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A serine protease inhibitor from the hornfaced bee, *Osmia cornifrons*, exhibits antimicrobial activities

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ABSTRACT

Serine protease inhibitors play a critical role in physiological processes and immune responses by regulating serine protease activities. Here we describe the molecular cloning and antimicrobial activities of a serine protease inhibitor from the hornfaced bee, *Osmia cornifrons* (OcSPI). OcSPI consists of 405 amino acid residues and contains a potential reactive center loop (RCL) region in its C-terminus. Recombinant OcSPI was produced as a 64-kDa glycoprotein in baculovirus-infected insect cells and exhibited inhibitory activity against chymotrypsin. Additionally, OcSPI demonstrated inhibitory activity against microbial serine proteases, such as subtilisin A and proteinase K, but not against tissue plasminogen activator, thrombin, or plasmin. Recombinant OcSPI bound directly to *Escherichia coli*, *Bacillus subtilis*, and *Beauveria bassiana* and exhibited antimicrobial activity against both bacteria and fungi. Our results demonstrated the antimicrobial functions of OcSPI and suggest a role for OcSPI in the immune response of *O. cornifrons* bees.

Introduction

Serine proteases are regulated by serine protease inhibitors (or serpins), which are a large superfamily of protease inhibitors (Irving et al., 2000). In insects, serpins play a key role in the innate immune response, including inhibiting serine protease cascades (Cerenius et al., 2010). The *Drosophila melanogaster* genome contains 29 serpin genes, many of which contain a conserved hinge domain in their C-termini (Garrett et al., 2009). Serpins typically consist of approximately 400 amino acid residues and contain a reactive center loop (RCL), which is located 30–40 residues from the C-terminus and includes hinge region and proteinase cleavage site (P1 and P1′) residues (Irving et al., 2000; Gettins, 2002; Osterwalder et al., 2004; Garrett et al., 2009). In *Drosophila*, serpins play a critical role in immune responses: regulating the Toll-mediated innate immune response (Levashina et al., 1999; Green et al., 2000) and the phenoloxidase cascade (De Gregorio et al., 2002; Ligoxygakis et al., 2002). In *Bombyx mori*, serpin-15 inhibited prophenoloxidase activation and the expression of antimicrobial peptides (Liu et al., 2015). In *Aedes aegypti*, *Anopheles gambiae*, and *Manduca sexta*, serpins have also been reported to be involved in the regulation of the Toll pathway and prophenoloxidase activation (Tong et al., 2005; Shin et al., 2006; An and Kanost, 2010; Zou et al., 2010; An et al., 2011; Chu et al., 2015).

Neuroserpin is a serine protease inhibitor that is expressed in the central and peripheral nervous systems (Stoeckli et al., 1989). Previous studies have demonstrated that neuroserpins inhibit tissue plasminogen activator (tPA), plasmin, and thrombin (Hastings et al., 1997; Krueger et al., 1997). In *Drosophila*, serpin 4 is the closest vertebrate neuroserpin homolog and functions as a neuroserpin-like inhibitor of subtilisin-like proprotein convertases (Osterwalder et al., 2004). A recent study demonstrated that the *D. melanogaster Spn42Da* (previously known as *serpin 4*) encodes eight protein isoforms (Ellisdon et al., 2014) that inhibit furin-, subtilase- and chymotrypsin-like serine proteases and papain-like cysteine proteases (Oley et al., 2004; Osterwalder et al., 2004; Richer et al., 2004). From these results, Spn42Da isoforms potentially improve immune defense by inhibiting pathogenic proteolytic enzymes (Brüning et al., 2007). Additionally, serpin27A is induced upon microbial infection (Ligoxygakis et al., 2002).

In the present study, we provide the molecular characterization of a serine protease inhibitor of the hornfaced bee, *Osmia cornifrons* (OcSPI), and demonstrate that OcSPI exhibits antimicrobial activities. We cloned the *OcSPI* cDNA and characterized the predicted OcSPI protein to consist of 405 amino acids containing a potential RCL region with the hinge region and proteinase cleavage site characteristic of active proteinase inhibitors (Garrett et al., 2009). We expressed recombinant OcSPI in baculovirus-infected insect cells and characterized the inhibitory activities of recombinant OcSPI against chymotrypsin, subtilisin A, and proteinase K. These inhibitory activities led us to hypothesize that OcSPI may be involved in antimicrobial functions by inhibiting microbial serine proteases (Kim et al., 2013b). We also assayed the

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microbial binding and antimicrobial activities of recombinant OcSPI. This study provides evidence that OcSPI acts as both an antimicrobial protein and serine protease inhibitor.

Materials and methods

cDNA cloning, nucleotide sequencing, and phylogenetic analysis

The cDNA encoding OcSPI was isolated from expressed sequence tags (ESTs) generated using the *O. cornifrons* cDNA library (Lee et al., 2015). Plasmid DNA extraction was performed using a Wizard Mini-Preparation kit (Promega, Madison, WI, USA) according to the manufacturer's protocols. The nucleotide sequences were determined by DNA sequencing. Pairwise comparisons of the *OcSPI* cDNA sequences were performed using the DNASIS and BLAST programs (http://www.ncbi.nlm.gov/BLAST). A multiple alignment of the predicted amino acid sequences of the *OcSPI* gene was performed using MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK). The signal peptide sequences in the OcSPI protein were predicted using the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP). Phylogenetic analysis was performed on the GenBank-registered serpin amino acid sequences using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2000).

Expression and purification of the recombinant OcSPI protein

For the production of the recombinant OcSPI protein, we used a baculovirus expression vector system (Je et al., 2001). The *OcSPI* cDNA was PCR-amplified using the plasmid *pBluescript-OcSPI* as a template. The oligonucleotide primer set used for *OcSPI* amplification was as follows: *OcSPI* forward primer (1–21) 5′-GGATCCATGAGTATATTAAAGA AAAGT-3′, which was designed to include a *Bam* HI restriction site (underlined), and *OcSPI* reverse primer (1198–1218) 5′-CTCGAGTTAA TGATGATGATGATGATGAACTTTCGGTTCATTAAC-3′, which was designed to include a *Xho* I restriction site (underlined) and a His-tag sequence. PCR was performed using an amplification program as follows: 94 °C for 3 min, 30 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min), and 72 °C for 5 min. The PCR products were cloned into the *pGem-T* vector (Promega) and verified by nucleotide sequence analysis. To insert the PCR fragment into the transfer vector, the *OcSPI* fragment excised from *pGemT–OcSPI* by digestion with *Bam* HI and *Xho* I was ligated into the same sites of the transfer vector *pBacPAK8* (Clontech, Palo Alto, CA, USA). For the production of recombinant *Autographa californica* nucleopolyhedrovirus (AcNPV–OcSPI), 500 ng of the construct (*pBacPAK8–OcSPI*) was co-transfected with 100 ng of AcNPV viral DNA (Je et al., 2001) into 1.0–1.5 × 10^6 *Spodoptera frugiperda*-derived Sf9 cells using the Lipofectin transfection reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. The transfected Sf9 cells were cultured at 27 °C in TC100 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) to produce recombinant viruses (AcNPV–OcSPI). The purification of the His-tagged recombinant OcSPI protein was performed using the MagneHis™ Protein Purification System (Promega) according to the manufacturer's protocol. Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the supplier's protocol.

Western blot analysis

Protein samples were separated by 12% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as described by Towbin et al. (1979). The membrane was blocked with a 1% bovine serum albumin (BSA) solution and incubated with an anti-His antibody [diluted 1:10,000 (v/v)] at room temperature for 1 h. After washing with Tris-buffered saline with Tween-20 (TBST, 10 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 0.05% (w/v) Tween-20), the membrane was then incubated with a horseradish peroxidase-conjugated anti-mouse IgG [diluted 1:5000 (v/v)] as the secondary antibody for 1 h. The immunoblot signal was detected using an enhanced chemiluminescence (ECL) western blotting system (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's protocol.

RNA extraction and northern blot analysis

Ten *O. cornifrons* bees (Lee et al., 2015) were dissected after anesthesia by chilling and collecting tissue samples, such as the epidermis, fat body, gut, muscle, and venom gland. Tissue samples were washed three times with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Total RNA extraction from tissue samples was performed using a Total RNA Extraction Kit (Promega) according to the manufacturer's protocol. Total RNA was subjected to a 1.0% formaldehyde agarose gel electrophoresis (5 μg of total RNA/lane) and then transferred onto a nylon membrane (Schleicher & Schuell). Using the *OcSPI* cDNA as a probe, which was labeled with [α-^{32}P] dCTP (Amersham Biosciences) using a Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocols, hybridization was performed at 42 °C in hybridization buffer (5× saline-sodium citrate, 5× Denhardt's solution, 0.5% SDS, and 100 mg/ml denatured salmon sperm DNA), as previously described (Lee et al., 2015). The membrane was washed with 0.1% SDS and 0.2× saline-sodium citrate at 65 °C and then exposed to autoradiography film at −70 °C.

Tunicamycin treatment

For tunicamycin experiments, recombinant baculovirus (AcNPV–OcSPI)-infected Sf9 cells were incubated with tunicamycin (Sigma), which is a specific inhibitor of *N*-linked oligosaccharide addition, as described previously (Kim and Jin, 2015). Briefly, insect Sf9 cells (1 × 10^6 cells/35-mm diameter dish) were infected with recombinant virus (AcNPV–OcSPI) for 2 h at 27 °C and replaced with TC100 medium containing 5 μg of tunicamycin/ml. At 2 days post-infection (p.i.), total cellular lysates were harvested and inhibition of the added *N*-linked oligosaccharide was determined by western blot analysis with the anti-His antibody [diluted 1:10,000 (v/v)] as described above.

Serine protease inhibition assay

The serine proteases used were commercially available preparations of bovine trypsin, bovine α-chymotrypsin, subtilisin A from *Bacillus licheniformis*, proteinase K from *Engyodontium album*, human thrombin, human tissue plasminogen activator (tPA), and human plasmin from Sigma (St. Louis, MO, USA). The serine protease inhibition assays of the recombinant OcSPI protein were performed according to Kim et al. (2013a,b) and Choo et al. (2012). Twenty nM of trypsin, α-chymotrypsin, subtilisin A, or proteinase K was incubated in 100 mM Tris–HCl (pH 8.0) containing 20 mM $CaCl_2$ and 0.05% Triton X-100 with recombinant OcSPI proteins (0–400 nM) at 37 °C for 30 min. The residual enzyme activities were determined at 405 nm or 410 nm. The substrates were commercially available preparations of *N*α-benzoyl-DL-arginine *p*-nitroanilide-hydrochloride (BApNA) and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) from Sigma and used 0.5 mM BApNA for trypsin and 0.5 mM Suc-AAPF-pNA for α-chymotrypsin, subtilisin A, and proteinase K. For the thrombin, tPA, or plasmin inhibition assays, 20 nM of thrombin, tPA, or plasmin were incubated with recombinant OcSPI proteins (0–400 nM) at 37 °C for 30 min in 50 mM Tris–HCl buffer (pH 7.4). The residual enzymatic activities were determined at 405 nm. The substrates were purchased from Chromogenix (Mölndal, Sweden) and used 0.5 mM of S-2238 for thrombin, S-2288