# Biological Characterization of A Kunitz-type Inhibitor from Malaysian King Cobra (*Ophiophagus hannah*) Venom †

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**Abstract:** Kunitz-type inhibitors (KTIs) are proteins that bear homology to the Bovine Pancreatic Trypsin Inhibitor (BPTI) and exhibit a wide variety of biological activities, including inhibition of various proteases, interference with hemostasis, and inflammation; showing their functional diversity. The KTIs have been isolated and identified as either chymotrypsin or trypsin inhibitors. This study aims to isolate and further characterize the pharmacological properties of a KTI from the Malaysian king cobra (*Ophiophagus hannah*) venom. The inhibitory effect on serine protease activity was determined using chromogenic substrates. The whole venom was fractionated by size exclusion HPLC, and the isolated peaks were identified with N-terminal sequencing. The fractions were then incubated with plasmin at different times, and the inhibition of its biological activities was tested. The whole venom reduced the trypsin activity towards its chromogenic substrate. After size exclusion chromatography, 13 fractions were isolated. After testing their effects on plasmin activity, F10 showed the most remarkable effect, preventing the fibrinolytic activity on fibrin plates, and partially inhibiting the fibrinogenolytic activity. These characterization studies will elucidate the biomedically relevant pharmacological properties intrinsic to a KTI from king cobra venom, leading to their potential use for biomedical applications.

**Keywords:** King cobra; Kunitz-type inhibitor; hemostasis; fibrinolysis; plasmin inhibitor

**Key Contribution:** A KTI, named OH-TCI, from the Malaysian king cobra venom was partially purified and characterized. Besides its inhibitory activity towards trypsin, here we showed for the first time that OH-TCI could inhibit the activity of a serine protease from the hemostatic system, plasmin, towards its biological substrates fibrin and fibrinogen

## 1. Introduction

Protease inhibitors are ubiquitous components expressed by various organisms and existing in multiple forms [1]. In general, they are proteins or peptides with the ability to inhibit proteolytic enzymes' activities, being the serine protease inhibitors the most studied and widely distributed of the protease inhibitor superfamily [1,2]. Among this group are the Kunitz-type inhibitors (KTIs), low molecular mass peptides, with a motif that usually contains approximately 60 amino acids residues, stabilized by three disulfide bridges, and are homologous with the conserved Kunitz motif present in bovine pancreatic trypsin inhibitor (BPTI) [1–4].

The KTIs have been widely isolated and characterized from snake venoms belonging to the families Elapidae and Viperidae. Even though the overall three-dimensional structures remain similar, minor differences in the amino acid sequences can modulate these

molecules' activity and promote the functional diversity found in snake venoms, increasing their toxicity towards the prey [3–7]. Furthermore, a single venom may contain several KTIs in non-covalent protein complexes acting synergistically or can form complexes with other toxins, thus inducing potent effects in the victims [8–12].

These properties are translated in a broad spectrum of physiological effects in different systems, including inhibition of various proteases, interference with blood coagulation, fibrinolysis, and inflammation, activation or blockade of acid-sensing ion channels (ASICs), and blocking of ion channels [1,3,4,8,12]. Moreover, these functions are becoming increasingly relevant as new information reveals their unique role in snakebite pathophysiology and possible biomedical applications and utility as diagnostic tools [1].

Several toxins have been identified from the king cobra (*Ophiophagus hannah*) venom, including phospholipases $A_2$ (PLA$_2$s), three-finger toxins (3FTxs), metalloproteinases (SVMP), and KTIs. Among the KTIs, OH-TCI and Oh11-1 have been isolated from these venoms, being OH-TCI, a dual trypsin/chymotrypsin inhibitor, found in all the venoms from different geographical areas [13,14]. This study aims to isolate and further characterize the pharmacological properties of a KTI from the Malaysian king cobra venom. The toxin was partially purified through a size-exclusion HPLC and characterized for its potential inhibitory effect on serine protease activity using different *in vitro* assays. Our results demonstrated that the partially pure OH-TCI was able to inhibit the plasmin activity towards its physiological substrates from the hemostatic system, revealing its potential as a therapeutic tool for bleeding disorders characterized by a dysregulation of the fibrinolytic system.

## 2. Results

### 2.1. Inhibition of the Amidolytic Activity of Trypsin by the Whole Venom of King Cobra

After incubation for 30 min at room temperature, the Malaysian king cobra venom significantly reduced the amidolytic activity of trypsin on L-BAPNA in a concentration-dependent manner ($p < 0.01$), however, it did not abrogate the enzymatic activity compared to the positive control, a cocktail of inhibitors (2%) (Figure 1 and Table 1).

**Table 1.** Effect of the Malaysian king cobra venom on the activity of trypsin at 20 min post-incubation.

| Sample | O.D. 405 nm (Mean ± SD) | Inhibition (%) |
| --- | --- | --- |
| Trypsin (8 μM) | 1.161 ± 0.064 | N/A |
| Trypsin (16 μM) + Cocktail of inhibitors (2%) | 0.058 ± 0.022 | 96[2] |
| Trypsin (16 μM) + KCV[1] (1 mg/mL) | 0.490 ± 0.074 | 61[2] |
| Trypsin (16 μM) + KCV[1] (2 mg/mL) | 0.299 ± 0.067 | 75[2] |

[1] Malaysian king cobra venom. [2] $p < 0.01$.

### 2.2. Partial Isolation and Purification of OH-TCI

The king cobra venom was run through a size-exclusion chromatography, and 13 fractions were collected (F1-F13) (Figure 2A). In F10, two bands were detected using electrophoresis and were identified through N-terminal sequencing as a long neurotoxin (upper band) and the KTI OH-TCI (lower band) (Figure 2B). The N-terminal sequence obtained for the KTI was GRPKF(**C**)ELPAVSGF.
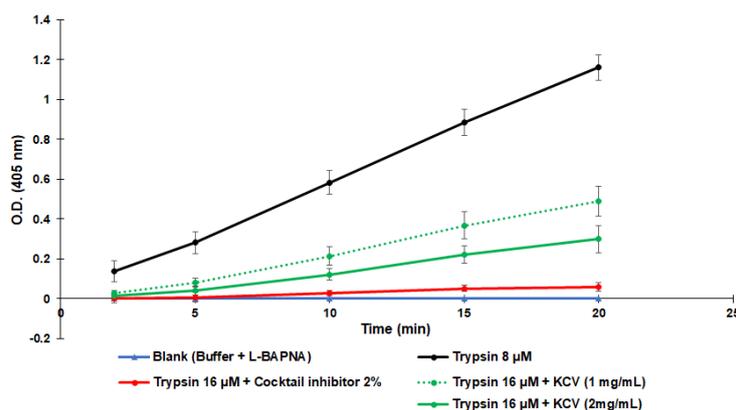
**Figure 1.** Effect of Malaysian king cobra (*O. hannah*) venom on the activity of trypsin. Trypsin was incubated with king cobra venom for 30 min at room temperature, the inhibition towards the serine protease was measured through the hydrolysis of the synthetic chromogenic substrate L-BAPNA by trypsin at different times. Results were expressed as the mean ± SD of the absorbances recorded at 405 nm over 20 min. Blank: Assay buffer (100 mM Tris-HCl, containing 10 mM of $CaCl_2$, pH 8.0). KCV: Malaysian king cobra venom.
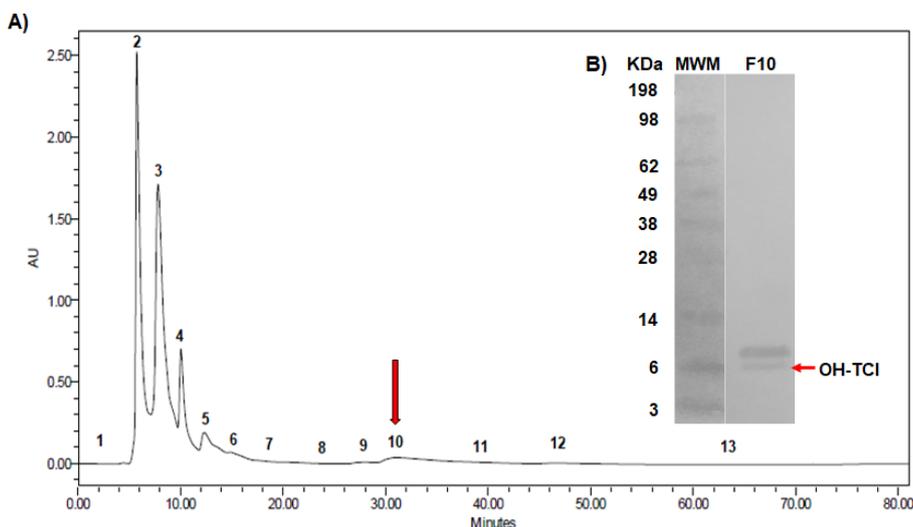


**Figure 2. A)** Fractionation of *O. hannah* crude venom (30 mg) by size-exclusion chromatography. Thirteen fractions were obtained, and the partially purified OH-TCI was achieved in F10 (arrow). **B)** SDS-PAGE analysis of F10. Lane 1: SeeBlue Plus2 Marker (Invitrogen™); Lane 2: F10.

### 2.3. Inhibitory Effect of Partially Pure OH-TCI on Plasmin Towards Its Physiological Substrates

We employed the fibrinogenolytic activity assay (Figure 3A) to determine the inhibitory effect on the cleavage of the three chains that form the fibrinogen molecule (lane 1). After 24 h of incubation, 0.26 nKat of plasmin degraded the fibrinogen into fragments of different sizes (lanes 2 and 6). This proteolytic activity was partially inhibited, preventing Bβ and γ chains' cleavage, when plasmin was pre-incubated with F10 containing OH-TCI (lane 4). The cocktail of inhibitors completely inhibited the fibrinogenolytic activity of plasmin (lane 3).

Meanwhile, in the presence of plasminogen, the partially purified OH-TCI decreased by 97% the fibrin lysis by plasmin ($10 \pm 4$ vs. $279 \pm 3$ $mm^2$); under the same conditions, the cocktail of inhibitors completely abrogated the fibrin lysis (Figure 3B).
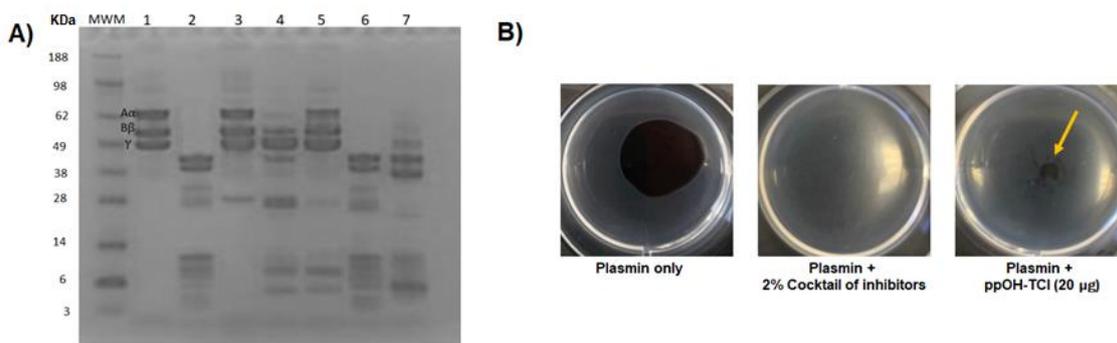
**Figure 3.** Inhibition of plasmin towards its physiological substrates by partially purified OH-TCI. **A)** Inhibition of the fibrinogenolytic activity of plasmin on fibrinogen. SDS-PAGE under reducing conditions. Lanes: MWM: Molecular weight marker; 1) Fibrinogen control; 2) Fibrinogen + plasmin; 3) Fibrinogen + (plasmin + 2% cocktail of inhibitors); 4) Fibrinogen + (plasmin + partially purified OH-TCI); 5) Fibrinogen + partially purified OH-TCI; 6) Fibrinogen + plasmin; Fibrinogen + king cobra venom. **B)** Inhibition of the fibrinolytic activity on fibrin plates of plasmin. Arrow points to the inhibitory effect of the partially purified OH-TCI.

## 3. Discussion

The Malaysian king cobra, *O. hannah*, belongs to the Elapidae family and is endemic from India through Southeast Asia [13]. Many toxins have been isolated from king cobra venom, including natriuretic peptides, short and long neurotoxins of the 3FTx family, cystatin inhibitors, PLA2s, cysteine-rich secretory proteins (CRiSPs), PIII-SVMPs, l-amino acid oxidase molecules (LAOOs), and KTIs [13,15]. The KTIs Oh-11.1 and OH-TCI shared 74% of identity, but OH-TCI is a unique KTI displaying a potent inhibitory activity against chymotrypsin and trypsin [14]. Here, we start the characterization of the pharmacological properties and activities of OH-TCI.

The KTIs are also known as Kunitz-type protease inhibitors and are highly conserved but display small differences in the region of the sequence that interacts with the protease, which defines their specificity towards serine proteases, and give rise to an array of biological functions [1,4,5,11]. In this sense, these toxins are classified, according to the amino acid residue in the P1 position, into two major classes: trypsin inhibitors and chymotrypsin inhibitors [16]. We used the measurement of the amidolytic activity to determine the presence of KTIs in snake venoms by testing the inhibitory activity on the hydrolysis of synthetic chromogenic substrates by the serine protease trypsin. The Malaysian king cobra venom inhibited trypsin activity on L-BAPNA (specific chromogenic substrate for this serine protease), suggesting the presence of OH-TCI. However, this inhibition was not complete due to other toxins in the venom with intrinsic proteolytic activity [13]. Based on these results, we proceeded to start the purification of OH-TCI, using size-exclusion chromatography. The partially purified KTI from the king cobra venom was obtained in F10, along with a long chain neurotoxin, and confirmed to be OH-TCI.

The KTIs have been found in the venoms of Elapidae and Viperidae families, suggesting that they interfere with the hemostatic system as a mechanism to disturb the prey's homeostasis [7,11]. We modified two *in vitro* assays, usually employed to test the hemostatic activity of snake venoms, to determine the inhibitory activity of OH-TCI on plasmin, a serine protease with trypsin-like activity that plays a major role in the modulation of hemostasis, thrombosis, fibrinolysis, inflammation, apoptosis, and the complement cascade [17]. Plasmin was pre-incubated with the partially purified OH-TCI and tested for its proteolytic activity on two substrates: fibrinogen and fibrin. We used the fibrinogenolytic activity assay to determine the inhibitory effect on the cleavage of the three chains that form the fibrinogen molecule (A$\alpha$, B$\beta$, and $\gamma$). OH-TCI partially inhibited the fibrinogenolytic activity of plasmin, preventing the cleavage of B$\beta$ and $\gamma$ chains. Meanwhile, it inhibited almost completely the degradation of a fibrin clot in the presence of

plasminogen. Plasmin is involved in various pathological processes, including thrombolysis, tumor progression, hyper-fibrinolysis states, and other diseases [18]. Here we showed for the first time that OH-TCI could inhibit this serine protease activity, suggesting that this toxin can be a candidate with therapeutic potential as an anti-fibrinolytic agent. Further studies will include more purification steps to obtain the pure form of this toxin and evaluate the modulation of plasmin activity against other substrates involved in physiopathologic states such as inflammation and metastasis.

## 4. Materials and Methods

### 4.1. Materials

#### 4.1.1. Reagents

Human fibrinogen and human fibrinogen with plasminogen was obtained from Hyphen Biomed (France). Protein-Pak 60 column was from Waters™ (Milford, MA, USA). Plasmin, Imidazole, Bovine Thrombin, Cocktail of inhibitors, Bovine pancreatic trypsin (type-1), L-BAPNA, $CaCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). PBS were from Dulbecco, Tris-HCl were from. Bis-Tris buffer solution 4X, NuPage reducing agent, MES SDS running buffer, 4–12% Bis-Tris SDS-PAGE gel, SeeBlue Plus2 Marker, Simply Blue Safestain were obtained from Invitrogen™ (Carlsbad, CA, USA).

#### 4.1.2. Venom

Malaysian king cobra (*O. hannah*) venom was obtained from MetTox, Deland, FL.

### 4.2. Methods

#### 4.2.1. Protein Concentration

The protein concentration was measured spectrophotometrically estimated by assuming that 1 unit of absorbance/cm of path length at 280nm corresponds to 1mg of protein/mL [19].

#### 4.2.2. Trypsin Inhibiting Activity of Whole Venom of Malaysian O. hannah

The inhibitory activity against trypsin was tested with a modified method from He et al. [14]. Briefly, trypsin (initial concentration of 8 μM) and different concentrations of the venom (0.2–2 mg/mL) were incubated for 30 min at room temperature in a 1:1 ratio. The addition of the synthetic chromogenic substrate L-BAPNA (initial concentration 50 mM) initiated the reaction. The release of p-nitroaniline was monitored at 405 nm for 25 min. Results were expressed as the mean ± SD of the absorbances, and the percentage of inhibition was calculated. The pre-incubation of trypsin with a cocktail of inhibitors (initial concentration 2%) was used as positive control.

#### 4.2.3. Size-exclusion Chromatography

Approximately 0.03 g of *O. hannah* venom was reconstituted in 1 mL of PBS buffer ($NaH_2PO_4$–$Na_2HPO_4$, pH 5.8, containing 0.1 M NaCl) and loaded onto a Protein-Pak 60 column equilibrated with the same buffer in a Waters™ 1525 binary HPLC system (Waters, USA). Venom was run for 80 min at a flow rate of 1 mL/min and read at a wavelength of 280 nm. Fractions were collected, pooled, dialyzed, and lyophilized.

#### 4.2.4. N-terminal Amino Acid Sequence Determination of Proteins Purified from King Cobra Venom

The N-terminal amino acid sequence determination was done as described by Salazar et al., in a PPSQ-33B protein sequencer (Shimadzu, Japan) [20].

#### 4.2.5. Inhibitory Activity of F10 Containing OH-TCI on Plasmin Towards Its Substrates

#### 4.2.5.1. Fibrinogenolytic Activity

The effect of F10 on the fibrinogenolytic activity of plasmin on fibrinogen was evaluated as described by Margres et al. [21]. Briefly, after the pre-incubation of plasmin (0.26 nKat) for 30 min at 37 °C with F10 (1 mg/mL), it was incubated for 24 h at 37°C with a fibrinogen solution (5 mg/mL), and the residual fibrinogenolytic activity was evaluated on a NuPAGE 4–12% Bis-Tris SDS PAGE gel.

#### 4.2.5.2. Fibrinolytic Activity

The effects of the fibrinolytic activity of plasmin on a fibrin plate was evaluated through a modification of the method described by Rodriguez-Acosta et al. [22]. Briefly, in 6-wells plates, 75 µL of thrombin (1000 IU/mL) were added to 1.5 mL of human fibrinogen with plasminogen solution in imidazole buffer (3 mg/mL), then the fibrin clot was allowed to form at room temperature. Samples were prepared as follows: 10 µL of plasmin (0.26 nKat) were mixed with 20 µL of F10 (1 mg/mL) and incubated for 30 min at 37°C. Plasmin mixed with assay buffer (PBS, pH 7.4) or 2% of cocktail of inhibitors were used as negative and positive control, respectively. Twenty microliters of each mix were placed in each well, and incubated at 37 °C in a humid atmosphere for 24 h. The area of lysis was measured and expressed as $mm^2$.

#### 4.2.6. Statistical Analysis

The data was expressed as the arithmetic means ± the standard deviation (SD) of 3 independent experiments. Student's t-test was employed for possible differences comparing to the negative control. Values of $p < 0.01$ were accepted as statistically significant for all the experiments.

**Conflicts of Interest:** The authors declare no conflict of interest.

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