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Chapter 11

Glutathione *S*-Transferase Genes from Ticks

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Additional information is available at the end of the chapter

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1. Introduction

For a long time before the discovery of glutathione S–transferases (GSTs; EC 2.5.1.18), it was a well known fact that some orally administered electrophilic compounds ultimately become excreted in the urines as a conjugates of N- acetyl cysteine, the so called mercapturic acids. Glutathione was then identified by [1] to be the source of cysteine used for biosynthesis of the mercapturic acids. As a consequence, the GSTs were discovered as enzymes catalyzing the first step in the formation of mercapturic acids. The first paper on GSTs was presented by [2], who described the partial purification and some properties of cytosolic rat liver enzymes capable of catalyzing the formation of GSH conjugation with halogenated aromatic compounds. GSTs form a group of ubiquitous enzymes that catalyze the conjugation between glutathione and several molecules, and play the most important role in the cellular detoxification pathway of endogenous and xenobiotic compounds [3].

GST family classified based on primary structure, substrate specificity and immunological properties. Presently, seven classes of GSTs are recognized in mammals, namely the specific Alpha, Mu, Pi and the common Sigma, Theta, Zeta and Omega. The classification of GSTs into different classes is also reflected in the chromosomal location of the genes. In human, each class is encoded by genes organized into clusters on different chromosomes. For example, the genes of all known class Mu GSTs are clustered on chromosome l, the genes of the class Alpha, Pi and Theta are clustered on chromosomes 6, 11, 22, respectively [4]. Polymorphisms have been identified in the GSTM1, GSTT1 and GSTPl genes coding for enzymes in the μ, θ, and π classes, respectively. The GSTM1 and the GSTT1 genes are polymorphic in humans, and the phenotypic absence of enzyme activity is due to a homozygous inherited deletion of the gene [5-7].

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Ticks are blood sucking ectoparasites that infest a wide array of species. They are vectors of diseases in humans and other animals. The southern cattle tick, *Rhipicephalus 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 Rhipicephalus ticks to all currently used organophosphates [8], synthetic pyrethroids and amidines [9]. Despite previous studies that suggested increased detoxification [10] and target site insensitivity may contribute to the increased tolerance to acaricides, the mechanisms conferring resistance on ticks are poorly understood.

In the past years, significant advancement has been made to determine the potential role of GSTs in toxicology. Besides the well established role of GSTs in detoxification of xenobiotic compounds, it has been observed that GSTs have other intracellular substrates including the metabolites released from cellular molecules. In ticks, GSTs have attracted attention because of their involvement in the defense towards insecticides mainly organophosphates, organochlorines and cyclodienes. This chapter will give highlight on some of the cloned GST genes in ticks and will discuss and review the folding and unfolding states of a GST mu class from the cattle tick *Rhipicephalus annulatus* distributed in Egypt.

2. Glutathione S-transferase genes in ticks

Ticks are blood feeding external parasites of mammals, birds, and reptiles throughout the world. Tick infestations of animals and especially farm ones like cattle and camels, economically impact food industry by reducing weight gain and milk production, and by transmitting pathogens that cause babesiosis (*Babesia bovis* and *Babesia bigemina*) and anaplasmosis (*Anaplasma marginale*). The most important and widely distributed ticks include American dog tick (*Dermacentor variabilis*) is the most commonly identified species responsible for transmitting *Rickettsia rickettsii*, which causes Rocky Mountain spotted fever in humans, *R. microplus* and *R. annulatus* which infest cattle and distributed in Asia, Latin America, and Africa, *Hyalomma dromedarii* which infest camels (Asia and Africa), and the blacklegged tick (*Ixodes scapularis*), commonly known as "deer tick" and can transmit the organisms responsible for anaplasmosis, babesiosis, and Lyme disease and is widely distributed in the northeastern and upper midwestern United States.

Acaricide application constitutes a major component of integrated tick control strategies [11]. However, use of acaricides has had limited efficacy in reducing tick infestations and is often accompanied by serious drawbacks, including the selection of acaricide-resistant ticks, environmental contamination, and contamination of milk and meat products with drug residues.

GST enzymes are one of the important supergene families that are involved in protecting the organism from oxidative stress and xenobiotics including the acaricides. Different studies have been carried out to explore the role of the different GST families in detoxification in ticks. The methods applied in these studies used biochemical approaches, direct cloning us-

ing consensus sequences or using the available information from whole genome sequence information. Niranjan Reddy et al. [12] studied the GST superfamily organization in *Ixodes scapularis* using the whole genome sequence information (IscaW1.1, December' 2008) by applying different phylogenetic and bioinformatic tools. They identified all the three broad GST classes, the canonical, mitochondrial, and microsomal forms. A total of 35 GST genes belong to five different canonical GST classes, namely Delta (7 genes), Epsilon (5), Mu (14), Omega (3), and Zeta (3 genes) GST classes, and two mitochondrial Kappa class GST genes, and a single microsomal GST gene were found. The analysis of these sequences identified members of the Delta- and Epsilon-classes which are thought to be specific to the Insecta. Surprisingly, Ixodes has lost two of the functionally important gene families, Theta-and Sigma-GSTs.

GSTs had been reported to play a major role in the organophosphate resistance pathway of the *Musca domestica* (Corrnell-HR strain) [13]. On the contrary, Li et al. [14] reported that GSTs play only a minor role against organophosphate toxicity in *R. microplus*. Several GST coding frames had been cloned from *R. microplus* as done by [15] (accession number AAL99403), and [16] described that the activity of this protein is enhanced by organophosphate and coumaphos.

Some GST genes were cloned from different tick species and are of the mu class. The conservation score is represented in figure 1, and three state secondary structure is in figure 2. However, several attempts were carried out to explore the distribution of the different GST classes in ticks. The most widely distributed and economically important; the cattle tick *R. microplus* was used to initiate a study of the genome using an expressed sequence tag (EST) approach [17]. They reported the construction of a gene index named BmiGI from 20417 ESTs derived from a normalized cDNA library. The BmiGI was used to identify genes which might be involved in the acaricide resistance including GSTs.

Gurrero et al. [17] reported 15 possible GST coding genes identified from the BmiGI. One of these sequences was reported to be similar to the human GST class Omega 1, and the other clone was similar to mouse GST of Zeta 1 class. The total 15 clones are listed in table 1.

3. Unfolding/refolding of *Rhipicephalus annulatus* GST mu class

GSTs are dimeric proteins composed of identical or structurally related subunits. Each subunit has a molecular weight of about 25 kDa and is built of two domains and contains a complete active site consisting of a highly conserved G-site (GSH binding site) and a divergent H-site (Hydrophobic substrate binding site). The functional soluble enzymatic forms are found in dimers and only subunits within the same class can form heterodimers as found in alpha subunits, but this would not happen with either pi or mu subunits.

The nature of protein folding mechanisms and the manner in which the compact native state is achieved are still not well understood. From a wide range of experiments, it is now evident that specific pathways of folding are involved, at least for many proteins. At equilibri-

um, most monomeric and many oligomeric proteins display essentially a two-state pathway upon folding/unfolding, for which thermodynamically stable folding intermediates do not exist. Other mechanisms result in the formation of stable intermediates. These monomeric intermediates sometimes have preserved tertiary structure or appear as molten globules [18-20]. For proteins composed of subunits, the intermediates are either partially folded oligomeric states or monomeric states.

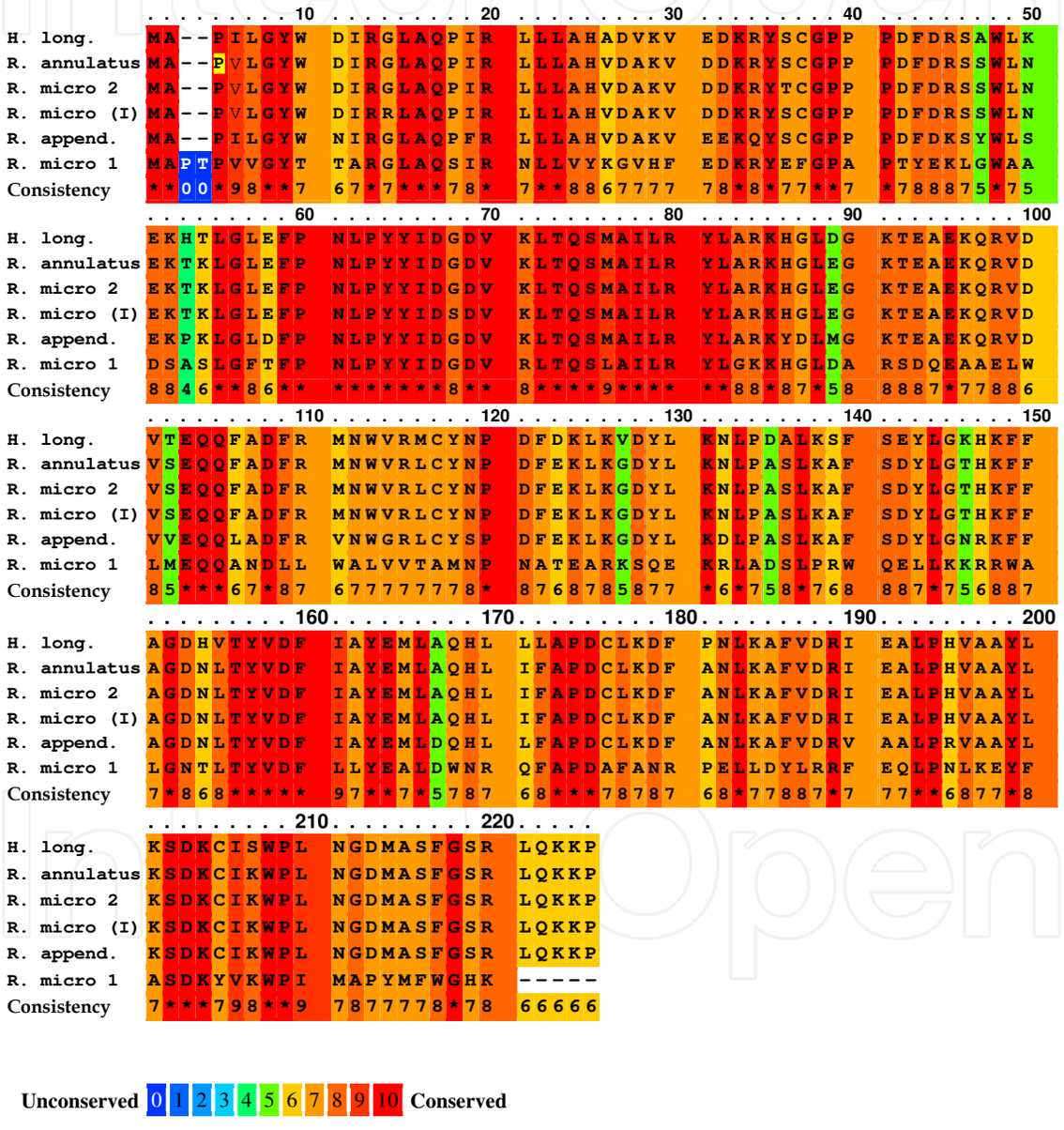

Figure 1. Amino acid conservation of Glutathione S-transferase mu class of different tick species. H. long. refers to *Haemaphysalis longicornis* GST (AAQ74441), R. annulatus is the *Rhipicephalus annulatus* GST (ABR24785), R. micro 2 refers to *Rhipicephalus microplus* GST (AAD15991), R. micro I is *Rhipicephalus microplus* Indian strain GST (ADQ01064),

R. micro 1 refers to *Rhipicephalus microplus* GST (AF366931_1), and R. append. refers to *Rhipicephalus appendiculatus* GST (AAQ74442). The conservation scoring was performed by PRALINE software (http://zeus.few.vu.nl). The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position.

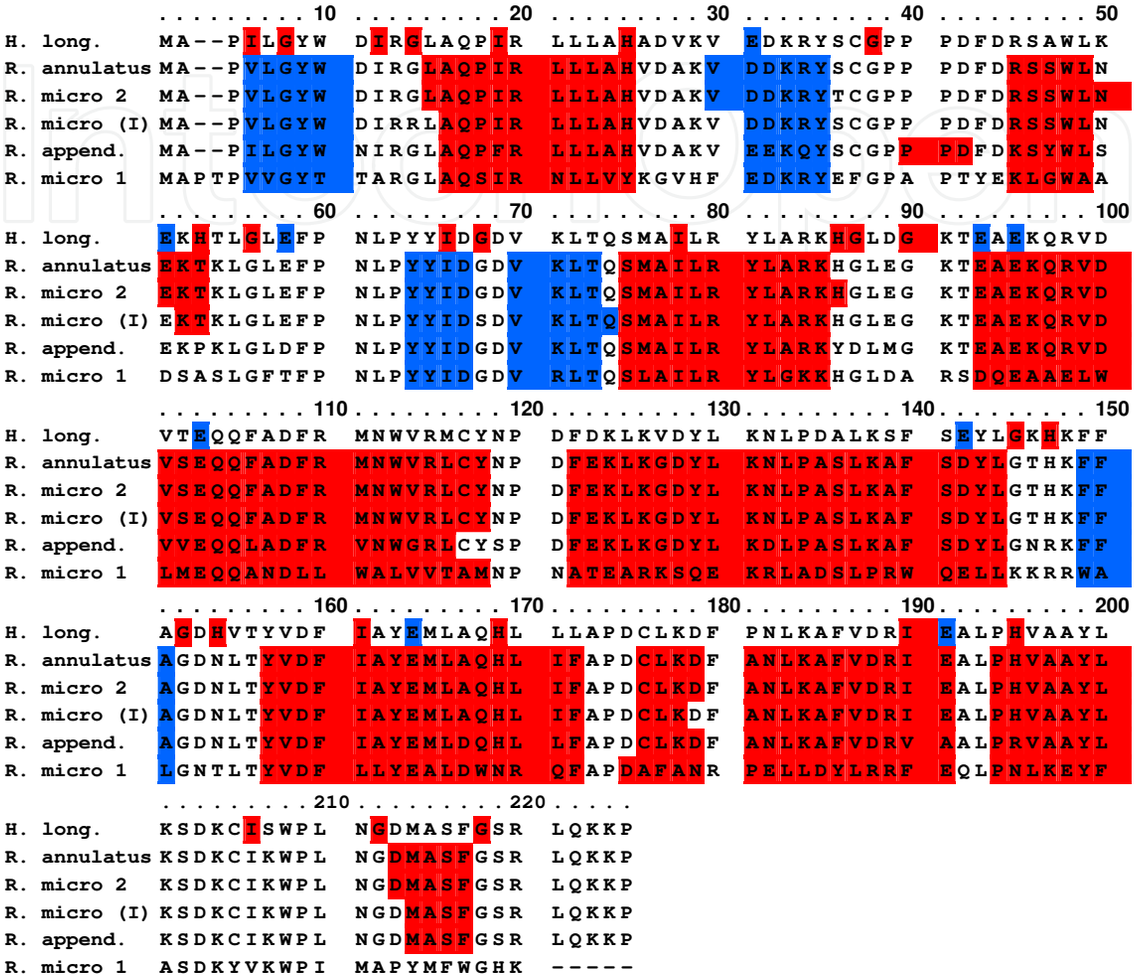

Figure 2. Three state (H, E C) secondary structure of Glutathione S-transferase mu class sequences from different tick species. H. long. refers to *Haemaphysalis longicornis* GST (AAQ74441), R. annulatus is *Rhipicephalus annulatus* GST (ABR24785), R. micro 2 refers to *Rhipicephalus microplus* GST (AAD15991), R. micro I is *Rhipicephalus microplus* Indian strain GST (ADQ01064), R. micro 1 refers to *Rhipicephalus microplus* GST (AF366931_1), and R. append. refers to *Rhipicephalus appendiculatus* GST (AAQ74442). The Helix (H) structure is in red and the Strand (E) is in blue. The sequence in the alignment has no color assigned for the coil (C) because there is no DSSP information available, or that no prediction was possible for that sequence.

Eftink et al. [18] proposed three models for proteins unfolding; the first is the two-state model. This model assumes that the protein exists only as a native dimer, D, and unfolded monomers, U, [D←→ 2U]. The second is three-state model (Folded monomers model). Unlike the first model, this one assumes that there is a two-step (three-state) unfolding process, with the formation of a folded monomeric intermediate, N. "folded" means that the intermediate can be further unfolded, in a cooperative manner, by addition of denaturant. However, it is not known to what extent the intermediate's structure actually resembles the

subunits of the native dimer [D←→2N←→2U]. The third model is also three-state model and also considers the existence of an intermediate in the unfolding process but the intermediate is a partially unfolded dimeric state, D', which can then be further unfolded to unfolded monomers [D←→ D'←→2U].

Clone	Length (bp)	Top Blastx Hit e value cutoff=0.001	(TC)-GST Class subfamily*	Accession number of the Hit
TC 213	861	*Galleria mellonella*	Delta or Epsilon	AAK64362
TC2718	808			
TC 298	908	*Homo sapiens* Omega 1	Omega 1	NP004823
TC 614	756	*Dermacentor variabilis*	Delta or Epsilon	AAO92279
TC3165	855			
TC 762	1061			
TC3881	831			
TC4914	718			
TC1038	742	*R. microplus*	Mu	AAL99403
TC2910	813			
TC3317	984			
TC3737	825	*R. microplus*	Mu	AAD15991
TC1082	1236	*Mus musculus* Zeta 1	Zeta	Q9WVL0
TC 811	859	*Anopheles gambiae*	Zeta	AAM61889
TC2689	1007	*Xenopus laevis*	Mu	CAD01094

Table 1. Glutathione S-transferase sequences identified in *Boophilus microplus* Gene Index (BmiGI). The asterisk means that there is no practical evidence of the subfamily. The assigned class is based on prediction similarity.

Full understanding of the protein folding process requires the identification and characterization of all intermediate steps, which are often very transient and detected by kinetic studies only. In these cases, some properties of the intermediates can be inferred, but little structural information can be derived from this approach. It is known that mild denaturing conditions, such as moderately high temperature or low pH, promote partially unfolded states that are similar to those observed at moderate concentrations of guanidinium chloride [19]. Therefore a large number of studies have been performed on these partially folded states [21]. Some of these more or less stable intermediates, called ''molten globules''are characterized by a largely conserved secondary structure but loss of tertiary structure and, due to the presence of a loosely packed hydrophobic core, binding of ANS is often observed

[22, 23]. Clear evidence of acidic pH induced stable folding intermediates has been obtained with some lipocalins, such as β-lactoglobulin [24, 25], retinol binding protein [26] and hGSTP1-1 [27].

Electrostatic interactions between charged residues on the surface of a protein play an important role in conferring stability to its folded structure. Change of pH alters the ionization state of these residues, causing intramolecular charge repulsion and possible disruption of salt bridges that can lead to destabilization of the native protein conformation [28]. pH is an important factor determining protein structure and function. Most proteins are stable and active at physiological pH and show varying degrees of denaturation in acid medium. However, as the acid concentration increases, a number of these proteins revert back to a compact conformation containing substantial secondary structure that resemble the folding intermediates known as molten globules [29, 30]. Study of the structural stability of a protein as a function of pH thus helps understand the thermodynamic or kinetic intermediates in its folding pathway and identifies the electrostatic interactions important for the stability of its folded state [31, 32].

In ticks, no data is available about the unfolding pathway of GST classes except for the *Rhipicephalus* (*Boophilus*) *annulatus* (*R. annulatus*) recombinant GST mu class (BaGSTM) [33]. Because of the non-identity of the different transitions monitored, the acid denaturation of BaGSTM does not appear to be a simple two-step transition, rather a multi-step process during which several intermediates coexist in equilibrium.

Shahein et al. [34] cloned the GST mu class from λZAP cDNA library of *R. annulatus*. The GST protein (Figure 3) contains four tryptophan residues (Try 7, Try 45, Try 110 and Try 214) and ten tyrosine residues [34]. 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 The authors showed that the *E. coli* expressed recombinant protein (BaGSTM) exhibited peroxidatic activity on cumene hydroperoxide sharing this property with GSTs belonging to the GST α class. The inhibition studies using cibacron blue and bromosulfophthalein showed that the *R. annuatus* GST shares this property with the mammalian GST mu class.

In its native state, the BaGSTM enzyme exhibits an emission spectrum with a maximum at 329 nm (excitation 280 nm). This feature characteristic of tryptophan residues partially buried in the protein matrix (Figure 4 a). The addition of increasing concentrations of GdmCl at equilibrium caused a red shift of λmax of the emission spectra from 329 nm to 352 nm. As shown in figure 4, compared with the native dimer, the fluorescence intensity increased with a slight red shift of λmax as the GdmCl concentration was increased. The intensity reached a maximum at approximately 1.45 M (partially unfolded dimer or nonnative dimeric intermediate). At GdmCl concentration between 1.5 M and 1.9 M there was no change in the fluorescence intensity or in λmax. The nonnative dimeric intermediate undergoes dissociation into monomeric intermediate at these GdmCl concentrations. Increasing the GdmCl concentration leads to another increase in the fluorescence intensity with a red shift of λmax and the intensity reached a maximum at approximately 2.4 M. This might be due to the formation of a partially unfolded monomer. After this concentration the intensity started to de-

crease with a red shift of λmax and at 4.0 M GdmCl, a λmax of 352 nm occurred, indicating the complete exposure of the tryptophan residues to the aqueous solvent which is consistence with the complete unfolding of the protein (unfolded monomer). From these results, at least two transition states between the native dimer and unfolded monomer could be identified for BaGSTM.

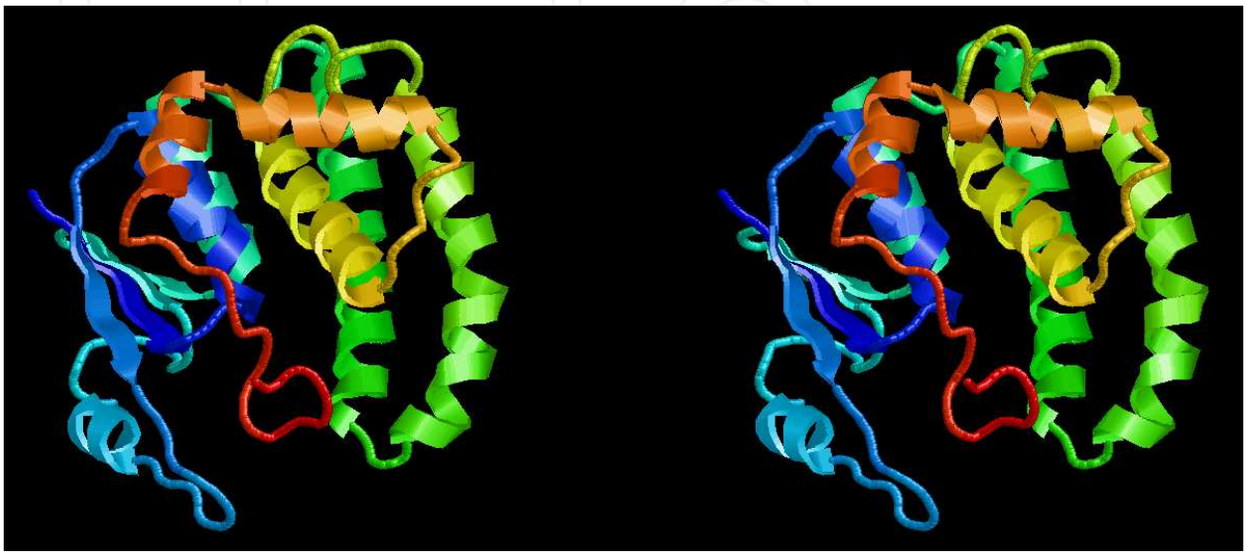

Figure 3. Theoretical model of *R. annulatus* GST mu class (ABR24785) built with M4T server. The numbers of groups are 218, atoms 1765, and bonds 1813 [35, 36].

As shown in the figure 5, at the concentration of urea between 0 and 1.75 M, there was an increase in the fluorescence intensity, as the concentration of urea increased, without any detectable shift of λmax. The intensity of fluorescence was increased by 50% at 1.75 M urea concentration compared with the native state of the protein indicating a partial exposure of the fluorophore (first phase). Increasing the concentration of urea, between 2.0 M and 3.0 M, resulted in a slight red shift (by 3 nm) of the emission maximum (second phase). Whereas, the increase in fluorescence intensity decreased as the concentration of urea was increased. The fluorescence intensity at the end of the first phase was higher than that at the end of the second phase. This indicates the movement of the fluorophore back into a more hydrophobic environment. At higher urea concentration, (between 3.25 and 4.5 M) the fluorescence intensity started to increase again with a shift of λmax (10 nm red shift). The fluorescence intensity was increased by three fold at 4.5 M urea compared to that of the native protein (third phase) [33].

Addition of higher concentrations of urea (5.0-7.0 M) did not change the intensity of the fluorescence significantly compared to that at 4.5 M urea but progressively shifted the λmax to 347 nm. At 8.0 M urea, the fluorescence intensity was decreased again with a shift of λmax to 352 nm indicating the complete unfolding of the protein. The present results indicate that three intermediates could be identified between the native dimer and unfolded monomer during the unfolding of BaGSTM.

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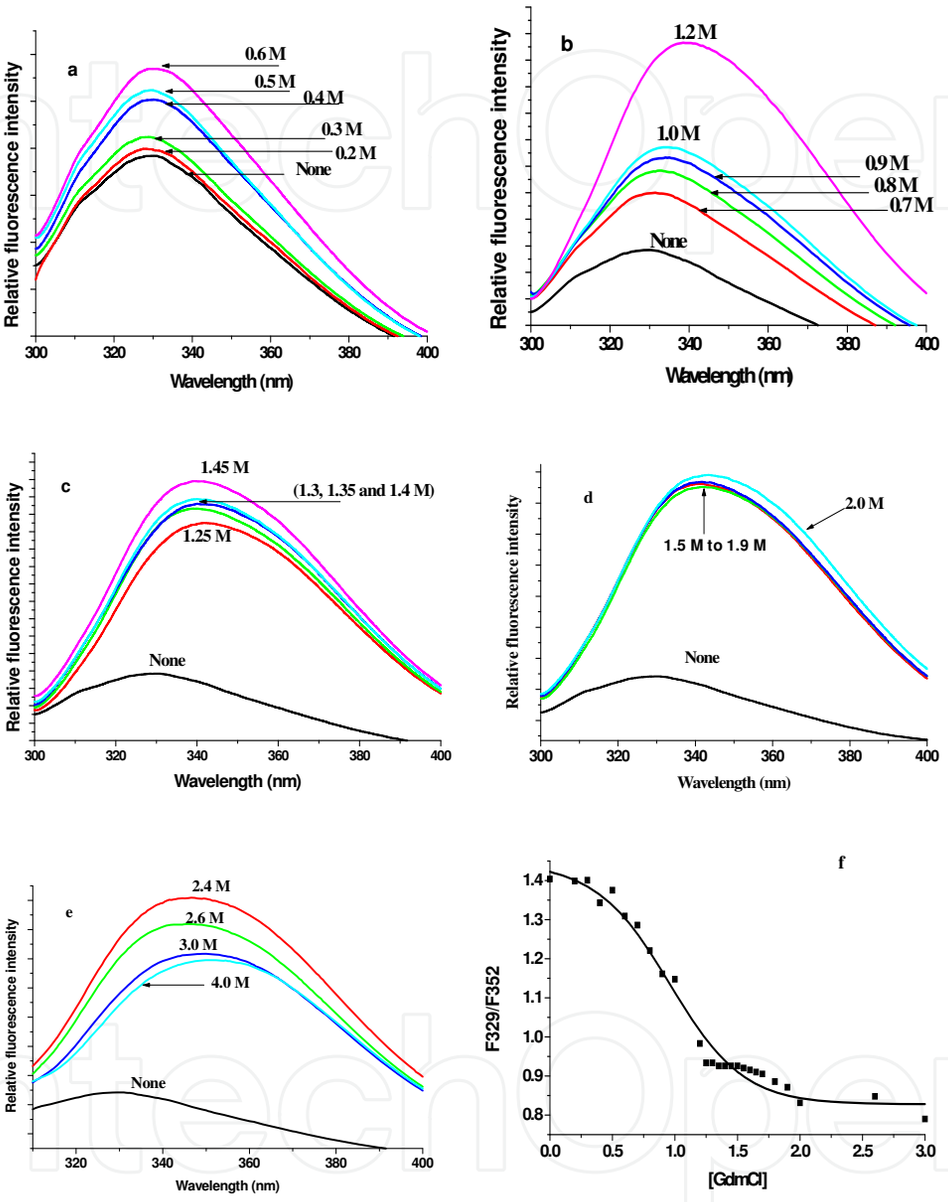

Figure 4. Fluorescence–emission spectra of BaGSTM (20 µg/ml) equilibrated in buffer A (20 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA/1 mM dithiothreitol) at different GdmCl concentrations ranging from 0 to 4.0 M at room temperature. Excitation was done at 280 nm and fluorescence was recorded from 300 to 400 nm (a to e). Unfolding was expressed as the ratio of fluorescence at 329 nm to the fluorescence at 352 nm (f).

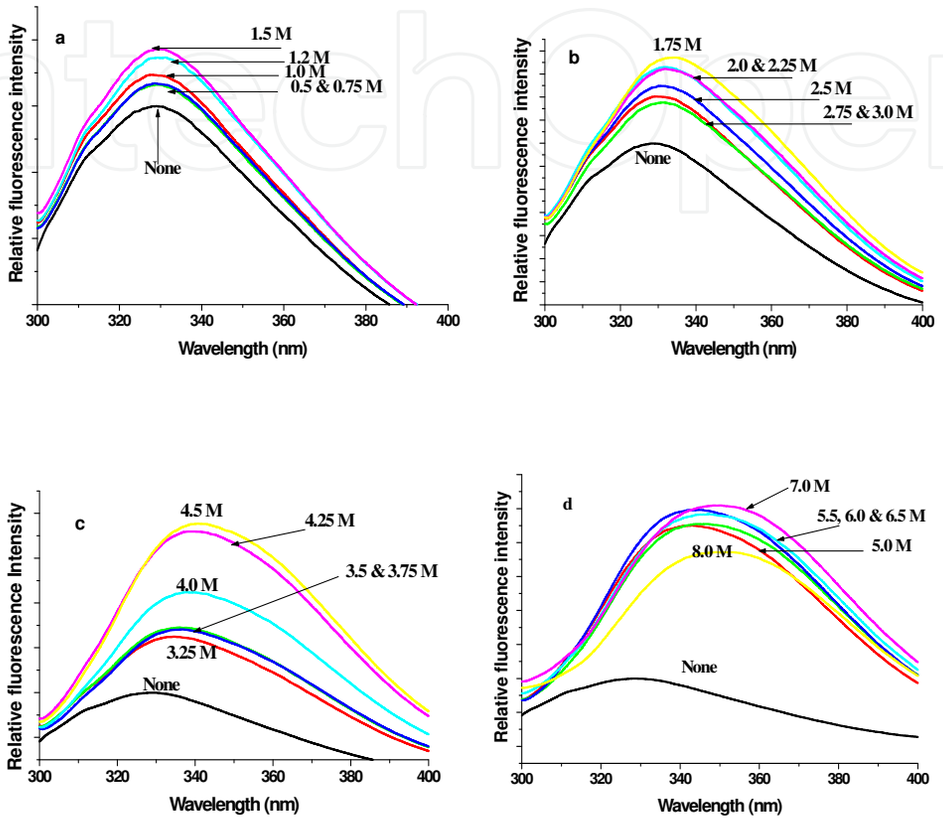

Figure 5. Urea-induced equilibrium unfolding for BaGSTM monitored by fluorescence. The protein (20µg/ml) was equilibrated in buffer A in the presence of the indicated concentration of urea at room temperature. Excitation was done at 280 nm and fluorescence was recorded from 300 to 400 nm.

In particular, the pH-dependent fluorescence transition of BaGSTM is clearly characterized by many distinct steps. The behavior of the protein in an acidic environment was investigated and analyses of fluorescence emission spectra of BaGSTM in solutions at different pH values were performed. The position of the emission maximum of a protein's fluorescence spectrum, upon excitation at 280 nm, was highly sensitive to the environment around its tryptophanyl and tyrosyl residues. As shown in figure 6, the acid denaturation of BaGSTM, as followed by the intrinsic fluorescence changes, was characterized by the presence of at least three transition states [33].

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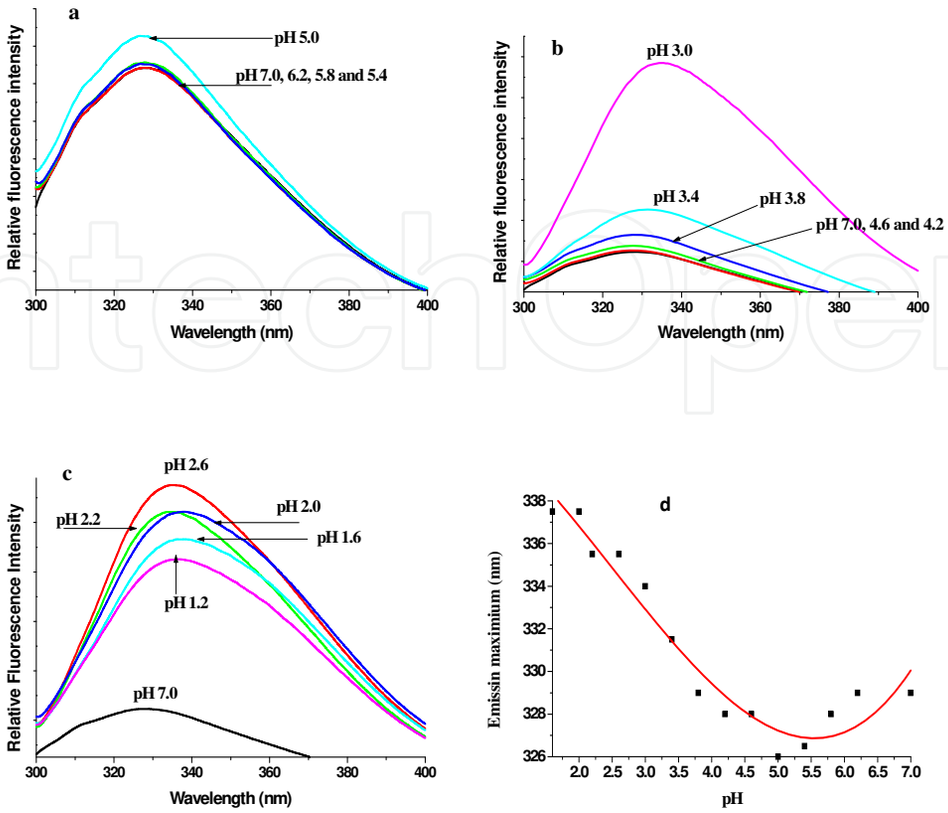

Figure 6. pH-induced equilibrium unfolding for BaGSTM monitored by fluorescence. The protein (20µg/ml) was equilibrated at room temperature in 0.02 M citrate-phosphate buffer at pH from 7.0 to 1.2 for 1 h before measurement. Excitation was done at 280 nm and fluorescence was recorded from 300 to 400 nm (a-c). Emission maxima were determined using the same excitation wavelength (d).

However, between pH 5.0 and 3.5, an initial red shift, from 338 to 342 nm of the maximum fluorescence emission, indicates a partial exposure of one or more tryptophanyl residues to the solvent. From pH 3.5 to pH 2.0 a second fluorescence transition occurs, characterized by a blue shift of λmax to 337 nm. This indicates the formation of a new type of structure in which the environment of the tryptophanyl residues is more hydrophobic. It has been proposed that the molten globule represents a common intermediate of the acid denaturation of many monomeric proteins.

GSTs are crystallized as dimers, but in solution class mu GST from rat its Asp97 mutant enzymes undergo reversible association and dissociation, the extent of which depends on protein concentration. Addition of 3 M potassium bromide to buffer solutions containing the wild-type rGSTM1-1 has generated monomers (GSTM1) [37]. A monomeric species of a human GSTpi has been constructed by introducing 10 site specific mutations. This drastically changed enzyme was structurally stable, but retained no activity [38].

The nonsubstrate ligand 8-anilino-1-naphthalene sulfonate (ANS) is a negatively charged hydrophobic fluorescent molecule, largely used to check the presence of compact partially folded intermediates. In fact, its very low fluorescence quantum yield in polar environment is strongly increased in non polar solvents [39]. Therefore, the binding of this molecule to

partially folded proteins, containing clusters of hydrophobic side chains accessible to solvent, is often observed in the presence of molten globules [23].

Unbound ANS emission spectra showed a maximum at 530 nm that was blue shifted upon binding of the dye to the protein. Binding of ANS to BaGSTM as a function of GdmCl concentration showed one peak centered at 1.5 M and one peak as a function of urea concentrations centred at 3.5 M (Figures 7 a and b). ANS binding fluorescence of BaGSTM as a function of pH did not show any transition peak. However, the fluorescence intensity was increased as the pH decreased. The fluorescence intensity about 2000 fold higher at pH 2.0 compared with that at neutral pH (Figure 7 c). At the neutral pH, the fluorescence of ANS in the presence of BaGSTM is perfectly super imposable to that of ANS alone. At less than pH 3.8, ANS binds to BaGSTM showed a blue shift displacement with an enhancement of fluorescence intensity. Binding of the dye occurs at the dimer interface and unfolded GST does not bind ANS. This makes ANS an excellent probe to monitor changes at the packing of hydrophobic cores in protein which undergoes structural changes and has been broadly used to study the presence of monomeric intermediates at the urea/GdmCl unfolding of several GSTs [38, 40-42]. ANS was also used to detect the presence of folding intermediates with hydrophobic patches such as the molten globule in penicillin G acylase [43] and apomyoglobin [44].

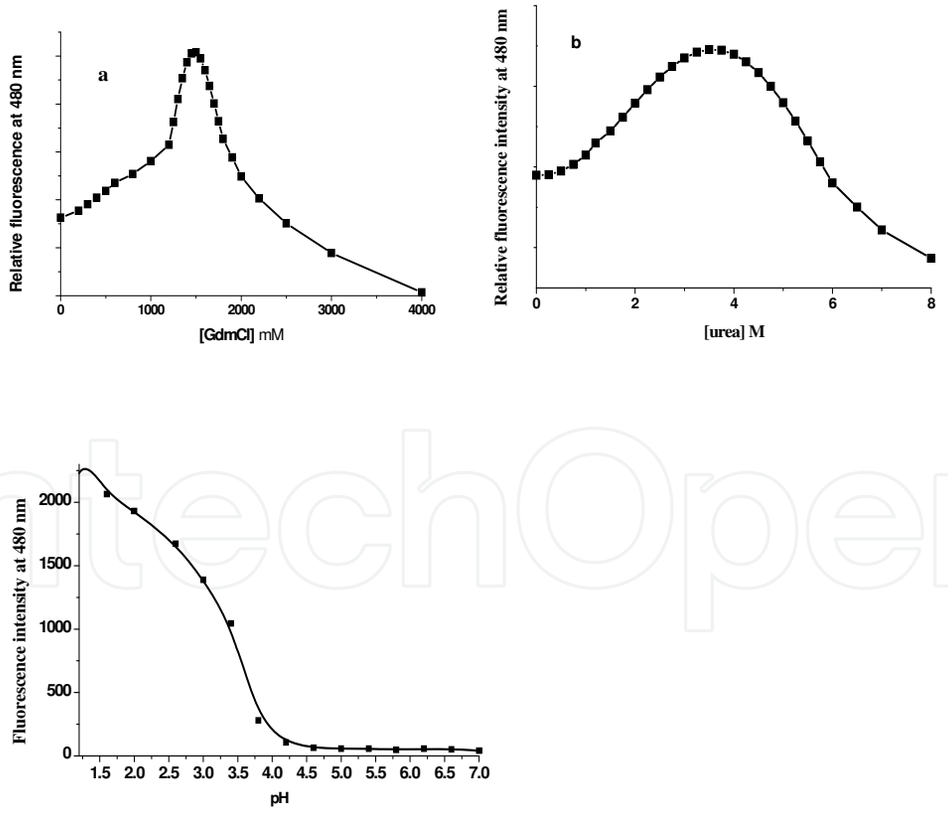

Figure 7. Variation of ANS binding fluorescence at 480 nm as a function of GdmCl concentrations (a) or urea (b) or as a function of pH (c). The proteins (20 µg/ml) were equilibrated in buffer A at room temperature in the presence of the indicated concentration of denaturant or pH. 100 µM ANS was added to the solution. Excitation was done at 380 nm and emission at 480 nm.

The unfolding results of the *R. (Boophilus) annulatus* (BaGSTM) demonstrate that the probes used (intrinsic fluorescence, excitation of tryptophan and tyrosine and ANS binding fluorescence) are differentially sensitive to various conformational states of BaGSTM. The presence of multiple nonsuperimposable transitions for this enzyme indicates that the two states unfolding mechanism is not applicable, and it is highly suggestion of the existence of at least two well-populated stable intermediates. Similar data were reported demonstrating the existence of stable intermediates at the unfolding of GSTs [42, 45-48]. However, only one transition intermediate was detected using ANS binding fluorescence for BaGSTM in presence of different concentrations of GdmCl or urea. This result is similar to that observed for sigma class GST in the presence of urea [46].

4. GST and immune system

Tick control is considered crucial all over the world to minimize the major drawbacks of tick infestations. The control strategies were adopted to use the acaricides which become less efficient. Seixas et al. [49] has stated the alternative approaches used in tick control and are classified into four groups: (i) biological control by tick pathogens or predators [50]; (ii) habitat alterations by planting tick-killing or repelling vegetation [51]; (iii) immunological control [52]; and (iv) development of tick-resistant breeds. They stated that although these methods have been proved to be theoretically valuable, most of them have been forsaken, since they did not afford acceptable cost/benefit ratios under field conditions, except for vaccines.

Many types of chemical tick control were started since 1893 including the application of arsenic acaricides, gamma BHC (organochlorine acaricide) and DDT. However, resistance to DDT was reported within 5 years of field application [53]. The toxicity plus the environmental awareness and other factors led to the removal of the previously mentioned acaricides from the tick control market.

In early 1960's, Diazinon and other organophosphate acaricides were applied and in 1970's, Triatix was the first amidine to be registered for tick control. In the 1980's, pyrethroids (Flumethrin and Permethrin) were also registered for tick control. The following table 2 shows the different acaricides used in tick control.

Along with the application of these chemicals with acaricidal properties as the predominant method of tick control throughout the world, resistance to the majority of the groups of chemicals had been evolved. Generally, the resistance may arise through several mechanisms including target sites, metabolic mechanism or reduced penetration. The target site resistance occurs when an allele of the gene coding for the target molecule is attacked by the acaricide. The penetration resistance occurs when an acaricide fails to penetrate the target individual. This type of resistance has not been reported in ticks. The third type of resistance mechanisms is the metabolic pathway which occurs through changes in the abilities of acaricide detoxification by an organism [17]. Three enzyme families including cytochrome P450s

(115 individual members), esterases (81 individual members), and GSTs (39 individual members), are involved in the metabolic resistance mechanism.

Type of Acaricide	Examples	Mode of Action
Arsenical Compounds	Arsenic Trioxide	Arsenite may bind to intracellular thiols, such as glutathione, and hence disrupting the ratio of reduced to oxidized glutathione leading to inhibition of cell division [54]
Chlorinated Hydrocarbons (Organochlorines)	DDT Gamma BHC Lindane Toxaphene	Interfere with the nerve conduction of ticks by affecting the ion channels, especially the voltage gated Na^+ channels [55]
Organophosphates	Diazinon Dichlorphos	Suppress the enzyme acetyl-cholinesterase [56]. This group of acaricides is used in the form of phosphorothionates which are converted by the ticks into an active more toxic ingredient named "phosphate" or the "Oxon".
Carbamates	Propoxure	Very similar to organophosphates
Amidines	Amitraz Cymiazol	They inhibit the monoamine oxidase enzyme which is responsible for the metabolism of neurotransmitter amines in tick nervous system. They probably interact with octopamine receptors causing an increase in nervous activity causing detaching of ticks from the animal [57]
Pyrethroides	Cypermethrin Flumethrin Cyhalothrin Alphamethrin	They interfere with nerve conduction [58]
Macrocyclic lactones (eg. Avermectin group)	Ivermectin	It affects neural transmission. In ticks, ivermectin inhibits female engorgement by reduction of body weight [59]

Table 2. Different acaricide groups used in tick control.

Anti-tick vaccine development is focused on the identification, molecular cloning and in vitro production of proteins playing key putative roles in tick physiology, such as cell signaling, modulation of host immune response, pathogen transmission, embryogenesis, digestion, and intermediary metabolism [60]. Of the different antigens used as an anti-tick vaccine was the GST molecule. GST was of special interest to stimulate cattle immune system and their critical role in the metabolic resistance of acaricides. The immunological bases of using GSTs as vaccines may be derived from the hypothesis that parasites can survive within their hosts for a period of time despite the complex immune environment surrounding them possibly accomplish this by adopting various immunomodulatory strategies, which include release of GSTs that counteract the oxidative reactive oxygen species (ROS) produced by the host activated cells and attach parasite cell membrane [61].

Since GSTs produced by parasites appear to be critical for the survival of parasites in the host, several studies evaluated the potential of parasite GSTs as vaccine candidates especially against schistosomiasis, fascioliasis and filarial parasites. However, several immunological studies were carried out to identify potential vaccines against helminth parasites including *Schistosoma mansoni* where the successful Sm28GST vaccine was developed by Capron et al. [62] and is in Phase II clinical trials. The injection of Sm28GST antigen elicited the production of immunoglobulins (especially IgE) and activation of eosinophils which could interfere with the function of parasitic GST [62]. Interestingly, the injection of Sm28GST in toxicity studies performed in dogs, rabbits and rats showed no system or local toxicity and no cross reactivity with rat or human GST [62]. Bushara et al. [63] and Morrison et al. [64] suggested that GST of *Schisotosoma spp*. and *Fasciola spp*. improved host immunity against these parasites. In this respect, GSTs were found to be up-regulated in response to rickettsial infection of *D. variabilis* ovaries [65]. They found that there is 0.25 fold increase in the mRNA expression of the GST gene.

Previous studies correlated the role of GSTs in insect innate immunity with increased GST expression in response to infection-induced oxidative stress [66-70]. Increasing numbers of insect GSTs are being characterized due to their roles in insecticide detoxification. Dreher-Lesnick et al. [71] had cloned two GST variants and sequenced from the American dog tick; *D. variabilis* tick. Their structural analysis revealed that one of them belongs to the theta class (Figure 8) but no data is available about their biological activities. The secondary structure prediction using the DSSP prediction is shown in figure 9. Comparison of these two GST molecules with those of other species indicates that GST1 is related to the mammalian class theta and insect class delta GSTs, while GST2 does not seem to fall in the same family. Northern blotting analyses revealed differential expression patterns, where GST1 and GST2 transcripts are found in the tick gut, with GST2 transcripts also present in the ovaries. Both *D. variabilis* GST transcripts are up-regulated upon tick feeding. The up-regulation of GST in this state is probably due to the stresses incurred during blood feeding. The authors could not rule out the possibility that up-regulation of GST in ticks may serve other purposes including cell protection from oxidative stress caused by infection with the intracellular bacterium Rickettsia.

D. variabilis serves as a host for an obligate intracellular cattle pathogen belongs to the genus Anaplasma; *Anaplasma marginale*. The developmental cycle of this pathogen begins in the gut cells of the host and the transmission to cattle occurs from the salivary glands during a second tick feeding. The *A. marginale* parasite has two stages occur within parasitophorous vacuole in the tick cell cytoplasm; the reticulated form (RF) which will transform to the infective dense form (DF). Kocan et al. [72] studied the characterization of the silencing effects of 4 different *D. variabilis* genes (separately) including the GST (DQ224235) on the development and infection levels of the *A. marginale*. They used the RNAi technology to silence these genes in male ticks and they showed that the *A. marginale* infection was inhibited both in tick guts after acquisition feeding and salivary glands after transmission feeding. *D. variabilis* ticks injected with GST dsRNA showed significant lower density of dense forms in guts after acquisition feeding. In general, the results of GST silencing demonstrate that GST is re-

quired for pathogen infection of *D. variabilis* guts and salivary glands and IDE8 cells. It would suggest that GST may reduce the harmful effects of the metabolites, produced by the cellular oxidative stress, which may affect the development of the pathogen. Surprisingly, Kocan et al. [72] noticed that *A. marginale* infection was increased in the fat body cells in the GST silenced ticks.

Vaccination studies using tick proteins like GST from *Haemaphysalis longicornis* (Hl-GST) demonstrated the immunogenicity and antigenicity of this protein in bovines. Ultimately, immunization with GST protein triggered a partial immune response against *R. microplus* infestation in cattle, manifested mainly as a reduction of 7.9% in egg fertility, 53% in the number of fully engorged ticks and 57% overall efficacy ratio [73]. These data suggest that GST proteins have potential to be used as antigens in an anti-tick vaccine.

In conclusion, the phylogenetic analysis of the different cloned GST genes from different tick species indicates that numerous GSTs are present in the tick genome, which may or may not belong to different classes. These sequences are distributed in different tick organs including ovaries, gut and salivary glands. However, it is clear that protective immunity against tick infestation can be achieved; demonstrating that vaccination is a realistic unconventional approach for tick control and GST would be a candidate.

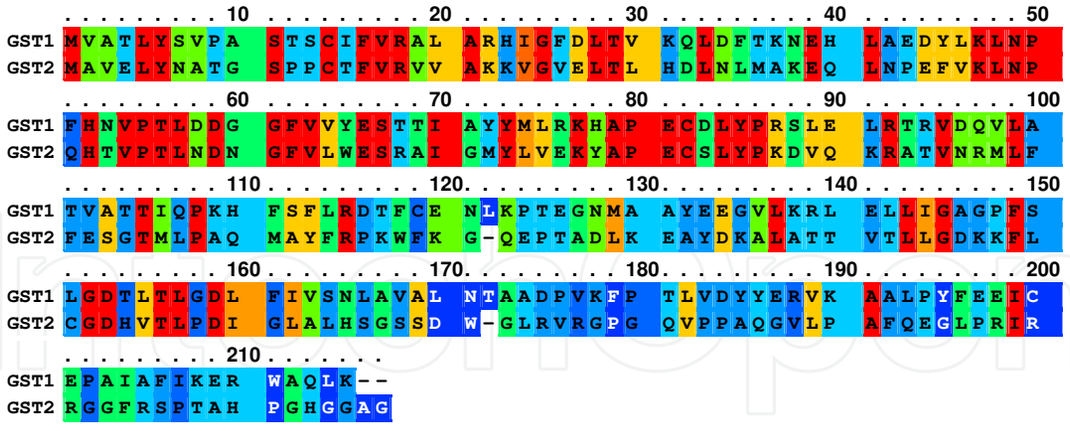

Figure 8. *Dermacentor variabilis* Glutathione S-transferase GST1 and GST2 (DQ224235 and AY241958, respectively) amino acid conservation. The conservation scoring is performed by PRALINE software (http://zeus.few.vu.nl). The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. The color assignments are as in figure 1.

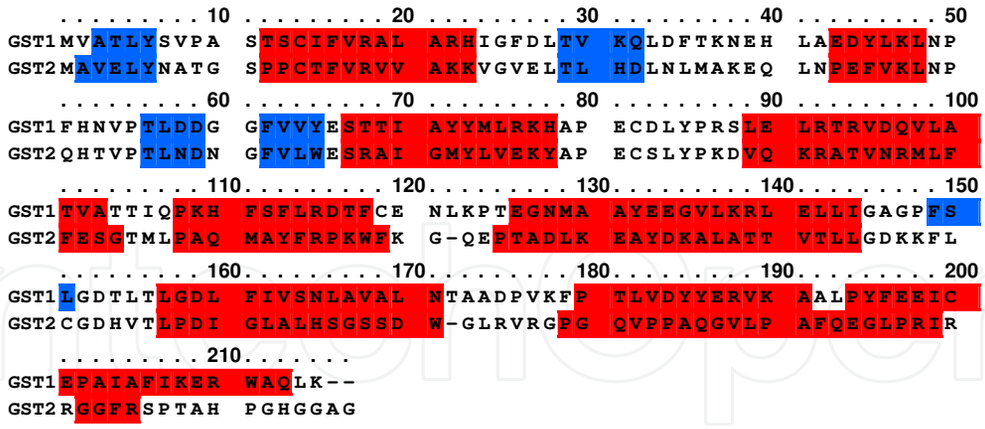

Figure 9. *Dermacentor variabilis* theta Glutathione S-transferase GST1 and GST2 (DQ224235 and AY241958, respectively) 3-state (H, E C) secondary structure. The Helix (H) structure is in red and the Strand (E) is in blue. The sequence in the alignment has no color assigned for the coil (C) because there is no DSSP information available, or that no prediction was possible for that sequence. The conservation scoring was performed by PRALINE software (http://zeus.few.vu.nl).

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