Identification and characterization of a novel elastase inhibitor from Hirudinaria manillensis

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[ABSTRACT] A large number of protease inhibitors have been found from leeches, which are essential in various physiological and biological processes. In the current study, a novel elastase inhibitor was purified and characterized from the leech of Hirudinaria manillensis, which was named HMEI-A. Primary structure analysis showed that HMEI-A belonged to a new family of proteins. HMEI-A exerted inhibitory effects on elastase and showed potent abilities to inhibit elastase with an inhibition constant ($K_i$) of $1.69 \times 10^{-5}$ mol·L$^{-1}$. Further study showed that HMEI-A inhibited the formation of neutrophil extracellular trap (NET). These results suggested that HMEI-A from the leech of H. manillensis is a novel elastase inhibitor which can suppress NET formation. It may play a significant role in blood-sucking of leeches and is a potential candidate as an anti-inflammatory agent.

[KEY WORDS] Elastase inhibitor; Hirudinaria manillensis; HMEI-A; NET formation; Anti-inflammation

Introduction

Bloodsuckers such as fleas and leeches need blood meals to survive and reproduce, which is achieved through releasing a variety of biologically active molecules after long-term evolution along with the host [1]. For example, desmolaris is identified as a novel FXIa inhibitor, a major anticoagulant from vampire bat saliva [2]. Anophelin belongs to a unique class of thrombin inhibitors, which was isolated and cloned from the salivary glands of the mosquito Anopheles albinus [3]. Moreover, variegin, which was isolated from the tropical bont tick Amblyomma variegatum, is identified as a new thrombin inhibitor [4].

Leeches secrete a number of bioactive molecules to help them feed. A leech bite can result in the release of these molecules to inhibit coagulation and avoid detection due to anticoagulant, analgesic and anti-inflammatory responses [5]. In the last twenty years, many bioactive molecules have been isolated and characterized from leeches, including bdellastasin (an inhibitor of plasmin and trypsin) [6], hirustasin (an inhibitor of trypsin, tissue kallikrein, granulocyte cathepsin G and α-chymotrypsin) [7], eglin (an inhibitor of subtilisin, α-chymotrypsin, chymosin, elastase and cathepsin G) [8], hirudin (a thrombin inhibitor) [9], guamericin (an inhibitor of chymotrypsin and elastase) [10] and bdellin-KL (an inhibitor of acrosin, plasmin and trypsin) [11].

Under inflammatory conditions, neutrophils can release an intracellular structure called neutrophil extracellular traps (NETs), which are composed of histones, chromatin DNA and granular proteins. The NETs can be released into the extracellular environment to capture and kill pathogens [12]. Although NET formation is a basic mechanism of immune system, it still causes many negative consequences. NET formation occurs in autoimmune diseases [13], chronic inflammatory diseases [14], diverse forms of vasculitis [15], transfusion-related acute lung injury [16], thrombosis [17], and even cancer-
associated thrombosis due to extracellular chromatin released by NETs [18].

NET formation is regulated by neutrophils elastase (NE) and NE inhibitors can suppress the NET formation of neutrophils. Upon NET formation, NEs get away from azurophilic granules and translocate to the nucleus, which then partially degrade specific histones and promote chromatin decondensation. Correspondingly, NE-knockout mice cannot form NETs in a pulmonary model induced by germ infection, which implies that the defect may contribute to the immune deficiency of these mice [19]. These findings reveal that NEs may be a new drug target in the treatment of such diseases.

In this study, we discovered a novel elastase inhibitor from *H. manillensis* which was named HMEI-A. Moreover, HMEI-A had the ability to inhibit NET formation and may play a crucial role in blood-sucking by suppressing the immune response of hosts.

**Materials and Methods**

**Collection of crude extracts**

The living *H. manillensis* were purchased from Guangxi Province, China. Crude extracts were prepared from the head of leeches as previously described [20]. Briefly, the heads were dissected from the bodies, washed in 0.9% saline, quickly frozen within liquid nitrogen and then crushed.

**Purification of HMEI**

The lyophilized crude extracts were dissolved in 20 mmol·L⁻¹ Tris-HCl buffer, pH 8.0, and then loaded on a G-75 column previously equilibrated with the same buffer. Elution was performed at a flow rate of 1 mL·min⁻¹ at 4 °C, and fractions were collected after 90 min of loading. The absorbance of the elution fractions was monitored at 215 nm. The fractions were pooled and lyophilized prior to further purification.

After the elastase-inhibiting activity test, the active fraction was resuspended and applied to RP-HPLC (reversed phase high-performance liquid chromatography) on a C₈ column (Waters, Milford, MA, USA, 5 μm particle size, 250 mm × 10 mm). Elution was carried out with a linear gradient of 5%–60% solution B (acetonitrile, added 0.1% TFA) for 55 min at a flow rate of 1 mL·min⁻¹. The eluted fraction with elastase-inhibiting activity was collected.

**Mass spectrometric analysis and sequencing of peptides**

The molecular weight and internal peptide sequences of HMEI-A were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS, AXIMA CFR, Kratos Analytical). Briefly, the disulfide bridges of HMEI-A were reduced and blocked. Reductive protein was hydrolyzed by trypsin. Finally, the hydrolyzed products of natural protein were detected. N-terminal amino acid sequence was determined by automatic Edman degradation on a pulsed liquid-phase sequencer (Applied PRISM 377, Shimadzu Corporation, Kyoto, Japan) was used for DNA sequencing. Subsequently, the blast search (NCBI) was performed for sequence alignment with other previously reported proteins. Multiple sequence alignments were performed by DNAMan software (Lynnon Biosoft, Quebec, QC, Canada).

**Isolation of Neutrophils**

Healthy human venous blood samples were collected from Kunning Blood Center and treated with heparin as an anti-coagulant. Then, 5 mL of blood and 5 mL of Polymorph-prep™ buffer (1114683, Axis-Shield/Alere Technologies AS, Dundee, Scotland) were carefully mixed in a 15-mL centrifuge tube before centrifugation at 500 × g at 20 °C for 30 min. Polymorphonuclear interface was carefully harvested, and finally the cells were washed twice in PBS at 300 × g at 20 °C for 5 min. The neutrophils were then suspended in the culture medium before experiments.

**NETs formation assay**

Neutrophils were seeded at a density of 5 × 10⁴ per well

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**Screening of cDNA encoding HMEI**

To screen the cDNA encoding HMEI, the synthesized cDNA was used as the template for PCR. Two pairs of oligonucleotide primers were used in PCR (Table 1). The first pair of primers included primer1 and primer 2. Primer 1 was designed according to the partial peptide sequence of HMEI determined by MALDI-TOF-MS, while primer 2 was from the SMART™ PCR cDNA Construction kit. The second pair of primers included primer 3 and primer 4. Primer 3 was designed on the basis of the PCR product using the first pair of primers, while primer 4 was from the kit mentioned above. The PCR conditions were listed as follows: 95 °C for 5 min; 30 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 30 s), and extension (72 °C for 60 s) and final extension at 72 °C for 10 min. The final PCR products were cloned into a pGEM®-T Easy vector (A1360, Promega, Madison, WI, USA). An applied Biosystems DNA sequencer (Model ABI PRISM 377, Shimadzu Corporation, Kyoto, Japan) was used for DNA sequencing. Subsequently, the blast search (NCBI) was performed for sequence alignment with other previously reported proteins. Multiple sequence alignments were performed by DNAMan software (Lynnon Biosoft, Quebec, QC, Canada).

**Elastase-inhibiting activity assay**

The elastase-inhibiting assay was performed using corresponding chromogenic substrates. Briefly, the enzyme was incubated with different concentrations (5, 10 and 20 nmol·L⁻¹) of HMEI-A in 60 μL of 50 mmol·L⁻¹ Tris buffer (pH 7.4) for 5 min followed by the addition of chromogenic substrate at a certain concentration. Then, a microplate reader (Epoch BioTek, Winooski, VT, USA) was used to measure the absorbance at 405 nm amd monitor under the kinetics model for 30 min. Then, 400 nmol·L⁻¹ elastase (E8140-1UN, Sigma, St. louis, MO, USA) was incubated with 10 or 20 μg·mL⁻¹ substrates, with the corresponding substrate of N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (M4765, Sigma, St. louis, MO, USA).

**Table 1** The primers used for cDNA cloning of HMEI

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>5'-ACCTTRCANCNCTCGNNTTRRCG-3'</td>
</tr>
<tr>
<td>2</td>
<td>5'-AAGVAGTGGTATATCAAGGCAAGACGT-3'</td>
</tr>
<tr>
<td>3</td>
<td>5'-GGTGGAAAGATGCTGCTACCAA-3'</td>
</tr>
<tr>
<td>4</td>
<td>5'-ATTCTAGAGCCGAGGCCGCA-3'</td>
</tr>
</tbody>
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Primers 1 and 2 for 5'-terminal cloning of HMEI; Primers 3 and 4 for 3'-terminal cloning of HMEI. (Y = C/T; R = A/G; N = A/C/T/G)
in 24-well plates in RPMI 1640 culture media (11835030, Thermo, MA, American) supplemented with 10% FCS (fetal calf serum, Z7010FBS, ETA LIFE, CA, American). The cells were stimulated with 100 ng mL⁻¹ phorbol-12-myristate-13-acetate (PMA, V1171, Promega, Beijing, China) for 3 h. The HMEI-A group was additionally added with 10 μg mL⁻¹ HMEI-A before stimulation. Then, the cells were fixed with 4% paraformaldehyde for 20 min, and washed twice in PBS. Finally, the cells were stained with DAPI (8961S, CST, MA, American) and photographed.

Results

Purification of HMEI

The crude extract of *H. manillensis* was separated into three fractions by G-75 column. As indicated in Fig. 1A, the fraction II had elastase-inhibiting activity. The active fraction was further applied to a C₈ RP-HPLC column. The protein (marked by the arrow in Fig. 1B) containing elastase-inhibiting activity was purified and named HMEI-A (*H. manillensis* elastase inhibitor). Its molecular weight (MW) was 13 388.5 Da, according to MALDI-TOF-MS analysis (Fig. 1C) under a positive ion and linear mode with specific operating parameters including a 20 kV ion acceleration voltage, 50-time accumulations for single scanning and 0.1% accuracy of mass determinations.

Primary Structure of HMEI

The partial N-terminal amino acid (aa) sequence of HMEI-A was determined as ESDAE by automatic Edman degradation, while other partial amino acid sequences of HMEI-A were identified as DCDTNFVVVTSHGTK and WPM-SYTNWNVGEPNNAK by MALDI-TOF-MS (Fig. 2). According to the N-terminal aa sequence, degenerate PCR primers were designed to clone the cDNA encoding the precursor of HMEI-A from the cDNA library, obtaining two homologous nucleotide sequences. Their encoding aa sequences were named HMEI-A and HMEI-B. The cDNA of HMEI-A had an open reading frame of 513 nucleotides coding a pro-protein of 170 amino acids including a signal peptide of 20 residues and mature HMEI-A of 150 residues (Fig. 3A). The cDNA of HMEI-B had an open reading frame of 507 nucleotides coding a pro-protein of 168 amino acids including a signal peptide of 21 residues predicted at SignalP-5.0 Server and mature HMEI-B of 147 residues (Fig. 3B). We also compared the predicted molecular weight (MW) and pI value (isoelectric point) of HMEI-A and HMEI-B by ProtParam. The predicted MW was 16 679.4 Da for HMEI-A, and 16 466.22 Da for HMEI-B. The predicted pI was 5.29 for HMEI-A, and 5.90 for HMEI-B. The two sequences belong to the same protein family (Fig. 3C). Sequences HMEI-A and HMEI-B showed high homology with 61.6% identity and the cysteine residues were highly conserved (indicated with an asterisk in Fig. 3C).

HMEI-A inhibited elastase and NET formation

Under experimental conditions, HMEI-A potently inhibited the activity of elastase. The enzyme kinetic study showed that HMEI-A was a competitive inhibitor with an inhibition constant (Kᵢ) of (1.69 ± 0.15) × 10⁻⁷ mol·L⁻¹ (Fig. 4A).

In previous studies, elastase inhibitor can inhibit NET formation [21]. Since HMEI-A inhibits the activity of elastase, we supposed that HMEI-A might have the ability to inhibit NET formation. PMA was described as one of the best inducer of NET formation, and DAPI was used to visualize the intracellular and extracellular DNA which is considered as a component of NETs [22]. We isolated neutrophils from healthy donors. The neutrophils were induced by PMA and treated with HMEI-A. We found that the cells formed NETs efficiently 3 h after stimulated with PMA. However, when treated with HMEI-A, the neutrophils failed to form NET by PMA stimulating (Fig. 4B−4C). The results indicated that HMEI-A can inhibit the NET formation by inhibiting the activity of elastase.

Discussion

To take a blood meal and adapt to variable environments, leeches have evolved to secret a variety of active proteins to inhibit the proteolytic enzymes from preys. To avoid detection by the host during feeding, leeches must secret anti-inflammator molecules, such as Eglin C, an inhibitor of hu-
H. manillensis. Furthermore, HMEI-A inhibited the formation of NETs by inhibiting elastase. The cDNA encoding HMEI-A precursor was cloned from the cDNA library. Mature HMEI-A is composed of 152 amino acid residues (Fig. 3A). Blast search in NCBI approved that HMEI-A is a novel toxin from leeches.

Neutrophils can eliminate extracellular microorganisms by releasing NETs. However, excessive NETs may cause adverse effects. For example, patients with cystic fibrosis (CF) and autoimmune diseases, such as systemic lupus erythematosus (SLE), develop antibodies to chromatin and granular components. These antibodies mediate pathological reactions themselves. For the treatment of these diseases, the elements of NETs have become the target for drug design. For example, the DNase have been used in the treatment of CF to cleave the excessive chromatin fragments. Bloodsuckers secrete the anti-inflammatory molecules to avoid detection by the host during feeding, indicating the role of these molecules as potential anti-inflammatory drugs [23]. As an elastase inhibitor, HMEI-A inhibits NET formation and may become a candidate for the treatment of such diseases.

In conclusion, we isolated and identified a new elastase inhibitor HMEI-A from the H. manillensis. The inhibitor helps leeches to successfully obtain blood meal by inhibiting NET formation, thereby inhibiting host immune response, and may become a potential anti-inflammatory drug candidate.

**Acknowledgments**

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**Fig. 2** The peptide sequence of HMEI-A by MALODI-TOF-MS. The determined amino acid sequences were DCDTTNVFYTVSHGT (A) and WPSMYTNWNGEPNNAK (B) respectively.

**Fig. 3** The ORF (Open Reading Frame) of HMEIs and the alignment of HMEI-A and HMEI-B. According to the N-terminal sequence, primers were designed and the cDNAs of HMEIs were obtained from the cDNA library by RT-PCR for HMEI-A (A) and HMEI-B (B). The signal peptides are shadowed. In A, the amino acid sequence determined by MALODI-TOF-MS in Fig. 2A and 2B are indicated with single underline and double underline, respectively. (C) The sequence alignment between HMEI-A and HMEI-B. The conserved residues and cysteine residues are indicated by shadow and asterisks, respectively.
HMEI-A concentration/(nmol·L$^{-1}$)

![Graph](image)

**Fig. 4** HMEI-A inhibits the activity of elastase and NET formation. (A) HMEI-A was a competitive inhibitor of elastase with an inhibition constant ($K_i$) of (1.69 ± 0.21) × 10$^8$ mol·L$^{-1}$ determined by the method of Dixon ($n = 3$) (B) Quantification of NET formation (DNA area, $n = 5$) in C using ImageJ software. Data obtained from ImageJ was analysed by one-way ANOVA. (C) Isolated neutrophils were cultured in complete RPMI-1640 medium, incubated with 10 μg·mL$^{-1}$ HMEI-A, 50 μg·mL$^{-1}$ crude extract or medium alone at 37 °C for 15 min. Then, the cells were stimulated with 100 ng·mL$^{-1}$ PMA for 3 h. The cells were fixed with 4% paraformaldehyde and stained with DAPI for fluorescence microscopy. The results showed that both HMEI-A and the crude extract have significant inhibitory effects, compared with normal saline treatment ($** P < 0.001$)

References


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