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Molecular cloning, expression and characterization of a functional GSTmu class from the cattle tick *Boophilus annulatus*

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Abstract

A full-length cDNA of a glutathione *S*-transferase (GST) was cloned from a cDNA library of the local Egyptian cattle tick *Boophilus annulatus*. The 672 bp cloned fragment was sequenced and showed an open reading frame encoding a protein of 223 amino acids. Comparison of the deduced amino acid sequence with GSTs from other species revealed that the sequence is closely related to the mammalian mu-class GST. The cloned gene was expressed in *E. coli* under T7 promotor of pET-30b vector, and purified under native conditions. The purified enzyme appeared as a single band on 12% SDS-PAGE and has a molecular weight of 30.8 kDa including the histidine tag of the vector. The purified enzyme was assayed upon the chromogenic substrate 1-chloro-2,4-dinitrobenzene (CDNB) and the recombinant enzyme showed high level of activity even in the presence of the β-galactosidase region on its 5′ end and showed maximum activity at pH 7.5. The K_m values for CDNB and GSH were 0.57 and 0.79 mM, respectively. The over expressed rBaGST showed high activity toward CDNB (121 units/mg protein) and less toward DCNB (29.3 units/mg protein). rBaGST exhibited peroxidatic activity on cumene hydroperoxide sharing this property with GSTs belonging to the GST α class. I_{50} values for cibacron blue and bromosulfophthalein were 0.22 and 8.45 μM, respectively, sharing this property with the mammalian GSTmu class. Immunoblotting revealed the presence of the GST molecule in *B. annulatus* protein extracts; whole tick, larvae, gut, salivary gland and ovary. Homologues to the GSTmu were also detected in other tick species as *Hyalomma dromedarii* and *Rhipicephalus* sp. while in *Ornithodoros moubata*, GSTmu homologue could not be detected.
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Keywords: Cattle ticks; *B. annulatus*; Cloning; Glutathine *S*-transferase; Class mu

1. Introduction

Glutathione *S*-transferase (GST, EC 2.5.1.18) is a family of multifunctional isoenzymes found in all eukaryotes. They are dimeric proteins composed of identical or structurally related subunits (Mannervik et al., 2005). Each subunit of 25 kDa is built of two domains and contains a complete active site consisting of a G-site (Glutathione binding site) and an H-site (Hydrophobic substrate binding site) (Stenberg et al., 2000). Based on their structure and biochemical properties, GSTs have been divided into the cytosolic alpha, mu, pi and theta classes, as well as a microsomal

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enzyme (Hayes and Pulford, 1995). One of the main functions of the enzyme is to catalyze a nucleophilic conjugation reaction of reduced glutathione (GSH) with a large variety of compounds bearing an electrophilic site, such as xenobiotics including pesticides, in the mercapturic acid pathway leading to the elimination of toxic compounds (Hayes and Pulford, 1995; Eaton and Bammler, 1999). In insects, this enzyme family has been implicated as one of the major mechanisms neutralizing the toxic effects of insecticides (Ranson et al., 1997; Huang et al., 1998; Wei et al., 2001).

Ticks are ectoparasites and many are vectors of diseases in humans and other animals. The southern cattle tick, *Boophilus microplus*, transmits the cattle fever pathogen (*Babesia* spp.) and is one of the most important cattle pests. Chemical pesticides continue to be the primary means of control for ectoparasites on livestock. Intensive use of these materials has led to the development of resistance in *Boophilus* ticks to all currently used organophosphates (Baxter et al., 1999), synthetic pyrethroids and amidines (Martinez et al., 2006). Despite previous studies that suggested increased detoxification (De La Fuente and Kocan, 2006) and target site insensitivity may contribute to the increased tolerance to acaricides, the mechanisms conferring resistance on ticks are poorly understood.

We here report the molecular cloning, expression and kinetic characterization of a mu-class GST from the Egyptian cattle tick *B. annulatus*. This study may provide a contribution for further studies on the role of tick GST in acaricide resistance.

2. Materials and methods

2.1. Screening of B. annulatus cDNA library

A 530 bp probe was generated by PCR from *B. annulatus* whole tick cDNA using two oligonucleotides GSTF and GSTR based on the consensus regions among the sequences of *B. microplus* and house dust mite (*Dermatophagoides pteronyssinus*) GST (Genbank, accession numbers AF077609 and S75286, respectively). The 530 bp PCR product was labelled with the Digoxigenin (Dig) system (Roche) according to the manufacturer's protocol. 2 μl of cDNA from *B. annulatus* was subjected to 35 amplification cycles. The labelled probe was purified and used to screen 500,000 plaque colonies of the *B. annulatus* λZAPII cDNA library, previously constructed from different *B. annulatus* tissues like salivary glands, ovaries, and gut, and other tick life cycle stages as eggs, larvae and adults. The average length of the cDNA inserts was 1.5 kb. The colonies were plated at 50,000 plaque forming units (pfu) per plate and grown on a lawn of XL1-Blue *E. coli*. Lifts were taken onto Nytran-nylon membranes, denatured, neutralized, and fixed by baking at 80 °C 2 h. Hybridization of the membranes with Dig-labelled probe and detection were carried out using the Dig detection kit (Roche) following the recommendations of the manufacturer. Positive plaques on membranes were identified, isolated in agar plugs, eluted and replated. The above screening protocol was then repeated. Individual positive plaques from the secondary screening were isolated. The cDNA inserts were recovered from PCR screen positive colonies using the Exassist/SOLR system (Stratagene). Individual bacterial colonies containing recombinant phagemid were grown up and phagemid DNA was purified and sequenced.

2.2. GST expression in BL21 (DE3) and purification

The prokaryotic expression vector pET30b (Novagen, Inc. Madison, USA) carries the T7 promotor and Kanamycin resistance gene was used to express the *B. annulatus* GST. From the sequence of *B. annulatus* GST clone, two primers, FEcoRV (5′-CCG **GAT ATC** GAT GGC TCC TGT GCT CGG CTA CTG G-3′) and RXhoI (5′-CCG **CTC GAG** TGC TTG TTT CAT GGC TTC TTC TGC-3′), were designed for PCR amplification of the full-length ORF of *B. annulatus* GST. The primers FEcoRV and RXhoI contained EcoRV and XhoI restriction sites, respectively. These sites were also present as unique sites in the cloning region of the pET30b expression vector, ensuring correct orientation of the insert. To ensure fidelity, PCR was performed using platinum pfx-DNA polymerase (Gibco) that has proofreading capacity. PCR product and vector were digested with EcoRV and XhoI before ligation. The ligated construct was transformed into BL21 (DE3) and colonies were picked and the plasmids were purified using the QIAprep spin plasmid kit (Qiagen). Before expression, the fidelity and orientation of *B. annulatus* GST cDNA in the vector was confirmed by sequencing.

After expression, the recombinant *B. annulatus* glutathione *S*-transferase (rBaGST) was affinity purified under native conditions using the MagneHis^{TM} Protein Purification System (Promega), following the instructions of the manufacturer. The histidine tagged protein was eluted using the elution buffer containing 100 mM HEPES, and 500 mM imidazole, pH 7.5.

2.3. DNA sequencing and data analysis

DNA sequencing was performed on an ABI-PRISM 310 automated DNA sequencer (PerkinElmer, Foster City, CA) at the DNA Sequencing Facility, *VACSERA*, Cairo, Egypt. Sequences were analyzed using the analysis software from the expasy web site (http://www.expacy.org).

2.4. Preparation of whole tick, larval, gut, salivary and ovarian antigens

Whole tick and larval antigens of *B. annulatus* were prepared according to the method of Ghosh et al. (1999). In brief, laboratory-reared, clean, 5–6-day-old unfed ticks or larvae were homogenized in cold buffer A which includes, 0.15 M phosphate-buffered saline (PBS) and 1 mM disodium EDTA, pH 7.2, containing cocktail protease inhibitors (PMSF, Aprotinin, Leupeptin and Bestatin, Sigma), filtered, sonicated, and centrifuged at $15{,}000 \times g$ for 60 min at 4 °C. The supernatant was designated as whole tick or larval antigen. The protein concentrations of the antigens were estimated according to the method of Bradford (1976). Gut, salivary glands and ovarian antigens were prepared according to the method of Das et al. (2000). In brief, tissues from the partially fed ticks were dissected out and homogenized in extraction buffer A, sonicated, and centrifuged. Supernatants were then collected as gut, salivary and ovarian antigens.

2.5. Enzyme activity assay

GST activity was assayed as described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction mixture was contained in a final volume of 1 ml which consisted of 1 mM CDNB, 1 mM glutathione, 100 mM potassium phosphate buffer, pH 6.5 and 20 μl of the protein sample. The activity was determined by measuring absorbance at 340 nm using a LABOMED spectrophotometer (USA) at 25 °C and one unit of transferase activity is defined as the amount of enzyme which catalyses the formation of one micromole of thio-ether per minute where the extension coefficient of thio-ether is 9.6 $mmol^{-1}\,cm^{-1}$. Protein was assayed by the method of Bradford (1976) using bovine serum albumin as standard.

2.6. Polyacrylamide gel electrophoresis

The electrophoretic analysis was performed in the Mini-Protean II Dual-Slab Cell (BioRad, USA). Preparation of gels, samples, and electrophoresis were performed according to the conditions described by Laemmli (1970).

2.7. Determination of kinetic parameters

The effect of pH on purified rBaGST was measured over a range of pH using 0.1 M potassium phosphate buffer for pH 6.5–8.0 and Tris HCl for pH 8.5–9.5. GST activity was assayed taking different concentrations of CDNB (0.125–2.0 mM) and holding GSH concentration at 1 mM, and different concentrations of GSH (0.125–2.0 mM) and holding CDNB concentration at 1 mM. The K_m and V_{max} were calculated from double reciprocal plot of $1/v$ versus $1/[S]$.

2.8. Inhibition studies

The I_{50} values for each inhibitor for GST were determined according to Yalcin et al. (1983), by measuring the specific activities at 25 °C in 0.1 M phosphate buffer, pH 6.5, in the presence of 1 mM GSH, 1 mM CDNB and different concentrations of inhibitor. Cibacron blue and bromosulfophthalein were dissolved in the assay buffer. I_{50} values were determined by measuring the activity of the enzyme in the presence of varying concentrations of the inhibitor. The I_{50} values were calculated by plotting percentage activity values versus log inhibitor concentration.

2.9. Preparation of rabbit anti-rBaGST

For raising anti-rBaGST antibodies, a male rabbit (3 kg) was immunized by intramuscular injection with 20 μg of purified rBaGST. The antigen dissolved in 0.5 ml of saline (0.9% NaCl) and mixed with an equal volume of Freund's complete adjuvant (Sigma, USA) was injected on day 0. The rabbit was boosted by 20 μg of the same antigen mixed with Freund's incomplete adjuvant on day 14 by the same route. Fourteen days after boosting, the rabbit was bled from the marginal ear vein; the serum was separated and used in immunoblotting.

2.10. Immunoblotting

Immunoblot analysis was performed using a NovaBlot semi-dry blotter (LKB, Bromma, Sweden). Preparation of buffers, samples, and the transfer procedure was carried out according to the method of Towbin et al. (1979) with slight modifications. The rabbit anti-rBaGST was used at dilution of 1:2000 in

0.01 M Tris buffered saline (TBS), pH 7.5 containing 0.5% BSA, while the anti-rabbit IgG peroxidase conjugate was used at dilution of 1:3000 in the same buffer and the reaction was developed using 4-chloro-1-naphthol as substrate.

3. Results

3.1. Characterization of the B. annulatus GST cDNA

Screening of *B. annulatus* cDNA library identified 4 positive clones from a total of 4.2×10^5 pfus screened. All clones were isolated and sequenced in their entirety. Three of them contained identical sequences of 829 bp within which a putative initial ATG codon (Kozak, 1991) is found 73 nucleotides downstream of the 5′ cDNA end, a single open reading frame of 672 bp encoding a polypeptide of 223 amino acids with calculated molecular weight of 25.589 Da and theoretical p*I* of 8.11, and a 85 bp 3′ UTR flanking region. The total nucleotide sequence of *B. annulatus* GST cDNA and its deduced amino acid sequence are shown in Fig. 1. The putative polyadenylation signal (Proudfoot and Brownlee, 1976), aataaa was found at nucleotides 789–794, and this position is 22-bp upstream from the poly A^+ tail (Fig. 1). The sequence of the entire clone was submitted to Genbank (Fig. 1; accession number EF440186). The fourth clone was partially similar to the other three clones but not identical (data not shown).

According to SWISS PROT/ScanProsite program (Hofmann et al., 1999), the deduced protein is composed predominantly of hydrophilic residues and a potential glycosylation site is present at residue Asn^{152} (Fig. 1). Comparison analysis of the *B. annulatus* GST protein with other sequences in Genbank showed high similarity with the class mu GSTs (Blast 2 version Blastp 2.0.5) (Altshul et al., 1997) (Fig. 2). Comparison of amino acid sequences between *B. annulatus* GST and other species had revealed 99.6% homology with *B. microplus* GSTmu, 53.7% with either human GSTM1 or GSTM2, 53.2% with rat GSTM1 or GSTM2, 54.1% with mouse GSTM1 and 54% with the dust mite GSTM. These data suggest that the isolated cDNA was homologue to GSTmu class and hence was designed BaGST.

3.2. Expression of BaGST in E. coli

The expression of tick GST was carried out in *E. coli* BL21 (DE3), using the prokaryotic expression vector pET30b. The coding region of the GST cDNA was introduced and expression was induced using IPTG at a final concentration of 1 mM at 37 °C. After induction of

cgcgcgtagcaaacggtcgtggtacttttccgtgcgtggtgtttcatttgagcgcgaactttatcatcaacc **ATG** GCT CCT GTG CTC
M A P V L
GGC TAC TGG GAC ATT CGC GGG TTG GCC CAG CCC ATC CGT CTG CTG CTT GCG CAC
G Y W D I R G L A Q P I R L L L A H
GTG GAC GCC AAG GTG GAC GAC AAG CGC TAC TCG TGC GGA CCC CCG CCG GAC TTT
V D A K V D D K R Y S C G P P P D F
GAC CGC AGC TCC TGG CTG AAC GAG AAG ACC AAG CTT GGC CTG GAG TTC CCC AAT
D R S S W L N E K T K L G L E F P N
CTG CCT TAC TAC ATT GAT GGC GAT GTC AAG CTG ACC CAG AGT ATG GCT ATC CTG
L P Y Y I D G D V K L T Q S M A I L
CGT TAC CTC GCC AGG AAG CAT GGT TTG GAG GGC AAG ACT GAA GCC GAG AAG
R Y L A R K H G L E G K T E A E K
CAG CGA GTG GAT GTC TCT GAG CAG CAG TTC GCA GAC TTC CGT ATG AAC TGG GTT
Q R V D V S E Q Q F A D F R M N W V
CGT CTC TGC TAC AAC CCT GAT TTC GAA AAG CTG AAG GGT GAC TAC CTC AAG AAC
R L C Y N P D F E K L K G D Y L K N
CTG CCG GCT TCT CTC AAG GCT TTC TCC GAC TAT TTG GGA ACC CAC AAG TTC TTT
L P A S L K A F S D Y L G T H K F F
GCT GGG GAC AAT CTC ACC TAT GTT GAC TTC ATT GCT TAC GAG ATG CTT GCC CAA
A G D **N*** L T Y V D F I A Y E M L A Q
CAT CTC ATT TTT GCT CCC GAC TGC CTG AAG GAC TTC GCC AAC CTC AAG GCC TTC
H L I F A P D C L K D F A N L K A F
GTC GAC CGT ATT GAG GCT CTT CCG CAC GTT GCG GCC TAC CTG AAG TCG GAC AAG
V D R I E A L P H V A A Y L K S D K
TGC ATT AAG TGG CCC CTC AAT GGC GAC ATG GCC AGC TTC GGC AGC AGG CTG CAG
C I K W P L N G D M A S F G S R L Q
AAG AAG CCA **TGA** aacaagcattcattctcttttattttcgaacaaaggtcttgac**aataaa**ataagcattaacttttaaaaaaaaaaaa
K K P **
aaaaaaa

Fig. 1. cDNA sequence and deduced amino acid sequence of the tick *B. annulatus* GSTmu class. Arrow-head lines indicate DNA primer locations used for probe design. The underlined lower case letters with bold type indicate the polyadenylation signal and the double asterisks (**) denote the stop codon. The N-linked glycosylation site is denoted in bold with single asterisk. This sequence has been submitted to Genbank, and assigned the accession number EF440186.

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         1                                                         59
BaGSTm   MAP-VLGYWDIRGLAQPIRLLLAHVDAKVDDKRYSCGPPPDFDRSSWLNEKTKLGLEFPN
BmGSTm   MAP-VLGYWDIRGLAQPIRLLLAHVDAKVDDKRYTCGPPPDFDRSSWLNEKTKLGLEFPN
HGSTm1   M-PMILGYWDIRGLAHAIRLLLEYTDSSYEEKKYTMGDAPDYDRSQWLNEKFKLGLDFPN
HGSTm2   M-PMTLGYWNIRGLAHSIRLLLEYTDSSYEEKKYTMGDAPDYDRSQWLNEKFKLGLDFPN
RGSTm2   M-PMTLGYWDIRGLAHAIRLFLEYTDTSYEDKKYSMGDAPDYDRSQWLSEKFKLGLDFPN
MGSTm1   M-PMILGYWNVRGLTHPIRMLLEYTDSSYDEKRYTMGDAPDFDRSQWLNEKFKLGLDFPN
RGSTm1   M-PMILGYWNVRGLTHPIRLLLEYTDSSYEEKRYAMGDAPDYDRSQWLNEKFKLGLDFPN
MiteGSTm MSQPILGYWDIRGYAQPIRLLLTYSGVDFVDKRYQIGPAPDFDRSEWLNEKFNLGLDFPN
         60                                                        119
BaGSTm   LPYYIDGDVKLTQSMAILRYLARKHGLEGKTEAEKQRVDVSEQQFADFRMNWVRLCYNPD
BmGSTm   LPYYIDGDVKLTQSMAILRYLARKHGLEGKTEAEKQRVDVSEQQFADFRMNWVRLCYNPD
HGSTm1   LPYLIDGAHKITQSNAILCYIARKHNLCGETEEEKIRVDILENQTMDNHMQLGMICYNPE
HGSTm2   LPYLIDGTHKITQSNAILRYIARKHNLCGESEKEQIREDILENQFMDSRMQLAKLCYDPD
RGSTm2   LPYLIDGSHKITQSNAILRYLGRKHNLCGETEEERIRVDVLENQAMDTRLQLAMVCYSPD
MGSTm1   LPYLIDGSHKITQSNAILRYLARKHHLDGETEEERIRADIVENQVMDTRMQLIMLCYNPD
RGSTm1   LPYLIDGSRKITQSNAIMRYLARKHHLCGETEEERIRADIVENQVMDNRMQLIMLCYNPD
MiteGSTm LPYYIDGDMKMTQTFAILRYLGRKYKLNGSNDHEEIRISMAEQQTEDMMAAMIRVCYDAN
         120                                                       179
BaGSTm   FEKLKGDYLKNLPASLKAFSDYLGTHKFFAGDNLTYVDFIAYEMLAQHLIFAPDCLKDFA
BmGSTm   FEKLKGDYLKNLPASLKAFSDYLGTHKFFAGDNLTYVDFIAYEMLAQHLIFAPDCLKDFA
HGSTm1   FEKLKPKYLEELPEKLKLYSEFLGKRPWFAGNKITFVDFLVYDVLDLHRIFEPKCLDAFP
HGSTm2   FEKLKPEYLQALPEMLKLYSQFLGKQPWFLGDKITFVDFIAYDVLERNQVFEPSCLDAFP
RGSTm2   FERKKPEYLEGLPEKMKLYSEFLGKQPWFAGNKITYVDFLVYDVLDQHRIFEPKCLDAFP
MGSTm1   FEKQKPEFLKTIPEKMKLYSEFLGKRPWFAGDKVTYVDFLAYDILDQYRMFEPKCLDAFP
RGSTm1   FEKQKPEFLKTIPEKMKLYSEFLGKRPWFAGDKVTYVDFLAYDILDQYHIFEPKCLDAFP
MiteGSTm CDKLKPDYLKSLPDCLKLMSKFVGEHAFIAGANISYVDFNLYEYLCHVKVMVPEVFGQFE
         180                                  223
BaGSTm   NLKAFVDRIEALPHVAAYLKSD--KCIKWPLNGDMASFGSRLQKKP
BmGSTm   NLKAFVDRIEALPHVAAYLKSD--KCIKWPLNGDMASFGSRLQKKP
HGSTm1   NLKDFISRFEGLEKISAYMKSS--RFLPRPVFSKMAVWGNK-----
HGSTm2   NLKDFISRFEGLEKISAYMKSS--RFLPRPVFTKMAVWGNK-----
RGSTm2   NLKDFVARFEGLKKISDYMKSG--RFLSKPIFAKMAFWNPK-----
MGSTm1   NLRDFLARFEGLKKISAYMKSS--RYIATPIFSKMAHWSNK-----
RGSTm1   NLKDFLARFEGLKKISAYMKSS--RYLSTPIFSKLAQWSNK-----
MiteGSTm NLKRYVERMESLPRVSDYIKKQQPKTFNAPTSKWNASYA-------
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Fig. 2. Alignment of deduced amino acid sequence of *B. annulatus* GST with *B. microplus*, dust mite and mammalian mu-class GSTs: *B. microplus* (Bm) with accession number (AF077609), Human (H) with accession numbers P09488 and P28161 for GSTM1 and GSTM2, respectively, Rat (R) with accession numbers P04905 and P08010 for GSTM1 and GSTM2, respectively, Mouse (M; P10649), and dust mite with accession number (P46419). Highlighted amino acids represent similarities between species.

the expression, affinity purification of the rBaGST was carried out under native conditions and the expression and purification was checked out using 12% SDS-PAGE (Fig. 3). The eluted protein concentration was measured by the method of Bradford (1976), and the concentration was 1.8 mg/ml. The purified rBaGST had an apparent molecular weight of 30.8 kDa after Coomassie Blue staining. rBaGST contains a fragment of the fusion protein β-galactosidase at the 5′ end that accounts for the difference between the purified fusion protein and the calculated GST molecular weight of 25.589 kDa.

3.3. Enzyme activity

The purified fusion rBaGST eluted from the Ni-NTA agarose column was assayed for its activity on the chromogenic substrate CNDB. The conjugation of CDNB was observed in assays with both rBaGST and GST from partially engorged *B. annulatus* females (Fig. 4). Endogenous bacterial GST control was included in the assays and no activity was detected. rBaGST (0.5 μg/assay) showed high GST activity and the specific activity was 121 units/mg protein, compared to the native GST activity from *B. annulatus* female (240 μg/assay) and the specific activity was 0.2 units/mg protein.

3.4. Enzyme kinetics

Enzyme kinetic constants are summarized in Table 1. The pH optimum for the rBaGST with CDNB as substrate was found to be 7.5 (Fig. 5). The effect of substrate concentration on GSH-CDNB conjugation activity was investigated at 25 °C for K_m determination. The rBaGST showed apparent Michaelis–Menten kinetics with respect to both substrates, GSH and CDNB. The K_m values of the rBaGST for GSH and

Table 1
Kinetic parameters of purified rBaGST

Kinetic parameters	rBaGST
K_m (CDNB)	0.57 mM
K_m (GSH)	0.79 mM
V_{max} (CDNB)	75.2 μmol/min/mg protein
V_{max} (GSH)	48.8 μmol/min/mg protein
pH optimum	7.5

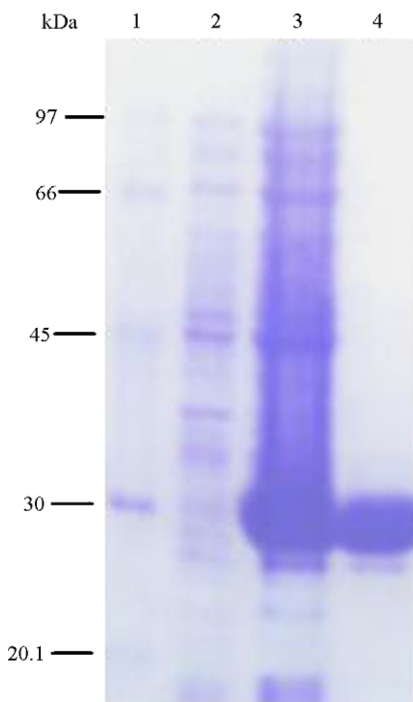


Fig. 3. 12% SDS-PAGE of expressed and purified recombinant GST from the cattle tick *B. annulatus*. Lane (1) molecular weight marker, lane (2) bacterial lysate of induced BL21/pET30b (empty vector), lane (3) bacterial lysate of induced BL21/pET30b with recombinant GST, and lane (4) purified GST.

CDNB were 0.79 and 0.57 mM, with V_{max} of 48.8 and 75.7 μmol/min/mg protein, respectively (Fig. 6).

3.5. Substrate specificity

The specific activities measured for rBaGST toward various substrates are listed in Table 1, which shows that

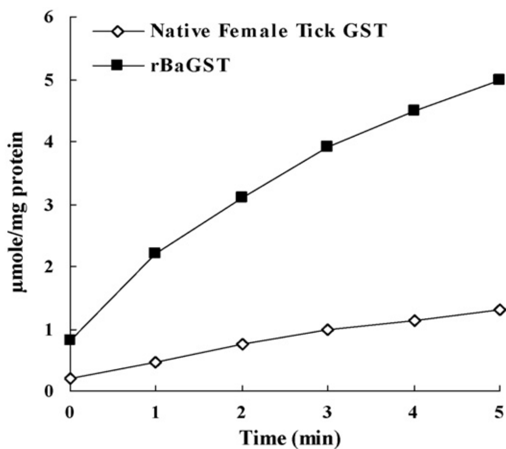


Fig. 4. Native GST activity in female *B. annulatus* total proteins and rBaGST purified after expression.

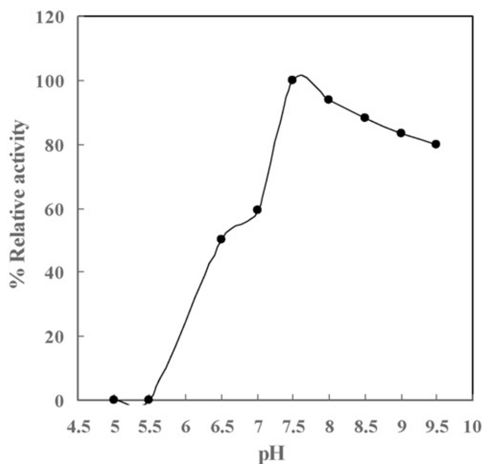
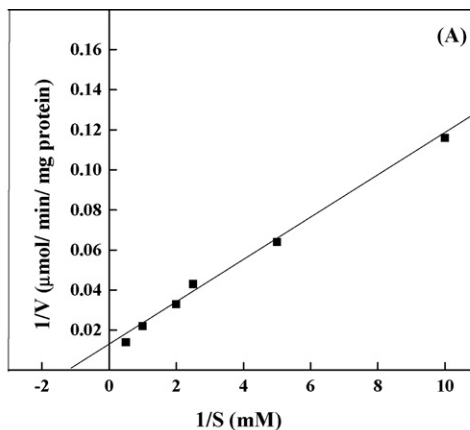


Fig. 5. Effect of pH on the enzymatic activity of rBaGST. The buffers used were 0.1 M citrate for pH 5, 0.1 M acetate for pH 5.5, 0.1 M potassium phosphate for pH 6.5–8 and Tris–HCl for pH 8.5–9.5.



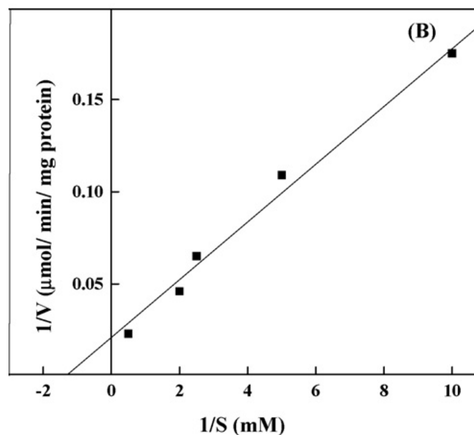


Fig. 6. Lineweaver–Burk plot relating the purified enzyme rBaGST activity to CDNB (A) and GSH (B) concentration.

Table 2
Substrate specificity for rBaGST purified enzyme

Substrate	Specific activity (units/mg protein)	% Relative activity
1-Chloro-2,4-dinitrobenzene (CDNB)	121	100
Bromosulfophthalein	0	0
1,2-Dichloro-4-nitrobenzene (DCNB)	29.3	24.6
p-Nitrophenethylbromide	56.4	46.6
Cumene hydroperoxide	62.4	51.6

the activity was highest for CDNB. The enzyme also had a peroxidatic activity with the substrate cumene hydroperoxide with specific activity of 62.43 μmol/min/mg protein. The activity of other substrates is summarized in Table 2.

3.6. Inhibition studies

Cibacron blue and bromosulfophthalein were tested for their ability to inhibit CDNB-conjugating activity of rBaGST (Fig. 7). I_{50} for cibacron blue and bromosulfophthalein were 0.22 and 8.45 μM, respectively.

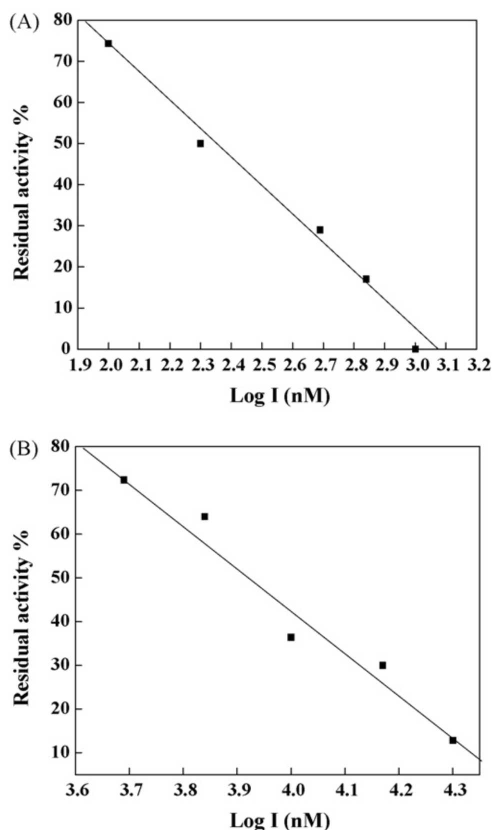


Fig. 7. Effect of cibacron blue (A) and bromosulfophthalein (B) on the enzymatic activity of the rBaGST. Cibacron concentration was varied from 0.1 to 1.0 μM, and bromosulfophthalein. concentration was varied from 5.0 to 20 μM.

3.7. Immunodetection of GSTmu homologues in B. annulatus tissues and other tick species

Rabbit anti-rBaGST antibodies were used to localize and estimate the native GST protein molecular mass in five different protein extracts from the hard tick *B. annulatus* including the whole tick, whole larval, gut, salivary glands and ovarian proteins (Fig. 8). The rabbit anti-rBaGST antibodies were able to detect very close double protein bands in the whole *B. annulatus* protein extract with molecular weight around 26 and 25.5 kDa, while a single protein band with molecular weight of approximately 26 kDa was detected in the other tissues.

To ascertain the presence of GSTmu-class homologues in other tick species distributed in Egypt, the rabbit anti-rBaGST antibodies were used in immunoblot analysis against the hard ticks; *Rhipicephalus* sp. and *Hyalomma dromedarii* and the soft tick; *Ornithodoros moubata*, protein extracts. A single protein band with molecular weight of 26 kDa, which corresponds to the estimated GST molecular weight, was detected in both *Rhipicephalus* sp. and *H. dromedarii* while the GST homologue could not be detected in *O. moubata* (Fig. 8, lane 6). The reaction of normal rabbit serum (as a

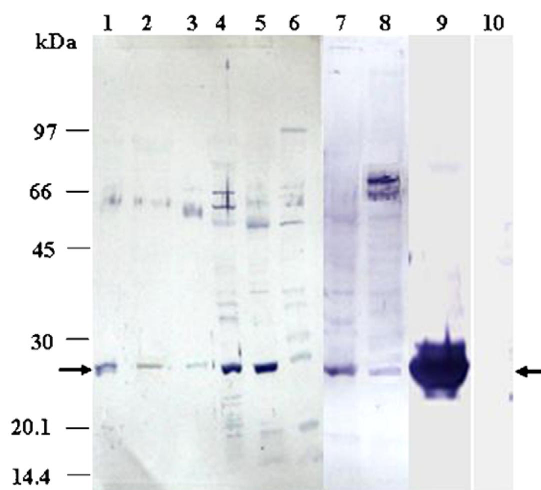


Fig. 8. Immunoblotting of 12% SDS-PAGE. Lane 1; whole *B. annulatus* proteins, lane 2; *B. annulatus* larval proteins, lane 3; *B. annulatus* gut proteins, lane 4; whole *H. dromedarii* proteins, lane 5; whole *Rhipicephalus* sp. proteins, lane 6; whole *O. moubata* proteins, lane 7; *B. annulatus* ovarian proteins, lane 8; *B. annulatus* salivary gland proteins, lane 9; and 10; *B. annulatus* rGST. Lanes from 1 to 9 were blotted against rabbit anti-recombinant *B. annulatus* GST, while lane 10 was blotted against normal rabbit serum. Arrows indicate the GST bands.

negative control) with the rBaGST shows no reactivity (Fig. 8, lane 10).

4. Discussion

In order to clone cattle tick GSTmu class, we used a *B. annulatus* cDNA library. The GSTmu probe was generated by PCR amplification of cDNA from whole *B. annulatus* tick using primers based on consensus regions among the sequences of *B. microplus*, human, rat and mouse GSTmu. Four clones were identified from different plates. The nucleotide sequences of GSTmu from *B. annulatus* (BaGST) included an ORF of 672 bp encoding a polypeptide of 223 amino acids. The Kozak sequence (Kozak, 1991) recognized by ribosomes as the translational start site and thus required for protein expression, conformed strongly to the sequence found within the GSTmu 5′ UTR. However, it is likely that this is the initiation site, based on the absence of a preceding initiation codon in any of the clones. In fact, it is reported that a GSTmu (accession number AF077609) was isolated from the larval stage of *B. microplus* and the translational start site, in this sequence, conformed strongly to the Kozak sequence (He et al., 1999). On the other hand, another *B. microplus* GSTmu-class sequence isolated from the salivary gland (accession number AF366931) was identified and conformed poorly to the Kozak sequence (Rosa de Lima et al., 2002). The putative polyadenylation site was found to be 22 bp upstream from the poly A^+ tail and did not overlap the translational stop codon as in the *B. microplus* salivary gland GST (Rosa de Lima et al., 2002) and in the spruce budworm, *Choristoneura fumiferana* (Feng et al., 1999).

The BaGST ORF encoded a predicted protein of 223 amino acids and p*I* of 8.11. The predicted molecular weight of the deduced protein is 25.589 Da. Similar GSTs molecular sizes and function, were found from larvae of the Australian sheep blowfly *Lucilia cuprina*, the nematode *Haemonchus contortus* (Sharp et al., 1991; van Rossum et al., 2004), house dust mite *D. pteronyssinus* (O'Neill et al., 1994), the tick *B. microplus* (He et al., 1999), the free living *Caenorhabditis elegans* (Campbell et al., 2001), and mouse (Guo et al., 2002).

Comparison of the deduced amino acid sequences of the BaGST protein with sequences in the Genbank shows that the BaGST is most similar to the class mu GSTs (BLAST 2 version Blastb 2.0.5) (Altshul et al., 1997). The BaGST deduced protein is composed predominantly of hydrophilic residues characteristic of cytosolic proteins. Four cysteine residues were found in BaGST protein and were consistent with the larval BmGST (Fig. 2). In the salivary gland BmGST, cysteine residues were absent as in sequence of GST of *C. fumiferana* (Feng et al., 1999) supporting the evidence that cysteine residues are not essential to the catalytic activity of the class mu GSTs (Widersten et al., 1991).

There are two active sites per dimer for cytosolic GST enzymes, the highly specific GSH binding site (G-site) that located in domain I close to the N-terminal sequence, and the H-site that interacts nonspecifically with the second hydrophobic substrate and is located in domain II at the C terminal end (Hansson et al., 1999; Stenberg et al., 2000). The BaGST protein has the conserved active site motif between residues 58 and 66, where GSH binds. Comparison of BaGST with the protein databank for GST sequences revealed the presence of the SMAILRYL motif that may play an important structural role in GSH binding site and the interface domain (Armstrong et al., 2001).

In the present study, the sequence of the predicted polypeptide was highly homologues with the BmGST (about 99.6% overall identity) (Fig. 2). The overall sequence homology shared between the cattle tick BaGST and the mammalian mu class is at least 53% and the degree of similarity is much higher at the N-terminus than the C-terminus among GSTs which is common in GST families (Hayes et al., 2005).

GSTs have been correlated with the detoxification of a wide range of electrophilic compounds (Stenberg et al., 2000). In insects, up regulated expression of GSTs have been associated with insect resistance to insecticides particularly, the organophosphorus compounds (Hayes and Pulford, 1995; Huang et al., 1998; Vararattanavech and Ketterman, 2003; Winayanuwattikun and Ketterman, 2004). In preliminary results with class mu GST of *B. microplus* larvae, He et al. (1999) showed no differences in mRNA levels between untreated larvae of susceptible and organophosphorus-resistant strains. They referred the lack of differences between the susceptible and resistance ticks to the presence of other GSTs in ticks involved in resistance. In our preliminary results, we have identified another GSTmu isoform (clone number four, data not shown) from the *B. annulatus* cDNA library that may be coincident with the previous hypothesis.

In order to study the enzymatic characteristics of the BaGST, we expressed the ORF of the BaGST, affinity purified rBaGST, measured the enzymatic activity of the eluted protein and compared it with the GST from *B. annulatus* female protein extract. The substrate CDNB is not class specific and can interact with alpha, mu, pi and sigma GSTs (Takamatsu and Inaba, 1994) but not to

class theta GST (Meyer et al., 1991). rBaGST showed GST activity even containing a fragment of β-galactosidase on its 5′ end, but possibly with an altered level of activity. rBaGST showed high GST activity (121 units/mg protein) compared to the native GST activity from *B. annulatus* females (0.2 units/mg protein). The results of GST enzymatic activity using the chromogenic substrate CDNB confirmed the presence of GST in the cattle tick *B. annulatus*.

Optimum pH values for GST with a variety of different substrates range from 6 to 9.5. When CDNB is considered, narrow range of pH 7.0–9.0 is obtained, but the most is in the vicinity of pH 8.0 (Clark, 1989). In the present investigation, rBaGST showed maximum activity at pH 7.5. The kinetic constants for the rBaGST toward CDNB and GSH were comparable to values reported from insects GST (Prapanthadara et al., 1996; Yu and Huang, 2000; Jirajaroenrat et al., 2001; Valles et al., 2003), and the tick recombinant *B. microplus* (Da Silva Vaz et al., 2004).

GSTs differ in their substrate specificities and variations among members of different classes are mirrored in the variations in the structure of the binding site for the electrophilic hydrophobic substrates (Mannervik and Widersten, 1995). In vertebrates the multiple forms of GST with narrow but overlapping substrate specificities have been suggested as being beneficial for excluding all possible foreign or endogenous compounds that the organisms may encounter. Most of the GST studies in insects have been done using CDNB or DCNB as benzene substrate and DCNB has been shown not to be sensitive substrate in these cases (Franciosa and Berge, 1995; Francis et al., 2001). rBaGST exhibited almost the same behavior. However, this was not the case for the purified GST of the grasshopper (*Zonocerus variegatus*); a polyphagous insect where DCNB appears to be a relatively sensitive substrate (Adewale and Afolayan, 2006). rBaGST exhibited peroxidatic activity on cumene hydroperoxide sharing this property with GSTs belonging to the GST α class.

In terms of inhibition, class α is noted for high I_{50} values for cibacron blue (5–20 μM), while class μ has a low values (0.05–0.7 μM) (Mannervik et al., 1985). Regarding the I_{50} for cibacron blue the value obtained for rBaGST enzyme (0.22 μM) resembles that of the class μ.

The broad distribution of the cloned GSTmu class in the different *B. annulatus* tissues indicates the importance of the mu class to this ectoparasite. The presence of GST homologues in other tick species as *H. dromedarii* and *Rhipicephalus* sp. with the almost similar molecular weights as in *B. annulatus* suggests that the GSTmu-class gene is conserved between these hard tick species and not in soft ticks as *O. moubata*.

In this paper, we have described the cloning, expression and characterization of *B. annulatus* GST similar to mammalian mu class. Further work will be conducted to understand the physiological role of GSTmu in cattle tick metabolism as well as, its possible role in the tick–host relationship.

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