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Anti-elastolytic activity of a honeybee (*Apis cerana*) chymotrypsin inhibitor

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ABSTRACT

The honeybee is an important insect species in global ecology, agriculture, and alternative medicine. While chymotrypsin and trypsin inhibitors from bees show activity against cathepsin G and plasmin, respectively, no anti-elastolytic role for these inhibitors has been elucidated. In this study, we identified an Asiatic honeybee (*Apis cerana*) chymotrypsin inhibitor (AcCI), which was shown to also act as an elastase inhibitor. AcCI was found to consist of a 65-amino acid mature peptide that displays ten cysteine residues. When expressed in baculovirus-infected insect cells, recombinant AcCI demonstrated inhibitory activity against chymotrypsin (K_i 11.27 nM), but not trypsin, defining a role for AcCI as a honeybee-derived chymotrypsin inhibitor. Additionally, AcCI showed no detectable inhibitory effects on factor Xa, thrombin, plasmin, or tissue plasminogen activator; however, AcCI inhibited human neutrophil elastase (K_i 61.05 nM), indicating that it acts as an anti-elastolytic factor. These findings constitute molecular evidence that AcCI acts as a chymotrypsin/elastase inhibitor.

1. Introduction

The honeybee is a social insect species and an economically important insect that plays an essential role in the pollination of flowering plants [1] and the supply of several products, such as honey, royal jelly, propolis, pollen, wax, and venom [2]. Honeybee-derived products have been used as a staple of traditional medicine for centuries in Asian countries such as Korea, Japan, and China [2]. Bee venom is a rich source of pharmacologically active compounds; it has been used as an alternative medicine to treat a variety of disorders, including arthritis, rheumatism, pain, tendonitis, cancer, inflammation, and skin diseases [2–4]. Bee venom also contains serine proteases, which show fibrinolytic/fibrinogenolytic activity [5,6], and serine protease inhibitors, which exhibit antifibrinolytic activity [7].

Serine protease inhibitors are found in numerous tissues of a wide variety of blood-sucking and venomous animals. Structurally, these protease inhibitors consist of 50–70 amino acid residues that display a disulfide-rich alpha/beta fold structure [7–12]. Functionally, serine protease inhibitors show inhibitory activity against trypsin and/or chymotrypsin [7,10–19]. In addition, serine protease inhibitors are involved in various physiological processes, such as potassium channel blocking, blood coagulation, fibrinolysis, and inflammation [7,10–13,19–24]. Thus, these serine protease inhibitors might represent useful lead compounds for the design of novel pharmaceuticals [7,20–24].

The protection of bees as pollinators of flowering plants [1] and the utilization of bees as suppliers of natural products have been subjects of worldwide focus [2]. Additionally, the entire genomic sequence of the honeybee *Apis mellifera* has already been published [25]. Although chymotrypsin/cathepsin G and trypsin/plasmin inhibitors have been isolated from *A. mellifera* [10,11] and *Bombus ignitus* [7], additional roles for bee serine protease inhibitors remain relatively unexplored. Serine protease inhibitors derived from blood-sucking and venomous animals show inhibitory activity against plasmin, plasma kallikrein, thrombin, and neutrophil elastase [7,18–23]. However, the anti-elastolytic activity of bee-derived serine protease inhibitors has not been described until now.

Here, we report the identification of an Asiatic honeybee (*Apis cerana*) chymotrypsin inhibitor (AcCI) that also exhibits inhibitory activity against elastases, which function as modulators of the inflammatory process [26]. Our results describe the molecular characterization of the first bee-derived serine protease inhibitor that acts as a chymotrypsin/elastase inhibitor.

2. Materials and methods

2.1. Gene cloning and sequence analysis

The Asiatic honeybee *A. cerana* (Hymenoptera: Apidae) used in this study was supplied by the Department of Agricultural Biology

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of the National Academy of Agricultural Science in the Republic of Korea. A clone encoding AcCI was selected from the expressed sequence tags (ESTs) that were generated from a cDNA library constructed using whole bodies of *A. cerana* worker bees. Plasmid DNA was extracted using the Wizard Miniprep Kit (Promega, Madison, WI, USA), and the cDNA sequence was analyzed using an ABI310 automated DNA sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA, USA). The cDNA sequence was compared to the sequences available in the online DNASIS and BLAST databanks from NCBI (http://www.ncbi.nlm.nih.gov/BLAST). MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the deduced amino acid sequences of the serine protease inhibitor genes, and the signal sequence was predicted by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP). Genomic DNA was extracted from the fat body tissues of a single *A. cerana* worker bee using a Wizard Genomic DNA Purification Kit (Promega). This DNA was then used as a template for polymerase chain reaction (PCR) amplification using oligonucleotide primers designed from the *AcCI* cDNA sequence (forward primer (1–18), 5′-ATGATTCGAAT AATAACT-3′; and reverse primer (258–241), 5′-TCAACAATCAC GTGTCAA-3′). PCR was performed as follows: 94 °C for 2 min, 30 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s), and 72 °C for 10 min. The nucleotide sequences of the PCR products were determined using a BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer as described above.

2.2. RNA extraction and reverse transcription-PCR (RT-PCR)

Apis cerana worker bees were dissected on ice using a stereo microscope (Zeiss, Jena, Germany). Tissue samples (epidermis, fat body, midgut, and venom gland) were collected and washed with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). Total RNA was isolated from the tissue samples using a Total RNA Extraction Kit (Promega). To examine *AcCI* expression in the *A. cerana* worker bee tissues, an *AcCI* cDNA fragment was amplified from total RNA by RT-PCR. The primers were designed from the *AcCI* cDNA sequence (GenBank accession number JX899417) (forward primer (1–18) 5′-ATGATTCGAATAATAACT-3′ and reverse primer (258–241) 5′-TCAACAATCACGTGTCAA-3′). As an internal control for the RT-PCR, a *β-actin* gene fragment of 840 bp (GenBank accession number JX899419) was amplified (forward primer 5′-ATGTGTGACGACG AAGTA-3′ and reverse primer 5′-GTACACGGTCTCGTGGAT-3′). RT-PCR was performed as follows: 94 °C for 2 min, 30 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s), and 72 °C for 10 min. The resulting fragments were analyzed by electrophoresis on a 1.2% agarose gel. The nucleotide sequences of the RT-PCR products were determined as described above.

2.3. Expression of recombinant AcCI

A baculovirus/insect cell expression system [27] was used for the production of recombinant AcCI. The *AcCI* cDNA, which encodes 85 amino acids, was PCR-amplified from *pBluescript-AcCI* (forward primer 5′-GGATCCATGATTCGAATAATAACTATT-3′ and reverse primer 5′-CTCGAGTCAATGATGATGATGATGACAATCACGTGTCAA AAC-3′). The reverse primer for the amplification of *AcCI* was engineered to include a His-tag sequence. The PCR cycling conditions were 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 sequenced using the BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (Perkin–Elmer Applied Biosystems). The *AcCI* fragment was inserted into the *pBacPAK8* vector (Clontech, Palo Alto, CA, USA) to generate an expression vector under the control of the *Autographa californica* nucleopolyhedrovirus (AcNPV) polyhedrin promoter. For the expression experiments, 500 ng of the construct (*pBacPAK8-AcCI*) and 100 ng of the AcNPV viral DNA [27] were co-transfected into $1.0–1.5 \times 10^6$ *Spodoptera frugiperda* (Sf9) cells for 5 h using Lipofectin transfection reagent (Gibco BRL, Gaithersburg, MD, USA). The transfected cells were cultured in TC100 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) at 27 °C for 5 days. Recombinant baculoviruses were propagated in Sf9 cells cultured in TC100 medium at 27 °C. The recombinant proteins were purified using the MagneHis™ Protein Purification System (Promega). Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Glycoprotein staining was performed using a Gel Code Glycoprotein Staining Kit (Pierce, Rockford, IL, USA).

2.4. Western blot analysis

Western blot analysis was performed using an enhanced chemiluminescence (ECL) Western blot system (Amersham Biosciences, Piscataway, NJ, USA). Protein samples were mixed with sample buffer, boiled for 5 min, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 14% gel. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), which was subsequently blocked with 1% bovine serum albumin. The membrane was then incubated with an anti-His antibody at room temperature for 1 h and 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). Next, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG at a dilution of 1:5000 (v/v). After repeated washes with TBST, the membrane was incubated with ECL detection reagents (Amersham Biosciences) and exposed to autoradiography film.

2.5. Serine protease inhibition assay

Bovine trypsin (100 nM) (Sigma, St. Louis, MO, USA) or bovine α-chymotrypsin (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 increasing amounts of recombinant AcCI (0–200 nM) at 37 °C for 30 min. The residual enzyme activity was determined at 405 nm using the following substrates: 0.5 mM BApNA (Sigma) for trypsin and 0.5 mM Suc-AAPF-pNA (Sigma) for α-chymotrypsin. Additionally, 100 nM of human neutrophil elastase (Sigma), porcine pancreatic elastase (Sigma), human thrombin (Sigma), human tissue plasminogen activator (tPA; Sigma), or bovine factor Xa (Novagen, Darmstadt, Germany) was incubated with increasing amounts of AcCI (0–200 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 (Sigma) for the elastases, S-2238 (Chromogenix, Mölndal, Sweden) for thrombin, S-2288 (Chromogenix) for tPA, or S-2222 (Chromogenix) for factor Xa [5–7]. The initial reaction rate was determined by calculating the slope of the linear portion of the kinetic curve. The inhibitory effect was expressed as the percent reduction in the initial hydrolysis rate; the reaction rate in the absence of inhibitor was defined as 100%. The inhibitor concentration that decreased the rate of hydrolysis by 50% (IC_{50}) was also determined. The values of the inhibition constants (K_i) were calculated using the equation $K_i = IC_{50}/(1 + S/K_m)$ [28].

2.6. Fibrin plate assay

The fibrin plate assay was performed with 10 ml of human fibrinogen (0.6%) that was clotted with three units of thrombin. Plasmin alone (1 μg) or a mixture of plasmin (1 μg) and