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Cloning and Expression of cDNA Encoding a Cysteine Protease Inhibitor from Clamworm and Its Possible Use in Managing *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae)

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A cDNA encoding a cysteine protease inhibitor (CPI) was isolated from the cDNA library of clamworm *Perinereis aibuhitensis* Grube. The deduced amino acid sequence analysis showed that the protein had 51%, 48%, and 48% identity with Zgc:153129 from *Danio rerio*, cystatin B from *Theromyzon tessulatum*, and the ChainA, stefin B tetramer from *Homo sapiens*, respectively. The gene was cloned into the intracellular expression vector pET-15b and expressed in *Escherichia coli*. The recombinant CPI (PA-CPI) was purified by affinity chromatography on Ni-charged resin and ion-exchange chromatography on DEAE-Sepharose FF. The relative molecular mass of PA-CPI was 16 kDa as deduced by SDS–PAGE. Activity analysis showed that the recombinant protein could inhibit the proteolytic activity of papain. A constitutive and secretive expression vector was also constructed, and the cDNA encoding CPI was subcloned into the vector for extracellular expression. Western blotting analysis results showed that the PA-CPI was secreted into the medium. Bioassay demonstrated that *E. coli* DH5α harboring pUC18ompAcat-CPI showed a significant difference in mortality to the Asian longhorned beetle *Anoplophora glabripennis* compared with untransformed *E. coli* DH5α and control.

Keywords: Cysteine protease inhibitor, constitutive and secretive expression vector, *Anoplophora glabripennis*, cDNA library, *Perinereis aibuhitensis* Grube

Wood-boring and bark beetles (WBBB) are among the most significant and worldwide forest pests [14]. With numerous invasive species, such beetles represent an important threat to the biosecurity of all forested countries [6, 17, 36]. The most taxa among invasive WBBB are bark and ambrosia beetles (Curculionidae: Scolytinae), longhorn beetles (Cerambycidae), and to a lesser extent other Curculionidae, jewel beetles (Buprestidae), and several other families [9]. The Asian longhorned beetle (ALB), *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae), is a wood-boring beetle in the family Cerambycidae, native to China and Korea [31] as a major tree killer. The ALB grows and develops in a broad range of hardwood trees [35], attacking apparently healthy, vigorous deciduous tree species that primarily include maple (*Acer* spp.), poplar (*Populus* spp.), and willow (*Salix* spp.) [20, 31]. The ALB has caused fatal damages in shelter forests and serious economic loss in North China [48].

With an increase in international trade that involves untreated solid wood packing materials [3, 18, 46], the ALB has been introduced into the North America [19], Europe [21, 34], and Japan [43]. From 1996 to 1998, most interceptions of beetles at ports-of-entry in the U.S.A. were associated with solid wood packing material. Coleoptera accounted for 94% of all pest interceptions, with the Cerambycidae accounting for 37% of all beetle interceptions [7]. The Japanese pine sawyer beetle, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), which is the transmission vector of pine wilt disease (PWD), is also a member of the family Cerambycidae. Both of them have caused heavy losses to the local economy and ecology.

To date, the primary methods used in the ALB eradication program include quarantine, removal of infested trees, and alternatively, trunk or soil injections of imidacloprid in host trees [44]. Because of the increasing public concern over the environmental impact of the pesticides, human health effects, and undesirable effects on non-target organisms, these management strategies are coming under

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more critical consideration [23]. As such, alternative methods need to be developed to control ALBs.

Disruption of insect protein digestion by dietary proteinase inhibitors (PIs) has been suggested as an alternative to pest control [42]. PIs are thought to regulate endogenous proteases that have been isolated and characterized from mammals, plants, insects, and microorganisms [32]. PIs occur naturally in various plants and are believed to be one class of plant defensive proteins against insects [11, 15, 47]. The inhibitors discovered so far are classified as serine, cysteine, aspartic, and metalloprotease inhibitors based on the active amino acids in their reaction centers [4]. The PIs act by binding directly with the proteinases in the insect gut, thereby reducing the insect's digestive capacity, and then causing a reduction in the availability of amino acids necessary for their growth and development [8, 38], or by interfering with important biochemical or physiological processes of insects, such as the proteolytic activation of enzymes or molting of insects [16, 29]. Previous reports showed that there are two major proteinase classes in the digestive systems of phytophagus insects: serine and cysteine proteinases. Lepidopterans have serine proteinases as the major digestive enzymes, whereas coleopterans and hemipterans have cysteine proteinases [39]. There also have been some reports that showed the successful cases for resistance of insects and pests using cysteine or serine PIs, such as the Colorado potato beetle [21] and root-lesion nematodes [34].

Cysteine proteinase inhibitors (CPIs) have been isolated from various animal [33] and plant tissues [25], and many of them have been characterized in terms of protein structure and inhibitory activity. Moreover, CPIs used for insect control mostly come from plants [42]. However, there are only a few reports describing the purification of CPIs from Annelida and applications of CPI in controlling insects. Lefebvre *et al.* [28] isolated a cystatin B from the leech *Theromyzon tessulatum*, which belongs to the Annelida *P. aibuhitensis* Grube, a widespread, shore-inhabiting marine clamworm in Asia. It has been used in Chinese traditional herbal medicine for hundreds of years. In recent years, much attention has focused on the clamworm because it has been found to contain several bioactive peptides [30].

In this study, we constructed a cDNA library of *P. aibuhitensis*, and a cDNA encoding CPI was identified. The gene encoding CPI was cloned into intracellular and extracellular expression vectors, respectively. Then the biological activity of the recombinant CPI and the insectcidal activity of *E. coli* harboring this gene were also investigated.

Materials and Methods

Clamworm

The specimens of *P. aibuhitensis* Grube used to construct the cDNA library were collected at Jiaozhou Bay (Qingdao, China) in June 2006.

Bacterial Strains, Plasmids, and Culture Conditions

E. coli DH10B, *E. coli* DH5α, *E. coli* BL21 (DE3) (Invitrogen, Grand Island, U.S.A.), and *E. coli* JM109 (Promega, Madison, U.S.A.) were used as the hosts for the construction of the cDNA library, cloning, and expression, respectively. Plasmid pAP3neo, pMD-18T Simple (TaKaRa, Dalian, China), and pET-15b (Novagen, Darmstadt, Germany) were used as cloning and expression vectors, and plasmid pUC18 (TaKaRa, Dalian, China) and pACYC184 (New England Biolab, Ipswich, U.S.A.) were used as templates to amplify the target DNA fragments by polymerase chain reaction (PCR). *E. coli* was grown at 37°C in Luria–Bertani (LB) medium supplemented with appropriate antibiotic when required.

Construction of the cDNA Library

P. aibuhitensis Grube was dissected and washed three times with sterile physiological saline solution. Total RNA was extracted with RNAiso Reagent (TaKaRa, Dalian, China), and mRNA was prepared from total RNA with the Oligotex-dT30 mRNA purify Kit (TaKaRa, Dalian, China). Then, total mRNA was reverse transcribed to the first strand of cDNA by using M-LMV reverse transcriptase (Promega, Madison, U.S.A.) and Oligo $(dT)_{16}$. The cDNA was blunted with T4 DNA polymerase and ligated with a *Not*I adaptor. The cDNA was cloned into pAP3neo after being digested with *Not*I. The recombinant plasmids were introduced into *E. coli* DH10B to construct a cDNA library of *P. aibuhitensis*.

Sequencing and Analysis

The transformants were incubated and selected on LB plates supplemented with 100 μg/ml ampicillin (Sigma, St. Louis, U.S.A.), for 12 h at 37°C. Colonies were picked and recombinant plasmids were purified from the transformants using a TaKaRa MiniBEST Plasmid Purification Kit Ver.2.0 (TaKaRa, Dalian, China), and the cDNA inserts were sequenced using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Courtaboeuf, France). The positive clone encoding CPI was screened out from the resulting sequences by homology search using BLAST at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/). The recombinant plasmid was designated as pAP3neo-CPI. The deduced amino acid sequence was assembled using the ExPASy translate tool. The signal sequence was predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP). Homology alignment was performed with the ClustalW program and DNAstar software.

Cloning for Intracellular Expression

According to the sequenced cDNA of CPI, primers CPI-F and CPI-R (Table 1) were designed. PCR was performed to amplify the open reading frame (ORF) encoding the CPI using pAP3neo-CPI as the template, and primers CPI-F and CPI-R. The PCR product was digested with *Nde* and *Xho*, and cloned into the pET-15b vector to generate plasmid pET-15bCPI. The recombinant plasmid was transformed into *E. coli* DH5α. The transformants were coated onto LB agar plates supplemented with 100 μg/ml ampicillin and cultured overnight. The recombinant plasmids were extracted and the insert was verified by restriction enzyme analysis and sequencing.

Intracellular Expression and Purification of CPI

Plasmid pET-15bCPI was introduced into *E. coli* BL21 (DE3) to construct engineering bacteria. A single colony of the engineered bacteria was inoculated into 20 ml of LB broth supplemented with 100 μg/ml ampicillin. The cells were grown overnight at 37°C in

Table 1. Oligonucleotide primers used in this study.

Primer designation[a]	Nucleotide sequence[b]	Description
CPI-F (*Nde*I)	5'-ACATGACTA**CATATG**GAGTCGGCAC-3'	For amplification of CPI from pAP3neo-CPI
CPI-R (*Xho*I)	5'-ACC**CTCGAG**CTAATTATTATTA-3'	
PUC-F (*Eco*RI)	5'-AAATCAATTC**GAATTC**GACGAAAGGGCCTCGTG-3'	For amplification of sequence from pUC18
PUC-R (*Hin*dIII)	5'-AAATCAATTC**AAGCTT**ATTAATGAATCGGCCAAC-3'	
CAT-F (*Hin*dIII)	5'-T**AAGCTT**TGAGACGTTGATC-3'	For amplification of CAT gene from pACYC184
CAT-R (*Pst*I)	5'-TAAGCTT**CTGCAG**GCTTATTATCAC-3'	

[a] The sites of restriction enzyme in parentheses were inserted in the primer for cloning.
[b] The sequences in bold letters are the restriction enzyme sites.

shake cultures. The cultured cells were transferred to 2 l of fresh LB broth medium supplemented with 100 μg/ml ampicillin. The cells were grown at 37°C until the A_{600} reached 1.0. Then, a final concentration of 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of recombinant CPI with 6 His-tag at the N-terminal. After incubation for another 4–6 h at 37°C, with vigorous shaking, the cultured cells were harvested by refrigerated centrifugation at 4°C, 8,000 ×*g* for 10 min.

Cell pellets were resuspended in 100 ml of ice-cold binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole) and disrupted by ultrasonication. The lysate was centrifuged at 12,000 ×*g* for 30 min to remove debris. Clear extracts was loaded onto a Ni-charged affinity chromatography column (GE Healthcare) equilibrated with binding buffer. The column was washed with 10 volumes of binding buffer and 6 volumes of wash buffer (20 mM Tris-HCl, 0.5 M NaCl, 60 mM imidazole) to remove unbound proteins. The bound proteins were eluted with 6 volumes of elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole). Subsequently, the purified protein was dialyzed against TH buffer (20 mM Tris-HCl, pH 8.0) and loaded onto DEAE-Sepharose FF (1.5×5 cm) equilibrated with TH buffer. After the column was washed thoroughly with TH buffer supplemented with 0.1 M NaCl, the recombinant protein was eluted with TH buffer supplemented with 0.3 M NaCl. Purified CPI was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12% polyacrylamide gel by the method of Laemmli [26], and then stained with Coomassie brilliant blue R-250. Protein concentration was determined by the Bradford method [5] using bovine serum albumin as a standard.

Assay of Cysteine Protease Inhibitory Activity

Chromogenic substrate L-BAPNA was used for the enzyme inhibition assays. The activity of papain was assayed using a slight modification of the method of Sharma *et al*. [41]. Briefly, papain (Sigma, Saint Louis, U.S.A.) was pre-incubated at 25°C for 15 min in 0.1 M phosphate buffer [pH 6.0, containing 0.2 mg/ml dithiothreitol (DTT) and 0.5 mg/ml Na_2EDTA] with PA-CPI for different molar ratio and without PA-CPI for control. The reaction was started by adding 50 μl of N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPNA) (2.0 mg/ml) and incubated at 37°C for 10 min, and then stopped by the addition of 200 μl of 30% acetic acid. The absorbance was measured at 405 nm to determine the relative activity of papain.

Construction of the Vector for Constitutive and Secretive Expressions

Initially, we synthesized a nucleotide sequence that sequentially included the Lac promoter (Plac) derived from the pUC18 vector (mutated to constitutive promoter P121 [1, 13]), ompA signal peptide from *E. coli* [12], and His-tag encoding region followed by the multiple cloning site (MCS) from the pET-15b vector, and with the *Eco*RI and *Hin*dIII sites at the two sides of the sequence, respectively. Subsequently, the sequence was inserted into the pMD-18T Simple vector and verified by sequencing. The resultant plasmid was designated as pMD18TompA.

Further on, the nucleotide sequence from 637 to 2,686 bp of the pUC18 vector was amplified by PCR using primers PUC-F and PUC-R (Table 1) as the backbone of the constitutive and secretive vector. The PCR product was gel-purified using a TaKaRa Agarose Gel DNA Purification Kit Ver .2.0 (TaKaRa, Dalian, China) and digested with *Eco*RI and *Hin*dIII. Then, it was ligated with the synthesized fragment isolated by digested plasmid pMD18TompA with the same restriction enzymes to construct pUC18ompA. Subsequently, pACYC184 vector was used as the template to amplify the chloramphenicol-resistant gene using primers CAT-F and CAT-R (Table 1). After being digested with *Hin*dIII and *Pst*I, the PCR product was inserted into the *Hin*dIII and *Pst*I sites of pUC18ompA yielding the constitutive and secretive vector pUC18ompAcat. All inserts were verified by DNA sequencing.

Subcloning for Extracellular Expression

The ORF encoding CPI was obtained by digesting plasmid pET-15bCPI with *Nde*I and *Xho*I, and subcloned into the pUC18ompAcat vector cut with the same enzymes. Therefore, the CPI-encoding gene was under the control of a constitutive promoter and ompA secretive signal peptide. The resultant recombinant plasmid was designated as pUC18ompAcat-CPI. The recombinant plasmid was transformed into *E. coli* DH5α to construct the secretive engineered bacteria.

Extracellular Expression and Purification of CPI

The secretive engineered bacteria harboring plasmid pUC18ompACat-CPI were cultivated for 12 h in LB broth medium supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol at 37°C. The culture was centrifuged at 4°C, 8,000 ×*g* for 10 min. The extracellular CPI in the cell-free culture was precipitated by adding ammonium sulfate slowly to 90% saturation. The precipitated proteins were then collected by centrifugation at 4°C, 10,000 ×*g* for 30 min, and the pellets were dissolved in TH buffer, and dialyzed against binding buffer. Ni-charged affinity chromatography was used for purification of the extracellular CPI, as described above. The eluted fraction was dialyzed against distilled water and then freeze-dried.

SDS–PAGE and Western Blotting

Protein was redissolved in TH buffer, separated by 12% SDS–PAGE, and then transferred onto a PVDF membrane. The membrane was

blocked with 5% skim milk in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), incubated with antiserum of rabbit anti-His-tag, and subsequently incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit immunoglobulin G before visualization using the ECL reagents (Amersham).

Insecticidal Bioassay

The secretive engineering bacteria expressing CPI extracellularly were cultivated at 37°C by shaking in LB broth medium until the final concentration of 2×10^{10} CFU/ml. For insecticidal bioassay, the cultures were sprayed onto short wood sections of poplar. Simultaneously, treatment of wood sections by spraying untransformed *E. coli* DH5α was also done. Those sprayed with LB broth medium were used as the negative control treatment. Third-instar larvae of *A. glabripennis* were placed on the wood sections. Twelve larvae were used for each treatment, and larval mortality was recorded 9 days later. The bioassay was replicated three times and the data were analyzed statistically by using one-way ANOVA and least significant difference (LSD) tests.

GenBank Accession Number

The nucleotide sequence of PA-CPI studied in this work has been deposited in the GenBank sequence database under Accession No. FJ485939.1.

Results

Construction of *P. aibuhitensis* cDNA Library and Gene Screening

The cDNA library was constructed using mRNA isolated from *P. aibuhitensis* tissue. The cDNA fragments were cloned into pAP3neo vector and transformed into *E. coli* DH10B. The library contained approximately 7.8×10^5 clones and the range of insert sizes was 0.5–3.0 kb. Three hundred clones were randomly selected from the cDNA library and sequenced. Homology search for all the sequences using BLAST revealed different classifications of the genes. Among them, we screened out a cDNA encoding CPI (Accession No. FJ485939.1).

```
  1 ATGGAGTCGGCACTTCAACAGAAATCTGTCATGATGTCTGGTGGTTTAGGCCCAACTCTT
    M  E  S  A  L  Q  Q  K  S  V  M  M  S  G  G  L  G  P  T  L   20
 61 CCTGCTGATCCGGAGACGCAAGTTATTTGTGATGAGGTAAAAAACCAGCTGGAGGGCGAA
    P  A  D  P  E  T  Q  V  I  C  D  E  V  K  N  Q  L  E  G  E   40
121 GTTGGACGTAACTTTGCTGAGTACAATGCCATCCTTTTTAGGAGTCAAGTAGTTGCTGGA
    V  G  R  N  F  A  E  Y  N  A  I  L  F  R  S  Q  V  V  A  G   60
181 ACCGTCCTCTTCAT CAAAGTGCACGTTGGCCATGCTGATTACAT TCATA TTCGCAT CTTC
    T  V  L  F  I  K  V  H  V  G  H  A  D  Y  I  H  I  R  I  F   80
241 ATACCACTGCCAGGTCCAGGAAAAACCAGTCTTGGAGGGTACCAACTACACAAGACTAAG
    I  P  L  P  G  P  G  K  T  S  L  G  G  Y  Q  L  H  K  T  K  100
301 GATGATCCAAT CAAAT ATTTT A ATAA TAATTAG
    D  D  P  I  K  Y  F  N  N  N  *                              110
```

Fig. 1. Nucleotide and deduced amino acid sequences of PA-CPI from *P. aibuhitensis.*
Numbers in the left and right margins correspond to the first nucleotide and last amino acid in the line, respectively. The stop codon is indicated by an asterisk.

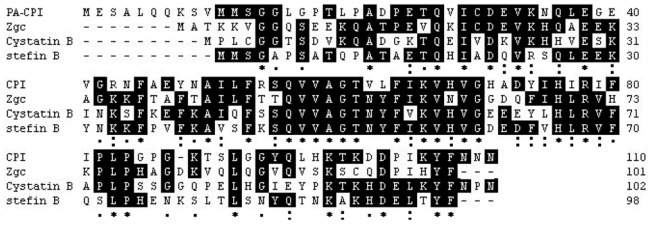

Fig. 2. Alignment of the deduced amino acid sequence of PA-CPI from *P. aibuhitensis* with other homologous proteins.
Asterisks and black shade indicate identity; colons and dots indicate decreasing degrees of conservative substitutions.

Nucleotide Sequence and Deduced Amino Acid Sequence

The nucleotide sequence of the CPI-encoding gene was submitted to GenBank under Accession No. FJ485939.1. Analysis of the nucleotide sequence revealed an ORF of 333 bp that encodes a protein of 110 amino acid residues with a calculated molecular mass of 16.1 kDa and pI value of 6.39 (Fig. 1). The deduced amino acid sequence of the PA-CPI was compared with the published sequences using BLASTP analysis, and the result showed a similarity of 51%, 48%, and 48% identities with Zgc:153129 from *Danio rerio* (Accession No. AAI24646), cystatin B from *Theromyzon tessulatum* (Accession No. AAN28679), and ChainA, stefin B tetramer from *Homo sapiens* (Accession No. 2OCT_A), respectively (Fig. 2).

Intracellular Expression and Purification of PA-CPI

The ORF encoding CPI was amplified by specific primers CPI-F and CPI-R (Table 1), and then cloned into expression vector pET-15b to construct pET-15bCPI. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) for expression. There was no signal peptide with the PA-CPI (predicted with SignalP) and thus it was expressed

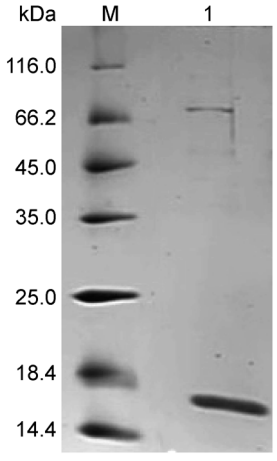

Fig. 3. 12% SDS–PAGE analysis of purified intracellular PA-CPI.
Lane M, molecular mass standards (Fermentas); Lane 1, recombinant CPI.

intracellularly. The recombinant protein was overexpressed by induction with 1.0 mM IPTG and the His-tag fused CPI was purified by Ni-charged affinity chromatography followed by ion-exchange chromatography. The apparent molecular mass was inferred to be approximately 16 kDa by SDS–PAGE (Fig. 3).

Inhibitory Activity of Recombinant CPI

The inhibitory activity of PA-CPI was investigated using papain as the proteolytic enzyme and L-BAPNA as the substrate. The protocol was performed as described previously. As shown in Fig. 4, the relative activity of papain decreased gradually with the increasing molar ratio between PA-CPI and papain. When the molar ratio reached to 2, only 6% of relative activity of papain remained.

Description of Constitutive and Secretive Vector pUC18ompAcat

To construct a constitutive and secretive vector, the lac operator was mutated with two nucleotides to turn the inducible promoter into a constitutive promoter (Fig. 5A), and the new promoter was designated as P121. The nucleotide sequence including P121, ompA signal peptide coding region, and His-tag coding region followed by MCS was synthesized as described above. The synthesized fragment with its size of 629 bp was to ensure the constitutive and secretive characteristics of the vector (Fig. 5B). Subsequently, the synthesized fragment was ligated into the pUC18 backbone amplified by primers PUC-F and PUC-R. The chloramphenicol-resistant gene was inserted into this plasmid to generate the final vector. The constructed vector, pUC18ompAcat, is a 3.5 kb plasmid with an ampicillin-resistant gene (Amp^R) and a chloramphenicol-resistant gene (Cm^R) as selection markers. Its encoded His-tag can make the purification of fusion protein simpler (Fig. 5C).

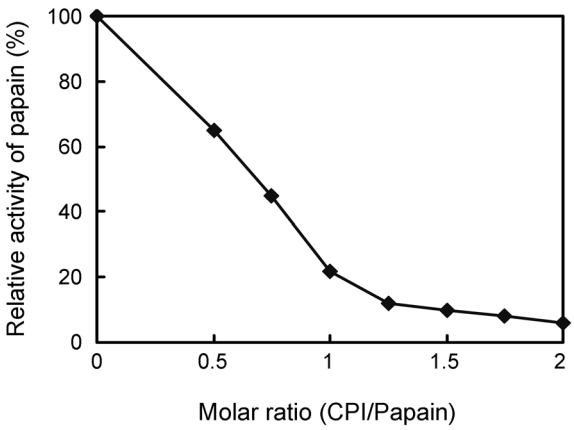

Fig. 4. Inhibition of recombinant CPI against papain.
With the increase of molar ratio between PA-CPI and papain, the relative activity of papain was assayed. L-BAPNA was used as the substrate.

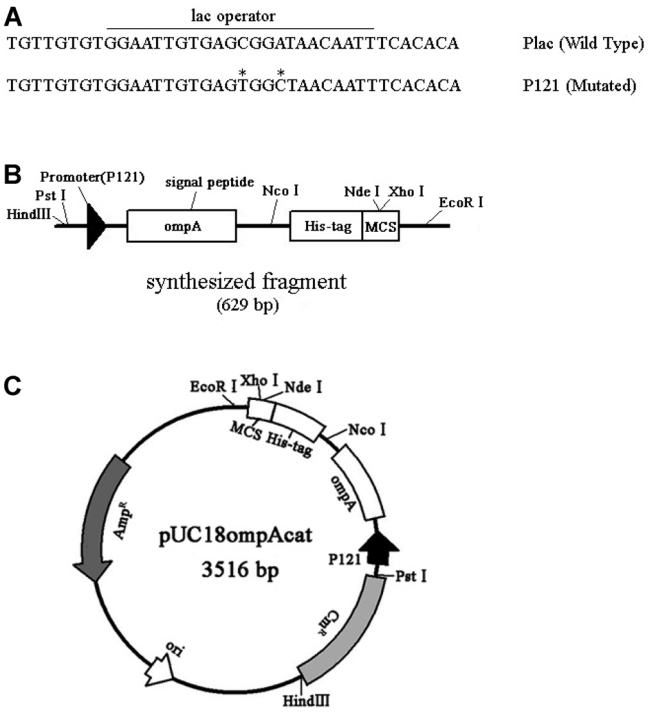

Fig. 5. Description of constructed vector pUC18ompAcat.
A. The mutated sequence in the lac operator. The overlined sequence is the lac operator, and the nucleotides with asterisks are the mutated nucleotides. **B.** The synthesized fragment and restriction enzyme sites. **C.** The map of constructed vector pUC18ompAcat.

Extracellular Expression and Purification of CPI

The ORF encoding CPI was subcloned into pUC18ompAcat to generate expression plasmid pUC18ompAcat-CPI. Consequently, the recombinant CPI was expressed constitutively and secretively under the control of P121 promoter in the absence of IPTG. The secreted PA-CPI fused with His-tag at its N-terminal in the cell-free culture was partially purified by ammonium sulfate precipitation and Ni-charged affinity chromatography. There was an apparent protein with molecular mass of approximately 16 kDa as analyzed by SDS–PAGE (Fig. 6A). To confirm that the partially purified protein was recombinant PA-CPI, the protein was analyzed by Western blotting using anti-His-tag antiserum. The results showed that the purified protein was indeed PA-CPI (Fig. 6B).

Bioassay

The insecticidal activity of the secretive engineered bacteria expressing CPI extracellularly was tested against the larvae of *A. glabripennis.* Twelve larvae were tested for each treatment and the mortality was recorded 9 days after treatment. As shown in Fig. 7, larval mortality of the group treated with the engineered bacteria was 46%, which was significantly different ($p<0.01$) from those in the groups treated with untransformed *E. coli* and LB medium.

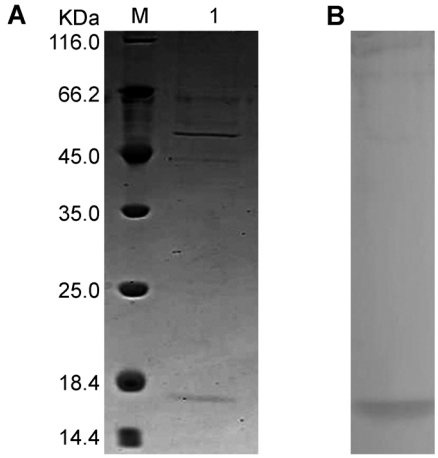

Fig. 6. Secretive expression of PA-CPI using vector pUC18ompAcat. **A.** Lane M, Proteins standard; Lane 1, Partially purified PA-CPI. **B.** Detection of PA-CPI by Western blotting analysis.

Discussion

Until now, several practices have been used to prevent or reduce the damage of *A. glabripennis*. However, there are no satisfactory methods when effect and security are both considered. CPIs have been widely used in controlling insect pests such as some coleopterans [27].

In the present study, a cDNA encoding CPI was screened out from the cDNA library of *P. aibuhitensis* Grube, which had an ORF of 333 bp and encoded a protein of 110 amino acid residues. The deduced amino acid sequence was analyzed with BLASTP, and the putative conserved domains were detected. The result showed that the protein belongs to the CY superfamily, which includes stefins, cystatins, kininogens, and phytocystatins [2, 45], and have a similarity of 51%, 48%, and 48% with Zgc:153129, cystatin B, and Chain A, stefin B tetramer, respectively. It was an intracellular protein, based on the lack of signal peptide deduced by signalP.

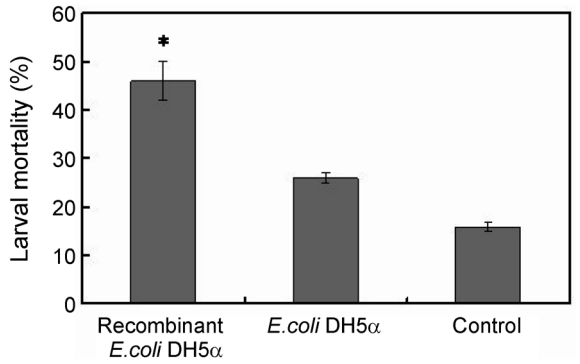

Fig. 7. Insecticidal bioassay of *E. coli* DH5α secretively expressing PA-CPI against larvae of *Monochamus alternatu.*
Significant difference from those in groups treated with untransformed *E. coli* DH5α and LB at $p<0.01$ is indicated as an asterisk in the figure.

For the purpose of insect control, constitutive expression and extracellular secretion are needed for PA-CPI. However, *E. coli*, with a few exceptions, does not secrete proteins out of the cells. To solve this problem, recombinant proteins were usually expressed fused with a signal peptide [10]. Yet, the availability and efficiency of active transport systems are not universal. In the present study, we constructed a secretive and constitutive plasmid, in which an ompA signal peptide was chosen for this purpose. OmpA is a signal sequence of the *E. coli* outer membrane protein, and the advantage of using the ompA signal system is that an expressed protein could have its native N-terminal amino acid sequence by signal peptide cleavage [22, 24, 37]. To achieve the constitutive expression, a lac operator was mutated with two nucleotides to change the inducible promoter into a constitutive promoter (P121). For easy purification and identification of secreted protein, a His-tag coding region was inserted between the coding region of the OmpA signal peptide and MCS. Based on our results, we can see that recombinant PA-CPI, fused with His-tag at the N-terminal, was constitutively expressed and secreted by *E. coli* harboring pUC18ompAcat-CPI. The transformed *E. coli* showed better insecticidal activity compared with *E. coli* alone. *E. coli* alone could also kill *A. glabripennis* larvae to some extent, probably due to toxins it secreted. For further study, we hope that transformed *E. coli* could also be applicable to control other Cerambycidae such as *Monochamus alternatu.*

Moreover, a chloramphenicol-resistant gene was inserted into the constructed vector so that it could be adapted to more bacteria. The work of transformation of more environmentally friendly microorganism such as *Bacillus subtilis* and attempts to increase the efficiency of expression and secretion of PA-CPI in engineered bacteria are in progress.

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