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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

recombinant AcCI (50 or 100 ng) was dropped onto the fibrin plates, and the plates were incubated at 37 °C for various time periods (15, 30, 60, 120, 180, or 240 min). The fibrinolytic activity was determined by measuring the formation of a clear area on the plates [5–7].

3. Results

3.1. AcCI is a honeybee chymotrypsin inhibitor

In our search for a honeybee-derived serine protease inhibitor, we identified an EST for a gene encoding a chymotrypsin inhibitor (AcCI) in an *A. cerana* cDNA library. An *AcCI* cDNA that included a full-length chymotrypsin inhibitor gene was then identified by the *A. cerana* EST search (GenBank accession number JX899417). The *AcCI* gene consists of three exons and encodes an 85-amino acid protein (Fig. 1A and B). We predicted the primary structure of AcCI based on the signal peptide identified by the SignalP program and the mature peptide predicted by alignment with other chymotrypsin inhibitors. The 85-amino acid AcCI protein includes a 20-amino acid signal peptide and a 65-amino acid mature peptide (Fig. 1C). Analysis of the peptide sequence of mature AcCI revealed distinctive features that were similar to members of other chymotrypsin inhibitor families, including ten conserved cysteine residues and a P1 site (Fig. 1C). These features suggest that AcCI is structurally and functionally similar to other chymotrypsin inhibitors.

We examined the expression pattern of *AcCI* in *A. cerana* worker bees to verify that this transcript is an *A. cerana*-derived serine protease inhibitor. RT-PCR was performed using mRNA prepared from the epidermis, fat body, midgut, and venom gland of *A. cerana* worker bees. Based on the RT-PCR results, the *AcCI* gene was found to be constitutively expressed in all of the tissues examined in this study (Fig. 2).

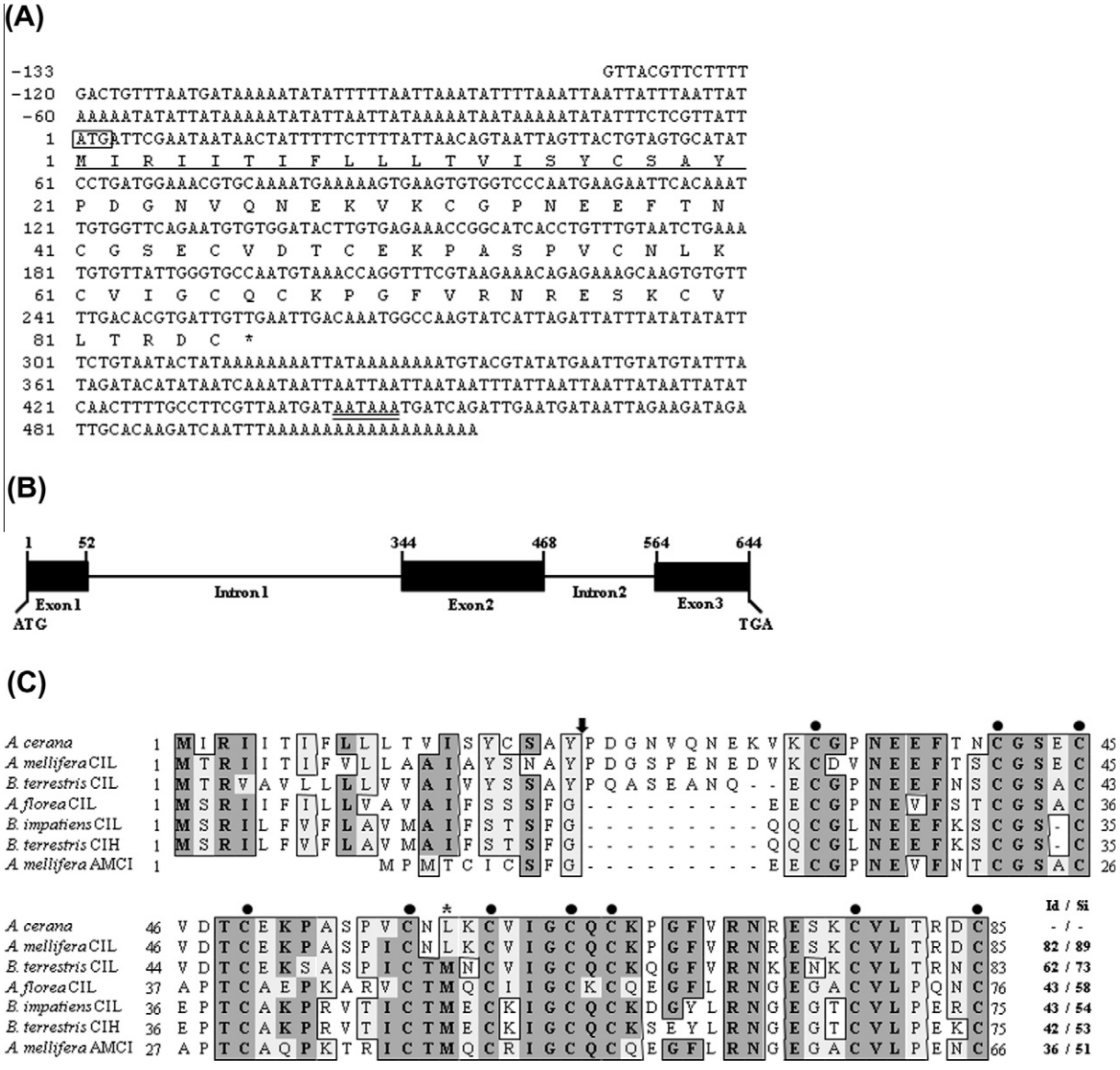

Fig. 1. AcCI is a bee serine protease inhibitor. (A) The nucleotide sequence of *AcCI* cDNA (GenBank accession no. JX899417) and its deduced amino acid sequence. The start codon (ATG) is boxed, and the termination codon is indicated with an asterisk. The putative polyadenylation signal is double-underlined. The predicted signal sequence is underlined. (B) Organization of the *AcCI* gene (GenBank accession no. JX899418). Numbers indicate the position in the genomic sequence. Exons are represented by solid boxes. (C) The alignment of the amino acid sequences of AcCI and other known serine protease inhibitors. The cleavage site of the predicted signal sequence is indicated by a vertical arrow. The conserved cysteine residues are indicated by solid circles. The P1 position is marked with an asterisk. The sources of the aligned sequences were *A. cerana* AcCI (this study, GenBank accession no. JX899417), *A. mellifera* CIL (XP_001121077), *B. terrestris* CIL (NP_001037044), *A. florea* CIL (XP_003696076), *B. impatiens* CIL (XP_003485573), *B. terrestris* CIH (JX667784), and *A. mellifera* AMCI (XP_001120243). The AcCI sequence was used as a reference for the identity/similarity (Id/Si) values.

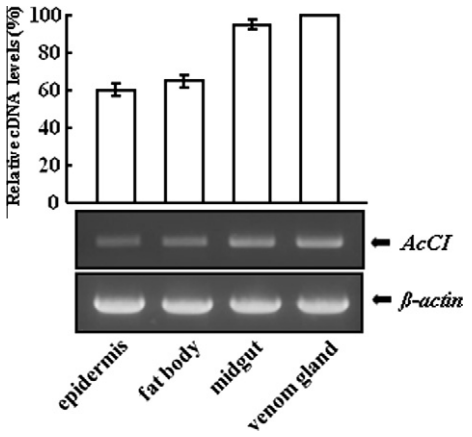

Fig. 2. Expression of *AcCI* in *A. cerana* worker bees. Total RNA was isolated from the epidermis, fat body, midgut, and venom gland of *A. cerana*. The *AcCI* cDNA was amplified by RT-PCR using the total RNA. The resulting fragment was analyzed by electrophoresis on a 1.2% agarose gel (upper panel). The *β-actin* gene was used as an internal control for the RT-PCR (lower panel). The levels of *AcCI* cDNA are the means of three assays, which are calculated relative to those of the expression recorded for the venom glans (shown as 100%). Bars represent the means ± SE.

To further characterize this inhibitor, we expressed recombinant AcCI in baculovirus-infected insect cells (Fig. 3A). The purified recombinant AcCI, which contained 6 additional His residues, was identified as a 16-kDa protein (Fig. 3B), which was much larger than its predicted molecular mass of 7.2 kDa. Several putative *O*-glycosylation sites, but no *N*-glycosylation sites, were found in the sequence of the mature peptide of AcCI. To determine whether AcCI was indeed glycosylated, glycoprotein staining of the recombinant AcCI was performed. The difference between the predicted molecular mass of 7.2 kDa for the mature AcCI peptide and the observed molecular mass of 16 kDa on the SDS–PAGE gel was shown to be due to the presence of carbohydrate moieties (Fig. 3C).

Upon further investigation, we found that recombinant AcCI could inhibit the activity of chymotrypsin (IC_{50}: 24.71 nM), but not trypsin (Fig. 3D). The inhibitory constant (K_i) of AcCI against chymotrypsin was 11.27 nM (Table 1).

3.2. AcCI also acts as an elastase inhibitor

We investigated whether AcCI functions as an antifibrinolytic factor and/or an anti-elastolytic factor. First, we assessed whether AcCI inhibits several other enzymes associated with the hemostatic system. AcCI had no detectable inhibitory effect on factor Xa, thrombin, tPA (Fig. 4A), or plasmin activity (Fig. 4B), indicating that AcCI is not an antifibrinolytic factor.

Next, we assessed whether AcCI exhibits anti-elastolytic activity and found that it inhibited both human and porcine elastases (Fig. 4C). The inhibitory ability of AcCI against human neutrophil elastase (IC_{50}: 38.50 nM) was 1.8-fold stronger than that against porcine pancreatic elastase (IC_{50}: 70.21 nM). Based on the ratio of

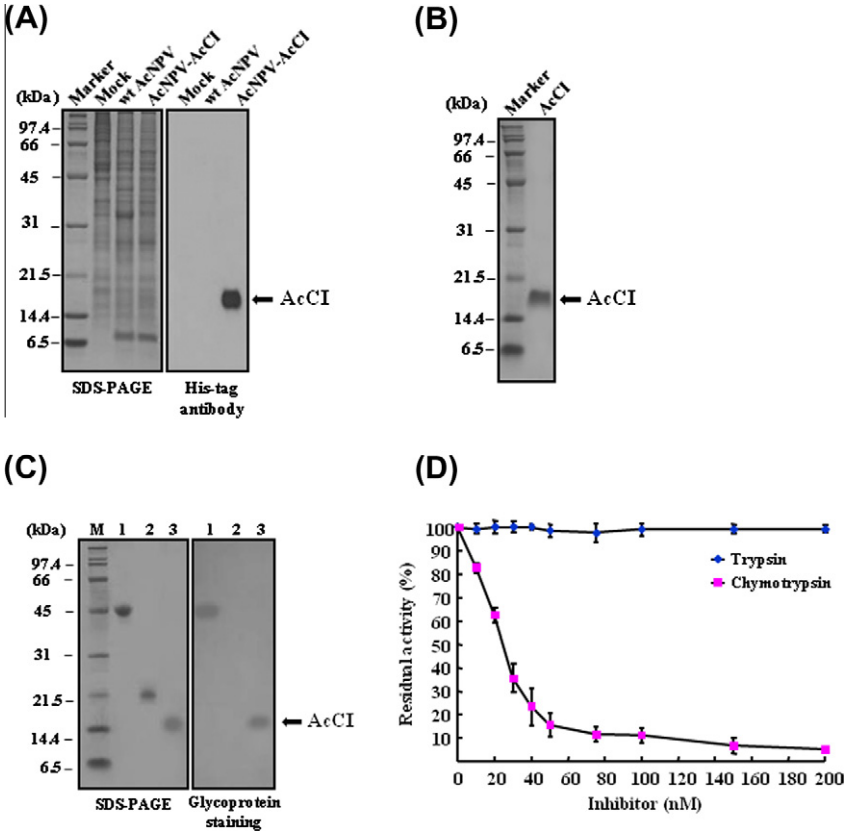

Fig. 3. AcCI is *O*-glycosylated and inhibits chymotrypsin. (A) The expression of recombinant AcCI in baculovirus-infected insect cells. Sf9 cells were infected with recombinant AcNPV-AcCI or wild-type AcNPV and collected at 3 days post-inoculation. Total cellular lysates were subjected to 14% SDS–PAGE (left), electroblotted, and incubated with an anti-His-tag antibody (right). A molecular weight standard was used for size comparisons. The recombinant AcCI is indicated with an arrow. (B) SDS–PAGE of purified recombinant AcCI expressed in baculovirus-infected Sf9 insect cells. (C) Glycoprotein staining of AcCI. Purified AcCI and control protein samples were subjected to 12% SDS–PAGE (left) and then analyzed by glycoprotein staining (right). Horseradish peroxidase (5 μg), a glycosylated protein, was used as a positive control (lane 1). Soybean trypsin inhibitor (5 μg), a non-glycosylated protein, was used as a negative control (lane 2). AcCI (lane 3) is indicated with an arrow. A molecular weight standard was used for size comparisons (lane M). (D) Enzyme inhibition by AcCI. Trypsin or chymotrypsin was incubated with increasing amounts of AcCI, and the residual enzyme activity was then determined (n = 3).

Table 1
Inhibitory activity of AcCI against serine proteases.

Enzyme	Concentration (nM)[a]	IC_{50} (nM)	Ratio[b]	K_i (nM)
Chymotrypsin	100	24.71	0.25	11.27
Human elastase	100	38.50	0.39	61.05
Porcine elastase	100	70.21	0.70	101.89

[a] The concentration of enzymes used in this experiment.
[b] The molar ratio of the IC_{50} to the enzyme concentration.

inhibitor to enzyme, chymotrypsin was inhibited by AcCI more effectively than the two elastases (Table 1). The K_i values of AcCI inhibition against the human and porcine elastases were 61.05 nM and 101.89 nM, respectively (Table 1). These results show that AcCI has both anti-elastolytic and antichymotrypsin functions.

4. Discussion

Although serine protease inhibitors have been isolated from a wide variety of venomous animals, few have been functionally characterized from bees. Among them, chymotrypsin/cathepsin G and trypsin/plasmin inhibitors have been isolated from the honeybee *A. mellifera* [10,11] and bumblebee *B. ignitus* [7], respectively. In this study, we identified AcCI as the first bee-derived serine protease inhibitor that acts as an elastase inhibitor. We hypothesized that AcCI is similar to chymotrypsin inhibitors because, in addition to its putative signal peptide, it contains a mature peptide domain with ten conserved cysteine residues and a P1 site, features that are also present in several chymotrypsin inhibitors [8–11]. The expression patterns of *AcCI* in the tissues examined in this study suggest that AcCI is a functional chymotrypsin inhibitor in both the body and venom of *A. cerana* worker bees. Additionally, AcCI shares protein sequence identities with other functionally characterized bee chymotrypsin inhibitors, such as the *B. terrestris* chymotrypsin inhibitor homolog, which is expressed in the venom gland and fat body [29], and *A. mellifera* AMCI, which has been isolated from the larval hemolymph [10].

Serine protease inhibitors can target trypsin and/or chymotrypsin [7,10]. We therefore expressed recombinant AcCI in baculovirus-infected insect cells and determined that it inhibits chymotrypsin activity. In addition, the recombinant AcCI was *O*-glycosylated, as has also been shown for the *B. terrestris* chymotrypsin inhibitor homolog [29], and was found to contain a Leu residue at the P1 site. Generally, chymotrypsin inhibitors possess a Leu-Met-Phe-Tyr-Trp, or Asn P1 residue [11,14,30]. For example, *A. mellifera* chymotrypsin/cathepsin G inhibitors [10,11] and *Ascaris* chymotrypsin/elastase inhibitors [8,9] have a Met residue at the P1 site. Therefore, our data indicate that AcCI is a member of the chymotrypsin inhibitor family.

In addition to their trypsin/chymotrypsin inhibitory functions, some serine protease inhibitors also exhibit antifibrinolytic [7,19–22] and/or anti-elastolytic activities [8,9,17–19]. We therefore tested whether AcCI may also target additional serine proteases and found that both human neutrophil elastase and porcine pancreatic elastase were inhibited by AcCI. Of note, human neutrophil elastase plays a major role in the regulation of vascular injury and inflammation [26]. However, AcCI had no detectable inhibitory effect on factor Xa, thrombin, plasmin, or tPA enzymes that are associated with the hemostatic system [7]. Our findings are similar to previous findings that *Ascaris* chymotrypsin inhibitors show inhibitory activity against elastases [8,9]. Taken together, our results suggest that AcCI can function as an anti-elastolytic factor.

In summary, we cloned and characterized the functional features of the first honeybee serine protease inhibitor that exhibits inhibitory activity against chymotrypsin and elastases. Given that trypsin/plasmin and chymotrypsin/cathepsin G inhibition are the only known functions of bee-derived serine protease inhibitors [7,10,11], the inhibition of elastases by AcCI appears to be novel.

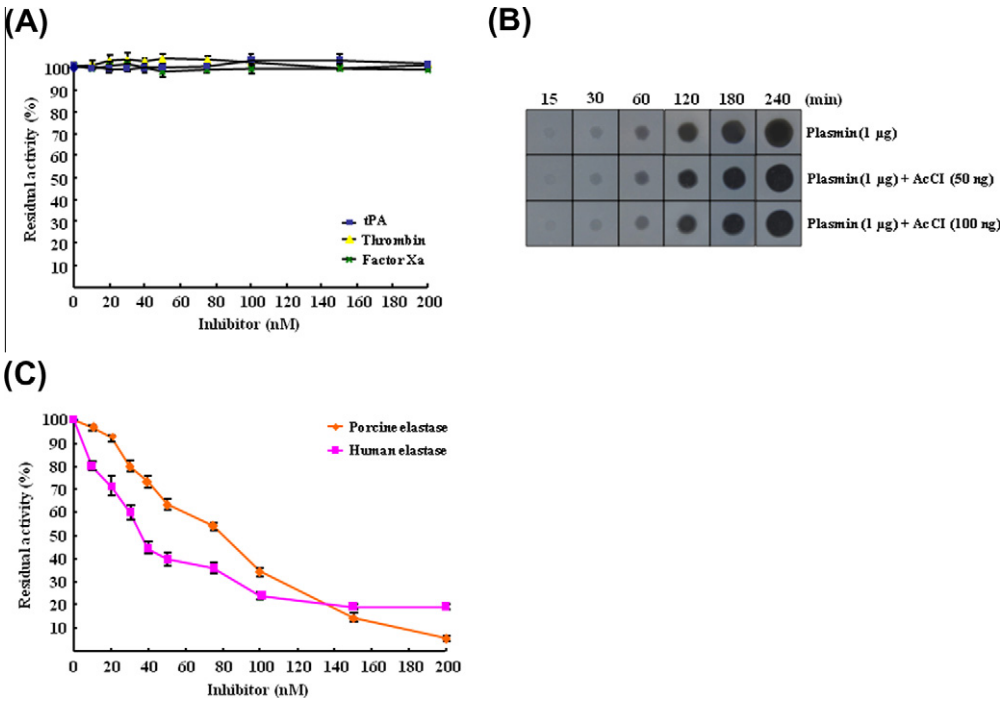

Fig. 4. AcCI inhibits elastase, but not factor Xa, thrombin, tPA, or plasmin. (A) Inhibitory activity of AcCI against several enzymes associated with the hemostatic system. Factor Xa, thrombin, or tPA was incubated with increasing amounts of AcCI, and the residual enzyme activity was determined ($n = 3$). (B) Inhibition assay of AcCI against plasmin. Plasmin alone or a mixture of plasmin and AcCI was dropped onto a fibrin plate and incubated at 37 °C for various periods of time. (C) Inhibitory activity of AcCI against elastases. Human or porcine elastase was incubated with increasing amounts of AcCI, and the residual enzyme activity was determined ($n = 3$).

The finding that AcCI exhibits anti-elastolytic activity not only highlights the potential physiological roles of bee-derived serine protease inhibitors but also provides a novel approach for anti-inflammatory therapies.

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