Isolation, expression and characterization of a novel dual serine protease inhibitor, OH-TCI, from king cobra venom

Ying-Ying He a,b,1, Shu-Bai Liu a,b,1, Wen-Hui Lee a,c, Jin-Qiao Qian a,b,c,d, Yun Zhang a,c,*

a Biotoxin Units, Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, 32 East Jiao Chang Road, Kunming, Yunnan 650223, China
b Graduate School of the Chinese Academy of Sciences, Beijing 100039, China
c Kunming Medical College, Kunming, Yunnan 650032, China
d Department of Anesthesiology, The First Affiliated Hospital of Kunming Medical College, Kunming, Yunnan 650031, China

Abstract
Snake venom Kunitz/BPTI members are good tools for understanding of structure–functional relationship between serine proteases and their inhibitors. A novel dual Kunitz/BPTI serine proteinase inhibitor named OH-TCI (trypsin- and chymotrypsin-dual inhibitor from Ophiophagus hannah) was isolated from king cobra venom by three chromatographic steps of gel filtration, trypsin affinity and reverse phase HPLC. OH-TCI is composed of 58 amino acid residues with a molecular mass of 6339 Da. Successful expression of OH-TCI was performed as the maltose-binding fusion protein in E. coli DH5α. Much different from Oh11-1, the purified native and recombinant OH-TCI both had strong inhibitory activities against trypsin and chymotrypsin although the sequence identity (74.1%) between them is very high. The inhibitor constants (Ki) of recombinant OH-TCI were 3.91 × 10⁻⁷ and 8.46 × 10⁻⁸ M for trypsin and chymotrypsin, respectively. To our knowledge, it was the first report of Kunitz/BPTI serine proteinase inhibitor from snake venom that had equivalent trypsin and chymotrypsin inhibitory activities.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

Protein inhibitors of serine proteinases are ubiquitous. They are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms [16,19–21]. The protein inhibitors have assumed crucial roles in physiological processes, such as blood coagulation and tissue remodeling [20,24,27], where the extent of proteolysis requires limitation. Among the structurally diverse inhibitor families have been identified, members of the Kunitz/BPTI (bovine pancreatic trypsin inhibitor) family consists of approximately 60 amino acids and is characterized by 6 conserved cysteine residues forming 3 disulfide linkages (1–6, 2–4, 3–5) that contribute to the compact and stable nature of the folded polypeptide [1,3,17,20].

Snake venoms contain serine protease inhibitors in addition to enzymes and toxins. Several Kunitz-type inhibitors from the venoms of Viperidae and Elapidae snakes have been isolated and sequenced [2,6,8,22,23,25,26,30–32]. Although the physiological role of the Kunitz-type inhibitors in snakes is not known, it has been proposed that they are involved in the processes of coagulation, fibrinolysis and inflammation through undefined...
interactions with proteases [37]. Up to date, only a chymotrypsin inhibitor (Oh11-1) has been reported in king cobra venom [6]. In the present study, we reported the purification, molecular cloning, recombinant expression and characterization of a novel Kunitz-type inhibitor named OH-TCI (trypsin- and chymotrypsin-dual inhibitor from Ophiophagus hannah) from king cobra venom. Although the sequence identity between OH-TCI and Oh11-1 is very high (74.1%), OH-TCI is quite different from Oh11-1 with the strong inhibitory activity against trypsin and chymotrypsin. The primary structure of OH-TCI and its unique inhibitory activity specificity will give important clues to understand the inhibitor interactions with different serine proteinases.

2. Materials and methods

2.1. Materials

Lyophilized crude venom from O. hannah was obtained from the stock of Kunming Institute of Zoology (China). Sephadex G-50 column from Pharmacia, Reverse phase high-performance liquid chromatography (RP-HPLC) C18 and C4 column was from Agilent Technologies (USA). Vector pMAL-p2X was from New England Biolabs (USA). The polymerase Pfu, Hind III were from Promega (USA). An agarose gel DNA purification kit and isopropyl β-D-thiogalactopyranoside (IPTG) were from Takara (Dalian, China). Bovine pancreatic trypsin (type I) (T8003), bovine α-chymotrypsin (type VII, C3142), Bacillus licheniformis subtilisin, human thrombin, chromogenic substrate Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPNA, B3279), N-succinyl-Ala-Ala-Pro-Phe-p-NA (S7388) and N-CBZ-Gly-Gly-Leu-p-NA (C-3022) were obtained from Sigma (USA). H-D-Phe-Pip-Arg-p-NA (S-2238) was from Kabi Vitrum (Sweden). Ampicillin and factor Xa were from Sigma. All other reagents were of analytical or sequencing grade. To determine the molar concentration of the active site enzyme, the trypsin was subjected to active site titration with p-nitrophenyl-ε-guanidinobenzoate hydrochloride (pNPGB, N8010, from Sigma), using a Lambda Bio 40 UV/VIS spectrometer (PerkinElmer). Titration was performed in 0.1 M veronal buffer (pH 8.6), containing 0.02 M CaCl₂ [7].

2.2. Isolation and purification of OH-TCI

The crude venom (0.5 g) of king cobra was dissolved in 3 ml PBS buffer (NaH₂PO₄–Na₂HPO₄, pH 5.8, containing 0.1 M NaCl) and was loaded onto a Sephadex G-50 column (2.6 cm × 100 cm) equilibrated with the same buffer. Collected fractions (2.5 ml/tube) were monitored by absorbance at 280 nm. Trypsin and

Fig. 1 – Purification of OH-TCI from king cobra crude venom. (A) Gel filtration chromatography. 0.5 g crude venom of Ophiophagus hannah dissolved in 3 ml PBS buffer was loaded onto a Sephadex G-50 column. (B) Trypsin-affinity column chromatography. Fractions of peak IV of Sephadex G-50 column, with both trypsin and chymotrypsin inhibitory activities, were pooled and then applied to a trypsin-affinity column (1 ml). After extensive washings with PBS buffer, acetate buffer and equilibrated with PBS buffer, 0.1N HCl was employed for elution. Protease inhibitory activities existed in peak IV. (C) RP-HPLC chromatography. Peak IV from the trypsin-affinity column was loaded onto a ZORBAX 300SB-C18 column and a linear acetonitrile gradient of 25–40% was employed for elution. (Inset) 20% SDS-PAGE analysis of purified OH-TCI. (Lane 1) Purified OH-TCI; (lane 2) protein marker. (D) Mass spectrometry of purified OH-TCI. Arrows in the panels (A)–(C) indicate OH-TCI containing peaks.
clones were selected by PCR and their nucleotide sequences were transformed into the formula: concentration (mg/ml) = (determined by UV absorbance at 215 and 225 nm using the concentrations of inhibitor. The slopes (m/s) of saturation curves at different inhibitor concentrations were confirmed by sequencing. The clone encoding OH-TCI was cultured at 37 °C in rich broth (RB) medium containing ampicillin (100 μg/ml) to a density of (2–4) × 10⁶ cells/ml (A₆₀₀ approximately 0.5). Protein expression was then initiated by adding IPTG to the culture medium to a final concentration of 1 mM. The induced cells were then incubated at 25 °C and harvested 16 h later.

2.6. Purification and characterization of recombinant OH-TCI

Cells were lysed by osmotic shock in 5 mM MgSO₄. After centrifugation, periplasmic extracts containing the MBP-OH-TCI fusion protein were applied to the trypsin-affinity column and eluted as described in Section 2.1. The fusion protein with dual inhibitory activities mainly recovered in the HCl-eluted fractions. After thorough dialysis against factor Xa working buffer (20 mM Tris–HCl, pH 8.0, containing 100 mM NaCl), the fusion protein was cleaved by factor Xa at 23 °C for 48 h at a ratio of 1:100 (w/w) of factor Xa to fusion protein. The digestion mixture was then subjected to reverse phase HPLC on a C₁₈ column (4.6 mm × 250 mm) and further purified on a C₁₈ column (4.6 mm × 250 mm). The primary structure of the recombinant OH-TCI was confirmed by N-terminal amino acid sequencing and molecular mass determination as described above.

2.7. Protease inhibiting activity of OH-TCI

The inhibitory effects of the sample tested on the hydrolysis of synthetic chromogenic substrates by serine proteases were assayed in 100 mM Tris–HCl buffer, containing 10 mM CaCl₂, pH 8.0 (for trypsin, chymotrypsin and thrombin) or pH 8.45 (for subtilisin) at 25 °C. The protease (final concentrations 400 nM trypsin, 40 nM chymotrypsin, 20 nM thrombin or 30 nM subtilisin) and different amounts of the recombinant inhibitor (final concentrations ranging from 0 to 130 nM) were pre-incubated at 25 °C for 30 min. 1-BapNA (final concentration 50 mM) and S7388 (20 mM) were used as a substrate for trypsin and chymotrypsin, respectively; S-2238 (0.02 mM), for thrombin and C-3022 (0.2 mM), for subtilisin. The reaction was initiated by the addition of the substrate. The formation of p-nitroanilide (pNA) was monitored continuously at 405 nm for 2 min. The effect of the inhibitor was estimated by setting the initial velocity obtained in the presence of enzyme alone (without inhibitor) as 100%. The slopes (Kᵣ/Vₘₐₓ) of lines obtained from the Lineweaver–Burk representation (1/V vs. 1/[S]) of saturation curves at different inhibitor concentrations were plotted against the concentrations of inhibitor. The inhibitory constant (Kᵢ) of the chymotrypsin/inhibitor complex or the trypsin/inhibitor was determined from the intercept point of the x-axis [9].

2.8. Electrophoretic mobility shift assays

10 μg bovine pancreatic trypsin or 10 μg bovine α-chymotrypsin in 100 mM Tris–HCl buffer (containing 10 mM CaCl₂, pH 8.0) were mixed with increasing concentrations of OH-TCI and incubated at 25 °C for 30 min before adding methylene blue (0.002%) as an indicator. In the parallel experiments,
trypsin-OH-TCI, chymotrypsin-OH-TCI, or trypsin-chymotrypsin were mixed with the molar ration 1:1 (for trypsin 10 μg; for chymotrypsin, 10 μg; for OH-TCI, 2.5 μg). The mixtures were incubated at 25 °C for 30 min and chymotrypsin, trypsin or OH-TCI was added respectively. The mixtures were incubated at 25 °C for another 30 min and the indicator methylene blue was added. Samples were loaded to 20% native-PAGE (low-pH conditions), performed electrophoresis [18] at 4 °C and silver stained [13].

3. Results

3.1. Purification of OH-TCI from king cobra venom

The crude king cobra venom was separated into four fractions by Sephadex G-50 gel filtration as indicated in Fig. 1A. The fraction IV, with the low-molecular mass and trypsin and chymotrypsin inhibitory activity, was further loaded to a trypsin-affinity column. Surprisingly, in the eluted fraction IV (in Fig. 1B), inhibition of trypsin as well as chymotrypsin was detected. The fraction was further purified using RP-HPLC by a column C18. The peak with dual inhibitory activity was indicated with an arrow in Fig. 1C and the final product was shown to be a single band on 20% SDS-PAGE (Fig. 1C, inset). So the peptide was named as OH-TCI. In the purification, an overall yield of 0.89 mg of purified OH-TCI was obtained from 0.5 g of crude venom with a purification factor of 189.8 (Table 1). The molecular mass and the purity of purified OH-TCI were determined by a MALDI-ToF mass spectroscopy assay, Fig. 1D. Based on these results, clone 16 was confirmed to code for OH-TCI.

As the Kunitz/BPTI protease inhibitors from snake venoms, OH-TCI possesses six cysteine residuals in the conservative positions to form three disulfide bridges. OH-TCI is basic, with the isoelectric point (pI) 8.61 predicted by Vector NTI 7.0. Alignment of amino acid sequence of OH-TCI deduced from clone 16 with that of the Kunitz-BPTI family peptides from snake venoms indicated that OH-TCI had high homology to snake venom Kunitz-BPTI peptides (Fig. 2C), especially serine proteinase inhibitors from Elapidae. The identity between OH-TCI and Oh11-1 was 74.1%. Surprisingly, except for Glu50, all the residues at the N-terminal (from Gly1 to Pro5) and the C-terminal (from Ala29 to Gly58) of the two peptides are identical (Fig. 2B).

3.3. Functional expression of OH-TCI in DH5α

Successful expression of OH-TCI-MBP fusion protein was performed in E. coli DH5α and purified on a trypsin-affinity column. The fusion protein was cleaved by factor Xa and loaded to RP-HPLC (C4 and C18) to purify the recombinant OH-TCI. The purified recombinant OH-TCI was identified by Edman degradation assay and MALDI-ToF-MS analysis. The first 15 N-terminal amino acid residues were shown to be the same as those of the native peptide, and MALDI-ToF-MS analysis showed a single peak with the molecular mass of exactly 6339 Da (data not shown).

3.4. Effects of OH-TCI on proteinases

The specificity of OH-TCI was determined by assaying inhibitory activity against four different proteinases. Both the native and recombinant OH-TCI proteins have strong inhibitory activity against trypsin and chymotrypsin (Table 2). However, no inhibition of the hydrolysis of S-2238 by thrombin and C-3022 by subtilisin could be observed. Interestingly, the inhibitor constants (K) for chymotrypsin and trypsin were very close, both around 1.7 × 10⁻⁷ M in the native OH-TCI and no more than 5-fold in the recombinant OH-TCI. As deter-

---

### Table 1 – Purification of OH-TCI

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein mg</th>
<th>%</th>
<th>Inhibition of trypsin (Total units)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude venom</td>
<td>500</td>
<td>100</td>
<td>88.65 0.18</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G-50</td>
<td>149.1</td>
<td>29.82</td>
<td>58.95 0.40</td>
<td>2.22</td>
<td>66.50</td>
</tr>
<tr>
<td>Trypsin affinity</td>
<td>4.06</td>
<td>0.81</td>
<td>41.56 10.24</td>
<td>56.9</td>
<td>46.88</td>
</tr>
<tr>
<td>HPLC (C18)</td>
<td>0.89</td>
<td>0.18</td>
<td>30.41 34.17</td>
<td>189.8</td>
<td>34.30</td>
</tr>
</tbody>
</table>

a The protein concentration was determined by a protein assay kit (Bio-Rad) with BSA as a standard.

b The protein concentration was determined using the formula: concentration (mg/ml) = (A215 – A225) × 0.144 [36]. Trypsin inhibitory activity was determined as described under Section 2.
Fig. 2 – OH-TCI cDNA and amino acid sequence analysis. (A) The cDNA sequence and the deduced amino acid sequence of OH-TCI. The determined N-terminal amino acid sequence is underlined. (B) Alignment of mature peptide sequences of snake venom BPTI/Kunitz family peptides. Identical residues are indicated by asterisks and the P1 position is properly labeled by an arrow and shadowed. The characteristic cysteine residues of Kunitz domain are boxed. Conserved residues between OH-TCI and Oh11-1 are shaded. (C) Phylogenetic relationships of snake venom BPTI/Kunitz family based on mature peptide sequences. Mature peptide sequences for the alignment and phylogenetic analysis were retrieved from 2008 1692–1699.
detected (Fig. 4C).

The convex protease-binding loop surrounding the reactive site exhibits an extended conformation and serves as a recognition motif for the loop of protease inhibitors generally corresponds to the specificity of the cognate enzymes, i.e., inhibitors with P1 of Lys and Arg tend to inhibit trypsin and those with P1 of Leu, Met, Phe, Tyr, Trp and Asn tend to inhibit chymotrypsin [20]. The presence of Lys in the P1 site (as indicated by the arrow in Fig. 2B) suggests that OH-TCI should have trypsin inhibitory activity. The convex protease-binding loop surrounding the reactive site exhibits an extended conformation and serves as a recognition motif for the inhibitor specificity [8]. Sequence differences between OH-TCI and the chymotrypsin inhibitor Oh11-1 were observed at positions 10–12, 14, 18–21 and 25–29 (Fig. 2B). This may explain the difference in their inhibitory activity against serine proteases.

Besides that the residues in the binding loop, residues from a sequentially remote region can also contact the enzyme and influence the association energy [4,16,21]. In the reported crystal structure of bovine α-chymotrypsin and bovine pancreatic trypsin inhibitor [5], the complex of the proteinase and the inhibitor forms a compact dimer with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2. Each BPTI molecule, at opposite ends, is contacting both proteinase molecules in the dimer, through the reactive site loop and through residues next to the inhibitor’s C-terminal region. Lys15, the P1 residue of BPTI, however, does not occupy the α-chymotrypsin S1 specificity pocket, being hydrogen bonded to backbone atoms of the enzyme surface residues Gly216 and Ser217 [5]. OH-TCI can inhibit chymotrypsin hydrolysis activity with the molar ratio 2:2.
With the quite high homology to other Kunitz BPTI inhibitors, we can deduce that OH-TCI binding chymotrypsin with the same way as BPTI binding chymotrypsin. That is to say, two OH-TCI molecules bind to two chymotrypsin molecules; the residuals in the reactive site loop and the C-terminal end are responsible for the interaction between OH-TCI and chymotrypsin. Although great variations in the reactive loop residuals, the fragment Gly\textsuperscript{14}-Ala\textsuperscript{18}, important for chymotrypsin functional binding as mentioned in BF9 (Bungarus fasciatus fraction IX) \[8\], can be found the corresponding fragment in OH-TCI (Gly\textsuperscript{13}-Ala\textsuperscript{17}). More importantly, all the residues at C-terminal (from Ala\textsuperscript{30} to Gly\textsuperscript{58}) have no any variations, which can maintain the interactions between the chymotrypsin and inhibitor C-terminal region. Taken together, this may explain the strong chymotrypsin inhibitory activity shown by OH-TCI.

The two Kunitz BPTI inhibitors from king cobra venom showed highly homology. However, OH-TCI gained new trypsin inhibitory activity and more stronger chymotrypsin inhibitory activity with only several residuals variation. This gives the support that all the Kunitz BPTI homologues came from a common ancestor \[37\] and developed their diversity functions by accumulated variations.

**Acknowledgements**

This work was supported by grants from Chinese National Natural Science Foundation (30630014, 30570359 and 30670412) and grants of “Key Research Direction-KSCX2-YW-R-088”, “Western Light” Projects from the Chinese Academy of Sciences.

**REFERENCES**


[12] Hokama Y, Iwanaga S, Tatsuki T, Suzuki T. Snake venom proteinase inhibitors. III. Isolation of five polypeptide inhibitors from the venoms of Hemachatus haemachatus (Ringhals’ cobra) and Naja nivea (Cape cobra) and the complete amino acid sequences of two of them. J Biochem (Tokyo) 1976;79:559–78.


[17] Lazdunski M. Calcicludine, a venom peptide of the Kunitz-type protease inhibitor family, is a potent blocker of high-threshold Ca2+ channels with a high affinity for L-type channels in cerebellar granule neurons. Proc Natl Acad Sci USA 1994;91:878–82.


