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Molecular Cloning and Characterization of a Glutathione S-Transferase Encoding Gene from *Opisthorchis viverrini*

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SUMMARY An adult stage *Opisthorchis viverrini* cDNA library was constructed and screened for abundant transcripts. One of the isolated cDNAs was found by sequence comparison to encode a glutathione S-transferase (GST) and was further analyzed for RNA expression, encoded protein function, tissue distribution and cross-reactivity of the encoded protein with other trematode protein counterparts. The cDNA has a size of 893 bp and encodes a GST of 213 amino acids length (OV28GST). The most closely-related GST of OV28GST among those published for trematodes is a 28 kDa GST of *Clonorchis sinensis* as shown by multiple sequence alignment and phylogenetic analysis. Northern analysis of total RNA with a gene-specific probe revealed a 900 nucleotide OV28GST transcriptional product in the adult parasite. Through RNA *in situ* hybridization OV28GST RNA was detected in the parenchymal cells of adult parasites. This result was confirmed by immunolocalization of OV28GST with an antiserum generated in a mouse against bacterially-produced recombinant OV28GST. Both, purified recombinant and purified native OV28GST were resolved as 28 kDa proteins by SDS-PAGE. Using the anti-recOV28GST antiserum, no or only weak cross-reactivity was observed in an immunoblot of crude worm extracts against the GSTs of *Schistosoma mansoni*, *S. japonicum*, *S. mekongi*, *Eurytrema spp.* and *Fasciola gigantica*. The enzyme activity of the purified recombinant OV28GST was verified by a standard 1-chloro-2, 4-dinitrobenzene (CDNB) based activity assay. The present results of our molecular analysis of OV28GST should be helpful in the ongoing development of diagnostic applications for opisthorchiasis viverrini.

Opisthorchis viverrini is the agent of opisthorchiasis and causes serious health problems among human populations in North and Northeast Thailand. The prevalence of opisthorchiasis in Northeastern Thailand is estimated to be 18.57% which means about 6 million people are infected.[1,2] Infection of humans takes place through consumption of raw or undercooked cyprinoid fish harboring infective metacercariae. The parasites remain in the bile ducts for many years causing hepatobiliary diseases such as obstructive jaundice, hepatomegaly, cholangitis, cholelithiasis and cholangiocarcinoma.[3-6] Infected persons develop strong humoral and cell mediated immune responses probably due to the contact of parasite products with the host immune system through the inflamed epithelial lining of the bile ducts.[7-9]

At present, knowledge about the host/parasite relationship and molecular biology of *O. viverrini* is limited. Several protein antigens have been characterized and used for immunodiagnosis and attempts have also been made to develop a method to detect parasite DNA in feces.[10-12] Both techniques have been found to be effective but have still not replaced microscopic examination. In order to develop an immunodiagnosis with high sensitivity and specificity, a good candidate

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antigenic protein must be studied. Moreover, this protein should be readily available with high quality and purity. Glutathione S-transferase (GST) is a catalytic enzyme which is active in the parasite's defense mechanism by detoxifying bioactive chemicals. Two isozymes of GST have been reported, a 26 kDa cytosolic enzyme and a 28 kDa tegumental enzyme.[13] GST is an antigenic protein of trematodes that induces a strong host immune response. Recombinant GST has been used for detecting IgG and IgE antibodies in clonorchiasis.[14] In the present study, the gene that codes for *O. viverrini* GST was cloned and the recombinant 28 kDa GST was characterized and compared with GSTs of other species.

MATERIALS AND METHODS

Construction of cDNA library

Adult *O. viverrini* (1 mg wet weight) obtained from infected hamsters (*Mesocricetus auratus*) were homogenized in TRIzol reagent (Invitrogen-Life Technologies, Carlsbad, USA) by use of an Ultra-Turrax T25 homogenizer (IKA, Staufen, Germany) and the total RNA was extracted. The RNA (1 µg) was used to construct a directional cDNA library in Lambda TriplEx2 (SMART cDNA synthesis kit, BD Biosciences-Clontech, Palo Alto, USA). The recombinant Lambda DNA was packaged in Gigapack III Gold packaging extracts (Stratagene, La Jolla, USA). The primary library (4×10^6 pfu/ml) was amplified to a titer of 1×10^9 pfu/ml and stored at -80°C in 7% DMSO.

Screening of cDNA library

The cDNA library was screened for abundant transcripts with a ^{32}P-dCTP labeled mixed cDNA probe (Hexalabel DNA labeling, Fermentas Life Sciences, Vilnius, Lithuania). Briefly, a plaque lift of 50,000 plaques was done to a nitrocellulose membrane (BA85, Schleicher & Schuell, Dassel, Germany) and the DNA of the transferred phage particles was released and denatured under alkaline conditions. The air-dried membrane was baked at 80°C for 1 hour, prehybridized and hybridized in 5x SSPE, 5x Denhardt's solution, 50% Formamide, 0.5% SDS, 1 mg/ml herring sperm DNA, and 20 ng/ml of the ^{32}P-dCTP labeled cDNA probe. The hybridization reaction was done at 55°C for 16 hours. Posthybridization washes of the membrane were done in 1x SSC, 0.1% SDS at room temperature for 10 minutes and in 0.1x SSC, 0.1% SDS at 50°C for 30 minutes to remove nonspecifically bound probe. An autoradiograph of the air-dried membrane was produced on Kodak XAR-5 film at -80°C for 16 hours. Positive plaques were recovered from the original plate and rescreened at low density (100-200 pfu/90 mm plate) to obtain single clones. Cre-recombinase-mediated recombination was used to release the pTriplEx2 phagemid carrying the cDNA from the viral DNA. Several single clones were obtained but only the analysis of clone C1A2 is reported in this publication.

Sequencing and sequence analysis of cDNA

Plasmid DNA was prepared using the Jet Star Plasmid Midi Kit (Genomed, Lohne, Germany). Sequencing of the cDNAs was done by MWG AG Biotech, Ebersberg, Germany. Sequence analysis was done using MacMolly Lite (Soft-Gene GmbH, Berlin, Germany). The BLAST program[15] was used to search the GenBank database for related sequences. Multiple sequence alignments were done using the CLUSTAL W program[16] through the emboss emma program.[17] A prediction of the secondary structure of the encoded protein was done by the PHD program.[18] A phylogenetic tree was constructed to examine relationships among selected homologous proteins using the program PAUP 4.0[19] and based on 1,000 replicates.

Northern hybridization

Twenty micrograms of total RNA was size separated in a 1.5% denaturing agarose gel (2.2 M formaldehyde) in 1x MOPS buffer (0.2 M MOPS [3-(N-morpholino) propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA adjusted to pH 7.0) and transferred to a nylon membrane (Nytran N, Schleicher & Schuell). The air-dried membrane was baked at 80°C for 1 hour, prehybridized and hybridized in hybridization buffer II (5x SSC, 2% blocking solution, 50% formamide and 0.02% (w/v) SDS) at 65°C for 16 hours. A DIG-labeled antisense RNA of OV28GST (DIG-RNA labeling and detection kit, Roche Diagnostics AG, Rotkreuz, Switzerland) was used as a hybridization probe at a concentration of 100 ng/ml. The hybridized probe was detected by an anti-DIG/alkaline phosphatase conjugate on the membrane using NBT/BCIP as substrates.

RNA *in situ* hybridization

RNA *in situ* hybridization was done as described before.[20] Adult parasites were fixed over-

night in 4% paraformaldehyde, 0.1 M PBS, pH 7.4, dehydrated through a series of ethanol steps and embedded in paraplast. Sections were cut at 6 µm (Leica RM2145, Wetzlar, Germany) mounted on coated slides (Histogrip, Zymed, San Francisco, USA) and dried at 42°C overnight. The sections were dewaxed in xylene, rehydrated and post-fixed in 4% paraformaldehyde, 0.1 M PBS. Afterwards the sections were treated two times in 0.1% DEPC, 0.1 M PBS for 15 minutes each and equilibrated in 5x SSC (0.75 M NaCl, 75 mM sodium citrate). The sections were hybridized at 60°C with 60 µl of 400 ng/ml DIG-labeled antisense OV28GST RNA probe (DIG-RNA labeling kit, Roche Diagnostics AG) in hybridization buffer II for 16 hours. After the hybridization reaction the sections were washed in 2x SSC for 30 minutes at 25°C, 1 hour at 65°C and finally in 0.1x SSC for 1 hour at 65°C. Enzymatic detection of the hybridized probe was done as described before.

Expression and purification of recombinant and native OV28GST

A subfragment of C1A2, beginning at the start codon in position 23 of AY057838 was subcloned into the pET21a expression vector (Novagen, Madison, USA) as follows: DNA of pTriplEx2-C1A2 was used as a template in a standard PCR (30 cycles at 94°C, 55°C, 72°C, 1 minute each step) using the forward primer 5'-CAT ATG AGT GGT GAA AAA TAC AA-3' and reverse primer 5'-GAC TCA CTA TAG GGC GAA TTG G-3' (pTriplEx2 bases 653 to 674, BD Biosciences-Clontech). The PCR product was subcloned into pGEM T-easy (Promega, Madison, USA) and the sequence verified (Bioservice Unit, Bangkok, Thailand). Afterwards it was cut with restriction endonucleases *Nde* I (introduced by the forward primer) and *Xho* I (contained in the multiple cloning site of pTriplEx2) and inserted into the *Nde* I/ *Xho* I recognition sites of pET21a.

The expression of recombinant OV28GST protein in the transformed *E. coli* strain BL21 was induced at an OD_{600} of 0.6 by adding IPTG to a final concentration of 1 mM into the medium. After 4-hour growth the bacterial cells were pelleted at 2,600 x *g* and 4°C for 10 minutes, resuspended in PBS (1.37 M NaCl, 27 mM KCl, 101.4 mM Na_2HPO_4, 17.6 KH_2PO_4 [pH 7.3]) and sonicated on ice in short bursts (Microson ultrasonic cell disruptor, Heat Systems, New York, USA). The soluble product of the lysate was separated by centrifugation for 5 minutes at 12,000 x *g* at 4°C from the insoluble cellular debris and used for purification of the recombinant OV28GST protein by the Bulk-GST Purification Module Kit (Amersham Biosciences, Piscataway, USA). Purification was done according to the manual supplied in the kit. In addition, the native OV28GST was also purified by the Bulk-GST Purification Module kit. Freshly obtained adult parasites were suspended in lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% Triton-X 100, 1 mM EDTA, pH 7.2, 1 mM phenyl-methyl-sulphonyl-fluoride) and homogenized on ice. The homogenate was rotated at 4°C for 1 hour, the insoluble material pelleted for 15 minutes at 12,000 x *g* and the native OV28GST purified from the supernatant.

The extraction of crude worm preparations from *O. viverrini*, *S. mansoni*, *S. japonicum*, *S. mekongi*, *F. gigantica* and *Eurytrema spp*. was done as described above for the native OV28GST protein. Fresh worms were suspended and homogenized in lysis buffer. The homogenate was rotated at 4°C for 1 hour and insoluble material pelleted for 15 minutes at 12,000 x *g*. The supernatant was used as crude worm protein extract.

Production of anti-recOV28GST polyclonal antiserum

A polyclonal antiserum against recombinant OV28GST protein (recOV28GST) was raised in a female BALB/c mouse. Three subcutaneous injections with doses of 50 µg recombinant OV28GST cut from gels after SDS-PAGE were done in weeks 0, 3, and 6. In the priming step, recombinant OV28GST was injected with complete Freund's adjuvant; the second and third immunization steps were done with incomplete Freund's adjuvant (http://dicty.com.nwu.edu/Chris_lab/Lab%20Manual/Immunological_techniques.htm). Blood was collected and the serum obtained in weeks 0 (preimmune), 2, 5, and 8 by centrifugation at 2,600 x *g* for 15 minutes. The sera were tested for their antibody titer by ELISA and were kept at -20°C before being used for EITB and immunolocalization.

Glutathione S-transferase activity assay

The enzyme activity of 1 µg recombinant OV28GST was measured in 1 ml reaction buffer (0.01 M potassium phosphate, pH 7.0, 10 mM 1-chloro-2, 4-dinitrobenzene, 10 mM reduced glutathione) at room temperature for 5 minutes. The photometric

absorption values at a wavelength of 340 nm were recorded in one-minute steps.[21]

Enzyme-linked electroimmunotransfer blot (EITB)

EITB was performed as previously described.[22] Crude worm extracts of 10 µg each of *O. viverrini*, *S. mansoni*, *S. japonicum*, *S. mekongi*, *F. gigantica* and *Eurytrema* spp., 0.5 µg each of purified native and recombinant OV28GST proteins were size separated by SDS-PAGE (12.5%) and transferred to a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences). The immunogenicity of the transferred proteins was determined by EITB using the raised polyclonal mouse anti-recOV28GST serum and an alkaline phosphatase-rabbit anti-mouse immunoglobulin conjugate. Enzymatic detection was done with the substrates NBT (0.3 mg/ml) and BCIP (0.15 mg/ml) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$.

Immunolocalization of OV28GST

Native OV28GST protein was localized in paraffin sections (5 µm) prepared from adult specimens as described above. The dewaxed and rehydrated sections were covered with 1 mM EDTA pH 8.0 and heated in a microwave oven at 700 watts for 5 minutes. Unspecific binding sites were blocked by incubation with 0.1% glycine in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) for 30 minutes at room temperature and in 4% bovine serum albumin in TBS for 30 minutes. The sections were incubated in a 1:200 dilution of polyclonal mouse anti-recOV28GST antiserum in TBS for 16 hours and washed in TBST (0.1% Tween-20 in TBS) three times for 5 minutes each. The sections were then incubated in a 1:50 dilution of biotinylated rabbit anti-mouse immunoglobulin in TBS for 30 minutes and avidin:biotinylated horseradish peroxidase complex (ABC complex-HRP, DAKO) for 30 minutes. Enzymatic detection was done using 3, 3' diaminobenzidine (Sigma-Aldrich, St. Louis, USA) as a substrate.

Species names, database entry names, and accession numbers of the GSTs used for comparisons in this publication

These included 28 kDa GST of *O. viverrini* (OV28GST AAL-23713), 28 kDa GST of *Clonorchis sinensis* (CS28GST AAD17488), mu class GST of *C. sinensis* (CsMuGST AAB46369), 28 kDa GST of *S. haematobium* (SH28GST AAA29892), 28 kDa GST of *S. bovis* (SB28GST AAA29893), 28 kDa GST of *Paragonimus westermani* (PW28GST AAB63382), alpha class GST of *Fasciola hepatica* (FhGST-alpha AAB28746), GST of *F. gigantica* (FgGST AAD23997), mu class GST of *F. hepatica* (FhMu-GST AAA29140), S-crystallin of *Loligo opalescens* (SQCRYS AAB-01054), GST of *Ommastrephes sloanei pacificus* (SQGST 1GSQ), GST of *Caenorhabditis elegans* (CEGST AAB65417), mu class GST of *Rattus norvegicus* (RatMuGST AAD00603), 28 kDa GST of *S. mansoni* (SM28GST P09792), 28 kDa GST of *S. japonicum* (SJ28-GST P26624), 26 kDa GST1 of *S. mansoni* (Sm26GST1 P15964), 26 kDa GST2 of *S. mansoni* (Sm26-GST2 P35661), GST of *Ascaris suum* (ASGST P46436), GST of *Onchocerca volvulus* (ONCVGST P46434), pi class GST of *Onchocerca volvulus* (OncvGSTP2 P-46427), GST of *Dirofilaria immitis* (DimmitisGST P4642), GST of *Blattella germanica* (BlageGST O18598), sigma class GST of *Musca domestica* (HouseFlyS1 P46437), pi class GST of *Macaca mulatta* (MonkeyGSTPi Q28514), pi class GST of *Mus musculus* (MouseP1 P19157), alpha class GST of *Rattus norvegicus* (RatGSTA8 P14942), GST-Yc1 of *Rattus norvegicus* (RatGSTYc P04904), pi class GST of *Rattus norvegicus* (Rat-GSTP7 P04906), Prostaglandin-D-synthase of *Rattus norvegicus* (Rat-PGDS P22057), alpha class GST of *Gallus gallus* (ChickGSTalpha Q08392), mu class GST of *Gallus gallus* (ChickMuGST P20136), Prostaglandin-D-synthase of *Gallus gallus* (ChickPGDS CAA07005), GST of *Brugia malayi* (Bmalay-iGST CAA73325), mu-5 class GST of *Mus musculus* (MouseM5 NP_034490), mu-2 class GST of *Mus musculus* (MouseM2 NP_032209), and pi class GST of *Sus scrofa domestica* (pigpiGST S13780).

RESULTS

Screening of cDNA library and sequence analysis of isolated cDNA

An adult stage cDNA library of *O. viverrini* was constructed in Lambda TriplEx2 (ClonTech) and screened for abundant transcripts with a mixed cDNA probe prepared from *O. viverrini* RNA. One of the positive clones detected in the primary screen (C1A2) was selected for further analysis. The cDNA insert of C1A2 is 893 bp in length. It contains a 22 bp 5' UTR, an open reading frame of 639 bp length and a 198 bp 3' UTR followed by the poly(A) tail. A putative polyadenylation signal (TATAAA) is located 23

bp upstream of the poly(A) tail. The encoded protein of 213 amino acid residues size has a computational predicted molecular weight of 24 kDa and is rich in leucine and glutamic acid residues (a total of 20.66%). A search of the GenBank database through the BLAST program[15] showed that the encoded protein is homologous to the 28 kDa glutathione S-transferases of other helminths and to the related S-crystallin of cephalopods. The percentages of identical residues shared between the newly found OV28GST and homologous proteins of trematodes, nematodes and cephalopods are shown in Fig. 1A. It varies between 57% for the 28 kDa GST of the liver fluke *C. sinensis* (GenBank AAD17488) and 29% for *Onchocerca volvulus* GST-1 (SWISS-PROT P46434). An alignment of GST amino acid sequences obtained through the CLUSTAL W program[16] shows eleven residues to be identical among the compared proteins. These are Tyr10*, Phe11*, Glu18, Arg21, Asp33, Trp41*, Pro54*, Asp88, Gly155, Asp162, and Arg208 (Fig. 1C). Individual amino acids forming part of the GSH-binding site are indicated by a star. In the GST-related squid lens protein S-crystallin, an additional stretch of 15 amino acids (KNGRFFENGKESEMN) exists. The prediction of the secondary structure of OV28GST by the PHD program[18] revealed a conserved structure of the active site of the protein responsible for glutathione binding. A phylogenetic analysis through the PAUP program[19] (Neighbor-Joining with 1,000 replicates) showed that OV28GST is in one cluster with the 28 kDa sized, sigma class GSTs (Fig. 1B). The next neighbor of OV28GST in the phylogenetic tree is the GST of

	OV28GST	CS28GST	SB28GST	SH28GST	SM28GST	SJ28GST	PW28GST	SQGST	ASGST	SQCRYS	CEGST
CS28GST	57.1										
SB28GST	44.6	42.7									
SH28GST	45.5	44.1	97.2								
SM28GST	46.0	43.1	91.9	90.0							
SJ28GST	45.4	41.9	78.2	76.7	77.2						
PW28GST	37.7	37.6	38.6	38.6	38.6	42.6					
SQGST	34.1	31.4	36.0	27.5	27.9	30.1	28.2				
ASGST	31.9	31.0	31.1	31.1	32.5	31.6	32.8	29.6			
SQCRYS	30.9	38.7	36.0	35.3	36.0	34.3	28.8	39.9	33.6		
CEGST	30.8	26.1	29.1	29.1	30.1	30.9	37.6	38.5	47.0	35.8	
ONCVGST	29.4	28.8	29.6	29.1	29.2	28.8	30.8	25.8	36.8	36.9	34.2

Fig. 1A Cross-species identity values (%) for amino acid sequences of OV28GST and homologous GST proteins. Details of species names and accession numbers of the compared glutathione S-transferases are given in the Materials and Methods section.

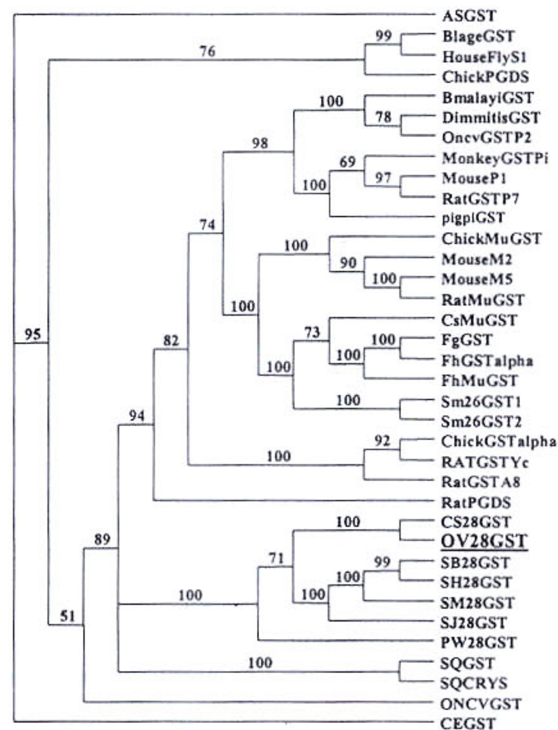

Fig. 1B A phylogenetic tree showing the relationship of *O. viverrini* OV28GST to glutathione S-transferases of other species. Details of species names and accession numbers of the compared glutathione S-transferases are given in the Materials and Methods section.

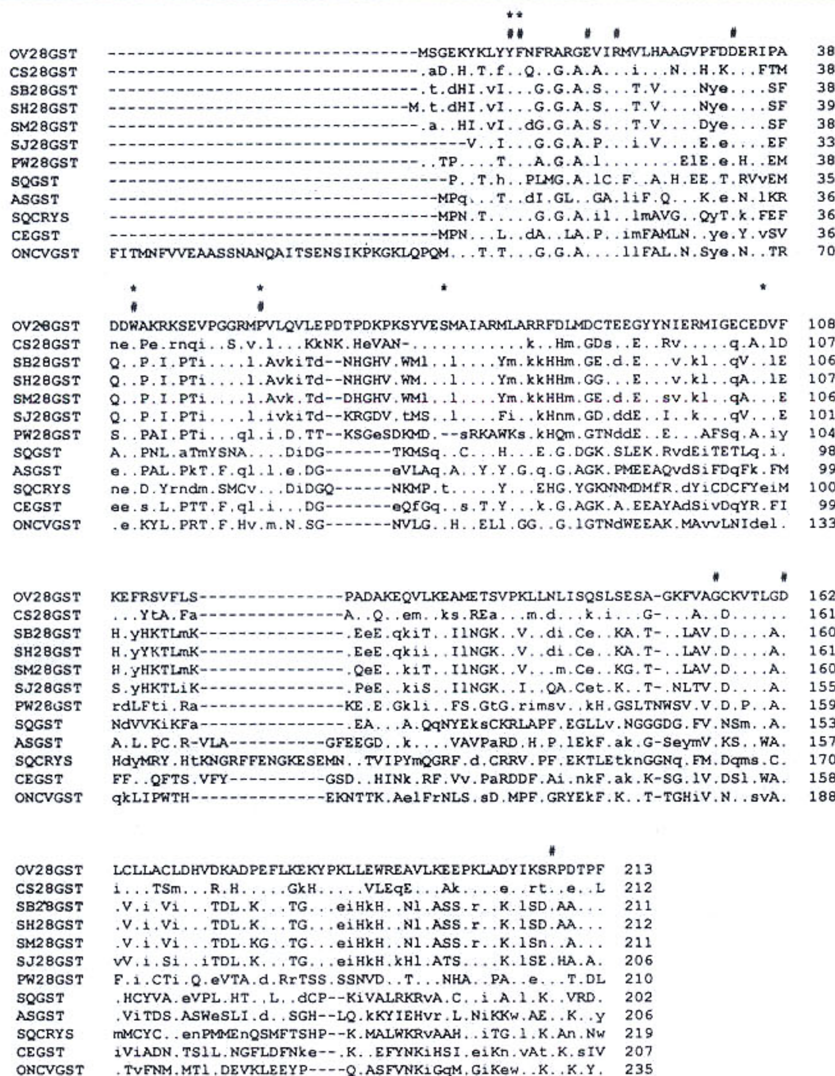

Fig. 1C Amino acid sequence alignment of OV28GST and homologous proteins. Identical residues are indicated by dots (.), similar residues by lower case letters and different residues by upper case letters. Gaps are indicated by a dash (-) and residues forming the GSH binding site are indicated by asterisks (*). Residues identical for all of the aligned sequences are indicated by hatch signs (#). Details of species names and accession numbers of the compared glutathione S-transferases are given in the Materials and Methods section.

C. sinensis (CS28GST)[13] confirming the sequence alignment.

Northern hybridization

Northern hybridization of *O. viverrini* total RNA with an antisense OV28GST RNA probe resulted in a single prominent hybridization signal at a transcript size of 900 nucleotides (Fig. 2). The transcript size indicates that the full length of the mRNA sequence is contained in the cloned OV28GST cDNA. The size is identical to the 28 kDa GST of *Paragonimus westermani*[23] whereas *C. sinensis* GST is encoded by a mRNA of 1 kb size.[13]

RNA *in situ* hybridization

Paraffin cross sections of adult *O. viverrini* were hybridized with an antisense OV28GST RNA probe. Positive hybridization signals were detected in the parenchymal cells (Fig. 3A). Hybridization signals were not observed in vitelline cells, tegument and caecum. A sense strand OV28GST RNA probe did not result in positive hybridization signals (Fig. 3B) as was true for a control with only the enzyme substrates to reveal endogenous phosphatase activity (data not shown).

Enzyme-linked electroimmunotransfer blot (EITB)

The crude worm protein extracts of *O. viverrini*, *S. mansoni*, *S. japonicum*, *S. mekongi*, *F. gigantica*,

and *Eurytrema* spp., and the purified native and recombinant OV28GST proteins were blotted onto a nitrocellulose membrane and probed with the anti-recOV28GST polyclonal antiserum. The antiserum detected the 28 kDa OV28GST in the crude worm protein extract of *O. viverrini* and the purified native and recombinant OV28GST proteins while no signals were obtained at 28 kDa in crude worm protein extracts of *S. mansoni*, *S. japonicum*, *S. mekongi*, *F. gigantica* and *Eurytrema* spp. (Fig. 4).

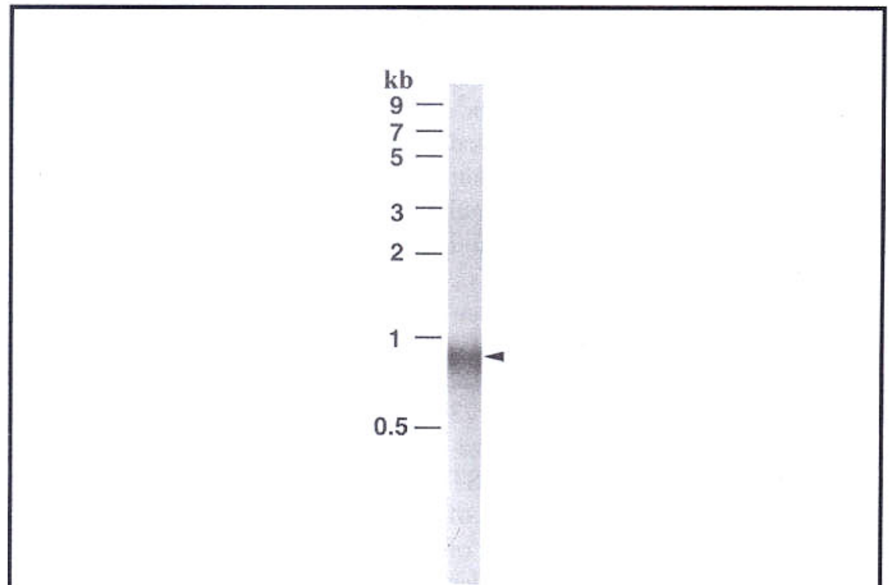

Fig. 2 Northern hybridization of total RNA of *O. viverrini* with an OV28GST RNA probe. Total RNA, 20 µg isolated from adult parasites, was size-separated in an 1.5% MOPS-agarose gel by electrophoresis. The hybridization signal was obtained after capillary transfer to a nylon membrane, hybridization with a DIG-labeled antisense OV28GST RNA probe and enzymatic detection. The fragment sizes of the RNA marker (BioLabs) are shown at the left side. The probe is hybridizing to a transcript of 900 nucleotides size (arrowhead).

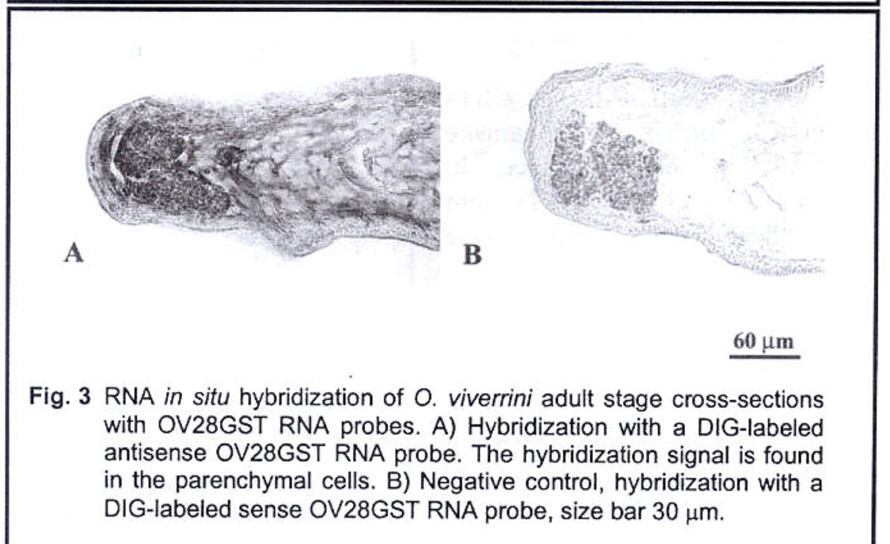

Fig. 3 RNA *in situ* hybridization of *O. viverrini* adult stage cross-sections with OV28GST RNA probes. A) Hybridization with a DIG-labeled antisense OV28GST RNA probe. The hybridization signal is found in the parenchymal cells. B) Negative control, hybridization with a DIG-labeled sense OV28GST RNA probe, size bar 30 µm.

Immunolocalization of OV28GST

The distribution of OV28GST was studied in paraffin cross sections of adult *O. viverrini* using the anti-recOV28GST polyclonal antiserum as a probe. OV28GST protein was localized in the parasite's parenchymal cells (Fig. 5A) matching the results of RNA *in situ* hybridization and the localization of GST as reported for other trematodes.[13,23-25] A negative control with preimmune serum did not show staining in the parenchymal cells (Fig. 5B).

Enzyme activity of recombinant OV28GST

The specific activity of recombinant OV28GST to the universal substrate CDNB was 5.73 µmol/minute/mg protein.

DISCUSSION

In this report we describe the isolation and characterization of a cDNA from *O. viverrini* that encodes a 28 kDa glutathione S-transferase protein. It was isolated from an adult stage *O. viverrini* cDNA library. The OV28GST deduced amino acid sequence shows overall identity with the highest score to the sigma class CS28GST of *C. sinensis* (57.1%). The deduced OV28GST protein is composed of 213 amino acids and therefore has nearly the same size as the GSTs of *C. sinensis*,[13] *P. westermani*[23] and *Schistosoma*.[26] OV28GST was expressed as a soluble recombinant protein in *E. coli* and purified by glutathione affinity chromatography. The observed enzymatic activity of recombinant OV28GST confirmed the function suggested by sequence similarity to other trematode GSTs. The result of EITB

after SDS-PAGE shows that both recombinant OV28GST and native OV28GST run as 28 kDa proteins. Also the predicted secondary structure of OV28GST is conserved when compared to those of other helminthic GSTs. The conserved amino acids shared between squid GST, nematodes (*O. volvulus*,[27] *C. elegans*[28] and *A. suum*[29]) and trematodes (lung, blood and liver flukes) are Tyr10, Phe11, Arg16, Glu18, Asp40 Trp41, Lys45, Pro54, Ser73 and Asp106. Northern hybridization using an antisense OV28GST RNA probe against total RNA of adult worms detected a transcript of approximately 900 nucleotides compatible with the 835 nucleotides size of the OV28GST cDNA. This cDNA obviously contains the full length mRNA of OV28GST. The mRNA distribution of OV28GST was observed by RNA *in situ* hybridization. The RNA was limited to the parenchymal cells in accordance with the protein location.

It is speculated that GSTs have an important role in parasite survival due to their abundance.[30] In mammalian liver cells, GSTs form 4% of the total soluble protein. Due to their activity, GSTs play a major role in detoxification of endogenous and exogenous toxins. With respect to *O. viverrini* these toxins may be generated by the parasite's own metabolism and/or through host responses to the infection.[28,30,31] In Mammalia, eight classes of cytosolic GST have been characterized. In trematodes, 26 kDa and 28 kDa GSTs can be distinguished.[26] With respect to amino acid sequence, substrate specificity and inhibitor effects, it has been shown that 28 and 26 kDa types are similar to the sigma and mu classes of mammalian cytosolic GSTs. In *S. japonicum*, no significant difference was observed between the tissue distributions of 26 and 28 kDa GST proteins by TEM.[24] Generally, parasitic trematodes seem to contain more 28 kDa than 26 kDa GSTs (molar ratio of 28 to 26 kDa is 20:1 in *S. mansoni*, *S. haematobium*, *S. bovis*, *S. japonicum*, *P. westermani*[23,26] and 14:1 in *C. sinensis*[13]). The functional speci-

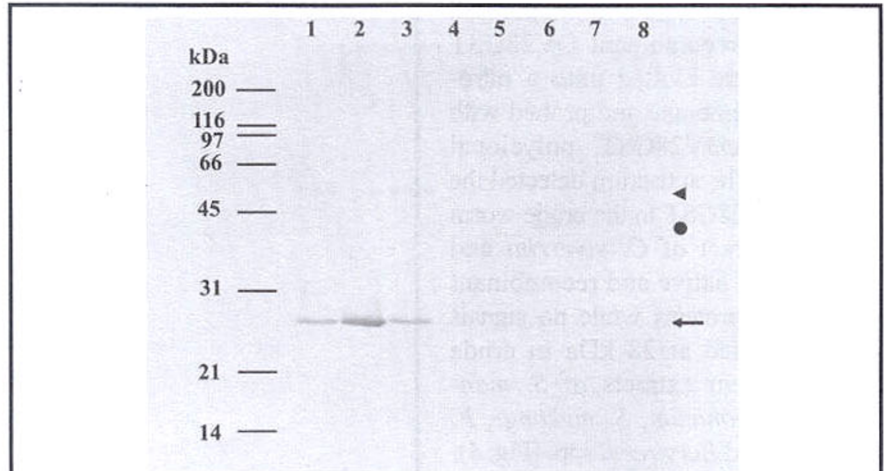

Fig. 4 Enzyme-linked immunoelectrotransfer blot (EITB) of crude worm extract, purified native and recombinant OV28GST. The preparations were separated by 12.5% SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with anti-recOV28GST polyclonal antiserum. The signal was obtained after further incubation with an alkaline phosphatase rabbit anti-mouse conjugate. Lane 1: Purified native OVGST28, lane 2: recombinant OVGST28, lanes 3-8: crude worm extracts of *O. viverrini*, *S. japonicum*, *S. mansoni*, *S. mekongi*, *F. gigantica* and *Eurytrema* spp., respectively. Arrow, 28 kDa GST protein; arrow head 56 kDa putative GST dimer; circle, cross-reacting proteins.

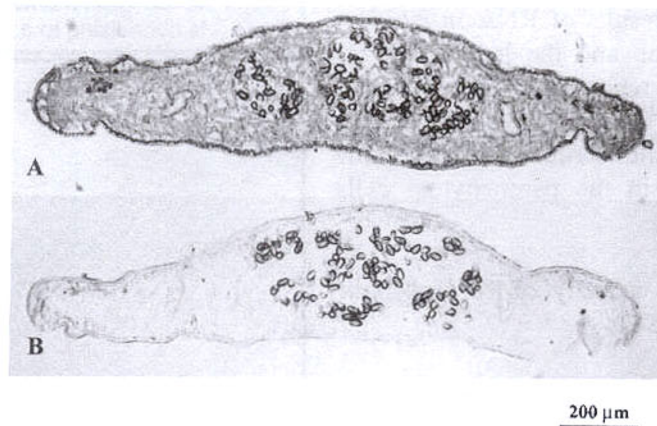

Fig. 5 Immunolocalization of OV28GST protein in cross sections of adult *O. viverrini*. The cross-sections were incubated with either preimmune serum or the anti-recOV28GST polyclonal antiserum. Detection was done by further incubation with horse radish peroxidase-conjugated ABC complex (DAKO) and diaminobenzidine substrate. A) anti-recOV28GST polyclonal antiserum, B) preimmune serum, size bar 200 μm. In consistence with RNA *in situ* hybridization (Fig. 3), OV28GST protein is observed in parenchymal cells only.

ficities of these two enzymes are unknown but it can be assumed that their preference of substrate and responses to inhibitors are different. Identity of amino acid sequences between the 28 kDa and 26 kDa GSTs of the same species is low while the identity within each class is higher between different species. We suppose that more than one class of GST does exist in *O. viverrini*. After purification of soluble *O. viverrini* proteins by glutathione affinity chromatography and separation of the products by SDS-PAGE, two similarily sized 28-29 kDa proteins were observed. In an immunoblot, the 28 kDa protein showed strong reaction with anti-OV28GST polyclonal antibodies while the 29 kDa protein showed only a weak reaction. Crude worm proteins probed with anti-OV28GST polyclonal antibodies confirmed this result. A prominent band at 28 kDa was observed. This result implies that there is no or only weak cross reactivity of the anti OV28GST antibodies with other GSTs of *O. viverrini*. As mentioned earlier only important amino acids such as those found at the active site are conserved between the 26 and 28 kDa GSTs. Further studies are needed to identify additional GSTs and their function in *O. viverrini*. Also a putative 56 kDa GST dimer that reacted with the anti-OV28GST polyclonal antibody was observed as a weak signal in immunoblots (Fig. 4). This 56 kDa protein occurs in crude worm protein extracts, purified recombinant and native OV28GST with the same intensity. OV28GST protein could be detected by immunohistochemistry techniques in parenchymal cells exclusively. There was no signal in cells of vitelline tissue, tegument and the intestine epithelial lining. This finding corresponds to results of TEM immunogold techniques applied to *S. mansoni* GST.[25] Using OV28GST for diagnostic application may be helpful for indicating opisthorchiasis. In several studies, the use of GST for diagnosis has been investigated.[14] GST is also a candidate protein for the generation of vaccines against trematodes.[32,33] Several studies have shown a significant decrease in infection rate after immunization with GST in experimental animals.[33-35] Our analysis of OV28GST at the nucleic acid and protein level confirms the phylogenetic position of *O. viverrini* next to *C. sinensis*. It indicates the importance of OV28GST as a major detoxifying protein in *O. viverrini* and therefore a possible use of this protein in the diagnosis of opisthorchiasis viverrini.

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