents on Hyper film, according to the manufacturer's instructions (Amersham Biosciences). The dog sera were also analysed for the presence of reactive IgE-antibodies using a goat anti-dog-IgE peroxidase conjugate (ICL, Newberg, OR). For the analysis of *S. scabiei* antigens, mites and protein extracts were prepared as previously described (Mattsson et al., 2001).

2.8. Determination of enzyme activity

After a one-step purification, the specific activity of the different varieties of ssGST was determined using the GST detection module according to the manufacturer's instruction (Amersham Biosciences). The GST activities were measured spectrophotometrically in an Ultrospec 1000 UV/Visible spectrophotometer (Amersham Biosciences) and 1 cm spectrophotometer cells. For the substrate 1-chloro-2,4-dinitrobenzene (CDNB) a molar extinction coefficient ($\Delta\varepsilon$) of 9.6 mM^{-1} cm^{-1} was used (Habig et al., 1974). The activities, μmol min^{-1} mg^{-1}, were calculated as $((A(t2)-A(t1))/(\Delta\varepsilon \times (t2-t1) \times b \times m)) \times V_{tot}$ where A is absorbance, t is time (min), $\Delta\varepsilon$ is the molar extinction coefficient (mM^{-1} cm^{-1}), b is the path length (cm), m is weight (mg) and V_{tot} is final volume in cuvette (l).

To determine the apparent V_{max}, K_M and k_{cat} values for ssGST, the enzyme activity towards 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 assay, a CDNB range from 0.05 to 1.5 mM, a fixed reduced glutathione (GSH) concentration of 5 mM and a fixed ssGST concentration of 0.38 μM were used. All reactions were performed at 21 °C and the absorbance at 340 nm was measured every 20 s 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 V_{max} and K_M 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 V_{max} and $[E]_{tot}$. Graphs and calculations were performed using GraphPad Prism v4 (http://www.graphpad.com).

2.9. Immunolocalisation of GST

Formalin fixed and paraffin embedded skin biopsies, 5–6 μm in thickness, from an *S. scabiei* infected fox were deparaffinised, rehydrated and rinsed in 50 mM Tris–HCl (pH 7.6) with 9% NaCl according to standard procedures. The material was then treated against endogenic peroxidases by impregnation with 1.5 ml 30% H$_2$O$_2$/50 ml 0.05 M Tris–HCl for 20 min in a darkroom, and against unspecific binding through microwave treatment (750 W) for 2×5 min in 10 mM Tris and 1 mM EDTA (pH 9.0), and thereafter for 15 min at RT in fresh 10 mM Tris and 1 mM EDTA (pH 9.0). The cross-sections were incubated with the ssGST specific polyclonal immune serum diluted 1:2000 in 50 mM Tris–HCl (pH 7.6) and after 30 min the samples were rinsed as above. For detection, a streptavidin–biotin system, DAKO LSAB+ (DAKOCytomation, Glostrup, Denmark), was used according to Dakopatt's instructions, the AEC Substrate kit for peroxidase (Vector Laboratories, Burlingame, CA) was used as substrate and Mayer's haematoxylin was used as counterstain.
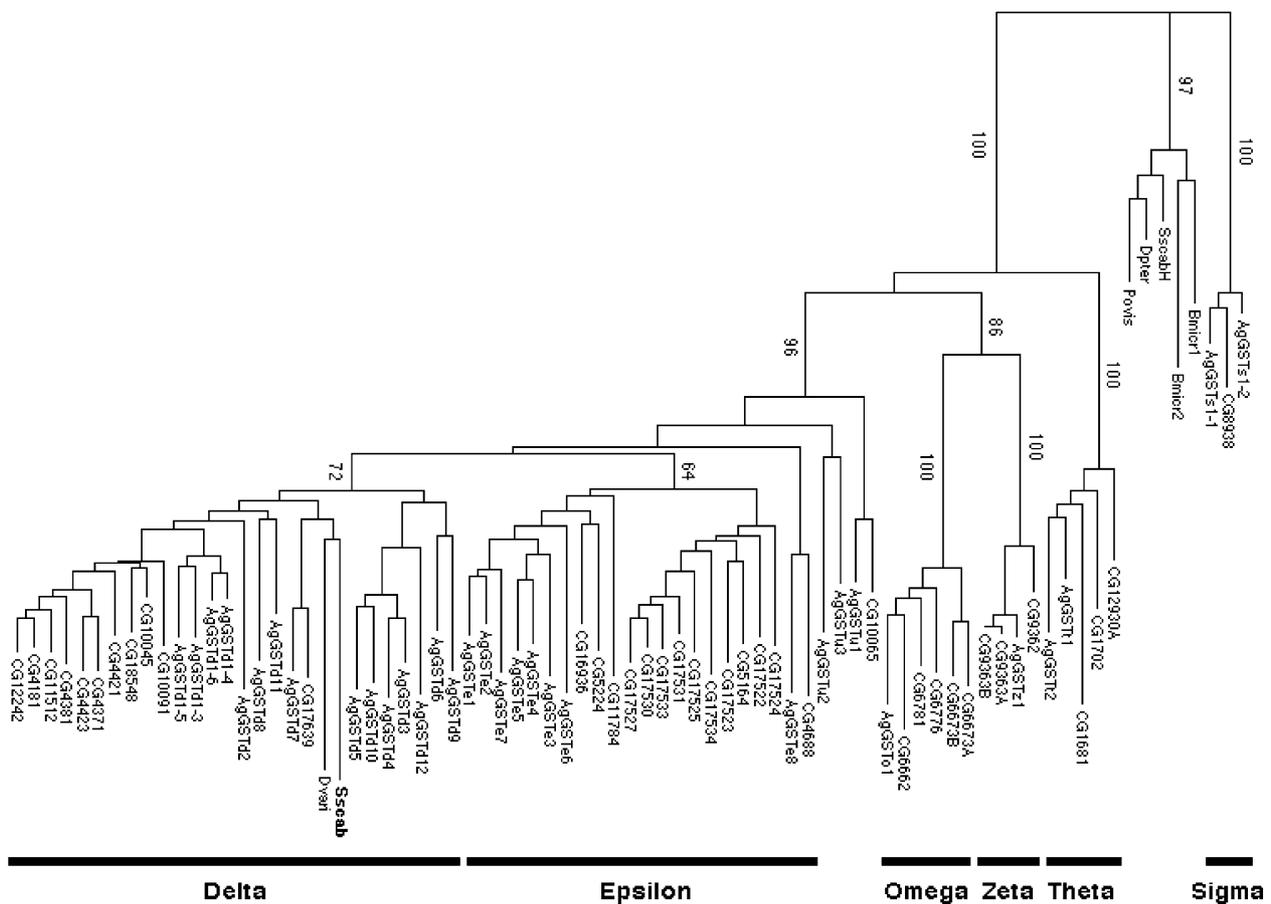
3. Results

3.1. Sequence analysis of the S. scabiei GST

The ssGST nucleotide sequence was identified through sequence analysis of EST SAS0751 (Ljunggren et al., 2003). The full cDNA sequence included a complete ORF of 684 bp that encoded a putative protein of 227 amino acids in length and with a predicted molecular weight of 26 kDa. The cDNA sequence corresponding to the complete ORF of *S. scabiei* GST has been deposited in GenBank under the accession number AY649788.

A preliminary analysis of 179 eukaryote GST protein sequences showed two clear gene groups when rooted on the microfungi gamma class, in accord with other recent extensive analyses (Marco et al., 2004), with the alpha, mu, pi and sigma/nematode classes forming one group and the delta, epsilon, omega, phi, tau/lambda, theta and zeta classes forming the other. This analysis clearly associated two of the Acari (tick and mite) GST sequences with the delta and/or epsilon classes and the other five sequences with the mu-class. Therefore, we separately analysed the relationships among each of these two groups of identified classes in more detail.

The first analysis involved all of the known GST sequences from *A. gambiae* and *D. melanogaster*, both of which have been thoroughly studied (Ding et al., 2003), along with the Acari sequences. This analysis unambiguously shows that both the new *S. scabiei* (mite) sequence described in the present work and the *Dermacentor variabilis* (tick) sequence are members of the delta-class of GSTs (Fig. 1). This is the first report that this class of GSTs occurs outside the class Insecta. The second analysis involved all of the known mu-class sequences from platyhelminths and a selection of vertebrates, along with selected representatives of the related classes and all of the Acari sequences. This analysis places the other *S. scabiei* (Fischer et al., 2003), the two *Boophilus microplus*,

Fig. 1. Neighbour-joining phylogenetic tree, based on distances derived from the Jones–Taylor–Thornton amino-acid substitution model, showing the relationships of the known Acari GST gene sequences to those of the complete *Anopheles gambiae* and *Drosophila melanogaster* GST gene superfamilies. Where relevant, sequence nomenclature follows Ding et al. (2003), with Ag: *A. gambiae*, CG: *D. melanogaster*, Bmicr1: *Boophilus microplus* larva, Bmicr2: *B. microplus* adult, Dpter: *Dermatophagoides pteronyssinus*, Dvar: *Dermacentor variabilis*, Povis: *Psoroptes ovis*, Sscab: *Sarcoptes scabiei* (this study), SscabH: *Sarcoptes scabiei* var. *hominis*. Shown at the bottom is the classification of the genes into GST classes. The numbers on the branches indicate the percentage support from 1000 bootstrap replicates, showing the relatively high bootstrap support for the relevant branches. The maximum-likelihood tree was similar to the one shown here.
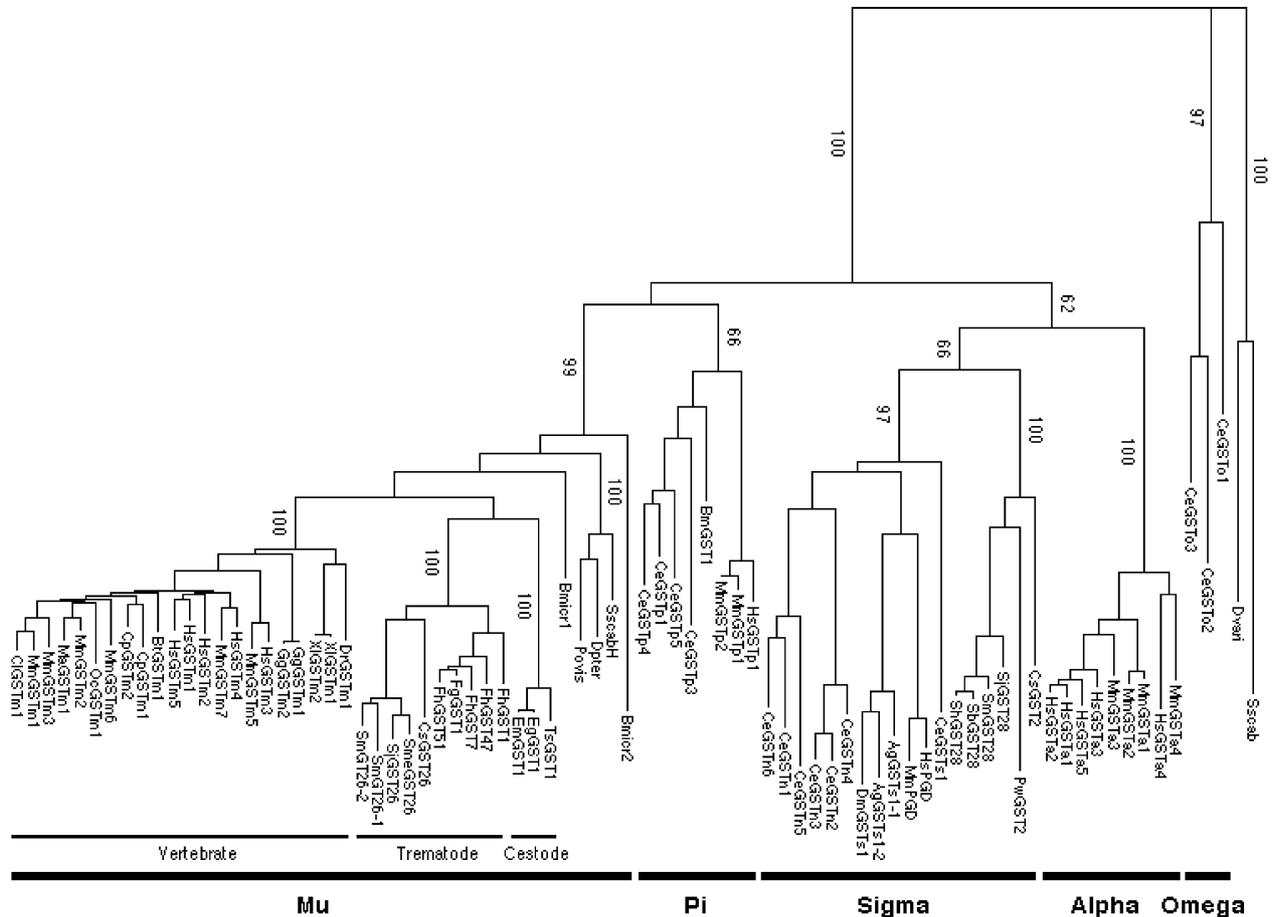
Fig. 2. Neighbour-joining phylogenetic tree, based on distances derived from the Jones–Taylor–Thornton amino-acid substitution model, showing the relationships of the known Acari GST gene sequences to those of the complete *Homo sapiens*, *Mus musculus* and *Caenorhabditis elegans* GST gene superfamilies for the displayed classes, plus selected platyhelminth and vertebrate-mu sequences. Where relevant, sequence nomenclature follows Marco et al. (2004), with Ag: *Anopheles gambiae*, Bm: *Brugia malayi*, Bt: *Bos taurus*, Ce: *C. elegans*, Cl: *Cricetulus longicaudatus*, Cp: *Cavia porcellus*, Cs: *Clonorchis sinensis*, Dm: *D. melanogaster*, Dr: *Danio rerio*, Eg: *Echinococcus granulosus*, Em: *Echinococcus multilocularis*, Fg: *Fasciola gigantica*, Fh: *Fasciola hepatica*, Gg: *Gallus gallus*, Hs: *H. sapiens*, Ma: *Mesocricetus auratus*, Mm: *M. musculus*, Oc: *Oryctolagus cuniculus*, Pw: *Paragonimus westermani*, Sb: *Schistosoma bovis*, Sh: *Schistosoma haematobium*, Sj: *Schistosoma japonicum*, Sm: *Schistosoma mansoni*, Sme: *Schistosoma mekongi*, Ts: *Taenia solium*, Xl: *Xenopus laevis*, Bmicr1: *Boophilus microplus* larva, Bmicr2: *Boophilus microplus* adult, Dpter: *Dermatophagoides pteronyssinus*, Dvar: *Dermacentor variabilis*, Povis: *Psoroptes ovis*, Sscab: *Sarcoptes scabiei* (this study), SscabH: *Sarcoptes scabiei* var. *hominis*. Shown at the bottom is the classification of the genes into GST classes. The numbers on the branches indicate the percentage support from 1000 bootstrap replicates, showing the relatively high bootstrap support for the relevant branches. The maximum-likelihood tree was similar to the one shown here.

the *Dermatophagoides pteronyssinus* and the *Psoroptes ovis* sequences as the basal branches within the mu GST class (Fig. 2). This is the first report that two classes of GSTs occur within the Acari, and also within a single species of the Acari.

3.2. Sub-cloning of the S. scabiei GST

To continue to characterise the ssGST, we sub-cloned the ORF into two different expression vectors. The first construct (pPU98) directed the expression of the ssGST as a fusion protein under the control of the tac-promoter. The ssGST fusion protein had the maltose binding protein (MBP) from *E. coli* as an N-terminal fusion partner and

a C-terminal His_6-tag. The predicted size of the recombinant protein was estimated to be 70 kDa. The second expression plasmid (pPU99) had the ssGST fused to an N-terminal His_6-tag under the control of the T7 promoter. The estimated molecular weight for the His-tagged ssGST was 28 kDa.

A third construct (pPU100) had the ssGST spliced to a multicloning-site followed by a sequence coding for a His_6-tag. The size of the recombinant protein was estimated as 29 kDa. During the construction of pPU100 the $+2$ codon of the ssGST sequence was exchanged from a GCT (alanine) to an AAA-codon (lysine) to enhance the expression of the recombinant protein (Stenström et al., 2001).
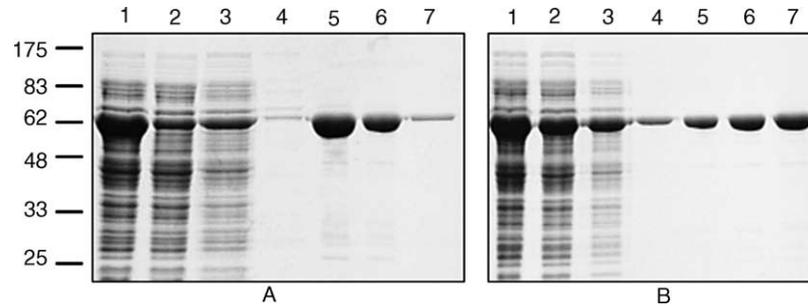
Fig. 3. Coomassie Blue-stained SDS-polyacrylamide gels after two independent affinity purifications. (A) Whole cell lysate and various fractions after purification on a 1 ml HiTrap chelating HP column. (B) Whole cell lysate and various fractions after purification on a 1 ml GSTrap FF column. Lane 1, *Escherichia coli* cell lysate; lane 2, flow-through; lane 3, wash; lanes 4–7, eluted fractions. The sizes of the molecular mass markers are given in kDa (New England Biolabs prestained protein marker).

3.3. Expression and purification of ssGST

Both pPU98 and pPU99 were transformed into BL21(DE3) for expression of the recombinant ssGST protein. Induction of expression by the addition of IPTG resulted in the production of very high yields of recombinant protein from both constructs, as indicated by SDS-PAGE analysis (data not shown). We could not detect any significant differences in expression between the different recombinant proteins. This suggests that MBP might not be necessary to stabilise the ssGST in *E. coli* (Hannig and Makrides, 1998).

After preparation of bacterial lysates, half of the amount of the recombinant ssGST produced from pPU98 was affinity purified on a glutathione column by means of the GST activity (ssGST1) and the rest was affinity purified by using His$_6$-tag (ssGST2). The same purification procedures were applied to the recombinant ssGST from pPU99 (ssGST3 and ssGST4). The use of the glutathione-GST affinity purification scheme gave better results than when using the His$_6$-tag strategy (Fig. 3). Nevertheless, both strategies yielded purities greater than 90%. Estimation of protein amounts showed that the level of recombinant protein in the expression experiments exceeded the binding capacity of the affinity columns.

We also purified ssGST3 to homogeneity. After the GST-glutathione affinity purification, ssGST3 was captured on a HiTrap chelating column via its His$_6$-tag. Before being loaded onto an anion exchanger column, the pH was adjusted to 7.0 according to the estimated pI (5.7) by the use of an NAP5 column for buffer exchange. In the final polishing step, the ssGST3 was purified to homogeneity on a HiTrap Q HP anion exchanger column. After the final step the purity was estimated to be about 99%.

The pPU100 plasmid was expressed and purified in the same manner as above but the yield from this pPU100 was lower than from the other constructs. The resulting proteins were designated ssGST5 and ssGST6, respectively.

3.4. Kinetic analysis of recombinant S. scabiei GST

All ssGST proteins showed specific activity in the enzyme assay with the GST detection module (Table 1). In contrast, a partially purified *E. coli* extract purified on a HiTrap chelating column did not demonstrate any activity. Higher specific activities were observed for the recombinant proteins that were purified using the GST binding properties. The MBP fusion partner will have an impact on the specific activity since the calculation is dependent on the amount of protein added to the assay. This was also observed for ssGST1 and ssGST2. The tag positioning might influence the down-stream performance of the target protein when constructing fusion–tag complexes. The initial comparison between ssGST3 and ssGST5 suggested that the position of the His$_6$-tag did not affect the kinetics (Table 1). To further study these two recombinant enzymes we performed a more thorough enzyme assay. The ssGST3

Table 1
The specific enzymatic activity towards CDNB for six different ssGST protein fractions as described in Section 3.4

ssGST fraction	Construct	Purification column	Specific activity (μmol min^{-1} mg^{-1})
ssGST1	MBP-ssGST-His$_6$-tag	GSTrap FF	6.10 ± 0.50
ssGST2	MBP-ssGST-His$_6$-tag	HiTrap chelating HP	3.60 ± 0.25
ssGST3	His$_6$-tag-ssGST	GSTrap FF	12.0 ± 0.50
ssGST4	His$_6$-tag-ssGST	HiTrap chelating HP	6.90 ± 0.55
ssGST5	ssGST-His$_6$-tag	GSTrap FF	12.0 ± 1.0
ssGST6	ssGST-His$_6$-tag	HiTrap chelating HP	4.30 ± 0.18

All values are from three independent experiments and the maximal deviations from the average values are given in the table.

Table 2
The kinetic properties for steady-state catalysis of CDNB by ssGST3 and ssGST5 as described in Section 2

Kinetic parameters	ssGST3	ssGST5
V_{max} (μmol min^{-1} mg^{-1})	405 ± 12	958 ± 39
K_M (mM)	0.28 ± 0.026	0.31 ± 0.038
k_{cat} (s^{-1})	193 ± 5.7	470 ± 19
k_{cat}/K_M (s^{-1} mM^{-1})	689 ± 67	1516 ± 196

Values are from three independent experiments and the standard errors are given in the table. ssGST3: His$_6$-tag-ssGST, purified with GSTrap FF column. ssGST5: ssGST-His$_6$-tag, purified with GSTrap FF column.

and ssGST5 were analysed under steady-state conditions using CDNB as substrate. From the Michaelis–Menten plot we could determine the various kinetic properties (Table 2). We detected differences in the turnover rate (k_{cat}) as well as in the catalytic efficiency (k_{cat}/K_M) between these recombinant enzymes.

3.5. Western blot analysis

In Western blot experiments, the rabbit hyperimmune sera reacted both with ssGST5 and with the native GST in *S. scabiei* protein extract derived from mites isolated from foxes (Fig. 4). The molecular mass of the recombinant GST (ssGST5) corresponded to the predicted value of 29 kDa. When analysing *S. scabiei* protein extracts, the polyclonal sera reacted with a band of the expected molecular mass for native *S. scabiei* GST (26 kDa). The polyclonal antibody against GST from *S. japonicum* did not cross-react with the recombinant ssGST. Interestingly, none of the sera from
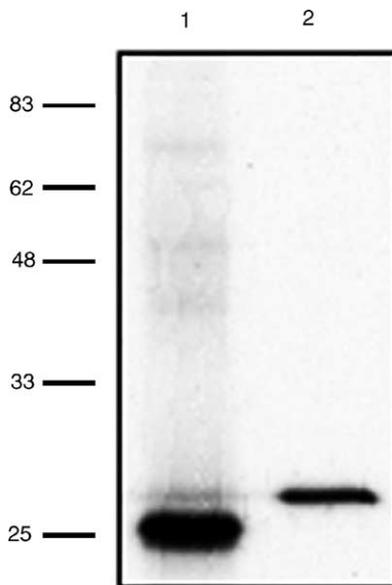

Fig. 4. Western blot of purified recombinant *Sarcoptes scabiei* GST and native *S. scabiei* proteins, derived from mites isolated from foxes, were probed with the polyclonal sera raised against recombinant *S. scabiei* GST. Lane 1, native *S. scabiei* protein extract; lane 2, recombinant ssGST5. The sizes of the molecular mass markers are given in kDa (New England Biolabs prestained protein marker).
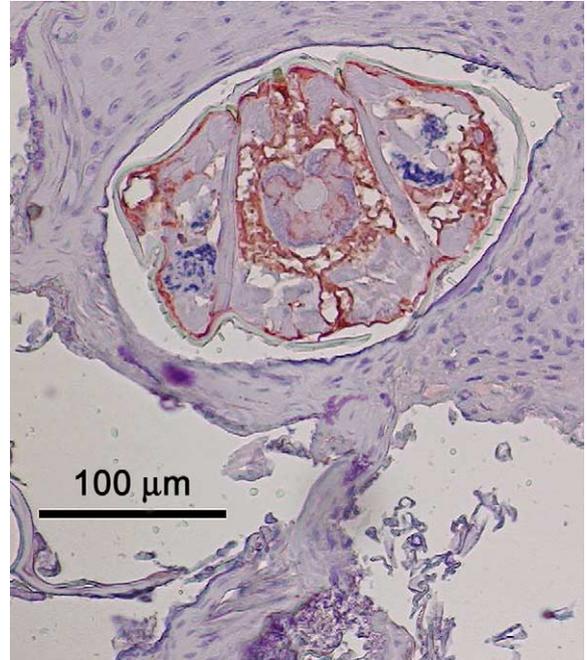

Fig. 5. Distribution of ssGST in adult *Sarcoptes scabiei* derived from a mange-infected fox. Immunostaining (indicated with brown/red colour) was noted in the integument of the epidermis and the cavities surrounding vital organs. Minor staining was observed in the intestine, and no labelling was detected in the host tissue outside the mite's body.

mange-infected dogs and pigs contained specific antibodies towards ssGST. Thus, no difference was obtained between dogs and pigs with sarcoptic mange and dogs and pigs without the disease (data not shown).

3.6. Immunolocalisation

The anti-ssGST sera stained discrete portions of the mites (Fig. 5), while preimmune serum showed no staining. Specifically, the integument of the epidermis and cavities surrounding vital organs were stained. Minor staining was observed in the intestine, and no labelling was detected in the host tissue outside the mite's body.

4. Discussion

In this paper, we report the identification and characterisation of a *S. scabiei* glutathione *S*-transferase (ssGST) belonging to the delta-class. Active versions of the enzyme were produced in large amounts in *E. coli* and purified to homogeneity. In our kinetic analysis with recombinant ssGST it appears as if the positioning of affinity tags influences the catalytic activity. With antibodies towards recombinant ssGST we have shown that the GST is expressed in *S. scabiei* and that the enzyme was localised in discrete areas in the parasite.

Eukaryote GSTs are currently arranged into 12 different classes: alpha, delta, epsilon, lambda, mu, omega, phi, pi,

tau, theta, sigma and zeta, as well as a mitochondrial kappa and a microsomal class (Chelvanayagam et al., 2001; Marco et al., 2004; Sheehan et al., 2001). Most well studied species have been shown to have genes for several of these GST classes, although some classes are still considered to be taxon-specific (e.g. phi, tau and lambda have been found only in plants, and delta and epsilon only in insects). However, ours is the first report of genes for two distinct GST classes in any member of the Acari (ticks and mites). Given that other species usually have genes for half-a-dozen GST classes, and frequently have several genes for each class, we expect that more genes will be found in *S. scabiei* as well as in other Acari.

The ssGST described in this work has been identified as belonging to the delta GST class, which has until now been considered to be insect-specific (Ding et al., 2003; Sawicki et al., 2003). Given that we have now reported this class from two unrelated species of Acari (both a tick and a mite), we predict that it is likely to be more widespread within this taxonomic group. It may be even more widespread among arthropods as a whole, given that Insecta and Acari are not usually considered to be closely related taxa. Therefore, it will be interesting to look at other members of the Arachnida, as well as the Crustacea, as no complete GST sequences have yet been reported from these groups. The epsilon GST class has not yet been reported from the Acari, but it is possible that it may also occur in this taxonomic group.

We have identified the other *S. scabiei* GST gene (Fischer et al., 2003) as belonging to the mu GST class, which is sometimes considered to be vertebrate-specific (Chelvanayagam et al., 2001). Our analyses indicate that this would only be true if the Platyhelminthes (flatworms) and Acari sequences are classified as new GST classes. It has long been suggested that these two taxonomic groups have mu-class GSTs (O'Neill et al., 1994; Panaccio et al., 1992), but no thorough analysis of the relationships has been reported until now.

All of the different ssGST preparations were catalytically active, as demonstrated by their ability to conjugate reduced glutathione to the substrate CDNB. Recombinant enzymes purified using the GST and glutathione interaction showed higher specific activity compared to those purified using the His-tag. This is probably due to the selective enrichment of correctly folded enzymes in the former protocol. The HiTrap purification in the other protocol does not discriminate between the active and inactive forms of the enzyme. During steady-state conditions both ssGST3 and ssGST5 had relatively high affinity towards CDNB ($K_{M(ssGST3)} = 0.28 \pm 0.026$ mM and $K_{M(ssGST5)} = 0.31 \pm 0.038$ mM) compared to some earlier studies on recombinant delta GSTs from mosquitoes (Jirajaroenrat et al., 2001; Prapanthadara et al., 1998), where two delta GSTs from *A. gambiae* had K_M between 0.099 and 0.123 mM, and three enzymes from *Anopheles dirus* had values between 0.10 and 0.21 mM, although a fourth member of the delta-class had a K_M of

0.52 ± 0.067 mM. However, the ssGST5 had the highest turnover rate ($k_{cat} = 470 \pm 19$ s^{-1}) and is the most reactive enzyme in catalysing CDNB conjugation ($k_{cat}/K_M = 1516 \pm 196$ mM^{-1} s^{-1}). This indicates that the construction of the recombinant protein might affect its enzymatic ability. The N-terminal domain of GST is the most conserved part of the enzyme whereas substrates bind to the C-terminal region (Ouaissi et al., 2002). In ssGST3, the His$_6$-tag is located N-terminally of the ssGST, and probably interferes with the enzyme function since the catalytically important amino acids are proposed to reside in the N-terminal domain of GST (Chen et al., 2003).

Immunolocalisation using specific antibodies against the recombinant protein identified unique locations in the parasite. The ssGST is immunolocalised to the integument of the epidermis and the cavities surrounding vital organs. A much weaker staining was observed in the intestine. However, we could not detect any staining on the external surface of *S. scabiei* or in the host tissue. In our sequence analysis we could not identify any signal peptide typical for a secretion signal. Thus, the absence of ssGST in the host tissue and the sequence data suggest that this ssGST is not exposed to the immune system of the host. Immunoblotting data also confirmed this observation, since we did not detect any reaction between *S. scabiei*-specific IgG or IgE with ssGST.

The existence of a delta-class GST and a mu-class GST in *S. scabiei* challenges some of the conceptions about the GST classification in general, but it also highlights the importance of more studies about *S. scabiei*. For instance, do other members of the *S. scabiei* GST family contribute to the immune response by the host or exert any immunomodulatory functions? Whatever the answers are, much more information is needed about the molecular pathways underlying pathogen biology and the host–pathogen interaction in scabies.

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