Comparative Biochemistry and Physiology, Part C 201 (2017) 11–18

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Anti-fibrinolytic and anti-microbial activities of a serine protease inhibitor from honeybee (*Apis cerana*) venom

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ARTICLE INFO

Keywords:
Anti-fibrinolytic agent
Anti-microbial agent
Apis cerana
Bee
Serine protease inhibitor

ABSTRACT

Bee venom contains a variety of peptide constituents, including low-molecular-weight protease inhibitors. While the putative low-molecular-weight serine protease inhibitor Api m 6 containing a trypsin inhibitor-like cysteine-rich domain was identified from honeybee (*Apis mellifera*) venom, no anti-fibrinolytic or anti-microbial roles for this inhibitor have been elucidated. In this study, we identified an Asiatic honeybee (*A. cerana*) venom serine protease inhibitor (AcVSPI) that was shown to act as a microbial serine protease inhibitor and plasmin inhibitor. AcVSPI was found to consist of a trypsin inhibitor-like domain that displays ten cysteine residues. Interestingly, the AcVSPI peptide sequence exhibited high similarity to the putative low-molecular-weight serine protease inhibitor Api m 6, which suggests that AcVSPI is an allergen Api m 6-like peptide. Recombinant AcVSPI was expressed in baculovirus-infected insect cells, and it demonstrated inhibitory activity against trypsin, but not chymotrypsin. Additionally, AcVSPI has inhibitory effects against plasmin and microbial serine proteases; however, it does not have any detectable inhibitory effects on thrombin or elastase. Consistent with these inhibitory effects, AcVSPI inhibited the plasmin-mediated degradation of fibrin to fibrin degradation products. AcVSPI also bound to bacterial and fungal surfaces and exhibited anti-microbial activity against fungi as well as gram-positive and gram-negative bacteria. These findings demonstrate the anti-fibrinolytic and anti-microbial roles of AcVSPI as a serine protease inhibitor.

1. Introduction

Bee venom is a complex mixture of biologically, toxicologically, and pharmacologically active compounds, including polypeptides, enzymes, and low-molecular-weight substances (Son et al., 2007; Chen and Lariviere, 2010; Danneels et al., 2015). Even though bee venom contains various allergens and several nonallergenic low-molecular-weight substances, it has been used as an alternative medicine for the treatment of several diseases, such as arthritis, pain, and inflammation (Son et al., 2007; Chen and Lariviere, 2010), and has been an effective method for immunotherapy (Ozdemir et al., 2011). Currently, many peptide components from bee venoms have been shown to have various biological, toxicological, and pharmacological actions (Chen and Lariviere, 2010; Choo et al., 2010a, 2010b, 2012; Qiu et al., 2011, 2013; Kim et al., 2013a, 2013b; Park et al., 2014; Wan et al., 2014; Kim and Jin, 2015; Lee et al., 2015, 2016). Serine proteases, which show fibrinolytic/fibrinogenolytic activity (Choo et al., 2010a), and serine protease inhibitors, which exhibit anti-fibrinolytic activity (Choo et al., 2012; Qiu et al., 2013), have also been identified in bee venoms. The low-molecular-weight peptide secapin, a component of bee venom, functions as a serine protease inhibitor-like peptide that exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities (Lee et al., 2016). Furthermore, the biological actions of bee venom serine protease inhibitors have been characterized, including its anti-elastolytic, anti-fibrinolytic, and anti-microbial activities (Choo et al., 2012; Kim et al., 2013a, 2013b; Qiu et al., 2013; Wan et al., 2014; Lee et al., 2015).

Serine protease inhibitors are cysteine-rich peptides of 50–70 amino acid residues that show inhibitory activity against trypsin and/or chymotrypsin (Bania et al., 1999; Cierpicki et al., 2000; Choo et al., 2012; Kim et al., 2013a; Qiu et al., 2013; Lee et al., 2015). Among honeybee (*Apis mellifera*) venom components, allergen Api m 6 is a low-molecular-weight protease inhibitor that is comprised of a trypsin inhibitor-like cysteine-rich domain, which is a typical feature of serine protease inhibitors (Kettner et al., 2001; Krowarsch et al., 2003). Consequently,

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http://dx.doi.org/10.1016/j.cbpc.2017.09.001
Received 22 June 2017; Received in revised form 29 August 2017; Accepted 9 September 2017
Available online 14 September 2017
1532-0456/

molecular modeling of Api m 6 predicted its function as a putative serine protease inhibitor (Michel et al., 2012). Considering that bee venom serine protease inhibitors have various biological actions, including anti-fibrinolytic, anti-elastolytic, and anti-microbial activities, Api m 6 may also exhibit biological actions that are similar to a low-molecular-weight serine protease inhibitor; however, the anti-fibrinolytic and anti-microbial roles of Api m 6 have not been elucidated until now.

In this study, we report the identification of an Asiatic honeybee (*A. cerana*) venom serine protease inhibitor (AcVSPI) that exhibits high similarity to the putative low-molecular-weight serine protease inhibitor Api m 6, which contains a trypsin-like cysteine-rich domain (Kettner et al., 2001; Michel et al., 2012). We provide evidence that AcVSPI is an allergen Api m 6-like peptide that acts as a serine protease inhibitor. Importantly, we found that AcVSPI has an anti-fibrinolytic role as a plasmin inhibitor. We also found that AcVSPI has an anti-microbial role that exhibits anti-bacterial and anti-fungal activities.

2. Materials and methods

2.1. cDNA cloning and sequence analysis

The cDNA encoding AcVSPI was identified from expressed sequence tags (ESTs) generated using a honeybee *A. cerana* cDNA library (Kim et al., 2013a, 2013b). Wizard Miniprep Kit for plasmid DNA extraction was purchased from Promega (Madison, WI, USA). The cDNA sequence was analyzed using a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Pairwise sequence comparisons were performed using the sequences available in the online DNASIS and BLAST databanks from NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Alignment of the deduced amino acid sequences of the serine protease inhibitor genes was performed using MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK). The signal sequence of AcVSPI was predicted using the SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP). Genomic DNA extraction from the fat body tissues of a single *A. cerana* worker bee was performed using a Wizard Genomic DNA Purification Kit (Promega). The genomic DNA was used as a template for PCR amplification using oligonucleotide primers designed from the *AcVSPI* cDNA sequence (forward primer (1–18), 5′-ATGCCTCGTCTTGTTCTT-3′; and reverse primer (264–247), 5′-TTATAAAAGTCTTCCTGA-3′). The PCR cycling conditions were performed as follows: 94 °C for 2 min, 30 cycles of amplification (94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min), and 72 °C for 10 min. The PCR products were sequenced as described above.

2.2. RNA extraction and northern blot analysis

Total RNA was isolated from epidermis, fat body, midgut, muscle, and venom gland of *A. cerana* worker bees using a Total RNA Extraction Kit (Promega). Total RNA was subjected to electrophoresis on a 1.0% formaldehyde agarose gel (5 μg of total RNA/lane) and was transferred onto a nylon membrane (Schleicher & Schuell, Dasell, Germany). For hybridization, the *AcVSPI* cDNA probe was labeled with [α-^{32}P] dCTP (Amersham Biosciences, Piscataway, NJ, USA) using a Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA). The hybridization and exposure methods were performed as previously described (Park et al., 2014; Kim and Jin, 2015).

2.3. Expression of recombinant AcVSPI

The recombinant AcVSPI peptides were produced using a baculovirus/insect cell expression system (Je et al., 2001). The *AcVSPI* cDNA was PCR-amplified from *pBluescript-AcVSPI* (forward primer, 5′-GGATCCATGCCTCGTCTTGTTCTTGTCTC-3′; and reverse primer, 5′-ACTAGTTTATTTCTCGAACTGGGGATGACTCCATAAAAGTCTTCCTGAGAAAC ATCT-3′). A Strep-tag sequence was included in the reverse primer for the amplification of *AcVSPI*. The PCR cycling conditions and sequencing of the PCR products were performed as described above. The *AcVSPI* cDNA was inserted into the *Autographa californica* nucleopolyhedrovirus (AcNPV) *pBacPAK8* vector (Clontech, Palo Alto, CA, USA). For the production of recombinant AcNPV expressing recombinant AcVSPI peptides, 500 ng of the construct (*pBacPAK8-AcVSPI*) and 100 ng of the AcNPV viral DNA (Je et al., 2001) were co-transfected into 1.0–1.5 × 10^6 *Spodoptera frugiperda* (Sf9) insect cells for 5 h using Lipofectin transfection reagent (Gibco BRL, Gaithersburg, MD, USA). The transfected cells were cultured in TC100 insect cell culture medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) at 27 °C for 5 days. Recombinant AcNPVs were propagated in Sf9 insect cells cultured in TC100 medium at 27 °C. The purification of recombinant AcVSPI peptides was performed using the Strep-tag® Starter Kit (IBA Solutions For Life Sciences, Goettingen, Germany). The amount of recombinant AcVSPI peptides was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Protein samples were separated by SDS-PAGE on a 14% gel. Western blot analysis was performed using an enhanced chemiluminescence (ECL) Western blot system (Amersham Biosciences, Piscataway, NJ, USA). Following SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell), which was subsequently blocked with 1% bovine serum albumin. The membrane was then incubated with anti-Strep-tag II antibodies (Abcam, Cambridge, UK) at room temperature for 1 h and was washed in Tris-buffered saline with Tween-20 (TBST; 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.05% (w/v) Tween-20). Subsequently, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:5000 (v/v). After washing with TBST, the membrane was incubated with ECL detection reagents (Amersham Biosciences) according to the manufacturer's recommendations and was exposed to autoradiography film.

2.5. Serine protease inhibition assay

Bovine trypsin, bovine α-chymotrypsin, *Engyodontium album* proteinase K, *Bacillus licheniformis* subtilisin A, human neutrophil elastase, human thrombin, and human plasmin were purchased from Sigma (St. Louis, MO, USA). Substrate *N*α-benzoyl-DL-arginine *p*-nitroanilide-hydrochloride (BApNA), succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA), and S4760 were obtained from Sigma. Substrates S-2238 and S-2251 were purchased from Chromogenix (Mölndal, Sweden). Bovine trypsin (100 nM), bovine α-chymotrypsin (10 nM), proteinase K (100 nM), or subtilisin A (100 nM) was incubated in 100 mM Tris-HCl (pH 8.0) containing 20 mM $CaCl_2$ and 0.05% Triton X-100 with recombinant AcVSPI peptides (0–500 nM) at 37 °C for 30 min. The residual enzyme activity was determined at 405 nm or 410 nm using 0.4 mM BApNA for trypsin and Suc-AAPF-pNA for α-chymotrypsin, proteinase K, or subtilisin A as previously described (Lee et al., 2016). Human neutrophil elastase (100 nM), human thrombin (100 nM), or human plasmin (100 nM) was incubated with recombinant AcVSPI peptides (0–1000 nM) at 37 °C for 30 min in 50 mM Tris-HCl buffer (pH 7.4), and the residual enzyme activity was determined at 405 nm using 0.5 mM of the substrate S4760 for the elastase, S-2238 for thrombin, and S-2251 for plasmin as previously described (Lee et al., 2016).

2.6. Fibrin plate assay

Human fibrinogen was purchased from Sigma. Fibrin plates were prepared using 5 ml of human fibrinogen (0.5%) that was clotted with three units of human thrombin. Human plasmin (0.5 μg) was incubated