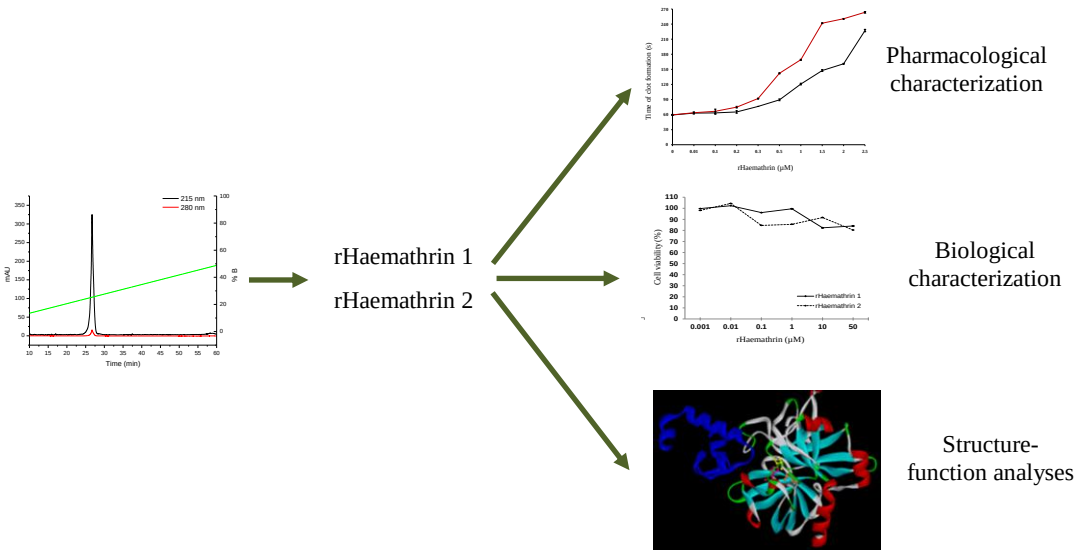
Chapter 5
Biochemical, pharmacological and biophysical characterization of recombinant haemathrins

Graphical abstract



5.1 Introduction

In the previous chapter identification and over-expression of two genes coding for thrombin inhibitors from the salivary gland of *Haemaphysalis bispinosa*, have been described. In this chapter, biochemical, biophysical and biological characterization of the recombinant thrombin inhibitors is carried out. rHaemathrins were found to inhibit *in vitro* coagulation of platelet poor plasma. The serine protease specificity of recombinant haemathrins was assayed using chromogenic substrates and these were found to specifically inhibit thrombin. The recombinant proteins inhibited thrombin with an IC_{50} of 46.13 ± 0.04 µM for rHaemathrin 1 and 40.057 ± 0.054 µM for rHaemathrin 2. However, when rHaemathrins were incubated for longer period of

time with thrombin the inhibitory activity was lost. Thus, it was found that haemathrins may inhibit thrombin at the initial stage, however thrombin expels the inhibitory activity by proteolytic cleavage of the inhibitors.

5.2 Materials and Methods

5.2.1 Materials

Chromogenic substrates were procured from Chromogenix (MA, USA) and coagulation factors were from Hematologic Technologies Inc. (Vermont, USA). RAW264.7 murine macrophage cell-line was kindly provided by Prof. A. K. Buragohain, Dibrugarh University. All other chemicals and reagents used were of analytical grade and purchased from Sigma (MO, USA), Merck (Mumbai, India) and Himedia (Mumbai, India).

5.2.2 Blood coagulation assay

rHaemathrins were tested for thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT). Briefly, citrated goat plasma (50 µl) was preincubated with different concentrations of the peptides (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 (TT: 50 µl of thrombin solution; PT: 50 µl of Uniplastin; APPT: 50 µl of Liquecelin added for 3 min and reaction started with 50 µl of 20 mM $CaCl_2$). The time of clot formation was recorded using a COAstat-1 coagulation analyzer (Tulip groups, Verna, India).

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

5.2.4 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^{-1})/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% [116].

5.2.5 Dose-dependent thrombin inhibition

Inhibition of amidolytic activity of thrombin by the peptides was assayed in 96-well microtiter plates in 20 mM Tris pH 7.4, 100 mM NaCl buffer containing 1 mg ml^{-1} 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.

5.2.6 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 preceeding section.

5.2.7 Chromatographic analysis of rHaemathrins treated with thrombin

rHaemathrin 1 or rHaemathrin 2 was incubated with human α-thrombin 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 (GL Biochem Ltd., 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. The coagulation assays of the peptidic fragment were performed as described in section 5.2.2.

5.2.8 Structure prediction and docking

Since no suitable structural template was found for structure prediction of haemathrins, QUARK tool (http://zhanglab.ccmb.med.umich.edu/QUARK/) was utilized for template-free protein structure prediction i.e. *Ab initio* protein structure building [285]. Query sequences were first broken into fragments of 1-20 residues where multiple fragment structures were retrieved at each position from unrelated experimental structures. Full-length structure models were then assembled from fragments using replica-exchange Monte Carlo simulations, which were guided by a composite knowledge-based force field. The 3-dimensional structure of haemathrins were also predicted using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) which uses a hierarchical protocol for automated protein structure prediction [286]. The predicted 3-D structures were subjected to RAMPAGE program (http://www-cryst.bioc.cam.ac.uk/rampage**)** to analyze their quality from the Ramachandran plots [287]. The protein-protein docking of haemathrin with α-thrombin (4BOH) was performed using Z-dock server (http://zdock.umassmed.edu/)

which utilizes the Fast Fourier Transform algorithm for an efficient global docking search on a 3D grid [288].

5.2.9 Hemolytic activity assay

The hemolytic activity of recombinant haemathrins was assayed using 5% RBC as described in section 3.2.8. 0 – 100 µM of rHaemathrin 1and 2 were incubated with 5% RBC for 1 h and the hemolysis of the cells were read spectrophotometrically.

5.2.10 Anti-microbial activity assay

For, anti-microbial activity assay, the bacterial inoculum (2 gram-positive bacteria: *Bacillus cereusa* and *Staphylacoccus aureus* and 2 gram-negative bacteria: *Pseudomonas aeruginosa* and *Klebsiella pneumonia*) was uniformly spread using sterile glass spreader on Mueller Hinton (MH) agar plate. 50 µl of rHaemathrins (1, 5 and 20 µg) were added to each of the wells (7 mm diameter holes bored in the agar medium). The plates were incubated for overnight at 37ºC. Tris-Cl (pH 7.4) was used as negative control and Gentamycin (0.4 µg μl^{-1}) was used as positive control. After incubation, the plates were observed for zone of clearance.

5.2.11 Cell cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to assess the *in vitro* cytotoxicity of rHaemathrins using RAW264.7 murine macrophage cell-line according to Borgohain *et. al.* [289]. Briefly, ~$1x10^4$ cells ml^{-1} were cultured in DMEM media supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS (Fetal Bovine Serum) and penicillin (100 units ml^{-1}), streptomycin (10 µg ml^{-1}) and incubated at 37^oC in a humidified 5% CO_2 environment. After 80% cell confluency, the cells were treated with different concentrations of rHaemathrin 1 and 2 and incubated for 24 h. After 24 h incubation, MTT was added to each well of the plates and incubated for 4 h. The formed formazan complex was dissolved properly in MTT solvent and the absorbance was taken at 580 nm. Percentage cell viability was calculated by taking the control cell viability as 100%.

5.3 Results

5.3.1 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. 5.1). 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. 5.2). 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. 5.3). This confirms that the haemathrins are anticoagulant in nature.

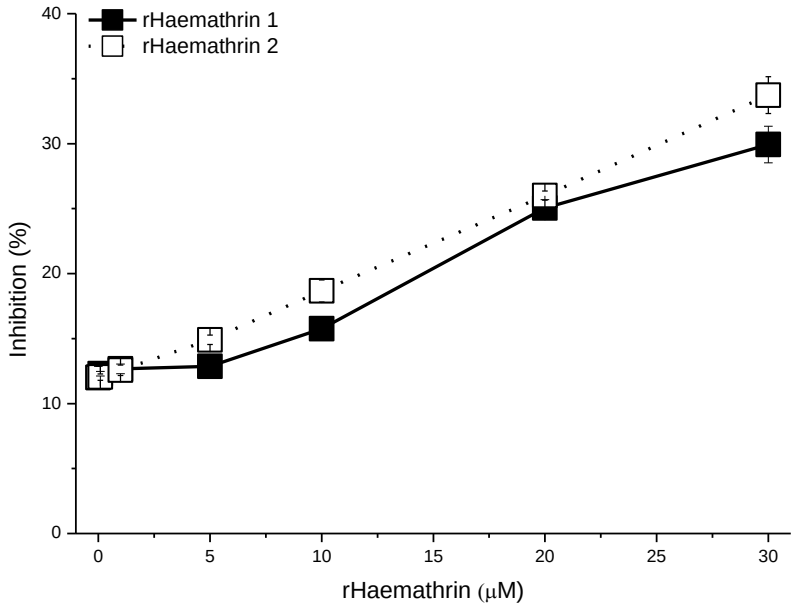


Figure 5.1 Graph showing increase in thrombin time of platelet poor plasma when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

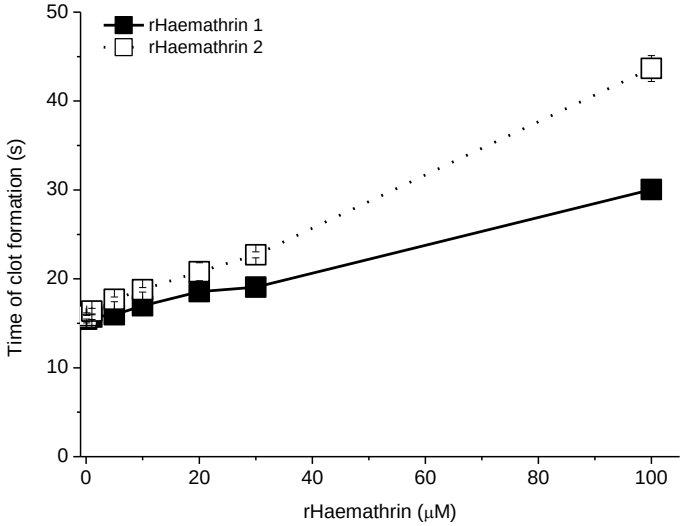


Figure 5.2 Graph showing increase in prothrombin time of platelet poor plasma when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

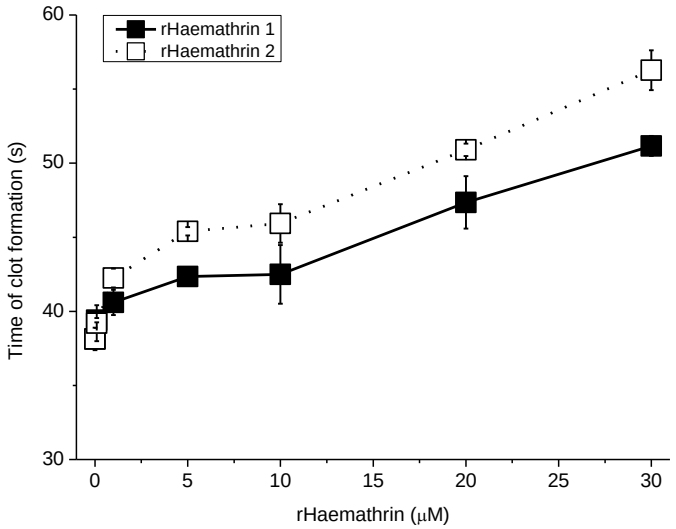


Figure 5.3 Graph showing increase in activated partial thromboplastin time of platelet poor plasma when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

5.3.2 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.

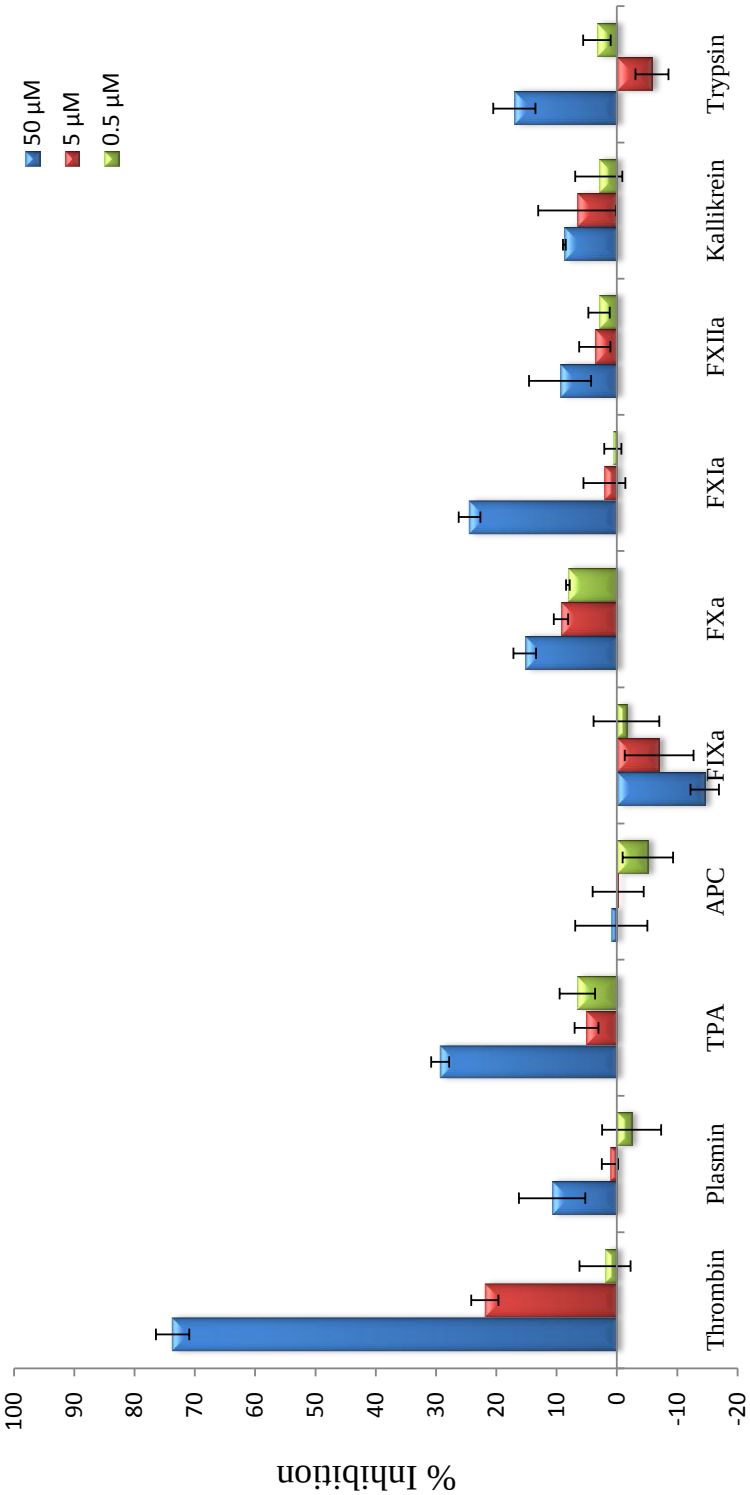


Figure 5.4 Selectivity profile of rHaemathrins 1. rHaemathrin 1 was screened against 10 proteases using chromogenic substrates (n=3, error bars represent ±S.D.).

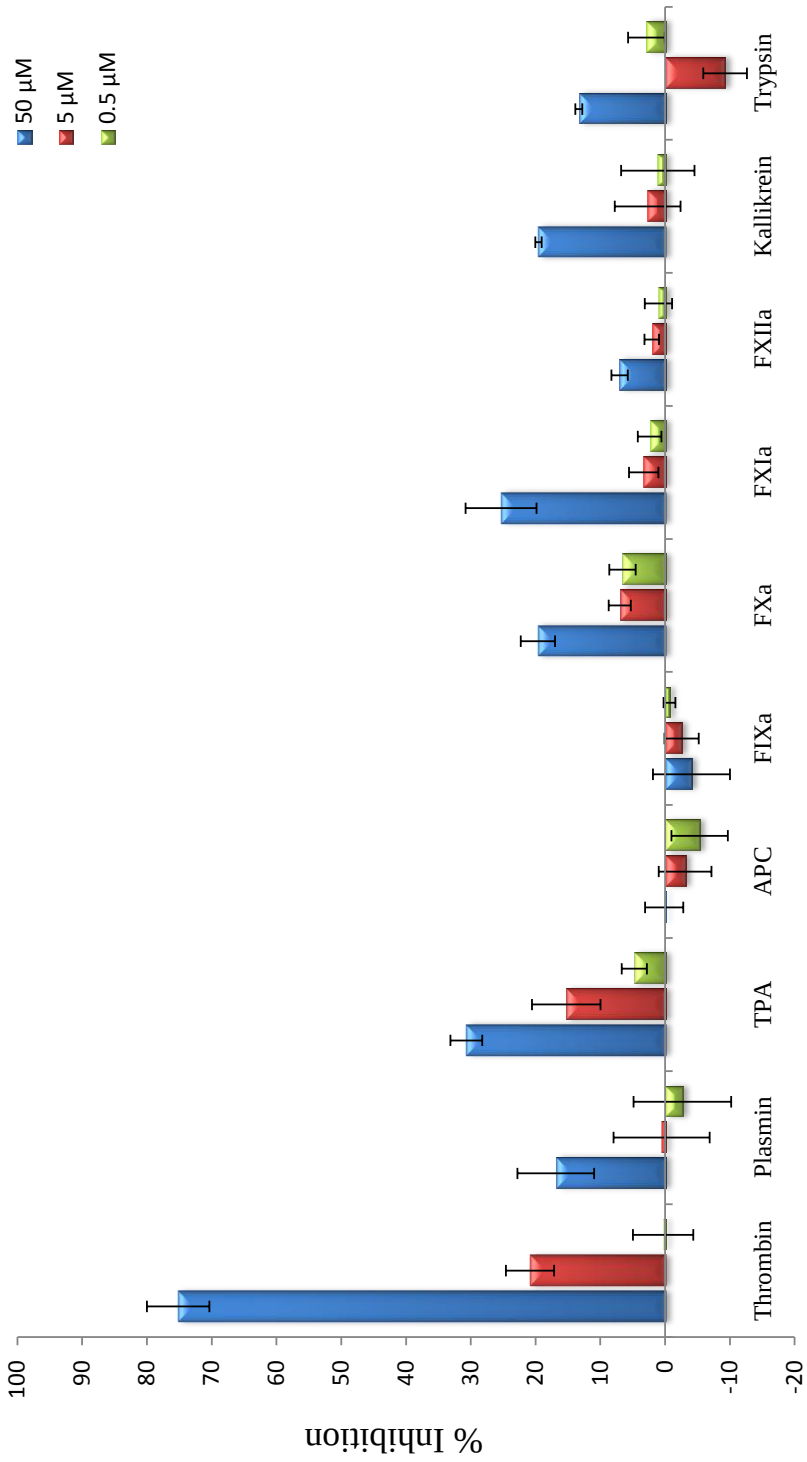


Figure 5.5 Selectivity profile of rHaemathrins 2. rHaemathrin 2 was screened against 10 proteases using chromogenic substrates (n=3, error bars represent ±S.D.).

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. 5.4 and Fig. 5.5). 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.

5.3.3 Fibrinogen clotting time

Haemathrin 1 and 2 both prolonged fibrinogen clotting time in a dose-dependent manner (Fig. 5.6). 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.

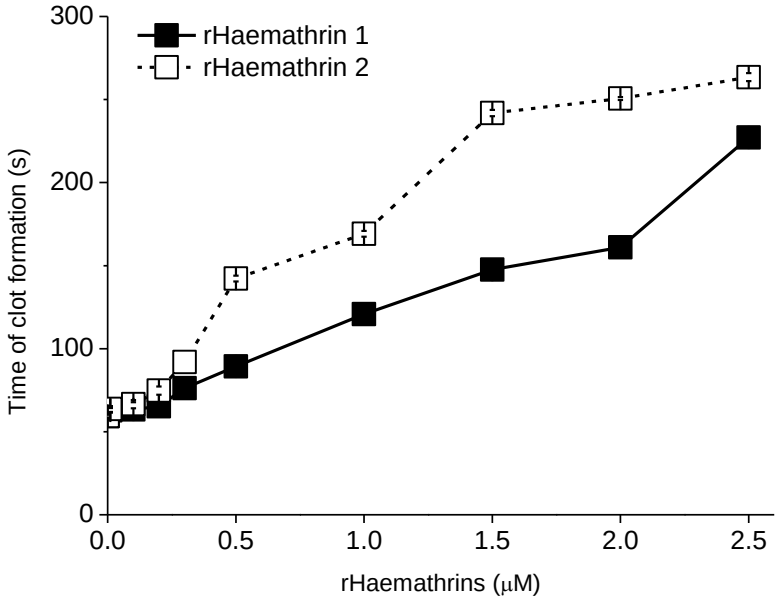
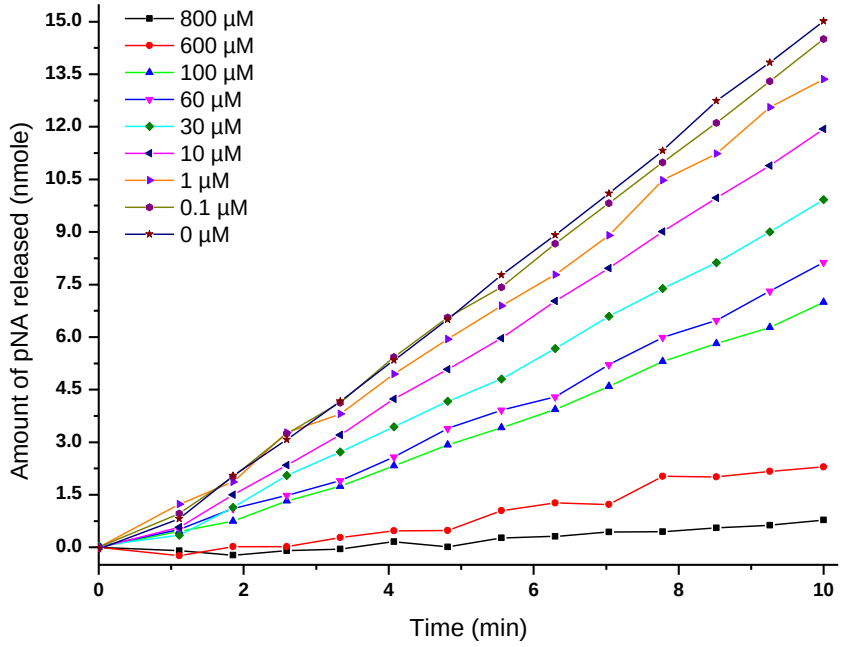


Figure 5.6 Graph showing increase in fibrinogen clotting time when incubated with rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

5.3.4 Thrombin inhibitory activity

rHaemathrins inhibited amidolytic activity of human α-thrombin dose-dependently (Fig 5.7). 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. 5.8). 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. 5.9).

a



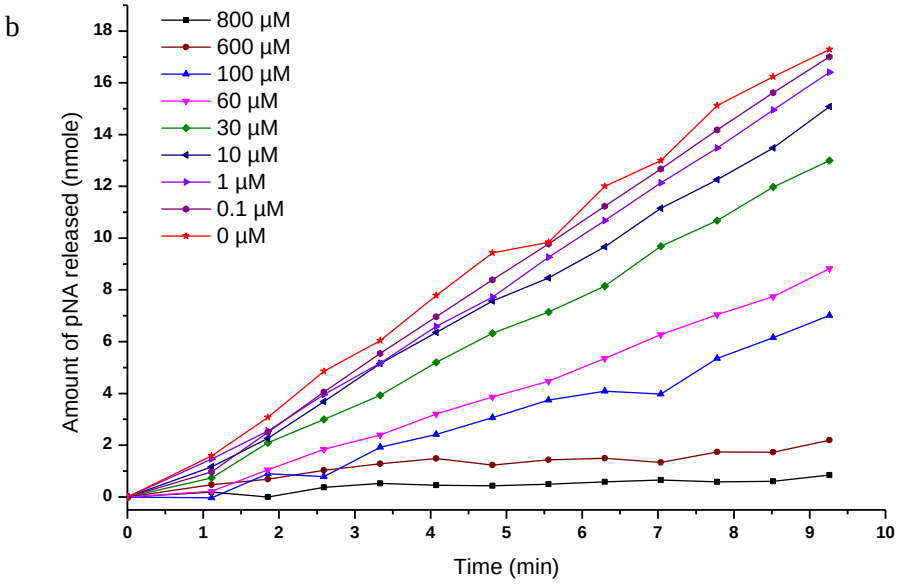


Figure 5.7 Linear progression curves of thrombin inhibition by (a) rHaemathrin 1 and (b) rHaemathrin 2 (inset: concentration in µM).

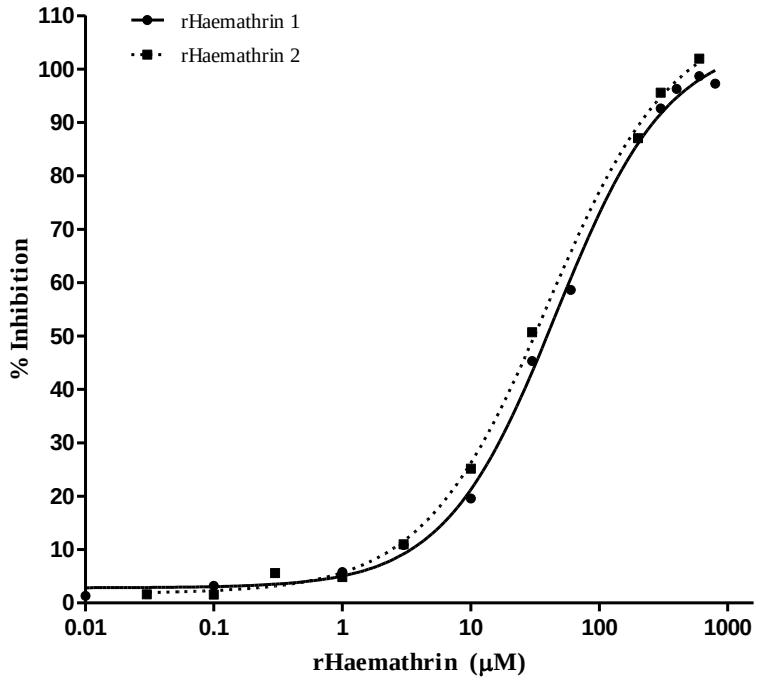
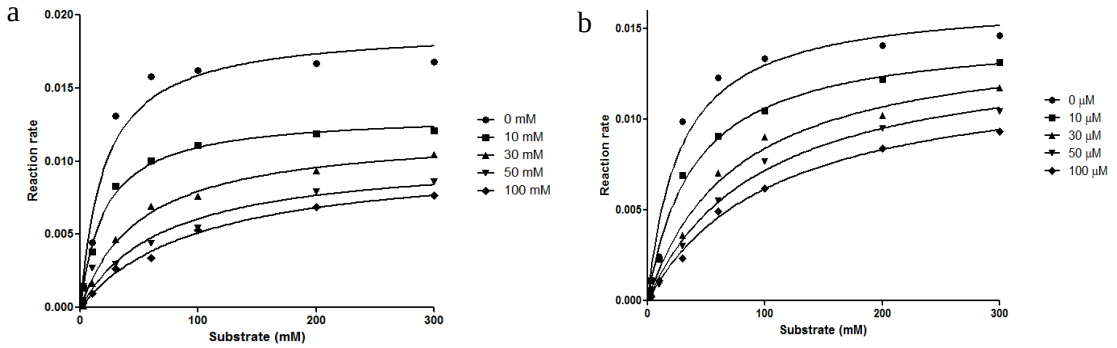


Figure 5.8 Dose-response curve of thrombin inhibition by rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed). The IC_{50} of haemathrin was calculated to be 46.13 ± 0.04 µM and that of haemathrin 2 to be 40.057±0.054 µM (n=3, values are mean ± S.D.).



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

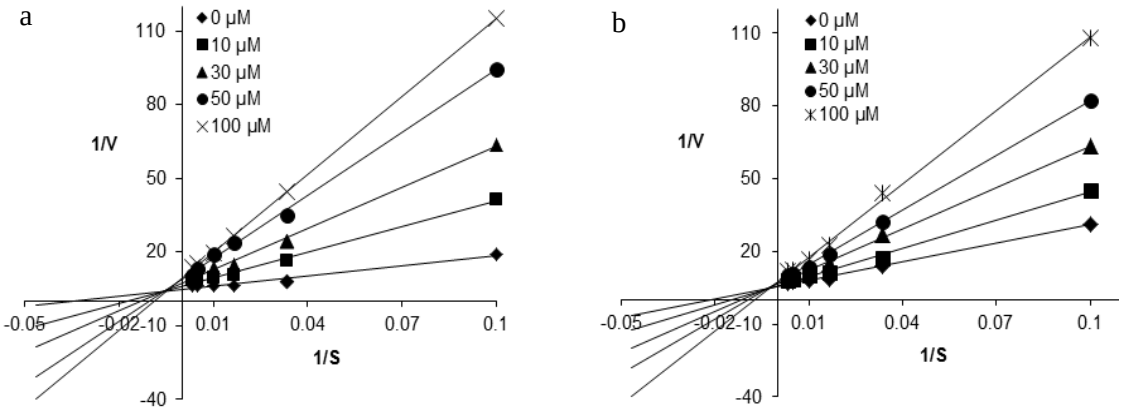


Figure 5.9 Michaelis-Menten curve of enzyme inhibition by (a) rHaemathrins 1 and (b) rHaemathrin 2 (n=3, values are mean ± S.D.). Lineweaver-Burk plot showing rHaemathrin 1 (a) and rHaemathrin 2 (b) as a mixed-type of thrombin inhibitor.

5.3.5 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. 5.10). 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.

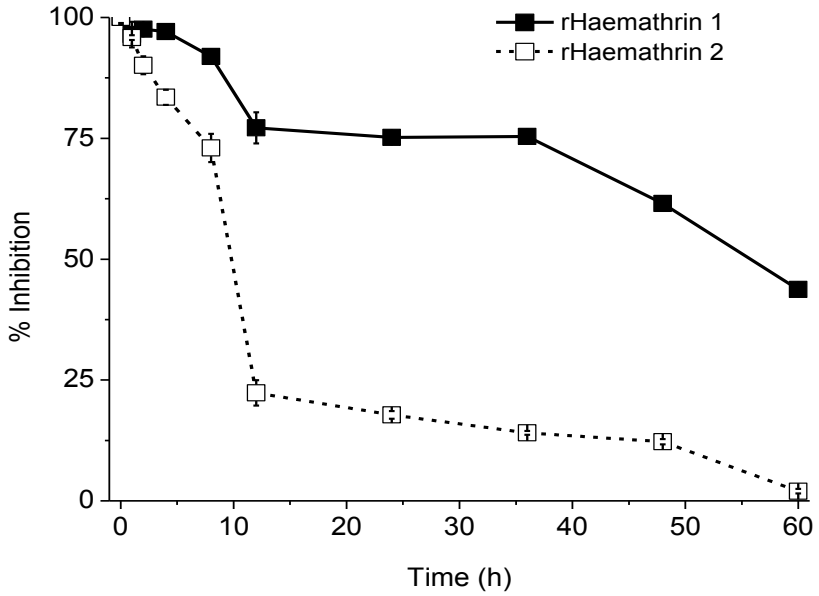


Figure 5.10 Graph showing decrease of percentage inhibition of thrombin amidolytic activity by rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) with time (n=3, error bars represent ±S.D.). Thrombin inhibition at 2 min was considered as 100%.

5.3.6 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. 5.11a, 5.11b). 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. 12a, 12b). Four fragments were identified for both rHaemathrin 1 and 2, which confirmed the cleavage site of thrombin (Table 5.1). 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.

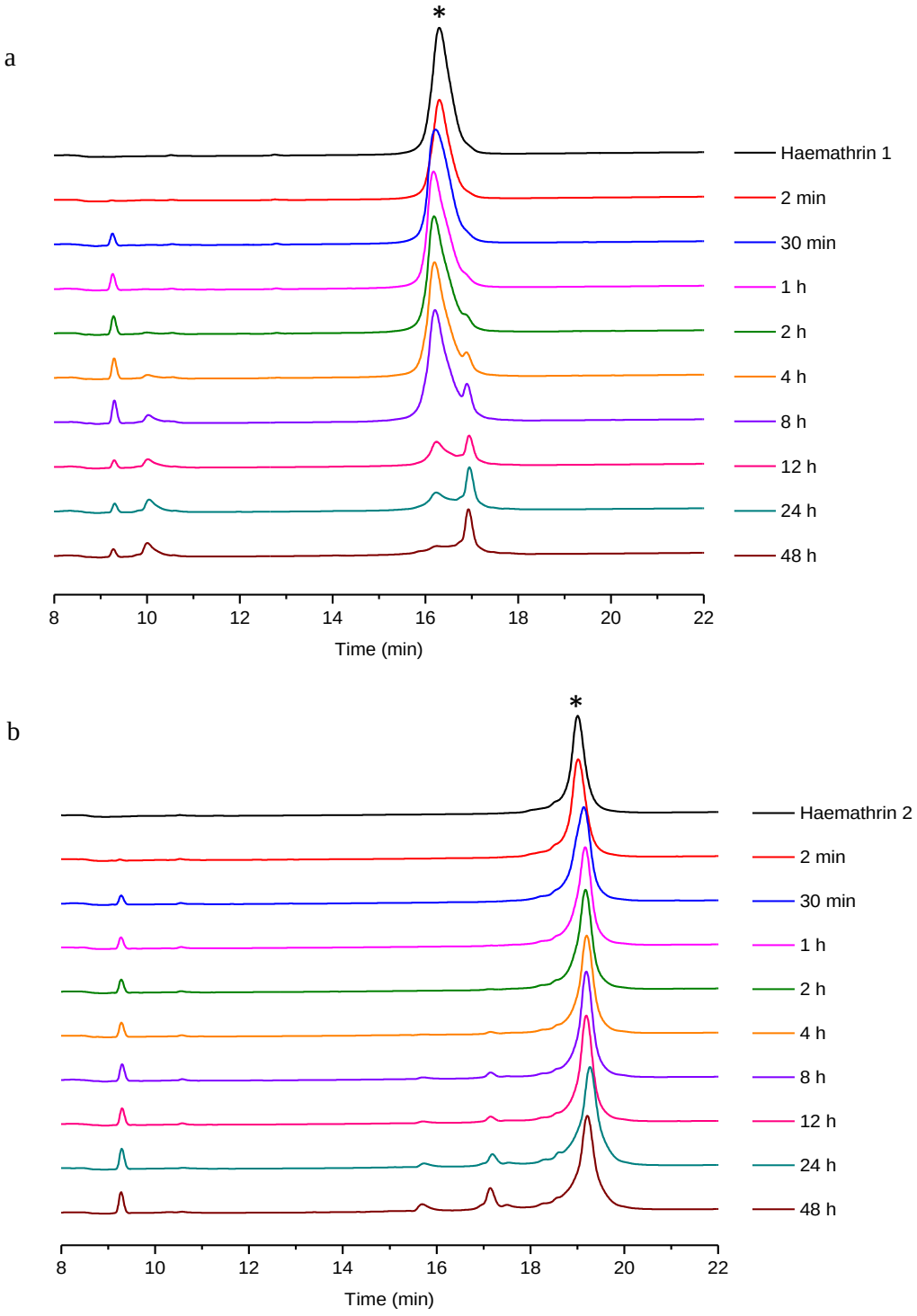


Figure 5.11 Reversed phase chromatogram showing cleavage of (a) rHaemathrin 1 and (b) rHaemathrin 2, when incubated with thrombin for different time intervals. * indicates peaks of rHaemathrin 1 and 2.

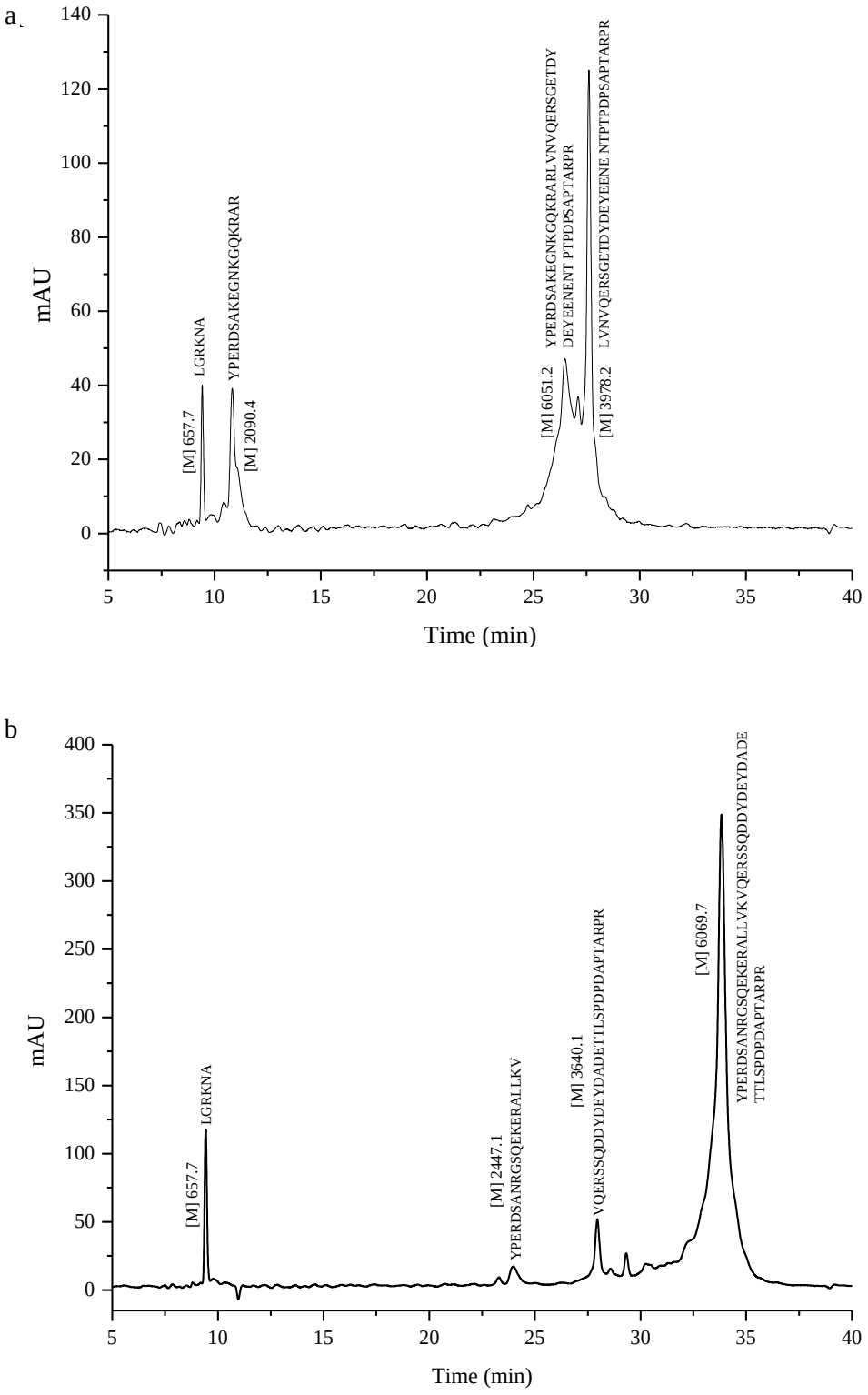


Figure 5.12 Chromatogram showing peaks of cleaved products of (a) rHaemathrin 1 and (b) rHaemathrin 2 corresponding to peptidic fragments identified by mass-spectrometry and FindPept tool.

5.3.7 Inhibitory activity of peptidic fragments

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 5.1). When tested for anti-coagulation activity using plasma, the fragments did not show inhibition of coagulation of the plasma (Table 5.2).

Table 5.1 Percentage inhibition of thrombin amidolytic activity by peptidic fragments of rHaemathrins.

Inhibitor	Peptide mass (*m/z*)		Peptide fragment	% Inhibition
	Experimental	Theoretical		
rHaemathrin 1 rHaemathrin 2	657.7 657.7	657.7 657.7	LGRKNA LGRKNA	14.96 ± 1.46 14.96 ± 1.46
	2090.4 2447.1	2090.2 2446.7	YPERDSAKEGNKGQKRAR YPERDSANRGSQEKERALLVK	15.42 ± 0.14 5.26 ± 2.21
	3978.2 3640.1	3978.1 3640.7	LVNVQERSGETDYDEYEENENTPTPDPSAPTARPR VQERSSQDDYDEYDADETTLSPDPDAPTARPR	29.40 ± 2.59 25.89 ± 2.82
	6051.2 6069.7	6050.4 6069.3	YPERDSAKEGNKGQKRARLVNVQERSGETDYDEYEENENTPTPDPSAPTARPR YPERDSANRGSQEKERALLVKVQERSSQDDYDEYDADETTLSPDPDAPTARPR	38.85 ± 2.38 16.02 ± 2.15
	6690.3 6709.1	6690.1 6709.1	YPERDSAKEGNKGQKRARLVNVQERSGETDYDEYEENENTPTPDPSAPTARPRL GRKNA (full-length) YPERDSANRGSQEKERALLVKVQERSSQDDYDEYDADETTLSPDPDAPTARPRL GRKNA (full-length)	52.50 ± 1.89 56.37 ± 0.82

Table 5.2 Anti-coagulation activity assay of haemathrin peptidic fragments. Fragment 53-59 is common for both haemathrin 1 and 2.

		Thrombin Time		Prothrombin Time		APTT	
	Fragments (30 µM)	**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
	54-59	12.07 ± 0.64	1.02	16.63 ± 0.64	1.07	38.20 ± 1.71	1.00

5.3.8 Computational docking of rHaemathrin to α-thrombin

The 3D structure of haemathrin 1 and 2 were predicted using Quark and I-TASSER online server. Though these were random coils in solution (section 4.3.17), predicted structure of the peptides showed alpha-helical coils (Fig. 5.13). However, the structure of haemathrin 1 predicted by I-TASSER was representative of random coils (Fig. 5.14a). Ramachandran plots of predicted structure of haemathrin 1 by Quark tool revealed only 52.6% residues in the favorable region (expected ~98%) and that of haemathrin 1 and 2 by I-TASSER revealed 40.4% and 52.6% residues in the favorable region, respectively (Fig. 5.15). This showed that these predicted structures were obsolete. However, the plot of predicted structure of haemathrin 2 by Quark tool revealed that ~90% of the residues were in the favorable region and ~5% in the allowed region (Fig. 5.15). Therefore, haemathrin 2 was taken as template for the docking studies and it was found that haemathrin 2 docked near the active site of α-thrombin; however, it did not interact with the active site residues of thrombin (Fig. 5.16)

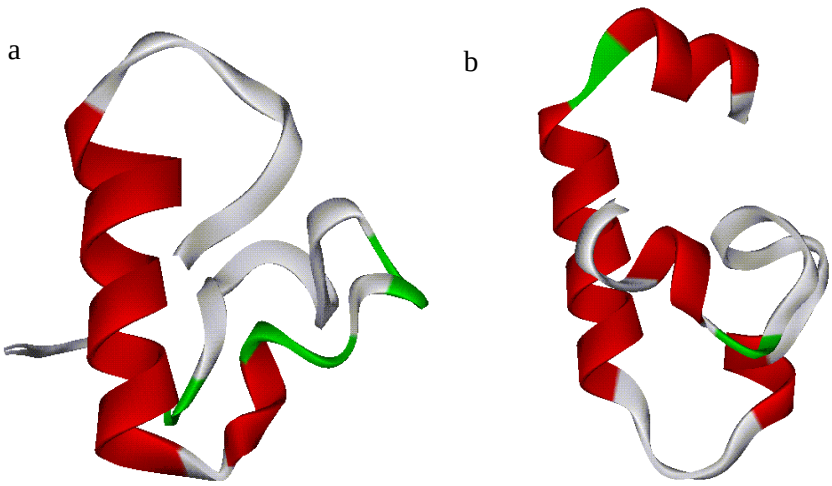


Figure 5.13 Predicted tertiary structure of (a) haemathrin 1 and (b) haemathrin 2 by Quark tool.

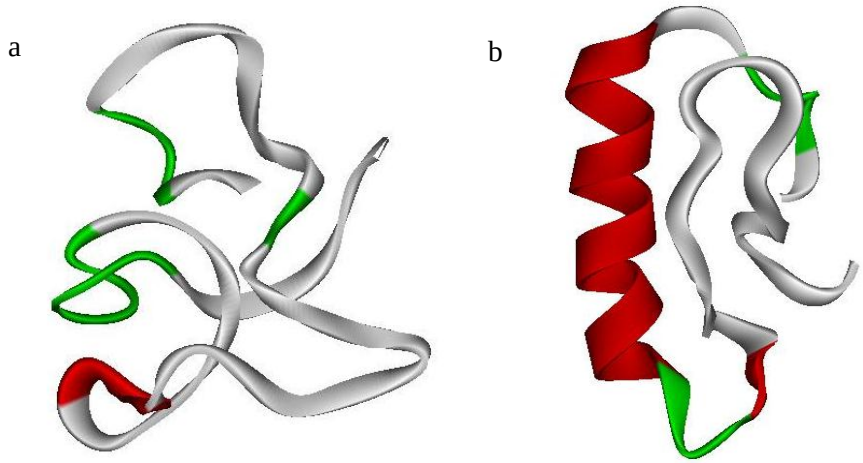
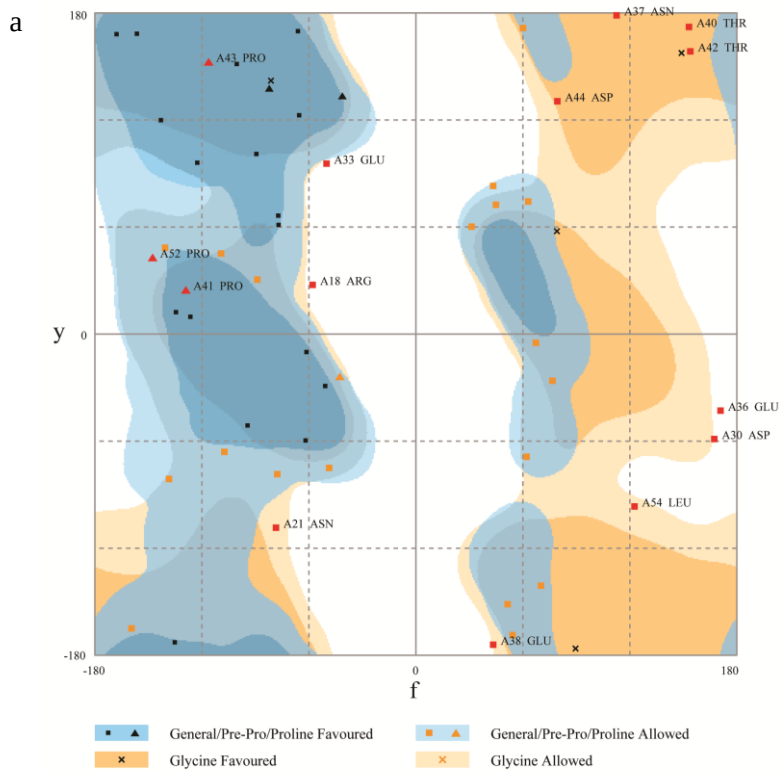
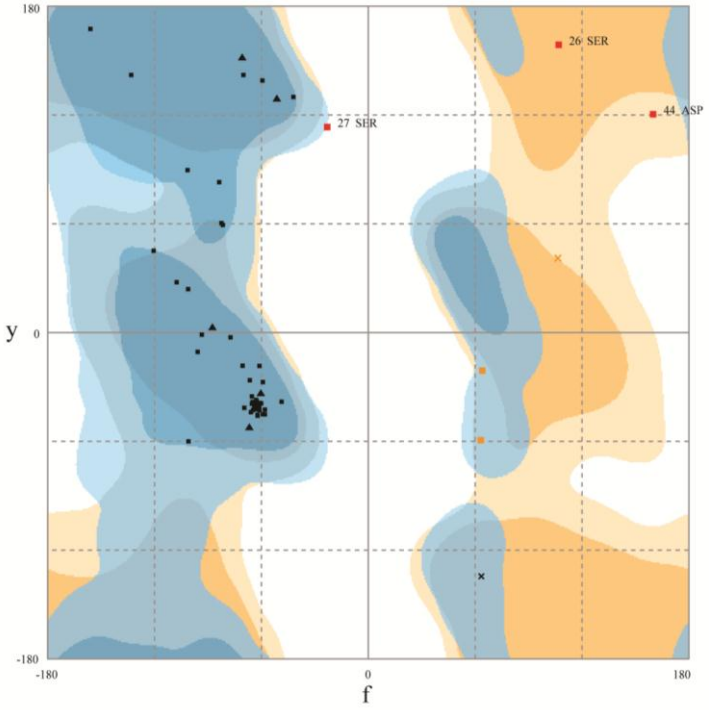


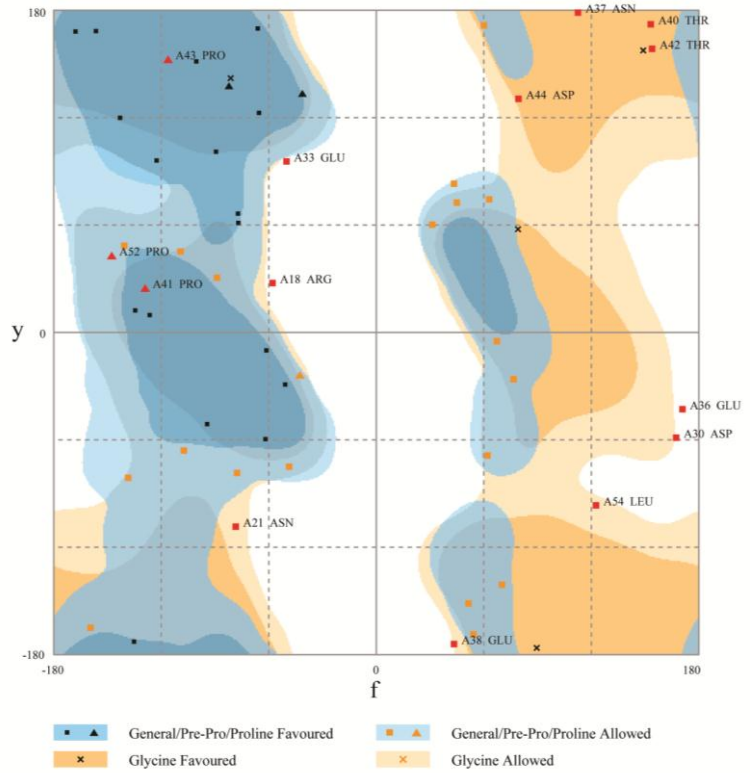
Figure 5.14 Predicted tertiary structure of (a) haemathrin 1 and (b) haemathrin 2 by I-TASSER server.



b


180
0
-180
y
-180
0
180
f
26 SER
27 SER
44 ASP

c


180
0
-180
y
-180
0
180
f
A37 ASN
A40 THR
A42 THR
A44 ASP
A43 PRO
A33 GLU
A52 PRO
A41 PRO
A18 ARG
A36 GLU
A30 ASP
A54 LEU
A21 ASN
A38 GLU
General/Pre-Pro/Proline Favoured
Glycine Favoured
General/Pre-Pro/Proline Allowed
Glycine Allowed

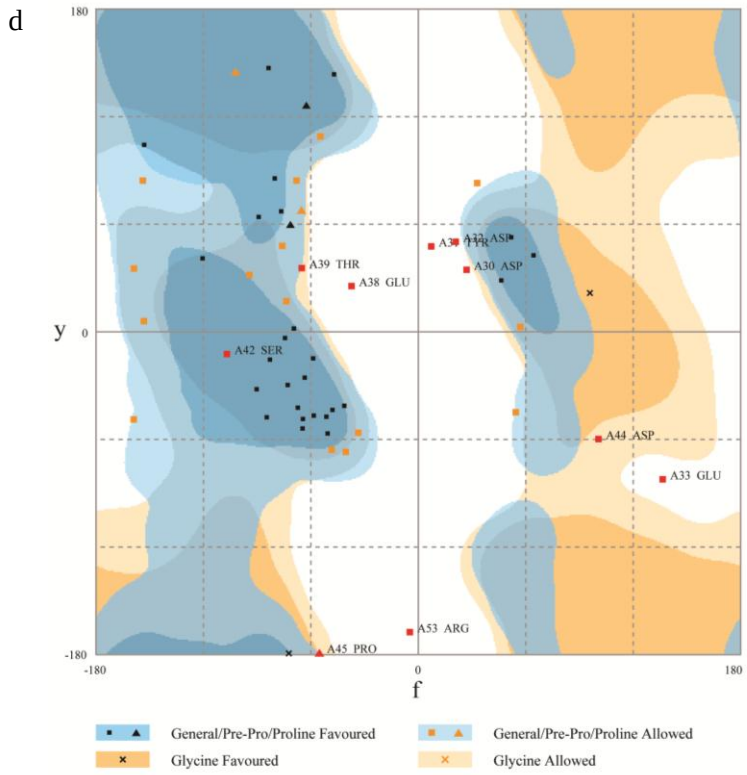
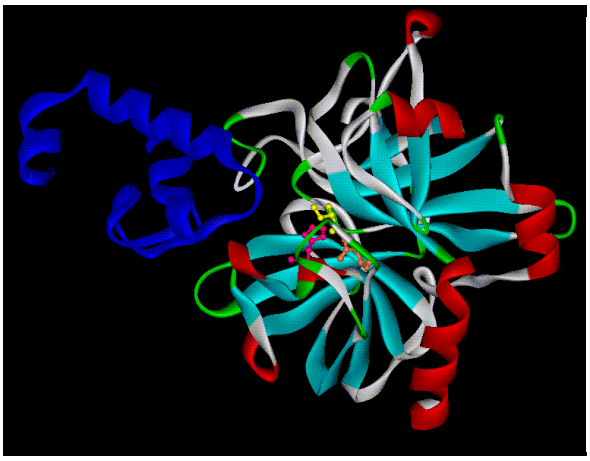


Figure 5.15 Ramachandran plots of protein structures predicted using Quark tool – (a) haemathrin 1, (b) haemathrin 2 and I-TASSER – (c) haemathrin 2, (d) haemathrin 2 (f=Phi angle, y=Psi angle).



1. **Cation-Pi Interactions**
   TRP- 370 of thrombin and **ARG-25** of haemathrin 2 form Cation-Pi Interactions with the distance of 5.92 angstrom and (cation-Pi) Angle of 89.45

2. **Ionic Interactions (within 6 Angstroms)**
   (thrombin) **ARG-340** : **ASP-46** (haemathrin 2)
   (thrombin) **GLU-522** : **ARG-51** (haemathrin 2)
   (thrombin) **GLU-522** : **ARG-56** (haemathrin 2)

3. **Hydrophobic Interactions (within 5 Angstroms)**
   (thrombin) **TRP-370** : **PRO-48** (haemathrin 2)
   (thrombin) **TRP-468** : **LEU-19** (haemathrin 2)
   (thrombin) **TRP-468** : **VAL-22** (haemathrin 2
   (thrombin) **TRP-468** : **LEU-54** (haemathrin 2)
   (thrombin) **VAL-472** : **VAL-22** (haemathrin 2)
   (thrombin) **VAL-472** : **LEU-54** (haemathrin 2)

Figure 5.16 Computational docking of haemathrin 2 to thrombin. Left panel: docked structure of haemathrin 2 with α-thrombin; Right panel: interacting residues of haemathrin 2 with thrombin.

5.3.9 Hemolytic activity assay

To check the effect of rHaemathrins on RBC, i.e., if they possess membrane-damaging property rHaemathrins were assayed for *in vitro* hemolysis. rHaemathrins did not show any hemolytic activity at even 100 µM of the peptides (Fig. 5.17), suggesting that these do not have any adverse effect on the cell-membrane.

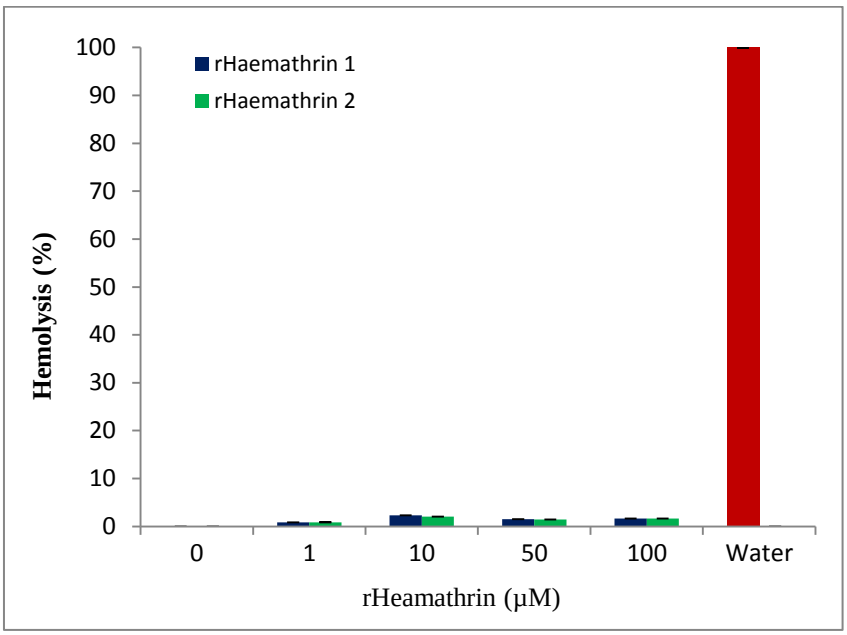


Figure 5.17 Graph showing percentage hemolysis of RBC by rHaemathrins. Hemolysis of RBC by water was taken as 100% (n=3, error bars represent ±S.D.).

5.3.10 Anti-microbial sensitivity test

The anti-microbial activity of the recombinant peptides was tested using bacterial cells. A zone of clearance (~5 mm) was observed in every plate for the positive control (Gentamycin). However, no zone of clearance was observed in any plate for both gram-positive and gram-negative bacteria treated with rHaemathrin 1 and 2 (Fig. 18a-h), showing that rHaemathrins do not have anti-bacterial cell and are non-toxic to microbial cells.

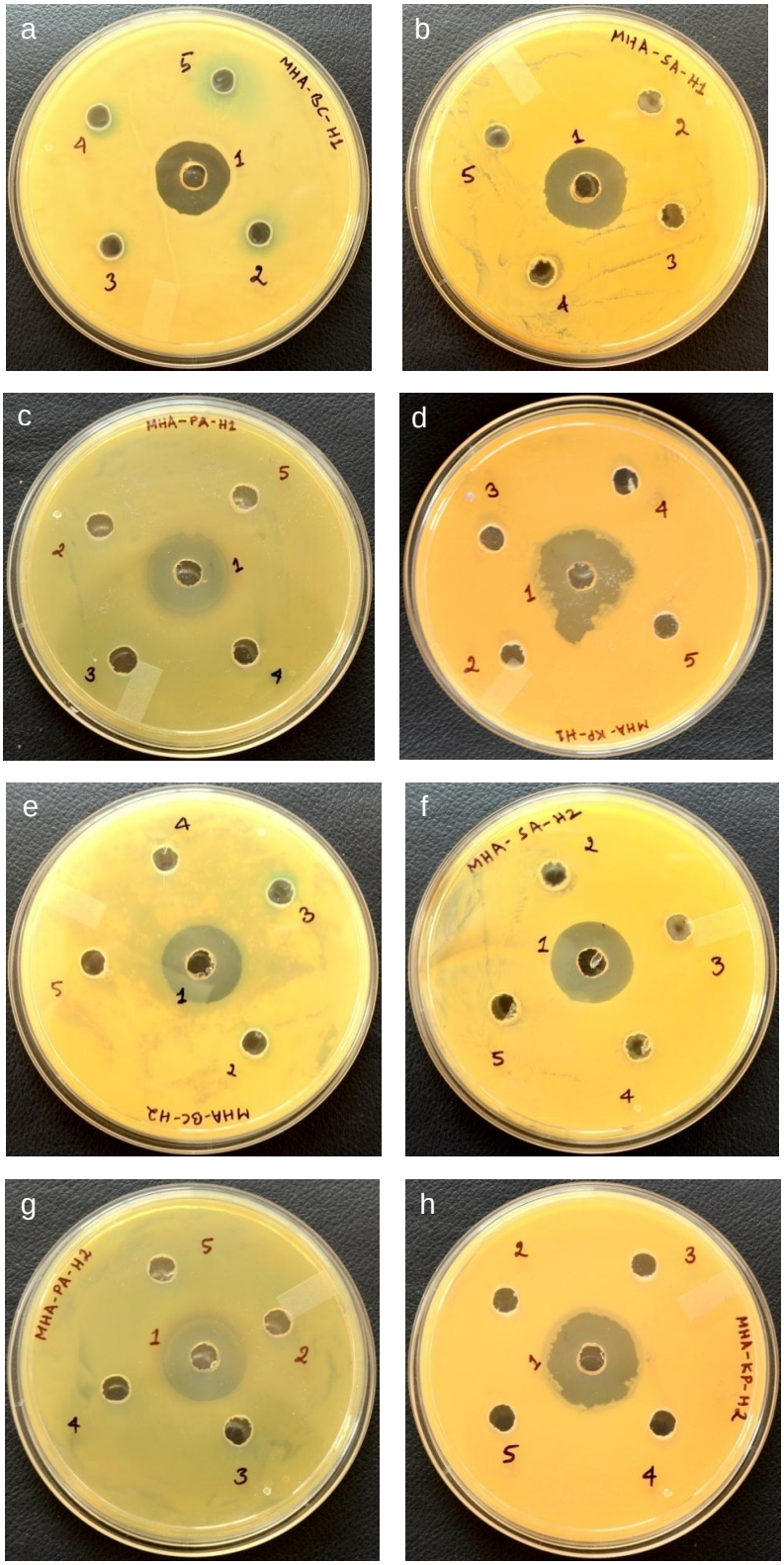


Figure 5.18 Anti-microbial sensitivity test of (a-d) rHaemathrin 1 and (e-h) rHaemathrin 2 by well-diffusion method (a, e: *Bacillus cereus*; b, f: *Staphylococcus aureus*; c, g: *Pseudomonas aeruginosa*; d, h: *Klebsiella pneumoniae*).

5.3.11 Cell cytotoxicity assay

rHaemathrins were tested in cell culture against murine macrophage cell-line to assess their cytotoxicity by MTT assay. It was found that the peptides were not toxic to the cells, as the cell viability was maintained above 80% for the highest concentration of the peptides in the experiment (Fig. 19).

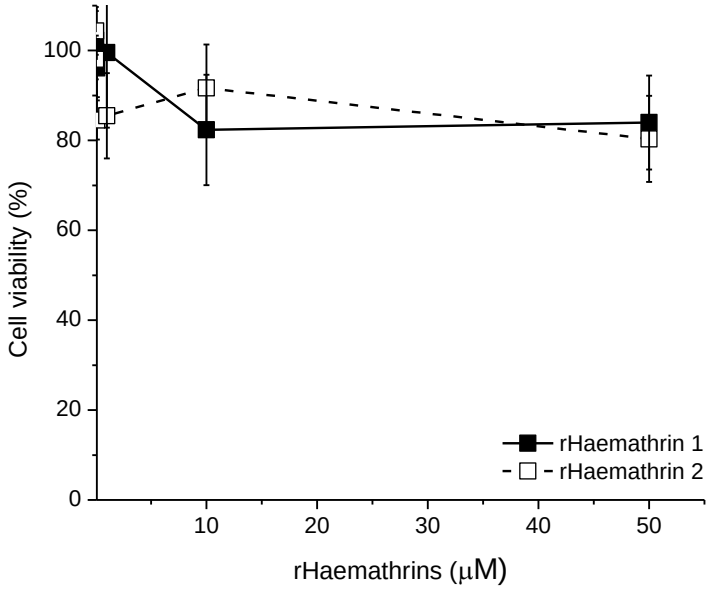


Figure 5.19 Graph showing percentage cell viability of murine macrophage cell line treated with different concentrations of rHaemathrin 1 (continuous) and rHaemathrin 2 (dashed) (n=3, error bars represent ±S.D.).

5.4 Discussions

This chapter reports the biological and biophysical characterization of recombinant haemathrin 1 and 2. This is the first description of anti-hemostatic protein from the salivary gland of *Haemaphysalis bispinosa*. As discussed in earlier chapters, rHaemathrins coded for mature peptides of ~6.7 kDa and showed about 60-70% similarity to madanins from *H. longicornis*. These peptides were recombinantly produced in *E. coli* cells by IPTG induction. 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. This shows that rHaemathrins either inhibit factor Xa (FXa) or thrombin of the coagulation pathway. rHaemathrins are found to be similar to recombinant madanins which prolonged the APTT and PT of plasma. Madanins were found to interact with thrombin/FXa, more specifically with thrombin [118]. Chimadanin, isolated from *H. longicornis* and recombinantly expressed in *E. coli*, is also found to prolonged APTT and PT of citrated sheep plasma to about 2 fold at a dose of 30 µM [224]. Thus rHaemathrins are anticoagulants similar to madanin and chimadanin.

To investigate if rHaemathrins target thrombin, like madanin, these were tested for inhibition of thrombin amidolytic activity using small chromogenic substrate. Progress curve of thrombin inhibition by rHaemathrins showed a time-dependent inhibition (Fig. 5.7), suggesting that rHaemathrins are slow binding inhibitors. Moreover, these required high concentration (600 – 800 µM) for complete inhibition of thrombin amidolytic activity. The inhibitory constants of the peptides for inhibition of amidolytic activity of thrombin against a small chromogenic substrate were too large to be potent thrombin inhibitor. Hence, these were further confirmed for inhibition of thrombin towards its natural substrate, fibrinogen. rHaemathrin 1 and 2 were found to prolong fibrinogen clotting time to about 3.8 and 4.4 folds, respectively, showing that they effect the fibrinogenolytic activity of thrombin. This suggests that they inhibit blood coagulation by inhibiting the function of thrombin. The binding of fibrinogen might not be inhibited by rHaemathrins; however, these might be blocking the interaction with the active site of thrombin. From the above results, it was evident that rHaemathrins target thrombin specifically, and rHaemathrin 2 was found to be functionally more potent than rHaemathrin 1 though they are isoforms. This is similar to madanins where madanin 1 was found to be functionally more potent than madanin 2 [118]. Though amino acid sequence of haemathrins showed high similarity to madanins, the number of negatively charged amino acid residues is higher which may be involved in interaction with the enzyme. Hence the mechanism of inhibition might be different in these enzymes.

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. Hence the inhibitor might be binding to

active as well as other sites. However, most of the thrombin inhibitors isolated and characterized from ticks are found to be competitive inhibitors of thrombin. Koh *et.al.* reported a novel fast, tight-binding thrombin inhibitor, variegin, from the salivary gland extract of *Amblyomma variegatum* [116]. Variegin is a competitive inhibitor of thrombin and binds to thrombin active site through residues 8–14; residues 15–32 binds to thrombin exosite-I; and the first seven N-terminal residues are needed for its fast binding, though these do not interact with thrombin directly [116]. Thrombin inhibitors, ornithodorin and savignin, isolated from salivary glands of *Ornithodoros moubata* and *Ornithodoros savignyi*, respectively, are slow, tight-binding competitive inhibitors and bind thrombin to both the active site and to exosite-1 [113,115,124]. Another thrombin inhibitor, boophilin, isolated from the salivary gland of *R. microplus* inhibited thrombin had a different mode of action; it inhibited thrombin in a non-canonical manner [110]. Modeling experiments showed that the C-terminal domain of boophilin interacts with exosite I of thrombin, while the N-terminal domain interacts with thrombin active site. In addition, the reactive-site loop of the bound inhibitor was found to be exposed in the ornithodorin-like conformation [115], and could engage a second trypsin-like serine protease in a canonical manner [110].

Most of the thrombin inhibitors isolated from hematophagous target the catalytic site and/or the exosite I/exosite II of thrombin [109,115,124,290-292] and exert their function by competitive inhibition. However, haemathrins showed characteristics of mixed-type inhibitors, which bind to the allosteric site of enzyme other than the substrate binding site. Therefore, to analyze the interaction of haemathrins with thrombin, computational docking was done using Quark tool. Docking studies revealed that haemathrin 2 binds near the active site cleft of thrombin. But, it did not interact with the active site residues (His57, Asp102, Ser195) of thrombin. Haemathrin 2 interacted with thrombin through Cation-Pi, ionic, and hydrophobic interactions. Trp370 of thrombin and Arg25 of haemathrin 2 formed a Cation-Pi interaction with a distance of 5.9 Å and (Cation-Pi) angle of 89.45. Ionic interaction were formed between Arg340-Asp46, Glu522-Arg51 and Glu522-Arg56 (thrombin-haemathrin 2), while hydrophobic interactions were between Trp370-Pro48, Trp468-Leu19, Trp468-Val22, Trp468-Leu54, Val472-Val22 and Val472-Leu54 (thrombin-haemathrin 2). Thus, rHaemathrins might be interacting with

thrombin such that it creates a steric hindrance and stalls thrombin function. It does not bind to substrate binding exosite I of thrombin, which substantiates our earlier result that it is a mixed-type inhibitor.

When subjected to incubation with thrombin, rHaemathrins were found to lose their function with increase in incubation time. This could possibly be due to instability of the peptides in solution, its degradation over time or post-processing by thrombin itself. The recombinant haemathrins were incubated with thrombin for 0 – 48 h, and the reactions at different time intervals were subjected to reversed phase-HPLC. The chromatograms indicated that rHaemathrins were processed by thrombin over time like madanins. The alignment of amino acid sequences of haemathrins with that of madanins was analyzed, and it was found that haemathrins had thrombin cleavage sites like that of madanins. Haemathrin 2 had similar cleavage pattern (Leu-Leu-Val-Lys | Val-Gln-Glu-Arg) as has been reported for madanin 2 (Leu-Leu-Val-Lys | Val-Gln-Glu-Arg), with a Leu at the P4 residue and a Lys at the P1 residue similar to that of the physiological substrate protease-activated receptor (PAR)-3 [117]. But the upstream cleavage pattern of haemathrin 1 (Lys-Arg-Ala-Arg | Lys-Val-Asn-Val) differed from the former with an Arg at the P1 position and Lys at the P4 position. It is known that thrombin has a strong preference for Arg at the P1 position and most of the thrombin-cleavable peptide sequences have this residue conserved [293,294]. The downstream cleavage sites of both haemathrin 1 (Ala-Arg-Pro-Arg | Leu-Gly-Arg-Lys) and haemathrin 2 (Ala-Arg-Pro-Arg | Leu-Gly-Arg-Lys) were similar to that of madanins and resembles the motif found in physiological substrates Protein C, factor XI, factor XII and insulin-like growth factor [295]. The post-processing of haemathrins may suggest to some evolutionary advantage of the peptidic inhibitors, or the peptidic fragments may be involved in some complex functional activity within the coagulation cascade. This could also be to the evolutionary advantage of the enzyme of the host species. However, these ticks complete their feeding cycle in about a week or longer period, even if the inhibitors are processed or digested by the enzyme itself; other anti-hemostatic compounds in the saliva are involved in a complex process of inhibition of the coagulation cascade, and the processing of the inhibitors in question might be a part of the process [103,107,170,256].

The peptidic fragments when tested for inhibition of thrombin amidolytic activity, did not significantly inhibit the function of thrombin; fragment 19-53 of haemathrin 1 and fragment 22-53 of haemathrin 2 showed about 50-55% inhibitory activity when compared to the full-length peptides. The peptidic fragments did not inhibit coagulation of goat plasma. However, it was found that fragment 19-53 of haemathrin 1 showed about 1.35 fold inhibition (full-length showed 2.51 fold inhibition) and 22-53 fragment of haemathrin 2 shoed 1.16 fold inhibition (full-length showed 2.83 fold inhibition) of thrombin time. But, these had no effect of PT and APTT of the plasma. It could most likely be that the amino acid residues of these fragments are involved in interaction with thrombin, and the fragments bind to thrombin; these fragments contain high number of negatively charged amino acid residues, Asp and Glu. The peptidic fragments of madanins, which are also processed by thrombin, marginally affected the amidolytic activity of thrombin and did not show inhibition of thrombin time [117]. Besides, madanins are competitive inhibitors; therefore, the peptidic fragments are unable to compete with the natural substrate, fibrinogen, of thrombin. Unlike madanins, haemathrins are mixed-type inhibitors, the peptides or the peptidic fragments may bind to the enzyme if or not the substrate is bound to it. The full mechanistic property of haemathrins is to be further dissected and established using other biophysical analyses.

For a molecule to be a suitable drug candidate, it must have certain attributes, one of which is its non-toxic property. Therefore, the toxicity of the recombinant haemathrins was assayed using red blood cell, bacterial cells and macrophage cell-line. rHaemathrins did not show any toxic effect to RBC, bacterial culture or the cell-line, indicating that these are non-toxic to cells. rHaemathrins directly targeted thrombin and are processed by the enzyme itself, which makes it a potential target for drug candidate. Hence, rHaemathrins can be targeted for design of novel thrombin inhibitors as drug candidates.

rHaemathrins are cleavable slow binding-type inhibitors of thrombin and specifically target thrombin through mixed inhibition mechanism. These may exert their inhibitory function at the initial stage of the coagulation cascade. When vascular tissue is injured, coagulation is initiated by the factor VIIa–tissue factor complex at the site of injury, which activates factor X [296]. This is followed by generation of

small amount of thrombin, that in turn activates factor V and VIII leading to amplification of thrombin generation. This is a crucial step in the coagulation cascade and haemathrins might inhibit blood coagulation by shutting down activation of factor V and VIII by thrombin. When rHaemathrins come in contact with thrombin, these may inhibit the function of thrombin at the initial stage. But, with time rHaemathrins are cleaved or disintegrated by thrombin and the inhibitory activity is suppressed. Ticks are known to have other anti-coagulant proteins/peptides in the saliva [107], which may be expressed at this stage of anti-coagulation process, thus stalling the host hemostatic system and receiving continuous flow of blood for feeding.