PROJECT COMPLETION REPORT

On

Title: Cloning, Expression and Structural Characterization of Anti-Thrombin from Cattle Tick Salivary Gland: Designing of Novel Anti-Thrombin Peptide

Sanction Order No. SB/EMEQ-009/2014

Under

Empowerment and Equity Opportunities for Excellence in Science Programme

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Principal Investigator:
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Title of the project: ***Cloning, Expression and Structural Characterization of Anti-Thrombin from Cattle Tick Salivary Gland: Designing of Novel Anti-Thrombin Peptide.***

1. Principal Investigator(s) and Co-Investigator(s): Dr. Robin Doley

2. Implementing Institution(s) and other collaborating Institution(s): Department of Molecular Biology and Biotechnology, Tezpur University
3. Date of commencement: 03/07/2014
4. Planned date of completion: 31 July 2018
5. Actual date of completion: 31 July 2018

6. Objectives as stated in the project proposal:
   - *Isolation of anti-thrombin from salivary gland extract (SGE).*
   - *Identification and isolation of cDNA encoding anti-thrombin from tick salivary gland.*
   - *Cloning and expression of recombinant anti-thrombin gene.*
   - *Biochemical and biological characterization of the recombinant protein*
   - *Three dimensional structure determination of the recombinant anti-thrombin protein using high resolution multidimensional NMR spectroscopy.*
   - *Mutagenesis study to establish the critical amino acid residues responsible for protein-protein interaction.*
   - *Design of novel anti-thrombin peptide.*

7. Deviation made from original objectives if any, while implementing the project and reasons thereof:

   - *Three dimensional structure of the recombinant protein was predicted by in-sillico method.*
   - *Interaction of thrombin and peptide was evaluated by docking studies*

8. Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams & photographs: **Annexure I**
9. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject: **Annexure I**
10. Conclusions summarizing the achievements and indication of scope for future work: **Annexure I**

11. S&T benefits accrued:
    i. List of Research publications

List of Publications from this Project (including title, author(s), journals & year(s)

1. Papers published only in cited Journals (SCI): 01 (Reprint enclosed)
    1. Brahma, R.K. et al. Identification and characterization of Rhipicephalus (Boophilus) microplus and Haemaphysalis bispinosa ticks (Acari: Ixodidae) of North East India by ITS2 and 16S rDNA sequences and morphological analysis, Exp.Appl.Acarol. 62 (2), 253--265, 2014.

    2. Rajeev Kungur Brahma, Guillaume Blanchet, Simran Kaur, R. Manjunatha Kini, **Robin Doley. (2017).** Expression and characterization of haemathrins, madanin-like thrombin inhibitors, isolated from the salivary gland of tick *Haemaphysalis bispinosa* (Acari: Ixodidae) **Thrombosis Research**. Jan 31;152:20-29. **[IF: 2.32]**

2. Papers published in Conference Proceedings, Popular Journals etc.: Nil
3. Conference attended/Delivered lecture:
    1. **Doley, R.** Expression and Characterization of Haemathrin, a Thrombin Inhibitor: Understanding the Structure function for design and development of antithrombotic drugs**. "Advances in Biotechnology and its Impact on Human Health**" Organized by Defence Research Laboratory, Tezpur during 7-11 Nov. 2016 **(Oral)**
    2. Rajeev K Brahma and Robin Doley. Molecular characterization of a novel thrombin inhibitor from Haemaphysalis bispinosa and expression in prokaryotic cells**. National seminar on recent advances in biotechnological research in North East India: Challenges and Prospects.** Organized by Department of Molecular Biology and Biotechnology, Tezpur University. November 27-29 2014. **(Oral)**
    3. Brahma, R.K and Doley, R. Molecular Characterization of cattle ticks from Sonitpur District, Assam based on ITS2 and 19\6S rDNA. **Recent Trends in Bioresource Management & Biodiversity Conservation**. Organized by Centre for Potential for Excellence in Biodiversity, Rajiv Gandhi University, Rono Hills, Doimukh, Arunachal Pradesh during 10-12 Dec. 2013. **(Poster)**

Patents filed/to be filed: Nil

ii. Manpower trained

PhDs produced no: 01 Mr. Rajeev Kungur Brahma, TZ121464 of 2012 **Title of the thesis:** Identification and characterization of thrombin inhibitor from the salivary gland of Tick (*Haemaphysalis bispinosa*)	Technical Personnel trained: 03	Research Publications arising out of the present project: 02

13. Financial Position:

No	Financial Position/ Budget Head	Funds Sanctioned (Rs)	Expenditure (Rs)	% of Total cost
I	Equipment	1300000.00	1299181.00	99.93
II	Salaries/ Manpower costs	2250000.00	909610.00	96.54
III	Supplies & Materials		1009237.00	
IV	Contingencies		80615.00	
V	Travel		111448.00	
VI	Overhead Expenses	500000.00	500000.00	100
VII	Others, if any	Bank Interest 12993.00		
	Total	4062993.00	3910091.00	97.76
	Amount refunded		152902.00	

14. Procurement/ Usage of Equipment

S. No	Name of the equipment	Make/Model	Cost (FE/Rs)	Date of installation	Utilization rate%	Remark regarding maintenance
1	UHPLC with accessories	Thermo Scientific/Dionex Ultimate 3000	12,70,985.00	19/03/2015	PI's lab= 77.6% Faculty of the dept. = 21.8% Faculty from University = 0.6%	No major breakdown till date

b) Plans for utilizing the equipment facilities in future: To use for on going projects

Name and Signature with Date:

a. Robin Doley

(Principal Investigator)

b. Nil

(Co-Investigator)

Annexure I

Methodology:

1. Collection of ticks:

The tick samples were collected from Amolapam Gaon, a village adjacent to Tezpur University, Assam. The ticks were detected using references from different identification keys/ features unique to each genera, viz. mouth parts, basis capitulum, scutum, eyes, festoons, adanal, subanal, and accessory anal plates, coxae and anal groove. The prevalent tick genera in the sampling village were found to be *Rhipicephalus* and *Haemaphysalis.*

2. Morphological characterization of tick:

Ultrastructural observations of the ticks were done by scanning electron microscopy. Briefly, the tick specimens were cleaned by sonication in 70% ethanol and washing in distilled water, and then fixed with 2.5% glutaryldehyde solution for 5 h. These were dehydrated with a gradient of 60-100% ethanol and critical-point dried. The tick specimens were then fixed to metal stub attached with a conductive carbon tape, and sputter coated with gold in an ion coater. The specimens were observed and photographed under a JEOL scanning electron microscope (model 6510, JEOL Ltd., Japan). The external features were recorded and species identified.

3. Isolation of genomic DNA:

Tick samples were washed in 70% ethanol and dissected into very fine pieces and homogenized using a homogenizer. Genomic DNA was then isolated using commercial kit (Qiagen) after incubating the samples with Proteinase K at 56ºC for 1 hr. To it 200 µl of Buffer AL was added followed by 200 µl 100% ethanol. The mixture was placed in a spin column and centrifuged at 8000 rpm for 1 min. Flow through was discarded and then washed with 500 µl Buffer AW1 at 8000 rpm for 1 min. Discard flow through and wash with buffer AW2 by centrifuging at 13000 rpm for 3 min. The bound DNA was eluted with 50 µl buffer AE.

4. Amplification and cloning of 5.8S ribosomal RNA gene

The 5.8S ribosomal RNA gene internal transcribed spacer 2 (ITS2) was amplified using specific primers, viz. F1-ITS2, R1-ITS2, F2-ITS2 and R2-ITS2 (Table 2)1. PCR cycler was programmed as follows: One cycle of 94ºC for 5 min; 30 cycles of 94ºC for 30 sec, 55ºC for 1 min, 68ºC for 2 min;

and final extension of 68ºC for 10 min. The amplified product was run on 0.8% agarose gel and the desired band was purified from gel using gel extraction kit. The gene was cloned into TA cloning vector pTZ57/R using PCR cloning kit.

Table 1: Primers for amplification of ITS21

Primer name	Sequence
F1-ITS2	5′-CTAAGCGGTGGATCACTCGG-3′
R1-ITS2	5′-GCACTATCAAGCAACACGACTC-3′
F2-ITS2	5′-CGAGACTTGGTGTGAATTGCA-3′
R2-ITS2	5′-TCCCATACACCACATTTCCCG-3′

5. Gel extraction of PCR products

DNA was extracted from agarose gel using QIAquick gel extraction. The DNA fragment was excised from the gel with a fresh scalpel. The gel slice was weighed in a clean eppendorf tube and 3 volumes of buffer QG was added to it. This was incubated at 50°C for about 10 min till the gel was completely dissolved. 1 gel volume of isopropanol was added to the tube. The sample was then applied to a spin column and centrifuged at 13,000 rpm for 30-60 s. The flow-through was discarded and the column washed with 750 µl buffer PE. The DNA was eluted with 30-50 µl of buffer EB in a clean tube by centrifugation. The eluted DNA was analyzed on 0.8% agarose gel.

6. Sequencing of genes

The genes were sequenced using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, MA, USA). 15 µl reactions were prepared using 3 pmol sequencing primer, containing approximately 3-10 ng of PCR template. Amplification was over 30 cycles at 95ºC for 10 s, 50ºC for 10 s, and 60ºC for 4 min. The PCR product was cleaned up by BigDye Terminator clean-up method. Briefly, 25 mM EDTA was added to the reaction, to which 2 µl 3 M sodium acetate (pH 4.6) and 50 µl of ethanol was added and incubated at room temperature for 15 min. The mixture was centrifuged at 12000 g for 20 min and the pellet washed with 70% ethanol. The pellet was dissolved in 15 µl Hi-Di formamide, transferred to sample tubes covered with septa, denatured and snap chilled. This was electrophoresed in an automated sequencer (Genetic Analyzer 3010, Applied Biosystems) and sequence obtained from the sequence analyzer (Applied Biosystems). The sequence obtained from the sequence analyzer was analysed using GENERUNNER.

7. Sequence analysis

The DNA sequences were submitted to GenBank using BankIt submission tool of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers were obtained (Table 2). The ITS2 and 16S rDNA sequences were aligned with sequences of different tick species obtained from the NCBI database using ClustalW program with default parameter settings [7]. Neighbor-Joining trees constructed with bootstraps of 1,000 replicates based upon the alignment of ITS2 and 16S rDNA using MEGA program (version 5) [8]. The evolutionary distances were computed using the Kimura 2-parameter method [9] and are in the units of the number of base substitutions per site. Pairwise distance was calculated using MEGA5. All ambiguous positions were removed for each sequence pair.

8. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of ITS2 to discriminate between tick species sharing the same host

The ITS2 rDNA of Rhipicephalus (B) microplus and Haemaphysalis bispinosa, which share the same host, was amplified using gene-specific primers. Restriction sites of the sequences were mapped using the online tools of Rebase® (http://rebase.neb.com/rebase/rebase.html) and suitable restriction enzyme was selected. PCR products were digested by HindIII enzyme (20 U) with 10 µl of amplified DNA in 30 µl reaction at 37ºC for 3 h. Restriction fragments were run on 1% agarose gel along with Generuler 1 kb plus DNA ladder (Thermoscientific), stained with ethidium bromide and documented

9. Isolation of Salivary Glands (SG)

For the isolation of the salivary glands the method of Edwards et. al. (2009) was followed with some modification. Fully-engorged tick was placed onto a petri-dish and the scutum was removed using a sterile blade under a stereo microscope. The blood meal was washed off with dissection buffer (PBS, pH 7.4/150 mM NaCl) and the salivary gland was dissected out using forceps (Figure 4). The extracted glands were stored at -80°C until use. For mRNA isolation, the glands were extracted and stored in RNA later and stored at -80°C.

10. Preparation of Salivary Gland Extract (SGE)

Salivary gland extract was prepared according to Koh et. al. (2007), with some modification. Salivary glands were thawed/incubated at ~90°C for 5 min (optional) and subjected to sonication (5x15 pulses, 30% cycle duty, 60% amplitude) in 500 µl of 20 mM Tris-Cl, 0.15 M NaCl buffer, pH 7.4 at 4°c. The cell lysate was centrifuged at 10,000 rpm for 5 min at 4°C and supernatant collected, constituting the SGE.

11. Preparation of salivary gland (SG) cDNA

Total mRNA was isolated from twenty SGs using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). First-strand synthesis was carried out using SMARTScribe reverse transcriptase at 42 °C for 1 h in the presence of the SMART IV and CDS III (3′) primers. Second-strand synthesis was performed using a long distance (LD) PCR-based protocol, using Advantage™Taq polymerase (Clontech) mix in the presence of the 5′ PCR primer and the CDS III (3′) primer. PCR conditions were as follows: 95 °C for 1 min; 21 cycles of 95 °C for 5 s, 68 °C for 6 min. A small portion of the cDNA obtained by PCR was analyzed on a 1.1% agarose gel to check quality and range of cDNA synthesized.

12. Amplification of gene of interest:

Primers were designed to amplify the transcript coding for anti-thrombin from the salivary gland cDNA. The cDNA encoding for the protein was amplified from the cDNA pool by PCR using the forward primer HbTI-F (5'-TTTGACCGCAATGAAGCAC-3') and two different reverse primers HbTI-R1 (5'-CTTCCAGCCTACAACATCAC-3') and HbTI-R2 (5'-TCTATAACCTACCGACGGC-3'). A total of 0.2 µM of the primer sets and about 200 ng of template DNA were used in a 30 µl PCR reaction mixture. PCR was performed as follows: one cycle of 94ºC for 2 min; 30 cycles of 94ºC for 20 s, 55ºC for 30 s, 72ºC for 30 s; and final extension of 72ºC for 10 min. The amplified DNA was electrophoresed on 1.1 % agarose gel and visualized under UV light.

13. Sequencing and analysis of the thrombin inhibitor genes

The cDNA was sequenced using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, MA, USA) in an automated sequencer (as described in section 2.2.8). The sequences were submitted to GenBank of National Center for Biotechnology Information (NCBI) using BankIt submission tool (https://www.ncbi.nlm.nih.gov/genbank/) as haemathrin 1 (KM086726) and haemathrin 2 (KM086725). The putative amino acid sequences were deduced using GENERUNNER. The

sequences were searched for sequence similarity using BLAST progam at the National Center for Biotechnology Information (NCBI). The predicted molecular weight and theoretical pI of the protein was calculated using ProtParam tool server program (http://web.expasy.org/protparam/). The signal peptide and the cleavage site for the mature protein was predicted using SignalP (Version 4) server program (http://www.cbs.dtu.dk/services/SignalP-4.0/) at the Center for Biological Sequence Analysis (CBS) [67]. Conserved domains were identified and analyzed by the Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The nucleotide and protein sequences of haemathrin 1 and 2 were aligned with sequences obtained from the NCBI database by ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

14. Preparation of Escherichia coli BL21(DE3)pLysS competent cells

Glycerol stock of E. coli BL21(DE3)pLysS was revived on Luria-Bertani Agar (LBA) plate. The starter culture of the bacterium was prepared and 2 ml of it was inoculated into 100 ml Luria-Bertani (LB) broth and grown at 37ºC till OD600 reached 0.6-0.8. The culture was aliquoted in 50 ml centrifuge tube and kept in ice for 10 min. The tubes were centrifuged at 3000 rpm at 4°C for 10 min, supernatant discarded and the pellet was re-suspended in 50 ml of 100 mM CaCl2. The suspension was centrifuged at 3000 rpm for 10 min at 4°C and the pellet was suspended in 6 ml of 100 mM CaCl2 and 2 ml 50% glycerol. Aliquots of 50 µl was transferred into sterilized pre-marked pre-chilled eppendorf tubes and stored at -80°C for later use.

15. Isolation of plasmids from bacterial cells

Single colonies of bacterial cells were picked and inoculated in 5 ml LB broth with Ampicillin and incubated with shaking at 37°C for overnight. Plasmids were isolated using QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instruction. Briefly, the overnight bacterial culture was centrifuged and the pellet was resuspended in 250 µl Buffer P1, RNase added and transferred to a microcentrifuge tube. 250 µl Buffer P2 was added and mixed thoroughly, followed by addition of 350 µl Buffer N3. The tube was centrifuged at 10,000 rpm for 10 min and the supernatant applied to QIAprep spin column and centrifuged for 30-60 sec. The flow-through was discarded and the column washed with wash buffer, Buffer PE. The flow-through was discarded and centrifuged for additional 1 min to remove the additional wash buffer. The DNA was eluted with 30-50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) and stored at -20ºC until use. The isolated plasmids were analyzed on 0.8% agarose gel.

16. Transformation of plasmids into competent cells

Plasmids (1-5 µl) was added to the E. coli BL21(DE3)pLysS competent cells and mixed gently. The tubes were incubated in ice for 45 min, followed by heat shock treatment at 42°C for 90 s in a heat block. 500 µl of LB broth without Ampicillin was added and incubated at 37°C for 1 h with shaking. The cells were plated on LB Agar plate with Ampicillin and incubated at 37°C for 16 h. The plates were observed for single colonies.

17. Cloning of cDNA coding for haemathrins

The DNA fragments encoding the mature peptide of haemathrin 1 and 2 were amplified (as described in section 2.2.6) using gene-specific primers (HbMl1-F: 5`-ATCCATGGCATACCCGGAGAGAGA-3` and HbMl1-32R: 5`-ATCTCGAGTCAAGCATTCTTTCGTCC-3`). The amplified product and pET32a(+) were digested with 5 U each of restriction enzymes XhoI and NcoI at 37°C for 1-3 h. The digested insert and vector were gel extracted using commercial kit (as described in section 2.3.7). The digested insert and vector (3:1) were ligated using T4 DNA ligase at 16°C for overnight. The ligation product was transformed into E. coli BL21(DE3)pLysS competent cells by heat-shock method as described in previous section (4.2.9).

18. Screening of clones by colony PCR

The clones were confirmed for insertion of the gene by colony PCR. Briefly, colonies were picked using sterilized tooth-pick, suspended in 10 µl distilled water and heated at 95°C for 2 min. The lysate was centrifuged at 10,000 rpm for 2 min and 1 µl of the supernatant was used as template for PCR (as described in section 2.2.6). The PCR products were analyzed on 1.1% agarose gel.

19. Expression of recombinant haemathrins (rHaemathrins)

Single colonies of E. coli BL21(DE3)pLysS transformed with recombinant pET-32a(+) (rpET-32a(+)) were picked and inoculated in 4 ml Luria Bertani (LB) broth with Ampicillin (Amp) and incubated with shaking at 37°C for overnight. The starter culture was then inoculated in 100 ml LB with Amp and grown at 37°C with shaking till OD600 reached 0.5 – 0.8. Cells transformed with pET-32a(+) was taken as control. IPTG was added to the culture to a final concentration of 0.1 mM and incubated at 37 °C for 3 hours. Before addition of IPTG 5 ml of the culture was kept aside

which served as uninduced cells. The cells were collected by centrifugation at 5,000 rpm for 10 min and lysed by heating at 95°C for 5 min in gel loading buffer, followed by loading on 12.5% SDS-PAGE gel. For optimization of the expression conditions, the cells were induced at different temperatures (16°C, 25°C, and 37°C), for different time intervals (2 and 4hours) and using different IPTG concentrations (0.05, 0.1, 0.5 and 1 mM). Cells were pellet down at 5000 rpm for 10 min. The cell pellet was suspended in 4 ml lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole, pH 8.0), and sonicated for 4 min (75% amplitude, 60% cycle duty, 3 min) in a Labsonic M Ultrasonic homogenizer (Sartorius Group, Bangalore, India). The cell lysate was centrifuged at 10,000 rpm at 4°C for 10 min. Pellet and supernatant were collected and run on 12.5% SDS-PAGE gel. The SDS-PAGE gels were stained and visualized as described in section 4.2.12.

20. Mass culture of recombinant protein for His-tag purification

The starter culture was inoculated to 1 L LB broth (with Amp) and grown at 37°C till OD600 reached 0.5-0.8. The cells were induced with 0.05 mM IPTG and grown at 37°C for 4 h. Cells were collected by centrifugation at 5000 rpm for 10 min. The cell pellet was suspended in 40 ml lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole, pH 8.0) and sonicated for 4 min (60% cycle duty). The cell lysate was centrifuged at 10,000 rpm at 4 °C for 20 min. Pellet and supernatant were collected and run on 12.5% SDS-Page gel.

21. His-tag purification of rHaemathrins

Fifteen ml Ni-NTA (Nickel-nitrilotriacetic acid) agarose slurry was taken and washed with distilled water and equilibrated with lysis buffer containing 10 mM imidazole (pH 8.0). The buffer was removed and 20 ml of the lysate was added to it. The mixture was incubated in ice with constant shaking for 2 h and then gently applied into column while keeping the outlet closed. The unbound fraction was collected in a fresh tube, followed by washing with 2 column volume of lysis buffer containing 10 mM or 20 mM imidazole. The bound recombinant protein was then eluted with 2 column volume of lysis buffer containing 100 mM imidazole. The fractions were collected and analyzed on 12.5% SDS-PAGE gel.

22. Dialysis of partially purified rHaemathrins

The partially purified rHaemathrins were dialyzed against (a) 25 mM NaH2PO4, 200 mM NaCl, 5 mM Imidazole (pH 7.4), (b) 10 mM NaH2PO4, 100 mM NaCl, 2 mM Imidazole (pH 7.4), (c) 5 mM NaH2PO4, 100 mM NaCl, 1 mM Imidazole (pH 7.4), and (d) finally the protein was dialyzed in 20

mM Tris-Cl (pH 7.4) for 8 h at 4°C using SnakeSkin™ Pleated Dialysis Tubing (Thermoscientific, MA, USA). The dialyzed sample was analyzed on 12.5% SDS-PAGE gel.

23. Cleavage of fusion protein by thrombin:

Haemathrin 1 or haemathrin 2 was incubated with human α-thrombin (Haematologic Technologies) or human factor Xa (Haematologic Technologies) at a ratio of 5:1 (thrombin) or 10:1 (FXa) for 0 – 48 h. The reactions were quenched by addition of 0.1% triflouroacetic acid (TFA) (v/v) and separated by reverse-phase chromatograhpy. The cleavage products were also loaded onto electrospray ion-trap mass spectrometer for MS/MS analysis. The sample (~40 µl) was injected into a Zorbax C18 column (150 x 4.6 mm, 5 µm, Thermo Scientific) pre-equilibrated with 0.1% formic acid and the eluent was directly fed to the mass spectrometer. The different molecular masses were obtained and the peptidic fragments were identified using FindPept server (http://web.expasy.org/findpept/).

24. Site-directed mutagenesis

Using Quick Change mutagenesis kit, mutation of the thrombin cleavage sites of rHaemathrins. Briefly, primers were designed with the desired change to R18N and R53N for rHaemathrins 1 and K21N and R53N for rHaemathrin 2 (given below). The oligonucleotide primers with the desired changes are extended by PfuUltra HF DNA polymerase which copies the recombinant plasmid. Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease (target sequence: 5′-Gm6ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. Dpn I treated nicked vector DNA is then transformed into competent cells and are selected. Plasmids are isolated from the transformed bacterial colonies and sequenced to confirm the mutation.

Haemathrin 1	**H1-RN18f**:	5`-AGAGCTAACCTAGTTAATGTACAAGAAC-3`
	H1-RN18r:	5`-CTTTTGCCCTTTGTTGCCTT-3`
	H1-RN53f:	5`-AACCTTGGACGAAAGAATGC-3`
	H1-RN53r:	5`-TGGTCTCGCCGTCGGTG-3`
Haemathrin 2	**H2-KN21f**:	5`-GTTAACGTACAAGAACGTTCTAGC-3`
	H2-KN21r:	5`-TAGCAGAGCTCTCTCTTTCTC-3`
	H2-RN53f:	5`-AACCTCGGACGAAAGAATGC-3`
	H2-RN53r:	5`-TGGTCTGGCAGTGGGTG-3`

25. Expression and purification of mutant proteins

Single colonies of *E. coli* BL21(DE3)pLysS transformed with recombinant with pET32a(+) [rpET32a(+)] were picked from LB agar plates and inoculated in 4 ml LB broth with ampicillin and and incubated overnight with shaking at 37°C. This starter culture was then inoculated in 100 ml LB broth with ampicillin and incubated at 37°C under continuous shaking condition till the OD_{600} reached 0.5-0.8. Before the induction process ~5 ml of culture was taken and kept separately to serve as uninduced cell sample. IPTG was added to the culture to a final concentration of 0.1 mM and incubated at 37°C for 3 hours. The cells were collected by centrifugation at 8,000 rpm for 3 min at 4°C, and supernatant was discarded. A small pinch from the cell pellet was taken out and kept separately to serve as the whole cell lysate sample. The cell pellet was then re-suspended in 4 ml of lysis buffer (50mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and sonicated for 5 min (60 amplitude, 60% cycle duty) in a Labsonic M Ultrasonic homogenizer (Sartorius Group, Bangalore, India). The cell lysate was centrifuged at 12,000 rpm for 10 min at 4°C. Pellet and the supernatant were collected separately and run on 12.5% SDS-PAGE gel. The SDS-PAGE gels were stained and visualized under white light to document the over-expression of the protein. The recombinant proteins were purified using His tag columns and characterized.

26. Cleavage of mutant haemathrins by thrombin and analysis by RP-HPLC

Cleavage of mutant haemathrin by thrombin and its analysis was carried out as described for rHaemathrins.

27. ESI-MS analysis of recombinant haemathrin 1 and 2

The molecular masses of the peptides were determined on Accela LCQ Fleet™ Mass Spectrometer (Thermo Scientific, CA, USA). The mass spectrometer was equipped with an electrospray ion source (ESI). Data were acquired in positive ion mode and scanned from m/z 500–2000. Peptides were diluted with MiliQ water to a final concentration of 10 µM and injected into an automated sampler. Xcalibur™ software (Thermo Scientific) was used to generate the intact mass spectra for the peptides and later deconvoluted using deconvolution software for intact molecular weight determinations.

28. Circular Dichroism (CD) measurements of haemathrins

Far-UV CD spectra (260 – 190 nm) of 20 µM peptides in 20 mM Sodium phosphate buffer pH 7.4 were recorded in a 0.2 mm path-length quartz cuvette at 20°C with a 0.2 nm resolution, a bandwidth of 2 nm, and a scan speed of 50 nm/min using a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan), with a Peltier system to control cell temperature. The CD intensities were expressed as molar ellipticity, [θ], with the unit deg cm^2 $dmol^{-1}$.

29. Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli [36] with some modifications. Briefly, for 12.5% resolving gel, 8.3 ml of Acrylamide/bisacrylamide (29:1) was added to a final volume of 20 ml containing 0.1% SDS, 4% glycerol, 5 ml 1.5 M Tris-Cl (pH 8.8), 0.1% Ammonium persulfate (APS) and 20µl N, N, N', N'-tetramethylethylenediamine (TEMED). 5% stacking gel was prepared by mixing 0.65 ml 30% Acrylamide/bisacrylamide, 0.1% SDS, 4% glycerol, 1.25 ml 0.5 M Tris-Cl (pH 6.8), 0.1% APS and 7.5 µl TEMED to a final volume of 5 ml. The samples were prepared by adding sample buffer (2% SDS, 10% glycerol, 0.2% Bromophenol blue, 0.25 M Tris-Cl, pH 6.8) containing 3% 2-mercaptoethanol and heated for 2 min in boiling water. After electrophoresis at constant current of 20-30 mA in Tris-Glycine-SDS buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS), the gel was silver-stained. The gel was photographed and documented.

30. Silver staining of SDS-PAGE gel

After electrophoresis, the gel was removed from the cassette and stained with silver stain (Pierce). Briefly, the gel was washed for 2 min in ultrapure water for two times. The gel was fixed in 30% ethanol: 10% acetic acid solution for 1 h, followed by washing twice in 10% ethanol for 2 min each, then twice in ultrapure water for 2 min each. The gel was then sensitized in the sensitizer solution (50 µl Sensitizer solution with 25 ml water) for 1 min and washed with water twice for 1 min each. The gel was stained with the staining solution (0.5 ml Enhancer with 25 ml Stain) for 30 min, washed twice with ultrapure water for 20 s each and developed for 2-3 min in developer solution (5 ml Enhancer with 25 ml Developer) until bands appeared. The reaction was stopped with 5% acetic acid for 10 min.

31. Preparation of platelet poor plasma from citrated goat plasma

For the preparation of platelet poor plasma (PPP), citrated goat blood was centrifuged twice at 2500 g for 20 min at room temperature. The PPP was then collected in 2 ml aliquots and stored at -20°C till further use [37]. The plasma was used within 4 h after thawing.

32. Coagulation assay

Recalcification time assay was done according to Doley and Mukherjee (2003) [37], with some modification. 100 µl of citrated goat plasma was incubated with/without SGE and 50 µl of Tris buffer (pH 7.4) at 37°C for 2 min. Coagulation was triggered by adding 100 µl of 100 mM CaCl2. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed by pre-incubating citrated goat plasma (50 µl) with different concentrations of the SGE (50 µl) or 20 mM Tris-Cl pH 7.4, 100 mM NaCl at 37°C for 2 min, followed by addition of corresponding reagents (PT: 50 µl of Uniplastin; APPT: 50 µl of Liquecelin added for 3 min and reaction started with 50 µl of 20 mM CaCl2) [38]. The time of clot formation was recorded using a COAstat-1 coagulation analyzer (Tulip groups, Verna, India).

33. Hemolytic activity assay

Hemolytic activity of SGE was evaluated according to Tambourgi et. al. [39], with slight modifications. SGE/water was added to 5% RBC and volume was adjusted to 2 ml by adding 0.9% NaCl. The mixture was incubated at 37°C for 1 h and centrifuged at 5000 rpm for 10 min. Supernatant was collected and absorbance was recorded at 540 nm. The percentage hemolytic activity was calculated taking hemolysis by water as 100%.

34. PLA2 activity assay

PLA2 activity of SGE was assayed by well-diffusion method [40]. 8% egg yolk was mixed with molten agar medium after cooling to about 45°C. The mixture was poured onto 90 mm petri-plate and allowed to solidify. Wells were punched using a sterilized borer and required amount of SGE/150 mM NaCl was added into the wells and incubated at 37°C for 3-5 h. 5µg of crude *Bungarus fasciatus* venom was used as positive control.

35. Clotting time

The peptides were tested for prolongation of fibrinogen clotting time using a spectrophotometer. 50 µl of fibrinogen solution in 50 mM Tris-Cl pH 7.4, 100 mM NaCl (buffer B) (3 mg ml^{-1}, final

concentration) was incubated with 50 µl of peptides in buffer B (various concentrations) at 37°C for 2 min. Fibrin clot formation was initiated by addition of 50 µl of thrombin solution in buffer B (20 nM, final concentration) and the increase in absorbance at 650 nm was followed for 30 min.

36. Selectivity of rHaemathrins against serine protease

rHaemathrin 1 and 2 were screened against 10 proteases using chromogenic substrates – final concentrations are given in parentheses in nanomolar and millimolar, respectively: plasmin (3.61)/S2251 (1.2), TPA (36.9)/S2288 (1), urokinase (40 units/ml)/S2444 (0.3), APC (2.14)/S2366 (0.67), FXIIa (20)/S2302 (1), FXIa (0.125)/S2366 (1), FXa (0.43)/S2765 (0.65), FIXa (333)/Spectrozyme FIXa (0.4), kallikrein (0.93)/S2302 (1.1), α-thrombin (0.81)/S2238 (0.1), and trypsin (0.87)/S2222 (0.1). 100 µl of peptides (0.5 µM, 5 µM and 50 µM) were pre-incubated with 100 µl of the proteases for 2 min, followed by addition of chromogenic substrate. The release of colored product *p*-nitroaniline was monitored at 405 nm for 10 min in Infinite® 200 PRO microplate reader (Tecan, Männedorf, Switzerland). Percentage inhibition was calculated by taking the rate of increase in absorbance in the absence inhibitor as 0% [38].

37. Dose-dependent thrombin inhibition

Inhibition of amidolytic activity of thrombin by the peptides were assayed in 96-well microtiter plates in 20 mM Tris pH 7.4, 100 mM NaCl buffer containing 1 mg/ml bovine serum albumin using S2238 as a chromogenic substrate for thrombin. 100 µl of peptides (0-800 µM) were preincubated with 100 µl of thrombin (0.81 nM, final concentration) for 2 min, followed by addition of S2238 (0-100 mM). The release of colored product *p*-nitroaniline (*p*-NA) was monitored at 405 nm for 10 min in Infinite® 200 PRO microplate reader. Percentage inhibition was calculated by taking the rate of increase in absorbance in the absence inhibitor as 0%. Dose-response curve and Michaelis-Menten curve were fitted using GraphPad Prism software (GraphPad Software, Inc.) to calculate the IC_{50} and other inhibition parameters.

38. Time-dependent thrombin inhibition

Thrombin was pre-incubated with 50 µM each of rHaemathrin 1 and 2 for different time intervals (0-60 h), followed by addition of S2238. The release of *p*-NA was recorded at 405 nm in a

microplate reader after 2 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h and 60 h, as described in earlier section.

39. Chromatographic analysis of rHaemathrins treated with thrombin

Haemathrin 1 or haemathrin 2 was incubated with human α-thrombin or human factor Xa (Haematologic Technologies) at a ratio of 5:1 for 0 – 48 h. The reactions were quenched by addition of 0.1% triflouroacetic acid (TFA) (v/v) and separated by reverse-phase chromatography. The cleavage products were loaded onto electrospray ion-trap mass spectrometer for MS/MS analysis. The sample (40 µl) was injected into a Zorbax C18 column (150 x 4.6 mm, 5 µm, Thermo Scientific) pre-equilibrated with 0.1% formic acid and the eluent was directly fed to the mass spectrometer. The different molecular masses were obtained and the peptidic fragments were identified using FindPept server (http://web.expasy.org/findpept/). The identified fragments were commercially synthesized (Shanghai, China) and tested for inhibition of amidolytic activity of thrombin against S2238 as described in section 5.2.5. 50 µM of the fragments were incubated with thrombin for 2 min, followed by addition of the substrate. The release of colored product *p*-NA was monitored at 405 nm for 10 min in a microplate reader.

40. *In silico* modeling of full length peptide and thrombin cleaved peptides:

Ab initio protein structure prediction was done in QUARK server (https://zhanglab.ccmb.med.umich.edu/QUARK/) implemented with Zhang lab where models are built from small fragments by replica-exchange Monte Carlo simulation.

Homology modelling for short peptide sequence of Haemathrin1 and Haemathrin 2 was done in ExPASy SWISS-MODEL online server (https://swissmodel.expasy.org/). Madanin1 in PDB ID 5L6N was used as a template to model the short peptide sequences of Haemathrin1 and Haemathrin2.

41. Energy minimization of modelled structures:

The models generated were subjected to energy minimization in GROMACS 4.6.5. CHARMM force field was used and SPC/E water model was used as solvent. Charges on the peptides were neutralized by adding appropriate ions. Energy was minimized in 1000 steps using steepest decent minimization. The energy minimized structures were then used for docking.

42. Docking of thrombin and modelled peptides:

Bovine thrombin was used for docking with the modelled peptides. Docking was done in ClusPro 2.0 protein-protein docking server (https://cluspro.bu.edu/) [3]. It uses Fourier correlation method to evaluate many putative complex structures considering large set of the translational and rotational space of relative positions between the two molecules. The docked structure with lowest binding energy was taken for visualization and interaction studies. Binding energy was calculated in PRODIGY (http://milou.science.uu.nl/services/PRODIGY/) implemented in HADDOCK2.2 webserver. Binding energy was obtained in kcal/mol.

43. Molecular Dynamics Simulation:

Molecular dynamic studies of docked protein-peptide complexes were done in GROMACS 4.6.5 package. GROMOS96 54a7 force field and SPC/E water model was used. Same force field and water model was used for protein and protein-peptide complexes. Solvation was done in a dodecahedron box. As the simulation was performed in solvent optimized at pH 7 so, the total charge on the protein was +10. This charge was neutralized by adding 10 Cl^- ions. Energy minimization was done using steepest descent minimization in 5000 steps. NVT equilibration was performed at 300 K for 100 ps using LINCS constraint algorithm and V-rescale thermostat for temperature coupling. NPT equilibration was done at 1 bar for 100 ps using LINCS constraint algorithm and Parrinello-Rahman barostat for pressure coupling. For long range electrostatic interactions, Particle-mesh Ewald algorithm was used and Van der Waals cut off was set at 1.4 nm for short range electrostatic interactions. After NVT and NPT equilibration, production MD was run for 10 ns using the same constraint algorithm, thermostat, barostat and electrostatic algorithm.

Results:

1. Collection and Morphological identification of cattle-ticks

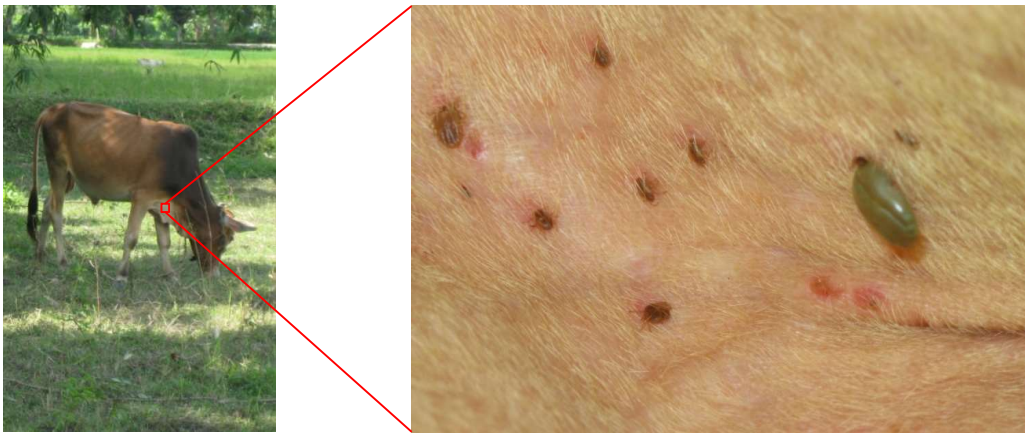


Figure 1: Representative of cattle for collection of ticks. Right panel: Ticks at different stages of feeding on host skin.

About 250 ticks (nymph, adult and full-engorged) were collected from throat and dewlap region of cattle (Fig. 1) and about 60 ticks (nymph, adult and full-engorged) from goats using tweezers. Based on morphological characters like shape of the capitulum, hypostomal dentition, bristle-bearing protuberance on palpal segment, spurs, cornua etc. these ticks were categorized. The male and female ticks were distinguished by the shape and position of genital operculum, presence of caudal appendages and adanal plates (Rhipicephaline ticks) and shape and size of festoons and spiracles (Haemaphysalinae ticks). The body weight of female Rhipicepahline ticks increased to about 130 times after feeding on host blood, while that of Haemaphysalinae ticks to about 110 times; the sizes of both the ticks increased to about 4-4.5 times after feeding (Fig. 2).

a

b

Figure 2: Female ticks (a: Rhipicephaline and b: Haemaphysalinae) at different feeding stages (Scale: 1mm).

Based on these characters the cattle ticks were morphologically identified as *Rhipiciphalus* (*Boophilus*) *microplus* and *Haemaphysalis bispinosa*, and the ticks collected from goats were found to be *H. bispinosa*. Further based on the scanning electron micrographs the adult ticks had 4/4 hypostomal dentition in both *R. microplus* and *H. bispinosa* (Fig. 3a, 3d). *R.* (*B*) *microplus* had a hexagonal basis capitulum, unlike that of *H. bispinosa* which had a rectangular basis capitulum. The ticks were identified to species level using following diagnostic morphological characters (Table 2).

Rhipicephalus (B) microplus

The species of genus *Rhipicephalus* was confirmed to be *R.* (*B*) *microplus* based on characteristics like hypostomal dentition in 4 + 4 columns and absence of bristle bearing protuberance on the internal margin of the palpal segment 1, both in male and females (Fig. 3a). Coxa 1 spurs were distinct and genital aperture posterior lip was U-shaped in female *R.* (*B*) *microplus* (Fig. 3b). Male *R.* (*B*) *microplus* was identified based on the presence of caudal appendage and lack of distinct spur like extension of the adanal plate (Fig. 3c).

Haemaphysalis bispinosa

This species was characterized by the absence of well-developed cornua. The lateral borders of the palpae were not widely salient and the tick showed prominent postero-dorsal and postero-ventral (Fig. 3d) spurs at palpal segment 3 in both, male and female tick. The postero-ventral spur of palpal segment 3 broadly triangular and blunt in both the sexes, overlapping anterior 1/3rd of palpal segment 2 in females (Fig. 3d) and anterior 1/2 of palpal segment 2 in males. The hypostome showed 4/4 rows of teeth (Fig. 3d) in both the sexes. Genital operculum in females was widely triangular in shape (Fig. 3e). The festoons in males were twice as long as broad. The spiracle in male was sub-oval, longer than broad with its dorsal and ventral sides parallel while in female spiracle was subcircular and as broad as long (Fig. 3f). Each coxa had a spure; coxa 1 possessed large spure (Fig. 3d and 3e) in both the sexes.

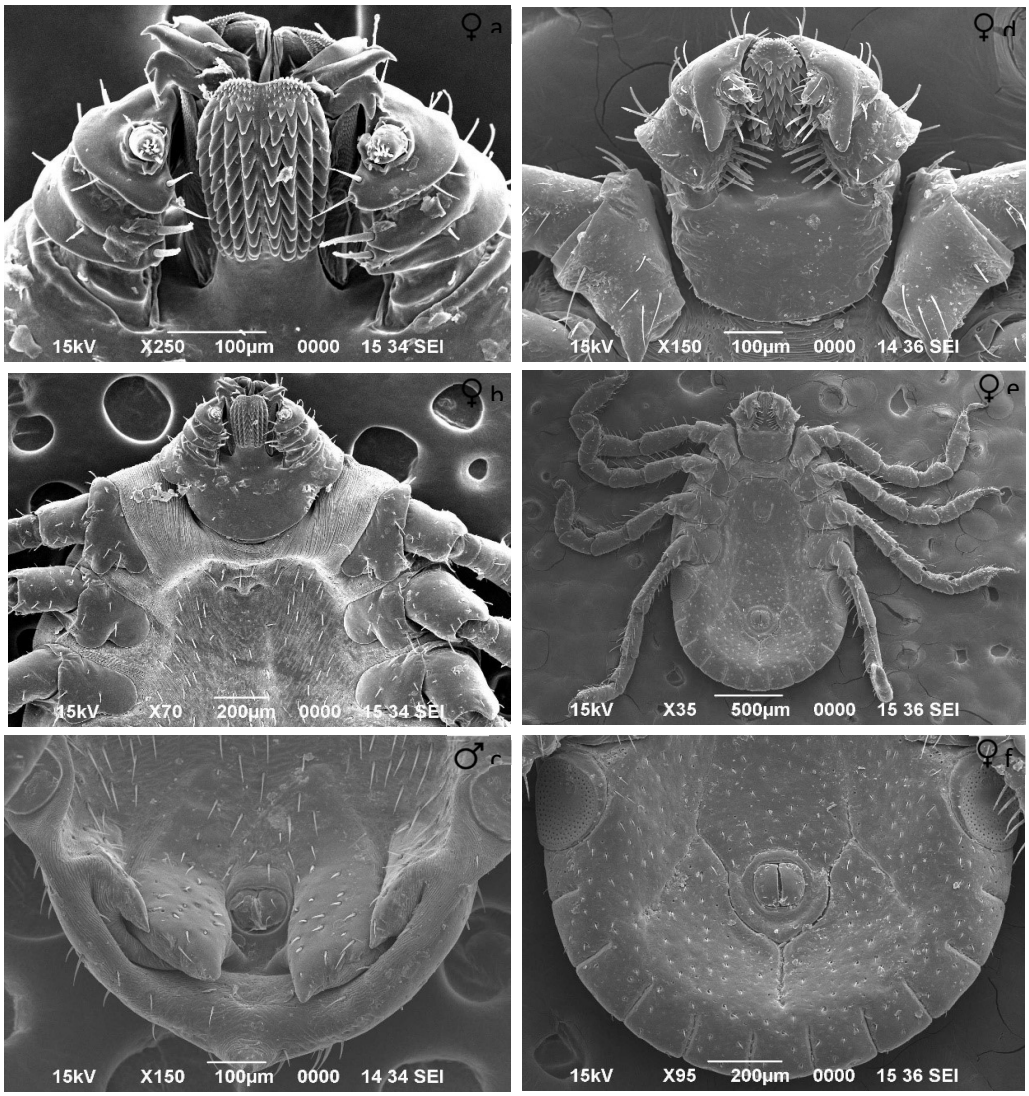


Figure 3: Electron micrographs of adult female *Rhipicephalus (B). microplus* (a, and b), adult male *R. (B). microplus* (c), and adult female *Haemaphysalis bispinosa* (d, e, and f).

Table 2: Differential morphological characters of *R*. (*B*) *microplus* and *H. bispinosa.*

Morphological keys	Tick species	
	R. microplus	***H. bispinosa***
Basis capituli	Hexagonal	Rectangular
Scutum	Inornate	Inornate
Hypostome	Very short	Short and broad
Eyes	Present	Absent
Hypostomal dentition	4+4	4+4
Palpi	Wider than long, no protuberence on palpal segment 1	Wider than long
Spurs	Distinct coxa 1 spurs	Prominent postero-dorsal and postero-ventral spurs at palpal segment 3
Anal groove	Below anus	Below anus
Festoons	None	Present, twice as long as broad in male
Ornation	None	None
Genitalia	U-shaped	Triangular in shape
Adanal plates	Present in males	Absent
Spiracles	Bluntly or elongate comma-shaped	Sub-oval in male, subcircular in female

Genomic DNA isolation from ticks

Genomic DNA was isolated from whole tick using DNeasy blood and tissue kit. The 260/280 ratios of isolated DNA samples from tick specimens were found to be 1-1.15. A prominent band for the isolated gDNA was observed at ~ 20 kb when run on 0.8% agarose gel (Fig. 4).

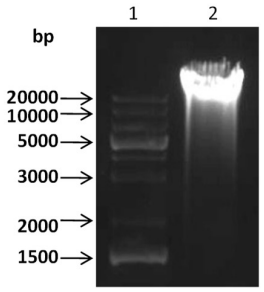


Figure 4: Analysis tick genomic DNA on 0.8% Agarose gel. Lane 1: 1 kb plus DNA ladder; lane 2: genomic DNA.

Amplification and sequencing of ITS2 and 16S rDNA

The ITS2 gene amplified from these tick specimens were found to be approximately 1500 bp and 1700 bp for *R.* (*B*) *microplus* and *H. bispinosa*, respectively (Fig. 5). While, amplified products of about 450 bp were obtained for mitochondrial 16S rDNA for each of these ticks (Fig. 6). The ITS2 and 16S rDNA genes of 3 individuals of both *R. microplus* and *H. bispinosa* were sequenced using BigDye terminator cycle sequencing (Fig. 7) and the sequences (Fig. 8&9) were submitted to GenBank of the National Center for Biotechnology Information (NCBI) (Table 3).

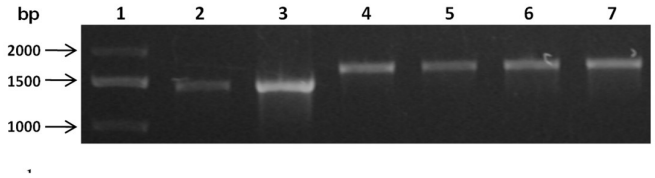


Figure 5: 0.8% agarose gel of ITS2 PCR products. Lane: 1 kb plus DNA ladder; lane 2-3: ITS2 amplified from *R.* (*B*) *microplus*; lane 4-5: ITS2 amplified from *H. bispinosa.*

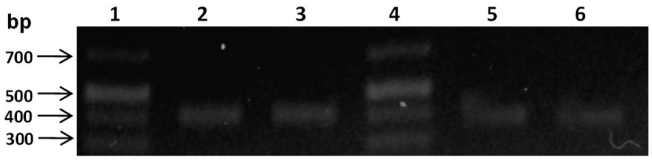


Figure 6: 0.8% agarose gel of 16S rDNA PCR products. Lane 1 and 4**:** 1 kb plus DNA ladder; lane 2-3: *R.* (*B*) *microplus* 16S rDNA PCR product; lane 5-6: *H. bispinosa* 16S rDNA PCR product.

Table 3: Sequences submitted to NCBI database with GenBank™ accession numbers.

Species	Specimen voucher no.	Host	GenBank accession no. ITS2	16S
Rhipicephalus (*B*) *microplus*	TUTEZ-121	Cattle	JX974346	JX974347
Rhipicephalus (*B*) *microplus*	TUTEZ-R1301	Cattle	KC853417	KC853421
Rhipicephalus (*B*) *microplus*	TUTEZ-R13V4	Cattle	KC879264	KC953868
Haemaphysalis bispinosa	TUTEZ-G135	Goat	KC853416	KC853420
Haemaphysalis bispinosa	TUTEZ-R1324	Cattle	KC853414	KC853419
Haemaphysalis bispinosa	TUTEZ-R1320	Cattle	KC853415	KC853418

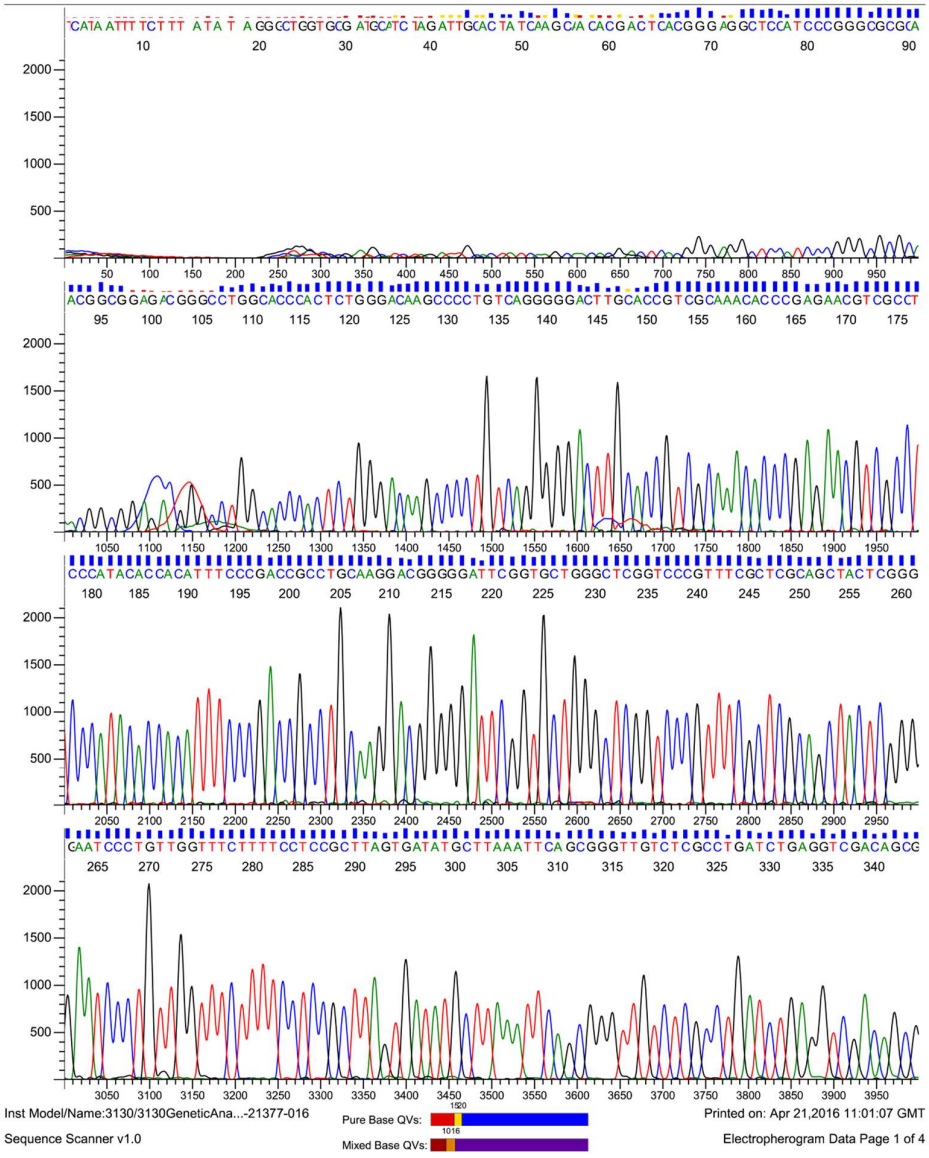


Figure 7 Chromatogram showing peaks corresponding to bases of ITS2 sequence from *R.* (*B*) *microplus* after Sanger sequencing.

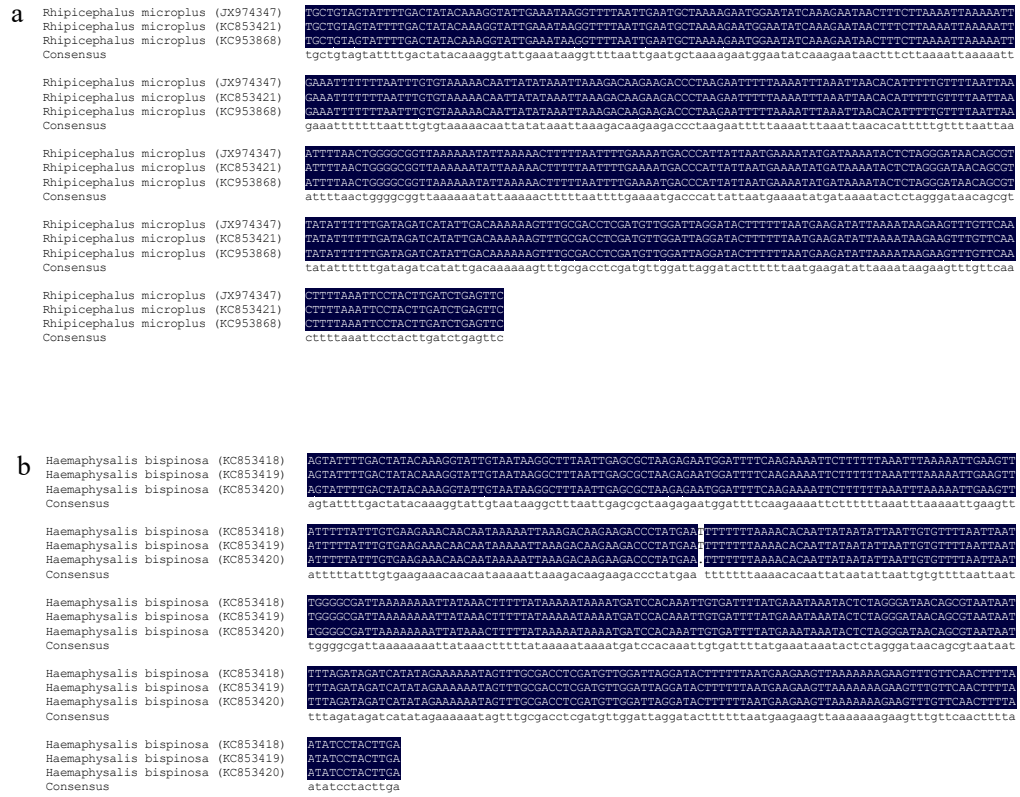
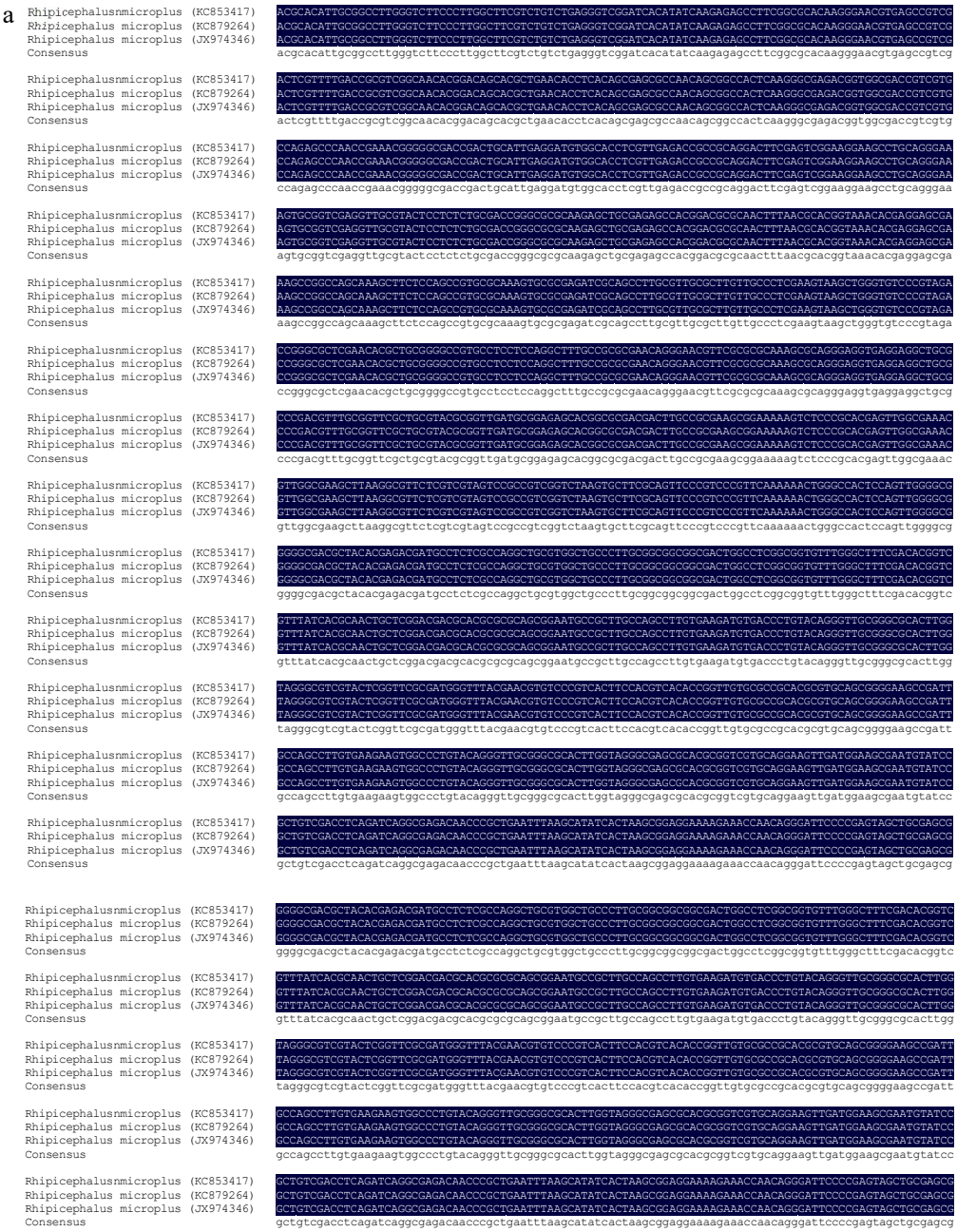


Figure 8 Nucleotide sequence alignment of 16S rDNA of (a) *R*. (*B*) *microplus* and (b) *H. bispinosa*.

a


Rhipicephalusnmicroplus (KC853417)
Rhipicephalus microplus (KC879264)
Rhipicephalus microplus (JX974346)
Consensus

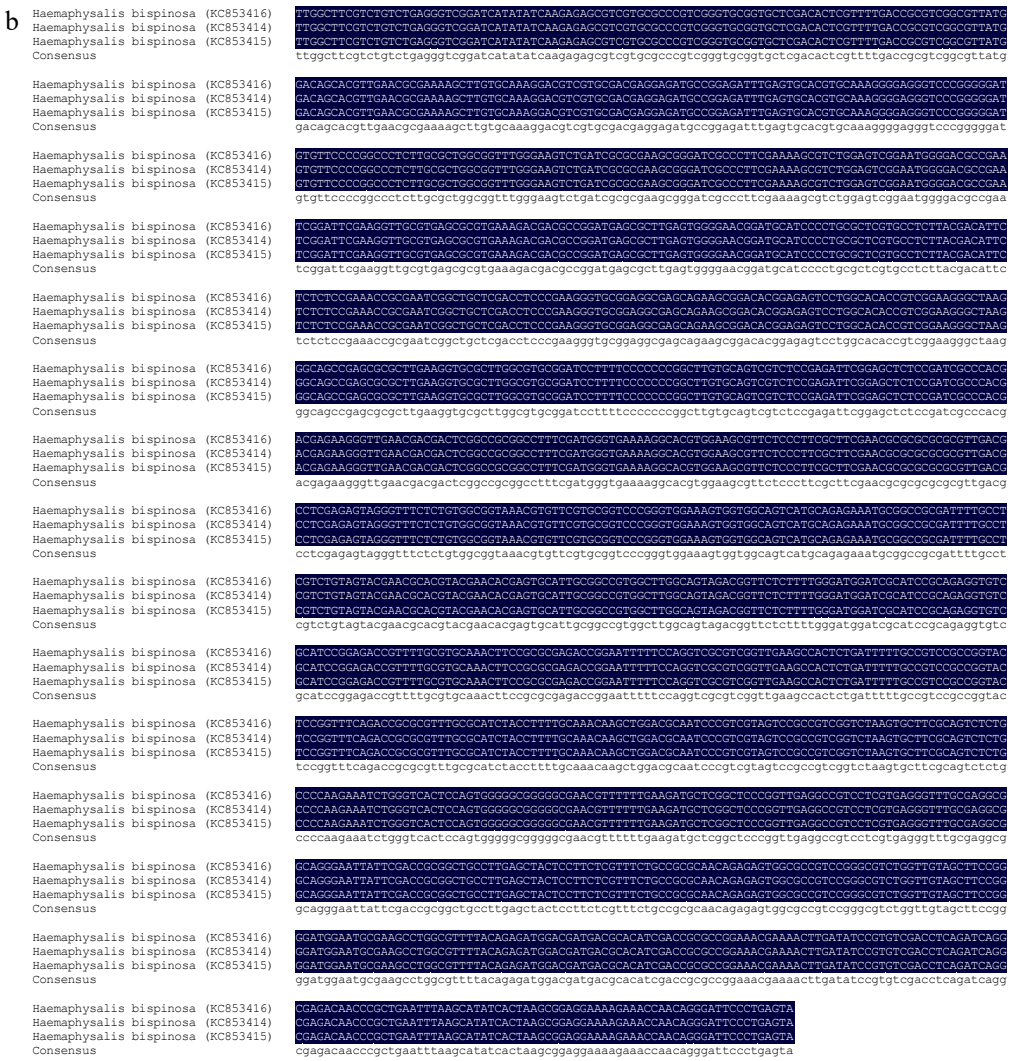


Figure 9 Nucleotide sequence alignment of ITS2 of (a) *R.* (*B*) *microplus* and (b) *H. bispinosa.*

Phylogenetic analysis

Phylogenetic relationships based on the alignment of ITS2 and 16S rDNA sequences were performed to analyze the evolutionary status of the two tick species in this study. The partial ITS2 sequences of *R.* (*B*) *microplus* and *H. bispinosa* were aligned to those of 17 tick species available in the nucleotide database and the NJ tree was constructed based on alignment of ITS2 sequences using *Ixodes scapularis* (GU319067.1) as outgroup. Phylogenetically the *Rhipicephalus* tick in the present

study is *R.* (*B*) *microplus* (Fig. 10, 11), which validates its identity concluded by morphological characterization. The tree based on ITS2 showed that *H. bispinosa* and *H. longicornis* are closely related as they were clustered together (Fig. 10). However, NJ tree constructed based on alignment of 16S rDNA sequences (Fig. 11) using *Dermanyssus gallinae* (L34326.1) as outgroup showed that *H. longicornis*, and *H. doenitzi* were clustered together with a bootstrap value of 62, while *H. bispinosa* branched out from these two suggesting that they may be closely related species. Pairwise distance analysis of the *Haemaphysalis* ticks showed that *H. bispinosa* is genetically closest to *H. longicornis* (Table 4).

Table 4: Pairwise distance of ITS2 of *Haemaphysalis bispinosa* with 5 *Haemaphysalis* and 1 *Ixodes* ticks.

	Species (accession number)	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	*Haemaphysalis bispinosa* (KC853414)	-						
2	*Haemaphysalis longicornis* (JQ346684)	0.104	-					
3	*Haemaphysalis doenitzi* (JQ346685)	0.210	0.245	-				
4	*Haemaphysalis qinghaiensis* (HQ005302)	0.253	0.272	0.270	-			
5	*Haemaphysali sflava* (JQ625712)	0.257	0.274	0.273	0.021	-		
6	*Haemaphysalis leporispalustris* (JQ868582)	0.263	0.281	0.274	0.135	0.146	-	
7	*Ixodes scapularis* (GU319067)	1.264	1.336	1.395	1.267	1.294	1.310	-

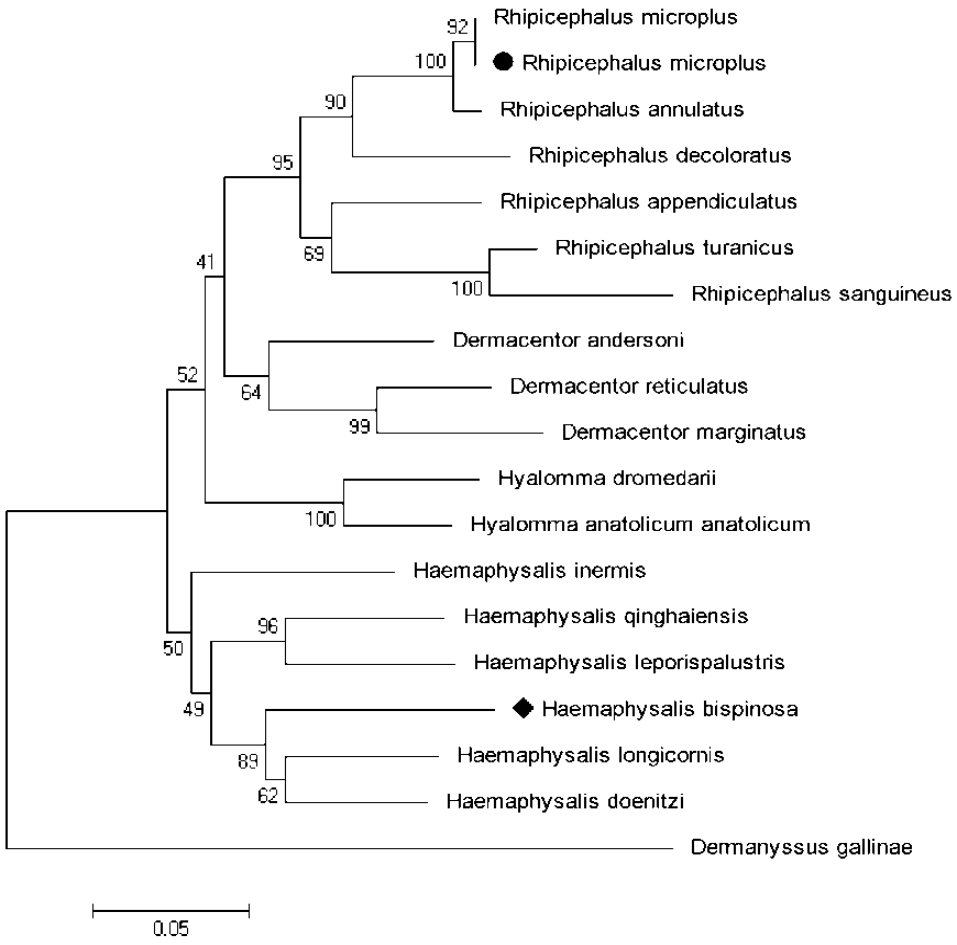


Figure 10 Neighbor-Joining tree constructed based on sequence alignment of ITS2 sequences with 1,000 bootstraps. ITS2 sequences from database are: *R. microplus* (JQ625709.1), *R. decoloratus* (U97716.1), *R. appendiculatus* (U97706.1), *R. zambeziensis* (DQ849261.1), *R. turanicus* (DQ849267.1), *R. sanguineus* (JQ625707.1), *R. punctatus* (AF271278.1), *D. andersoni* (EU520395.1), *D. reticulatus* (FM212280.1), *D. occidentalis* (DQ248056.1), *D. marginatus* (JF758644.1), *Hyalomma dromedarii* (JQ733570.1), *Hy. anatolicum anatolicum* (HQ005303.1), *Haemaphysalis flava* (JQ625712.1), *H. doenitzi* (JQ346685.1), *H. longicornis* (JQ346684.1), *H. qinghaiensis* (HQ005302.1), *H. humerosa* (AF199115.1), *H. leporispalustris* (JQ868582.1), and *I. scapularis* (GU319067.1).

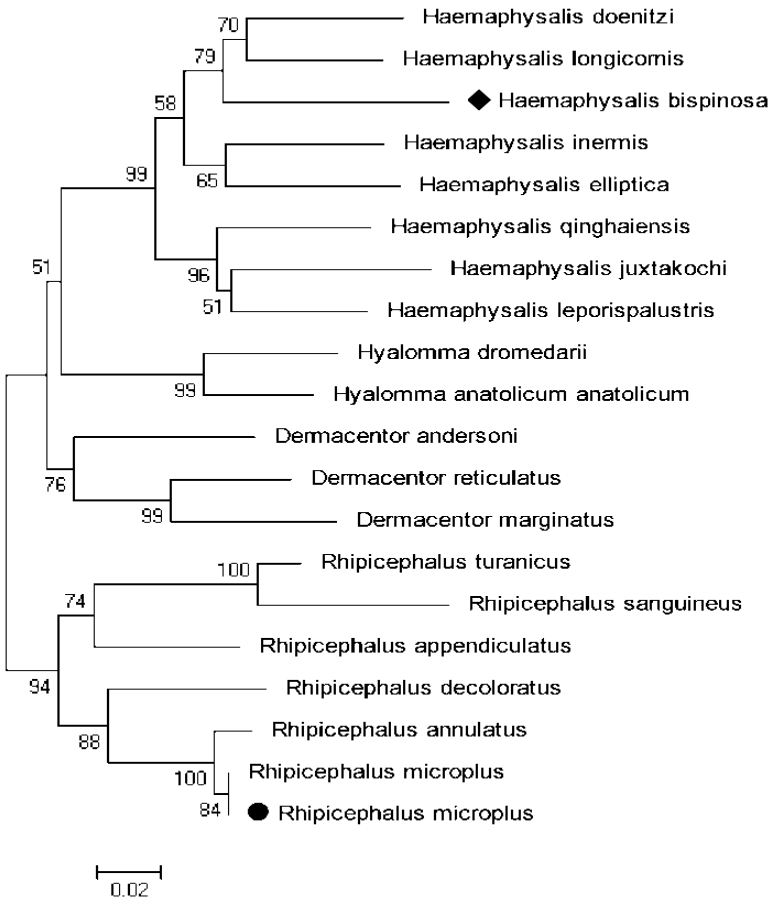


Figure 11 Neighbor-Joining tree constructed based on sequence alignment of 16S rDNA sequences with 1,000 bootstraps. 16S rDNA sequences are *R. microplus* (EU918188.1), *R. decoloratus* (EU918193.1), *R. annulatus* (Z97877.1), *R. appendiculatus* (L34301.1), *R. turanicus* (L34303.1), *R. sanguineus* (KC243838.1), *D. andersoni* (L34299.1), *D. reticulatus* (JF928516.1), *D. marginatus* (Z97879.1), *Hy. dromedarii* (L34306.1), *Hy. anatolicum anatolicum* (JX392003.1), *H. doenitzi* (JF979402.2), *H. longicornis* (FJ712721.1), *H. qinghaiensis* (FJ712720.1), *H. leporispalustris* (L34309.1), *H. elliptica* (HM068961.1), *H. juxtakochi* (AY762324.1), and *H. inermis* (U95872.1).

PCR-RFLP analysis of ITS2

Restriction maps analyzed using online tools revealed *Hind*III to be suitable enzyme for distinguishing the two species in the present study, as ITS2 sequence of *R.* (*B*) *microplus* has three and *H. bispinosa* has two *Hind*III restriction sites (Fig. 12). The results of PCR-RFLP assay could differentiate between the two ticks based on the sequence differences of ITS2. Digestion of ITS2 of *R.* (*B*) *microplus* by *Hind*III resulted into three bands (around 700 bp, 500 bp and 300 bp), whereas that of *H. bispinosa* was digested into two bands (around 1500 and 200 bp) (Fig. 13). The PCR-RFLP assay had identical profiles for the two developmental stages of *H. bispinosa*

Name: Haemaphysalis bispinosa ITS2

Conformation: linear

Enzymes: HindIII

Noncutters:

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
HindIII	AAGCTT	6	five_prime	1	121

Name: Rhipicephalus microplus ITS2

Conformation: linear

Enzymes: HindIII

Noncutters:

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
HindIII	AAGCTT	6	five_prime	2	512, 806

(Fig. 13; Lane 3 and 4 represents nymphal ticks, Lane 5 and 6 represents adult ticks).

Figure 12 Restriction sties were mapped using online tool RestrictionMapper.

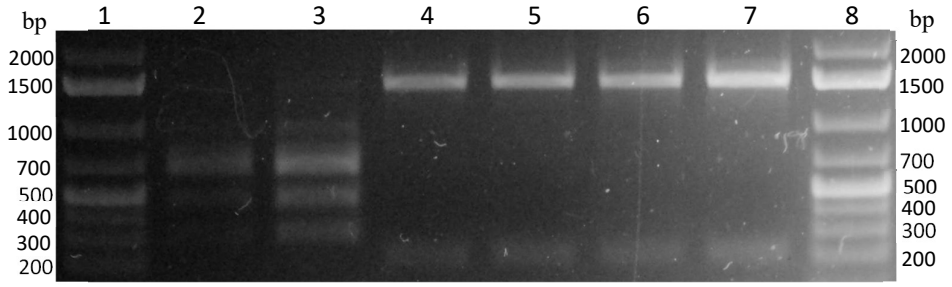


Figure 13 PCR-RFLP analysis of ITS2 gene using restriction enzyme *Hind*III. Lanes 2 and 3: *R. microplus*; lanes 4-7: *H. bispinosa* samples; lane 1 and 8 represents 1 kb plus DNA molecular weight marker.

2. *Isolation of salivary gland and preparation of Salivary Gland Extract*

The salivary glands from the fully fed ticks were successfully dissected out under a microscope (Fig. 14). The glands appeared as clear, grape-like structures. A pair of glands was extracted from each tick specimen.

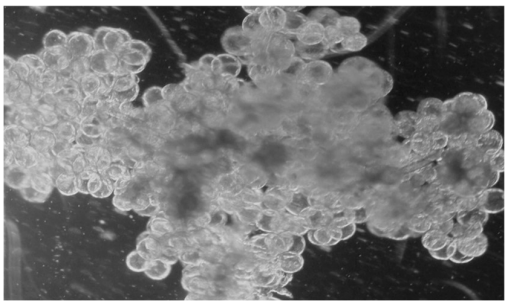


Figure 14 Representative of a salivary gland dissected out from *H. bispinosa* female tick (10x magnification).

SDS-PAGE gel analysis of Salivary Gland Extract

Homogenized and clarified lysate of salivary glands, which constituted the SGE, was run on 12.5% SDS-PAGE gel (Fig. 15). Abundance of a large number of proteins ranging from 25 kDa to 250 kDa was observed on the gel. Bands of size ranging from 10-20 kDa were also observed on the gel, which represent the size of most of the anti-coagulant proteins, mainly thrombin inhibitors, isolated from ticks.

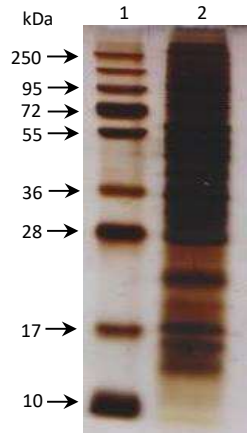


Figure 15 SDS-PAGE gel profile of salivary gland extract. Lane 1: Protein molecular weight marker; lane 2: *H. bispinosa* salivary gland extract.

Recalcification time of SGE

SGE delayed clot time of the platelet poor plasma. The recalcification time of platelet poor plasma was found to be 118 ± 4.24 s. However, the same was prolonged to 233.55 ± 1.34 s when PPP was incubated with 8 µg ml^{-1} of SGE (Fig. 16).

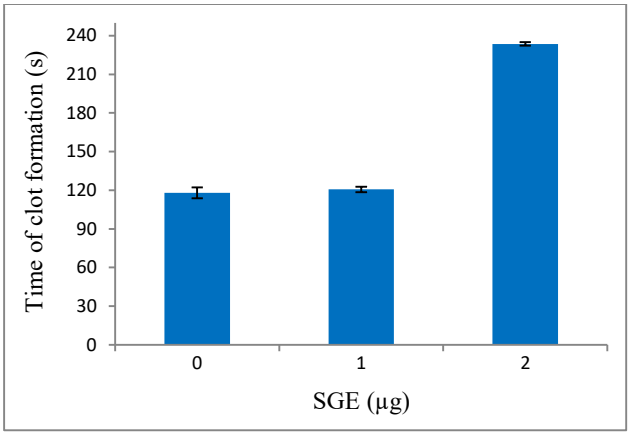


Figure 16 Recalcification time of PP plasma when pre-incubated with SGE (Salivary Gland Extract) of *H. bispinosa* (n = 3, error bars represent ±S.D.).

Activated partial thromboplastin time (APTT) of SGE

Salivary gland extract of *H. bispinosa* delayed APTT of platelet poor plasma (Fig. 17). The APPT of PPP was recorded to be 31.96 ± 0.47 s, while that for PPP treated with 8 µg ml^{-1} of SGE was found to be 86.45 ± 6.15 s, which is about 2.7 fold the normal clotting time.

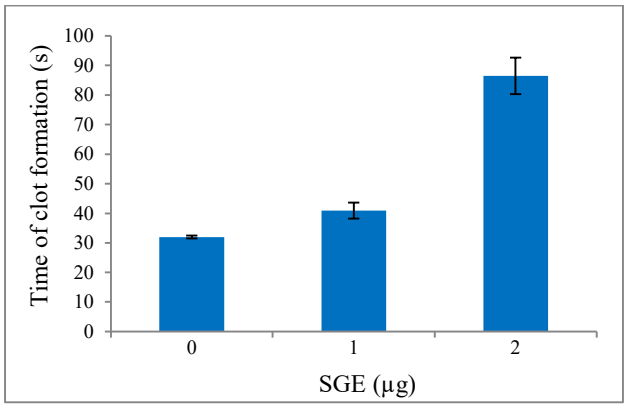


Figure 17 Activated partial thromboplastin time of PP plasma pre-incubated with SGE (Salivary Gland Extract) of *H. bispinosa* (n = 3, error bars represent ±S.D.).

Prothrombin time (PT) of SGE

Salivary gland extract of *H. bispinosa* did not delay PT of platelet poor plasma (Fig. 18). There was no visible change in the PT of PPP, when it was incubated with 8 µg ml^{-1} of SGE.

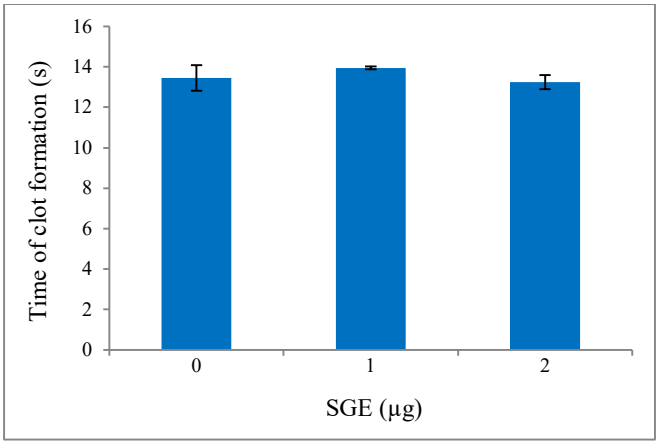


Figure 18 Prothrombin time of PP plasma pre-incubated with SGE (Salivary Gland Extract) of *H. bispinosa* (n = 3, error bars represent ±S.D.).

Hemolytic activity of SGE

Salivary gland extract of *H. bispinosa* did not show any hemolysis of RBC when tested up to 5 µg (Fig. 19), suggesting that it does not have any membrane damaging property. The lysis of RBC (positive control) in the presence of water was taken as 100%.

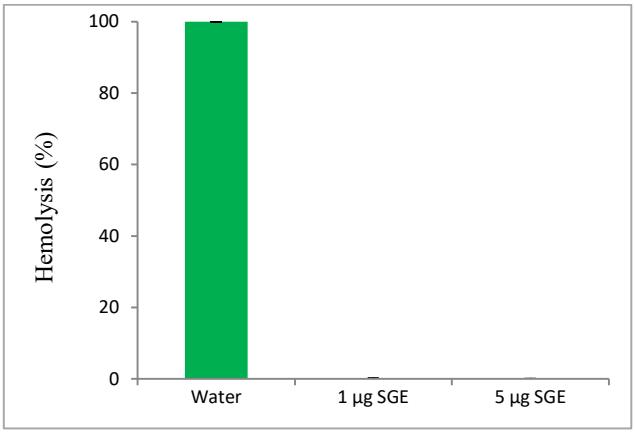


Figure 19 Percentage hemolysis of RBC by SGE (Salivary Gland Extract) of *H. bispinosa*. Percent hemolysis was calculated considering the hemolysis by water as 100% (n = 3, error bars represent ±S.D.).

3. *Isolation of total RNA from H. bispinosa salivary gland and amplification of thrombin inhibitor*

Isolation of total RNA

Total RNA was successfully isolated from salivary gland of *H. bispinosa* as observed in 1.1% agarose gel (Fig 20). The presence of bright band at about 1500 bp and 750 bp corresponding to 28S rRNA and 18S rRNA and smear indicates the quality of the total RNA isolated. The concentration of RNA was measured using NanoDrop 2000 and found to be 146 ng μl^{-1} with an A260/A280 of 1.952.

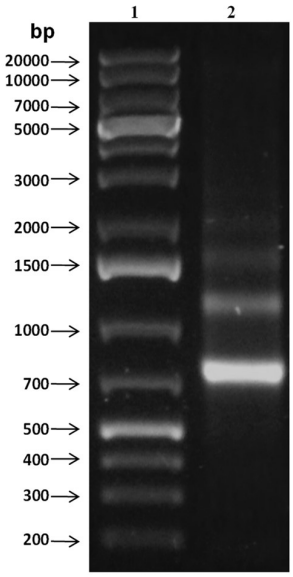


Figure 20 1.1% agarose gel profile of total RNA isolated from salivary gland. Lane 1: 1 kb plus DNA ladder; lane 2: total RNA.

cDNA synthesis from salivary gland total RNA

cDNA synthesized using Clontech cDNA library construction kit, appeared as a smear ranging from ~0.5 to ~4.5 kb on 1.1% agarose gel (Fig. 21), with few bands showing abundant mRNA at about 700-1500 kb. The concentration of synthesized cDNA was measured to be 377.2 ng μl^{-1} with A260/A280 ratio of 1.63.

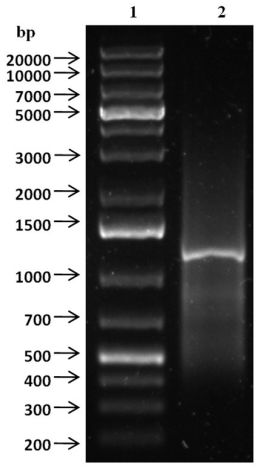


Figure 21 1.1% agarose gel profile of synthesized cDNA from salivary gland total RNA. Lane 1: 1 kb plus DNA ladder; lane 2: cDNA.

Amplification of thrombin inhibitors from H. bispinosa

cDNA coding for thrombin inhibitors were amplified from the cDNA synthesized from salivary gland total RNA of partially fed female ticks. Two amplicons of ~300 bp were obtained using the gene-specific primers (Fig. 22). The amplicon obtained using primer HBTI-F and HBTI-R1 was named as amplicon 1 and that using HBTI-F and HBTI-R2 names as amplicon 2. The concentration of amplicon 1 and amplicon 2 was calculated to be 52.5 ng μl^{-1} and 78.75 ng μl^{-1} respectively.

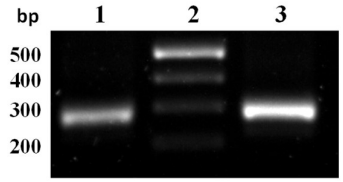


Figure 22: 1.1% agarose gel profile of amplified gene. Lane 1: Amplicon 1 amplified using HbTI-F and HbTI-R1 primers; lane 2: 1 kb plus DNA ladder; lane 3: Amplicon 2 amplified using HbTI-F and HbTI-R2 primers.

Sequencing and analysis of haemathrin 1 and 2

Sequencing of the amplified products using BigDye Terminator reagents reveals the nucleotide sequence of 331 bases. This nucleotide sequence includes the

5`and 3`UTRs and the open reading frame. The amino acid translation of the cDNA sequences had coding sequence of 234 bp encoding a protein of 78 amino acid residues and was devoid of cysteine residues (Fig. 23). Similarity searches of the two translated proteins were performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Blast search results indicated that haemathrins were similar to thrombin inhibitors, madanins (acc. no. AAP04349 and AAP04359) and madanin-like proteins (acc. no. BAE00175 and BAE00067) isolated from *Haemaphysalis longicornis* and had the Inhibitor I53 superfamily putative conserved domain (Figure 24a & b). The genes were named as haemathrin 1 and haemathrin 2. The first 19 amino acids were predicted to be signal peptide by SignalP program at CBS, Technical University of Denmark (http://www.cbs.dtu.dk/services/SignalP/). The predicted mature peptide of the proteins had calculated molecular weight of 6690.1 Da and 6709.1 Da for haemathrin 1 and 2, respectively.

Haemathrin 1

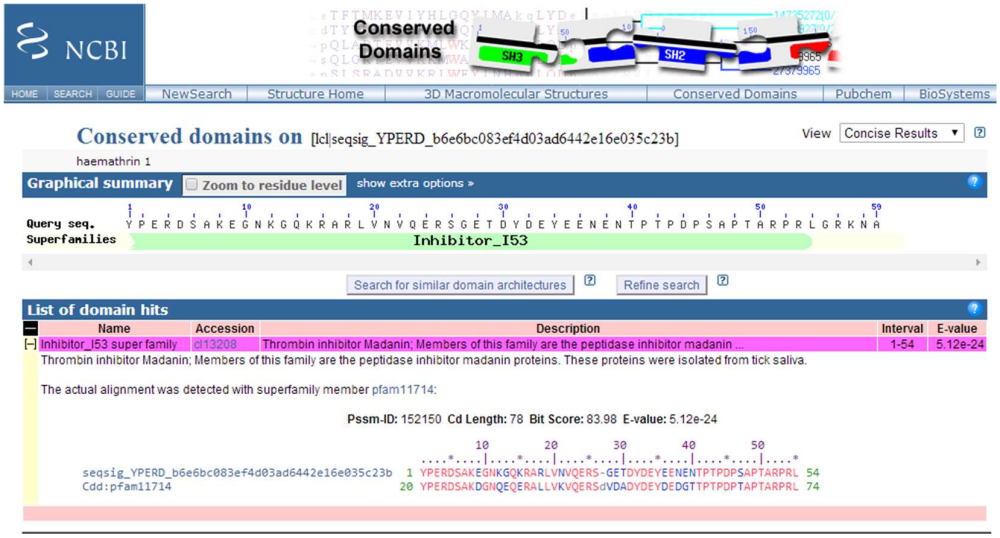
```
GGACGAAAAGATATTTTGACCGCAATGAAGCACTTCGCAATTTTTATTCTTGCTGTTGTG    60
                          M  K  H  F  A  I  F  I  L  A  V  V
GCCAGTGCCGTGGTGATGGCATACCCGGAGAGAGATTCAGCGAAGGAAGGCAACAAAGGG   120
 A  S  A  V  V  M  A  Y  P  E  R  D  S  A  K  E  G  N  K  G
CAAAAGAGAGCTCGGCTAGTTAATGTACAAGAACGTTCAGGTGAAACTGACTATGATGAA   180
 Q  K  R  A  R  L  V  N  V  Q  E  R  S  G  E  T  D  Y  D  E
TATGAAGAAAATGAAAACACTCCTACTCCGGATCCAAGTGCACCGACGGCGAGACCACGG   240
 Y  E  E  N  E  N  T  P  T  P  D  P  S  A  P  T  A  R  P  R
CTTGGACGAAAGAATGCTTGAATCAATGGTGCTCTTGATTTCTATAACCTACCGATGGCG   300
 L  G  R  K  N  A  *
GTGATGTTGTAGGCTGGAAGAAAACATCTCC                                 331
```

Haemathrin 2

```
GGACGAAAAGATATTTTGACCGCAATGAAGCACTTCGCAATTTTTATTCTTGCTGTTGTG    60
                          M  K  H  F  A  I  F  I  L  A  V  V
GCCAGTGCCGTGGTGATGGCATACCCGGAGAGAGATTCAGCAAATAGAGGCAGCCAAGAG   120
 A  S  A  V  V  M  A  Y  P  E  R  D  S  A  N  R  G  S  Q  E
AAAGAGAGAGCTCTGCTAGTTAAAGTACAAGAACGTTCTAGCCAAGATGACTACGATGAA   180
 K  E  R  A  L  L  V  K  V  Q  E  R  S  S  Q  D  D  Y  D  E
TATGATGCAGATGAGACCACTCTTTCTCCGGATCCAGATGCACCCACTGCCAGACCACGG   240
 Y  D  A  D  E  T  T  L  S  P  D  P  D  A  P  T  A  R  P  R
CTCGGACGAAAGAATGCTTGAATCAATGGTGCTCTTGATTTCTATAACCTACCGATGGCG   300
 L  G  R  K  N  A  *
GTGATGTTGTAGGCTGGAAGAAAACATCTCC                                 331
```

Figure 23: Nucleotide sequences and deduced amino acid sequences of haemathrin 1 and haemathrin 2. The first 19 amino acids (**bold**) were predicted to be signal peptide for both the peptides and the *italicized* nucleotide base are untranslated region (UTR).

a



b

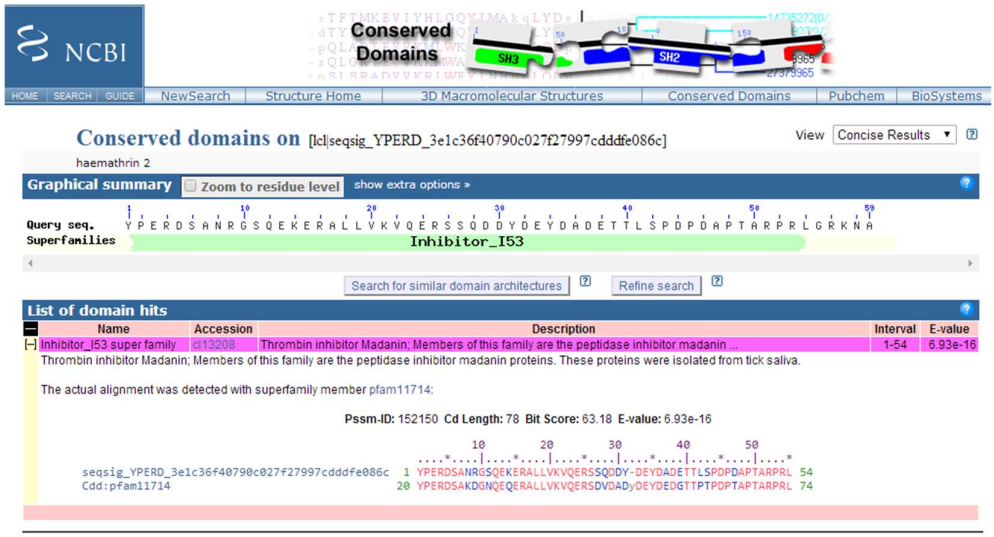


Figure 24: Conserved domains search of (a) haemathrin 1 and (b) haemathrin 2 showed that these belong to I53 superfamily of inhibitors.

Amino acid sequence alignment of haemathrin 1 and 2

The deduced amino acid sequence of mature peptide of haemathrin 1 showed 67.8 % similarity to that of haemathrin 2 (Fig. 25), which was ~3% less than the similarity between madanins (70.49 %). Haemathrin 1 showed 65% similarity to madanin 1, while haemathrin 2 was 70.9% similar to madanin 2 (Fig. 25). Hence,

haemathrins are madanin-like isoforms expressed in the salivary gland of *H. bispinosa*.

```
Haemathrin 1 YPERDSAKEGNKGQKRARLVNVQERSGETDYDEYEENENTPTPDPSAPTARPRLGRKNA 59
Haemathrin 2 YPERDSANRGSQEKERALLVKVQERSSQDDYDEYDADETTLSPDPDAPTARPRLGRKNA 59
             *******:.*.: ::** **:*****.: *****: :*.* :***.************

Haemathrin 1 YPERDSAKEGNKGQKRARLVNVQERS-GETDYDEYEENENTPTPDPSAPTARPRLGRKNA 59
Madanin 1    YPERDSAKEGNQEQERALHVKVQKRTDGDADYDEYEEDGTTPTPDPTAPTAKPRLRGNKP 60
             ***********: *:** *:**:*: *::*******: .******:****:*** ::.

Haemathrin 2 YPERDSANRGSQEKERALLVKVQERS--SQDDYDEYDADETTLSPDPDAPTARPRLGRKNA 59
Madanin 2    YPERDSAKDGNQEKERALLVKVQERYQGNQGDYDEYDQDETTPPPDPTAQTARPRLRQNQD 61
             *******: *.**************   .*.****** **** .*** * ****** :::
```

Figure 25: Alignment of amino acid sequence of mature peptides of haemathrins and madanins.

4. Cloning and Expression of Haemathrins

Cloning of cDNA coding for haemathrins

The nucleotide sequence of the mature peptide was re-amplified using gene-specific primers flanked with *Nco*I and *Xho*I restriction sites (Fig. 26). The double digested product was successfully sub-cloned into the *Nco*I and *Xho*I restriction sites of pET32a(+) expression vector. The recombinant plasmids were transformed into *E. coli* BL21(DE3)pLysS competent cells. Colony PCR of the bacterial colony confirmed the insertion of the gene of interest. Three clones each of haemathrin 1 and haemathrin 2 were found to be positive (Fig. 27 and 28). Insertion of the correct open reading frame (ORF) of the genes into the expression vector was further confirmed by sequencing of the recombinant plasmids using T7 promoter and T7 terminator universal primers.

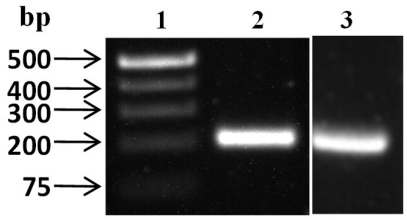


Figure 26: 1.1% agarose gel profile of PCR products of gene coding for mature peptides of haemathrin 1 and 2. Lane 1: 1 kb plus DNA ladder; lane 2: haemathrin 1 PCR product; lane 3: haemathrin 2 PCR product.

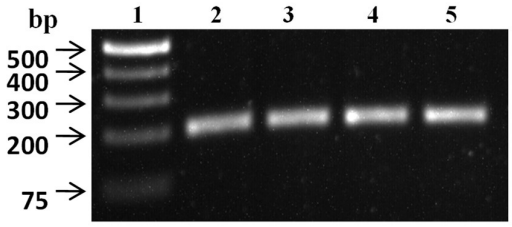


Figure 27: 1.1% agarose gel profile of colony PCR products (haemathrin 1). Lane 1: 1 kb plus DNA ladder; lane 2: Positive control; lane 3: clone 1; lane 4: clone 2; lane 5: clone 3.

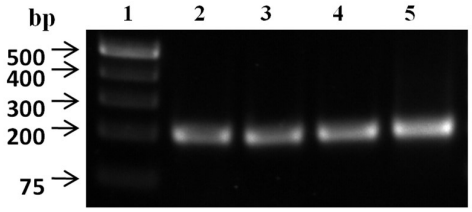


Figure 28: 1.1% agarose gel profile of colony PCR products (haemathrin 2). Lane 1: DNA molecular weight marker; lane 2: Positive control; lane 3: clone 1; lane 4: clone 2; lane 5: clone 3.

Expression of recombinant haemathrins (rHaemathrins)

E. coli BL21(DE3)pLysS transformed with recombinant vectors were induced with IPTG and the cell lysate was analyzed on SDS-PAGE. Bands of about 25 kDa were observed on coomassie-stained SDS-PAGE gel which was absent in uninduced cells. This is the expected size of rHaemathrins with the fusion tag (Fig. 29, lane 4 and 5). The molecular mass of the fusion tag is about 18 kDa (Fig. 29, lane 3) while that of haemathrins is about 6.7 kDa, which sums up to about 25 kDa.

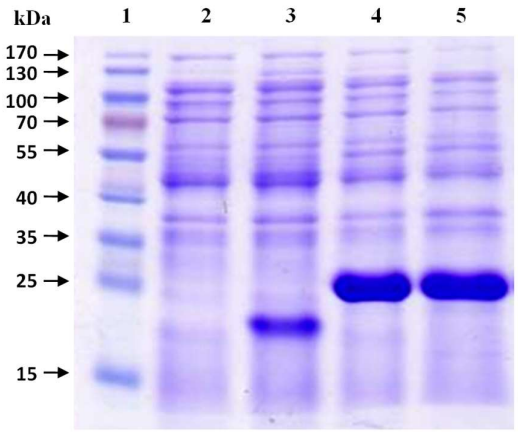


Figure 29: 12.5% SDS-PAGE gel profile of expressed rHaemathrins. Lane 1: Protein molecular weight marker; lane 2: cell lysate of uninduced cells; lane 3: cell lysate of induced cells with pET32a; lane 4: cell lysate of induced cell with recombinant plasmid (haemathrin 1); lane 5: cell lysate of induced cells with recombinant plasmid (haemathrin 2).

Effect of temperature on expression of rHaemathrin

E. coli BL21(DE3)pLysS cells transformed with rHaemathrin 2 were grown at 37ºC till the mid log phase. For induction, 0.1 mM IPTG (final concentration) was added to the culture and grown at different temperatures (16ºC, 25ºC and 37ºC). It was observed that there was no significant difference in expression of rHaemathrins at different temperatures (Fig. 30).

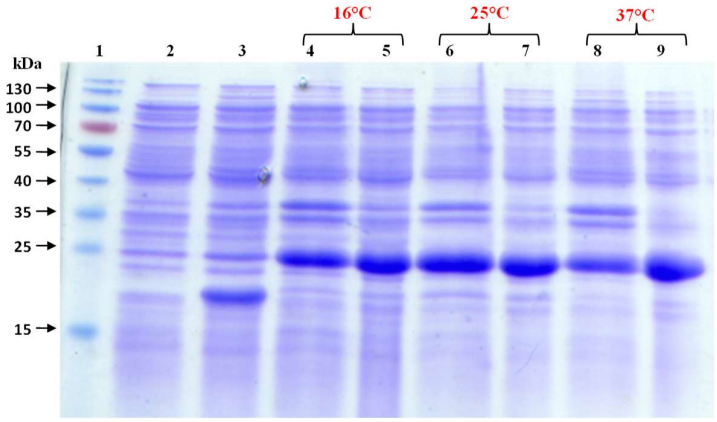


Figure 30: 12.5% SDS-PAGE gel profile of expression of rHaemathrin 2 at different temperatures. Lane 1: Protein molecular weight marker; lane 2: cell lysate of uninduced cells; lane 3: cell lysate of induced cells with pET32a; lane 4: cell pellet of induced cell at 16ºC; lane 5: cell lysate of induced cells at 16ºC; lane 6: cell pellet of induced cell at 25ºC; lane 5: cell lysate of induced cells at 25ºC; lane 8: cell pellet of induced cell at 37ºC; lane 9: cell lysate of induced cells at 37ºC.

Effect of IPTG concentrations on overexpression of rHaemathrin 2

Varying concentration of IPTG was used for over-expression recombinant haemathrins keeping the temperature constant at 37ºC. It was observed that 0.05 mM IPTG was adequate for expression of rHaemathrin (Fig. 31). Higher concentration of IPTG did not have any effect on the expression of the recombinant protein.

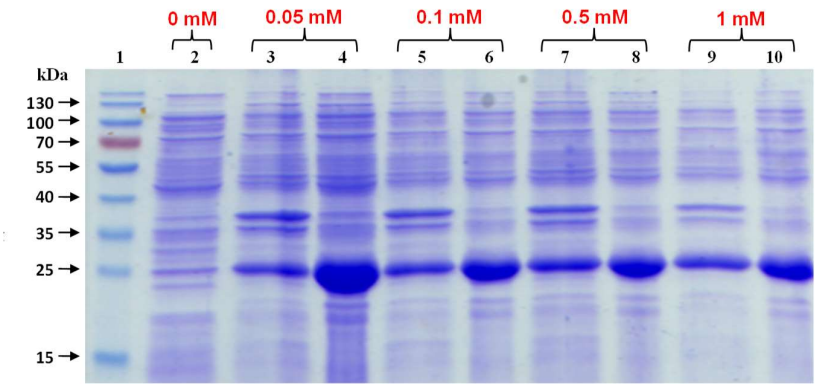


Figure 31: 12.5% SDS-PAGE gel profile of expression of rHaemathrin 2 using different concentration of IPTG. Lane 1: Protein molecular weight marker; lane 2: cell lysate of uninduced cells; lane 3: cell pellet of cells induced by 0.05 mM IPTG; lane 4: cell lysate of cells induced by 0.05 mM IPTG; lane 5: cell pellet of cells induced by 0.1 mM IPTG; lane 6: cell lysate of cells induced by 0.1 mM IPTG; lane 7: cell pellet of cells induced by 0.5 mM IPTG; lane 8: cell lysate of cells induced by 0.5 mM IPTG; lane 9: cell pellet of cells induced by 1 mM IPTG; lane 10: cell lysate of cells induced by 1 mM IPTG.

Effect of time of induction o expression of rHaemathrins

Over-expression of the recombinant proteins was also tested for different time interval at 37ºC. It was observed that the recombinant proteins were expressed as soluble and insoluble fraction at 2 h and 4 h incubation time with no visible difference in expression level (Fig. 32, lane 4 and 6).

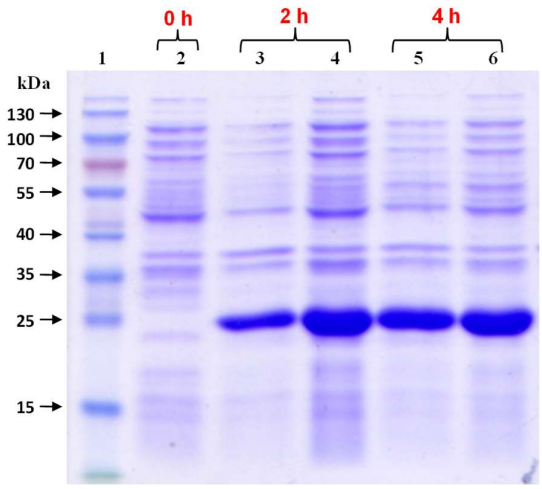


Figure 32: 12.5% SDS-PAGE gel profile of expression of rHaemathrin 2 using different time intervals of induction. Lane 1: Protein molecular weight marker; lane 2: cell lysate of uninduced cells; lane 3: cell pellet of cells induced for 2 h; lane 4: cell lysate of cells induced for 2 h; lane 5: cell pellet of cells induced for 4 h; lane 6: cell lysate of cells induced for 4 h.

Mass culture of recombinant protein for His-tag purification

For isolation of the recombinant fusion protein, 1 L culture media using standard conditions (0.05 mM IPTG induction, 37ºC incubation temperature and 4 h incubation time) was prepared. The recombinant fusion proteins of about 25 kDa (Fig. 33, lane 3 and 4) were produced on induction with 0.05 mM IPTG.

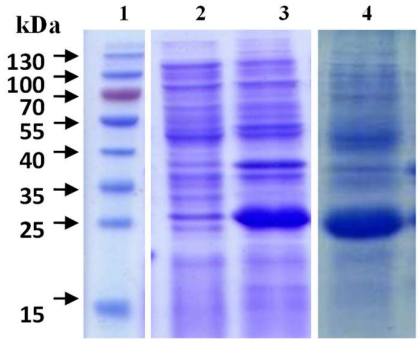


Figure 33: 12.5% SDS-PAGE gel profile of expression of rHaemathrins using optimized conditions. Lane 1: Protein molecular weight marker; lane 2: cell lysate of uninduced cells; lane 3: cell lysate of cells (rHaemathrin 1); lane 4: cell lysate of cells (rHaemathrin 2).

His-tag purification of rHaemathrins

The soluble fraction obtained from the mass culture was used for purification of the recombinant protein using Ni-NTA agrose beads. The bound recombinant proteins were eluted using 100 mM imidazole from the Ni-NTA agrose beads. Bands of ~25 kDa of partially purified rHaemathrins were observed when eluents was analyzed on SDS-PAGE gel (Fig. 34, lane 5 and Fig. 35, lane 7).

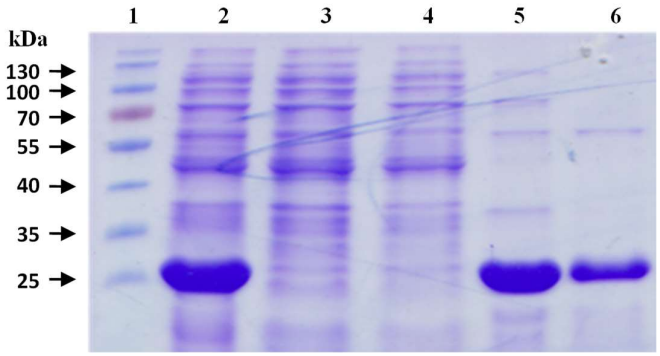


Figure 34: 12.5% SDS-PAGE gel profile of His-tag purification of rHaemathrin 1. Lane 1: Protein molecular weight marker; lane 2: cell lysate; lane 3: unbound fraction; lane 4: wash fraction 1 (20 mM imidazole); lane 5: elute fraction 1; lane 6: elute fraction 2.

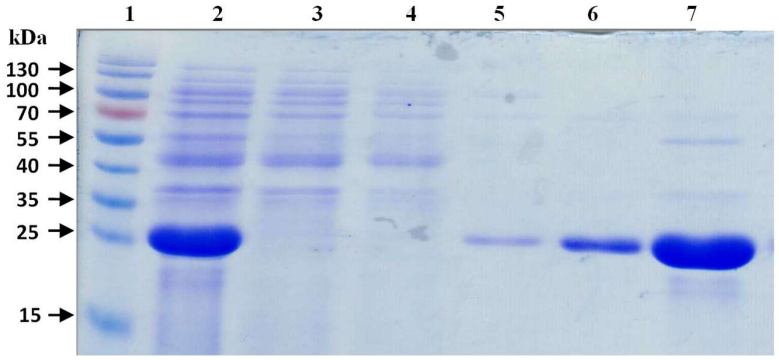


Figure 35: 12.5% SDS-PAGE gel profile of His-tag purification of rHaemathrin 2. Lane 1: Protein molecular weight marker; lane 2: cell lysate; lane 3: unbound fraction; lane 4: wash fraction 1 (10 mM imidazole); lane 4: wash fraction 2 (20 mM imidazole); lane 5: elute fraction 1; lane 6: elute fraction 2; lane 7: elute fraction 3.

Dialysis of partially purified rHaemathrins

The partially purified rHaemathrins were dialyzed using SnakeSkin™ pleated dialysis tubing to remove salts. Dialyzed rHaemathrin 1 and rHaemathrin 2 were centrifuged and analyzed on 12.5% SDS-PAGE, which showed a prominent bands at ~25 kDa which confirmed that the protein did not aggregate during this process (Fig. 36).

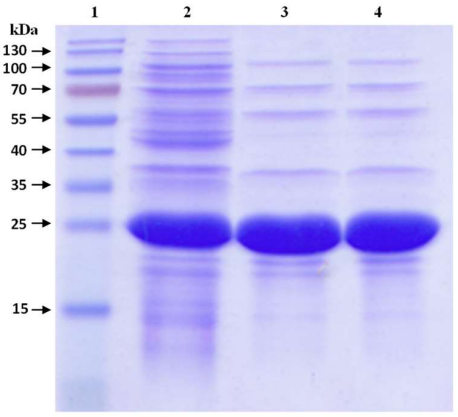


Figure 36: 12.5% SDS-PAGE gel profile of dialyzed rHaemathrins. Lane 1: Protein molecular weight marker; lane 2: cell lysate; lane 3: dialyzed rHaemathrin 1; lane 4: dialyzed rHaemathrin 2.

Cleavage of rHaemathrins by enterokinase (EK)

The fusion protein was digested with enterokinase which revealed the release of peptidic products of ~7 kDa (Circled, red) and fusion partner of ~18 kDa on Tris-tricine SDS-PAGE gel (Fig. 37a and 37b). This corresponds to rHaemathrin 1 and rHaemathrin 2 and the fusion tag, respectively. The cleaved peptides were further purified using RP-HPLC. Single peaks corresponding to rHaemathrin 1 (Fig. 38a) and rHaemathrin 2 (Fig. 38b) were obtained showing the homogeneity of the purified peptides.

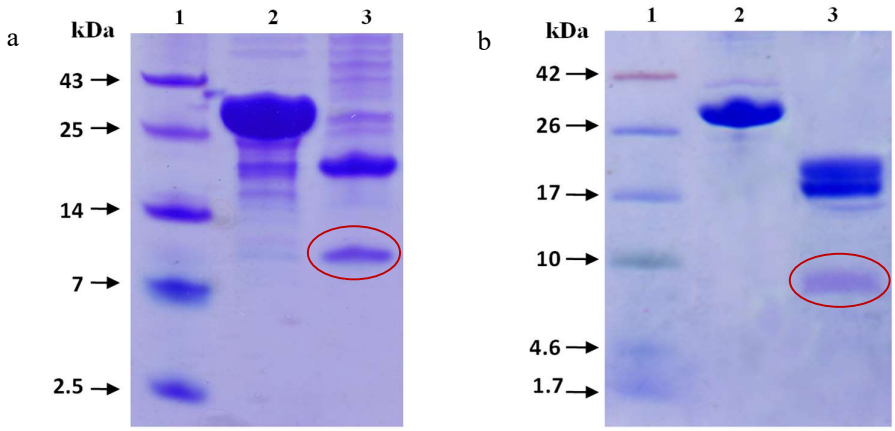


Figure 37: 18% tricine-SDS-PAGE gel profile enterokinase digested (a) rHaemathrin 1 and (b) rHaemathrin 2. Lane 1: Protein molecular weight marker; lane 2: undigested protein; lane 3: enterokinase digested protein (peptides of interest are circled).

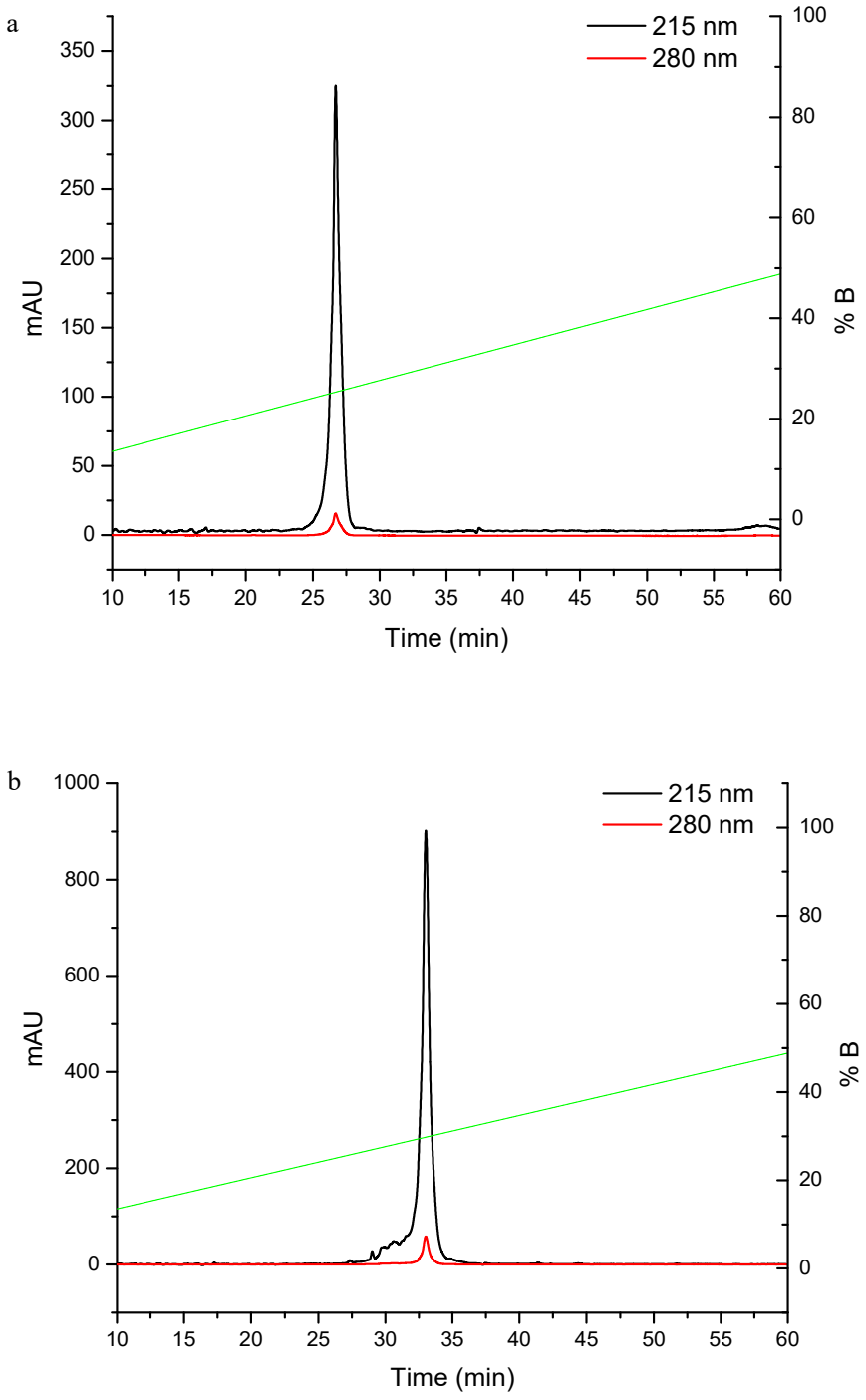


Figure 38: Chromatogram showing peaks corresponding to (a) rHaemathrin 1 and (b) rHaemathrin 2.

ESI-MS analysis of recombinant haemathrin 1 and 2

The integrity and molecular masses of the recombinant haemathrins were verified by mass spectrometry. The ESI-MS spectra of recombinant haemathrin 1 and 2 revealed a deconvulated mass of 6690.3 Da and 6709.1 Da, respectively (Fig. 39), which were in agreement with the predicted molecular masses of the proteins (6690.1 Da for haemathirn 1 and 6709.1 for haemathrin 2).

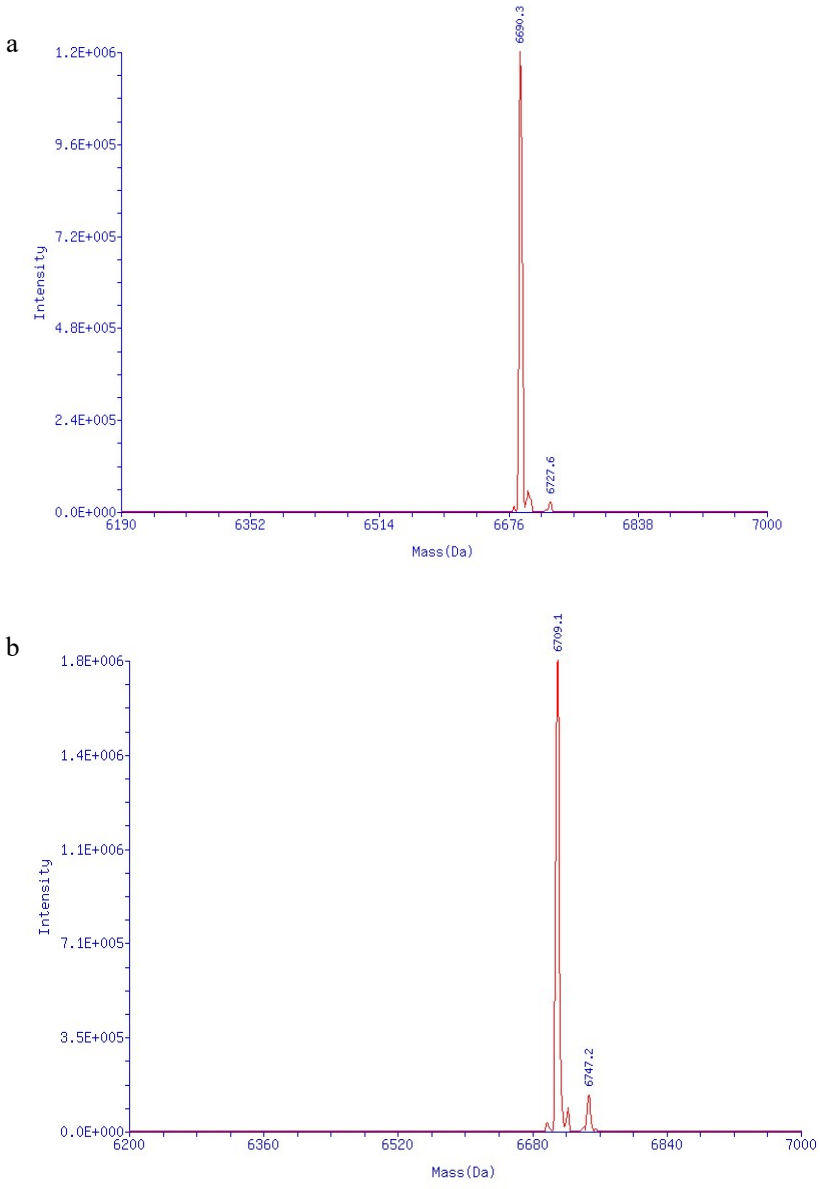


Figure 39: Deconvulated ESI-MS spectra of (a) rHaemathrin 1 & (b) rHaemathrin 2.

Circular Dichroism (CD) measurements of rHaemathrins

rHaemathrins were found to lack ordered secondary structure, as shown by their CD spectra that are characteristic of random coils (Fig. 40). This indicates that the recombinant peptides are intrinsically disordered in solution.

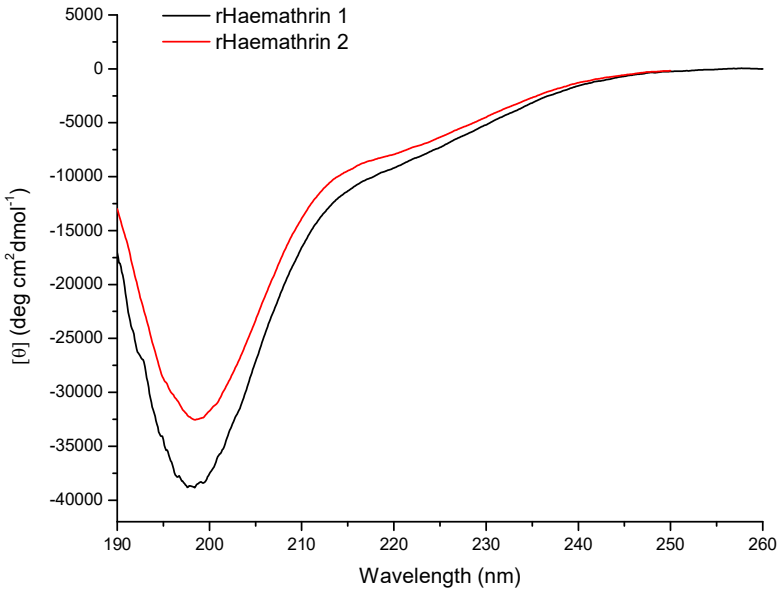


Figure 40: CD spectra of purified rHaemathrin 1 (black) and rHaemathrin 2 (red) were recorded in the far-UV region (190-260 nm).

5. *Biochemical, pharmacological and biophysical characterization of recombinant haemathrins*

Blood coagulation assay

rHaemathrins were tested for its effect on the coagulation cascade using goat platelet poor plasma and found to delay clotting time of platelet poor plasma (PPP). Thrombin time of PPP was found to be 29.93 ± 1.40 s and 33.73 ± 1.41 s in presence of 30 µM of rHaemathrin 1 and 2, respectively, whereas the normal thrombin time of PPP is 12 ± 0.25 s (Fig. 41). The PT of PPP was prolonged to 30.03 ± 0.90 s and 43.66 ± 1.45 s by 100 µM of rHaemathrin 1 and 2, respectively from 15.60 ± 0.41 s which was the normal PT of PPP (Fig. 42). Similarly the APTT of PPP was also prolonged to 51.16 ± 0.66 s and 52.26 ± 1.34 s by 30 µM of rHaemathrin 1 and rHaemathrin 2, respectively as compared to normal APTT of PPP which is 38.13 ± 0.75 s (Fig. 43). This confirms that the haemathrins are anticoagulant in nature.

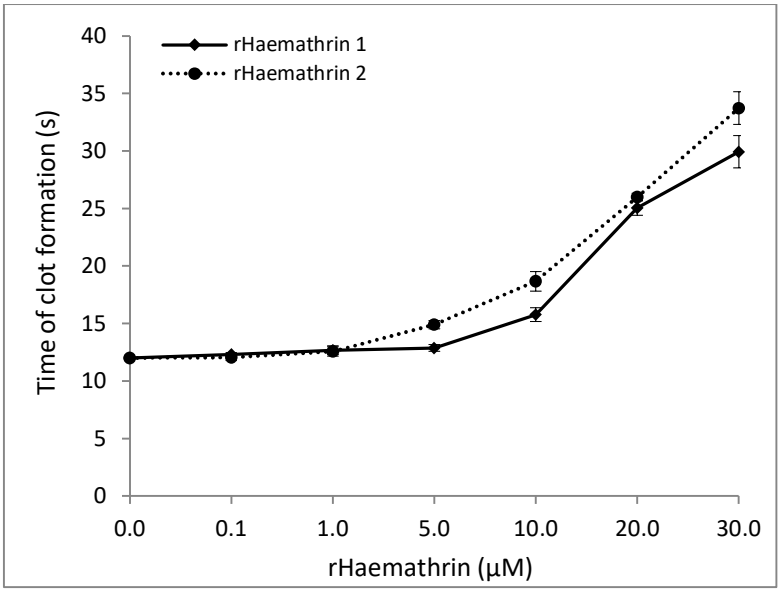


Figure 41: Graph showing increase in thrombin time of platelet poor plasma when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

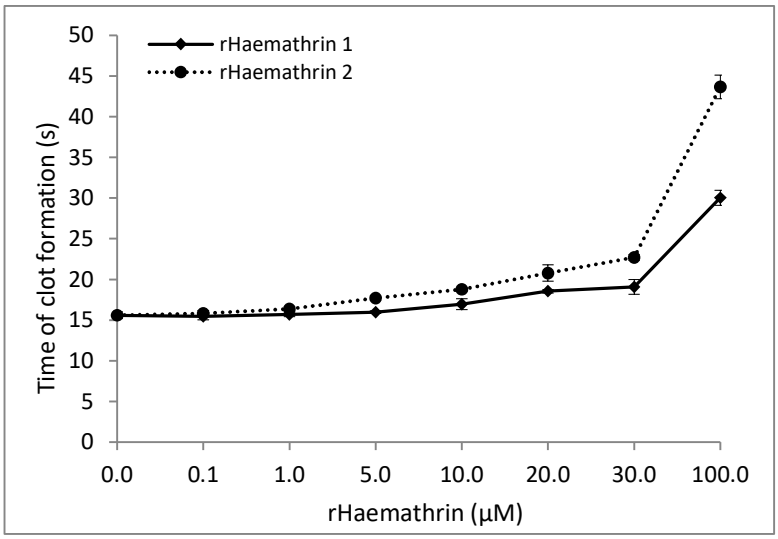


Figure 42: Graph showing increase in prothrombin time of platelet poor plasma when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

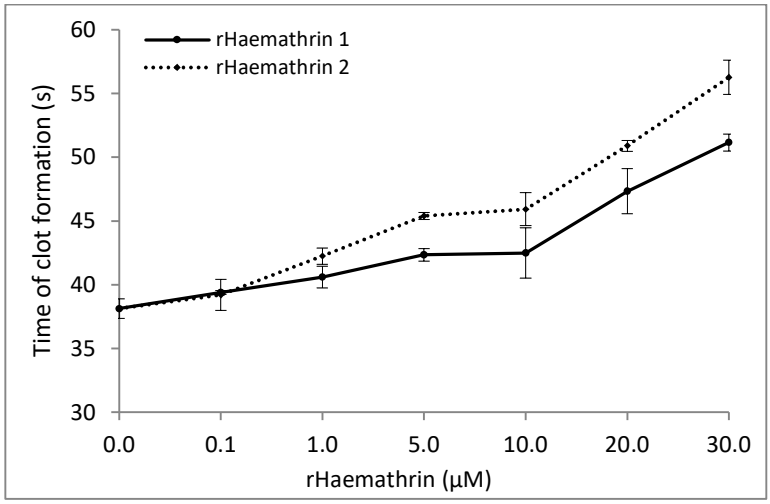


Figure 43: Graph showing increase in activated partial thromboplastin time of platelet poor plasma when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

Fibrinogen clotting time

Haemathrin 1 and 2 both prolonged fibrinogen clotting time in a dose-dependent manner (Fig. 44). At 2.5 μM concentration, haemathrin 1 prolonged fibrinogen clotting time to 227.14 ± 1.42 s and haemathrin 2 prolonged the same to

263.48 ± 2.45 s from 59.43 ± 0.86 s (normal clotting time). Though rHaemathrins are isoforms with 68% identity, rHaemathrin 2 was found to be more potent anticoagulant.

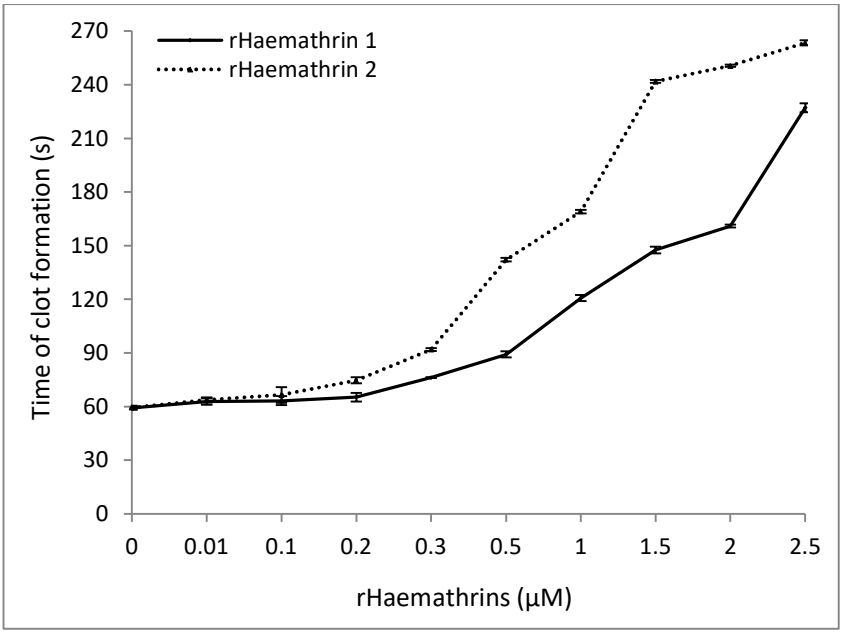


Figure 44: Graph showing increase in fibrinogen clotting time when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

Selectivity of haemathrins against serine protease

rHaemathrins were screened for specificity against 10 serine proteases, including classical serine protease trypsin. Apart from thrombin, rHaemathrin 1 and 2 did not show any significant inhibition against the tested serine proteases. rHaemathrins inhibited ~10-30% amidolytic activity of Plasmin, TPA, Fxa, FXIa and Kallikrein at a concentration of 50 µM, as compared to 70-75% inhibition against thrombin (Fig. 45 and Fig. 46). On the other hand, it was observed that haemathrins activated FIXa to some extent. The action of the proteins against APC was not conclusive though it showed negative values in both the cases.

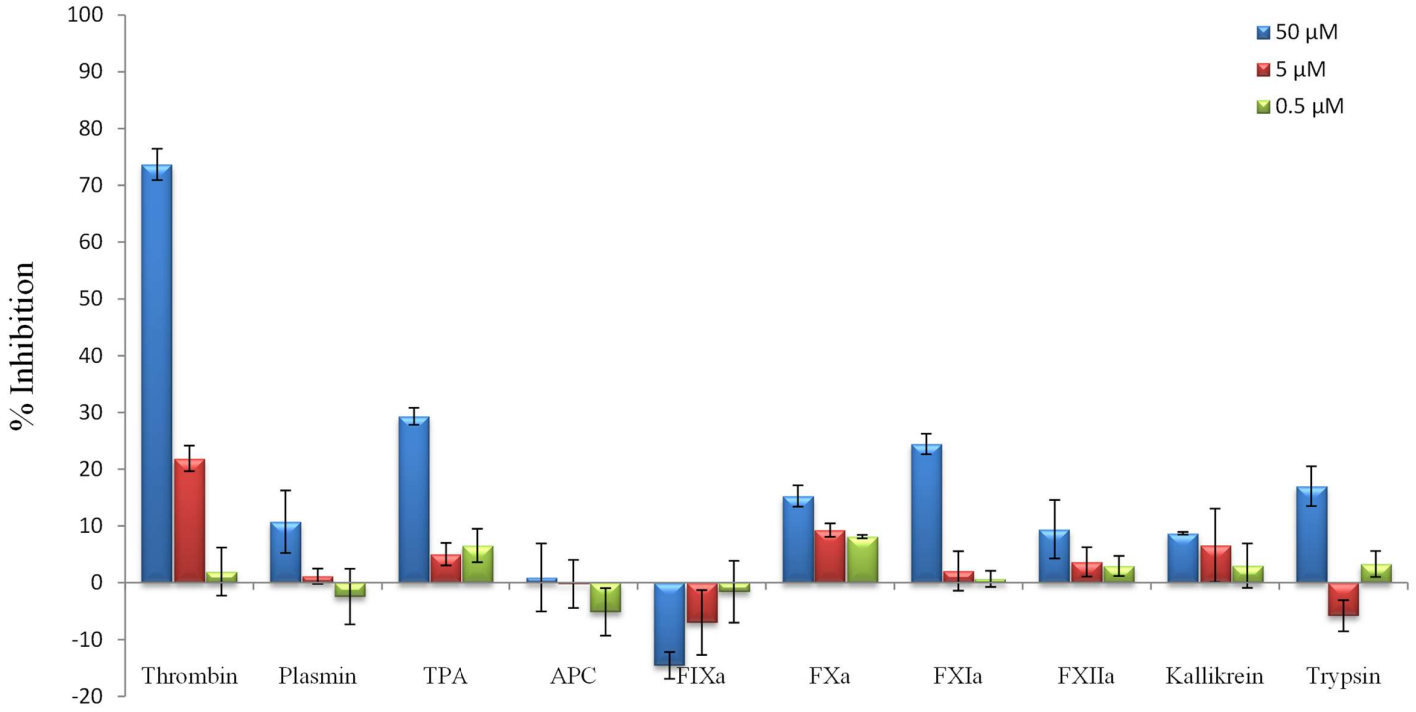


Figure 45: Selectivity profile of rHaemathrins 1. rHaemathrin 1 was screened against 10 proteases using chromogenic substrates (n=3, error bars represent ±S.D.).

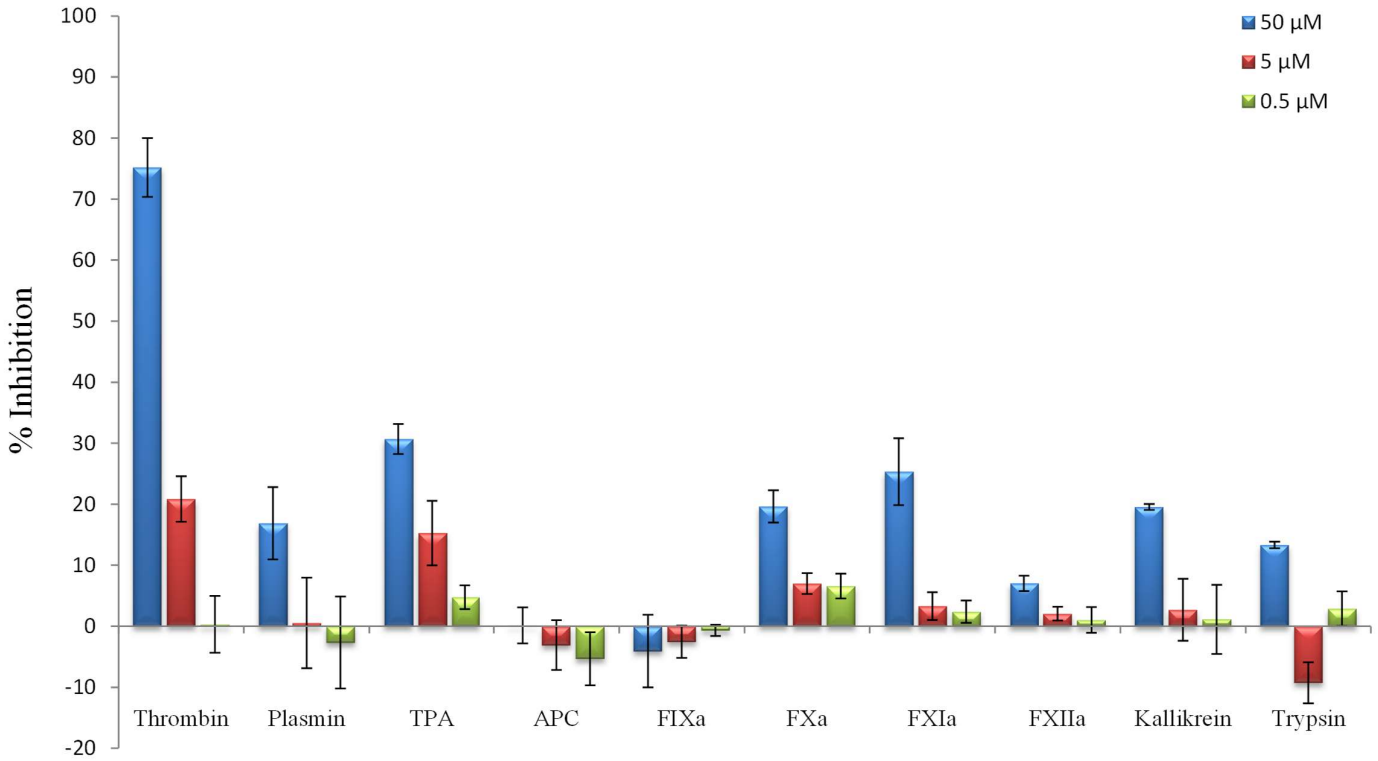
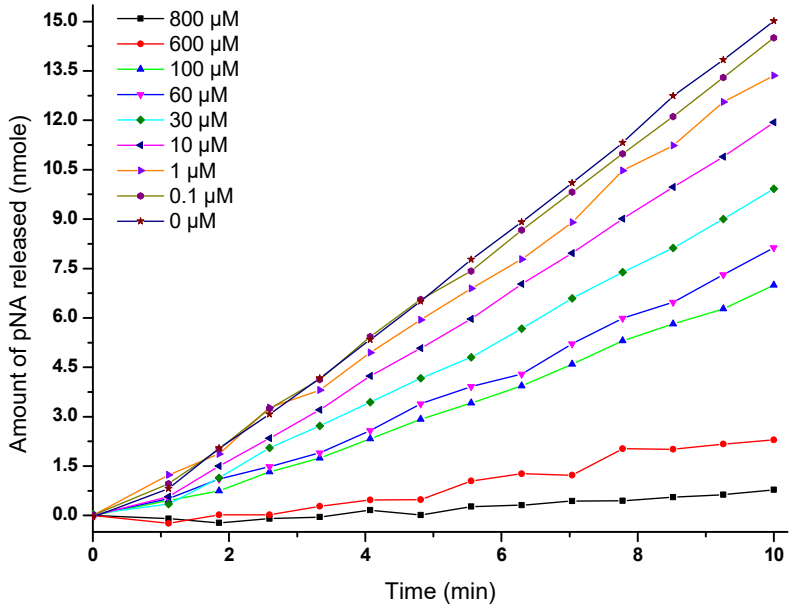


Figure 46: Selectivity profile of rHaemathrins 2. rHaemathrin 2 was screened against 10 proteases using chromogenic substrates (n=3, error bars represent ±S.D.).

Thrombin inhibitory activity

rHaemathrins inhibited amidolytic activity of human α-thrombin dose-dependently (Fig 47a&b). In addition to that, the inhibitory activity was time dependent, which indicated that rHaemathrins are slow binding-type inhibitors. It was observed that at 600-800 µM of rHaemathrin 1 and 2, 95-100% of thrombin's activity towards its chromogenic substrate was inhibited. Thus rHaemathrins are thrombin inhibitors. The IC_{50} of inhibition was calculated to be 46.13 ± 0.04 µM (R^2=0.9985) for haemathrin 1 and 40.057±0.054 µM (R^2=0.9988) for haemathrin 2 (Fig. 48). From the Michaeles-Menten curve, it was found that the Vmax of enzyme inhibition of rHaemathrin 1 and 2 decreased with increase in Km of the same, which are characteristics of mixed-typed inhibitors (Fig. 49 and 50).

a



b

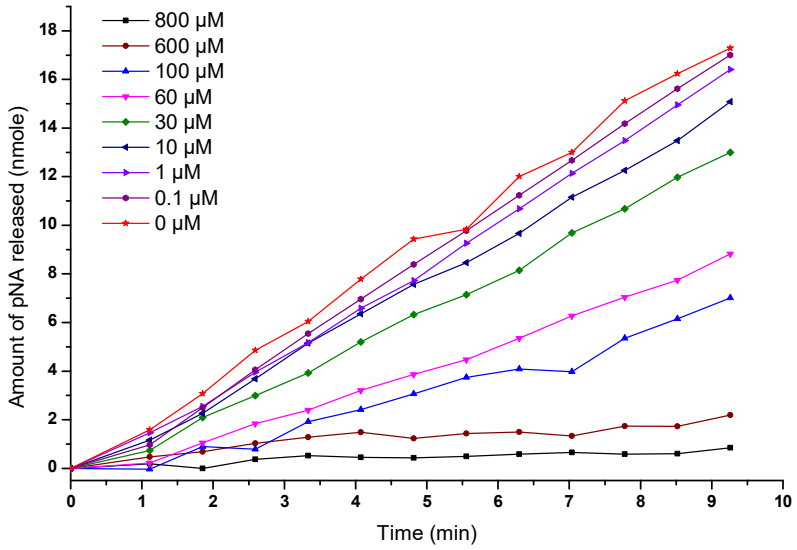


Figure 47: Linear progression curves of thrombin inhibition by (a) rHaemathrin 1 and (b) rHaemathrin 2 (inset: concentration in µM).

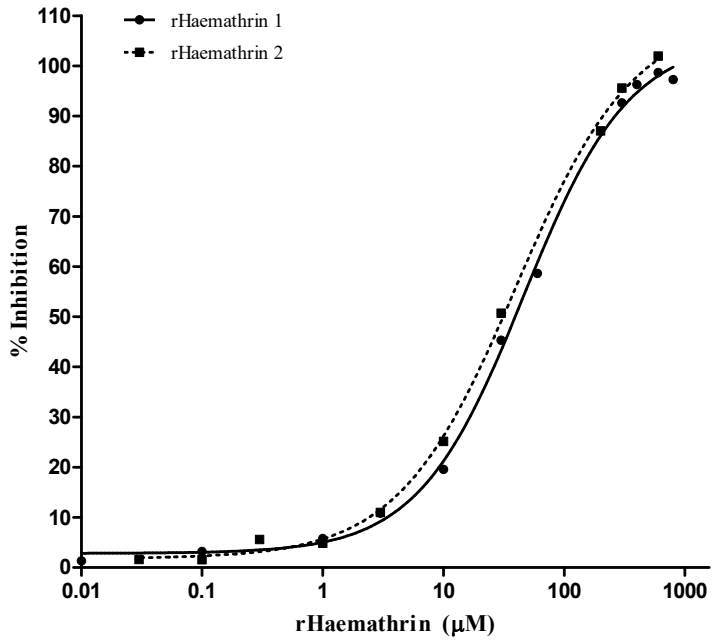
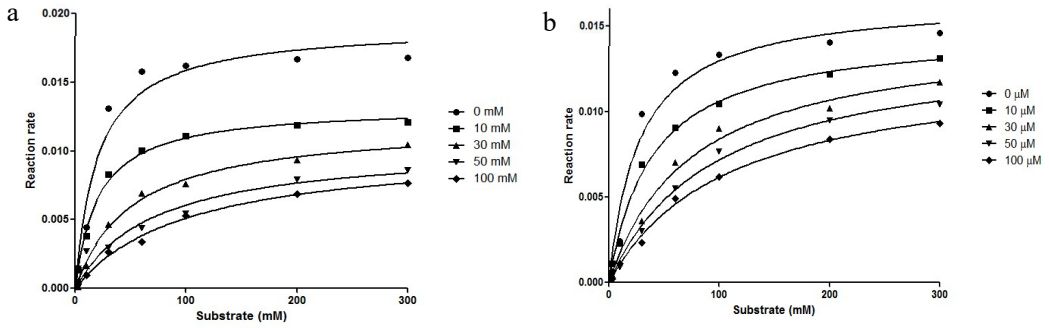


Figure 48: Dose-response curve of thrombin inhibition by rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed). The IC_{50} of haemathrin was calculated to be 46.13 ± 0.04 µM and that of haemathrin 2 to be 40.057±0.054 µM (n=3, values are mean ± S.D.).



Substrate		0 µM	10 µM	30 µM	50 µM	100 µM
rHaemathrin 1	Vmax	0.01910	0.01321	0.01199	0.01036	0.01022
	Km	20.36	20.71	50.92	70.45	99.70

Substrate		0 µM	10 µM	30 µM	50 µM	100 µM
rHaemathrin 2	Vmax	0.01655	0.01473	0.01466	0.01398	0.01278
	Km	27.53	38.80	74.71	94.55	107.3

Figure 49: Michaelis-Menten curve of enzyme inhibition by (a) rHaemathrins 1 and (b) rHaemathrin 2 (n=3, values are mean ± S.D.).

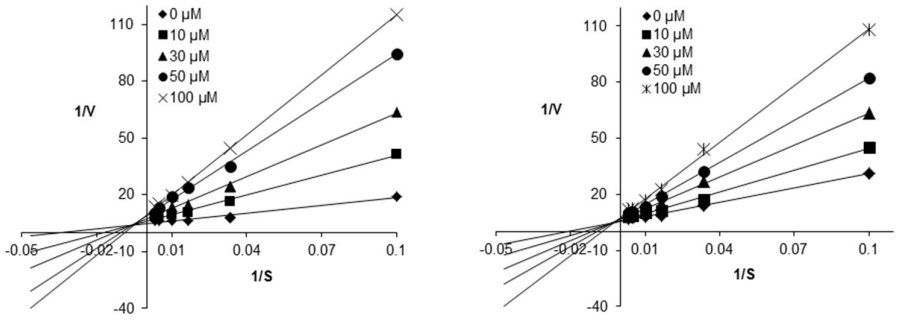


Figure 50: Lineweaver-Burk plot showing rHaemathrin 1 (a) and rHaemathrin 2 (b) as a mixed-type of thrombin inhibitor.

Time-dependent thrombin inhibition

To analyze how rHaemathrins exhibited thrombin inhibition over time, they were tested for inhibition of thrombin amidolytic activity against its chromogenic substrate for different time interval (0 – 60 h). It was observed that the inhibitory activity of the peptides decreased gradually with time; thrombin inhibition at 2 min was considered as 100% (Fig. 51). The inhibitory activity

of rHaemathrin 1 decreased by about 56%, while that of rHaemathrin 2 decreased by about 98% after incubation of rHaemathrins with thrombin for 60 h.

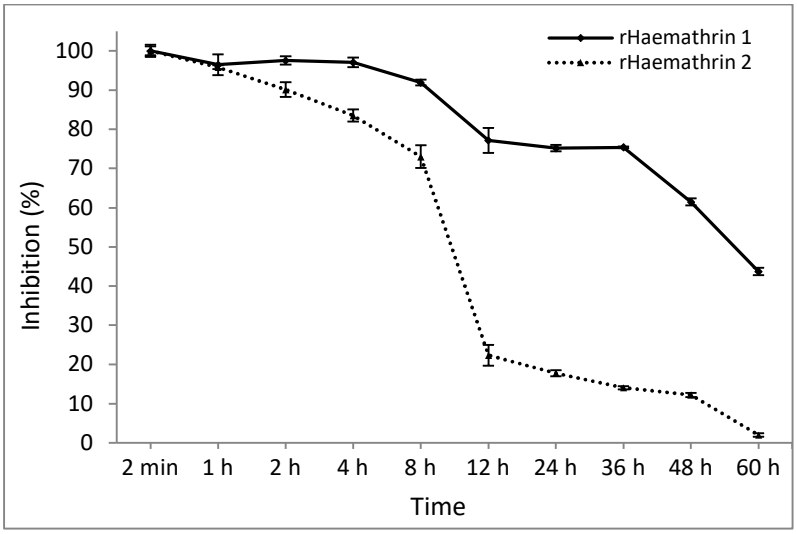


Figure 51: Graph showing decrease of percentage inhibition of thrombin amidolytic activity by rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) with time (n=3, error bars represent ±S.D.). Thrombin inhibition at 2 min was considered as 100%.

Chromatographic analysis of rHaemathrins treated with thrombin

To understand the loss of inhibitory activity of rHaemathrins, they were incubated with thrombin for different time intervals, and the reactions were subjected to RP-HPLC. Both rHaemathrin 1 and 2 were found to be hydrolyzed by thrombin with increase in time of incubation. The RP-HPLC profile showed depletion of the rHaemathrin peaks (*) and appearance of other minor peaks (Fig. 52a, 52b). The appearance of minor peaks is the hydrolysis products of rHaemathrins which were confirmed by mass spectrometric analysis. The probable peptide sequences were identified by submitting the peptide masses and searching against the protein sequences (Fig. 53A&B). Four fragments were identified for both rHaemathrin 1 and 2, which confirmed the cleavage site of thrombin (Table 5). For rHaemathrin 1, the four fragments corresponded to residues 1-18, 19-53, 1-53 and 54-59 and that for haemathrin 2 were residues 1-21, 22-53, 1-53, and 54-59 were obtained (Fig. 54).

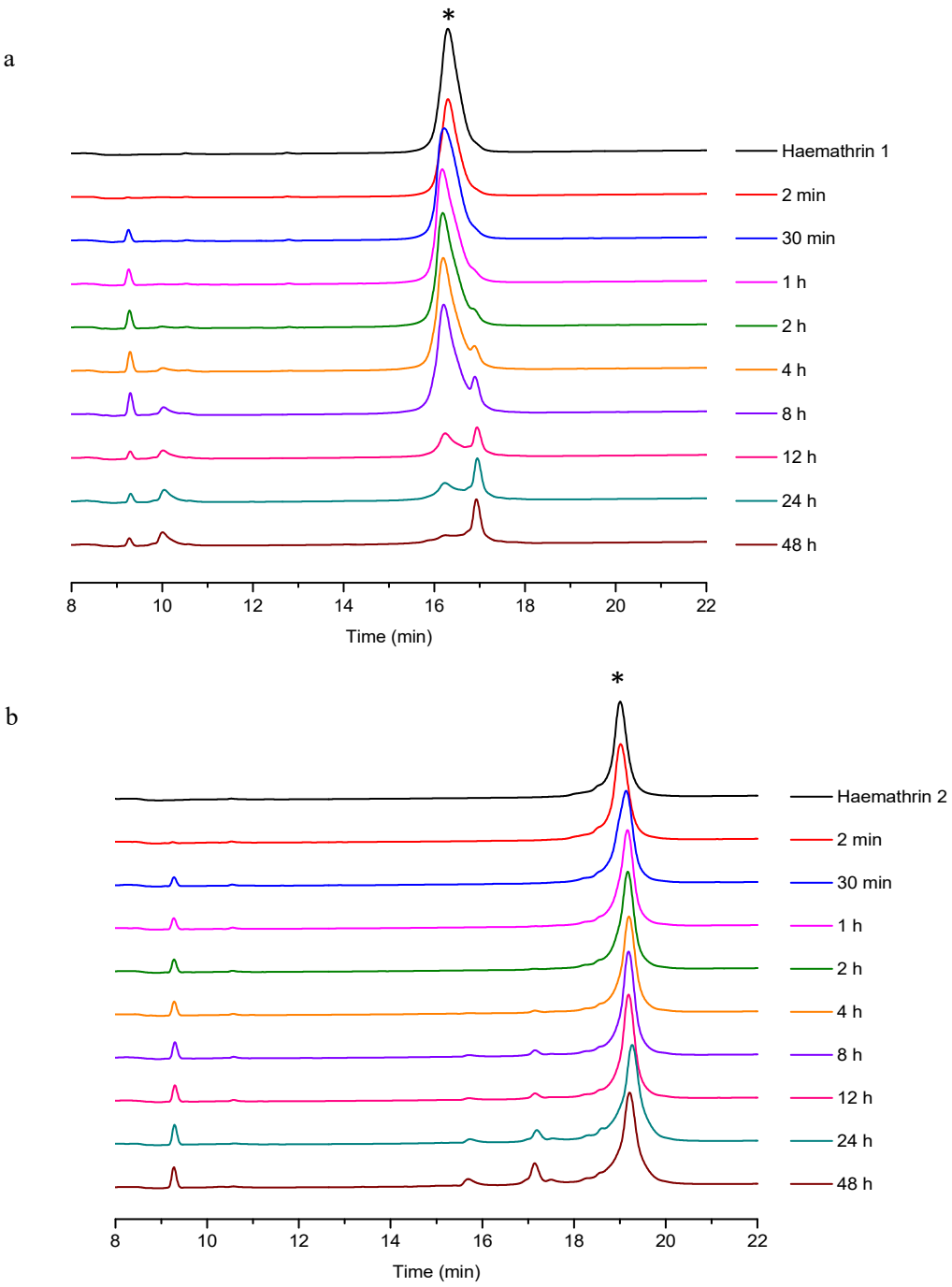


Figure 52: Chromatogram showing cleavage of (a) rHaemathrin 1 and (b) rHaemathrin 2, when incubated with thrombin for different time intervals.

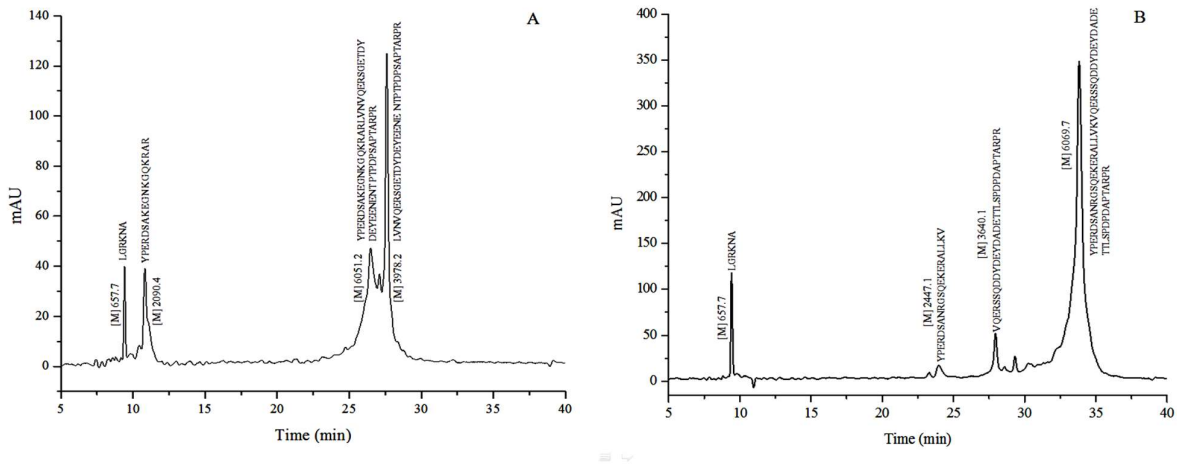


Figure 53: Reverse-phase chromatogram of cleaved fragments of (A) haemathrin 1 and (B) haemathrin 2.

Table 5: Identification of the cleavage products of rHaemthrins by using FindPept server (http://web.expasy.org/findpept/).

Inhibitor	Peptide fragment	Peptide mass (*m/z*)	
		Experimental	Theoretical
Haemathrin 1 **Haemathrin 2**	LGRKNA	657.7	657.7
	LGRKNA	**657.7**	**657.7**
	YPERDSAKEGNKGQKRAR	2090.4	2090.2
	YPERDSANRGSQEKERALLVK	**2446.9**	**2446.7**
	LVNVQERSGETDYDEYEENENTPTPDPSAPTARPR	3978.2	3978.1
	VQERSSQDDYDEYDADETTLSPDPDAPTARPR	**3640.8**	**3640.7**
	YPERDSAKEGNKGQKRARLVNVQERSGETDYDEY EENENTPTPDPSAPTARPR	6050.7	6050.4
	YPERDSANRGSQEKERALLVKVQERSSQDDYDE YDADETTLSPDPDAPTARPR	**6069.8**	**6069.4**

rHaemathrin 1	Observed(Da)	Calculated (Da)
YPERDSAKEGNKGQKRARLVNVQERSGETDYDEYEENENTPTPDPSAPTARPRLGRKNA	657.7	657.7
YPERDSAKEGNKGQKRARLVNVQERSGETDYDEYEENENTPTPDPSAPTARPRLGRKNA	2090.4	2090.2
YPERDSAKEGNKGQKRARLVNVQERSGETDYDEYEENENTPTPDPSAPTARPRLGRKNA	3978.2	3978.1
rHaemathrin 2		
YPERDSANRGSQEKERALLVKVQERSSQDDYDEYDADETTLSPDPDAPTARPRLGRKNA	657.7	657.7
YPERDSANRGSQEKERALLVKVQERSSQDDYDEYDADETTLSPDPDAPTARPRLGRKNA	2446.9	2446.7
YPERDSANRGSQEKERALLVKVQERSSQDDYDEYDADETTLSPDPDAPTARPRLGRKNA	3640.8	3640.7

Figure 54: Mapping of thrombin cleavage site on rHaemathrin 1 &2. Arrows indicate site of thrombin cleavage.

The peptidic fragments were also tested for its inhibitory activity against thrombin amidolytic activity, however they were found to be insignificant as compared to the full-length recombinant peptides (Table 6). When tested for anti-coagulation activity using plasma, the fragments did not show inhibition of coagulation of the plasma.

Table 6: Anti-coagulation activity assay of haemathrin peptidic fragments. Fragment 53-59 is common for both rhaemathrin 1 and 2.

Fragments (30 µM)		Thrombin Time		Prothrombin Time		APTT	
		Time (s)	Fold	Time (s)	Fold	Time (s)	Fold
Normal clot time	-	11.83 ± 0.45	1.00	15.60 ± 0.41	1.00	38.13 ± 0.75	1.00
rHaemathrin 1	Full-length (1-59)	29.78 ± 1.40	2.51	19.06 ± 0.90	1.22	51.16 ± 0.66	1.34
	1-18	12.17 ± 0.21	1.03	15.09 ± 0.15	0.96	38.23 ± 1.36	1.00
	19-53	16.00 ± 0.95	1.35	16.53 ± 0.23	1.04	39.23 ± 2.46	1.03
	1-53	14.53 ± 0.61	1.23	15.77 ± 0.36	0.95	38.9 ± 0.85	0.99
rHaemathrin 2	Full-length (1-59)	33.58 ± 1.41	2.83	22.70 ± 0.34	1.45	56.26 ± 1.34	1.47
	1-21	12.17 ± 0.60	1.03	15.90 ± 1.40	1.01	38.4 ± 1.56	0.99
	22-53	13.77 ± 0.60	1.16	15.30 ± 0.45	0.96	38.93 ± 1.78	1.01
	1-53	14.93 ± 0.42	1.26	15.70 ± 0.21	1.03	38.05 ± 0.78	0.98
	53-59	12.07 ± 0.64	1.02	16.63 ± 0.64	1.07	38.20 ± 1.71	1.00

6. *Over-expression of the mutant proteins and characterization*

Overexpression of mutant proteins

E. coli BL21(DE3)pLysS competent cells transformed with recombinant vectors containing the coding sequence for mutant Haemathrin 1 (mHaemathrin 1) and mutant Haemathrin 2 (mHaemathrin 2) were induced with IPTG and the cell lysate was analysed on SDS-PAGE. Bands of over-expressed protein of about 25 kDa were observed on commassie- stained SDS-PAGE gel, which were absent in the uninduced cells. This is the expected size of the recombinant Haemathrins with the fusion tag. Mass culture was carried out and the recombinant proteins were purified by His tag columns (Fig. 4 & 5).

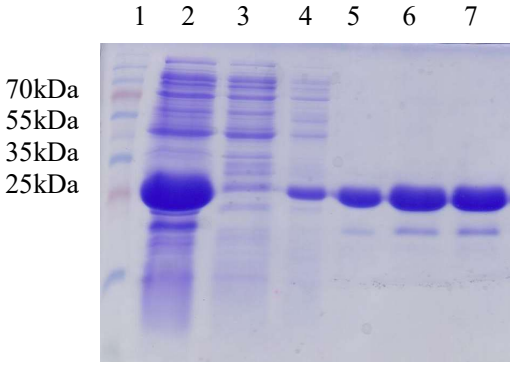


Figure 55: 12.5% SDS-PAGE gel profile of His-tag purification of mHaemathrin 1. Lane 1: Protein molecular weight marker; lane 2: cell lysate; lane 3: unbound fraction; lane 4: wash fraction 1(20mM imidazole); lane 5: wash fraction 6(20mM imidazole); lane 6: wash fraction 7(20mM imidazole); lane 7: fraction (250mM imidazole).

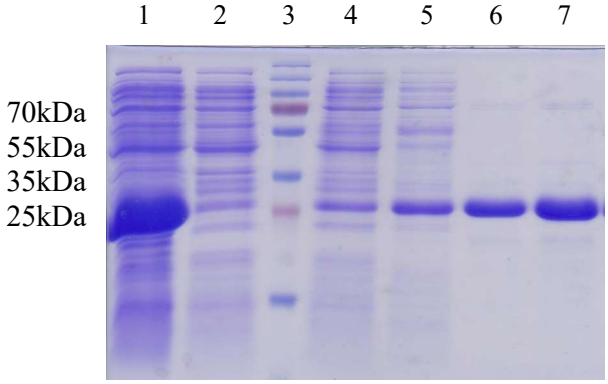


Figure 56: 12.5% SDS-PAGE gel profile for His-tag purification of mHaemathrin 2. Lane 1: cell lysate; lane 2: unbound fraction; lane 3: Protein molecular weight marker; lane 4: wash fraction 1(20mM imidazole); lane 5: wash fraction 2(20mM imidazole); lane 6: wash fraction 7(20mM imidazole); lane 7: fraction 1(250mM imidazole).

Characterization of the mutant proteins

The purified mHaemathrins were tested for its effect on the anticoagulant activity. Both the mutant proteins did not exhibit any inhibitory activity when tested for APTT and PT using goat plasma (Table 7). Therefore the mHaemathrins were assayed for its inhibitory activity against thrombin amidolytic activity and thrombin time. With increase in dose mHaemathrins were able to inhibit the catalytic activity of thrombin (Fig. 57). At 400 µM of the mHaemathrins the thrombin lost is hydrolytic activity when S2238 was used as the substrate. Moreover the thrombin time was delayed significantly when the mHaemathrins were pre-incubated with thrombin (Fig. 58). After 36 hr of incubation the fold delay in thrombin time was found to be greater than 20 fold. In some of the cases the plasma did not clot, suggesting complete loss of the thrombin activity. This is in contrasting to the wild type Haemathrins which losses its inhibitory activity on incubation with thrombin for 48 hrs.

Table 7: Effect of mHaemathrins on blood coagulation

Concentration	mHaemathrin 1				mHaemathrin 2			
	APTT	Fold change	PT	Fold Change	APTT	Fold Change	PT	Fold Change
NCT	43.90 ± 1.56	1.0	24.57 ± 1.91	1.0	43.90 ± 1.56	1.0	24.57 ± 1.91	1.0
50 µM	36.60 ± 1.49	0.83	20.9 ± 1.27	0.85	36.16 ± 0.27	0.82	21.67 ± 0.56	0.88
100 µM	36.06 ± 0.58	0.82	20.07 ± 0.58	0.81	35.90 ± 2.56	0.81	20.5 ± 1.05	0.84
200 µM	34.66 ± 1.02	0.79	19.07 ± 1.11	0.77	37.76 ± 0.91	0.86	19.23 ± 0.31	0.78
400 µM	38.90 ± 2.20	0.88	ND	NA	42.10 ± 1.0	0.95	ND	NA

:* ND: Not determined; NA: Not applicable

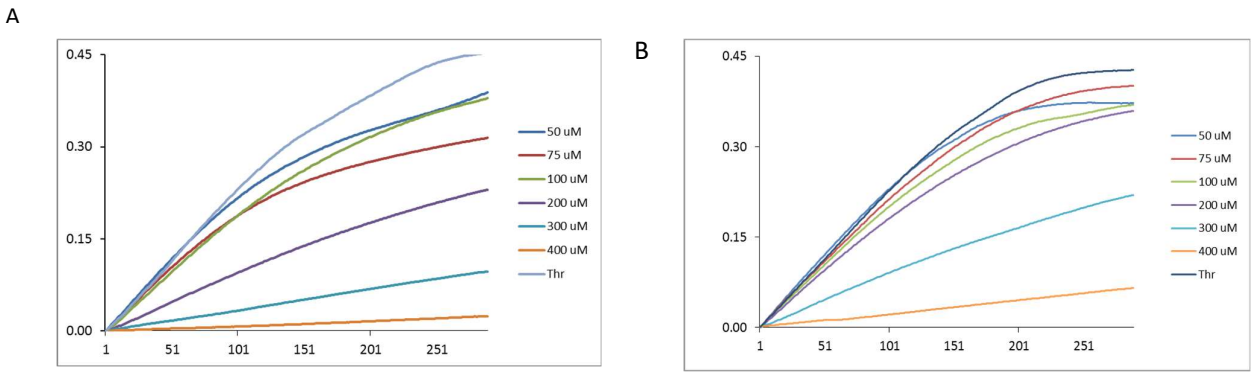


Figure 57: Dose dependent inhibition of amidolytic activity of thrombin by mHaemathrins 1 [A] and mHaemathrin 2 [B].

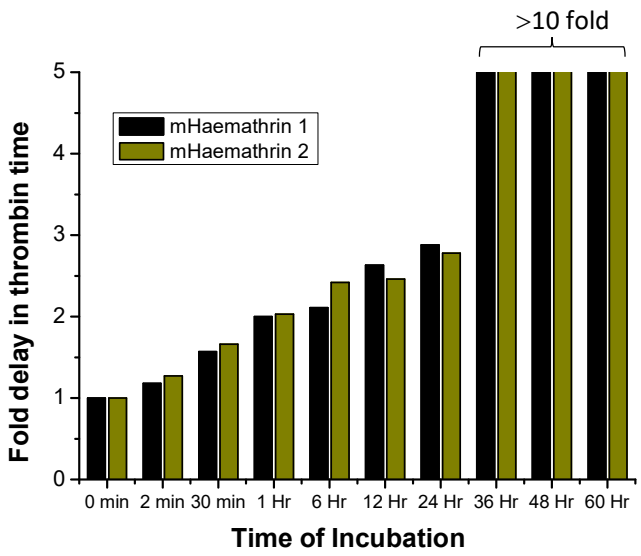


Figure 58: Time dependent inhibition of thrombin time by mHaemathrins. Thrombin and mHaemathrins were pre-incubated at 37 °C for various time intervals and inhibition of thrombin activity was assayed.

The rHaemathrins when incubated with thrombin, it was observed that it loses its inhibitory activity. Further using RP-HPLC we have shown that with increase in incubation time, rHaemathrins are cleaved by thrombin. However the mutant proteins were found to inhibit the thrombin activity when pre-incubated with thrombin. After 36 Hr of incubation, thrombin completely lost is activity (plasma did not clot) (Fig. 58). Since the thrombin cleavage sites of rHaemathrins were mutated, hence thrombin could not cleave the proteins resulting in loss of its activity. This was further confirmed by RP-HPLC analysis of the thrombin-mHaemathrin incubated mixtures (Fig. 8). Under identical conditions, the retention time of mHaemathrins before and after incubation with thrombin was found to be same.

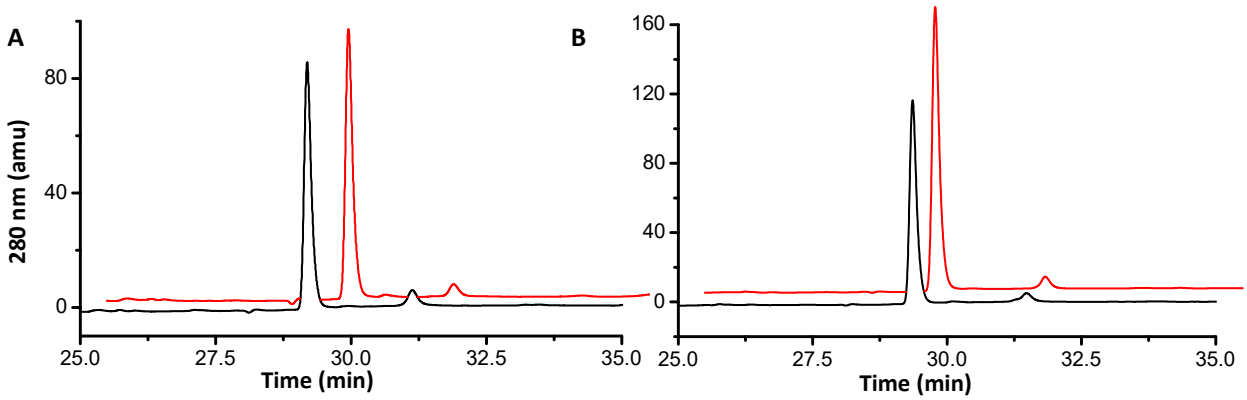


Figure 59: Analysis of mutant haemathrin 1 [Panel A] and mutant haemathrin 2 [Panel B] by RP-HPLC before [red line] and after incubation with thrombin for 48 hrs [black line]. Elution of the protein was monitored at 280 nm.

7 In-silico characterization of Haemathrins

Structure of modeled peptides

In the previous study we have shown by *in-vitro* experiments that Haemathrin1 and 2 are cleaved by thrombin resulting into three peptides. The full length and all the thrombin cleaved peptides showed different folding pattern and secondary structural when modelled. The modelled structures were refined by energy minimized which showed slight variation from the modelled structures (Figure 60 & 61). These minimized structures were used for the *in-silico* studies.

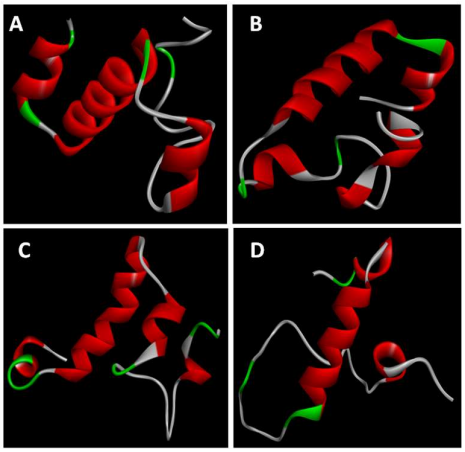
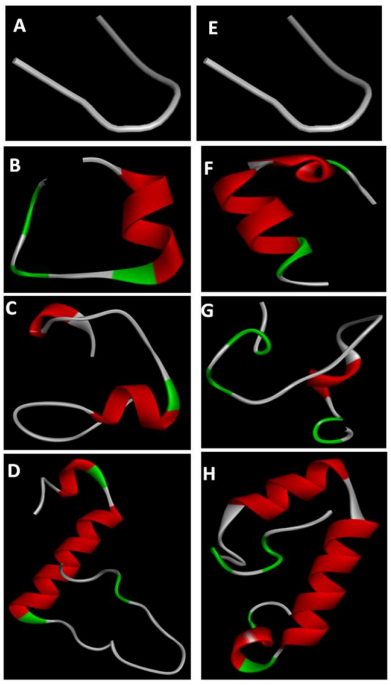


Figure 60: A: Modelled structure of Haemathrin 1. B: Haemathrin 2 before energy minimization. C & D are Haemathrin 1 and Haemathrin 2 after energy minimization.



Amino sequences of peptides	
A & E	LGRKNA (54^{th} -59^{th})
B	YPERDSAKEGNKGQKRAR (1^{st} -18^{th})
C	LVNVQERSGETDYDEYEENENTPTPD PSAPTARPR (19^{th} – 53^{th})
D	YPERDSAKEGNKGQKRARLVNVQER SGETDYDEYEENENTPTPDPSAPTARP R (1^{st} -53^{rd})
F	YPERDSANRGSQEKERALLVK (1^{st} -21^{st})
G	VQERSSQDDYDEYDADETTLSPDPDA PTARPR (22^{nd} -53^{rd})
H	YPERDSANRGSQEKERALLVKVQERS SQDDYDEYDADETTLSPDPDAPTARP R (1^{st} – 53^{rd})

Figure 61: Modelled structure of thrombin cleaved Haemathrin 1 peptides (A-D) and Haemathrin 2 peptides (E-H) after energy minimization.

Interaction study of thrombin and Haemathrins

In-silico docking study reveals that C-terminal fragment, LGRKNA (54-59) of full length Haemathrin 1 interact with both active site and central part to the Exosite II of thrombin. The fragment without the C-terminal (1-53 residues) is found to interacts at the Exosite II and amino acid sequence DYDEY(D/E)(E/A)(D/N)E(N/T) is involved (Fig. 62). This sequence was found to be conserved in other thrombin inhibitors (Madanin1, Madanin2 and chimadanin) obtained from different species of cattle tick. However Haemathrin 1 (both full length as well as fragments) has less binding energy as compared to Madanin 1.

Similarly, full length Haemathrin 2 also interacts with both active site and Exosite II of thrombin (Fig 63). The detailed interactions i.e. the number of hydrogen bonds, hydrophobic bonds as well as electrostatic bonds formed between Thrombin and Haemathrins are shown in Table 8.

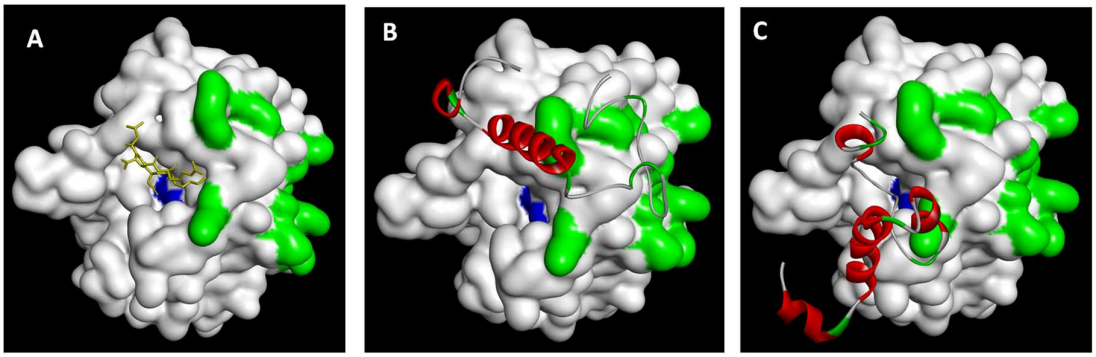


Figure 62: Interaction of Thrombin and Haemathrin. A: C-terminal (54-59 residues), B: peptide without the C-terminal (1-53 residues) and C: Full length. The green colour in thrombin depicts Exosite II and blue colour depicts active site.

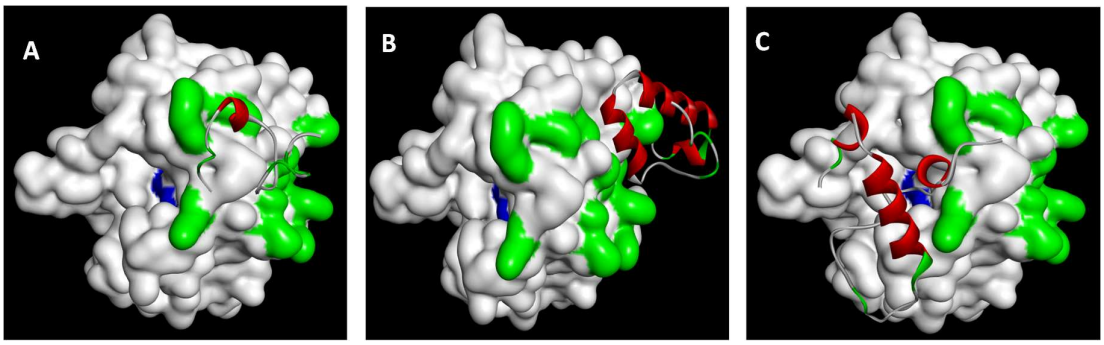


Figure 63: Interaction of Thrombin and Haemathrin 2 peptides. A: Without N- and C- terminal (21-53 residue), B: without the C-terminal (1-53 residue) and C: Full length. The green colour in thrombin depicts Exosite II and blue colour depicts active site.

Table 8: Details of full length Haemathrin 1 and 2 and thrombin cleaved peptides interaction with thrombin.

Peptide	Binding site	Binding energy (kcal/mol)	Amino acids involved in interacting with Exosite/ Active site	Total Bonds involved
Madanin 1 (5L6N)	Exosite II and active site	-7.4	D29,Y30,D31,E32,E34, E35,T41,D43,R53	7 Hydrogen bonds 2 Hydrophobic bonds 12 Electrostatic bonds
Haemathrin 1 Full length	Exosite II and active site	-10.6	N37,T40,P45 P48 with active site	10 Hydrogen bonds 10 Hydrophobic bonds 5 Electrostatic bonds
Haemathrin 1 19-53 residue	Exosite II	-9.1	E35,T40,P41,P52	5 Hydrogen bonds 6 Hydrophobic bonds 4 Electrostatic bonds
Haemathrin 1 and 2 54-59 residue (LGRKNA)	Active site	-8.1	L54	5 Hydrogen bonds 5 Hydrophobic bonds 4 Electrostatic bonds
Haemathrin 2 Full length	Exosite II and active site	-8.9	E24 P48 with active site	7 Hydrogen bonds 7 Hydrophobic bonds 3 Electrostatic bonds
Haemathrin 2 21-53 residue	Exosite II	-8.3	Q23,S27,D29,D32,D37, E38	9 Hydrogen bonds 1 Hydrophobic bonds 4 Electrostatic bonds
Haemathrin 2 1-53 residue	Exosite II	-9.4	D30,Y31,E33,Y34,D35,T39	7 Hydrogen bonds 7 Hydrophobic bonds 9 Electrostatic bonds

Rational redesign of thrombin inhibitor:

Docking studies with full length as well as thrombin cleaved peptides of Haemathrins showed that the N-terminal sequence doesn't interact with thrombin whereas the residue 21st to 53rd is involved in binding to Exosite II of thrombin with a binding energy of -8.3 kcal/mol and the C-terminal end (LGRKNA) binds to the active site. Hence to redesign thrombin inhibitor from haemathrin residue 22nd to 59th was considered as it contains the conserve sequence as well as active site binding domain (VQERSSQDDYDEYDEDETTLSPDPDAPTARPRLGRKNA). The structure of this peptide was modelled and energy minimized structures were used for docking with thrombin (Fig. 64). Residues 22nd-59th peptide interacted with thrombin at the exosite II and active site with a binding energy of -11.7 kcal/mol.

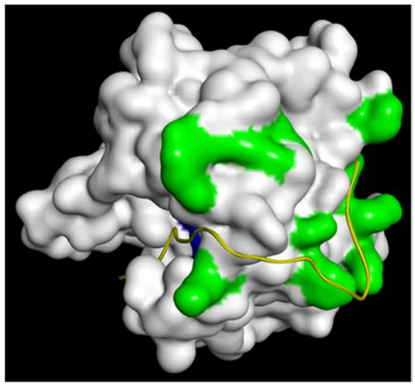


Figure 64: Interaction of Thrombin cleaved haemathrin peptides (22nd to 59th) with thrombin.

6.1 ***Amino acid substitution (R53K and R53A)***

Haemathrin 2 :VQERSSQDDYDEYDEDETTLSPDPDAPTARPRLGRKNA
Haemathrin 2 R53K:VQERSSQDDYDEYDEDETTLSPDPDAPTARPKLGRKNA
Haemathrin 2 R53A:VQERSSQDDYDEYDEDETTLSPDPDAPTARPALGRKNA

Figure 65: Alignment of amino acid sequence of wild type and mutant peptides.

Sequence PRL (highlight in figure 65) at the C-terminal end is a conserved sequence present in all characterized anti-thrombin peptides. Arginine at 53rd position is the site of cleavage by thrombin. As soon as anti-thrombin peptides are cleaved at this position, the peptides are released, thus decreasing the inhibitory property of the peptides. This residue was changed to Lysine to interfere

with the normal cleavage process so that the complex becomes more stable. But substituting the Arginine residue with Lysine changed the orientation of the peptide resulting in C-terminal end leaving the active site though the Exosite II binding sequence interacted with the Exosite II. The same result was obtained when Arginine was substitutes with Alanine, a neutral amino acid (Fig 66).

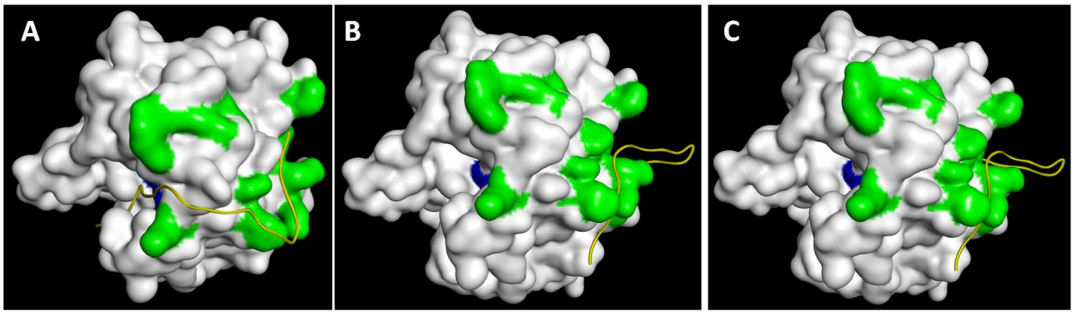


Figure 66: Interaction of Thrombin with Haemathrin 2 (A); Haemathrin 2 R53K (B); and Haemathrin 2 R53A (C).

Minimization of peptide

To obtain a minimized peptide that could bind to both exosite II and active site, two peptides were designed. In one of the peptide the thrombin cleaved peptide (LGRKNA) was removed whereas in the other peptide the N-terminal (VQERSSQD) was removed (Fig 67). These peptides were modelled and docked with thrombin to study their interaction.

Haemathrin 2 (22-59 residues): VQERSSQDDYDEYDEDETTLSPDPDAPTARPRLGRKNA
Haemathrin 2 (22-53 residues): VQERSSQDDYDEYDADETTLSPDPDAPTARPR----------
Haemathrin 2 (30-59 residues): ----------DYDEYDADETTLSPDPDAPTARPRLGRKNA

Figure 67: Alignment of amino acid sequence of wild type and mutant peptides. The removed amino acid residues are shown in dash.

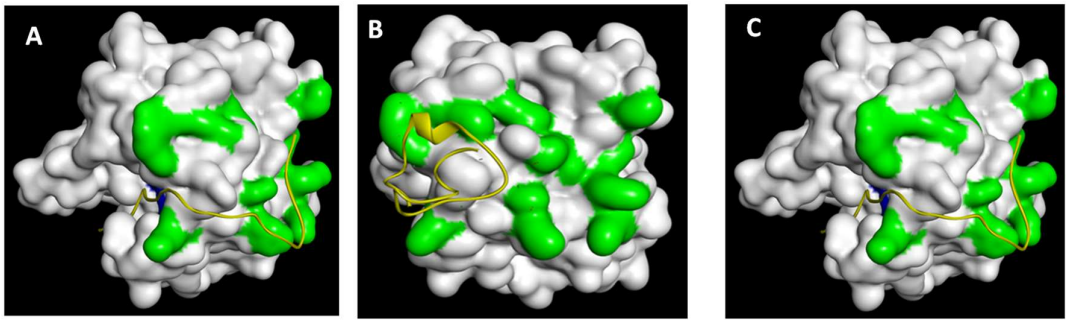


Figure 68: Interaction of mutated haemathrin peptides with thrombin. A: Residues 22nd-59th (binding energy: -11.7 kcl/mol); B: Residue 22nd-53rd (Binding energy: -8.3 kcal/mol); C: Residues 30th-59th (-12.2 kcal/mol). The green colour in thrombin depicts Exosite II and blue colour depicts active site.

The N-terminal removed peptide was found to be interacting with thrombin but its binding energy (-8.3 kcal/mol) was less than the original peptide (Fig. 68). Whereas when N-terminal removed and the C-terminal (31-59 residues) was retained, the binding energy was found to be -12.2 kcal/mol and binds to both Exosite II as well as the active site (Fig 69). This redesigned peptide was named as minimized haemathrin (mHaemathrin).

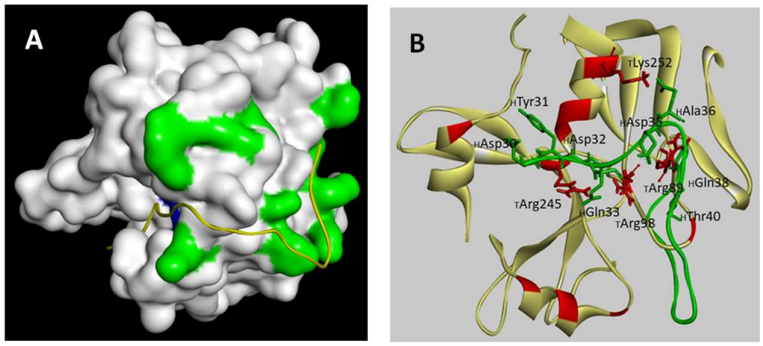


Figure 69: A: Docking of mHaemathrin with Thrombin. B: Hydrogen bond between mHaemathrin with Thrombin.

Tyrosine sulfation

Anti-thrombin peptides occur in two forms: sulfated and non-sulfated. Hirudin was the first anti-thrombin peptide to be isolated in sulfated form. The sulfation occurs in the tyrosine residues which increases the affinity of the anti-thrombin peptides towards thrombin. However it has been reported that the peptides undergo desulfation during isolation.

In-silico sulfation of the peptides were carried out by replacing the –OH group in the tyrosine ring by a sulfate group ($SO4^{2-}$). The peptide residues from 30th to 59th (DYDEYDEDETTLSPDPDAPTARPRLGRKNA) was considered as it was showing the maximum binding energy. The Y31 and Y34 residues were sulfated and modelled for docking study. Double sulfation of Y31 and Y34 of Madanin 1 was also modelled in this study to check the interaction. Sulfated madanin showed structural change but in case of haemathrins no such changes were observed (Fig 70).

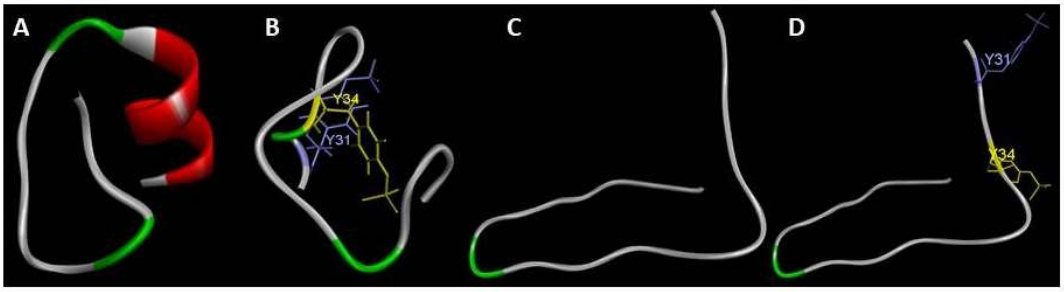


Figure 70: Modelled structure of non-sulfated (A) and sulphated (B) Madanin 1 peptides and 22nd to 59th non-sulfated (C) and sulfated (D) haemathrin peptides.

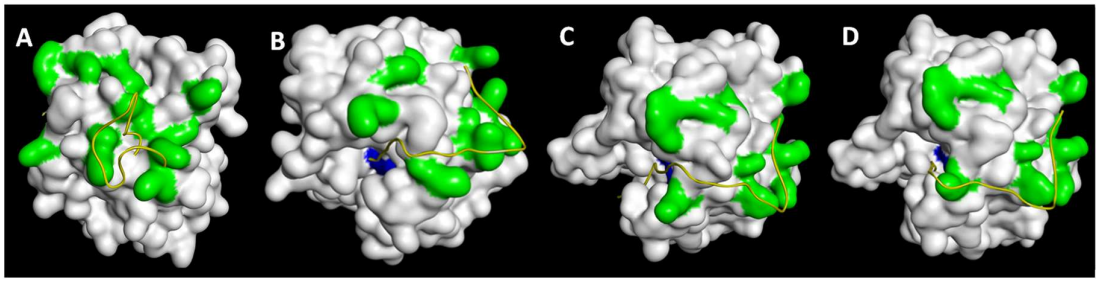


Figure 71: Docking of modelled non-sulfated (A), sulphated (B) Madanin peptides, non-sulphated 30th to 59th (C) and sulfated 30th to 59th haemathrin peptides (D).

After docking the binding energy of the double sulfation at Y31 and Y34 of both the peptides were found to be higher than the non-sulfated peptides. The details of interaction and binding energy of the sulfated and non-sulfated is shown in Table 9.

Table 9: Table showing interaction of non-sulfated and sulfated Madanin 1 peptide and 30th to 59th haemathrin peptides with thrombin.

Peptide	Binding site	Binding energy (kcal/mol)
Madanin 1 (sulfated)	Exosite II and active site	-12.2
Madanin 1 (Non-sulfated)	Exosite II and active site	-12.7
Haemathrin 2 (sulfated):	Exosite II and active site	-14.3
Haemathrin 2 (Non-sulfated)	Exosite II and active site	-12.1

```
Madanin1 (sulfated)          : ADYDEYEEDGTTPTPDPTAPTAKPRLRGNKP
Madanin1 (Non-sulfated)      : ADYDEYEEDGTTPTPDPTAPTAKPRLRGNKP
Haemathrin2 (sulfated)       :  DYDEYDADETTLSPDPDAPTARPRLGRKNA
Haemathrin2 (Non-sulfated)   :  DYDEYDADETTLSPDPDAPTARPRLGRKNA
```

Figure 72: Comparison of residues of non-sulfated and sulfated Madanin interacting with thrombin. Sulfated The arginine residue involved in interacting with active site residue of thrombin is highlighted in red and residues involved in interacting with the residues of thrombin Exosite II is shown in blue color.

Table 10: Summary of interactions

Peptide	Binding site	Binding energy (kcal/mol)	Amino acids involved in interaction with ExositeII/active site	Total bonds involved
Haemathrin 2 Full length (1-59 residues)	Exosite II and active site	-8.9	E24 P48 with active site	7 Hydrogen bonds 7 Hydrophobic bonds 3 Electrostatic bonds
Haemathrin 2 (22-59 residues)	Exosite II and active Site	-11.7	D30, Y31, D32, E33, D35, A36, E38, T40, P48, T49 R53 with active site	12 Hydrogen bonds 4 Hydrophobic bonds 5 Electrostatic bonds
Haemathrin 2 R53K (22-59 residues)	Exosite II	-10.6	E33, Y34, D35, A36, D37	8 Hydrogen bonds 1 Hydrophobic bonds 6 Electrostatic bonds
Haemathrin 2 R53A (22-59 residues)	Exosite II	-11.4	E33, Y34, D35, A36, D37	9 Hydrogen bonds 1 Hydrophobic bonds 5 Electrostatic bonds
Haemathrin 2 (22-53 residues)	Exosite II	-8.3	Q23, S27, D29, D32, D37, E38	9 Hydrogen bonds 1 Hydrophobic bonds 4 Electrostatic bonds
Haemathrin 2 (30-59 residues)	Exosite II and active site	-12.1	D30, Y31, D32, E33, D35, A36, E38, T40, P48, T49, A50 R53 with active site	14 Hydrogen bonds 4 Hydrophobic bonds 6 Electrostatic bonds
Haemathrin 2 Y31/Y34 sulfated (30-59 residues)	Exosite II and active site	-14.3	D30, D32, E33, Y34, D35, P43, D44, A47, P48, T49, A50 R53 with active site	12 Hydrogen bonds 4 Hydrophobic bonds 6 Electrostatic bonds

7. Salient Research Achievements:

- In the present study, two tick species, *Rhipicephalus* (*B*) *microplus* and *Haemaphysalis bispinosa*, were identified and characterized using both morphological and genetic tools.
- *R.* (*B*) *microplus* is primarily a cattle tick, while *H. bispinosa* was found to parasitize both cattle and goats.
- Both the ticks when full-engorged appeared dark in color, similar in size and festoons were not apparent.
- *R.* (*B*) *microplus* ITS2 sequence (JX974346.1) showed more than 98% homology with that of *R. microplus* (JQ625709.1), containing 14 transversions, 4 transitions, 1 deletion and 3 additions. While ITS2 sequence of *H. bispinosa* was found to be 64.53% and 62.75% similar to that of *H. longicornis* and *H. doenitzi*, respectively.
- The partial 16S rDNA of *R. microplus* showed 100% similarity to *R. microplus* (EU918188.1) with no deletion or addition of nucleotide bases. The 16S rDNA of *H. bispinosa* showed 88.11% similarity to *H. longicornis* (FJ712721.1) and 87.79% similarity to *H. doenitzi* (JF979402.1).
- This is the first phylogenetic analyses of *H. bispinosa* based on 16S rDNA or ITS2 sequences.
- The PCR-RFLP profile of ITS2 of *R. (B) microplus* and *H. bispinosa* showed clear distinction in the digestion pattern of the ITS2 sequence. Hence this PCR based tool could be used to quickly differentiate the tick species prevalent in this region which differs marginally when fully fed.
- Salivary gland from the collected ticks were successfully isolated which exhibited dose dependent anticoagulant activity,
- Analysis of SGE on SDS-PAGE gel, bands of proteins ranging from about 10 to 250 kDa were observed which represent anticoagulant and proteins expressed in the salivary glands.
- Salivary gland cDNA was successfully prepared and using gene specific primers Madanin like sequence was amplified
- The isolated genes were christened as haemathrin (***Haema****physalis* **thr**ombin **in**hibitor). Haemathrins coded for mature peptides of about 6.7 kDa and showed about 60-70% similarity to madanins from *H. longicornis.*
- The cDNA coding for the mature peptides of haemathrin 1 and 2 were successfully cloned into pET32a(+) expression vector and the recombinant proteins were over-expressed using *E. coli* BL21(DE3)pLysS expression host.

- 50% of the protein was found in the soluble fraction at the optimum condition of overexpression of 0.05 mM IPTG concentration and 37°C of culture condition.
- rHaemathrin 1 and 2 prolonged thrombin time of the plasma significantly to about 2.5 and 2.8 folds, respectively at a concentration of 30 μM. It also prolonged PT and APTT, indicating that they are anti-coagulants and acts on common pathway of the coagulation cascade. rHaemathrin 1 and 2 delayed APTT to about 1.3 and 1.5 folds, respectively, while rHaemathrin delayed PT to about 1.2 and rHaemathrin 2 to about 1.5 folds at a concentration of 30 μM.
- rHaemathrins showed a time-dependent inhibition suggesting that rHaemathrins are slow binding thrombin inhibitors.
- When the recombinant inhibitors were tested for inhibition of thrombin against different substrate concentrations and Michaelis-Menten curve of enzyme inhibition was plotted, it was observed that with decrease in Vmax, the Km increased. This is characteristic of mixed type of inhibition
- Haemathrins targets thrombin for its anticoagulant activity. However, with increase in time of incubation, thrombin cleaves Haemathrins and it losses its inhibitory activity.
- Using proteomic approach, the thrombin cleavage sites on Haemathrins were mapped.
- Using site directed mutagenesis approach the thrombin cleavage residue R18 and R53 of rHaemathrins 1 and K21 and R53 for rHaemathrin 2 where changed to A.
- Mutant Haemathrins (mHaemathrins) were successfully over-expressed in bacterial expression system and purified.
- mHaemathrins did not inhibit the APTT and PT time of goat plasma, however the thrombin time was inhibited both dose dependently and time dependently.
- RP-HPLC analysis of the pre-incubated thrombin-mHaemathrin revealed that the mHaemathrins are resistant to thrombin cleavage.
- In-silico study reveals Full length Haemathrin 1 and 2 (59 residues) interacts with thrombin at Exosite II and active site.
- DYDEY(D/E)(E/A)(D/N)E(N/T) sequence binds at Exosite II whereas the C-terminal end (LGRKNA) interacts with the active site of thrombin.
- To remodel Haemathrin as more efficient thrombin inhibitor, three modifications: (a) Deletion of N-terminal/C-terminal, (b) Mutation and (c) Tyrosine sulfation were carried out.

- Removal of the N-terminal and C-terminal of Haemathrin 2 retaining 21st to 53rd residues, we have found that it binds to Exosite II.
- Retaining the C-terminal (21-59 residues), remodeled Haemathrin 2 interacted with both Exosite II and active site with a binding energy of -12.2 kcal/mol.
- Amino acid substitution at A36 and R51 of Haemathrin 2 (21-53 residues) increase the binding affinity of Haemathrin 2 towards thrombin. However it did not interact with the active site.
- Sulfation of the Tyrosine residues increased the binding energy from -12.1 to -14.3 kcal/mol. However since sulfation is not a strong PTM which get desulfated during isolation hence sulfated peptides are not considered for further studies.
- The minimized Haemathrin (mHaemathrin) from 30th to 59th residue is DYDEYDADETTLSPDPDAPTARPRLGRKNA which will be synthesised for further studies

10. Work to be done

1. Synthesis of the optimized peptide and further characterization

11. New observations:

1. ***Salivary gland of Haemaphysalis bispinosa expresses cleavable thrombin inhibitors for feeding on blood meal.***
2. ***In- silico studies reveals minimization of thrombin cleaved peptide binds to thrombin at exosite II***

12. Innovations: Nil

13. Application Potential:

13.1 Long Term: **Design of thrombin inhibitors**

13.2 Immediate: **Validation of in-silico designed thrombin inhibitor**

14. Research work which remains to be done under the project (for on-going projects):

Inhibition of thrombin by in-silico designed minimized Haemathrins.

Annexure-II

REQUEST FOR ANNUAL INSTALMENT WITH UP-TO-DATE STATEMENT OF EXPENDITURE

(Two copies)

1. SERB Sanction Order No and date: SERB/F/2406/2014-15 dated 03.07.2014
2. Name of the PI : Dr. Robin Doley
3. Total Project Cost : Rs. 41,06,000/-
4. Revised Project Cost (if applicable) : NA
5. Date of Commencement : July 2014
6. Statement of Expenditure : 1st April 2018 to 31st July 2018
   (month wise expenditure incurred during current financial year)

Month & year	Expenditure incurred/committed (Rs)
April 2018	0.00
May 2018	56250.00
June 2018	25658.00
July 2018	0.00
Total	**81935.00**

7. Grant received in each year:
   a. 1st Year: Rs. 20,00,000.00
   b. 2nd Year: Rs. 5,50,000.00
   c. 3rd Year: Rs. 7,00,000.00
   d. 4th Year: Rs. 8,00,000.00
   e. Interest, if any: Rs. 12993.00
   f. Total (a+b+c+d+e): Rs. 40,62,993.00

Finance Officer
Tezpur University

Statement of Expenditure

(1st April 2017 to 31st July 2018)

Sl. No.	Sanctioned Heads	Total funds allocated (1st year)	Total funds allocated (2nd year)	Total funds allocated (3rd year)	Total funds allocated (4th year)	Total Fund received	Expenditure incurred					Total Expenditure	Balance as on 31 July 2018
							1st Year (25 July 2014 to 31st March, 2015)	2nd Year (1st April 2015 to 31st March 2016)	3rd Year (1st April 2016 to 31st March 2017)	4th Year (1st April 2017 to 31st March 2018)	4th Year (1st April 2018 to 31st July 2018)	Total Expenditure till 31st July 2018	
(I)	(II)	(III)	(IV)	(V)	(VI)		(VI)	(VII)	(VIII)	(IX)	(X)	(XI)	(XII)
1	Manpower costs	575000.00	425000.00	575000.00	675000.00	2250000.00	90134.00	290484.00	325000.00	178307.00	25685.00	909610.00	139090.00
2	Consumables						320367.00	179130.00	223748.00	285992.00	0	1009237.00	
3	Travel						4207.00	37679.00	12152.00	26577.00	0	80615.00	
4	Contingencies						35677.00	17321.00	21804.00	36646.00	0	111448.00	
5	Others if any						-	-	-	0	0	0	
6	Equipment	1300000.00	0.00	0.00		1300000.00	45000.00	1254181.00	0.00	0	0	1299181.00	819.00
7	Overhead expenses	125000.00	125000.00	125000.00	125000.00	500000.00	122115.00	127885.00	115625.00	78125.00	56250.00	500000.00	00
8	**Total**	**2000000.00**	**550000.00**	**700000.00**	**800000.00**	**4050000.00**	**617500.00**	**1906680.00**	**698329.00**	**605647.00**	**81935.00**	**3910091.00**	**139909.00**
												Interest Earned	**12993.00**
													152902.00

Name and Signature of Principal Investigator:

Date:

Dr. Robin Doley
Associate Professor
Dept. of Molecular Biology and Biotechnology
Tezpur University
(A Central University)
Napaam, Tezpur-784028
Sonitpur, Assam

22/11/18

Signature of Competent financial authority: _____
(with seal) Date: _____

Finance Officer
Tezpur University

*** DOS – Date of Start of project**

Note :

1. Expenditure under the sanctioned heads, at any point of time, should not exceed funds allocated under that head, without prior approval of SERB i.e. Figures in Column (VIII) should not exceed corresponding figures in Column (III)
2. Utilisation Certificate (Annexure III) for each financial year ending 31st March has to be enclosed along with request for carry-forward permission to the next financial year.

Annexure-III

UTILISATION CERTIFICATE (2 COPIES)

[FOR THE FINANCIAL YEAR – 1st April to 31ST July 2018]

1. Title of the Project/ Scheme : **Cloning, expression and structural characterization of anti-thrombin from cattle tick salivary gland: designing of novel anti-thrombin peptide.**

2. Name of the Institution : **Tezpur University**

3. Name of the Principal Investigator: **Dr. Robin Doley**

4. Science & Engineering Research Board (SERB) Sanction order No & date sanctioning the project: **SERB/F/2406/2014-15 dated 03.07.2014** (First financial sanction order)

5. Head of account as given in the original sanction order: 1. **Non-recurring Items (Equipments)**
   2. **Recurring Items (Manpower, consumables, Travels, Contingencies, Overhead charges)**

6. Amount brought forward from the previous Financial year quoting SERB letter no and date in which the authority to carry forward the said amount was given
   i. Amount: **2,21,844.00**
   ii. Letter No: **NA**
   iii. Date: **NA**

7. Amount received during the financial year (Please give SERB Sanction order no and date)
   i. Amount: **Nil**
   ii. Order No: NA

8. Total amount that was available for expenditure (excluding commitments) during the financial year (Sr. No. 6+7) **Rs. 2,21,844.00**

9. Actual Expenditure (excluding commitments) Incurred during the financial year (upto 1st April to 31st July 2018) : **Rs. 81935.00**

10. Balance amount available at the end of the financial year: **Rs. 139909.00**

11. Unspent balance refunded, if any (please give details of cheque no etc.): **Rs. 139909.00+12993.00 (Bank Interest)= Rs. 152902.00 Cheque No.**

12. Amount to be carried forward to the next financial year (if applicable): NA

Finance Officer
Tezpur University

UTILISATION CERTIFICATE

Certified that out of the **NIL** of grant–in-aid sanctioned during the year **2017-18** in favour of NA vide SERB order **NA** and **Rs. 221844.00** on account of unspend balance of the previous year, a sum of **Rs. 81935.00** has been utilized for which it was sanctioned and that the balance of Rs. **139909.00 Plus Rs 12993.00 (Interest earned) = Rs. 152902.00** remaining unutilised at the end of the year has been refunded/returned to SERB (vide DD/Cheque No. 532769 dated 11-12-2018) /~~will be adjusted towards the grants-in-aid payable during the next year i.e.~~

Signature of PI
Date:

Dr. Robin Doley
Associate Professor
Dept. of Molecular Biology and Biotechnology
Tezpur University
(a Central University)
Napaam, Tezpur- 784 028
Sonitpur, Assam (India)

Signature of Registrar/
Head of the Institute
Date:

Registrar
Tezpur University

Accounts Officer of the
Institute
Date: 22/11/18

Finance Officer
Tezpur University

(Countersigned in SERB)

Signature:_____
Designation:_____
Date:_____

जारी करने वाली शाखा
Issuing Branch: TEZPUR UNIVERSITY
कोड नं /CODE No: 14259
Tel No. 03712-267285

State Bank of India

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DEMAND DRAFT

Key: VEFDES
Sr. No: 247337

1	1	1	2	2	0	1	8
D	D	M	M	Y	Y	Y	Y

मांगे जानेपर FUND FOR SCIENCE & ENGINEERING RESEARCH**********

या उनके आदेश पर
OR ORDER

ON DEMAND PAY

One Lakh Fifty Two Thousand Nine Hundred and Two Only

रुपये RUPEES

152902.00

अदा करें ₹

IOI 000471532769 Key: VEFDES Sr. No: 247337 AMOUNT BELOW 152903(176) मूल्य प्राप्त / VALUE RECEIVED
Name of Applicant TEZPUR UNIVERSITY R& D

भारतीय स्टेट बैंक
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अदाकर्ता शाखा / DRAWEE BRANCH:NEW DELHI MAIN BRANCH
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