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# Trypsin and chymotrypsin inhibitor peptides from the venom of Chinese *Daboia russellii siamensis*

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ABSTRACT

Two trypsin inhibitors and one chymotrypsin inhibitor from Chinese *Daboia russellii siamensis* venom, denoted as CBPTI-1, CBPTI-2 and CBPTI-3 were purified, characterized and cloned from lyophilized venom-derived cDNA libraries. The N-terminus of CBPTI-1 was modified and not amenable to Edman degradation sequencing, however an internal partial sequence was found to be SGRCRGHLRRIYYNPDSNKCE. The N-termini of CBPTI-2 and CBPTI-3 were unmodified and their partial sequences were established as HDRPTFCNLAPESGRCRAH and HDRPKFCYLPADPGECMAYIRSFYYDS respectively. From cloning studies CBPTI-1 was found to consist of 66 amino acid residues, while CBPTI-2 and CBPTI-3 precursors consist of 60 amino acid residues, including 6 cysteine residues. Another cDNA sequence (CBPTI-4) was also obtained. Alignment of cDNA sequences showed that CBPTI-3 exhibited similar sequence homology to CBPTI-4 cDNA except for an 8 nucleotide deletion in the open-reading frame. CBPTI-1 and CBPTI-2 were demonstrated to be potent trypsin inhibitors, but were also shown to be effectively potent in chymotrypsin inhibition. The $K_i$ values of CBPTI-1 and CBPTI-2 for trypsin inhibition were $4.07 \times 10^{-7}$ M and $6.66 \times 10^{-7}$ M, respectively, and they were non-competitive in their activity. CBPTI-3 showed chymotrypsin inhibition activity with a $K_i$ value of $2.55 \times 10^{-9}$ M, but did not show trypsin inhibitor activity.



## 1. Introduction

Russell's viper (*Daboia russelli*) is distributed in South East Asia including India, Bangladesh, Sri Lanka, Bhutan, Nepal, Pakistan, Burma, Thailand, Cambodia, China and Taiwan (Wuster et al., 1992a,b). According to its geographic origin, Russell's viper has been classified into at least four subspecies: *D. russelli formosensis* from Taiwan, *D. russelli pulchella* from Sri Lanka and South India, *D. russelli russelli* from North India and Pakistan, and *D. russelli siamensis* from China and South east Asia (Jayanthi and Gowda, 1988; Woodhams et al., 1990; Lee, 1944). Tsai et al. (1996) provided evidence for classifying Russell's viper into two types depending on the first N-terminal amino acid residue asparagine or serine of phospholipase A2 (PLA2). Wuster et al. (1997) classified them into two distinct subspecies based on their morphological characteristics. The snake venom BPTI peptides contain about 60 amino acids and are basic polypeptides with six cysteine residues forming three conserved-fold disulphide bridges. Based on protein structure, the Kunitz superfamily is classified into two families; small Kunitz-type inhibitors/BPTI-like toxins and soft tick anticoagulant proteins (Chen et al., 2001). According to their functions, snake venom Kunitz/BPTIs are

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classified as being either neurotoxic or non-neurotoxic with trypsin and chymotrypsin inhibitors belonging to the latter group. Other BPTIs have little or no inhibitory activity and act as $K^+$ and $Ca^{2+}$ channel blockers (Cardle and Dufton, 1997). The inhibitors of proteases are grouped primarily as serine-, cysteine-, aspartic- or metalloprotease inhibitors. Laskowski and Kato (1980) classified serine proteinase inhibitors into about a dozen families based on sequence homologies. Rawlings et al. (2004) described a new taxonomy to classify the inhibitor units of the peptidase inhibitors to 48 families based on similarities in their amino acid sequences.

Protease inhibitors such as the polypeptides aprotinin and ulinastatin, have been used as pharmacological agents. In agriculture, transgenic crops producing protease inhibitory activity against digestive enzymes of insect pests have been investigated (Samac and Smigocki, 2003; Telang et al., 2003). Textilinins, trypsin inhibitors (RVV II) and Prmulgins, members of the BPTI superfamily from *Pseudonaja textilis textilis*, Russell's viper and Papuan pigmy mulga snake venoms, respectively, have been found to inhibit activities of trypsin and plasmin (Masci et al., 2000; Takahashi et al., 1972; Inagaki et al., 2012) and reduce blood loss in a murine bleeding model (Masci et al., 2000; Flight et al., 2009). Snake venom Kunitz/BPTI inhibitors are thought to affect the processes of coagulation, fibrinolysis and inflammation (Shafqat et al., 1990b).

In this study, we describe the purification, characterization and cloning of trypsin and chymotrypsin inhibitors belonging to the BPTI superfamily, from Chinese *D. russelli siamensis* venom.

## 2. Materials and methods

### 2.1. Materials

Lyophilized *D. russellii siamensis* venom originated from Guangxi Zhuang Autonomous Region, China and was obtained from Xinyuan Jiayu management department for snake venoms (Guangzhou, China), an agency of Rainbow Snake Farm (Yingtan City, Jiangxi Province, China). A Superdex™ Peptide 10/300 GL column was purchased from Pharmacia (Uppsala, Sweden). POROS 50 HS and 50 HQ gels were bought from Perkin Elmer (Wellesley, MA, USA). A Jupiter C-18 column was purchased from Phenomenex Inc. (Torrance, CA, USA). Spherigel ODS gel was obtained from Dalian Yi Li Te Co. (Dalian, China). GluC (*Staphylococcus aureus* protease V8) was bought from New England Biolabs Ltd. (Beverly, Massachusetts, USA). Benzoyl-D, L-4-arginine-*p*-nitroanilide (BAPNA) was purchased from Shanghai Shui Yuan Biotechnology Co. Ltd. (Shanghai, China). Bovine trypsin and chymotrypsin were from Shanghai Sheng Gong Biological Engineering and Technology Service Co., Ltd. (Shanghai, China) and Amesco (Carson, CA, USA), respectively. Micro BCA™ Protein Assay Kit was from Pierce Biotechnology Inc. (Rockford, Illinois, USA). The pGEM-T Easy vector and PCR Rapid Purification Kits were purchased from Promega (Madison, WI, USA) and DNA Sequencing Kits (BigDye Terminator v3.1) was from ABI (Foster City, California, USA). All reagents used were of analytical grade.

### 2.2. Isolation and purification

#### 2.2.1. Pretreatment of crude venom

One hundred milligrams of Chinese *D. russellii siamensis* venom were dissolved in 1.0 ml of 0.4/99.6 (v/v) trifluoroacetic acid (TFA)/water. The venom solution was heated at 80 °C for 30 min in a water bath and a supernatant prepared by centrifugation at 10,000 × *g* for 10 min. The precipitate was washed twice using 0.4/99.6 (v/v) TFA/water and supernatants pooled and concentrated with a rotating vacuum concentration centrifuge (Martin Christ, Osterode am Harz, Germany).

#### 2.2.2. Gel filtration chromatography

The concentrated sample was dissolved in distilled water and applied to a Superdex™ Peptide 10/300 GL column (1.5 × 30.0 cm), which was equilibrated with 0.02 M PBS buffer, pH 7.0, containing 0.25 M NaCl at a flow rate of 0.5 ml/min. The chromatography was conducted using a Beckman Gold HPLC system (Beckman, CA, USA) at room temperature, and the eluate monitored at 214 nm using a Beckman 166 UV detector (Beckman, CA, USA). The buffer used in the HPLC study was filtered through a 0.22 μm membrane filter from Xing Ya Co. Ltd. (Shanghai, China).

#### 2.2.3. POROS 50 HS column chromatography

Collected fractions from the gel filtration purification that exhibited trypsin and chymotrypsin inhibitor activities were collected and diluted four-fold (v/v) in 0.02 M phosphate buffer, pH 7.0. The sample was then loaded onto a self-packed POROS 50 HS column (0.6 × 15.0 cm), which was equilibrated with 0.02 M phosphate buffer, pH 7.0 at a flow rate of 1.0 ml/min. The absorbed components were eluted with a linear gradient of NaCl from 0 to 0.2 M in the equilibrium buffer for 80 min and 0.35–1.0 M NaCl for 120 min. The chromatography was conducted using a CCPD 8000 HPLC system (Toyosoda Co. Ltd., Tokyo, Japan) at room temperature, and the eluate was monitored at 214 nm with a Beckman 166 UV detector.

#### 2.2.4. Reversed phase C-18 column chromatography

The fractions with inhibitory activities against trypsin and chymotrypsin from the POROS 50 HS column chromatography purification were loaded onto a Jupiter C-18 column (0.46 × 15 cm) equilibrated with 0.05/99.95 (v/v) TFA/water at a flow rate of 0.5 ml/min. The absorbed components were eluted by a gradient of acetonitrile containing 0.05% TFA using a CCPD 8000 HPLC system as described above.

#### 2.2.5. POROS 50 HQ column chromatography

The appropriate active fraction, as purified by C-18 column chromatography, was concentrated using a ZFQ 85A rotating vacuum concentrator (Shanghai Medical Machine Co., Shanghai, China). The concentrated fraction was dissolved in 0.01 M Tris–HCl, pH 9.0, and HPLC of dissolved samples was performed using a POROS 50 HQ self-packed column (0.6 × 15 cm). The column was equilibrated with Tris–HCl buffer of 0.01 M, pH 9.0 at a flow rate