Comparative Biochemistry and Physiology, Part B 153 (2009) 116–120

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

A novel serine protease inhibitor from the venom of *Vespa bicolor* Fabricius

Xinbo Yang [a,b,1], Yakun Wang [a,1], Zekuan Lu [a], Lei Zhai [a], Juguo Jiang [c], Jingze Liu [a,*], Haining Yu [a,*]

[a] College of Life Sciences, Hebei Normal University, Shijiazhuang 050016, Hebei, China
[b] Biochemistry & Biology College, Yantai University, Yantai 264005, Shandong; China
[c] Shandong Shengli Co., Ltd., 2238 Tianchen Street, Jinan 250101, Shandong, China

ARTICLE INFO

Article history:
Received 20 December 2008
Received in revised form 6 February 2009
Accepted 16 February 2009
Available online 1 March 2009

Keywords:
Bioactive peptides
Serine protease inhibitor
Vespa bicolor
Wasp venom

ABSTRACT

Hornets possess highly toxic venoms, which are rich in toxin, enzymes and biologically active peptides. Many bioactive substances have been identified from wasp venoms but only a few serine protease inhibitors have been identified from two kinds of wasp venoms. In this work, a serine protease inhibitor named bicolin was purified and characterized from the venom of the wasp, *Vespa bicolor* Fabricius. The precursor encoding bicolin was cloned from the cDNA library of the venomous glands. It is a cysteine-rich small protein containing 54 amino acid residues including 6 half-cysteines. The peptide is homologous to serine protease inhibitors isolated from venoms of *Anoplius samariensis* and *Pimpla hypochondriaca*. Bicolin showed inhibitory ability against trypsin and thrombin.

1. Introduction

The venoms of arthropods have attracted considerable interest as a potential source of pharmacological substances (Habermann, 1972; Hirai et al., 1979a,b; Nakajima, 1984). Wasp venom gland is biochemically, pharmacologically, and physiologically complex organ which fulfills a wide range of functions necessary for wasp survival, including predatoriness, defense, etc (Han et al., 2008; Yang et al., 2008). Over the past several decades, studies have focused on the bioactive compounds present in wasp venoms (Yasuhara et al., 1983; Argiolas and Pisano, 1984, 1985; Yu et al., 2007). These compounds include amines, small peptides, and proteins of high molecular mass such as enzymes, allergens and toxins (Konno et al., 2000; Mendes et al., 2004; Mendes et al., 2005; Xu et al., 2006a,b; Zhou et al., 2006). Only three serine protease inhibitors (peptide toxin As-fr-19, cysteine-rich venom protein 2 (CVP2) and CVP4) are identified from two kinds of wasp venoms (Parkinson et al., 2004; Hisada et al., 2005). *Vespa bicolor* is one of the most dangerous species of vespine wasps, found in most provinces of China. It is a kind of social wasp. This hornet is aggressive and predatory. Stings by this hornet generally produce severe pain, local damage, cardiovascular system disorder and occasionally death in large vertebrates including man (Evans and Summers, 1986; Sakhuja et al., 1988; Korman et al., 1990; Watemberg et al., 1995; Chao and Lee, 1999; Chen et al., 2004). Little was reported on the chemical constituents of *V. bicolor* venom. Herein, we reported purification, characterization and biological activities of a serine protease inhibitor from *V. bicolor* venom.

2. Materials and methods

2.1. Wasp venom

The wasps of *V. bicolor* were collected in Hebei province of China. The collected wasps were stimulated by alternative current (6 V) lasting for 6–10 s. The wasp venom was secreted onto a clean glass plate (50×50 cm), immediately collected and stored at −20 °C.

2.2. Peptide purification

The lyophilized wasp venom sample (0.1 g) was dissolved in 5 mL 0.1 M phosphate buffer solution, pH 6.0, and filtered through a 10-kDa cut-off Centriprep filter (Millipore, Bedford, CA). The yield of this filtration was about 30%. The filtrate was next lyophilized. Lyophilized filtrate was applied to a 5×250-mm Vydac C_{18} RP-HPLC (reversed-phase high-performance liquid chromatography) column (Sigma) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. Elution (0.7 mL/min) with collecting fraction of 3 mL was performed using 0.1% (v/v) TFA/water over 10 min, followed by a linear gradient of 0%–80% acetonitrile containing 0.1% (v/v) TFA in 0.1% (v/v) TFA /water over 100 min, and final elution with 80% acetonitrile containing 0.1% (v/v) TFA. The absorbance at 220 nm was monitored. UV-absorbing peaks were collected and lyophilized, and their effects on serine proteases were detected.

2.3. Structural analysis

Peptide sample (1 mg) was first dissolved in 1 mL 0.1 M Tris–HCl buffer (pH 8.6) containing 6 M ultra-pure urea and 0.02 M 2-mercaptoethanol. Following flushing with nitrogen, 5 mg of iodoacetic acid was

* Corresponding authors. Tel.: +86 311 86268842; fax: +86 311 86268313.
E-mail addresses: jzliu21@heinfo.net (J. Liu), yuhaining@mail.hebtu.edu.cn (H. Yu).
[1] These authors contributed equally to this paper.

1096-4959/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2009.02.010

added into the reaction mixture while maintaining the pH at 8.6 via the addition of 0.1 M NaOH and incubation for another 3 h. Finally, carboxymethylated (CM) peptide was desalted on a Vydac C8 RP-HPLC column (200×4.6 mm) and lyophilized. N-terminal amino acid sequence was directly determined by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Mass measurement was performed on a Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry instrument (Bruker Reflex), using α-cyano-4-hydroxycinnamic acid (HCCA) suspended in 100% acetonitrile as matrix.

2.4. SMART cDNA synthesis

Total RNA was extracted using TRIzol (Life Technologies, Ltd.) from the 0.1 g venomous glands of *V. bicolor*. 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′-AAGCAGTGGTATCAACGCAGAG-TACT (30) N-1 N-3′ (N = A, C, G or T; N-1 = A, G or C), and SMART II A oligonucleotide, 5′-AAGCAGTGGTATCAAC GCAGAGTACGCGGG-3′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT- 3′. The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 62 °C, 1 min at 72 °C.

2.5. Screening of cDNA encoding bicolin

The cDNA synthesized by SMART™ techniques was used as template for PCR to screen the cDNAs encoding bicolin. Two pairs of oligonucleotide primers were used to screen the cDNA encoding biocolin. One pair of them is S1 (5′-gc(A/t/C/G)ca(t/C)cc(A/T/C/G)(C/T) T(A/T/C/G)TG(C/T)(C/T)T(A/T/C/G) (C/T)T(A/T/C/G)GA(C/T)-3′ based on the mature peptides of bicolin in the sense direction) and primer II A as mentioned in "SMART cDNA synthesis" in the antisense direction were used in PCR reactions. Another pair is S2 (5′ (G/A)TC(A/T/C/G)A(A/G)(A/T/C/G)A(A/G)(A/G)CA(A/T/C/G)A(A/G)(A/T/C/G)GG (A/G)TG(A/t/C/G)CG-3′) and primer II A. 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.

2.6. Serine protease inhibitory assay

The inhibition effects of the sample on the hydrolysis of synthetic chromogenic substrates by serine proteases (All the serine proteases are from Sigma) were assayed in 50 mM Tris–HCl, pH 7.8 at 37 °C according to previous method (Lai et al., 2004). The protease (final concentrations 10 nM for trypsin and thrombin, 21 nM and 23 nM for chymotrypsin, and 20 nM for elastase, respectively) and the inhibitor (final concentration 0.5 μM) were pre-incubated for 10 min at 37 °C (Lai et al., 2004). S-2238 (H-D-Phe-Pip-Arg-pNA, Kabi Vitrum, Stockholm, Sweden) and B-3133 (N-Benzoyl-Arg-4-Nitroanilide-hydrochride-pNA, Sigma) were used as substrates for trypsin and thrombin, and elastase, respectively. The reaction was initiated by the addition of the substrate to a final concentration of 0.5 mM. The formation of p-nitroaniline was monitored continuously at 405 nm for 5 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 5 min. The effect of the inhibitor was estimated by setting the initial velocity as 100%, obtained with enzyme alone (without inhibitor). The inhibition assay was carried out as described above and the *K*i value was obtained by reciprocal plotting of the reaction velocity vs. inhibitor concentration under different chromogenic substrate concentrations (0–2 mM). The peptides were quantified by UV absorbance at 215 and 225 nm using the formula: concentration (mg/mL) = (A_{215} nm − A_{225} nm) × 0.144.

2.7. Recalcification time assay

Platelet-poor plasma (PPP) was prepared by centrifuging the citrated whole blood twice at 2500 g for 15 min at 4 °C and used within 4 h. For the recalcification time assay (RT), certain amount of sample in 0.1 mL 0.9% NaCl was added to 0.1 mL PPP at 37 °C, and the mixture was incubated at 37 °C for 5 min. The time from the addition of 0.1 mL of 25 mM $CaCl_2$ until the appearance of the first clot was recorded. The plasma aliquots incubated only with 0.1 mL of 0.9% NaCl were tested as controls (Xu et al., 2007).

3. Results

3.1. Purification of serine protease inhibitor peptide

As illustrated in Fig. 1, the components from venoms of *V. bicolor* were considerably complex. About 40 peaks were eluted from venoms by C18 RP-HPLC (Fig. 1). The peak indicated as B (Bicolin) in Fig. 1 was found to exert significant trypsin-inhibitory activity. Ten batches of the purified peptide peaks were pooled and studied further.

3.2. Structural analysis

Purified serine protease inhibitor indicated as B in Fig. 1 was named bicolin. It was subjected to amino acid sequence analysis by automated Edman degradation. Its amino acid sequence was `, and it molecular mass was 5749.4 Da analyzed by MALDI-TOF mass spectrometry (Fig. 2).

3.3. cDNA cloning

A cDNA clone containing an insert around 360-base pairs was identified and isolated (GenBank accession number FJ749250). Both strands of the clone were sequenced (Fig. 3). It was found to have an open reading frame that encodes a polypeptide composed of 77 amino acids including the predicted signal peptide and the mature bicolin

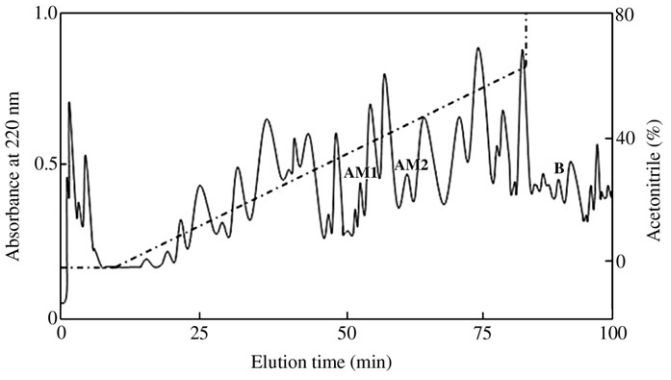

Fig. 1. Isolation of serine protease inhibitor from the venoms of *V. bicolor*. The lyophilized venom sample (0.1 g) was dissolved in 5 mL 0.1 M phosphate buffer solution, pH 6.0, and filtered through a 10-kDa cut-off Centriprep filter (Millipore, Bedford, CA) and the filtrate was and lyophilized. Lyophilized filtrate was applied to a Hypersil BDS C18 RP-HPLC column (5×250-mm) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The elution was performed with the indicated gradient of acetonitrile in Fig. 1 at a flow rate of 0.7 mL/min, and purified serine protease inhibitor was indicated by a "B". AM1 and AM2 are antimicrobial peptides (Chen et al., 2008).