Structural and functional analysis of a glutathione S-transferase from *Ascaris suum*

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A recombinant glutathione S-transferase (GST) (EC 2.5.1.18) from the parasitic nematode *Ascaris suum* (AsGST1) displays specific activity with a variety of model substrates and secondary products of lipid peroxidation. The AsGST1 interacts with a range of model inhibitors, haematin-related compounds, bile acids and anthelmintics. The reported variations in biochemical activity correlate with structural differences observed by homology modelling. Here, differences in the topography of the proposed substrate binding site between the AsGST1 and the host GSTs were identified. A rabbit polyclonal antiserum was raised against the glutathione-binding proteins of *A. suum* and specific antibodies against AsGST1 were affinity-purified using the recombinant protein. These antibodies were used to localize

the AsGST1 in adult worms by immunohistochemical staining. The strongest immunostaining for AsGST1 was localized in the intestine in all worms examined. This suggests that the enzyme may be responsible for the metabolism of materials that are incorporated from the environment, as well as for molecules that are excreted or secreted from the parasite to the environment. It also demonstrates the accessibility of the enzyme to an inhibitor or blocking antibody. In addition, the structure and sequence of the gene encoding AsGST1 have been determined. Southern-blot analyses of the AsGST1 gene suggests that it is a single-copy gene. The nucleotide sequence analysis revealed that the gene is composed of four exons and three introns, and potential regulatory elements were identified in the 5' flanking sequence.

INTRODUCTION

In this investigation, the roundworm *Ascaris suum* was used as a model for the closely related human pathogenic nematode *Ascaris lumbricoides*, the most prevalent human intestinal helminth. Crompton [1] estimates that more than one billion people are infected, which represents approx. 22% of the world's population. Chronic infection with adult worms may lead to impaired nutrition, especially in children, whereas the acute phase involves allergic bronchopneumonia, intestinal obstruction, biliary or pancreatic duct obstruction and hepatic abscesses. Elimination of an *Ascaris* infection involves chemotherapy, as well as the modification of living conditions, thereby reducing the risk of re-infection [2]. Currently, one of the drugs most widely used to treat ascariasis is albendazole, which is highly effective against both adult and larval stages of *Ascaris*, with relatively few side effects. However, due to the extremely high infection rate, the development of immunoprophylaxis against ascariasis is also required.

The glutathione S-transferases (GSTs) (EC 2.5.1.18) are detoxification enzymes that catalyse the nucleophilic addition of the tripeptide GSH to endogenous and xenobiotic electrophilic toxins. The GSTs have been implicated in the development of resistance to pesticides, herbicides, antibiotics, anthelmintics and a number of additional drugs [3–7]. The role of GST as a protective antigen in vaccination against trematode infections is well established. Therefore the development of a parasite GST inhibitor or neutralizing antibody, to supplement existing chemotherapeutic strategies, is a major goal of parasite GST research.

We have previously reported the isolation, sequence and

expression of a cDNA encoding an *A. suum* GST (AsGST1) [8]. To complement our structural analysis of the AsGST1, we have further analysed the substrate specificities of recombinant AsGST1 (rAsGST1) and conducted a survey of the inhibitor sensitivities. To assess the functional realm of this enzyme in the parasite, AsGST1 has been localized by immunohistochemistry. This also indicates the accessibility of the enzyme to an inhibitor or blocking antibody. Finally, since the expression of GSTs is inducible, we have begun to dissect the molecular regulators governing expression of this enzyme. We have analysed the genomic structure and potential promoter regions of the AsGST1 gene. Induction of the expression of this enzyme by the parasite may be linked to survival, in a response to immunological or drug-related stress.

EXPERIMENTAL

Parasites

Adult *A. suum*, which represents a convenient model for parasitic nematodes in general, were collected from a local slaughterhouse. For construction of the *A. suum* genomic library, the worms were snap-frozen at -70°C and homogenized. For Western blotting, adult worms were washed with sterile RPMI 1640 medium, the adult *A. suum* (two worms per flask) were transferred to 20 ml of medium containing penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) and incubated for 12 h. Subsequently the medium was replaced with fresh medium, also supplemented with penicillin and streptomycin, and the worms were incubated for another 12 h to reduce their gut content. At the end of the

Abbreviations used: GST, glutathione S-transferase; AsGST1, glutathione S-transferase from *Ascaris suum*; rAsGST1, recombinant AsGST1; CDNB, 1-chloro-2,4-dinitrobenzene; rOvGST1/rOvGST2, GSTs from the nematode *Onchocerca volvulus*; PG, prostaglandin; APAAP, alkaline phosphatase-anti-(alkaline phosphatase).

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incubation, the reproductive and muscle tissue and the gut of *A. suum* were isolated and homogenized in PBS [150 mM NaCl/16 mM Na_2HPO_4 /4 mM NaH_2PO_4 (pH 7.3)] containing 1% (v/v) Triton X-100.

Production of recombinant AsGST1 and enzyme assays

The complete AsGST1 cDNA was ligated into the expression vector pJC20 and transformed into the *Escherichia coli* strain BL21. The rAsGST1 was purified using GSH–Sepharose chromatography as previously described [8].

The standard second substrate used was 1-chloro-2,4-dinitrobenzene (CDNB) [9], and conjugating activities with other second substrates (obtained from Aldrich-Chemie) were determined as described in the literature [10]. The assay used to determine the glutathione-dependent conjugation of 4-hydroxynonenal was performed according to Alin et al. [11]. The GSH peroxidase activity of the GST was determined according to the method of Günzler and Flohe [12]. Linoleic acid hydroperoxide was synthesized by autooxidation of linoleic acid as described by Brophy and Barrett [13].

Protein concentrations were determined by the method of Bradford [14] using BSA as a standard.

Inhibition studies

Inhibition was estimated graphically as the IC_{50} value, which is the concentration of an inhibitor that is required for 50% inhibition of enzymic activity in a standard assay, using CDNB as the second substrate [15]. Inhibitors were dissolved in 100% ethanol/0.2 M NaOH or DMSO.

Gel-filtration chromatography

FPLC was performed on a High Load Superdex 75 column (16 cm $\times$ 1.6 cm; Pharmacia). The column was equilibrated with 50 mM Tris/HCl (pH 8)/150 mM NaCl and the rAsGST1 was loaded on to the column at a flow rate of 60 ml $\cdot$ h⁻¹. Fractions (2 ml) were collected and the conjugating activity with CDNB was determined.

Production of *A. suum* GST rabbit polyclonal antiserum and affinity purification of anti-AsGST1 antibodies

A. suum homogenate was loaded on to a GSH–Sepharose column. After washing with PBS [150 mM NaCl/16 mM Na_2HPO_4 /4 mM NaH_2PO_4 (pH 7.3)], specifically bound material was eluted with a GSH gradient (0–20 mM, in 50 mM Tris/HCl, pH 8.0). The fractions containing the highest level of GST activity were pooled and analysed by SDS/PAGE, and 100 μ g of the pooled GSH-binding proteins were used for the immunization.

To raise rabbit polyclonal antiserum, the proteins were emulsified in Freund's complete adjuvant (day 0) and given by subcutaneous injection, followed by three immunizations [100 μ g (each) in Freund's incomplete adjuvant on days 14, 28 and 56]. The rabbit serum produced was tested for specificity by immunoblot analysis with total *A. suum* extracts. For the immunolocalization, the antiserum was affinity-purified using purified rAsGST1 immobilized on nitrocellulose filters. Here, approx. 500 μ g of rAsGST1 [in 50 mM Tris buffer (pH 7.6)/0.1% (v/v) SDS] was heated for 5 min at 95 °C and applied to a nitrocellulose filter. The antiserum was incubated with the filter overnight with gentle shaking. The filter was washed with 0.15 M NaCl (30 min) and PBS (2 $\times$ 10 min), and the rAsGST1-specific antibodies were eluted using 0.2 M glycine (pH 3)/1 mM EGTA (15 min), followed by neutralization with 0.1 vol. of 1 M Tris

base. The specificity of these antibodies was confirmed by Western-blot analysis on *A. suum* total protein extract.

SDS/PAGE and Western blot

Gut, reproductive and muscle tissue from *A. suum* were homogenized (glass/glass homogenizer) and centrifuged at 14000 *g*. Aliquots of tissue with the same protein concentrations were heated at 95 °C for 10 min in SDS/PAGE sample buffer. The proteins were separated by SDS/PAGE [12.5% (w/v) gel] and electrophoretically transferred on to a nitrocellulose membrane. The AsGST1 was revealed by immunostaining, using affinity-purified rabbit anti-AsGST1 primary antiserum, anti(rabbit IgG)–alkaline phosphatase-labelled antibody conjugate, and the chromogenic substrates Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside.

Homology modelling

Homology modelling was undertaken using Modeller interfaced with Quanta. Following standard protocol, the model was built using a representative range of GSTs for which crystal structures were available (Brookhaven database files 1hna.pdb, 1glp.pdb, 1gne.pdb, 1gsr.pdb) and which have the highest degree of homology with the AsGST1. Following averaging of the template co-ordinates, the model was built and refined using simulated annealing and conjugate gradient minimization. The model was verified by comparison with Alpha-, Mu- and Pi-class GST crystal structures. The Pi-class GSTs show the highest degree of sequence identity with AsGST1. In this class the residues Tyr-7, Pro-53, Asp-57, Ile-68, Gly-145 and Asp-152 have been found to be conserved in all known GST sequences. For the helminth GST homology model, these residues generally superimpose within 2 Å of those from known crystal structures.

Immunolocalization of AsGST1

Adult female (7) and male (7) *A. suum* were collected and immediately fixed in 4% (v/v) buffered formaldehyde or 80% (v/v) ethanol for 10 min and then injected with the same fixative, cut in 2 cm pieces, and fixed for another 24 h. Embedding was performed in paraffin by standard methods. For immunohistochemistry, the alkaline phosphatase–anti(alkaline phosphatase) method (APAAP) was applied according to the recommendations provided by the manufacturer (Dako Diagnostika GmbH, Hamburg, Germany) and as described by Cordell et al. [16]. As primary antibody, the affinity-purified rabbit anti-AsGST1 serum was used at a dilution of 1:30. Preimmune serum (also absorbed against rAsGST1) was used as the control at the same dilution, as well as a negative control (PBS buffer). As secondary antibodies, anti-rabbit monoclonal antibody and anti-mouse polyclonal antibody (DAKO) were applied. Fast Red TR salt (Sigma, St. Louis, MO, U.S.A.) was used as the chromogen, and haematoxylin functioned as the counterstain. The best labelling results were achieved on ethanol-fixed sections.

Southern blot, screening of phage library and DNA sequencing

Genomic DNA was isolated from *A. suum* by the procedure of Jowett et al. [17], digested with restriction enzymes and size-fractionated by agarose-gel electrophoresis. The DNA was blotted on to nylon membranes (GeneScreen Plus; Du Pont) and probed with ³²P-labelled AsGST1 cDNA probes [8]. Southern blot hybridization and wash procedures were performed as previously described [18].

An *A. suum* adult worm lambda fix II (Stratagene) genomic DNA library was screened with the ³²P-labelled AsGST1 cDNA

using standard high-stringency conditions. Twelve positive clones were identified and purified, and the DNA inserts from three independent lambda fix II AsGST1 clones were analysed.

The dideoxy chain-termination method (Sequenase Sequencing Kit, USB/Amersham) using vector-specific and gene-specific oligonucleotide primers (Applied Biosystems) was applied for DNA sequencing of both strands. DNA and amino acid sequence analyses were carried out using DNASIS and PROSIS computer software (Hitachi).

Northern blot

Poly(A)⁺-enriched RNA (5 µg) was separated by agarose (1.0 %, w/v)/formaldehyde gel electrophoresis and transferred to a GeneScreen Plus membrane. The Northern blot filter was hybridized for 20 h at 45 °C in the hybridization solution [5 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate)/1 % SDS/50 % (v/v) formamide/5 × Denhardt's (1 × Denhardt's = 0.02 % Ficoll/0.02 % polyvinylpyrrolidone/0.002 % BSA)/100 µg of tRNA] in the presence of 100 ng of ³²P-labelled DNA at 1 × 10⁶ c.p.m./ml. The filter was washed at 45 °C in 1 × SSC/1 % SDS (3 × 20 min).

RESULTS AND DISCUSSION

Substrate specificities, interaction with secondary products of lipid peroxidation and sensitivity to various inhibitors

The absence of post-translational modification of AsGST1 and the cytosolic localization have been ideal for its high-level expression in *E. coli*. Following affinity purification of rAsGST1, substrate specificities with model substrates that are representative of the different mammalian GST classes, organic hydroperoxides and reactive carbonyls were determined. Specific activities were calculated and are listed in Table 1. To confirm that the activities measured are not due to the activity of a co-purified *E. coli* GST, glutathione-binding proteins were purified from untransformed *E. coli* BL21 using the same purification method. Endogenous GST activity could not be detected.

Table 1 Substrate specificities of rAsGST1

Values for specific activity are expressed as nmol·min⁻¹·mg⁻¹ of protein. All values are means ± S.D. of at least triplicate determinations (N.D., not detectable).

Substrate	[Substrate] (mM)	[GSH] (mM)	Specific activity (nmol·min ⁻¹ ·mg ⁻¹)
CDNB	1.0	1.0	38200 ± 2300
Model substrate			
Bromosulphophthalein	0.03	5.0	N.D. (< 5)
1,2-Dichloro-4-nitrobenzene	1.0	5.0	N.D. (< 5)
Ethacrynic acid	0.2	5.0	260 ± 12
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane	5.0	5.0	N.D. (< 5)
Lipid hydroperoxides			
<i>t</i> -Butyl hydroperoxide	0.25	1.0	N.D. (< 10)
Cumene hydroperoxide	0.25	1.0	312 ± 30
Linoleic hydroperoxide	0.05	1.0	N.D. (< 10)
Reactive carbonyls			
Hexa-2,4-dienal	0.05	2.5	65 ± 14
<i>trans,trans</i> -Hepta-2,4-dienal	0.05	2.5	186 ± 33
<i>trans,trans</i> -Nona-2,4-dienal	0.05	2.5	424 ± 2
<i>trans,trans</i> -Deca-2,4-dienal	0.05	2.5	204 ± 12
<i>trans</i> -Hex-2-enal	0.025	1.0	87 ± 4
<i>trans</i> -Oct-2-enal	0.025	1.0	446 ± 11
<i>trans</i> -Non-2-enal	0.025	1.0	553 ± 21
4-Hydroxynon-2-enal	0.010	0.5	87 ± 45

rAsGST1 has high specific activity with the universal model substrate CDBN, 38200 nmol·min⁻¹·mg⁻¹. When comparing this value with the activity of two recombinant GSTs from the nematode *Onchocerca volvulus* (rOvGST1, rOvGST2) [19,20], both of which were also produced in prokaryotic expression systems, it is approx. 15 times more active with this substrate. Recombinant GSTs from trematodes [21,22] show values ranging from approx. 5000 to 120000 nmol·min⁻¹·mg⁻¹. The highest activity observed was with the *Schistosoma mansoni* GST Sm28 [23], followed by *Fasciola hepatica* rGST47 and rGST51, whereas the recombinant GST from the cestode *Echinococcus multilocularis* (rEMGST1) displays approx. 13-fold lower activity with CDBN [24]. The apparent *K_m* value of rAsGST1, determined for CDBN, was calculated to be 2.67 ± 0.6 mM (mean ± S.E.M). This *K_m* value is similar to that determined for the rOvGST2 and the schistosomal recombinant GSTs [21]. rAsGST1 is moderately active with the model substrate ethacrynic acid, a substrate preferentially conjugated by the mammalian GST Alpha- and Pi-classes, but there is no specific activity detectable with the model substrates for the other GST classes.

It has been proposed that GSTs are part of an interception system, following free-radical damage to membrane phospholipids that also involves the successive action of phospholipase A₂, GSH peroxidase and lysophosphatide fatty acyl-CoA transferase. Failure to detoxify lipid peroxides will provoke metal-catalysed decomposition, resulting in the release of further reactive carbonyl compounds, and it is likely that their efficient detoxification by GSTs is essential for survival [25]. The rAsGST1 was able to catalyse the reduction of cumene hydroperoxide, with a specific activity in the range found for mammalian Mu-class GSTs [26]. The Alpha-class GSTs have a much higher activity with cumene hydroperoxide. Furthermore, rAsGST1 was shown to detoxify members of the *trans,trans*-alk-2,4-dienal and *trans*-alk-2-enal series such as 4-hydroxynon-2-enal, which is a powerful cytotoxin [27].

Recently a prostaglandin (PG)-H D-isomerase was isolated from rat spleen, and the partial amino acid sequence was shown to be related to the sequence of GSTs from parasitic helminths [28]. This similarity, in turn, suggests that certain helminth GSTs may be involved in eicosanoid metabolism. The production of prostaglandins by these parasites could be an important component of their mode of subversion or suppression of host immunity [29]. A Sigma-class-related GST has also been recently isolated from *Ascaridia galli*, which shows a high level of specific activity in the GSH-dependent isomerization of PGH to PGE and comparable activity in its isomerization of PGH to PGD to the rat Sigma-class GST [30]. Although, like most GSTs, AsGST1 was shown to have some activity, it does not have the high specificity of the *A. galli* Sigma-class GST (D. J. Meyer, personal communication).

Gel-filtration chromatography demonstrated that rAsGST1 is active as a dimer. This is consistent with the quaternary structure of cytosolic GSTs from other organisms that are described as being active as homo- or heterodimeric proteins, with the exception of a possible monomeric GST found in *F. hepatica* [31].

rAsGST1 was inhibited by several model inhibitors (Table 2) of mammalian GSTs, including natural breakdown products of haem and bile acids. In addition to their catalytic activity, GSTs are known to function as intracellular transporters of non-substrate hydrophobic compounds and to detoxify the compounds by passive binding. A result of this interaction is the inhibition of GST activity. Helminth GSTs were also shown to potentially neutralize anthelmintics by binding these toxic ligands, i.e. acting as 'biochemical sponges' [32]. However, the

Table 2 Inhibitor sensitivities of rAsGST1

The assays were performed in duplicate and at least four concentrations of inhibitors were used. The standard assay was carried out using a final concentrations of 1 mM GSH, and 1 mM CDNB as the second substrate.

Inhibitor	IC ₅₀ (μM)
Model inhibitors	
Triphenyltin chloride	0.3
Bromosulphophthalein	1.0
Cibacron Blue	0.09
Haematin-related compounds	
Bilirubin	9.0
Protoporphyrin IX	30.0
Bile acids	
Chenodeoxycholate	(15)*
Lithocholate	100.0
Mammalian GST inhibitors	
Chalcone	45.0
Alizarin	4.2
Purpur gallin	5.2
Quercetin	0.6
Ellagic acid	0.6
Anthelmintics	
Albendazole	520.0
Bithionol	7.7
Hexachlorophen	0.34

* 15% inhibition at 1000 μM.

Figure 1 MOLSCRIPT ribbon diagram showing the conserved GST residues of AsGST1

benzimidazole carbamate derivative albendazole shows limited inhibition of rAsGST1 activity, whereas the dichlorophenol bithionol and the diphenyl derivative hexachlorophen were more potent inhibitors of rAsGST1. Plant phenols are better inhibitors of rAsGST1 than they are for the schistosomal and the *Moniezia expansa* major GSTs [21]. The IC₅₀ values of AsGST1 show more similarity to those obtained for rOvGST2 [20].

Human mu	1	10	20	30	40	50	60
Mouse pi	---	P-MTLGYWNI	RGLAHSIR	LLLETTD	SSYEKKY	TMGDAP	YDRSQWLN
Schistosomal	---	PP-YTIVYFP	VRGRCEAM	RMLLAD	QGSWK	EVVTI	---
Pig pi	---	SP-I-LGYWK	IKGLVQ	PTRLLE	LYLEEK	YEEHLY	ERDEG
Human alpha	---	PP-YTITYFP	VRGRCEAM	RMLLAD	QGSWK	EVVTM	---
AsGST1.pdb	---	AEKP	---	KLHYFN	ARGRMEST	RLMLAA	AGVFE
Human mu	65	75	85	95	105	115	125
Mouse pi	---	PYLI-DGTH	KITQSN	AILRYI	ARKHNL	CGESEKE	QIRED
Schistosomal	---	PKFE-DGDL	TLTYSN	AILRHL	GRSLGL	YGKQRE	AAQMD
Pig pi	---	YYTI-DGDL	TLTYSN	AILRHL	GRSLGL	YGKQRE	AAQMD
Human alpha	---	PMVEID	GM-KLVQ	TRAILN	YASKYN	LYGKDI	KEALID
AsGST1.pdb	---	PLLEVD	GGEV-LAQ	SAATYR	YLGRQF	GLAGKT	PMEEAQ
Human mu	130	140	150	160	170	180	190
Mouse pi	---	DFEKL-KPEY	LQA-L--P	--E-M-L	KLYSQFL	---	GKQFW
Schistosomal	---	NYENG-KNDY	VKA-L--P	--G-H-L	KPFETL	LSQNGG	KAFIVG
Pig pi	---	DFEFL-KVD	FLSK-L--P	--E-M-L	KMFEDRL	---	CHRTY
Human alpha	---	NYEAG-KEKY	VKE-L--P	--E-H-L	KPFETL	LSQNGG	QAFVVG
AsGST1.pdb	---	EKDA-KLAL	IKEKI--K	--N-RYF	PAFEKVL	--KSHG	QDYL
Human mu	195	205	215	225	235	245	255
Mouse pi	---	PSCL-DAF	---	PN-LKDF	ISRFEG	LEKISAY	MK--SSRF
Schistosomal	---	PGCL-DNF	---	PL-LSAY	VARLSAR	PKIKAF	LS--SPEH
Pig pi	---	PMCL-DAF	---	PK-LVCF	KRIEAI	POIDKYL	---
Human alpha	---	PSCL-DAF	---	PL-LSAY	VARLSAR	PKIKAF	LS--SPEH
AsGST1.pdb	---	SS-LISSF	---	PL-LKAL	KTRISN	LPTVKFL	QPGSP

Figure 2 Alignment of AsGST1 with other known GST sequences used to construct the homology model

The conserved GST residues of AsGST1 are indicated by asterisks. pdb, protein database (Brookhaven).

Figure 3 Overlay of Alpha-carbon traces of AsGST1 and pig lung Pi GST (piGST)

Two perpendicular views are shown.

Structural model of AsGST1

In the homology model of AsGST1 presented in Figure 1, the commonly conserved residues are indicated on the ribbon backbone. Although AsGST1 exhibits a low level of sequence homology to the mammalian enzymes, homology modelling can be undertaken with some confidence, since GSTs display remarkable topological similarity, independent of their class or sequence homology [33]. In order to avoid biasing the AsGST1 model to one particular topology, the homology model was built using a representative range of GSTs for which crystal structures were available. The sequence identities were: pig lung Pi (37%), mouse liver Pi (34%), human liver Alpha (29%), human muscle Mu (28%) and *Schistosoma japonicum*, Sj26GST (26%). Allowing conservative replacements, the sequence similarities were brought to 49%, 45%, 45%, 40% and 37% respectively. The degree of homology is uniform across the entire AsGST1 sequence. The homology model lacked the typical structural features of Alpha- or Mu-class GSTs [34], the extra helix over the active site and the Mu-loop respectively. It did, however, exhibit a strong topological relationship with mammalian Pi-class GSTs. Figure 3 shows a structural overlay of pig lung Pi GST and

Figure 4 Detection of AsGST1 in various *A. suum* tissue preparations by Western-blot analysis

Equivalent protein concentrations were applied to the gel: lane 1, gut; lane 2, muscle tissue; lane 3, reproductive tissue. AsGST1 was revealed by immunostaining, using the affinity-purified antiserum (anti-AsGST1) as primary antibody. Molecular-mass markers are shown on the left.

AsGST1 Alpha carbon traces. In contrast with the Pi-class GST, the AsGST1 C-terminal coil does not stretch along the back of the hydrophobic substrate binding site, resulting in an open cleft at the active site. In the mammalian counterparts the hydrophobic substrate binding pocket is a closed tunnel-like feature. This difference in the topography of the proposed substrate binding site would explain the variation in biochemical activity between AsGST1 and the mammalian counterparts.

Although a large number of inhibitors exist for GSTs, a parasite-specific inhibitor has not been described. Our continuing structural studies of rAsGST1 have the aim of defining the three-dimensional conformation of AsGST1, this being the basis for the rational design of inhibitors and for the identification of specific epitopes for vaccine development.

Localization of AsGST1 in parasite tissues

Equivalent amounts of protein from three different worm tissues were run on SDS/PAGE (12.5% gel) and immobilized on a nylon membrane. The native AsGST1 was specifically detected with the affinity-purified anti-AsGST1 serum (Figure 4). This serum is specific to AsGST1 and does not cross-react with other *A. suum* GSTs [8]. The quantitative evaluation of the immunostaining with the AsGST1 in the tissues analysed clearly shows that the distribution of AsGST1 in different tissues is not uniform. The gut possessed the highest amount of AsGST1 (lane 1), followed by the muscles (lane 2) and finally the reproductive tissue (lane 3). To further analyse this pattern of AsGST1 expression, the enzyme was localized in adult worm cross-sections via immunohistochemical procedures.

The anti-AsGST1 serum stained discrete portions of the adult worms, while preimmune serum and negative controls showed no staining (Figures 5A and 5B). Satisfying labelling results for AsGST1 were obtained at a dilution of 1:30 of the affinity-purified serum. Therefore the following results are based on experiments using this dilution. No labelling was detected in the cuticle, the oviducts and the ovaries (Figure 5B), nor in their germinal or in their growth zone. The cytoplasmic part of the longitudinal muscle cells showed moderate staining; almost no staining was observed in the muscle fibres (Figure 5B). The large uterus epithelial cells, as well as the uterine musculature and basal lamina, were also completely unlabelled. Eggs in the uterus, and sometimes in the oviduct, showed moderate immunostaining for AsGST1 (Figure 5E). Sometimes the lateral chords of the hypodermis showed weak to moderate staining, but no overall labelling was observed in the hypodermis. Dorsal and ventral chords as well as the nerve fibres (chords) in their poaches revealed no staining for AsGST1.

The strongest immunostaining for AsGST1 was observed in the intestine of all worms examined (Figures 5B and 5D), especially in its inner zone, which is formed as a brush border of microvilli (Figure 5D). No staining was detected in the basal lamina of the intestine (Figure 5D). Otherwise, a homogeneous staining of the cytoplasm in the very long and narrow cylindrical intestinal cells was observed. Sometimes their apical portion showed stronger labelling. Vesicles originating from the microvilli of the intestinal cells showed, in most cases, positive immunolabelling for AsGST1 (Figures 5B and 5D). It is unlikely that localization of the AsGST in the microvilli is an experimental artifact, due to cross-reactivity of the antiserum with carbohydrate epitopes on the microvilli glycocalyx, since the antiserum used was affinity-purified using recombinant non-glycosylated protein.

The localization of AsGST1 in the intestine is in contrast with the localization of the GSTs in *O. volvulus*. Neither *O. volvulus* GST is found in the intestine. OvGST2 is located throughout the syncytial hypodermis, as well as in the uterine epithelium [20], whereas OvGST1 is localized distinctly in the outer portion of the hypodermis [35]. With respect to filarial nematodes, transcuticular absorption is universally accepted, and the outer region of the filarial hypodermis might facilitate the exchange of material with the cuticle and the environment. Striebel [36] speculated that the intestine of adult female *Onchocerca* has lost its functional importance and progressively degenerates with age. Hence the OvGSTs are localized in the hypodermis, which is probably responsible for the metabolism of materials that are incorporated from the environment, as well as molecules that are excreted or secreted from the parasite to the environment. These are functions that are normally performed by the nematode's intestine, hence the localization of AsGST1 in the intestine.

Tissue specificity of GSTs may be related to the particular detoxification requirement of each tissue. The expression of GSTs in mammals has been reported to be tissue-specific and developmentally regulated [37]. Each organ possesses a unique profile of GST forms. Developmental changes in GST isoenzyme composition and levels of expression occur in various tissues as they progress from fetal to adult stage [38]. The tissue distribution of GSTs has also been examined in trematodes. In *S. mansoni*, Sm26GST and Sm28GST have been found in parenchymal cells of adults and are absent in musculature, gut and reproductive tissue [39–41]. They have been described to be present in the tegument and/or subtegumental tissues, although this observation is controversial. In *F. hepatica*, GSTs are found in the tegument, muscular tissue, parenchymal cells and in the lamellae of the intestine [41]. In a more recent study [42], antisera were generated to synthetic peptides of regions unique to each of the four isolated GST proteins. The antisera raised against the GST-51 peptide, which cross-react with two other GSTs from *F. hepatica*, localized the GSTs in the parenchyma and in the lamellae of the intestinal caeca. The authors suggest that the GSTs of the adult fluke may play a role in the absorptive function of the gut.

Characterization of the AsGST1 gene

The AsGST1 gene and its flanking sequences were mapped by genomic Southern-blot analysis (Figure 6A). The complete cDNA [8] was hybridized with *A. suum* genomic DNA that had been digested with *Hae*III (2.8 kb fragment; lane 1), *Eco*RI (1.0 and 3.0 kb fragment; lane 2) and *Dra*I (0.5 and 1.5 kb fragment; lane 3). Northern-blot analysis (Figure 6B), using the complete AsGST1 cDNA as the probe demonstrated a single transcript from this gene, approx. 700 bp, that is consistent with the size of the cDNA.

Figure 5 Cross-sections of female *A. suum* immunohistochemically stained using the APAAP technique with the affinity-purified polyclonal antibody against AsGST1 (1:30) and with preimmune serum as a control

(A) Preimmune serum, showing completely unlabelled intestine (in), uterus (ut), oviduct (od) and eggs (eg); scale bar 100 μ m. (B) Anti-AsGST1, showing the intensely labelled intestine (in), the moderately labelled cytoplasm of the musculature (mu) and the unlabelled ovary (ov). Cuticle and hypodermis were also unlabelled; scale bar 100 μ m. (C) Preimmune serum; detail of the intestine; no staining was observed; basal lamina (bl), microvilli (mv), terminal fibrillar net (tfn); scale bar 25 μ m. (D) Anti-AsGST1; detail of the intestine; strong labelling was observed in the cylindrical cells; note the intense labelling of the brush border of microvilli as indicated by the arrow; no staining in the basal lamina (bl) and nuclei (n); scale bar 25 μ m. (E) Anti-AsGST1; detail of the uterus lumen; showing fertilized eggs with moderately labelled embryonic stages as indicated by the arrow; scale bar 50 μ m.

Figure 6 Genomic DNA Southern-blot and Northern-blot analysis of *A. suum*

(A) Genomic DNA Southern blot analysis. *A. suum* genomic DNA was digested with: lane 1, *Hae*III; lane 2, *Eco*RI; lane 3, *Dra*I. The DNA fragments were separated on a 0.9% agarose gel and transferred to a GeneScreen Plus membrane. The filter was hybridized with the same probe used above, washed and exposed overnight to X-ray film. (B) Northern blot analysis of *A. suum* GST1 mRNA. Poly (A)⁺ RNA of adult *A. suum* was subjected to agarose (1.0%) formaldehyde gel electrophoresis; 5 µg was transferred to GeneScreen Plus membranes and hybridized with the radiolabelled complete cDNA of AsGST1. Nucleotide markers are shown on the left in each case.

The same cDNA probe was also used to isolate and partially map four distinct genomic phage clones from an *A. suum* lambda fix II library. The 1.0 and 3.0 kb *Eco*RI fragments were isolated, which encompasses the entire AsGST1 gene. The restriction-enzyme pattern of the isolated clones is consistent with that observed in the genomic Southern blot experiment. Both *Eco*RI fragments were cloned into pBluescript SK and subjected to sequence analysis. The nucleotide sequence of the entire coding region, comprising 1855 bp, was determined. In addition, the sequences 750 bp upstream of the start codon and 450 bp downstream from the stop codon were determined (Figure 7).

The sequence analysis of the AsGST1 gene (gAsGST1) indicated that it is composed of four exons (exon I, 133 bp; exon II, 93 bp; exon III, 125 bp; exon IV, 272 bp) and three introns (Figure 4). The 5' and the 3' splice junctions agree with the consensus GT-AG sequences. Since the AsGST1 mRNA [7] possesses the *Caenorhabditis elegans* 5' spliced leader sequence (SL1), analysis of its single-copy gene indicates that the mechanism of mature AsGST1 mRNA production involves both *cis*- and *trans*-splicing processes. The mammalian GST genes usually possess seven to eight exons [43]. The other nematode GST genes described to date are those encoding the 24 kDa GST (OvGST2) from *O. volvulus* [20] and the two GSTs from *C. elegans*. The OvGST2 gene has seven exons. One of the *C. elegans* GST genes has three exons (*C. elegans* cosmid R107, product R107.7), and the other has two exons (*C. elegans* cosmid R03D7, product R03D7.6).

Sequence analyses aimed at the identification of general eukaryotic promoter elements, as well as GST-specific regulatory sequences [44], were performed. Regulatory sequences such as the xenobiotic responsive element [45] and the antioxidant-responsive element [46] have been described in GST gene promoter regions. However, only a potential Oct-2 element (position 116–122, Figure 7) and a putative HNF-5 recognition site, a transcription factor involved in the control of expression of liver specific genes (position 596–602, Figure 7), were identified

tctgtgacct	tggttgacag	ttgttaagcc	agcccgat	ccactccaac	50
attacaagtt	tctatgcgct	aagttgcgca	ttactgcact	ctccgcaatt	100
tcattcactc	ggatctcatg	agcagtcagt	tcattatagga	tcgcacgaa	150
tttttaagat	cacactttgc	gctttcattt	tcocccgttt	ttatattcctt	200
atgttaatgg	ccctttacat	gcattgttct	ttggcctatg	catattattt	250
tggaatgaa	caagtgaat	tggtcgagag	cgatttgcgt	ctatgcagtg	300
tttagtaact	ttgcaatagc	attggccgct	tatgcagatg	tgtggcgaagc	350
cacttatgcc	aagaatttcg	tttttcattc	acactcgaag	caacgcaaaa	400
tgcaactttt	tttctcagaa	attcgctcca	aattttgcag	cactaaaaac	450
ttttataact	tatagagaaa	gccttccatt	agaattaaat	accgggacga	500
aatggagtgc	aaaatgtcat	cttaagctgt	agttatgtct	atcaaatctc	550
atcgccacag	tctttcttgc	gtcaataact	gtgacctaca	gacttgcaaa	600
cacatacata	cataggtgta	taggcacaga	ggtgatgttg	ctttagtccc	650
actatggaga	aatttgcaga	agcgtcattt	aacaatgcgt	ttaaagctct	700
DraI					
aggctgtagc	accatcgac	atcctaagtt	tccttttttt	agcaaggaat	750
ATGCCGAGT	ACAAGCTCAC	GTACTTCGAT	ATTCTGGCC	TGGGTGAGGG	800
M P Q Y K L T Y F D I R G L G E G					17
TGCACGCTA	ATTTTCCACC	AAGCCGCGT	CAAAATTCGAG	GACAAACGCT	850
A R L I F H Q A G V A F E D N R					33
TCAAGAGGGA	AGACTGGCCT	GCGCTCAAC	CAAGtatgtc	taccgcttga	900
L K R E D W P A L K P					44
tctgcattga	gttgtacata	tttctttgtg	gggacacttc	tcattgtttag	950
cttttcgccc	acctgtcat	atatgaaac	tggtttgtgc	caacaaacat	1000
ccatagtgtc	ttgaagtcgt	aaagatcgtg	aaaattcagg	taaaagctga	1050
aataaaaagc	tcaataaaaa	gcacagcaag	aaactactgc	agaaggaagt	1100
gtttttcaga	GACTCCTTTT	GCTCAATGC	CATTGCTCGA	GTGGATGGA	1150
K T P F G Q L P L L E V D G					58
GAGGTGCTTG	CACAGAGTGC	TGCTATCTAC	AGATCTCTCG	GCAGCAAAAT	1200
E V L A Q S A A I Y R A Y L G R Q F					75
CGtaagtgg	ctgttcagct	tttaaagcaa	cacattcaat	tcaagaatgt	1250
DraI					
tcccgcctga	gttcaccggc	acagcggtag	gaatctatca	gccctttatt	1300
tcccatgtta	atctctgggc	tcattgcgtg	cagcagccaa	cggttattaa	1350
cttaccctac	ttgccgacag	ttgcagtgat	aagcatctgc	agtcattttt	1400
gctgcgatac	accactgcc	aggatgcga	agacatgcac	tgtattctct	1450
tgcatgtg	gagctcgag	attgtcctgt	gatggatcag	cggtacacct	1500
tgccaaacac	gtttccgaaa	caaagtatat	gagtggtg	ttcatcaca	1550
attcctcagt	ggttggttga	atgtctgagc	tcgtgtgttc	acatgctatt	1600
ttcccttag	atacctgagc	attgccggtt	ttcggcgacc	tgctatagcc	1650
taaaaacaga	tgtccggagt	cttcaacatt	gttctctttt	ctctctgtag	1700
gcgatattat	ggattgcaat	gcgaagaagg	cttgaggctt	gtcatcatca	1750
cgaggatttg	tctgttttagg	TCTGGCTGGG	AAAACGCCAA	TGGAGGAGGC	1800
G L A G K T P M E E A					86
ACAAGTGGAT	TCGATATTTG	ACCAGTTCAA	AGACTTCATG	GCAGAATTAA	1850
Q V D S I F D Q F K D F M A E L					102
GACCATGCTT	CAGGTGCTC	GCCGGCTTCG	AAGAGGCGCA	CAAAgttggc	1900
R P C F R V L A G F E E D K					117
tgcttaactt	tttcgtttat	ttcttcagtg	ttatttatct	gttaaaaggc	1950
aacaaacatc	aaagaaatga	gtacaaacaa	ataagagaat	cgcattcgaag	2000
ggtagacagg	ctattagctga	agtgtcatgt	ttttggcgaa	gagcgtgcac	2050
cttttcattc	ttgatatttc	caagcacatc	gaaggaaatga	gccgattact	2100
tttgggactt	cgcagtgccg	aggcacagtt	ttgaagtga	tttactctct	2150
EcoRI					
agattaccgt	ttgcttcac	gcacagcatg	tgaattctga	aaacttttgt	2200
cccaaggaat	ctgaactggg	ctgctaattt	ccaagtatgc	tatcagttgt	2250
ttcctagaat	gacttcatgc	attttctgct	tttagGAGAAG	GTGCTGAAAG	2300
E K V L K					122
AGGTTGCCGT	GCCGGCACGC	GACAAGCATC	TGCCACTACT	GGAGAAATTC	2350
E V A V P A R D K H L P L L E K F					139
CTTGCGAAAT	CGGGTAGCGA	GTACATGGTG	GGCAATTCGG	TCACGTGGGC	2400
L A K S G S E Y M V G K A S V T W A					156
GGATCTCGTG	ATCACCGATT	CCTTGGCTTC	TTGGGAGTCA	TTAATCCCCG	2450
D L V I T D S L A S W E S L I P					172
ACTTCCTTAG	CGGTATCTTT	CAGCTAAAGA	AGTACATTGA	ACACGTTCGC	2500
D F L S G H L Q L K K Y I E H V R					189
GAAGTGCCTA	ACATCAAGAA	ATGGATAGCT	GAGCGACCGA	AGACGTCATA	2550
E L P N I K K W I A E R P K T P Y					206
TTAGAGTCTC	TTCTGCTTTT	GTGAATTTAT	GTATTTCGAT	AAACACTTTT	2600
*					
ATTTTCcgcg	tctgtctagc	cagacttgga	caatctgtca	ggcagcaatt	2650
cacagcagat	cttctattaa	cacttgcctg	ctcaccoccat	ccccctcccc	2700
cgccacacaag	actcgagaat	gctgcgagta	atatagtgat	tgctaaaaat	2750
tgctttatac	ttgtctgcgc	gcgcagtgct	tcttttaaaa	gcagcaatga	2800
DraI					
ttgcttgacc	ttccgctgtc	ggcaccctca	gtcctttgtc	cgaagaagga	2850
gtgaactgct	tactgtctaa	taggtctgag	ctggtttccg	ccccctctcc	2900
tccctcttta	ctgtctccaca	ccgttccaca	atggttttcc	tgtaatgctt	2950
tccatccttg	cccttcccat	cttctctttg	aacgctctgc	tagtacttgc	3000
tcttgatttc	ctctcatcgg	aaggagtcgc	ggggattttg	tagaaatcaa	3050
tgcaaatcg	tgtaatgtct	gttcgggttaa	gcacatcatg	attactcatg	3100
tttgactcat	gaaaataacg	acatttcttg	gacgtgggat	gatgatgcca	3150
tctgctcgga	atcagaaaaa	tcgtggtgaa	ttc		3183
EcoRI					

Figure 7 The complete nucleotide sequence of the *A. suum* GST1 gene

Numbers indicate the positions in the nucleotide and amino acid sequences. Lower-case letters represent introns and flanking sequences; exons are given in capital letters. The *Dra*I and *Eco*RI sites are given in bold.

in the AsGST1 gene sequence. A comparison of the 5' non-coding regions of the known nematode GST genes with one another did not reveal any common sequence elements or motifs.

Detailed surveys of the metabolic pathways in helminths have identified the GSTs as the major phase II detoxification system required for the parasite's survival in the host [32]. This enzyme, which differs in its primary amino acid sequence, gene structure, biochemical properties and most likely in the structure of its active site from those GST classes described in the host, can therefore be considered a potential parasite target for chemo- or immuno-therapy. By neutralizing the activity of this defence protein, it may be possible to tip the molecular balance in favour of the host during a chronic parasitic infection.

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