Chapter 6
Conclusion and future prospects

6.1 Conclusion

In the present work, we have identified, isolated and characterized thrombin inhibitors from the salivary gland of *Haemaphysalis bispinosa*. Two ticks were identified from the study area, i.e. Napaam village, Sonitpur, India using morphological and molecular methods. The ticks were *Rhipiciphalus* (*B*) *microplus* and *Haemaphysalis bispinosa*; this is the first report of phylogenetic analysis of the two ticks from this region and that of ITS2 and 16S ribosomal DNA from *H. bispinosa*. A PCR-RFLP based diagnostic tool was developed to distinguish between the two ticks, as these are difficult to be identified visually at the nymphal and fully engorged stages.

Salivary gland extract of *H. bispinosa* showed significant anti-coagulant activity, when tested for its effect of *in vitro* coagulation of goat plasma. Using genomic approach cDNAs coding for thrombin inhibitors, haemathrin 1 and haemathrin 2, were amplified from the cDNA pool of the salivary gland. Nucleotide analysis reveals that these are similar to madanins which belong to a unique class of I53 superfamily of inhibitors. The mature peptides of haemathrin 1 and 2 were expressed in *E. coli* expression host and purified using metal-affinity and reversed phase chromatography. The expressed recombinant haemathrin 1 and 2 (rHaemathrins 1 and 2) had molecular masses of 6690.3 and 6709.1 Da, respectively and were disordered in solution. These showed significant prolongation of clotting of goat plasma and rHaemathrin 2 was found to be more potent than rHaemathrin 1. rHaemathrins dose-dependently inhibited amidolytic activity of thrombin against a small chromogenic substrate, and delayed the clot time of its natural substrate, fibrinogen, significantly, showing it specifically targets thrombin. rHaemathrins inhibited thrombin in a slow binding, and mixed inhibition manner, unlike most of the thrombin inhibitors isolated from ticks which are competitive inhibitors.

Computational docking results showed that haemathrins interacts with thrombin near the active site, but did not interact with the active site residues. This is unlike other thrombin inhibitors like madanin, microphilin and variegin from ticks, which bind to the exosite I of thrombin and competitively inhibit the function of thrombin.

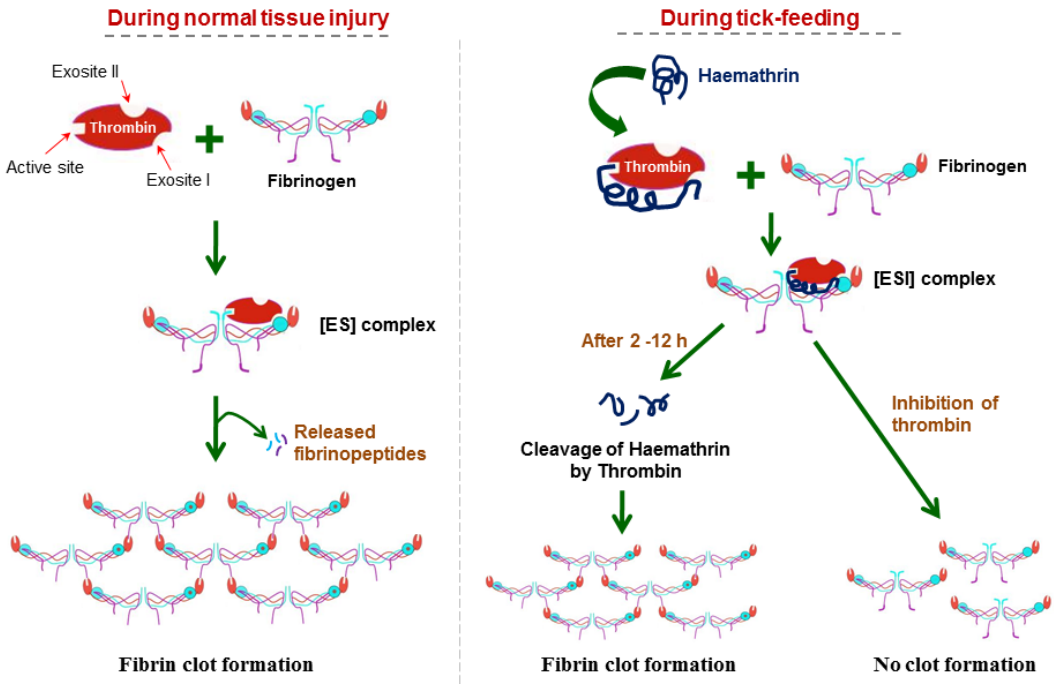

Figure 6.1 Schematic representation of mechanism of action of haemathrins.

The thrombin inhibitory activity of rHaemathrins decreased when incubated with thrombin for longer period of time. When the reactions of the peptides with thrombin at different time intervals were run on RP-HPLC, they were found to be cleaved by throsumbin itself, which was confirmed by mass-spectrometric analysis. Haemathrins were cleaved at the Arg-Leu and Lys-Val points, which are thrombin cleavage recognition sites.

Present study demonstrates the presence of thrombin inhibitors in the salivary gland of *H. bispinosa*. Haemathrins are cleavable thrombin inhibitors which might be involved in initial blood feeding mechanism, but loses its inhibitory activity during the feeding process. The mechanism of action of rHaemathrins is summarized in Figure 6.1.

6.2 Future prospects

6.2.1 Site directed mutagenesis and thrombin inhibition

The activity of haemathrins was found to decrease on incubation with thrombin for different time intervals. Biophysical characterization reveals that due to cleavage of the peptides by thrombin it loses its inhibitory activity. Therefore, to understand the role of cleavage of the peptides by thrombin, the cleavage recognition residues of thrombin can be substituted with neutral amino acid using site-directed mutagenesis. The inhibitory activity of the mutated peptides can be assessed to understand if the cleavage of the peptides really contributes to the loss of inhibitory activity of haemathrins.

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6.2.2 Structure determination of haemathrin and co-crystallization with thrombin

The CD spectra reveal that the secondary structure in solution is predominantly random coils while *in silico* prediction reveals the presence of alpha-helix. This discrepancy can be resolved by determining the three dimensional structure using X-ray diffraction. Moreover the exact site of interaction with thrombin is not clearly understood though it is showing mixed type of inhibition, as revealed from the biochemical studies. The molecular docking shows that it binds in close proximity to the active site of α-thrombin. However, this data needs to be validated by determining the three dimensional structure of the complex. Co-crystallization of the full length peptide and thrombin would reveal the position and detail account of mechanism of interaction. This study can be carried out in future to understand the structure-function correlation of thrombin inhibition by haemathrins.

6.2.3 Design of synthetic peptide (thrombin inhibitor)

Based on the crystal structure of haemathrin and thrombin complex, the amino acid residues involved in interaction can be identified. This would lead to design of thrombin inhibitors based on haemathrins. The peptides involved in interaction can be synthesized and further studies can be carried out to validate the application of these peptides as thrombin inhibitors.