

Research Brief

Brugia malayi and *Wuchereria bancrofti*: gene comparison and recombinant expression of π -class related glutathione *S*-transferases

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Abstract

The nucleotide sequence for *Brugia malayi* and *Wuchereria bancrofti* GST have been submitted to EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under Accession Nos. Y12788 and AY195867.

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Index Descriptors and Abbreviations: GST, glutathione transferase; GSH, reduced glutathione

Keywords: Glutathione transferase; *Wuchereria bancrofti*; *Brugia malayi*; Glutathione; Filariasis

Human lymphatic filariasis is mainly caused by the parasites *Wuchereria bancrofti* and *Brugia malayi* and currently affects 120 million people worldwide. One-third of people living in India, one-third live in Africa, and the remainder in Southeast Asia, the Pacific, and Latin America. Despite a vigorous host immune response, the parasites persist for long periods of time in the human lymphatic system. The pathology of filariasis is a complex interaction of acute and chronic inflammatory conditions caused by the parasite and secondary opportunistic infections. The clinical symptoms of this chronic disease range from hydrocele in *W. bancrofti* infection to lymphoedema that can lead to severe elephantiasis (Ottesen, 1992).

Glutathione *S*-transferases (GSTs) are multifunctional proteins that are encoded by a multigene family. They catalyse the addition of reduced glutathione (GSH) to endogenous or xenobiotic, often toxic electrophilic compounds. This catalysis primarily involves nucleophilic substitution or addition reactions. However, GSTs can also act as peroxidases, isomerases, or just as binding proteins (ligandins) sequestering hydrophobic molecules (Sheehan et al., 2001). The natural substrates of GSTs range from molecules of foreign origin to by-products of the normal cellular metabolism. Within a parasitic context it is especially important to analyse the possible function of the GSTs in drug resistance, in the regulation of stress response, and in the modulation of the host immune response. It has been proposed that

host immune-dependent damage to helminth parasites is mediated by several mechanisms also including the production of reactive oxygen species by stimulated host phagocytes (Henkle-Dührsen and Kampkötter, 2001; Selkirk et al., 1998). Here the GSTs can effectively neutralize primary and secondary products of lipid peroxidation that arise during the attack by host immune effector molecules. Furthermore, it has been shown that they can bind/conjugate commercial anthelmintics (Brophy et al., 1989). The GSTs appear to be the major phase II detoxification system present in helminths since there is no evidence that the oxygen-dependent P-450 system is expressed in adult worms (Brophy and Pritchard, 1994).

Here we report the isolation and cloning of the GSTs from *B. malayi* (BmGST) and *W. bancrofti* (WbGST). Screening of a *B. malayi* Lambda Screen (Novagen) library made from adult female worms (constructed by T.H. Dafa'alla, University of Giessen, Germany) was performed as described by Sambrook et al. (1989). As probe, the complete cDNA of the cytosolic *Onchocerca volvulus* GST2 (OvGST2; Liebau et al., 1996b) was ³²P-labelled by random priming and hybridizations were performed using standard high stringency conditions. The nucleotide sequences of both strands of the cDNA were determined by terminator cycle sequencing using Ampli Taq DNA polymerase (Applied Biosystems) on an ABI PRISM™ automated sequencer (Perkin-Elmer). The WbGST was amplified by PCR from a *W. bancrofti* lambda ZAP cDNA library made from infective larvae (constructed by M.R. Lizotte-Waniewski, Smith College, Northampton, USA).

The comparison of the cDNAs derived from *B. malayi* and *W. bancrofti* GST revealed an extremely high sequence identity, with

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A

gBmGST ...CTATTCTGAGACATCTTGCTCGCAAACATAgtaatagtttgtg-----
 gWbGST ...CTATTCTGAGACATCTTGCTCGCAAACATAgtaactgtttgtgctgttttgatctct

gBmGST -----ttcgttgtcgaatttttagtag
 gWbGST tgaattttcttgatagaatccagtgc aaaatagagcaatttgttgtgaattttcagtag

gBmGST ttgcagattacaggtagtta-agcaatcgttctgtttatgcagttaacattgatgattc
 gWbGST ttgcagattacaggtagttacagcagtcattctgtttatgcagttaacattgatgattc

gBmGST tttcatatagATTAAATGGTGGAAATGAAC TGAAACGACACATATTGATATGTTCTGT
 gWbGST tttcatatagATTAAATGGTGGAAATGAAC TGAAACGACACATATTGATATGTTCTGT

gBmGST GAAGGCGTCCGTGATCTGCACACCAAATATACGAAAATGATTACCAGGCTTATgtaaac
 gWbGST GAAGGCATCCGTGATCTACATACCAAATATGC AAAATGATTACCAGGCTTATgtaaac

gBmGST atc---tttcacctattaattatgctatcgtttacgaaattttagaattgtggactcg
 gWbGST atcatctttacctattaattatgctatcgtttacgaaattttagaattgtggactcg

gBmGST ttttcattagggcgg-----tctcgtcattttcaaacaggcaactaaactggtataga
 gWbGST ttttcattaggggtgggttttaattctcgtcattttcaaacaggcaactaaagtgtataga

gBmGST ttatttggctcattttcatattttctgcatatgaatgaatttagttcatttcgttgaag
 gWbGST ttatttggctcattttcatattttctgcatataaaatgaatttagttcatttcatttgaag

gBmGST tggtagcattccagaatgctaaaaatctctttgt-cagagataatgctaaaaatctctt
 gWbGST tgg-tcgca-tccagaatgctaaaaatctctttgtaca-----

gBmGST tgtgacagagataaataatagaggaaaatttgaacaattaagta caataatcattgtat
 gWbGST -----gagataagtaatagaggaaaatta-aacaattaagta caataatcattgtat

gBmGST tatgaccaattattactgaacctgttcatcaatgg-tcattccgtcaaaagcacgc-tta
 gWbGST tatgatcaattattgctgaacctgttcatcaatgattcattctctgcaaaagcacgcatta

gBmGST tattcattgcttttttagGACACTGAAAAGGATTCCTACATAAAAGATATATTGCCAGTAG
 gWbGST tattcattgcttttttagGACACAGAAAAGGATTCCTACATAAAAGATATATTGCCAGTAG

gBmGST AAGCGCGAAAATTTGAAAAGTTTCTTGCTACACGAGACGATGGAAAAATTTTCATTCTAG
 gWbGST AGTTGGCGAAAATTTGAAAAGTTGCTTGCTACACGAGACGATGGAAAAATTTTCATTCTAG

gBmGST GAGAAAAGgtccaggaaatcttggttaactgtaatt---taa-actataggaatgc
 gWbGST GAGAAAAGgtccaggaaatcttgattaaactgtaattattttaactacatagaataac

gBmGST ttgttaaggatactatca--actgagatgatcaattgatggcgaaataatattaattcc
 gWbGST ttgttatggatactatattgattgagatgataaagttgatgacgaaataatattaattcc

gBmGST agctttaaaagtattgcttttaactaaactatgagacgttttcagATTTCATACGTAG...
 gWbGST agctttaaaagtattgcattaactaaactatga---gttttttagATTTCATACGTAG...

B

BmGST 1 MSYKLTYPFIRGLAEPRIVLVDQGIKFTDDRINASDWPSMKSHHFGQLPCLYDGDHQT
 WbGST 1 MSYKLTYPFIRGLAEPRIVLVDQGIKFTDDRINASDWPSMKSHHFGQLPCLYDGDHQT
 OvGST2 1 MSYKLTYPFIRGLAEPRIVLVDQGIKFTDDRINAKDDESSIKSQFQGLPCLYDGDHQT

GG * * * * *
 BmGST 61 VQSGAILRHLARKENLNGNELETHIDMFCEGVRLDHTKYTKMIYQAYLTEKDSYIKDI
 WbGST 61 VQSGAILRHLARKENLNGNELETHIDMFCEGIRLDHTKYAKMIYQAYLTEKDSYIKDI
 OvGST2 61 VQSGAILRHLARKYNLNGNEMETTYIDMFCEGVRLDHLVKYTRMIYMAYTEKDPYIKSI

** * * * *
 BmGST 121 LEVEAAKFEKFLATRDDGKNFILGKISYVDFVLFEELDIHQILDPHCLDKFPLLKAYHQ
 WbGST 121 LEVELAKFEKLLATRDDGKNFILGKISYVDFVLFEELDIHQILDPHCLDKFPLLKAYHQ
 OvGST2 121 LEVELAKFEKLLATRNGRNILGDKISYADYALFEELDVHQILDPHCLDKFPLLKAYHQ

* *
 BmGST 181 RMEDRPGLKEYCKQRNRAKTPVNGNGKQ
 WbGST 181 RMEDRPGLKEYCKQRNRAKTPVNGNGKQ
 OvGST2 181 RMKDRPKLKEYCKRDAKTPVNGNGKQ

Fig. 1. (A) Comparison of two intronic sequences of *B. malayi* (gBmGST) and *W. bancrofti* (gWbGST). Introns are indicated by lower case letters, the exons by bold capital letters. Nucleotide differences are shaded in grey. (B) Alignment of *W. bancrofti* GST, *B. malayi* GST, and *O. volvulus* GST2. The sequences are shown in single letter code. Identical amino acids are shaded in black and conserved amino acid changes in grey. Differences between *W. bancrofti* and *B. malayi* are indicated by “#” and highly conserved residues throughout different GST classes are indicated by asterisk. Residues located in the GSH-binding site are marked by the letter “G.”

only 17 nucleotide differences (97%), resulting in 4 amino acid changes (98%) (Fig. 1). Cross reactivity of *B. malayi* and *W. bancrofti* antibodies raised against housekeeping proteins has been reported frequently and sequence identities of more than 95% (98%) on nucleotide and amino acid level, respectively, have been described elsewhere (Dong and Carlow, 1999; Gnanasekar et al., 2002). The amino acid sequence identity to the *O. volvulus* GST2 is 79% (Fig. 1) (Liebau et al., 1996b). The highly conserved residues Y 7, P 51, D 57, A 65, I 66, G 144, and D 151, that have been found in all known GST sequences, are present in the filarial GSTs. The three sequences are topologically related to the π -class GSTs, reiterating earlier observations that the π -class GSTs are highly conserved across organisms. Furthermore, all three proteins lack the extra helix of the α -class and the μ -loop characteristic of the μ -class GSTs. Whereas the GSH-binding site of all three filarial π -class GSTs is closely related to their mammalian counterpart, they have a more open hydrophobic-binding cleft. This critical structural difference in the active site has been exploited for the design of selective inhibitors of the π -class GST from *O. volvulus* (OvGST2). Here the tyrosine residue 115, also present in BmGST and WbGST, is a target for binding π -class GST inhibitors all bearing a carbonyl moiety at β -position to the point where GSH conjugation occurs (Brophy et al., 2000).

Recently, we showed that the *O. volvulus* GST1 (OvGST1) is an extracellular GSH-dependent prostaglandin D synthase, that catalyses the isomerization of the 9,11-endoperoxide group of the precursor prostaglandin H₂ directly at the parasite–host interface (manuscript in press). Blast analysis of the currently available 23,887 *B. malayi* ESTs demonstrated no significant homology to the OvGST1. When screening the *B. malayi* and *W. bancrofti* cDNA libraries with OvGST1 cDNA as a probe, no signal was obtained. Western blot analysis of *B. malayi* extract using polyclonal antisera raised against the recombinant OvGST1 did not show any high degree of cross-reactivity. Furthermore, isolation of the GSH-binding proteins from *Brugia pahangi* homogenate, using identical purification conditions (Liebau et al., 1994), yielded only one protein corresponding to the BmGST described here (data not shown). Other isolation procedures need to be carried out by us to clarify whether lymphatic filarial parasites express a homologous protein at very low levels or not at all. Whereas in *Dirofilaria immitis* the occurrence of a single GSH-binding protein has been described by Jaffe and Lambert (1986), Rao et al. (2000) observed three different GSH-binding proteins in *B. pahangi*.

To compare the intronic structure of *B. malayi* and *W. bancrofti* GSTs, partial genomic PCR products spanning three introns were obtained. The two fragments have identical intron/exon organizations. In the intronic region, single nucleotide differences and major nucleotide insertions/deletions are responsible for a 78% sequence identity. The nucleotide sequences of the GSH-peroxidase gene (gp29) from *B. malayi* and *W. bancrofti* show a 97% identity in the exons and only 72.3% in the introns (Cookson et al., 1993).

In order to synthesize the WbGST coding region for expression in *Escherichia coli*, the sense oligonucleotide 6943 (5'-GGAATTCCATATGAGTTATAAACTGACGTATTTCTATCCGA-3') encoding the first 8 amino acids and the antisense oligonucleotide 6945 (5'-CCGCTCGAGTCACTGTTTTCCATTTCCTCACTGG-3') encoding the last 8 amino acids of the WbGST cDNA were used in PCR with the complete cDNA as template. The sense primer contained a *Nde*I-restriction site and the antisense primer a *Xho*I-restriction site to simplify directed, in-frame cloning into pJC20 (Clos and Brandau, 1994). The constructs were transformed into *E. coli* BL21 (DE3) and the transformed bacterial cells were grown overnight in Luria–Bertani medium containing 50 μ g/ml ampicillin. Following 1:100 dilution in the same medium, the bacteria were grown to OD₆₀₀ 0.5 before induction with 1.5 mM isopropyl-thiogalactoside (IPTG). After 3 h at 37°C, bacterial cells were harvested and the recombinant enzymes were isolated by resuspending the bacterial cells in phosphate-buffered saline (PBS; 150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, pH 7.3), followed by sonication and centrifugation at 100,000g (1 h, 4°C; TBT

55.38 rotor, Centrikon T-1065; Kontron). Both recombinant GSTs were purified as previously described (Liebau et al., 1996a). The yield of both GSTs was 10–15 mg per litre of bacterial culture. Representative of recombinant expression and purification of both GSTs, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the WbGST is documented in Fig. 2.

The substrate-specificity of the recombinant WbGST was determined with a range of various electrophilic substrates (Table 1). Bromosulphophthalein, 1,2-dichloro-4-nitrobenzene, ethacrynic acid, and reactive carbonyls were determined as described in the literature (Brophy et al., 1989; Jaffe and Lambert, 1986). The GPx activity was determined according to the method of Flohe and Günzler (1984). The protein concentration was determined using the Bradford method (Bradford, 1976). 1-Chloro-2,4-dinitrobenzene (CDNB) was used as the standard second substrate (Mannervik and Guthenberg, 1981) and showed the highest activity. The K_m value for this substrate was determined to be 1.28 ± 0.2 mM. Another substrate that was preferentially conjugated by the WbGST was ethacrynic acid, a typical π -class substrate. Ethacrynic acid is a phenylacetic acid derivative, used as a diuretic drug and displays an electrophilic group that is similar to the α,β -alkenals that are generated under oxidative stress (Ahokas et al., 1985; Berhane et al., 1994). Furthermore, the enzyme had moderate GPx with the cumene hydroperoxide, the model substrate for organic hydroperoxide substrates and displayed low GSH-conjugating activity with secondary products of lipid peroxidation.

To characterize the GSTs by the immune response they may provoke in their human host, the serological reactivity of WbGST with sera from *W. bancrofti* microfilaria carriers or patients with clinical bancroftian filariasis from eastern Indonesia was analysed. As negative controls two serum pools containing 10 samples of individuals not infected with filarial parasites were used. SDS–PAGE and Western blotting were performed according to standard techniques (Burnette, 1981). Briefly, 5–15% polyacrylamide linear gradient slab gels were run using recombinant WbGST as loading protein (4 μ g/cm). The protein was then blotted onto nitrocellulose, the blot was cut into strips, and the strips were blocked for 1 h with non-fat milk powder (5%). Overnight incubation was carried out at 4°C with patients' sera at a dilution of 1:20 as primary antibody. As secondary antibodies peroxidase-conjugated anti-IgG or anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4 in combination with peroxidase-conjugated goat anti-mouse antibodies (all Calbiochem, La Jolla, CA, USA) were used at a dilution of 1:1000 and incubated for 1 h at RT. Nitrocellulose strips were then developed with 4-chloro-1-naphthol and the enzymatic reaction was stopped with double-distilled water. All incubation steps were separated by washing the nitrocellulose strips with alkaline

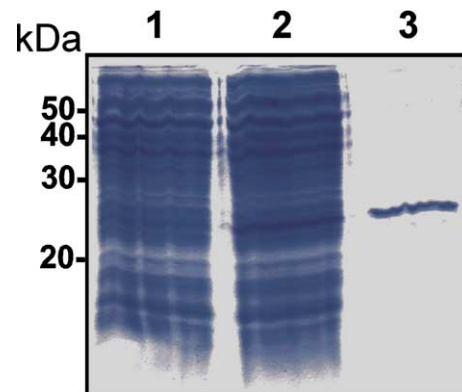


Fig. 2. Production and purification of rWbGST. Coomassie-stained 12.5% SDS–PAGE. Lane 1, lysate of *E. coli* BL21 (DE3) containing pJC20. Lane 2, lysate of *E. coli* BL21 pLys containing pJC20-WbGST with IPTG induction. Lane 3, affinity-purified rWbGST.

Table 1
Substrate specificities of rWbGST

Substrate	[Substrate] (mM)	[GSH] (mM)	Specific activity (nmol min ⁻¹ mg ⁻¹)
CDNB	1.0	1.0	1280 ± 70
<i>Model substrate</i>			
Bromosulphophthalein	0.03	5.0	ND
Ethacrynic acid	0.2	5.0	571 ± 30
1,2-Dichloro-4-nitrobenzene	1.0	5.0	ND
<i>Lipid hydroperoxides</i>			
<i>t</i> -Butyl hydroperoxide	0.25	1.0	ND
Cumene hydroperoxide	0.25	1.0	157 ± 21
<i>Reactive carbonyls</i>			
Hexa-2,4-dienal	0.05	2.5	ND
<i>trans,trans</i> -Hepta-2,4-dienal	0.05	2.5	32 ± 8
<i>trans,trans</i> -Nona-2,4-dienal	0.05	2.5	28 ± 2
<i>trans</i> -Non-2-enal	0.025	1.0	131 ± 11

Values for specific activity are expressed as nmol min⁻¹ mg⁻¹ of protein. All values are means ± SD of triplicate determinations (ND, not detectable).

phosphate buffer. For the screening of sera for IgG1 antibodies an ELISA was carried out using WbGST coated microtiter plates as described previously (Brattig et al., 1997). The cut-off was defined as the arithmetic mean of four negative control samples plus 3 standard deviations.

Western blot analysis was performed using sera from microfilaraemic, clinically asymptomatic individuals living on Alor Island, Indonesia. A strong IgG antibody reaction with the recombinant WbGST antigen was detected in 3 of 15 sera (Fig. 3A), whereas 6 additional sera showed only a weak reaction. The pool sera used as negative controls were negative, indicating that antigen recognition was filariasis specific. In comparison with our results, patients with generalized onchocerciasis showed a similar frequency of antibody reaction with the recombinant OvGST2 antigen (Brattig et al., 1997). Here it was shown that these antibodies belong to either the IgG1 and IgG3 isotype and not the IgG4 isotype, which is the typical antibody isotype found in response to antigens derived from adult filaria. To confirm this pattern of recognition of the π -class GST antigens, we tested four IgG positive sera with the recombinant WbGST antigen (Fig. 3B). All four sera contained IgG1 antibodies that reacted with the antigen, but no IgG2, IgG3, and IgG4 reaction was detected by Western blot. To determine the prevalence of IgG1 antibodies, 40 patients sera were tested with the WbGST antigen by ELISA. Using a

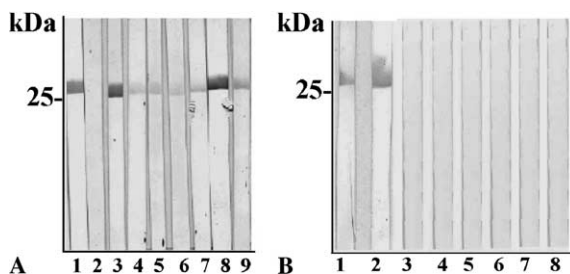


Fig. 3. Western blot detection of antibodies in patients' sera reactive with the recombinant WbGST antigen. (A) Total IgG antibodies in sera from nine different patients (lanes 1–9). (B) IgG isotyping using two total IgG positive sera (lanes 1–2 IgG1, lanes 3–4 IgG2, lanes 5–6 IgG3, and lanes 7–8 IgG4).

conservative cut-off of OD 0.232, seven (18%) samples were found to be IgG1 positive. These results show a moderate antigenicity of WbGST and indicate that the recognition of π -class GST antigens by IgG subclasses is similar in lymphatic filariasis and onchocerciasis. The effector mechanisms that are responsible for protective immunity in filarial infections are not fully understood; eosinophils, elevated levels of serum IgE and IgG4 and a pronounced bias towards either a Th₁ or Th₂ response have been described. Under the assumption that the balance of antibody isotype and subclass response are correlated to the level of immunity, the filarial π -class GSTs do not appear to be suitable immunoprophylactic antigens.

GSTs have long been considered as targets for the development of antiparasitic drugs and lead compounds have been discovered in the context of cancer therapy (Lazdins and Kron, 1999). Furthermore, β -carbonyl substituted GSH-conjugates, developed by Brophy et al. (2000), display high activity and selectivity towards the filarial OvGST2. Additionally, these GSH-conjugates have activity against nematodes in in vitro culture. Our observation that the π -class GSTs from human-infective filariae are highly conserved is encouraging with respect to the ongoing development of specific antifilarial drugs.

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