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Research paper

A small trypsin inhibitor from the frog of *Odorrana grahami*

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Abstract

A novel peptide inhibitor (OGTI) of serine protease with a molecular weight of 1949.8, was purified from the skin secretion of the frog, *Odorrana grahami*. Of the tested serine proteases, OGTI only inhibited the hydrolysis activity of trypsin on synthetic chromogenic substrate. This precursor deduced from the cDNA sequence is composed of 70 amino acid residues. The mature OGTI contains 17 amino acid residues including a six-residue loop disulfided by two half-cysteines (AVNIPFKVHFRCKAAFC). In addition to its unique six-residue loop, the overall structure and precursor of OGTI are different from those of other serine protease inhibitors. It is also one of the smallest serine protease inhibitors ever found.
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Keywords: Serine protease inhibitor; Amphibian; *Odorrana grahami*

1. Introduction

Serine protease inhibitors with molecular weights larger than 5 kDa are found widely in animals, plants and microorganisms [1,2]. Many pathogens are known to produce extracellular proteases, and recent findings suggest that proteases play an active role in the development of diseases [3]. Various lines of evidence suggest that a major function of protease inhibitors would be to combat the proteases of pests and pathogens [3]. A few of small peptide inhibitors have been found in plants such as SFTI-1 composed of 14 amino acid residues from sunflower seeds [4,5], three macrocyclic trypsin inhibitors composed of 34 amino acid residues from squash seeds [6] and marionstation composed of 14 amino acid residues from marine bacterium [6,7]. SFTI-1 is a cyclic peptide containing a single disulfide bridge [4,5] and squash macrocyclic trypsin inhibitors contain 3 disulfide bridges [6]. Marionstation is a linear peptide without a disulfide bridge in its primary structure [7]. SFTI-1 belongs to the BBI family of protease inhibitors because of its disulfide-linked nine-residue loop that adopts the characteristic canonical conformation formed from a short type VIb β-turn [4,5,8]. The canonical nine-residue loop of Bowman–Birk inhibitors (BBIs)/SFTI-1 provides an ideal template for drug design of specific inhibitors to target their respective proteases [9]. The inhibitor SFTI-1 is the focus of the current study. It is the smallest known cyclic plant peptide. Compared with the inhibitors found in plants, no such a small

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peptide inhibitor has been found from animals. All protease inhibitors found in animals have a molecular weight larger than 5 kDa and contain at least two disulfide bridges.

In this report, a small peptidic trypsin inhibitor composed of a 17 aa-containing single disulfide bridge was purified from the skin secretions of the frog of *O. grahami*. It has a different structure characteristic from other serine proteases, and this is one of the smallest serine protease inhibitor ever found.

2. Materials and methods

2.1. Collection of frog skin secretions

Adult specimens of *O. grahami* of both sexes ($n = 30$; weight range 30–40 g) were collected in Yunnan Province of China. Skin secretions were collected as follows: frogs were put into a cylinder container containing a piece of absorbent cotton saturated with anhydrous ether. Following exposure to anhydrous ether for 1–2 min, the frog skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each frog with a 0.1 M NaCl solution (containing 0.01 M EDTA). The collected solutions (500 ml total volume) were quickly centrifuged and the supernatants were lyophilized.

2.2. Peptide purification

Lyophilized skin secretion sample of *O. grahami* (3.5 g, total $OD_{280\ nm}$ of 1000) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0 as previously reported [10]. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the elution was monitored at 280 nm. The trypsin inhibitory activities of fractions were determined as indicated below. The protein peak containing trypsin inhibitory activity was pooled (30 ml) lyophilized, and re-suspended in 2 ml 0.1 M phosphate buffer solution, pH 6.0, and purified further by C_{18} reverse phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18}, 30 × 0.46 cm) column as illustrated in Fig. 1A,B.

2.3. Amino acid sequencing and mass spectrometry analysis

Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Fast atom bombardment (FAB) mass spectrometry was carried out on an Autospec-3000 spectrometer, equipped with a high field magnet, using glycerol:3-nitrobenzyl alcohol:dimethyl sulphoxide (1:1:1, v:v:v) as mixed matrix. The ion gun was operated at 25 kV with a current of 1 μA, using Cs^+ as the bombarding gas.

2.4. Serine protease inhibition assays

The inhibition effects on the hydrolysis of synthetic chromogenic substrates by serine proteases were assayed in 50 mM Tris–HCl, pH 7.8 (for trypsin, Sigma T4665; thrombin, Sigma T4393; chymotrypsin, Sigma C4129; elastase, Sigma E1250; plasmin, Sigma P1867) or pH 8.45 (for substilisin, Sigma P5380) buffer at room temperature. The protease (final concentrations 10 nM for trypsin and thrombin, 21 nM and 23 nM for chymotrypsin and elastase, 30 nM for substilisin, respectively) and different amounts of the inhibitor (final concentrations ranging from 0.01 to 10 μM) were preincubated for 10 min at room temperature. S-2238 (H-D-Phe-Pip-Arg-pNA, Kabi Vitrum, Stockholm, Sweden) was used as a substrate for trypsin and thrombin, S-2251 (Val-Leu-Lys-pNA, Kabi Vitrum, Stockholm, Sweden) for plasmin, B-3133 (*N*-Benzoyl-Arg-4-Nitroanilide-hydrochride-pNA, Sigma) for elastase and C-3022 (*N*-CBZ-Gly-Gly-Leu-pNA, Sigma) for substilisin. The reaction was initiated by the addition of the substrate with a final concentration of 0.5 mM. The formation of *p*-nitroaniline was monitored continuously at 405 or 410 nm (for C-3022) for 2 min. In the case of chymotrypsin, BTEE (*N*-benzoyl-tyrosine ethyl ester, Sigma) was used as the substrate and the reaction was monitored continuously at 253 nm for 2 min. MCA (pyrArg-Thr-Lys-Arg-7-amino-4-methylcoumarin, Bachem Bioscience, San Diego, CA, USA) was used for the furin activity assay as described by Han et al. [11]. The samples were preincubated with a fixed amount of furin (Sigma, F2677-50UN) (2×10^{-3} units) at 37 °C in 100 mM Hepes buffer, pH 7.5, containing 1 mM $CaCl_2$ for 5 min, the residual enzyme activity was then measured. The final substrate concentration was 1 μM. The fluorescence of the released MCA was measured on-line with a Perkin Elmer luminescence spectrometer (LS50B). The excitation and emission wavelength are 370 nm (slit width, 10 nm) and 460 nm (slit width, 10 nm), respectively. The inhibition constant K_i of OGTI was determined according to the method of Dixon (1953) [12]. The thermal stability of the inhibitor was also tested by assaying a sample that had been incubated at 100 °C for 3 min. In order to determine if OGTI acts as a substrate of trypsin, OGTI was incubated with trypsin for 2 h before adding the substrate. The hydrolyzed products were purified and analyzed by Edman degradation.

2.5. SMART cDNA synthesis

Total RNA was extracted using TRIzol (Life Technologies, Ltd.) from the skin of single sample of *O. grahami*. CDNA was synthesized by SMART™ techniques by using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The first strand was synthesized by using cDNA 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1N-3′ (N = A, C, G or T; N-1 = A, G or C), and SMART II An oligonucleotide, 5′-AAGCAGTGGTATCAAC GCAGAGTACGCGGG-3′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′.

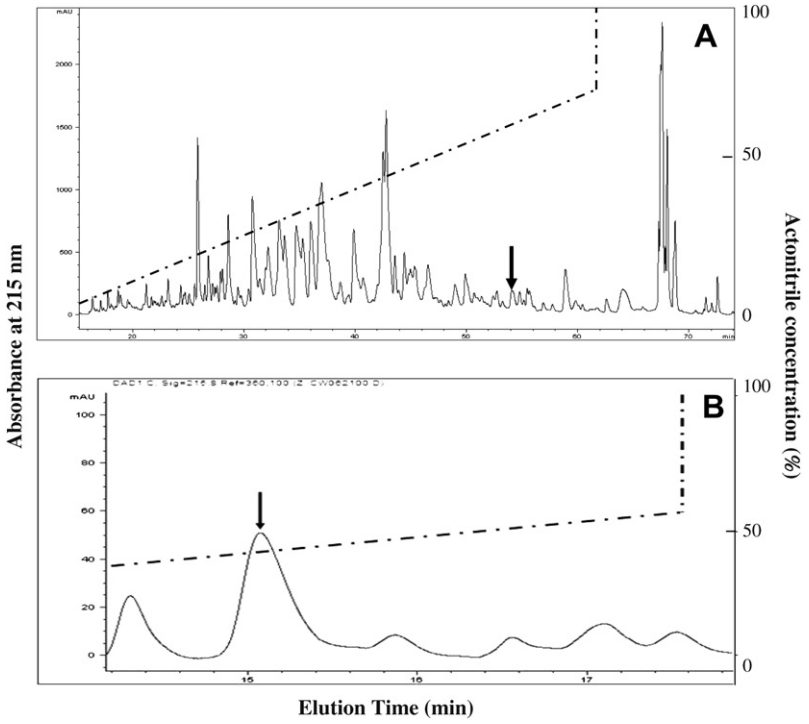

Fig. 1. Purification of OGTI from *O. grahami* skin secretions. The peak VI with trypsin inhibitory activity from Sephadex G-50 [10] was further purified on a Hypersil BDS C_{18} RP-HPLC column (30 × 0.5 cm) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The elution was performed with the indicated gradient of acetonitrile in (A) at a flow rate of 0.7 ml/min, and fractions were tested for trypsin inhibitory activities. The fraction with trypsin inhibitory activity indicated by an arrow in (A) was further purified by RP-HPLC with the indicated gradient of acetonitrile in (B) at a flow rate of 0.7 ml/min. The purified trypsin inhibitor was indicated by an arrow in (B).

2.6. Screening of cDNA encoding serine protease inhibitor

The cDNA synthesized by SMART™ techniques was used as template for PCR to screen the cDNAs encoding serine protease inhibitor. Two oligonucleotide primers, S_1 5′-(G/A)CA (G/A)AA(A/T/C/G)GC(A/T/C/G)GC(C/T)TT(G/A)CA(A/G/C/T)CC (G/T)(A/G)AA-3′, in the reverse direction, a specific primer designed according to the amino acid sequence determined by Edman degradation and primer II A as mentioned in "*SMART cDNA synthesis*" in the sense direction were used in PCR reactions. The DNA polymerase was Advantage polymerase from Clontech (Palo Alto, CA) The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

2.7. Circular dichroism (CD) spectroscopy

CD data were acquired with a Jasco J-810 CD spectrophotometer using a 0.2 mm path length cylindrical cuvette. The response was measured using wavelengths from 190 to 250 nm with a 0.2 nm step resolution and a 1 nm bandwidth. The rate of 100 nm/min and a response time of 0.25 s were used, and the spectra were averaged over eight scans. Spectra were recorded at a peptide concentration of 70 μM in three different environments: water, 5 mM Tris–HCl (pH 8.0), and 5 mM Tris–HCl containing 160 μM SDS (pH 8.0). The experimental temperature was 25 °C. In each case, the circular dichroism spectrum of the solvent was subtracted from the spectrum of the peptide.

2.8. Synthetic peptides

The peptide OGTI (AVNIPFKVHFRCKAAFC) and OGTI analog (AVNIPFKVHFRCA^{13}AAFC, replace Lys13 by Ala) used for the assay bioactivities and CD analysis in this paper was synthesized by AC SCIENTIFIC (Xi An) INC. (Xi An, China) and analyzed by HPLC and MALDI-TOF mass spectrometry to confirmed purity higher than 95%. All peptides were dissolved in water.

3. Results and discussion

3.1. Purification and characterization of serine protease inhibitor OGTI

The supernatant of *O. grahami* skin secretions was fractionated into several peaks by Sephadex G-50 as our previous report [10]. The inhibitory activity on trypsin was concentrated on the peak VI. Peak VI was collected and further fractionated by RP-HPLC. More than 50 peaks were obtained from this separation as indicated in Fig. 1A. The peak with trypsin inhibitory activity (marked by an arrow) was collected and

purified further by RP-HPLC. The purified trypsin inhibitor was indicated by an arrow in Fig. 1B.

The amino acid sequence of the purified serine protease inhibitor named OGTI was completely determined by Edman degradation. It is composed of 17 amino acid residues. The amino acid sequence is AVNIPFKVHFRCKAAFC. Its molecular weight analyzed by FAB-MS is 1949.8 that matched well with the theoretical molecular weight for disulfided OGTI of 1949.4. This result suggested OGTI likely contains intramolecular disulfide-linked bridge. The synthetic OGTI containing an intramolecular disulfide-linked bridge exerted the same trypsin inhibitory activity as the native OGTI. In our current experiments, there is no other trypsin inhibitor other than OGTI in the fraction VI.

3.2. Trypsin inhibition of OGTI

The inhibitory effects of OGTI on the hydrolysis of synthetic chromogenic substrates catalyzed by serine proteases as trypsin, thrombin, chymotrypsin, elastase and substilisin, were investigated. The amidolytic activity of trypsin was blocked by OGTI. The K_i value was determined to be 0.4 μM under the assay conditions. No inhibition of the hydrolysis of S-2238 by thrombin, BTEE by chymotrypsin, B-3133 by elastase, C-3022 by substilisin, S-2251 for plasmin, or MCA for furin could be observed, even with an inhibitor concentration up to 10 μM. After incubation in boiling water for 3 min, the trypsin inhibitory activity of OGTI was not lost, revealing that OGTI has high thermal stability.

OGTI was incubated with trypsin for 2 h. The hydrolyzed products were purified and analyzed by Edman degradation. Three peptide fragments were found from the hydrolyzed products. There are ANVIPFK, VHFR, and AAFC. It proved that the peptide bonds of Lys7–Val8, Arg11–Cys12, and Lys13–Ala14 could be cleaved by trypsin during long time incubation with the inhibitor (2 h). The synthesized OGTI analog (AVNIPFKVHFRCA^{13}AAFC, replace Lys13 by Ala) did not show trypsin-inhibitory activity, suggesting that the reactive site of this inhibitor is most likely located within the loop, namely, being Lys13–Ala14.

3.3. cDNA cloning of OGTI

Upon screening of a skin cDNA library, about 50 clones containing inserts of around 300 base pairs, were identified and isolated. Both strands of these clones were sequenced. One of the cDNA encoding the precursor of OGTI has a length of 303 base pairs as shown in Fig. 2A. It encodes a precursor containing 70 amino acids including a predicted signal peptide composed of 22 amino acid residues, an acidic spacer peptide containing multiple acidic amino acid resides, and a mature OGTI composed of 17 amino acid residues. A dibasic Arg-Lys motif is located at the C-terminal of OGTI propeptide. It is a processing site for propeptidase. A BLAST search revealed no similarity to any other sequence in the database. Total 50 positive clones encode five different OGTI precursors as shown in Fig. 2B. All the five precursors contain 70 amino acid residues while the precursor of SFTI-1, another serine protease inhibitor with single disulfide-linked bridge, has 56 amino acid residues. The five OGTI precursors are highly conserved. The amino acid replacement only occurred in three sites located in the predicted signal regions while other regions including the acidic peptide and mature OGTI are identical (Fig. 2B). The precursor sequences of OGTI and SFTI-1 are compared as in Fig. 2B. The overall structures of these precursors are different.

The superfamily of serine protease inhibitors are involved in a number of fundamental biological processes such as blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation and tumor suppression [13–16]. They have received considerable attention not only as helpful study tools but also for clinical use in human medicine [2]. Recently,

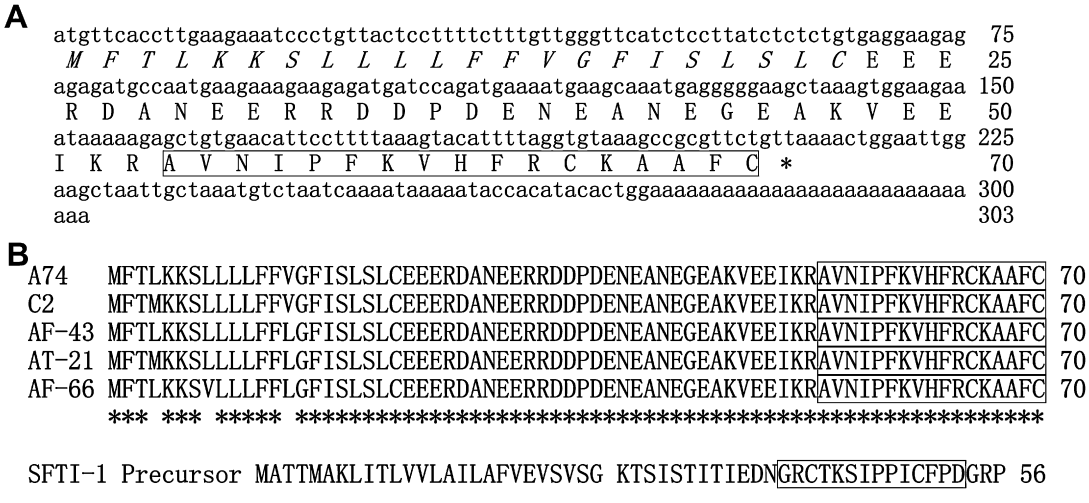

Fig. 2. (A) The nucleotide sequences encoding OGTI from *O. grahami* skin and the deduced amino acid sequence of the precursor polypeptide. The signal peptide is italicized, and the sequence of mature OGTI is boxed. The star (*) indicates stop condon; (B) peptide alignments of the precursors of OGTI and SFTI-1. The clone names are marked on the left. The sequences of mature OGTI and SFTI-1 are boxed. The SFTI-1 is from ref. [5]. The star (*) indicates the identical amino acid residues.

several small serine protease inhibitors with molecular weights less than 5 kDa have been identified from plants and microorganisms [4–6], respectively. Among these small serine protease inhibitors, SFTI-1 is the most potent known naturally occurring Bowman–Birk inhibitor. It has recently attracted much attention due to its small size and cyclized-backbone [4,5]. SFTI-1 is also the first serine protease inhibitor known to contain a single disulfide-linked bridge. No similar small serine protease inhibitors have been found in animals. During the past several decades, numerous studies have been focused on the bioactive compounds present in amphibian skin secretions [17,18]. A large amount of bioactive peptides are found in skin secretions of amphibians including antimicrobial peptides, bombesin-like peptides, bradykinin-like peptides etc. [19–22]. Several serine ptoteinase inhibitors with molecular weights of 60–80 kDa have also been identified from amphibian skins [23–25]. All the known serine protease inhibitors from amphibians contain 3–5 conserved disulfide bridges. In this paper, OGTI, a trypsin inhibitor composed of 17 amino acid residues, is identified and cloned from the skin secretions of the frog, *O. grahami*. The unique loop structure suggests that OGTI is a novel type of serine protease inhibitor. In addition, the overall structures of the predicted signal peptide, pro-peptide, possible processing sites and mature peptides of OGTI and SFTI-1 precursors are different (Fig. 2B). This suggests that OGTI and SFTI-1 belong to different serine protease inhibitor families although both contain a single disulfide-linked loop. The disulfide-linked nine-residue loop (CTKSIPPIC) of SFTI-1 has received considerable attention for design clinical medicines [4,5]. Compared with the nine-residue loop (CTKSIPPIC) of SFTI-1, the six-residue loop (CKAAFC) of OGTI may provide a novel and simple template for use in the design of clinical medicines.

3.4. Secondary structure analysis of OGTI

The conformation distribution of OGTI molecule was analyzed. Circular dichroism (CD) spectroscopy was used to study secondary structure of OGTI (Fig. 3). The patterns of CD in both water and Tris–HCl were almost same. They have no characteristic negative absorption at the 210 and 225 nm, suggesting that this peptide has no α-helix in these two kinds of solutions. The curve exhibits only the negative absorption from 190 to 250 nm, and has a maximum absorption at 203 nm, showing that the conformation distribution is random (94.8%).

ORB2K with an amino acid sequence of LKGCWTK SIPPKPCFGK, a SFTI-1-like trypsin inhibitor, has also been identified and characterized from the skin secretions of *O. gramhami*. The crystal structures of complex ORB2K-trypsin indicated that ORB2K has an anti-parallel β-sheet conformation and binds to trypsin by an internal hydrogen network. The side chain of Lys(P1) residue of ORB2K projects into the S1 pocket of trypsin, and directly interacts with the carboxyl group of Asp189, the main-chain carbonyl group of Ser190, and two water molecules. This Lys(P1) determines the specificity of the inhibitor for trypsin [10]. OGTI has

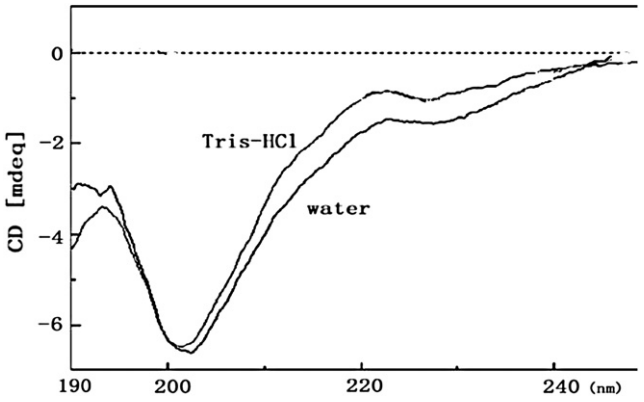

Fig. 3. The CD spectrum of OGTI in different solutions. A total of 70 μM OGTI was resolved in 5 mM Tris–HCl, pH 8.0 or water.

a six-residue loop (CKAAFC) that is different from the nine-residue loop (CTKSIPPIC) in SFTI-1 and the 11-residue loop (CWTKSIPPKPC) in ORB2K. In SFTI-1 and ORB2K, the Lys(P1) residues are located at the N-terminal part of these loops [4,5,10]. In OGTI, we also found that the lysine residue in the loop is likely the Lys(P1) residue.

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