

Expression of cysteine proteinases and cystatins in parasites and use of cysteine proteinase inhibitors in parasitic diseases. Part II: Arthropods

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ABSTRACT

Most of the cysteine proteases (CPs) expressed by ectoparasites belong to the papain-like superfamily (clan CA, family C1) and are associated with the physiological events during different developmental life stages. Cathepsins, legumains/asparaginyl endopeptidases and caspases (clan CA, family C1, C13 and C14, respectively) play a major role in mosquito biology. There is accumulating evidence that CPs expressed by arthropods may play a role in clinical presentations of some transmitted parasitic disease, e.g. babesiosis. During blood meals, salivary glands secrete bioactive substances, including CPs, to exert several pharmacological properties that assist ectoparasites to evade host defense responses and to induce inflammatory reactions. Furthermore, they play an important role in extracellular and intracellular proteins degradation as well as their processing. In this regard, cystatins (CYSs) were found to inhibit insect CPs suggesting its use as targets for control of mites and ticks. The present review aims to identify different CPs expressed in ectoparasites, the main vectors of several endemic diseases such as malaria, babesiosis, Chagas' and Lyme disease. Meanwhile, it highlights different CYSs that could be used as biological insecticides to control diseases transmitted by mites and ticks.

Keywords: arthropods, cathepsin, cystatins, vaccine, vector control.

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Abbreviations

AEP: Asparaginyl endopeptidase; **BFA:** Blood feeding arthropod; **Cath:** Cathepsin; **CP:** Cysteine proteinase; **CPI:** Cysteine proteinase inhibitor; **CYS:** Cystatin; **RNAi:** RNA interference method for gene knock out.

Mosquitoes

Ectoparasites, especially blood feeding arthropods (BFAs), possess salivary glands secreting bioactive substances, among which are cysteine proteinases (CPs) that allow evasion of host response and the production of inflammatory reactions⁽¹⁾. In addition, the secreted CPs assist in hemoglobin digestion during blood meals essential for egg production. It was shown that E64, a general cysteine proteinase inhibitor (CPI) was used to reduce fecundity of *C. pipiens* females and results revealed the presence of an enzyme band, most probably that of a CP, in untreated mosquitoes⁽²⁾.

Cathepsin-B-like (Cath-B-like) was characterized from yellow fever and malaria transmitting mosquitoes, and named as vitellogenic cath B (VCB). It degrades vitellogenin, the major yolk protein precursor⁽³⁾. In 2001, examination of CPs stored in the ovaries of *Culex pipiens* showed that Cath B- and L-like have a major role in development and maturation of *Culex* ovaries. It was shown that both Caths facilitate degeneration of developing follicles (follicular atresia) or oocysts resorption (oosorption)⁽⁴⁾. The investigators suggested that follicular atresia and oosorption should occur to maintain the balance between number of eggs

production and physiological as well as environmental factors such as insufficient blood meals, absence of males, lower temperatures, and lack of suitable oviposition sites. Three years later, the same investigators confirmed the effects of ovarian CPs using electron microscopy. Results revealed gradual degradation of the internal structures including yolk granules in the oocyte with appearance of irregularly shaped epithelial cells and signs of apoptotic cells⁽⁵⁾.

In a review article published in 2006, the reviewers discussed self-defense mechanism(s) involved in *Plasmodium*-infected mosquitoes to repair invaded mid-gut epithelium and to balance mosquitoes' survival. Death of *Plasmodium* ookinetes in infected mosquitoes was associated with caspase-like activity. Although it is homologous to mammalian caspases, it was not detected in *Plasmodium* genome, suggesting the role of other mosquitoes CPs, e.g. Caths, in repair and survival⁽⁶⁾. Based on a recent study conducted in Brazil⁽⁷⁾ on *Culex quinquefasciatus*, the investigators identified the activity of Cath B in egg extracts 24 h after oviposition. It was shown that Cath B significantly decreased in fertilized eggs compared to extracts from vitellogenic ovaries or unfertilized eggs, suggesting its role in yolk

degradation during embryogenesis. This result was confirmed using a specific Cath B inhibitor, C-074, but not with other tested CPIs in extracts of unfertilized eggs. In addition, the transcriptional profile of two Cath B genes was determined, and their enzymatic activities were expressed simultaneously in the vitellogenic females. Based on the data obtained from VectorBase (vectorbase.org), the investigators found significant similarity between the transcriptional profiles of both Caths and vitellogenin, the primary source of amino acids and lipids for embryonic development. Accordingly, the investigators hypothesized that Cath B cooperate in vitellin degradation during embryogenesis.

A novel caspase (*AeDredd*) was identified and characterized from all developmental stages of *Aedes aegypti* with highest expression in early pupae. It proved to have an essential role of apoptosis in the innate immune response of vectors towards intracellular parasites such as viruses⁽⁸⁾.

Tsetse fly

In an attempt to investigate gut enzymes involved in digestion of dietary proteins in *Glossina morsitans*, the vector of African trypanosomiasis, Yan *et al.*,⁽⁹⁾ molecularly characterized the gene encoding Cath B (*GmCatB*). They also characterized two other genes encoding zinc-metalloprotease and zinc-carboxypeptidase. Gene sequence analysis of *GmCatB* revealed 254 amino acids with a molecular weight of 29 kDa. Later, recombinant *GmCatB* was found to produce the highest proteolytic activity at pH 4.0. It degraded bovine hemoglobin and serum albumin, and was inhibited by E-64, i.e. it belongs to papain-like CP family⁽¹⁰⁾.

Bloodsucking bug (*Triatoma* spp.)

Cath D as well as Cath B- and Cath L-like activities were identified in the gut extracts of *Triatoma* spp.^(11,12). In addition, Cath-like enzymes were also involved in the proteolytic activation of canatoxin that results in production of entomotoxic peptide(s) involved in the deleterious effects in Chagas disease⁽¹³⁾.

Mites

Several studies were conducted to evaluate the efficacy of CPIs and/or CYS A in treatment of allergic syndromes caused by house dust mites (HDMs), *Dermatophagoides* species. Sequence analyses of allergens (*Der p 1* and *Der f 1*) expressed by *D. pteronyssinus* and *D. farinae*, the most common HDMs causing allergic asthma and rhinitis as well as atopic dermatitis, showed similarity with that of CPs⁽¹⁴⁾. Group 1 allergen (*Der-1*) is a glycoprotein which exhibits CP activity as determined by comparative modeling with papain, actinidin and papaya peptidases (clan CA, family C1)⁽¹⁵⁾.

In atopic dermatitis, *Der-1* was found to have major roles in cleavage of human CD23 and CD25, enhancement of total and specific IgE production, inactivation of the endogenous human cystatins (CYSs), and stimulation of human keratinocytes to produce IL-8 involved in the pathogenesis of atopic dermatitis⁽¹⁶⁻¹⁸⁾. The results of a study conducted in UK revealed the significant potency of PTL11028 as a selective irreversible inhibitor of CP activity of *Der-1*⁽¹⁹⁾. On the other hand, CYS A is a skin-derived dominant inhibitor against proteolytic activity of major HDMs allergens. Cystatin A (CYS A) also was used to inhibit keratinocytes stimulation as well as IL-8 production⁽²⁰⁾. One year later, Ogawa *et al.*,⁽²¹⁾ reported that *Der-1*-stimulated keratinocytes caused upregulation of the release of granulocyte-macrophage colony-stimulating factor which contributes in the pathogenesis of atopic dermatitis. The investigators showed that CYS A had a major homeostatic role against atopic dermatitis.

In respiratory asthmatic attacks caused by HDMs, *Der-1* was found to target multiple proteins involved in the control of IgE synthesis and production⁽²²⁾, degradation of endogenous protease inhibitors⁽²³⁾, and surfactant proteins⁽²⁴⁾, increasing the contact between allergens and dendritic cells (antigen presenting cells) beneath the bronchial epithelial barrier. To discover new drugs for treatment of asthma caused by HDMs, a study was conducted in 2014. The investigators described the mechanisms by which HDMs *Der-1* allergens promote allergic sensitization in asthma; cleavage of the epithelial tight junctions by their proteolytic activities followed by release of chemokines and other mediators (e.g., IL-13, IL-33 and IL-25). In their study, the investigators designed a proposal to identify reversible *Der-1* inhibitors and claimed that using this generated program might lead to emergence of promising candidates for treatment of allergic asthma caused by HDMs⁽²⁵⁾. Recently, it was shown that *Der-1* activates human receptor MRGPRX1 enhancing IL-6 production which contributes in pathogenesis of allergic rhinitis and allergic asthma⁽²⁶⁾.

Degradome sequencing, a method used to provide a comprehensive analysis of RNA degradation and to identify micro-RNA cleavage sites, showed that *Der-1* accounts for 50% of protease transcription and 22% of the total protease transcripts⁽²⁷⁾. Two years later, Randall and his colleagues⁽²⁸⁾ conducted a comparative analysis of proteases from *D. farinae* and found that *Der-1* showed high simultaneous expression as well as high stability than other proteases in *D. farinae*. In addition, they detected two other allergens (*Der-3* and *Der-6*) and two metallo-proteases with similar stability and expression. Therefore, it was recommended to characterize the detected proteases and to determine if they can stimulate human IgE production.

Regarding scabies' *Sarcoptes* spp. mites, Australian investigators discovered a unique multi-copy family of

genes encoding CPs with their catalytic sites inactivated by mutation. As they are absent in non-burrowing, free living mites, the investigators postulated that these encoding genes are evolved to adapt *Sarcoptes* spp. to the parasitic lifestyle to invade host tissues, and they were named SMIPP-Cs (scabies mite inactivated cysteine protease paralogs). Five SMIPP-Cs homologs were identified (a-e). Sequence gene analysis, prior and after activation, revealed replacement of glutamine (homologs a and b), and leucine (homologs c-e), by active histidine⁽²⁹⁾. Two years earlier (2002), another group of British investigators detected close similarity between *S. scabiei* CPs and HDMs *Der-1* allergens. Interestingly, genomic analysis of two *Dermatophagoides* spp. and sheep scab mite, *Psoroptes ovis* showed closed relatives in a single encoding gene⁽³⁰⁾.

In order to elucidate SMIPP-Cs function(s) of *Sarcoptes* spp. in different hosts with relation to its host specificity, Fernando *et al.*,⁽³¹⁾ bioinformatically analyzed SMIPP-Cs from human, pig and dog mites. The investigators detected five activated homologs in the three *Sarcoptes* spp. of medical and veterinary importance. Although SMIPP-Cf was detected only in mites invading pigs and dogs, human mites showed two different versions of SMIPP-Cc with absence of SMIPP-Cf. Accordingly, it was postulated that homolog's amplification and sequence variation occurred prior and after host adaptation, respectively. Amino acid sequences of genes encoding CPs are conserved among each other and phylogenetic alignment analysis with previous studies suggested that they belong to monophyletic gene family. In addition, the investigators detected SMIPP-Cs expression in all burrowing stages with the highest in female mites. Localization studies using recombinant antibodies showed constant intense localization in the intestinal tract and feces indicating their gut origin. Although the investigators assigned proteolytic activity related to digestion and providing nutrients as well as inhibition of host complement activation and evasion of host immune response to serine proteases, they recommended further studies to assign the definite role of SMIPP-Cs in interaction with host epidermal tissue and host immune response⁽³¹⁾.

Ticks

In a report published in 2011, the reviewers summarized the differences between soft and hard ticks as obligate blood feeders: 1) nymphs and adults of both sexes of soft ticks feed rapidly and several times during their life stages while hard ticks take a single large blood meal during their whole life cycle; 2) soft ticks feed within 30-60 min, while the process is slow for 6-9 days in hard ticks; 3) feeding and oviposition are repeated, lasting for 150-200 days and are not related to mating in soft ticks, while only mated female hard ticks die after oviposition of a single large batch of eggs. The reviewers also discussed all CPs expressed in several species of soft and hard ticks. In addition, they

claimed that the feeding process involving degradation of host hemoglobin is a complex process that requires involvement of several gut associated CPs as well as aspartic proteases. Beside hemoglobin degradation, they discussed the major role played by CPs in embryogenesis with special emphasis on degradation of yolk proteins, mainly vitellin. In that issue, they claimed that the cascade or pathway employed in ticks is more conserved among ticks compared to other BFAs⁽¹⁾.

Two other reviews were published to express the importance of tick CYs in development of either a vaccine candidate for vector control instead of acaricidal compounds, or novel drug target to treat human diseases transmitted by ticks. In the first review⁽³²⁾, overall 21 tick CYs were reviewed and only nine of them were functionally characterized in the reviewed literature. Interestingly, up to that date (2012), all CYs were identified as potent inhibitors of the proteolytic activity of papain-like CPs (Caths), but not legumains (asparaginyl endopeptidase; AEP) due to lack of binding site in their protein sequence, e.g. legumains from *Ixodes ricinus* (*IrAE*) and *Haemaphysalis longicornis* (*Hllgm1* and *2*). The reviewers postulated this observation to the involvement of these CPs in tick physiology rather than blood feeding process. The identified CYs were categorized according to their types. Type I (stefins) were assigned to *Rhipicephalus haemaphysaloides* (*RhCys-1*), *H. longicornis* (*HilCys-1*), and *Dermacentor variabilis* (*DvM602*). On the other hand, type II CYs (sialostatins) were identified in *I. scapularis* (*IsSL* and *IsSL-2*), *H. longicornis* (*HilCys 2* and *3*), *Ornithodoros moubata* (*OmC 1* and *2*), *Amblyomma americanum* (*AaSL*), *D. variabilis* (*DvM226* and *DvM334*), and *R. haemaphysaloides* (*RhCys-2*). In the second review⁽³³⁾, it was claimed that ~ 85% of the identified transcripts of the detected tick CYs belonged to the extracellular group, suggesting predominant immunomodulatory role.

Concerning the use of CPis of natural herbs, amaranth CYs proved to have effective proteolytic activity against a wide range of CPs detected in ticks infestation of plants, suggesting its role in control of insect pests⁽³⁴⁾. The present review will only focus on the role and function(s) of CPs and CYs, of medically important ticks which transmit or potentially transmit human diseases.

Degradation of host proteins in tick gut cells is a complex process that is not performed only by hemoglobinase enzyme activity. The digestive network consists of AEP (clan CD, family C13) and Caths B, C and L (clan CA, family C1) as well as gut-related aspartic and leucine aminopeptidases⁽³⁵⁾. Accordingly, the overall hemoglobinolytic activity measured in tick gut tissue extracts during feeding was highly elevated from the 6th day after attachment to the host, which corresponds to expression of these CPs⁽³⁶⁾. From

clan CD, AEP was the first CP identified, localized, characterized and fully sequenced in *I. ricinus*; the major European hard tick species which transmits Lyme disease and tick-borne encephalitis. It was observed that IrAEP was exported from the lysosomal vesicles, and extracellularly localized within the peritrophic matrix of engorged females at the 5th day of feeding. Its proteolytic activity on host hemoglobin degradation was effectively inhibited with a legumain-specific inhibitor. The investigators discussed their results and claimed that hemoglobin degradation was strongly similar to that observed in the regurgitates of *S. mansoni* adult worms⁽³⁶⁾. One year later, the same investigators confirmed the AEP role in hemoglobin degradation, in contrast to other BFAs which rely on serine peptidases⁽³⁷⁾.

Another report characterized cDNA of the genes encoding AEP from *H. longicornis* (*Hllgm* and *Hllgm2*), a vector incriminated in transmission of babesiosis. Endogenous *Hllgm* was immunolocalized in the midgut epithelium of all developmental stages with a 38 kD molecular weight. In addition, the investigators found that its potent proteolytic activity on both bovine hemoglobin and serum albumin was inhibited by the thiol blocking reagents, suggesting its major role in blood meal process⁽³⁸⁾. To elucidate legumains functions in *H. longicornis*, the same investigators utilized RNA interference (RNAi) techniques for gene knock down. Results showed significant damage of the midgut tissues with reduction in tick body weight. Moreover, there was significant delayed onset of oviposition with reduced egg number and increased structurally deformed eggs. Therefore, the investigators concluded the use of specific legumains inhibitors as vector control strategy to control diseases transmitted by *H. longicornis*⁽³⁹⁾. In another trial for tick population control, a group of American scientists conducted transcriptomic studies on expressed sequence tag (ESTs) from cDNA libraries from female tick midguts at varying stages of feeding to identify transcripts involved in blood meal digestion in *D. variabilis*. It was observed that ~25% (19 out of 82 ESTs) were expressed in the 6th day fed midguts. Moreover, phylogenetic analysis with significant sequence similarity to the reported CPs in other ticks revealed presence of three major groups of CPs; legumain, Caths B and L. The investigators recommended further studies to investigate the role of these peptidases in development of new strategy in tick control⁽³⁵⁾.

Endogenous longipain (known later as Cath B) was immunolocalized at lysosomal vacuoles of the midgut epithelium of *H. longicornis*. Utilizing gene knock down techniques, the investigators showed that ticks lacking endogenous Cath B significantly increased the number of *Babesia* parasites both *in vitro* and *in vivo*, compared to control, through specific adherence to the parasite membranes. Accordingly, the investigators hypothesized that Cath B showed pivotal role in *H.*

longicornis survival, host hemoglobin degradation and as defense response against *Babesia* merozoites. Therefore, they recommended further studies to elucidate the structural features of endogenous Cath B of *H. longicornis* to be used to control babesiosis transmission in ticks⁽⁴⁰⁾. Another CP localized in the midgut of *H. longicornis* was molecularly characterized and termed HICPL-A due to its sequence similarity to Cath L. The HICPL-A gene expression was up-regulated by the blood-feeding process. Its proteolytic activity in degrading bovine hemoglobin was inhibited by three CPs including E-64⁽⁴¹⁾.

Another important role played by CPs in ticks is embryogenesis, specifically the degradation of yolk proteins. In that way, CPs might help tick survival by providing amino acids for protein catabolism enabling larval survival until the first blood meal is achieved. In fact, vertebrate blood provides an essential source of nutrients for energy metabolism to support demanding activities including embryogenesis⁽⁴²⁾.

Salivary gland transcriptomic studies were conducted in unfed and fed *R. haemaphysaloides* which is a common ectoparasite in cattle, horse, sheep, and dogs, and the primary vector of bovine babesiosis as well as potential vector of human Kyasanur forest disease (Asian viral hemorrhagic fever). In their publication, sialo-transcriptome was analyzed, and the expressed CPs were confirmed by real-time PCR in salivary glands of different developmental stages during blood feeding. Results revealed successful cloning of four genes encoding expression of Caths B and L, and caspases 1 and 8 as well as autophagy-related genes (ATGs). As Caths and caspases were expressed at early and late phases of tick engorgement, respectively, the investigators suggested assignment of hemoglobin degradation and inhibition of host cytokines for the role and function(s) of the identified CPs, respectively. They also recommended further studies to characterize these CPs which may help in development of novel drug targets and/or a vaccine candidate for vector control⁽⁴³⁾.

On the other hand, autophagy is another pathway leading to apoptosis, with activity involvement of CPs, but controlled by ATG4 and ATG8. Recently, Yu *et al.*⁽⁴⁴⁾ succeeded to identify two ATG8s. It is well known that caspase 1 plays a major role in salivary gland apoptosis of *R. haemaphysaloides* 3–4 days after tick attachment. Although the investigators performed silencing RNAi technique to knock out the genes encoding caspases 1 and 8, they still observed apoptosis, but delayed. Accordingly, the investigators suggested that salivary gland apoptosis of *R. haemaphysaloides* is affected by both pathways; genes encoding caspases and ATGs⁽⁴⁴⁾.

During the period 2002-2015, a group of scientists working in National Institute of Health (NIH) published several reports^(35,45-51). In addition, Ribeiro and Franischetti⁽⁵²⁾ reviewed strategies involved in

hard ticks during blood feeding. One year earlier, they conducted transcriptome studies for saliva components of the black-legged tick (*I. scapularis*), the main USA vector of Lyme disease. They showed the presence of sialome protein, and its sequence analysis revealed homologous similarity with several protease inhibitors with different conserved cysteine residues⁽⁴⁵⁾. In 2006, Kotsyfakis and his colleagues⁽⁴⁶⁾ named it sialostatin-L (*IsSL*) because of its significant inhibitory proteolytic activity against human Cath L, and to a lesser extent Cath C. In addition, their results showed that *IsSL* targeted several essential enzymes required for both *in vitro* and *in vivo* proliferation of cytotoxic T lymphocyte (CTL) in cell line and mouse model, respectively. The investigators attributed the significant reduction of extra- and intracellular compartments to *IsSL* potent anti-hemostatic and anti-inflammatory activities. One year later, the same investigators discovered two genes encoding SL involved in proteolytic actions against host papain-like CPs during blood meals, suggesting expression of two CYSSs. In addition, they observed an important difference between their transcript abundance during blood meals, as SL is required to reduce proliferation at the bite site, SL2 accumulates to perform its actions later as observed by increased transcription. When the investigators compared the actions of both SLs in hard and soft ticks (*Ornithodoros moubata*), they observed that those of the latter (rapid BFA) perform their main role in the midgut rather than in the salivary gland. Accordingly, the investigators hypothesized the immunosuppressive activity of SL2 during the prolonged blood meal. To confirm their hypothesis, the investigators utilized reverse genetic approach employing RNAi to knock out both genes, and they observed increased numbers of dead ticks due to strong host primary immune response. Meanwhile, ticks' rejection was observed in subsequent booster infestation. Therefore, the investigators concluded that development of inhibitor(s) against both SLs could be valuable vaccine against Lyme disease⁽⁴⁷⁾.

To elucidate the immunosuppressive effect(s) of *IsSL2*, two further studies were conducted in 2015 in which the investigators demonstrated its mechanisms of action to inhibit interferon (IFN) signaling in host dendritic cells. Their results postulated three mechanism; interference with IFN-triggered signal transduction through decreasing phosphorylation process, inhibition of IFN- β -mediated induction of IFN-sensitive genes in stimulated-dendritic cells and *in vitro* promotion of virus replication in dendritic cells^(48,49). However, other investigators from Czech Republic also observed the efficacy of *IsSL2* as an immunosuppressive CYSS in treatment of experimental asthma through its potent impairment of IL-9 production by Th9 cells and reduced expression of other asthmatic factors in mast cells. It was found that experimental application of *IsSL2* in asthmatic mice (challenged with OVA antigen) significantly blocked eosinophilia and airway hyper-responsiveness⁽⁵³⁾.

For *A. americanum*, a common USA vector for several human diseases and known also as turkey tick, another SL (*AaSL*) detected in the salivary glands and midgut was partially sequenced due to design error of the cloning primers based on the SL gene, however, its partial sequence was ~100% identical to *IsSL*. When *AaSL* was investigated for its immunomodulatory role, it showed significant inhibition of immune response(s), suggesting capability of *A. americanum* utilizing its secretory *AaSL* to evade host immune response through disruption of normal antigen processing in host antigen-presenting cells. It was shown that knock out of the gene encoding *AaSL* led to 90 and 50% reduction in transcript abundance in the early and late phases of feeding, respectively, as well as decreased tick body weight, detachment of 35% ticks after 1 day and over 50% mortality of attached ticks, compared to control ticks⁽⁵⁰⁾.

It is worth mentioning that a single CYS type 1 (*DvM602*) and two of type II (*DvM334* and *DvM226*) were identified in *D. variabilis* and showed high amino acid sequence similarity to the other described CYSSs. However, their inhibitory functions were not determined⁽³⁵⁾. Another group of investigators from Czech Republic also, described two CYSSs (*OmC1* and 2) in soft ticks, *O. moubata*, the vector transmitting African swine fever virus and African tick-borne relapsing fever. While *OmC1* transcripts were found only in the midguts of unfed ticks, *OmC2* transcripts were found in all tissues and both possessed inhibitory proteolytic activity against Caths B, C, and H as well as papain⁽⁵⁴⁾. In 2010, the crystal structure of *OmC2* was characterized, and the investigators found its efficient inhibition of the proteolytic activity of endogenous (tick) Caths L and S as well as exogenous (host) Caths B, C and H⁽⁵¹⁾. Both *in vitro* and *in vivo* studies were conducted to demonstrate its significant role in reduction of pro-inflammatory cytokines (TNF- α and IL-12), and antigen-specific CD4+ T cells. Accordingly, the investigators hypothesized that *OmC2* is capable of suppressing host recognition of salivary antigens (endogenous Caths L and S) to manipulate the adaptive immunity which efficiently facilitates repeated blood meals on the same host. Meanwhile its ability to inhibit host Caths B, C and H, *OmC2* contributes to decrease host innate immunity. Therefore, a vaccination experiment utilizing *OmC2* antibodies was performed and showed significant increase in mortality rate of *O. moubata* nymphs after blood meals from immunized animals. The investigators concluded its usefulness as a candidate vaccine to control disease transmission⁽⁵¹⁾.

Apart from the NIH publications, three CYSSs were molecularly characterized, and identified in salivary content of *H. longicornis*. The identified CYSSs (*HICys* 1-3) showed efficient inhibition of the proteolytic activity of papain and Cath L, but not to Cath B. Their endogenous nature was immunolocalized in the acini of the salivary gland of adult ticks, their expressed transcripts were highly up-regulated in the early blood feeding processes,

and their genes were sequenced⁽⁵⁵⁻⁵⁷⁾. Moreover, another two CYSs were isolated from *R. haemaphysaloides* (*RhCys*-1 and 2) and cloned to investigate their inhibitory activities against different Caths. To assess their role and function(s), expression analyses in different developmental stages and RNAi studies were conducted. Results revealed their efficient inhibitory activity against all tested Caths, with strongest affinity to Cath S, high expression in egg stage, and essential role in embryogenesis^(58,59).

Recently, *in vitro* and *in vivo* effects of *RhCyst*-1 in four tumor cell lines and mouse tumor therapy model, respectively were investigated. It was the first report of using CYS as a potential drug in cancer therapy. It showed potent significant inhibitory effects on proliferation, migration, and invasion of all tumor cell lines *in vitro*. It also *in vivo* inhibited tumor growth and improved mice survival. The investigators attributed their results to the effects of *RhCyst*-1 in decreasing the number of myeloid-derived suppressor cells (MDSCs) from the peripheral blood mononuclear cells (PBMCs). However, the investigators recommended further studies to discover the link between *RhCyst*-1 and MDSCs regulation from the spleens and PBMCs⁽⁶⁰⁾.

As regards *I. ovatus*, a vector transmitting viral encephalitis in Japan, Parizi *et al.*⁽⁶¹⁾ succeeded to identify CYS type II (five variants, *JploCys*2a-e). Their expression differs among various developmental stages and tissues (e.g. salivary gland, gut, ovaries, and fat bodies), and showed variable inhibitory efficacy against the proteolytic activity of Caths B, C, and L. The investigators also showed their essential role during modulation of initial hemoglobin degradation in blood feeding process through its significant variable inhibitory activity against different Caths. *In silico* and *in vitro* comparative cross-antigenicity analyses of *JploCys*2 and two CYS isolated from *Rhipicephalus microplus*, a cattle ectoparasite with veterinary importance, showed high cross-antigenicity between native and recombinant CYSs. Based on the established development of a vaccine utilizing *RmCys*-2 in immunization of cattle for livestock control, the investigators suggested a similar vaccine to control transmission of viral encephalitis in Japan. Similarly, another three variants of CYS2 were recently described and successfully sequenced from Japanese tick transmitting viral encephalitis in Japan, *I. persulcatus* (*JpIpCys*2a-c). Their structural analysis and expression profile were demonstrated, and the investigators showed that their transcripts, except for *JpIpCys*2c, were detected in almost all tissues of all the developmental stages. They noticed the absence of the latter transcripts in unfed larvae⁽⁶²⁾.

Concluding remarks

- Salivary glands in all BFAs secrete CPs, mainly Caths, essentially for hemoglobin degradation prior to egg production. Three other roles were assigned; evasion of host defense response, production of inflammatory reactions, and embryogenesis.

- Caspase is the second expressed CP which has an essential role in apoptosis of elements involved in vector's innate immune response against intracellular transmitting microorganisms.
- Use of specific CPIs would be a new strategy for vector control instead of routine insecticides.
- Group 1 allergen (*Der*-1), the main antigenic element of house dust mites, exhibits CP proteolytic activities, and proved to have a major role in pathogenesis of atopic dermatitis as well as respiratory asthmatic attacks.
- Sequence analysis of gene(s) encoding CPs in scabies mites, identified five SMIPP-Cs homologs with close similarity to *Der*-1 allergens. They are intensely localized in the intestinal tract and feces, indicating their gut origin, of all burrowing stages with the highest in female mites. Further studies are recommended to assign their exclusive role in interaction with host epidermal tissue and host immune response.
- Ticks CYSs, as components of salivary glands, attract much attention in several publications. The most common identified and characterized CYSs belong to type II (sialostatins), that are essentially involved in impairment of the functions of host immune response through their roles in interference with intracellular and extracellular pathways leading to inhibition of hemostasis.
- Due to the modulatory inhibitory effects on Caths, ticks CYSs are considered potential targets for development of novel drugs and vaccine candidates for several human-tick-borne diseases such as Lyme disease and encephalitis. In addition, several studies recommended use of tick CYSs in cancer therapy, neurodegenerative disorders as well as psoriasis, muscular dystrophy, osteoporosis and rheumatoid arthritis.

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