A Novel Gene Encoding a Thrombin Inhibitory Protein in a cDNA Library from Haemaphysalis longicornis Salivary Gland

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ABSTRACT. A novel thrombin inhibitory protein coding gene was identified from a cDNA library derived from salivary gland of partially-fed Haemaphysalis longicornis (hard tick). The gene encoded a 93-amino acid protein, designated chimadanin, which had a signal peptide sequence and was predicted to be a secretory protein. It showed no similarity to any other previously identified proteins or conserved domain sequences. The gene was expressed during blood feeding and suggested to be expressed mainly in the salivary gland. The predicted mature region of chimadanin was expressed in Escherichia coli and characteristics of the recombinant chimadanin were determined. The activated partial thromboplastin time and the prothrombin time in sheep plasma were significantly prolonged by chimadanin in a dose dependent manner. Amidolytic activity of thrombin was also inhibited by chimadanin in a dose dependent manner and it suggested that chimadanin was an anticoagulant with thrombin inhibitory activity. This newly identified thrombin inhibitor may play an important role in tick blood feeding.

KEY WORDS: anticoagulant, chimadanin, Haemaphysalis longicornis, thrombin inhibitor, tick.

Blood sucking animals produce many kinds of bioactive substances and inject them to the host during infestation to create an optimal feeding environment [12, 18]. Coagulation control may be the most important matter for them since it occurs in the earliest stage of the infestation. Numerous anticoagulants have been isolated from hematophagous animals and some of them were well characterized [2, 26]. The most famous substance is hirudin, a specific thrombin inhibitor derived from Hirudo medicinalis (medical leech), which has been used as a commercial medicine for thrombosis [13]. There are also many kinds of anticoagulant substances identified from hard and soft tick species [12, 14]. Within the blood coagulation cascade, several serine protease inhibitors such as factor Xa inhibitors [16, 24], a factor VIIa/tissue factor complex inhibitor [7] and thrombin inhibitors [9, 11, 23] have been described.

Ticks are of medical and veterinary significant hematophagous arthropods, especially as vectors of important tick-borne disease causing agents. These pathogens are transmitted to the vertebrate hosts during tick blood feeding. Some molecules in tick saliva, for example anti-inflammatory substances or immunosuppressive proteins, are suspected to be helpful for the pathogen transmission [25]. Blood clotting is also thought to be important for the prevention of disease causative agents' invasion. Thus, investigation of anti-clotting molecules in the blood-sucking arthropods' saliva may provide us some useful information for prevention of tick-borne diseases.

In our previous study, a cDNA library of partially fed Haemaphysalis longicornis salivary glands was constructed and the randomly chosen cDNAs were sequenced [15]. Among the predicted protein coding sequences, we found a novel anti-coagulant protein gene that the expressed recombinant protein showed anti-coagulant and thrombin-inhibitory properties. This protein may help further understanding of tick-host interactions.

MATERIALS AND METHODS

Tick sample collection: Adult female ticks (Haemaphysalis longicornis) were fed on a rabbit and partially engorged ticks were collected on the 5th day for cDNA library construction. For gene expression analysis, adult ticks were collected before feeding, on the 3rd day and 6th day of blood feeding, and after engorgement. Nymphal ticks were collected on the 3rd day of feeding. Salivary gland, midgut and the carcass samples were obtained from adult ticks collected on the 3rd day. Salivary gland and midgut were washed three times with ice cold phosphate buffered saline (PBS) under the microscope to prevent other tissue contamination. From the organs and whole body of ticks, total RNA was extracted with TRIZOL (Invitrogen, U.S.A.) reagent according to the manufacturer's protocol.

cDNA library construction and random sequencing: Details were described elsewhere [15]. Briefly, salivary gland cDNA library was constructed from the extracted mRNA using the Creator SMART cDNA Library Construction Kit (Becton, Dickinson and Company, U.S.A.) according to the manufacturer's protocol. Randomly chosen clones were sequenced from 5' prime end by single passage using a specially designed primer.

Sequence analysis: Sequences were compared to those in
the non-redundant protein database for homology search using the National Center for Biotechnology Information (NCBI) blast program (http://www.ncbi.nlm.gov/BLAST) [1]. Secretory signal peptide and cleavage site for the mature protein were predicted by the SignalP server program (http://www.cbs.dtu.dk/services/SignalP) [17]. Predicted molecular weight and pI were calculated by ProtParam tool server programs (http://www.expasy.org/tools/protparam.html) [3].

**Gene expression analysis:** Gene expression analysis of different tick stages and organs was done by conventional reverse transcription-PCR (RT-PCR) assay with gene specific primers designed for the coding sequence (CDS) amplification. Tick beta actin was used as the positive control [5]. For determination of the levels of the gene expression at different stages of blood feeding, real-time RT-PCR assay was carried out with the same primer set by LightCycler (Roche Diagnostics, Germany) using LightCycler FastStart DNA Master SYBER Green I (Roche Diagnostics). Sample preparation and analysis were carried out according to the manufacturer’s protocol. The relative expression values were normalized to the expression value of the beta-actin.

**Expression and purification of recombinant protein:** Predicted mature protein sequence region was amplified by PCR with a specific primer set harboring a restriction enzyme site (forward primer: 5'-AAT AAT CAT ATG CAA CCA AAA GAG AAA ACC AAA GGC-3', reverse primer: 5'-ATA AGA ATG CGG CTC ATG AAC ATC GGC ATT CCG AGC TG-3'). The amplified fragments were digested with the enzymes (NdeI and NotI) and subcloned into an expression vector, pET44a (Merck KGaA, Germany). The vector was transformed into an *E. coli* strain Rosetta-gami B (Merck KGaA, Germany) and the recombinant protein was expressed according to the manufacturer’s procedure. Bacterial cells were suspended in water and the cell wall was destroyed by freeze and thaw repeat. The cell lysate was sonicated and centrifuged, and the supernatant was applied to the UNO Q1 anion exchange column (7 cell wall was destroyed by freeze and thaw repeat. The cell procedure. Bacterial cells were suspended in water and the nant protein was expressed according to the manufacturer’s many). The vector was transformed into an expression vector, pET44a (Merck KGaA, Germany).

**Plasma coagulation studies:** Sheep citrated plasma (600 µl) was mixed with different concentrations (0–30 µM, final concentration) of 100 µl recombinant protein in PBS. Determination of the activated partial thromboplastin time (APTT) and the prothrombin time (PT) was outsourced to a clinical laboratory testing company (SRL, Inc., Japan) and carried out according to the company’s procedures. Briefly, the citrated plasma mixed with the recombinant protein was incubated at 37°C with actin in APTT assay and with thromboplastin in PT assay. After adding CaCl₂, clotting time was measured by the Symex CA-1500 coagulation analyzer (Sysmex Corporation, Japan).

**Thrombin amidolytic activity inhibition assay:** Bovine thrombin (Sigma-Aldrich Co., U.S.A.) dissolved in 0.1 M Tris-HCl buffer (pH 8.0) was pre-incubated with recombinant protein samples in a 96-well plate for 20 min at room temperature. After adding Boc-Val-Pro-Arg-MCA (α-thrombin specific chromogenic substrate; Peptide Institute, Inc., Japan), the plate was incubated for another 6 min at room temperature and the fluorescence intensity was measured by MTP-650FA microplate reader (Corona Electric Co., Ltd., Japan) with excitation wavelength of 365 nm and emission wavelength of 450 nm. Accumulated fluorescent intensity during 6-min incubation period was considered as substrate cleavage velocity. The fluorescent intensities were adjusted with the background fluorescence without the recombinant protein and thrombin. Final concentrations of thrombin, recombinant protein and chromogenic substrate in the reaction solution (total 100 µl) were 1.5 nM, 0-2 µM and 7 µM, respectively.

**RESULTS**

cDNA cloning and analysis: From the constructed salivary gland cDNA library, 633 clones were randomly chosen and sequenced [15]. We focused on a major cDNA group that consisted of 25 clones of almost identical sequences. The sequence showed no similarity to any other previously identified proteins, nucleic acid sequences or conserved domains by NCBI blast programs and considered to be a novel protein coding gene. We submitted the sequence to the DNA Data Bank of Japan (DDBJ) as HLSG-g21 (accession number AB218911). The full-length cDNA was 421 bp long with the predicted CDS of 282 bp encoding a 93-amino acid residue protein. The nucleic acid sequence and the deduced protein sequence were shown in Fig. 1. The first 23 amino acid residues were predicted to be the signal peptide for secretion by SignalP program [17]. The protein was named chimadanin.

The predicted mature region of chimadanin was relatively small (70 amino acids, calculated molecular weight: 7472.1), and the theoretical isoelectric point was low (acidic, pI=4.64). Furthermore, the sequence contained no cysteine residues, indicating that the protein had no three-dimensional structures stabilized by disulfide bonds. These characteristics were similar to the thrombin inhibitory proteins that have been previously isolated from blood feeding arthropods [4, 11, 21, 27].

**Gene expression analysis studies:** The gene expression profiles were shown in Fig. 2. Chimadanin gene was expressed in both of nympha1 and adult stages on the 3rd day of blood feeding. In adults, the gene was mainly expressed in the salivary gland, and the expression was not observed in the midgut. A faint band was observed in the carcass sample (Fig. 2A). With the real time RT-PCR results, chimadanin gene was expressed significantly on the 3rd day and the expression level was decreased on the 6th
day. The gene expression was not detected before blood feeding (Fig. 2B).

Recombinant protein expression: The recombinant protein expressed in E. coli was confirmed by SDS-PAGE. The protein band was observed at the molecular mass of approximately 15 kDa (Fig. 3). The apparent size was about two times higher than the expected mass (7.6 kDa) calculated from its constituent amino acids. However, this phenomenon was consistent with the observation of thrombostasin, a thrombin inhibitor isolated from horn fly saliva.

Fig. 1. Nucleic acid sequence and deduced amino acid sequence of chimadanin (DDBJ accession number: AB218911). Predicted signal peptide sequence was found on the N terminus of the protein sequence (underlined). Polyadenilation signal is shown in boldface.

Fig. 2. Chimadanin gene expression analysis. The gene expression in the tick samples was analyzed by RT-PCR methods. Beta-actin gene was used as a control. (A) Gene expression in different tick stages and organs. For Nymph and Adult samples, whole body of the ticks on the 3rd day of blood feeding was used. Organs were collected from adult ticks on the 3rd day of blood feeding. The size of the amplified CDS fragment with adapter primers was 307 bp. (B) Gene expression levels at the different stages of blood feeding. Whole body samples of adult ticks were analyzed by Real-time RT-PCR.

Fig. 3. Expressed and purified chimadanin. The expressed recombinant chimadanin was purified by ion exchange column fractionation and dialysis. In the 17% polyacrylamide gel, the band was observed at the molecular mass of approximately 15 kDa (arrow).
Thrombostasin also showed significantly higher molecular mass in SDS-PAGE, apparent molecular mass of 16.7 kDa was observed for the expected 9.2 kDa protein because of its acidic character [27]. Chimadanin coding DNA sequence on the expression vector was confirmed by sequencing and no error was found. Thus, the higher molecular mass band shown in the SDS-PAGE might be attributed to the acidic feature of chimadanin or some other structural characteristics. No other protein bands were found in the purified chimadanin sample.

**Anticoagulation studies:** The results of APTT and PT with sheep plasma were shown in Fig. 4. Chimadanin prolonged the coagulation time in both of the assays significantly in a dose dependent manner. At the dose of 30 µM, it prolonged the coagulation period about two times longer than that of the PBS negative control; from 32.6 sec to 63.8 sec in APTT assay and from 21.1 sec to 38.6 sec in PT assay. Assays were done in duplicate. APTT is a screening assay for deficiency of coagulation factors associated with the intrinsic pathway of coagulation; on the other hand, PT is an assay for the extrinsic pathway. The above results indicated that chimadanin inhibited the blood coagulation factor that contributed to the common pathway of intrinsic and extrinsic coagulation pathways [11].

**Thrombin amidolytic activity inhibition:** Thrombin is a major coagulation factor which contributes to the final step of the common pathway of intrinsic and extrinsic coagulation pathways. Chimadanin dose dependently inhibited the thrombin amidolytic activity in the chromogenic substrate assay with α-thrombin specific small substrate (Fig. 5). The inhibitory activity was shown in the ratio of substrate cleavage velocity Vi/V0 (Vi: velocity in chimadanin existing sample, V0: velocity in chimadanin absent control). Under the current assay condition with 1.5 nM thrombin and 7 µM substrate, thrombin amidolytic activity was inhibited approximately 90% by 0.5 µM of chimadanin.

**DISCUSSION**

A novel thrombin inhibitory protein, named chimadanin, encoding gene was identified from *Haemaphysalis longicornis* salivary gland cDNA library. The sequence had no similarity to any of the proteins which have been previously identified. Expressed recombinant protein showed obvious anticoagulant activity and inhibition of thrombin amidolytic activity.

Madanin 1 and 2 were previously identified thrombin...
inhibitors from *H. longicornis* [11]. They prevented thrombin from cleaving fibrinogen without inhibiting thrombin amidolytic activity toward a small synthetic substrate. It was suggested that madanins were competitive inhibitors of not the catalytic site but the anion-binding exosite-1 of thrombin that was important for binding to fibrinogen and other target molecules. With negatively charged acidic residue clusters observed in madanins’ sequences, they could bind to the exosite-1 of thrombin and inhibit its physiological activity. Chimadanin and other blood feeding organism-derived thrombin inhibitors; hirudin [19], tssetse thrombin inhibitor [4], anophelin [6, 21] and thrombostasin [27], all of which had anti-thrombin amidolytic activity, also had similar acidic regions in the protein sequences. About hirudin and anophelin, their ability to bind to the exosite-1 and consequent inhibition of thrombin activity has been demonstrated [6, 8, 19]. Therefore, chimadanin may also be an inhibitor of both of the catalytic site and exosite-1 on thrombin.

Thrombin inhibition seemed to be very important for blood feeding animals since numerous kinds of thrombin inhibitors have been isolated from their saliva [12, 26]. In horn fly, the thrombin inhibitor thrombostasin was indicated to be the only one anticoagulant having anti-clotting ability in the saliva [27]. Thrombin is one of the coagulation factors in the final phase of blood coagulation cascades. However, it also activates factor V and factor VIII that are in the upper cascade and supposed to generate larger amount of thrombin. This means thrombin has a positive feedback loop for its amplification. It indicates that thrombin inhibition means not only inhibiting the final phase of the cascade but also the earlier phase of coagulation [11, 27].

By the RT-PCR results, chimadanin coding gene was highly expressed in the salivary gland during blood feeding, especially in the earlier stage. It was not expressed before feeding and the expression was not observed in the midgut. Taking account of the existence of a secretory signal sequence, the results indicated that chimadanin may have contribution during blood feeding as a portion of the tick saliva to control blood coagulation.

Sugino and Imamura have reported that vaccination of rabbits with HLS1 or HLS2, anticoagulant serine protease inhibitors identified from *H. longicornis*, showed anti-tick efficacy [10, 20]. However, these anticoagulants were concealed proteins and not exposed to the hosts [14]. Anti-tick vaccine targeting tick salivary bioactive molecules has a possibility for prevention of tick-borne disease pathogen transmission [22]. Further study of chimadanin and other tick derivable molecules will widen our knowledge about hematophagous animal biology.

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REFERENCES


