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Expression and characterization of haemathrins, madanin-like thrombin inhibitors, isolated from the salivary gland of tick *Haemaphysalis bispinosa* (Acari: Ixodidae)

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ABSTRACT

Saliva of hematophagous animals, such as ticks, is an excellent source of anticoagulant proteins and polypeptides. Here we describe the identification and characterization of two thrombin inhibitors named as haemathrin 1 and 2 from the salivary gland of tick *Haemaphysalis bispinosa* using genomic approach. Haemathrins are cysteine-less peptide anticoagulants, which share about 65–70% identity with madanins, and belong to inhibitor I53 superfamily of inhibitors of the MEROPS database. Haemathrins were overexpressed in *E. coli* and characterized to understand its mechanism of anticoagulant activity. Recombinant haemathrins (rHaemathrins) delayed the thrombin time, prothrombin time, activated partial thromboplastin time and fibrinogen clotting time. Selectivity screening against serine proteases of coagulation cascade reveals that rHaemathrins 1 and 2 specifically inhibit thrombin with an IC_{50} of 46.13 ± 0.04 μM and 40.05 ± 0.05 μM respectively. Similar to madanin, rHaemathrin 1 and 2 were cleaved by thrombin and consequently lost their inhibitory function over time. Analyses of the cleavage products revealed that the first cleavage, which occurs at the C-terminal end of rHaemathrins, drastically reduced their inhibitory activity. The synthetic peptides corresponding to the cleaved fragments showed significant loss in their ability to prolong plasma clotting times and to inhibit the amidolytic activity of thrombin. Thus haemathrins are the first cleavable thrombin inhibitors characterized from the salivary glands of *H. bispinosa*.

1. Introduction

Blood coagulation cascade, a physiological response to vascular injury, is activated by sequential proteolysis of circulating zymogens of serine proteinases resulting in fibrin clot formation [1]. Within the cascade, thrombin plays a central role in interacting with most of the zymogens, co-factors and receptors through its primary catalytic site and secondary anion-binding sites, exosite-I and -II. Therefore, owing to its central role in hemostasis, thrombin is one of the most targeted proteases for the inhibition of coagulation by hematophagous animals [2,3]. Most of the thrombin inhibitors characterized from ticks are Kunitz-type proteinase inhibitors. These are found in both soft (ornithodorin from *Ornithodoros moubata*, savignin from *O. savignyi*, monobin from *Argas monolakensis*) and hard ticks (boophilin from *Rhipicephalus* (*Boophilus*) *microplus*, amblin from *Amblyomma hebraeum*, hemalin from *Haemaphysalis longicornis*) [4–10]. These are double domain Kunitz-type thrombin inhibitors and function by non-canonically inhibiting the active site with the N-terminal domain and binding to the exosite-I with their C-terminal domain [5,10]. Although soft tick thrombin inhibitors are more potent and specific, they are kinetically slow, tight-binding competitive inhibitors [11]. Recently a new group of thrombin inhibitors, madanins, were isolated and characterized from the salivary glands of *Haemaphysalis longicornis*. Madanins are cysteine-less thrombin inhibitors and belong to a unique family of inhibitors, family I53 of MEROPS database [12,13]. They bind to the thrombin's substrate binding site, but lack the covalent stabilization of the reactive loop, which is commonly observed among other thrombin inhibitors [12]. These are unable to establish a secondary interaction with the enzyme due to absence of a compact core. Thus, madanins are easily cleaved by thrombin resulting in loss of activity. This is unlike the other characterized cysteine-less thrombin inhibitors, variegin [14] and anophelin [15] which evade loss of activity due to thrombin cleavage.

Haemaphysalis bispinosa is a hard-tick of Ixodidae family and is the most common tick species under the genus *Haemaphysalis* in India [16]. These ticks feed on host blood for several days and during this prolonged blood meal acquisition the ticks (except males) increase in size so does their salivary glands that produce saliva containing varying amounts and composition of bioactive compounds [17]. In the present study we have identified two isoforms of madanin-like thrombin inhibitors, haemathrins, from the salivary glands of *H. bispinosa*, which was previously characterized using morphological characters and molecular tools [17]. The cDNAs encoding both haemathrins were cloned into expression vector and produced in prokaryotic expression host as soluble

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proteins. Recombinant haemathrins (rHaemathrins) exhibited anticoagulant activity and thrombin-selective inhibition. Similar to madanins, rHaemathrins were cleaved by thrombin over time and the cleaved peptides did not inhibit the thrombin activity.

2. Materials and methods

2.1. Materials

Chromogenic substrates were procured from Chromogenix (Monza, Milano, Italy) and coagulation factors were from Hematologic Technologies Inc. (Essex Junction, VT, USA). All other chemicals and reagents used were of analytical grade and purchased from Sigma (St. Louis, MO, USA), Merck (Mumbai, Maharastra, India) and Himedia (Mumbai, Maharastra, India).

2.2. Collection of ticks

Partially fed adult female *Haemaphysalis bispinosa* ticks were collected from cattle (Napaam, Near Tezpur University Campus). Salivary glands were carefully dissected out using sterile forceps under microscope to prevent contamination of other tissues and washed 3–4 times with Tris Buffered Saline (TBS). The glands were either stored in *RNAlater* (Qiagen, Hilden, Germany) or directly used for isolation of total RNA using RNA isolation kit (Qiagen) following manufacturer's instructions.

2.3. Salivary gland cDNA synthesis

Total RNA was isolated from eight pairs of *Haemaphysalis bispinosa* salivary glands using the Qiagen RNeasy kit (Qiagen). First-strand synthesis was carried out using SMARTScribe reverse transcriptase (Clontech, Mountain View, CA, USA) 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.

2.4. Amplification of the gene by polymerase chain reaction (PCR)

The cDNA encoding haemathrins were amplified from the cDNA pool by PCR using the forward primer HbTI-F, designed from the 5′ untranslated region (UTR) of madanins (5′-TTTGACCGCAATGAAGCAC-3′) and reverse primers HbTI-R1 (5′-CTTCCAGCCTACAACATCAC-3′) and HbTI-R2 (5′-TCTATAACCTACCGACGGC-3′), designed from the 3′ UTR of madanin 1 and madanin 2, respectively. 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 94 °C for 2 min; 30 cycles of 94 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s; and final extension of 72 °C for 10 min. The amplified DNA was electrophoresed on 1.1% agarose gel and visualized under UV light.

2.5. Sequencing and analysis of the genes

The amplified cDNAs were sequenced directly using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Waltham, MA, USA). 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 program at the National Center for Biotechnology Information (NCBI). The predicted molecular weight and pI of the proteins 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) [18]. The nucleotide and protein sequences of haemathrins 1 and 2 were aligned with sequences obtained from the NCBI database using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.6. Cloning and expression of recombinant haemathrins

The cDNA encoding the mature regions of haemathrins 1 and 2 were subcloned into the *Nco*I and *Xho*I restriction sites of the expression vector pET32a(+) (Novagen, Darmstadt, Germany) using T4 DNA ligase. The ligation of the gene into the plasmids was confirmed by Sanger sequencing. *E. coli* BL21(DE3)pLysS cells containing recombinant plasmids were induced with 0.1 μM IPTG (final concentration) at 37 °C for 4 h, following which the cells were lysed in 50 mM phosphate pH 8.0, 300 mM NaCl, 20 mM Imidazole (buffer A). The clarified lysate was then loaded to Ni-NTA agarose beads (Qiagen) pre-equilibrated with buffer A, and eluted with buffer A containing 100 mM imidazole. The fractions were then further purified on Jupiter C18 column (4.6 mm × 250 mm, Phenomenex, CA, USA) pre-equilibrated with 0.1% trifluoroacetic acid (TFA) using a gradient of 0–80% acetonitrile. The recombinant proteins are expressed with a fusion protein of about 18 kDa (Trx-tag, His-tag, S-tag) which has enterokinase cleavage site. The fusion partner was then cleaved by enterokinase (Novagen, Darmstadt, Germany) and the recombinant haemathrins (rHaemathrins) were recovered by chromatographic separation using Jupiter C18 column (4.6 mm × 250 mm). The molecular masses of the peptides were determined on Accela LCQ Fleet™ electrospray ion-trap mass Spectrometer (Thermo Scientific, Waltham, CA, USA).

2.7. Circular dichroism (CD) measurements of haemathrins

Far-UV CD spectra (260–190 nm) of 20 μM protein solution 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].

2.8. Blood coagulation assay

The rHaemathrins were tested for thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT). Briefly, citrated goat plasma (50 μl) was pre-incubated with various 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).

2.9. Fibrinogen clotting time

The rHaemathrins 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, final concentration) was incubated with 50 μl of rHaemathrins 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.