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Protease inhibitor in scorpion (*Mesobuthus eupeus*) venom prolongs the biological activities of the crude venom

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[ABSTRACT] It is hypothesized that protease inhibitors play an essential role in survival of venomous animals through protecting peptide/protein toxins from degradation by proteases in their prey or predators. However, the biological function of protease inhibitors in scorpion venoms remains unknown. In the present study, a trypsin inhibitor was purified and characterized from the venom of scorpion *Mesobuthus eupeus*, which enhanced the biological activities of crude venom components in mice when injected in combination with crude venom. This protease inhibitor, named MeKTT-1, belonged to Kunitz-type toxins subfamily. Native MeKTT-1 selectively inhibited trypsin with a K_i value of 130 nmol·L^{-1}. Furthermore, MeKTT-1 was shown to be a thermo-stable peptide. In animal behavioral tests, MeKTT-1 prolonged the pain behavior induced by scorpion crude venom, suggesting that protease inhibitors in scorpion venom inhibited proteases and protect the functionally important peptide/protein toxins from degradation, consequently keeping them active longer. In conclusion, this was the first experimental evidence about the natural existence of serine protease inhibitor in the venom of scorpion *Mesobuthus eupeus,* which preserved the activity of venom components, suggests that scorpions may use protease inhibitors for survival.

[KEY WORDS] Scorpion *Mesobuthus eupeus*; Venom; Protease inhibitor; MeKTT-1

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Introduction

Serine protease inhibitors (SPIs) have been identified in a variety of organisms in nature, being present in not only the bodies of various animals but also the secretions of parasites, hematophagous invertebrates, amphibian skins, and the venom glands of poisonous animals [1-4]. Protease inhibitors are capable of inhibiting the catalytic activity of proteolytic enzymes and are extensively distributed in all kingdoms of cellular life [5-6], signifying the importance of inhibiting proteolysis in their biological processes for survival. Among peptide protease inhibitors, the Kunitz-type protease inhibitors are a well-characterized subfamily, because of their abundance in several organisms, which usually consist of about 60 amino acid residues possessing three disulfide bridges and can strongly inhibit trypsin and chymotrypsin [7].

Venomous creatures are considered as a very distinctive class of species among animals. Evolution has developed the venomous animals with venom glands and venoms, providing them with extraordinary advantages for their existence. Peptidic toxins from scorpions have been well studied and some of them have been proven as important tools for predation as well as useful candidate for drug design [8-9]. Moreover, it has been well reported that animal venoms contain Kunitz-type serine protease inhibitors [8, 10-12].

Many Kunitz-type serine protease inhibitors have been

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reported to be present in scorpion venoms, and most of them have been identified by cDNA cloning and transcriptomic analyses [13-17]. Till date, little has been established to reveal the biological functions of these protease inhibitors.

In the present study, we purified a native serine protease inhibitor, from the venom of *Mesobuthus eupeus,* a specific Kunitz-type trypsin inhibitor. To the best of our knowledge, this was the first report of natural occurrence of protease inhibitor in this species. Our results demonstrated the *in vivo* biological effects of MeKTT-1, suggesting an important role of the protease inhibitors in scorpion venom in protecting some other proteins from degradation and retaining their activity.

Materials and Methods

Venom collection

Adult scorpions *Mesobuthus eupeus* (both sexes, n = 5 000) were purchased from Shandong Province of China. Crude venoms were collected manually by stimulating the venom glands in the telson of scorpions using a 6 V alternating current and 1 mL of venom was mixed with 20 μL of proteinase inhibitor cocktail (Sigma, P8340-5, St. Lois, MO, USA). Following collection, the venoms were stored at –80 °C until use. The scorpions were fed on worms and kept in the plastic cages with open tops for easy monitoring. The milking was carried out every 2 weeks.

Fractionation of venom and peptide purification

The crude venom (0.8 mL) was diluted with 3.2 mL of 0.1 mol·L^{-1} phosphate buffered saline (PBS), pH 6.0, and centrifuged at 12 000 r·min^{-1} for 10 min. The supernatant was collected and then applied to a Sephadex G-50 gel filtration column (Superfine, 2.6 cm × 100 cm, Amersham Biosciences, Sunnyvale, CA, USA) equilibrated with 0.1 mol·L^{-1} PBS. The elution was performed with the same buffer and fractions were collected as 3.0 mL per tube. All the fractions were analyzed by monitoring under UV absorption at 280 nm to obtain the gel filtration profile. The fraction from gel filtration profile with major peak intensity was lyophilized, re-suspended in 5 ml of 0.1 mol·L^{-1} PBS, and purified further using C_{18} reverse-phase high-performance liquid chromatography column (Gemini, 5-μm particle size, 110 Å pore size, 250 mm × 4.6 mm) with linear gradient of 5%–100% solution of 99.9% acetonitrile with 0.1% TFA over 95 min at a flow rate of 1.5 mL·min^{-1}. The elution was monitored under UV at 280 nm and all the eluted peptides were collected and lyophilized separately. The peptide fractions having the desired function were further purified on an analytical C_{18} RP-HPLC column as described above.

Mass spectrometric analysis and peptide sequencing

The molecular mass of purified peptide was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. To determine the *N*-terminal sequence, the purified peptide was first subjected to an automatic Edman degradation in Applied ProciseTM 491-A protein sequencer (Shimadzu Corporation, Kyoto, Japan). Because of failure to determine the sequence by Edman degradation, the partial interior amino acid sequence of purified peptide was then determined by ISD MALDI MS. Following determination, a Blast (NCBI) search was carried out to check the sequence similarity with other previously reported peptides.

cDNA library construction and cloning for the gene encoding MeKTT-1

cDNA was synthesized as previously described [18]. Briefly, total RNA was extracted from the venom glands of scorpions using TRIzol (Life Technologies, Carlsbad, CA, USA) and used to prepare cDNA using a SMART™ PCR cDNA synthesis kit (Clontech Laboratories Inc., Mountain View, CA, USA). The cDNA was prepared using total RNA extracted from *M. eupeus* venom glands and served as the template to amplify the gene that encodes target peptide. PCR was performed with degenerate primers designed from a combination of peptide sequence (determined by ISD MALDI MS) to amplify cDNA encoding MeKTT-1. Following amplification, the fragment was sequenced by ABI 3730 automatic DNA sequencer (Applied Biosystems, Foster city, CA, USA), according to the manufacturer's instructions.

Serine protease inhibition assays

The inhibition effects of MeKTT-1 on the hydrolysis of synthetic chromogenic substrates by serine proteases were assayed at room temperature. Trypsin (bovine pancreatic trypsin; EC 3.4.21.4), chymotrypsin (bovine pancreatic α-chymotrypsin; EC 3.4.21.1), elastase (porcine pancreatic elastase; EC 3.4.21.36), Factor-Xa (human F-Xa; *EC* 3.4.21.6), Factor-XIIa (human F-XIIa; *EC* 3.4.21.38), thrombin (thrombin from bovine plasma; EC 232-648-7) and their respective chromogenic substrates Na-benzoyl-L-arginine 4-nitroanilide hydrochloride, *N*-succinyl-Ala-Ala-Pro-Phe p-nitroanilide, *N*-succinyl-Ala-Ala-Ala-p-nitroanilide, CH3OCO-D-CHA-Gly-Arg-pNA-AcOH, H-D-Pro-Phe-Arg-pNA.2HC, and H-D-Phe-Pip-Arg-pNA.2HC were purchased from Sigma. The inhibitory assay was performed in 50 mmol·L^{-1} Tris-HCl, pH 7.8, in a total volume of 100 μL. Trypsin (final concentration of 350 nmol·L^{-1}) was pre-incubated with various concentrations of MeKTT-1 (0 to 350 nmol·L^{-1}), whereas chymotrypsin, elasatase, F-Xa, F-XIIa and thrombin (final concentration of 400 nmol·L^{-1}) were pre-incubated with different concentrations of MeKTT-1 (0 to 450 nmol·L^{-1}) for 10 min at room temperature. The reactions were initiated by adding the substrates ranging from 0.1 to 0.8 mmol·L^{-1}. The initial rate of product formation in each reaction was monitored continuously at 405 nm for 5 min. The inhibitory activity of MeKTT-1 was determined by setting the initial velocity obtained in the presence of enzyme alone (devoid of inhibitor) as 100% [19]. Lineweaver–Burk plots ($1/V$ vas $1/[S]$) were used to determine the K_m/V_{max} values of trypsin activity on Na-benzoyl L-arginine 4-nitroanilide in the presence of different inhibitor concentrations. The slopes (K_m/V_{max}) of