ABSTRACT

Ticks are obligate hematophagous ectoparasites of terrestrial vertebrates, comprising of three families, the Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttalliellidae. To acquire blood meal, ticks insert their hypostome into the host's skin and thus damage the host tissue, capillary and small blood vessels. As a result, the host hemostatic system and immune system is activated. Therefore, for continuous feeding ticks inject through their saliva a cocktail of anti-hemostatic, anti-inflammatory and immunomodulatory substances. During the feeding process, ticks transmit several disease causing viruses and bacteria. Ticks and tick-borne diseases are of immense economic importance, as these cause serious loss to dairy and meat industries. Besides, ticks are vectors of several human diseases such as tick relapsing fever, rickettsioses, Lyme borreliosis, Rocky Mountain spotted fever and Mediterranean spotted fever. Studying tick salivary proteins may help in understanding pathogen transmission and also in developing new vaccines against ticks and tick-borne diseases. In addition, tick anti-hemostatic components, which includes anti-coagulants, platelet aggregation and adhesion inhibitors, vasodilators and figrin(ogen)olytic agents, can be targeted for development of novel therapeutic leads for the prevention and treatment of cardiovascular disorders and diseases. Less than 5% of the tick salivary proteins have been expressed and their function described for any tick species and so far, no drug molecule has been developed from tick salivary component. In addition, the current anti-coagulant drugs available in the market have several limitations. Therefore, exploration of tick salivary proteins is a viable domain and this may aid in discovery of novel therapeutic molecules.

The present work describes the identification, isolation and characterization of thrombin inhibitors from the salivary gland of the tick *Haemaphysalis bispinosa*. Firstly, two predominant 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 identify visually at the nymphal and fully engorged stages.

As there is no report of identification and characterization of anti-coagulant activities of salivary gland extract of *H. bispinosa*, the salivary gland extract (SGE) of the tick was assessed for its anti-coagulation properties. SGE prolonged recalcification time and activated partial thromboplastin time (APTT) of goat plasma, showing that it has anti-coagulant properties. Using genomic approach, the cDNAs coding for two isoforms of thrombin inhibitors were isolated using gene-specific PCR amplification. Sequence analysis of the cDNAs revealed that these are similar to madanins, thrombin inhibitors of I53 superfamily inhibitors, isolated from *H. longicornis*. These were christened as haemathrin 1 and 2 (***Haema****physalis* **thr**ombin **in**hibitor). Haemathrins coded for peptides of 78 amino acid residues, the first 19 of which were the signal peptides. The mature peptides had molecular mass of ~6.7 kDa, and were expressed in *E. coli* BL21(DE3)pLysS, followed by purification of the His-tagged proteins using metal affinity chromatography. The ESI-MS spectra of recombinant haemathrin 1 and 2 revealed major *m/z* signal of 6690.3 Da and 6709.1 Da respectively, which are in agreement with the predicted molecular masses of the proteins (6690.1 Da for haemathirn 1 and 6709.1 Da for haemathrin 2). The CD spectra of the proteins revealed that haemathrins are disordered in solution.

Recombinant hamathrin (rHaemathrin) 1 and 2 prolonged thrombin time, APTT and Prothrombin time of goat plasma significantly. When screened for specificity against 10 serine proteases, including classical serine protease trypsin, rHaemathrins were found to inhibit thrombin specifically. These were also found to prolong fibrinogen clotting time in a dose-dependent manner, showing that they specifically target thrombin. rHaemathrins inhibited amidolytic activity of human α-thrombin with IC_{50} of 46.13 ± 0.04 µM for haemathrin 1 and 40.057 ± 0.054 µM for haemathrin 2 and the inhibition was time-dependent, showing that the peptides inhibited thrombin in a slow binding manner. The inhibition was mixed-type, unlike most of the thrombin inhibitors isolated from ticks which are competitive inhibitors. Computational docking substantiated this result; docking revealed that haemathrins interacted to thrombin near the active site and not the substrate binding site, but did

not interact with the active site residues of the enzyme. Additionally it was observed that when rHaemathrins were incubated with thrombin for several hours, the inhibitory activity decreased significantly with time. When the reaction mix of rHaemathrin and thrombin was analyzed by RP-HPLC, it was observed that with increasing time of incubation, the rHaemathrins were disintegrated. Mass spectrometric analyses revealed that the fractions are the peptide fragments of rHaemathrins and the cleavage points of the peptides were Arg-Leu or Lys-Val, which are recognition sites of thrombin cleavage. This indicated that the recombinant peptides are processed by thrombin and thus it loses its activity. The peptidic fragments did not inhibit the coagulation of goat plasma.

rHaemathrins are cleavable slow binding-type inhibitors of thrombin and specifically target thrombin through mixed inhibition mechanism. These may exert their inhibitory function at the initial stage of the coagulation cascade. When vascular tissue is injured, coagulation is initiated by the factor VIIa–tissue factor complex at the site of injury, which activates factor X. This is followed by generation of small amount of thrombin, that in turn activates factor V and VIII leading to amplification of thrombin generation. This is a crucial step in the coagulation cascade and haemathrins might inhibit blood coagulation by shutting down activation of factor V and VIII by thrombin. When rHaemathrins come in contact with thrombin, these may inhibit the function of thrombin at the initial stage. But, with time rHaemathrins are cleaved or disintegrated by thrombin and the inhibitory activity is suppressed.