Developmental and Comparative Immunology 63 (2016) 27–35

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Secapin, a bee venom peptide, exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities

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ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form
15 May 2016
Accepted 16 May 2016
Available online 18 May 2016

Keywords:
Anti-fibrinolytic agent
Anti-elastolytic agent
Anti-microbial peptide
Honeybee
Secapin
Venom

ABSTRACT

Bee venom contains a variety of peptide constituents that have various biological, toxicological, and pharmacological actions. However, the biological actions of secapin, a venom peptide in bee venom, remain largely unknown. Here, we provide the evidence that Asiatic honeybee (*Apis cerana*) secapin (AcSecapin-1) exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities. The recombinant mature AcSecapin-1 peptide was expressed in baculovirus-infected insect cells. AcSecapin-1 functions as a serine protease inhibitor-like peptide that has inhibitory effects against plasmin, elastases, microbial serine proteases, trypsin, and chymotrypsin. Consistent with these functions, AcSecapin-1 inhibited the plasmin-mediated degradation of fibrin to fibrin degradation products, thus indicating the role of AcSecapin-1 as an anti-fibrinolytic agent. AcSecapin-1 also inhibited both human neutrophil and porcine pancreatic elastases. Furthermore, AcSecapin-1 bound to bacterial and fungal surfaces and exhibited anti-microbial activity against fungi and gram-positive and gram-negative bacteria. Taken together, our data demonstrated that the bee venom peptide secapin has multifunctional roles as an anti-fibrinolytic agent during fibrinolysis and an anti-microbial agent in the innate immune response.

1. Introduction

Bee venom is a complex mixture of biologically active compounds, including polypeptides, enzymes, amines, lipids, and amino acids (Chen and Lariviere, 2010; Danneels et al., 2015; Son et al., 2007). Although it is common knowledge that bee venom components are responsible for local and systemic allergic reactions, they are used as traditional and alternative medicine methods for anti-arthritis, pain relief, and anti-inflammation effects (Chen and Lariviere, 2010; Son et al., 2007); furthermore, they are the treatment of choice for venom allergy (Ozdemir et al., 2011). A number of recent studies have focused on various biological, toxicological, and pharmacological actions of bee venom components (Chen and Lariviere, 2010; Ozdemir et al., 2011; Son et al., 2007). The two major components of honeybee venom are melittin and phospholipase A_2 (PLA_2). Melittin, which is the main constituent of bee venom, possesses anti-microbial and lytic activities (Cruciani et al., 1991; Habermann, 1972), and PLA_2, which is the most commonly studied toxic enzyme, has nociceptive and inflammatory effects (Hartman et al., 1991; Landucci et al., 2000; Six and Dennis, 2000). Melittin, apamin, mast cell degranulating (MCD) peptide, adolapin, secapin, procamine, tertiapin, cardiopep, melittin F, and minimine are among the peptide constituents of bee venom (Chen and Lariviere, 2010; Son et al., 2007). Compared with investigations of the biological actions of melittin, apamin, MCD peptide, and adolapin, the biological actions of the other bee venom peptide components remain unknown.

Secapin, a peptide component of bee venom, was discovered approximately four decades ago. Secapin displays no toxicity in mice but exhibits signs of sedation, piloerection, and hypothermia when a high dose is injected (Gauldie et al., 1976). Subsequent studies have generated a complete sequence of cDNA coding for preprosecapin, and mature secapin is composed of 25 amino acid residues that contain a disulfide link (Gauldie et al., 1978; Vlasak and Kreil, 1984). Although secapin has been demonstrated to act as a potent neurotoxin (Taylor et al., 1984), additional roles for secapin remain relatively unexplored. Most recently, several properties of secapin have been reported, including hyperalgesic, edematogenic (Mourelle et al., 2014), and anti-bacterial properties (Hou et al., 2014). Considering that all of the bee venom peptide

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http://dx.doi.org/10.1016/j.dci.2016.05.011
0145-305X/

components described to date have various biological effects (Chen and Lariviere, 2010; Son et al., 2007), secapin may also possess various functions as a venom peptide; however, the biological actions of secapin are poorly understood.

Recently, we demonstrated that the Kazal-type serine protease inhibitor and the inhibitor cysteine knot (ICK) peptide in Asiatic honeybee (*Apis cerana*) venom exhibit anti-microbial activities (Kim et al., 2013b; Park et al., 2014). Furthermore, our previous study suggested the presence of a serine protease with fibrin(ogen) olytic activities in European honeybee (*Apis mellifera*) venom (Qiu et al., 2011). However, the component with the anti-fibrinolytic role in honeybee venom has not been elucidated until now.

Here, we show the evidence that *Apis cerana* secapin-1 (AcSecapin-1) is a multifunctional bee venom peptide that exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities. Because *A. mellifera* secapin exhibits anti-bacterial activity (Hou et al., 2014) and induces inflammation and pain (Mourelle et al., 2014), we hypothesized that secapin in bee venom is a multifunctional peptide. We focused our studies on the biological actions of AcSecapin-1 in *A. cerana* bee venom. Importantly, we found that AcSecapin-1 functions as a serine protease inhibitor-like peptide and acts as a plasmin inhibitor that has an anti-fibrinolytic role. Furthermore, we found that AcSecapin-1 acts as an anti-elastolytic factor that inhibits human and porcine elastases. Finally, we demonstrated that AcSecapin-1 is an anti-microbial peptide that exhibits anti-fungal activity as well as anti-bacterial activity.

2. Materials and methods

2.1. cDNA cloning

The cDNA encoding preproAcSecapin-1 was cloned from expressed sequence tags (ESTs) that were generated using an *A. cerana* cDNA library (Kim et al., 2013a, 2013b). The plasmid DNA was extracted using a Wizard Mini-Preparation Kit (Promega, Madison, WI, USA) and sequenced using a BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

2.2. Sequence alignment and phylogenetic analysis

Pairwise sequence comparisons were performed using the DNASIS and BLAST programs (http://www.ncbi.nlm.gov/BLAST). A multiple amino acid sequence alignment was performed using MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK). The signal peptide sequence of AcSecapin-1 was predicted using the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP). A phylogenetic analysis of the secapin amino acid sequences was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2000). The amino acid sequences of the hymenopteran secapins used in this study were *A. cerana* AcSecapin-1 (GenBank accession no.: KR732613), *A. dorsata* secapin (XM 006620083.1), *A. florea* secapin (XM 012493999.1), *A. mellifera* secapin-3 (JN541205.1), *Polistes hebraeus* secapin (AF488557.1), *A. mellifera* secapin (NM 001011618.1), *Vespa magnifica* secapin (AF488556.1), *Vespa velutina nigrithorax* secapin (AF488555.1), *A. florea* secapin-like (XM 003689775.2), *A. dorsata* secapin-like (XM 006617260.1), *A. cerana* secapin (AF487551.1), *A. mellifera* secapin-2 (JN541204.1), *Vespula maculifrons* secapin (AF488554.1), *Camponotus floridanus* secapin (HQ829846.1), *Harpegnathos saltator* secapin (GL451483.1), *Atta cephalotes* secapin (XM 012207756.1), *Acromyrmex echinatior* secapin (XM 011066690.1), *Solenopsis invicta* secapin (XM 011164365.1), *Vollenhovia emeryi* secapin (XM 012009579.1), *Cerapachys biroi* secapin (XM 011335175.1), and *Wasmannia auropunctata* secapin (XM 011688866.1). Phylogenetic trees were constructed using a bootstrap analysis with the option of heuristic search (bootstrap = 1000).

2.3. Recombinant protein expression and purification

The production of recombinant mature AcSecapin-1 was performed using a baculovirus expression system (Je et al., 2001). The 25-amino acid mature peptide sequence of AcSecapin-1 was PCR-amplified from *pBluescript-AcSecapin-1* using the following primers: forward primer (419–436), 5′-AGGTACTGTAATGAGTTGAGCTGAAACGATGTTCGT-3′, and reverse primer (476–493), 5′-TCTAGATTAATGATGATGATGATGATGTGGAATTTTCTCGCGACA-3'. A His-tag sequence was included in the *AcSecapin-1* cDNA sequence. The honeybee inhibitor cysteine knot signal sequence (Qiu et al., 2011) was incorporated into the *AcSecapin-1* cDNA sequence to secrete recombinant mature AcSecapin-1. The *AcSecapin-1* fragment corresponding to the mature peptide sequence was inserted into the *pBacPAK8* transfer vector (Clontech, Palo Alto, CA, USA) under the control of the *Autographa californica* nucleopolyhedrovirus (AcNPV) polyhedrin promoter. Co-transfection of 500 ng of the construct (*pBacPAK8-AcSecapin-1*) and 100 ng of AcNPV viral DNA (Je et al., 2001) was performed as previously described (Kim and Jin, 2015; Park et al., 2014). Recombinant baculoviruses expressing recombinant mature AcSecapin-1 were propagated in the *Spodoptera frugiperda* (Sf9) insect cell line that was cultured in TC100 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL) at 27 °C. The recombinant mature AcSecapin-1 peptides were purified using the MagneHis™ Protein Purification System (Promega) according to the manufacturer's protocols. The recombinant mature AcSecapin-1 peptides were identified by 14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis using an enhanced chemiluminescence western blotting system (Amersham Biosciences, Piscataway, NJ, USA) with anti-His antibodies (BETHYL Laboratories, TX, USA) and horseradish peroxidase-conjugated anti-mouse IgG diluted 1:5000 (v/v). The amount of recombinant mature AcSecapin-1 peptides was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the supplier's protocol.

2.4. Protease inhibition assay

Bovine trypsin (100 nM, Sigma, St. Louis, MO, USA), bovine α-chymotrypsin (10 nM, Sigma), *Engyodontium album* proproteinase K (100 nM, Sigma), or *Bacillus licheniformis* subtilisin A (100 nM, Sigma) was incubated in 100 mM Tris-HCl (pH 8.0) containing 20 mM $CaCl_2$ and 0.05% Triton X-100 with recombinant mature AcSecapin-1 (0–600 nM) at 37 °C for 30 min. The residual enzyme activity was estimated at 405 nm or 410 nm using 0.4 mM *N*α-benzoyl-DL-arginine *p*-nitroanilide-hydrochloride (BApNA, Sigma) for trypsin and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA, Sigma) for α-chymotrypsin, proteinase K, or subtilisin A. Human neutrophil elastase (100 nM, Sigma), porcine pancreatic elastase (100 nM, Sigma), human thrombin (100 nM, Sigma), or human plasmin (10 nM, Sigma) was incubated with recombinant mature AcSecapin-1 (0–600 nM) at 37 °C for 30 min in 50 mM Tris-HCl buffer (pH 7.4), and the residual enzyme activity was estimated at 405 nm using 0.5 mM of the substrate S4760 (Sigma) for the elastases, S-2238 (Chromogenix, Mölndal, Sweden) for thrombin, and S-2251 (Chromogenix) for plasmin. The inhibition constant (K_i) values were determined using the following equation: $K_i = IC_{50}/(1 + S/K_m)$ (Sinauridze et al., 2011).