



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



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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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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 data-banks 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'-ATGCCTCGTCTTGTCTT-3'; and reverse primer (264–247), 5'-TTATAAAAGTCTTCTCTGA-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, Dassel, Germany). For hybridization, the AcVSPI cDNA probe was labeled with [α -³²P] 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'-GGATCCATGCCTCGTCTTGTCTTGTCTC-3'; and reverse primer, 5'-ACTAGTTTATTTCTCGAACTGGGGATGACTCCATAAAAGTCTTCTGAGAAACATCT-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 $1.0\text{--}1.5 \times 10^6$ *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 (Mölnådal, 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 CaCl₂ 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

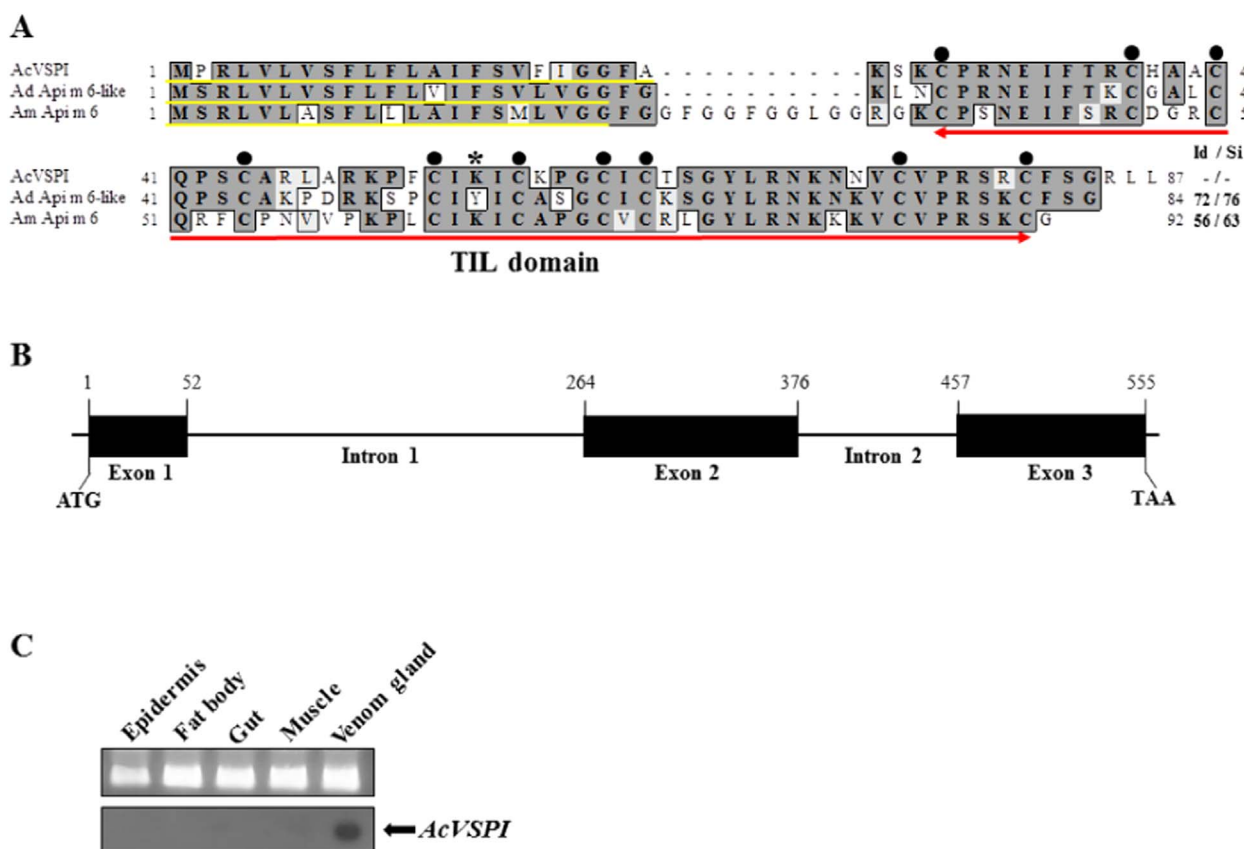


Fig. 1. AcVSPI is a low-molecular-weight serine protease inhibitor Api m 6-like peptide. (A) Alignment of the amino acid sequences of AcVSPI, *A. mellifera* Api m 6, and the *A. dorsata* Api m 6-like peptide. The predicted signal sequence is underlined in yellow. The conserved cysteine residues are indicated by solid circles. The P1 position is marked with an asterisk. The TIL domain is indicated by a red arrow. The sources of the aligned sequences were *A. cerana* AcVSPI (this study, GenBank accession no. MF281990), *A. mellifera* Api m 6 (NP 001035360), and *A. dorsata* Api m 6-like peptide (XP 006610831). The AcVSPI sequence was used as a reference for the identity/similarity (Id/Si) values. (B) Organization of the AcVSPI gene (GenBank accession no. MF281991). Numbers indicate the position in the genomic sequence. Exons are represented by solid boxes. (C) Expression of AcVSPI in *A. cerana* worker bees. Total RNA was isolated from the epidermis, fat body, midgut, muscle, and venom gland of *A. cerana* worker bees. RNA was separated by 1.2% formaldehyde agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with radiolabeled AcVSPI cDNA (lower panel). The ethidium bromide-stained RNA gel shows uniform loading (upper panel). AcVSPI transcripts are indicated with an arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with or without recombinant AcVSPI peptides (100 ng or 500 ng) on the fibrin plates at 37 °C for 30, 60, 120, 180, or 240 min. The fibrinolytic activity was determined by the clear area formed on the fibrin plates (Choo et al., 2012; Qiu et al., 2013; Lee et al., 2016).

2.7. Fibrinolytic cleavage assay

Human fibrinogen (200 µg) was treated with 1 unit of human thrombin in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM CaCl₂. The mixture of human fibrinogen and human plasmin (500 ng) was then incubated with or without recombinant AcVSPI peptides (500 ng) at 37 °C for 0, 30, 60, 120, or 240 min. After incubation, the samples were analyzed by 12% SDS-PAGE to observe the fibrinolytic cleavage (Choo et al., 2012; Qiu et al., 2013; Lee et al., 2016).

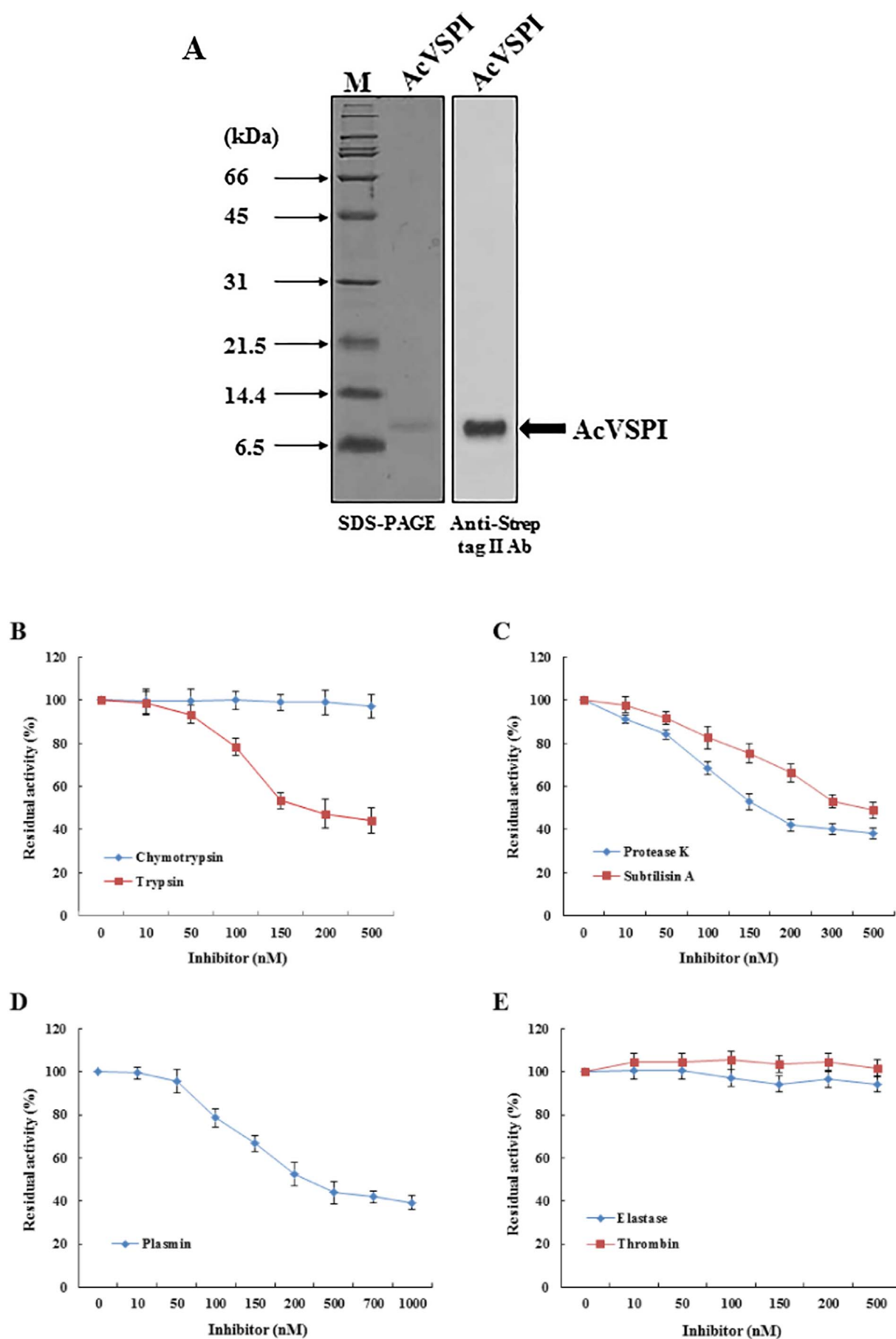
2.8. Microbial binding assay

Gram-negative bacterium *E. coli* DH5α (Lee et al., 2016), gram-positive bacterium *B. thuringiensis* strain 656-3 (Lee et al., 2016), and entomopathogenic fungus *B. bassiana* SFB-205 (Lee et al., 2016) were used in this study. The bacteria were cultured in Luria-Bertani (LB) medium, and the fungus was grown in potato dextrose broth. When the cultures reached an OD₆₀₀ of 0.4, 4 ml of each bacterial or fungal culture was harvested and washed twice with PBS. The bacterial or fungal samples were then resuspended with 40 µl of PBS and were incubated with recombinant AcVSPI peptides (0.8 µg) at room temperature for

10 min. Following incubation, samples were centrifuged at 4000 rpm for 5 min and were harvested separately as pellets and supernatant. The supernatants were directly used in this study. The pellets were washed with PBS and were resuspended in 40 µl of PBS. The pellets and supernatant samples were subjected to SDS-PAGE on a 14% gel. Western blot analysis was performed using an ECL chemiluminescence Western blot system (Amersham Biosciences) with anti-Strep-tag II antibody [diluted 1:10,000 (v/v)] as described above.

2.9. Immunofluorescence staining

Microorganisms were used as described above. *E. coli*, *B. thuringiensis*, and *B. bassiana* samples were washed twice with PBS and were incubated in 40 µl of PBS containing recombinant AcVSPI peptides (0.8 µg) at room temperature for 10 min. Following fixation with acetone (−20 °C) for 2 min, the microorganism samples were washed with PBS and were incubated in a 2% BSA solution at room temperature for 20 min. After PBS washing, the microorganism samples were incubated with rabbit anti-Strep-tag II antibody [diluted 1:500 (v/v)] as the primary antibody and were then incubated with fluorescein-conjugated goat anti-rabbit antibody [diluted 1:400 (v/v); Santa Cruz Biotech, Inc., Santa Cruz, CA, USA] as the secondary antibody. The recombinant AcVSPI peptides on the bacterial and fungal cell surfaces were visualized using laser-scanning confocal microscopy (Carl Zeiss LSM 510, Zeiss, Jena, Germany).



(caption on next page)

Fig. 2. AcVSPI functions as a serine protease inhibitor. (A) The expression of recombinant AcVSPI in baculovirus-infected Sf9 insect cells. The purified recombinant AcVSPI was identified by 14% SDS-PAGE (left) and Western blot analysis using anti-Strep-tag II antibodies (right). A molecular weight standard was used for size comparisons. The recombinant AcVSPI is indicated with an arrow. (B–E) Inhibitory activity of recombinant AcVSPI against serine proteases. Trypsin (B), chymotrypsin (B), proteinase K (C), subtilisin A (C), plasmin (D), elastase (E), or thrombin (E) was incubated with increasing amounts of recombinant AcVSPI, and the residual enzyme activity was determined ($n = 3$).

Table 1
Inhibitory activity of recombinant AcVSPI against serine proteases.

Enzyme	Concentration (nM) ^a	IC ₅₀ (nM)
Chymotrypsin	10	ND
Trypsin	100	195.75 ± 28.61
Subtilisin A	100	451.36 ± 59.6
Protease K	100	163.11 ± 10.28
Plasmin	100	319.69 ± 72.96
Elastase	100	ND
Thrombin	100	ND

ND, not detected.

^a The concentration of enzymes used in this experiment.

2.10. Anti-microbial activity assay

The anti-microbial activity assay of recombinant AcVSPI peptides was performed using a liquid growth inhibition assay as previously described (Lee et al., 2016). The microorganisms that were used were *E. coli*, *B. thuringiensis*, and *B. bassiana* as described above. Bacterial inoculum (2×10^3 cfu per well) or fungal inoculum (1×10^3 conidia per well) was placed in each well of a 96-well plate. Serial dilutions of recombinant AcVSPI peptides were added to each well containing the bacterial cells or fungal conidia. The 96-well plates were incubated at 37 °C for 24 h (for bacteria cells) or at 22 °C for 48 h (for fungal conidia) with shaking at 220 rpm. Following incubation, the bacterial or fungal growth inhibition was determined by the absorbance at 595 nm using a microplate reader (Bio-Rad Model 3550). The minimal inhibitory concentration (MIC) was measured as the lowest concentration of recombinant AcVSPI peptides showing 50% bacterial growth inhibition. The inhibition concentration value of recombinant AcVSPI peptides showing 50% fungal growth inhibition (IC₅₀) was measured. The MIC and IC₅₀ values are expressed as the mean ± standard deviation (SD) of three independent replicates.

2.11. Scanning electron microscopy

As described above, *E. coli*, *B. thuringiensis*, or *B. bassiana* was placed in each well of a 96-well plate and was cultured with or without recombinant AcVSPI peptides (0.8 µg/well) at 37 °C (for bacteria cells) or 22 °C (for fungal conidia) for 24 h with shaking at 220 rpm. Bacterial and fungal samples were fixed in 2.5% glutaraldehyde (EM grade, Sigma) in TBS (pH 7.3) at room temperature for 15 min. Following fixation, the samples were dehydrated with a series of grade ethanol, were air dried, and were coated with gold. The bacterial and fungal cell surfaces were visualized using a Hitachi S-530 scanning electron microscope (SEM) (Hitachi, Japan).

3. Results

3.1. AcVSPI is a low-molecular-weight serine protease inhibitor Api m 6-like peptide

In our search for a low-molecular-weight venom component, we identified a cDNA encoding AcVSPI in *A. cerana*. AcVSPI consists of 87 amino acids, which include a predicted 23-amino acid signal peptide and a 64-amino acid mature peptide (Fig. 1A). Comparison of the

amplicon size between the genomic DNA and cDNA sequences revealed that AcVSPI gene spans 555 bp and contains two introns and three exons (Fig. 1B). Database searches using the peptide sequence of AcVSPI showed similarity to the putative low-molecular-weight serine protease inhibitor Api m 6, a honeybee *A. mellifera* venom component (Fig. 1A). In addition, AcVSPI contains a trypsin inhibitor-like (TIL) cysteine-rich domain that is consistent with allergen Api m 6. AcVSPI exhibited high similarity to *A. dorsata* Api m 6-like peptide (72% peptide sequence identity) and *A. mellifera* allergen Api m 6 (56% peptide sequence identity) (Fig. 1A). Analysis of the peptide sequence of AcVSPI revealed typical features that were similar to Api m 6, including ten conserved cysteine residues and a P1 site (Fig. 1A). These features strongly suggest that AcVSPI is a low-molecular-weight serine protease inhibitor Api m 6-like peptide.

We also examined the expression pattern of AcVSPI in *A. cerana* worker bees to verify that AcVSPI is a honeybee *A. cerana* venom component. Northern blot analysis was performed using total RNA prepared from the epidermis, fat body, midgut, muscle, and venom gland of *A. cerana* worker bees. Based on the northern blot result, the AcVSPI transcripts were found to be specifically expressed in the venom gland (Fig. 1C).

3.2. AcVSPI functions as a serine protease inhibitor

To assess the functional role of AcVSPI, we expressed recombinant AcVSPI in baculovirus-infected insect cells. The purified recombinant AcVSPI, which contains additional 8 × Strep-tag residues at the C-terminus of the 64-amino acid mature AcVSPI, was identified as an 8.2-kDa peptide (Fig. 2A). Recombinant AcVSPI without additional 8 × Strep-tag residues has a molecular weight of approximately 7.0 kDa.

Using purified recombinant AcVSPI, we first determined whether AcVSPI has inhibitory effects against trypsin and/or chymotrypsin. We found that recombinant AcVSPI inhibits trypsin, but not chymotrypsin (Table 1 and Fig. 2B). To further assess the functional role of AcVSPI as a serine protease inhibitor, we next examined the inhibitory effects of AcVSPI against microbial serine proteases and found that recombinant AcVSPI exhibits inhibitory activities against subtilisin A and proteinase K (Table 1 and Fig. 2C). Interestingly, we also found that recombinant AcVSPI exhibited inhibitory activity against plasmin, but it had no inhibitory effect against elastase or thrombin (Table 1 and Fig. 2C–D). Taken together, these results demonstrate that AcVSPI functions as a serine protease inhibitor.

3.3. AcVSPI functions as an anti-fibrinolytic factor

Because recombinant AcVSPI inhibited plasmin in this study (Table 1), we determined whether AcVSPI inhibits the plasmin-mediated degradation of fibrin to fibrin degradation products (FDPs). The plasmin inhibitory assay using SDS-PAGE revealed that recombinant AcVSPI is a plasmin inhibitor that inhibits the degradation of human fibrin to FDPs (Fig. 3A). The fibrin plate assay also revealed that recombinant AcVSPI exhibits an inhibitory effect against plasmin in a dose- and time-dependent manner (Fig. 3B). Our results demonstrated that AcVSPI functions as a plasmin inhibitor that exhibits anti-fibrinolytic activity.

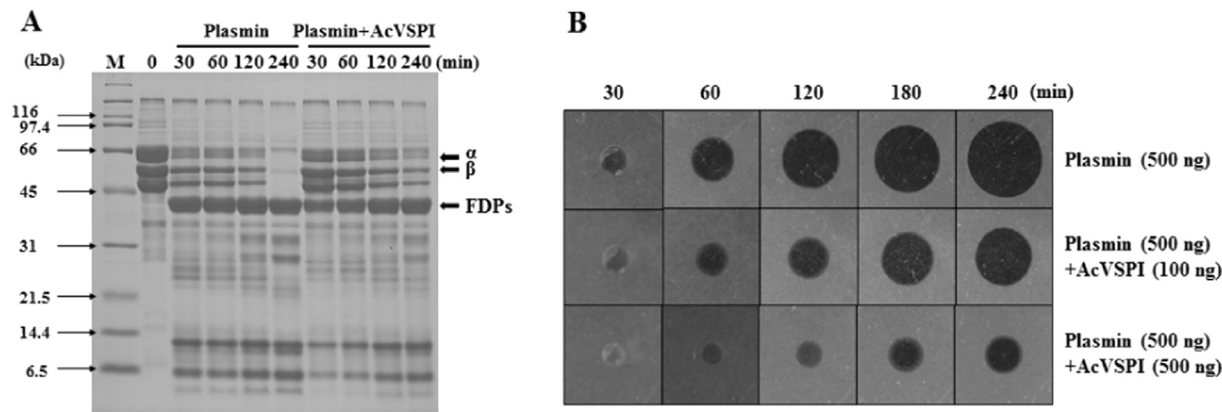


Fig. 3. AcVSPI functions as an anti-fibrinolytic factor. (A) AcVSPI-mediated human plasmin inhibition assay. The mixture of human fibrin and plasmin was incubated with or without recombinant AcVSPI for 30, 60, 120, or 240 min. AcVSPI-mediated human plasmin inhibition was analyzed using 12% SDS-PAGE. FDPs are indicated. (B) The fibrin plate assay of the inhibitory activity of AcVSPI against human plasmin. Plasmin or the mixture of plasmin and recombinant AcVSPI was incubated on fibrin plates at 37 °C for various periods of time (30, 60, 120, 180, or 240 min).

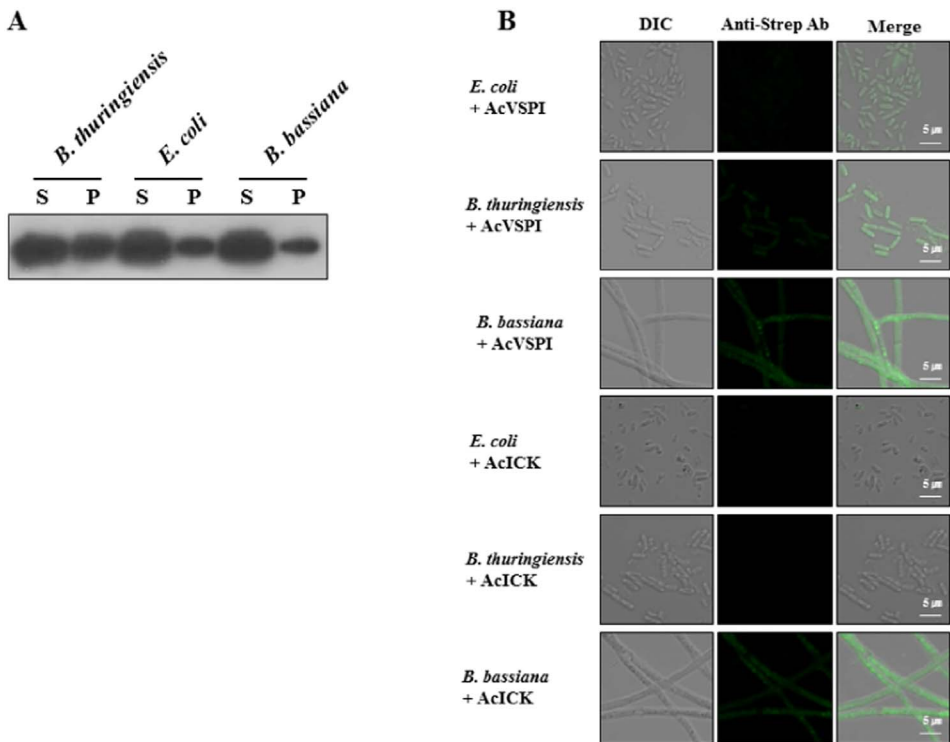


Fig. 4. Microbial binding ability of recombinant AcVSPI. (A) Western blot analysis of the binding of AcVSPI to bacterial and fungal cells. *E. coli*, *B. thuringiensis*, or *B. bassiana* was incubated with recombinant AcVSPI for 10 min. Bound AcVSPI (P) of the pellet was separated from free AcVSPI in the supernatant (S) using centrifugation, and the samples were analyzed using Western blotting with anti-Strep-tag II antibody. (B) Immunofluorescence staining of the binding of AcVSPI to bacterial and fungal cells. *E. coli*, *B. thuringiensis*, or *B. bassiana* was incubated with recombinant AcVSPI for 10 min. Confocal microscopy was performed to visualize AcVSPI binding (green) to the cell walls of *E. coli*, *B. thuringiensis*, and *B. bassiana*. *A. cerana* ICK peptide (Park et al., 2014) was used as a negative control (AcICK). The third column shows merged confocal images. Scale bar, 5 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. AcVSPI functions as an anti-microbial agent

Because recombinant AcVSPI has an inhibitory effect against microbial serine proteases (Table 1), we assessed whether AcVSPI has anti-microbial activity; to determine this, we first examined the microbial binding activity of AcVSPI. The microbial binding assay using Western blot analysis revealed that recombinant AcVSPI was bound to live microorganisms, such as *E. coli*, *B. thuringiensis*, and *B. bassiana* (Fig. 4A). Consistent with the microbial binding ability, immunofluorescence staining also revealed the localization of recombinant AcVSPI on the bacterial and fungal cell walls (Fig. 4B).

We therefore assessed whether the microbial binding ability of

recombinant AcVSPI correlates with the anti-microbial effect against bacteria and fungi. The SEM results revealed that recombinant AcVSPI induced structural damage to the bacterial and fungal cell walls (Fig. 5). These results suggest that AcVSPI-induced cell wall disruption leads to anti-microbial activity.

Finally, we assayed the anti-microbial activity of recombinant AcVSPI against bacteria and fungi. The MIC and IC₅₀ values indicated that recombinant AcVSPI exhibits a broad spectrum of anti-microbial activities against gram-negative bacterium (*E. coli*), gram-positive bacterium (*B. thuringiensis*), and entomopathogenic fungus (*B. bassiana*) (Table 2). Thus, these results demonstrate that AcVSPI acts as an anti-microbial agent that exhibits anti-microbial activity.

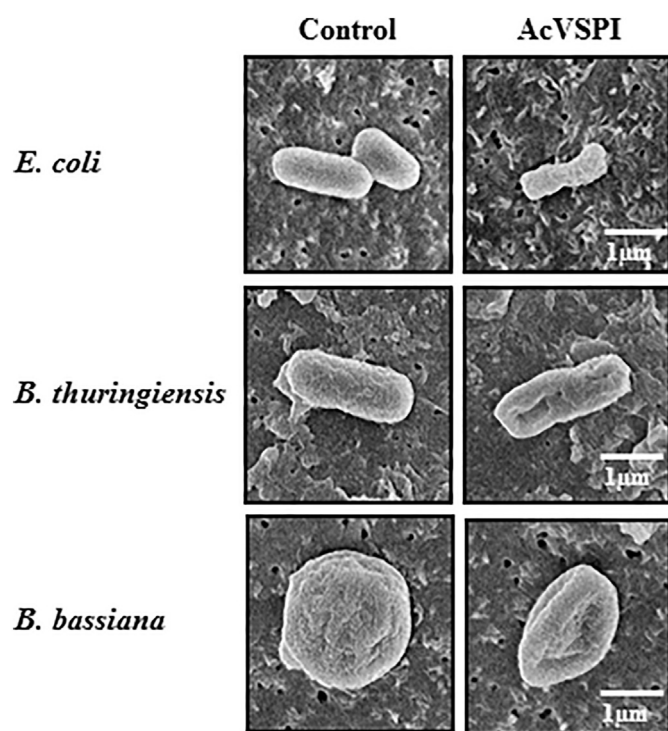


Fig. 5. SEM images of AcVSPI-induced structural damage to bacterial and fungal cell walls. *E. coli*, *B. thuringiensis*, or *B. bassiana* was treated with (right) or without (left) recombinant AcVSPI. Scale bar, 1 μ m.

Table 2

The anti-microbial activity of recombinant AcVSPI against bacteria and fungus.

	Microorganism	MIC ₅₀ (μ M)
Gram-positive bacterium	<i>B. thuringiensis</i>	1.21 $\pm$ 0.095
Gram-negative bacterium	<i>E. coli</i>	2.4 $\pm$ 0.18
		IC ₅₀ (μ M)
Entomopathogenic fungus	<i>B. bassiana</i>	1.4 $\pm$ 0.17

4. Discussion

Bee venom is a complex mixture of nonallergenic substances and allergens (Son et al., 2007; Chen and Lariviere, 2010; Danneels et al., 2015). The honeybee (*A. mellifera*) venom allergen Api m 6 is a putative low-molecular-weight serine protease inhibitor (Kettner et al., 2001; Michel et al., 2012). Although Api m 6 demonstrated T-cell proliferation and the recognition of specific IgE (Kettner et al., 2001) and was suggested as a putative serine protease inhibitor (Michel et al., 2012), the biological actions of Api m 6 remained unknown. Here, we report the functional roles of AcVSPI, which exhibits structural similarity to the allergen Api m 6.

In this study, we identified AcVSPI as an *A. cerana* venom serine protease inhibitor that exhibits anti-fibrinolytic and anti-microbial activities. We hypothesized that AcVSPI is an Api m 6-like peptide because it contains a 55-amino acid TIL domain with ten conserved cysteine residues and a P1 site, which are features that are also present at identical positions in the TIL domain of Api m 6 (Michel et al., 2012). Notably, the P1 (lysine residue) and P₃-P₃' (cysteine residues) positions of Api m 6 were identically observed in AcVSPI. These features are usually indicative of serine protease inhibition, as observed in serine protease inhibitors of the class of canonical inhibitors (Krowarsch et al., 2003). Considering the peptide sequence homology of AcVSPI with Api m 6 and *A. dorsata* Api m 6-like peptide, AcVSPI is highly likely to be a

putative serine protease inhibitor Api m 6-like peptide. Additionally, the expression pattern of AcVSPI suggests that AcVSPI is a low-molecular-weight serine protease inhibitor in the venom of *A. cerana* worker bees.

Among serine protease inhibitors, chymotrypsin/cathepsin G and trypsin/plasmin inhibitors have been isolated from honeybees (Banja et al., 1999; Cierpicki et al., 2000; Kim et al., 2013a) and bumblebees (Choo et al., 2012; Qiu et al., 2013). We therefore produced recombinant AcVSPI in baculovirus-infected insect cells and demonstrated that it inhibits trypsin, but not chymotrypsin. In addition, we found that the recombinant AcVSPI exhibited inhibitory activities against plasmin and microbial serine proteases, but it had no inhibitory effect against elastase and thrombin. Together, these results clearly demonstrate the functional role of AcVSPI as a serine protease inhibitor.

Some serine protease inhibitors from bee venom also exhibit anti-fibrinolytic (Choo et al., 2012; Qiu et al., 2013) and anti-microbial activities (Kim et al., 2013b; Wan et al., 2014). Because recombinant AcVSPI is a plasmin inhibitor, we determined whether AcVSPI may also inhibit the plasmin-mediated degradation of fibrin to FDPs and found that recombinant AcVSPI inhibits the degradation of human fibrin to FDPs. This finding is consistent with the results that bumblebee venom Kunitz-type serine protease inhibitors, which act as a plasmin inhibitor, exhibit anti-fibrinolytic activity (Choo et al., 2012; Qiu et al., 2013). Thus, our results demonstrate that AcVSPI can function as an anti-fibrinolytic factor during fibrinolysis, defining a functional role of AcVSPI as a plasmin inhibitor.

Bee venom serine protease inhibitors inhibit microbial serine proteases, and their anti-microbial activities against bacteria and fungi have been demonstrated (Kim et al., 2013b; Wan et al., 2014). In the present study, we also demonstrated that recombinant AcVSPI inhibits microbial serine proteases and exhibits anti-fungal activity as well as anti-bacterial activity, thus indicating that AcVSPI has a broad spectrum anti-microbial effect. Moreover, these results are consistent with the previous reports that bee venom serine protease inhibitors directly bind to fungal and bacterial cell walls, resulting in anti-microbial activity (Kim et al., 2013b; Wan et al., 2014). In this study, SEM analysis revealed that AcVSPI-induced structural damage to bacterial and fungal cell walls led to anti-microbial activity, which is likely to be an anti-microbial action of AcVSPI, as has been shown to be a common property of anti-microbial peptides (Brogden, 2005; Jenssen et al., 2006; Brudzynski and Sjaarda, 2015). Collectively, our results demonstrate that AcVSPI can function as an anti-microbial peptide, defining a functional role of AcVSPI as an anti-microbial agent.

In conclusion, we identified the functional features of AcVSPI, which exhibits high similarity to Api m 6 and acts as a serine protease inhibitor. Our results showed that AcVSPI has inhibitory effects against trypsin, plasmin, and microbial serine proteases and demonstrated that AcVSPI exhibits anti-fibrinolytic and anti-microbial activities. Given that allergen Api m 6 is a putative low-molecular-weight serine protease inhibitor (Kettner et al., 2001; Michel et al., 2012) and that AcVSPI is an allergen Api m 6-like peptide, the anti-fibrinolytic and anti-microbial roles of AcVSPI appear to be novel. Despite its putative low-molecular-weight serine protease inhibitor, Api m 6 is an allergen; thus, further studies will be needed to address the potential of AcVSPI to elicit IgE. The finding that AcVSPI exhibits anti-fibrinolytic and anti-microbial activities not only highlights the potential biological actions of low-molecular-weight bee venom serine protease inhibitors but may also provide novel insights into allergen Api m 6 and Api m 6-like peptides.

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