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Purification, characterization and molecular cloning of chymotrypsin inhibitor peptides from the venom of Burmese *Daboia russelii siamensis*

Chun-teng Guo[a,b], Stephen McClean[a,*], Chris Shaw[c], Ping-fan Rao[b], Ming-yu Ye[b], Anthony J. Bjourson[a]

[a] School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, UK
[b] The Institute of Biotechnology, Fuzhou University, Fuzhou 350002, Fujian Province, People's Republic of China
[c] School of Pharmacy, Queen's University, Belfast BT9 7BL, Northern Ireland, UK

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ABSTRACT

One novel Kunitz BPTI-like peptide designated as BBPTI-1, with chymotrypsin inhibitory activity was identified from the venom of Burmese *Daboia russelii siamensis*. It was purified by three steps of chromatography including gel filtration, cation exchange and reversed phase. A partial N-terminal sequence of BBPTI-1, HDRPKFCYLPADPGECLAHMRSF was obtained by automated Edman degradation and a Ki value of 4.77 nM determined. Cloning of BBPTI-1 including the open reading frame and 3′ untranslated region was achieved from cDNA libraries derived from lyophilized venom using a 3′ RACE strategy. In addition a cDNA sequence, designated as BBPTI-5, was also obtained. Alignment of cDNA sequences showed that BBPTI-5 exhibited an identical sequence to BBPTI-1 cDNA except for an eight nucleotide deletion in the open reading frame. Gene variations that represented deletions in the BBPTI-5 cDNA resulted in a novel protease inhibitor analog. Amino acid sequence alignment revealed that deduced peptides derived from cloning of their respective precursor cDNAs from libraries showed high similarity and homology with other Kunitz BPTI proteinase inhibitors. BBPTI-1 and BBPTI-5 consist of 60 and 66 amino acid residues respectively, including six conserved cysteine residues. As these peptides have been reported to have influence on the processes of coagulation, fibrinolysis and inflammation, their potential application in biomedical contexts warrants further investigation.

1. Introduction

Protease inhibitors are part of the composition of Russell's viper venom, in which they exhibit varying concentrations depending on their geographical distribution [21]. Trypsin and chymotrypsin inhibitors from snake venoms are members of the functionally diverse BPTI-like (bovine pancreatic trypsin inhibitor) superfamily. The snake venom Kunitz/BPTI inhibitors are basic polypeptides with approximately 60 amino acids in which there are six cysteine residues forming three conserved disulfide bridges. They exhibit diverse functions for inhibiting serine proteinase enzymes. This superfamily is classified into two families based on protein structure; small Kunitz-type inhibitors and BPTI-like toxins and soft tick anticoagulant proteins [5]. The Kunitz BPTI proteinase inhibitor family is divisible into subgroups according to source and bioactivity. Snake Kunitz/BPTI inhibitors have been divided into non-neurotoxic and neurotoxic groups according to their functions [2]. The non-neurotoxic snake Kunitz/BPTI inhibitors include trypsin and chymotrypsin inhibitors. Another group acting as K^+ and Ca^{2+} channel blockers with little or no inhibitory activity is the neurotoxic snake Kunitz/BPTI. This group includes α-dentrotoxin, toxin K, toxin I, calcicludine, and the small subunit of β-bungarotoxin from various snake sources [11,14]. Moreover, a trypsin inhibitor occuring in a complex containing a phospholipase A2 (PLA2) and a protease has also been reported [22]. The characteristically folded domain like that of a BPTI-like peptides is also found in several proteins, for example a human Alzheimer amyloid precursor protein [17], type VI collagen alpha3 (VI) [27,41], tissue factor pathway inhibitor [1] and bikunin [38].

Although trypsin and chymotrypsin inhibitors are widely distributed in Russell's viper venom, to date only some inhibitors have been cloned. Peptides belonging to the Kunitz BPTI proteinase inhibitor family have very similar amino acid sequences, but display various functions. The present study describes the purification, properties, structure and cloning of chymotrypsin inhibitors from Burmese *Daboia russelii siamensis* venom.

2. Materials and methods

2.1. Materials

Burmese *D. russelii siamensis* venom was purchased from Xinyuan Jiayu management department for snake venoms (Guangzhou, China), an agency of Rainbow Snake Farm (Yingtan

* Corresponding author. Tel.: +44 2870124406; fax: +44 2870 124965.
E-mail address: s.mcclean@ulster.ac.uk (S. McClean).

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City, Jiangxi Province, China). A Superdex™ Peptide 10/300 GL column was purchased from Pharmacia (Uppsala, Sweden). POROS 50HS gel was bought from Perkin Elmer (Wellesley, MA, USA). A Jupiter C_{18} column was purchased from Phenomenex Inc. (Torrance, CA, USA). Benzoyl-D, L-4-arginine-*p*-nitroanilide (BAPNA) was obtained from Shanghai ShuiYuan Biotechnology Co. Ltd. (Shanghai, China). *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was bought from Sigma (MO, USA). Bovine trypsin and chymotrypsin were bought from Shanghai Sheng Gong Biological Engineering and Technology Service Co. Ltd. (Shanghai, China) and Amesco (Carson, CA, USA), respectively. A Micro BCA™ Protein Assay Kit was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). A pGEM-T Easy vector and PCR Rapid Purification Kit were purchased from Promega (Madison, WI, USA) while a DNA Sequencing Kit (BigDye Terminator v3.1) was obtained from Applied Biosystems (Foster City, CA, USA). All reagents used were of analytical grade.

2.2. Isolation and purification

2.2.1. Pretreatment of crude venom by heating

One hundred milligrams of Burmese *D. russelii 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. The supernatant was prepared by centrifugation at 10,000 × *g* for 10 min. The precipitate was washed twice using 0.4/99.6 (v/v) TFA/water, then the supernatant was pooled and concentrated using a Christ Alpha rotating vacuum concentration centrifuge (Martin Christ, Osterode am Harz, German).

2.2.2. Superdex™ peptide 10/300 GL column chromatography

The concentrated supernatant was applied to a Superdex™ peptide 10/300 GL column (1.5 cm × 30.0 cm) equilibrated with 0.02 M PBS buffer, pH 7.0, containing 0.25 M NaCl at a flow rate of 0.6 ml/min. The chromatography was conducted using a Beckman Gold HPLC system (Beckman Instruments, Brea, CA, USA) at room temperature, the eluate monitored at 214 nm using a Beckman 166 UV detector and fractions collected.

2.2.3. POROS 50HS column chromatography

The fraction containing chymotrypsin inhibitor activity from gel filtration chromatography was collected and diluted four-fold (v/v) with 0.02 M phosphate buffer, pH 7.0. High performance liquid chromatography (HPLC) of the diluted fractions was performed using a self-packed POROS 50HS column (0.6 cm × 15.0 cm). The column 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 0.35 M in the equilibrium buffer for 140 min and 0.35–0.8 M NaCl for 100 min. The chromatography was conducted using a CCPD 8000 HPLC system from Tosoh Co. (Tokyo, Japan) at room temperature, the eluate was monitored at 214 nm with a Beckman 166 UV detector and fractions were collected.

2.2.4. Purification of BBPTI-1 on reverse phase C-18 column

The fractions with inhibitory activity against chymotrypsin from the POROS 50HS column chromatography separation were loaded onto a Jupiter C-18 column (0.46 cm × 15 cm) equilibrated with 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water at a flow rate of 0.5 ml/min. The absorbed components were eluted with a linear gradient from 0.05/79.95/20 (v/v/v) TFA/water/80% acetonitrile to 0.05/65.45/34.5 (v/v/v) TFA/water/80% acetonitrile for 155 min. The chymotrypsin inhibitors were concentrated by using a ZFQ 85A rotating vacuum concentrator (Shanghai Medical Machine Ltd., China) and a Christ RVC rotating vacuum evaporator (Martin Christ, Osterode am Harz, Germany) for further characterization. POROS 50HS column chromatography was then performed as described above.

2.3. Characterization of BBPTI-1

2.3.1. Mass spectrometry

MALDI-TOF MS of positive ions of trypsin inhibitors was carried out using a Voyager DE mass spectrometer (Voyager, Applied Biosystems, Warrington, UK) operated in linear mode. Alpha-cyano-4-hydroxycinnamic acid (Sigma, UK) (10 mg/ml) was prepared in a solution containing 0.1/49.9/50 TFA/water/acetonitrile. Calibration took place using a mixture of peptides of known mass (Laser Biolabs, Sophia-Antipolis, Cedex, France).

2.3.2. Determination of peptide concentration

A Micro BCA™ Protein Assay Kit (Pierce, USA) was used for determination of peptide concentrations using BSA (supplied with the kit) as the standard according to the manufacturer's protocol.

2.3.3. Amino acid sequence from automated Edman degradation

Sequence determination of purified peptide was performed by Edman degradation using a protein sequencer (Applied Biosystems Model 476A, Perkin Elmer, MA, USA). The primary structure was obtained by comparing the elution position of standard PTH-AAs.

2.3.4. Determination of chymotrypsin inhibitory activity and chymotrypsin inhibition constants (K_i)

Chymotrypsin inhibitor activities were measured according to the Mikola and Mikkonen method [28] with modifications. Assays were performed by adding together 30 μl of 5.0 μg/ml chymotrypsin solution (Amesco, USA), 10.0 μl of sample solution, 300.0 μl of 0.2 M Tris–HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ and 210.0 μl of distilled water. After the mixture was preincubated at 37 °C for 20 min in a water bath, 50 μl of preheated substrate (1.5 mg/ml), *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma, USA) were added to the assay solution and the mixture was further incubated at 37 °C for 5 min. The enzymatic reaction was terminated by the addition of 60.0 μl of 30% acetic acid. Distilled water was used as the control. The mixture was centrifuged at 10,000 × *g* for 5 min, and its absorbance read at 405 nm.

For the determination of the chymotrypsin inhibitor K_i value, an appropriate amount of chymotrypsin was incubated with two concentrations of substrate. Briefly, the mixture contained 50 μl of bovine chymotrypsin (5.0 μg/ml), varying volumes of inhibitor also at a concentration of 5.0 μg/ml, 300.0 μl of 0.2 M Tris–HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ and an appropriate amount of distilled water to provide a total volume of 550.0 μl. After the mixture was incubated at 37 °C for 20 min in a water bath, 50.0 μl of preheated substrate (1.2 mg/ml or 0.6 mg/ml) was added to the assay solution for a further incubation of 5 min. The enzymatic reaction was terminated by the addition of 60.0 μl of 30% acetic acid. References were obtained by adding acetic acid before the substrate was added to mixture. After the mixture was centrifuged at 10,000 × *g* for 5 min, its absorbance was read at 405 nm. A Dixon plot [10] of $1/v$ *versus* I inhibitor concentration at two substrate concentrations was performed to estimate the inhibition constant and to determine if any activity was competitive or non-competitive. In such plots non-competitive activity is observed by lines converging on the *x* axis while for competitive activity lines converge above the *x* axis.

2.3.5. Detection of trypsin inhibition activity

Trypsin inhibitor activity was measured according to the Mikola and Mikkonen method [28] with modifications. Assays were performed by adding 50 μl of trypsin solution (5.0 μg/ml), varying concentrations of inhibitor, 300.0 μl of 0.2 M Tris–HCl buffer (pH