Tick lysozymes

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Ticks are blood feeding arthropod parasites transmitting a wide variety of pathogens to their vertebrate hosts. The vector capability of ticks is tightly linked to their immune system. Ticks can defend themselves against microbial infection with a variety of antimicrobial peptides comprising lysozymes, defensins, and unique molecules not found in other arthropods. Lysozymes are hydrolytic enzymes, characterized by their ability to cleave the β-(1, 4)-glycosidic bond between N-acetylmuramic acid and N-acetyglucosamine in peptidoglycan, the major bacterial cell wall polymer. A full-length cDNA encoding lysozymes was obtained from cDNA libraries of salivary glands of the ixodid hard tick *Haemaphysalis longicornis* and designated as HlLysozyme. The HlLysozyme sequence represents an open reading frame for a putative signal peptide and the mature protein composed of 121 amino acids. The calculated molecular weight of the protein is 13.7 kDa, and the theoretical isoelectric point is 9.85. HlLysozyme shares a 41-79% amino acid sequence identity with the lysozymes of other organisms. Elevated gene expression of HlLysozyme was observed when female ticks were challenged with bacteria, suggesting the possible roles of lysozymes in the innate immunity of ticks against microorganisms. This study would provide a starting point for an immunological examination of tick-pathogen interactions.

Keywords: lysozyme; innate immunity; tick; *Haemaphysalis longicornis*; arthropod

1. Introduction

Ticks (Acari: Ixodida) are parasitic mites that suck from their vertebrate host. Systematically, they belong to the Phylum Arthropoda, Class Arachnida, Subclass Acari, Order Parasitoformes, and Suborder Ixodida [1]. Until now, more than 900 tick species have been described and divided into two major families, Argasidae (argasids, soft ticks) and Ixodidae (ixodids, hard ticks) [2]. Soft ticks, Argas or *Ornithodoros*, are multi-host parasites with several nymphal stages (2-8), all of which feed rapidly on their hosts (from minutes to hours). The adult ticks can feed repeatedly, and the females, which mate away from the host, deposit a limited number of eggs (a few hundred) after each feeding. The life span of argasids is long (up to several years), and they can live without food for years between individual blood meals as nymphs or adults. Hard ticks, *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes*, and most *Rhipicephalus*, are three-host ticks; in each stage, they can feed on a different host. The hard ticks feed slowly for several days, depending on the stage and species. The adult females typically mate on the host, and, after full engorgement, they drop off. The blood meal is digested within several weeks, and the female oviposits thousands of eggs and dies.

   Invertebrate arthropods lack an adaptive immune system, and, for their defense against potential pathogens, they rely on a network of cellular immune reactions and humoral factors involved in pathogen recognition and elimination. The molecular details of this innate immune system are elucidated in *Drosophila* fruit fly [3] and provide a base for further investigation of immune responses and parasite transmission in important insect disease vectors, such as mosquitoes [4] and tsetse flies [5].

   In contrast to model and/or blood-sucking insects, the innate immunity of ticks of ticks has not been thoroughly understood. Early studies emphasized the role of hemocytes and cellular immunity in innate defense of ticks against infection [6]. However, this has changed in recent years, since numerous studies have identified the sequences of a large number of tick immune proteins and peptides. In ticks, the invasion of foreign organisms induces or upregulates expression of molecules that act in concert to regulate the infectants [7]. They are secreted into the hemolymph plasma and termed humoral factors. There are a variety of antimicrobial peptides, lectins, lysozymes, coagulation factors, proteases, and protease inhibitors in nature. Particularly, many aspects concerning the biological role of lysozymes, such as the impact of peptideglycan fragments released by the lytic action of this enzyme in bacteria-host interactions, are not completely understood. In this review, different types of lysozymes recently found in several species of ticks are discussed, particularly in terms of their biological role.

2. Different types of lysozymes

The bacteriolytic function of lysozymes is widely employed in the innate immunities of both animals and plants; therefore, this enzyme is probably one of the defense molecules investigated in greatest detail [8]. Lysozymes hydrolyze the 1, 4-glycosidic bound between N-acetylmuramic acid and N-acetylglucosamine in cell-wall peptide glycans of Gram-positive bacteria. However, it was recently demonstrated that the antibacterial activity of lysozymes operates

independently of its muramidase activity [9]. Animal lysozymes have been categorized into the chicken (c)-, the goose (g)-, and the invertebrate (i)-type, according to their possession of type-specific amino acid sequence features and their species of origin [10]. The expression of c-type multi-gene families of a number of animal species is known to be regulated in response to bacterial challenge on the innate immunity of humoral defense. In a recent study, eight c-type lysozymes have been characterized in *Anopheles gambiae* mosquitoe [11]. The transcript abundance of each c-type lysozyme was determined by RT-PCR. Lysozymes c-1, c-6, and c-7 are expressed constitutively in all developmental stages from egg to adult. Lysozymes c-2 and c-4 are also found in all stages but with relatively much higher levels in adults. Conversely, lysozyme c-3 and c-8 transcripts are the highest in larvae. Lysozyme c-1, c-6, and c-7 transcripts are present in nearly all the adult tissue samples, while lysozymes c-2 and Lys c-4 are more restricted in their expression. Lysozyme c-1 and c-2 transcripts are clearly immune-responsive and increase significantly 6-12 hours post-challenge with bacteria. The functional adaptive changes that may have evolved during the expansion of this gene family are briefly discussed in terms of the expression patterns and gene and protein structures.

Lysozymes are ubiquitous proteins that have been studied in many species of invertebrates and vertebrates and have been characterized and demonstrated to be immune-associated molecules [12], digestive enzymes [13], and multifunctional molecules [14]. Lysozymes (HlLysozyme) isolated from ixodid *Haemaphysalis longicornis* ticks showed the closest alignment to a group of immune-related lysozymes isolated from *Bombyx mori* [15] and other ixodid ticks, *Dermacentor andersoni* and *D. variabilis* [16]. A comparison of the amino acid sequence reveals that arthropod lysozymes, except *Triatoma infestans*-2 [17], share the common characteristic of having 8 cystein residues that form 4 disulfide bridges as well as the conserved catalytic sites of the glutamic acid and asparatic acid residues. These conserved common structural features in insect and tick lysozymes suggest that they play a major role in arthropod survival. Lysozymes in the arthropod midgut play a role in carbohydrate digestion while simultaneously regulating the proliferation of Gram-negative bacteria in this region.

Through comparative molecular BLAST analyses, we established a classification for the highly conserved expressed products identified as c-type lysozymes from the ixodid *H. longicornis* and *D. variabilis* ticks and the argasid *Ornithodoros moubata* tick (Fig. 1). NCBI database searches and multiple-sequence alignment analyses demonstrated that the translated lysozyme sequences of *H. longicornis*, *D. variabilis*, and *O. moubata* are more similar to various c-type lysozymes than to those of the α-lactoalbumins and the other i- and g-type lysozymes [18]. However, some differences were observed between the translated sequences of the HlLysozyme and other tick lysozyme sequences reported to date. The most notable difference was the presence of the conserved aspartic acid residues required for Ca^{2+} binding in *Ornithodoros* lysozyme-translated sequence. However, these residues were not present in the hard tick *Haemaphysali*s and *Dermacentor* sequences.
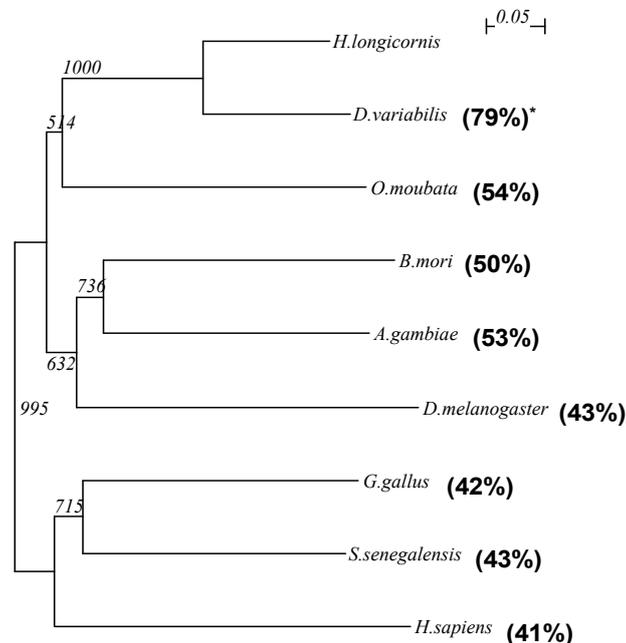
Fig. 1 Phylogenetic analysis of the amino acid sequences of lysozymes from *Haemaphysalis longicornis* (GenBank accession number AB558270), *Dermacentor variabilis* (AAO23571), *Ornithodoros moubata* (AAL17868), *Anopheles gambiae* (AAC47326), *Triatoma infestans* (ABI94387), *Drosophila melanogaster* (NP_476828), *Bombyx mori* (NP_001037448), *Streptopelia senegalensis* (ABC49680), *Gallus gallus* (NP_990612), and *Homo sapiens* (AAP97222). *Identity

3. Biological function of lysozymes

Lysozymes of different types are widespread among animals. Most organisms have the genetic capacity to produce multiple lysozymes of different types, and it is presumed that these lysozymes may have complementary or even different functions. A widely recognized function of lysozymes is their contribution to antibacterial defense. The distinct role of lysozymes is their participation as a digestive enzyme in some animals. In general, indications for the function of lysozymes can be derived from the special expression and regulation patterns of genes and enzyme activity. Lysozymes contributing to antibacterial defense are generally expressed and upregulated in tissues and body fluids after being exposed to microbial environments, while those potentially involved in a digestive function are highly expressed in the stomach or gut. Typically, defensive lysozymes have a neutral pH optimum and pI values of 8.0 or higher, while digestive lysozymes tend to have a low pH optimum and a higher resistance to proteases [19].

The lysozyme gene in salivary glands and fat bodies of adult *H. longicornis* ticks was up-regulated at the transcriptional level by blood feeding [20]. Alekseev et al. [21] examined the lysozyme lytic activity for hemolymph, gut contents, and homogenates from the whole body of the soft ticks *Ornithodoros* (*Alveonasus*) lahorensis and *Ornithodoros papillipes* against the bacterium *Micrococcus lysodeikticus*. They found that the lysozyme activity increased (a typical increase of 8–10-fold over the control with sterile blood) only in ticks infected with *M. lysodeikticus*, whereas the level of lysozymes did not change in sterile fed animals. Certainly, further experimental work based on well-defined sterile vs. infectious feeding conditions and comparison with the regulation of other tick gut-specific genes of digestive proteinases, such as longepsin [22], has to be conducted to obtain a basic understanding of this complex problem.

Recombinant HlLysozyme expressed in *Escherichia coli* displayed lysozyme-like activity in lytic-zone assays using the bacteria *M. lysodeikticus*. Heat stability is a commonly found feature in the majority of insect lysozymes [23]. Alekseev et al. [21] reported that heating at 100°C for 3 minutes reduced the activity of *Ornithodoros* lysozymes by 10% and 45% when performed in an acidic (pH 5–6) and an alkaline (pH 8–9) medium, respectively. We have also investigated the heat stability of recombinant lysozymes [20]. After heat treatment for 30 minutes, recombinant HllLysozyme was stable from 10 to 60°C, whereas its activity was destroyed at a temperature of about 90°C. The optimum pH of recombinant HlLysozyme was found to be 3 and 6, whereas the optimum pH of chicken egg lysozyme was reported to be pH 4 and 7 [8]. Our results indicate that recombinant HlLysozyme is hardly active at pH above 8; thus, an increase in pH would theoretically suppress the lysozyme activity. To explain the optical pH of HlLysozyme, we need to know the exact distribution of pH and the localization of lysozyme activity as they occur in the tick *in vivo*.

4. Lysozymes in the defense against bacteria

As opposed to the highly specific adaptive immune system of vertebrates, the immune defense in invertebrate arthropods cannot rely on a memory effect to combat invaders. However, arthropods have a very competent inducible immune defense system, which is regarded as a useful model for studying innate immune reactions. On bacterial challenge, insects synthesize a number of bactericidal proteins and peptides in the hemolymph. Inducible lysozyme activity in the hemolymph has been demonstrated for many insect orders, including *Diptera* [24], *Lepidoptera* [25], *Orthoptera* [26], and *Coleoptera* [27]. In addition to their activity against Gram-positive bacteria, some insect c-type lysozymes are antibacterial against Gram-negative bacteria. Moreover, other antimicrobial proteins and peptides, such as cecropins, defensins, and attacin, which are also induced by bacterial infection, can broaden the antibacterial spectrum of lysozymes through synergistic effects. These antimicrobial proteins and peptides are known to adversely affect bacterial cell membranes [28]. Therefore, together with these molecules, at least the c-type lysozyme is likely to play an important role in the insect's defense against bacteria.

In holometabolous arthropods, basically all larval tissues are degraded and replaced by new structures of the adult animal during metamorphosis [29]. Since this is a vulnerable stage in the insect's development, several immune functions, including lysozyme production, are ubiquitously upregulated to prevent the systemic spread of bacteria. Accordingly, high lysozyme levels are detected in the midgut in full-grown larvae of the cotton bollworm, *Helicoverpa armigera* [30]. Lysozymes are stored in granules in midgut cells and released into the midgut lumen just before metamorphosis is initiated [31]. Although the process of metamorphosis is less radical for hemimetabolous arthropods, upregulation of lysozyme expression in the bug *Triatoma infestans* and the soft tick *O. moubata* was also found immediately after moulting [32, 33].

Clear evidence for the role for lysozymes in tick innate immunity was demonstrated in hard ticks, *D. variabilis*, in which the transcription levels of a 121 amino acid-long (mature protein) C-type lysozyme were elevated in hemolymph following injection of *E. coli*, reaching a 17-fold increase within 72 hours post-challenge [16]. A comparison of tissue transcription levels showed that *D. variabilis* lysozyme was most abundant in the hemolymph but expressed in very low levels in the midgut or other organs. Blood feeding did not result in increased lysozyme expression in the *D. variabilis* tick, consistently with the findings of the lysozyme transcript but not of the active protein in the midgut of this species [16, 34]. In the hemolymph, lysozymes of *D. variabilis* may act synergistically with defensins in disrupting the bacterial cell wall, thereby greatly accelerating the killing action and perhaps broadening the range of bacteria inhibited by the

activity of the antimicrobial peptides [35]. Lysozyme antimicrobial activity has been reported from the hemocytes of hard ticks *Ixodes ricinus*, from cell lines derived from *Ixodes scapularis* and *D. andesoni*, and from homogenates of *Ixodes persulcatus* [36, 37]. Most reports showed that lysozymes acted on the cell wall of Gram-positive bacteria. A noteworthy finding was that lysozyme expression was not upregulated in *I. scapularis* or *D. andersoni* cell cultures in response to challenge with *Rickettsia peacockii*, but expression was upregulated after stimulation of the cells with *E. coli* and *M. luteus* [37]. This may indicate that endosymbionts in ticks, such as *R. peacockii*, could avoid recognition by the host innate immune system.

The immune-inducible characteristics of HILysozyme were confirmed by injection of *Staphylococcus aureus* into the hemocoel [20]. The HILysozyme gene was upregulated transiently in the fat bodies, midguts, ovaries, and hemolymph at 8 hours and 12 hours after *S. aureus* injection into the hemocoel, and the gene transcript levels decreased at 24 hours post-injection, and, by 24 hours, the transcript levels dropped back to those of 0 hour. On the other hand, the HILysozyme transcript levels in the fat bodies, midguts, ovaries, and hemolymph increased to a maximum at 24 hours after *E. coli* injection into the hemocoel. These results suggest that *E. coli* might survive in fat bodies, midguts, ovaries, and the hemocoel and is not more adversely affected than *S. aureus* by the normal physiological levels of HILysozyme or other molecules expressed in these tissues. However, the reason that *S. aureus* or *E. coli* injection showed no difference in the HILysozyme transcript levels in the salivary glands from 0 to 24 hours is not clear. They may not stimulate an increase HILysozyme gene expression level even though they can survive in the salivary glands. Further studies are required to resolve whether hemocoel components, such as HILysozyme, can reduce *E. coli* viability at a later time post-injection.

5. Lysozymes as a digestive enzyme

In the ruminant stomach, the function of lysozymes was determined to be to digest symbiotic bacteria that provide nitrogen and phosphorus molecules for milk production [38]. A similar adaptation of lysozymes to a digestive function has been reported from the midgut of *D. melanogaster* [39] and *Musca domestica* [40]. In general, these digestive lysozymes are the c-type [41] and are different from non-digestive lysozymes with regard to several features, namely, a lower number of basic amino acids (low pI values), resistance to proteases present in the alimentary tract (such as pepsin in the vertebrate stomach or cathepsin D in the insect midgut), and optimum pH in the acidic range.

Kang et al. [42] noticed a striking similarity between *A. gambiae* lysozyme c-1 and *Drosophila* Lys P. Reverse transcriptase PCR analysis of the *A. gambiae* lysozyme gene revealed much higher expression levels in sugar-fed adults compared to female adults that imbibed a blood meal. In contrast, Li et al. [11] described this lysozyme c-1, together with the lysozyme c-2, among the now known seven c-type lysozymes of *A. gambiae*, as the best candidates for an immune function. Since their research revealed highest transcript levels for lysozymes c-3 and c-8 in larvae that ingest bacteria as part of their diet, Li et al. [11] proposed a digestive function for these lysozymes. Adaptations resulting in an acidic pI were not present (pI value of 8.46 for both lysozymes c-3 and c-8); however, they were not expected either, since larval mosquito guts are weakly to strongly alkaline [43]. Besides the eight c-type lysozyme genes that exist in *A. gambiae* [11], Paskewitz et al. [44] characterized two i-type lysozyme genes in this mosquito species. An increased transcript level of these lysozymes was found in the midguts after a blood meal [45]; thus, a digestive role for these i-type lysozymes is possible.

In the lysozyme from the midgut of the soft tick *O. moubata*, the features of an antibacterial activity seem to be combined with a digestive function. Grunclova et al. [13] reported an upregulation of the lysozyme at the transcriptional level by a blood meal uptake, and Kopácek et al. [32] showed an 8-10-fold increase in lysozyme activity in whole body homogenates 24 hours after feeding on blood infected with different bacterial species than after sterile blood meal. Although phylogenetic analysis showed this lysozyme is more closely related to the family of the *Drosophila* digestive lysozymes than the other c-type lysozymes and the pH optimum of the *O. moubata* lysozyme is rather in the acidic range (pH 6.0), some characteristics, including a more acidic pI attributed to digestive lysozymes, are not present [13, 32].

Recent findings in *D. variabilis* demonstrated a differential regulation of defensin-like peptides in response to the oral vs. hemocoel route of bacterial challenge [46]. Therefore, there may be a differential regulation of c-type lysozymes as well. However, HILysozyme is independent of blood meal uptake and digestion. It was not shown to have a significant mRNA abundance level of these c-type lysozymes in the midgut. These findings might be very important because they demonstrate a crucial difference in the transcriptional regulation of HILysozyme from that of *Ornithodoros* lysozyme, which was reported to reach maximum levels of transcript abundance at 16 hours post-blood meal uptake in the gut tissues [13]. Based on these findings, the amino acid sequence DRCSLA within the *Ornithodoros* lysozyme is conserved in the digestive lysozymes of several Dipteran insect species, leading to the conclusion that the *O. moubata* lysozyme may act as a digestive adaptive c-type molecule [13, 32].

6. Conclusion and perspectives

Ticks are exposed to a variety of microorganisms in their life cycles, and, to avoid the pestiferous growth of microorganisms, such as bacteria, viruses, and parasites, they possess and develop efficient protective defense mechanisms. Until now, several antibacterial molecules have been shown to play a defensive role in ticks. The expression of defensin-like antibacterial molecules has been found in hard and soft ticks [47, 48. 49]. However, the precise nature of the anti-microorganism mechanisms in ticks remains unclear. The molecular structures of the innate immune systems of numerous arthropod vectors [50] have potential for the development of effective chemotherapeutic measures against arthropod-borne diseases.

Eighty years after its discovery and 40 years after the resolution of its three-dimensional structure, lysozymes continue to attract the interest of scientists in disciplines such as structural enzymology, evolutionary biology, immunology, and microbiology. Although the role of lysozymes in antibacterial defense is now well established in some experimental models (using lysozyme knockout animals), a more detailed insight in the spatio-temporal expression of lysozymes and the precise contribution of lysozymes to antibacterial defense in different tissues is lacking. Furthermore, the observation that most animals have two different types of lysozymes, at least at the gene level, raises the question of whether these would have a complementary role in antibacterial defense. An interesting development is the increasing number of indications that lysozymes may have acquired other functions besides their role in innate immunity. For example, there is good evidence that, following one of more events of gene duplication, the enzyme has been recruited for a digestive function in several animals, both invertebrate and vertebrate. Furthermore, lysozyme-specific peptidoglycan fragments released by the action of lysozymes may act as agonists of inflammatory pathways by binding to molecular pattern receptors of the innate immune system. Finally, the important role of lysozymes is also reflected by the discovery of an increasing number of specific lysozyme-inhibitor proteins in bacteria. The role of these inhibitors in bacteria-host interactions needs to be further established, but, in view of their specific inhibition of the c-, g-, or i-type lysozyme, these inhibitors will be useful tools to help in distinguishing the role of the different lysozymes produced by most animals.

Here, we confirmed through molecular characterizations that HlLysozyme underwent differential regulation specific to bacterial challenge. Our specific interests are the functional elucidation of immunity-related molecules of ticks and tick-borne pathogen transmissions. This study would provide a starting point for an immunological examination of tick–pathogen interactions.

References

[1] Nava S, Guglielmone AA, Mangold AJ. An overview of systematics and evolution of ticks. *Front Biosci.* 2009;14:2857-2877.
[2] Sonenshine DE. *Biology of ticks.* Vol 1. New York, Oxford University Press; 1991.
[3] Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat. Rev. Immunol.* 2007;7: 862-874.
[4] Osta MA, Christophides GK, Vlachou D, Kafatos FC. Innate immunity in the malaria vector *Anopheles gambiae*: comparative and functional genomics. *J. Exp. Biol.* 2004;207:2551-2563.
[5] Lehane MJ, Aksoy S, Levashina E. Immune responses and parasite transmission in blood-feeding insects. *Trends Parasitol.* 2004;20:433-439.
[6] Kadota K, Walter S, Claveria FG, Igarashi I, Taylor D, Fujisaki K. Morphological and populational characteristics of hemocytes of *Ornithodoros moubata* nymphs during the ecdysial phase. *J. Med. Entomol.* 2003;40:770-776.
[7] Tsuji N, Miyoshi T, Battsetseg B, Matsuo T, Xuan X, Fujisaki K. A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. *PLoS Pathog.* 2008;4:e1000062.
[8] Jolles P, Jolles J. What's new in lysozyme research? Always a model system, today as yesterday. *Mol. Cell Biochem.* 1984;63:165-189.
[9] Ibrahim HR, Thomas U, Pellegrini A. α helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J. Biol. Chem.* 2001;276:43767-43774.
[10] Nilsen IW, Myrnes B. The gene of chlamysin, a marine invertebrate-type lysozyme, is organized similar to vertebrate but different from invertebrate chicken-type lysozyme genes. *Gene* 2001;269:27-32.
[11] Li B, Calvo E, Marinotti O, James AA, Paskewitz SM. Characterization of the c-type lysozyme gene family in *Anopheles gambiae*. *Gene* 2005;7:131-139.
[12] Roxström-Lindquist K, Terenius O, Faye I. Parasite-specific immune response in adult *Drosophila melanogaster*: a genomic study. *EMBO Reports* 2004;5:207-212.
[13] Grunclovà L, Fouquier H, Hypsa V, Kopácek P. Lysozyme from the gut of the soft tick *Ornithodoros moubata*: the sequence, phylogeny and post-feeding regulation. *Dev. Comp. Immunol.* 2003;27: 651-660.
[14] Ursic Bedoya RJ, Mitzey AM, Obraztsova M, Lowenberger C. Molecular cloning and transcriptional activation of lysozyme-encoding cDNAs in the mosquito *Aedes aegypti*. *Insect Mol. Biol.* 2005;14:89-94.

[15] Lee WJ, Brey PT. Isolation and characterization of the lysozyme-encoding gene from the silkworm *Bombyx mori. Gene* 1995;161:199-203.

[16] Simser JA, Macaluso KR, Mulenga A, Azad AF. Immune-responsive lysozymes from hemocytes of the American dog tick, *Dermacentor variabilis* and an embryonic cell line of the Rocky Mountain wood tick, *D. andersoni*. I*nsect Biochem. Mol. Biol.* 2004;34:1235-1246.

[17] Balczun C, Knorr E, Topal H, Meiser CK, Kollien AH, Schaub GA. Sequence characterization of an unusual lysozyme gene expressed in the intestinal tract of the reduviid bug *Triatoma infestans* (Insecta). *Parasitol. Res.* 2008;102:229-232.

[18] Qasba PK, Kumar S. Molecular divergence of lysozymes and α-lactoalbumin. *Crit. Rev. Biochem. Mol. Biol.* 1997;32:255-306.

[19] Callewaert L, Michiels CW. Lysozymes in the animal kingdom. *J. Biosci.* 2010;35:127-160.

[20] Tanaka T, Kawano S, Nakao S, Umemiya-Shirafuji R, Rahman MM, Boldbaatar D, Battur B, Liao M, Fujisaki K. The identification and characterization of lysozyme from the hard tick *Haemaphysalis longicornis*. Ticks Tick Borne Dis. 2010;1:178-185.

[21] Alekseev A, Burenkova, LA, Podboronov VM, Chunikhin SP. Bacteriocidal qualities of ixodid tick (Acarina: Ixodidae) salivary cement plugs and their changes under the influence of a viral tick-borne pathogen. *J. Med. Entomol.* 1995;32:578-582.

[22] Boldbaatar D, Sikalizyo Sikasunge C, Battsetseg B, Xuan X, Fujisaki K. Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis. Insect Biochem. Mol. Biol.* 2006;36:25-36.

[23] Sotelo-Mundo RR, López-Zavala AA, Garcia-Orozco KD, Arvizu-Flores AA, Velázquez-Contreras EF, Valenzuela-Soto EM, Rojo-Dominguez A, Kanost MR. The lysozyme from insect (*Manduca sexta*) is a cold-adapted enzyme. *Protein Pept. Lett.* 2007;14:774-778.

[24] Hernandez VP, Higgins L, Fallon AM. Characterization and cDNA cloning of an immune-induced lysozyme from cultured A*edes albopictus* mosquito cells. *Dev. Comp. Immunol.* 2003;27:11-20.

[25] Gandhe AS, Arunkumar KP, John SH, Nagaraju J. Analysis of bacteria-challenged wild silkmoth, *Antheraea mylitta* (lepidoptera) transcriptome reveals potential immune genes. *BMC Genomics.* 2006;7:184.

[26] Adamo SA. Estimating disease resistance in insects: phenoloxidase andlysozyme-like activity and disease resistance in the cricket Gryllus texensis. *J. Insect Physiol.* 2004;50:209-216.

[27] Ourth DD, Jones BR. Lysozyme in eggs of the cotton boll weevil, *Anthonomus grandis Boheman* (*Coleoptera*: Curculionidae). *Experientia* 1980;36:196.

[28] Bulet P, Hetru C, Dimarcq JL, Hoffmann D. Antimicrobial peptides in insects; structure and function. *Dev. Comp. Immunol.* 1999;23:329-344.

[29] Hultmark D, Engström A, Andersson K, Steiner H, Bennich H, Boman HG. Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia. EMBO J.* 1983;2:571-576.

[30] Zhang Y, Huang J, Zhou B, Zhang C, Liu W, Miao X, Huang Y. Up-regulation oflysozyme gene expression during metamorphosis and immune challenge of the cotton bollworm, *Helicoverpa armigera. Arch. Insect Biochem. Physiol.* 2009;70:18-29.

[31] Russell VW, Dunn PE. Lysozyme in the midgut of *Manduca sexta* during metamorphosis. *Arch. Insect Biochem. Physiol.* 1991;17:67-80.

[32] Kopácek P, Vogt R, Jindrák L, Weise C, Safarík I. Purification and characterization of the lysozyme from the soft tick, *Orinithodoros moubata. Insect Biochem. Mol. Biol.* 1999;29:989-997.

[33] Kollien AH, Fechner S, Waniek PJ, Schaub GA. Isolation and characterization of a cDNA encoding for a lysozyme from the gut of the reduviid bug *Triatoma infestans. Arch. Insect Biochem. Physiol.* 2003;53:134-145.

[34] Sonenshine DE, Hynes WL, Ceraul SM, Mitchell R, Benzine T. Host blood proteins and peptides in the midgut of the tick *Dermacentor variabilis* contribute to bacterial control. *Exp. Appl. Acarol.* 2005;36:207-223.

[35] Johns R, Sonenshine DE, Hynes WL. Identification of a defensin from the hemolymph of the American dog tick, *Dermacentor variabilis. Insect Biochem. Mol. Biol.* 2001;31:857-865.

[36] Kuhn KH, Haug T. Ultrastructure, cytochemical, and immunocytochemical characterization of hemocytes of the hard tick *Ixodes ricinu*s (Acari; Chelicerata). *Cell Tissue Res.* 1994;277:493-504.

[37] Mattila JT, Munderloh UG, Kurtti TJ. *Rickettsia peacockii*, an endosymbiont of *Dermacentor andersoni*, does not elicit or inhibit humoral immune responses from immunocompetent *D. andersoni* or *Ixodes scapularis* cell lines. *Dev. Comp. Immunol.* 2007;31:1095-1106.

[38] Dobson DE, Prager EM, Wilson AC. Stomach lysozymes of ruminants. I. Distribution and catalytic properties. *J. Biol. Chem.* 1984;259:11607-11616.

[39] Regel R, Matioli SR, Terra WR. Molecular adaptation of *Drosophila melanogaster* lysozyme to digestive function. *Insect Biochem. Mol. Biol.* 1998;28:309-319.

[40] Lemos FJA, Ribeiro AF, Terra WA. A bacteria-digesting midgut-lysozyme from *Musca domestica* (Diptera) larvae purification, properties and secretory mechanism. *Insect Biochem. Mol. Biol.* 1993;23:533-541.

[41] Bachali S, Jager M, Hassanin A, Schoentgen F, Jollès P, Fiala-Medioni A, Deutsch JS. Phylogenetic analysis of invertebrate lysozymes and the evolution of lysozyme function. *J. Mol. Evol.* 2002;54:652-664.

[42] Kang D, Romans P, Lee JY. Analysis of a lysozyme gene from the malaria vector mosquito, *Anopheles gambiae. Gene* 1996;174:239-244.

[43] Zhuang Z, Linser PJ, Harvey WR. Antibody to H(+) V-ATPase subunit E colocalizes with portasomes in alkaline larval midgut of a freshwater mosquito (*Aedes aegypti*). *J. Exp. Biol.* 1999;202:2449-2460.

[44] Paskewitz SM, Li B, Kajla MK. Cloning and molecular characterization of two invertebrate-type lysozymes from *Anopheles gambiae. Insect Mol. Biol.* 2008;17:217-225.

[45] Demaio J, Pumpuni CB, Kent M, Beier JC. The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. *Am. J. Trop. Med. Hyg.* 1996;54:219-223.

[46] Sonenshine DE, Ceraul SM, Hynes WE, Macaluso KR, Azad AF. Expression of defensin-like peptides in tick hemolymph and midgut in response to challenge with *Borrelia burgdorferi*, *E. coli* and *Bacillus subtilis. Exp. Appl. Acarol.* 2002;28:127-134.

[47] Tsuji N, Fujisaki K. Longicin plays a crucial role in inhibiting the transmission of *Babesia* parasites in the vector tick *Haemaphysalis longicornis*. *Future Microbiol.* 2007;2:575-578.

[48] Zhou J, Liao M, Ueda M, Gong H, Xuan X, Fujisaki K. Sequence characterization and expression patterns of two defensin-like antimicrobial peptides from the tick *Haemaphysalis longicornis*. *Peptides* 2007;28:1304-1310.

[49] Chrudimská T, Chrudimský T, Golovchenko M, Rudenko N, Grubhoffer L. New defensins from hard and soft ticks: Similarities, differences, and phylogenetic analyses. *Vet. Parasitol.* 2010;167: 298-303.

[50] Lehrer RI, Ganz T. Antimicrobial peptides in mammalian and insect host defense. *Curr. Opin. Immunol.* 1999;11:23-27.