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Abstract Antimicrobial peptides (AMP), both synthetic and from natural sources, have raised interest recently as potential alternatives to antibiotics. Cyto-insectotoxin (*Cit1a*) is a 69-amino-acid antimicrobial peptide isolated from the venom of the central Asian spider *Lachesana tarabaevi*. The synthetic gene *Cit1a* fused with the EGFP gene was expressed as the EGFP-Cit1a fusion protein using a cysteine protease deleted *Bombyx mori* nucleopolyhedrovirus (BmNPV CP^-) bacmid in silkworm larva and pupa. The antimicrobial effect of the purified protein was assayed using disk diffusion and broth microdilution methods. The minimum inhibitory concentration of EGFP-Cit1a was also measured against several bacterial strains and showed similar antimicrobial activity to that of the synthetic Cit1a reported earlier. The EGFP-Cit1a fusion protein showed antibiotic activity toward Gram-positive and Gram-negative bacteria at the micromolar concentration level. These results show that active Cit1a can be produced and purified in silkworm, although this peptide is insecticidal. This study demonstrates the potential of active Cit1a purified from silkworms to use as an antimicrobial agent.

Keywords Cyto-insectotoxin (Cit1a) · Antimicrobial peptides · Silkworm · *Bombyx mori* nucleopolyhedrovirus

Introduction

The widespread overuse and inappropriate use of of antibiotics in medical practice inevitably leads to the emergence of resistant bacterial strains (Wright 2007) and antibiotic resistance is increasing at a rate that far exceeds the pace of the development of new antibiotics (Giuliani et al. 2007). The emergence of multidrug-resistant strains of different pathogens makes the need for the discovery of new antimicrobial agents increasingly important (Aziz and Wright 2005; Hayakawa et al. 2012). To overcome this problem, the development and adoption of new antibiotics is required. Antimicrobial peptides (AMP), both synthetic and native forms, have raised interest as antimicrobial agents (Giuliani et al. 2007). Among potential candidates for new antimicrobial agents, AMPs deserve special attention (Yount and Yeaman 2012; Hancock and Sahl 2006). AMPs are small polypeptide molecules (Yeaman and Yount 2003) and are found in a broad spectrum of organisms, from bacteria to vertebrates. AMPs most likely belong to the most ancient defense systems of multicellular organisms. Natural AMPs have been isolated from different organisms, ranging from bacteria to higher eukaryotes (Lazarev et al. 2011). In most cases, AMPs are believed to directly bind to target cell membranes at micromolar concentrations, which lead to functional and/or structural disturbance of the cell membrane; this mechanism implies a low probability of bacteria acquiring resistance to AMPs (Yeaman and Yount 2003).

In particular, Spider venoms may concurrently contain several dozen AMPs with different structures and consequently, possess a broad spectrum of activity (Kozlov et al. 2006; Vassilevski et al. 2008; Vassilevski et al. 2009). Recently, Vasslevski et al. (2008) identified cyto-insectotoxin (Cit1a), a novel AMP from the venom of the Central Asian spider (*Lachesana tarabaevi*), which represents a unique class of sider venom constituents. Cit1a is a linear cationic peptide with 69 amino acid residues and

represents an attractive molecule to combat intracellular pathogens as Cit1a has shown high antibacterial activity and a significant decrease in *Chlamydia trachomatis* viability inside infected cells (Polina et al. 2012). Lazarev et al. (Polina et al. 2012; Lazarev et al. 2013) characterized Cit1a as an antimicrobial and insecticidal peptide. Cit1a has low toxicity as shown by negligible toxicity to HEK293 cells and suppressed *Chlamydia* infection in the HEK293 cell line. Therefore, Cit1a is a potential agent for gene therapy for *Chlamydia* infection (Lazarev et al. 2011). Cit1a has the potential to provide an important breakthrough and form the basis for a new class of antibiotics belonging to the linear amphipatic peptide class.

The wide range of Cit1a activity suggests that this peptide may be used as an antimicrobial and pesticidal agent in the future. Since AMPs are usually short peptides, chemical synthesis could be one approach for producing them. However, a cost-effective and scalable method for large-scale production is required in order to commercialize the AMP (Ramos et al. 2013). AMPs can be prepared by solid phase peptide synthesis (Merrifield 1963), although to produce peptides in this manner involves significant synthesis costs, particularly for large-scale purposes (Wang et al. 2011). Preparative isolation of AMPs from natural sources and chemical synthesis is not economical (Hancock and Sahl 2006). Recombinant production systems would enable the production of peptides and proteins in various expression systems and allow for the large-scale production of AMPs to be economically viable. Antimicrobial peptides are produced as a fusion protein in heterologous hosts to neutralize their innate toxic activity and increase their expression levels (Wang et al. 2011). Large quantities of AMPs are required for pharmaceutical applications (Fan et al. 2010). Numerous expression systems currently have been used for the economical production of antimicrobial peptides (Ingham and Moore 2007).

Silkworm (*Bombyx mori*) is one of the most promising systems used for the production of recombinant AMPs (Liu et al. 2013; Fukushima et al. 2013). Recombinant proteins and peptides have been successfully produced in silkworm larvae or pupae and have been used for academic and industrial purposes, with several recombinant proteins having already been commercialized (Kato et al. 2010). There have been two systems, *Bombyx mori* nucleopolyhedrovirus and transgenic systems, which used silkworms for recombinant protein expression (Kato et al, 2010; Tomita, 2011). In this study, we used the silkworm in BmNPV bacmid system, for the expression and production of an AMP (Cit1a) which could potentially be used as a therapeutic agent for *Chlamydia* infection and as a potential pesticide. Green fluorescent protein, (EGFP) which has no antimicrobial activity, was fused with Cit1a for expression in silkworms.

Materials and Methods

Construction of recombinant BmNPV bacmid

The oligonucleotide sequences of *Cit1a* (accession number FM165474) was purchased from Eurofins MWG Operon (Tokyo, Japan) and the *Cit1a* gene was amplified by polymerase chain reaction (PCR) using the primer set FLAG-Cit1a-F and Cit1a-xba-R (Table 1, primer 1, 2). The EGFP fragment was also amplified as a DNA template from HPV174-EGFP *E. coli* BmDH10Bac (Palaniyandi et al. 2013) by PCR using the primer set Eco-EGFP-F and EGFP-FLAG-R (Table 1, primer 3, 4). Each amplified fragment was purified using GFX PCR and Gel Band Purification Kit (GE Healthcare, Chicago, USA) and fused to each other by PCR to obtain an *EGFP-Cit1a* fusion gene. After 10 cycles of PCR, the two primer sets (Eco-EGFP-F and Cit1a-xba-R, primer 1, 5) were added for amplification of the fusion fragment (EGFP-

111 Cit1a). The amplified fusion fragment was purified using GFX PCR and Gel Band
112 Purification Kit (GE Healthcare, Chicago, USA) and inserted at the *Eco*R1-*Xba*1 site
113 in pFastBac1 (Life Technologies, Carlsbad, CA, USA) following the ligation protocol.
114 The amplified *EGFP-Cit1a* fragment and pFastBac1 fragment were ligated in a
115 reaction mixture containing 30 ng of *EGFP-Cit1a* fragment, 78 ng of pFastBac1
116 fragment and 1 μl of T4 DNA ligase, followed by incubation at 16°C for 16 h.
117 Recombinant pFastBac1 was checked by PCR, electrophoresis, and sequencing. The
118 resulting recombinant pFastBac1 was transformed into the *E. coli* strain BmDH10Bac
119 CP^- (Hiyoshi et al. 2007) and cultivated at 37°C for 36 h. The recombinant BmNPV
120 CP^- bacmid DNA was extracted from *E. coli* cells, confirmed by PCR and was
121 designated as rBmNPV CP^-/EGFP-Cit1a bacmid.

122 Expression of EGFP-Cit1a fusion protein in silkworm

123 A recombinant BmNPV CP^- bacmid DNA was prepared by alkaline extraction, as
124 described in the Bac-to-Bac manual (Life Technologies). Ten micrograms of
125 extracted rBmNPV CP^-/EGFP-Cit1a bacmid, together with a helper plasmid, were
126 mixed with 1/10 volume of 1, 2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl
127 ammonium bromide (DMRIE-C) reagent (Life Technologies) and incubated at room
128 temperature for 30 min. This mixture (10 μg of DNA, 50 μl) was injected into the
129 abdominal section of the silkworm pupa with a needle (26 gauge) and syringe. The
130 DNA-injected silkworm pupae were incubated at 25°C in a humidified (65%)
131 environment for 4 to 6 days. The infected pupa was homogenated with Tris-buffered
132 saline (TBS, pH 7.4) containing 0.1% Triton X-100 (TBS-TX100) followed by
133 sonication and the homogenate was stored at −80°C until use. For silkworm larva, 50
134 μl of pupae homogenate diluted with phosphate-buffered saline (PBS, pH7.4) by 25

135 times was injected into each larva. The injected silkworm larvae were reared using
136 Silkmate 2S (NOSAN Co. Yokohama, Japan) as a diet at 25°C in a humidified (65%)
137 environment for 3 to 5 days, followed by collection of the hemolymph and fat body
138 from the silkworm larvae. Collected hemolymph and fat body were also stored at
139 −80°C until use.

140 Confocal laser scanning microscopy

141 Small pieces of fat body were collected from rBmNPV CP$^-$/EGFP-Cit1a bacmid-
142 injected silkworm larva and pupa for detecting the expressed EGFP-Cit1a fusion
143 protein. The samples were taken from both rBmNPV CP$^-$/EGFP-Cit1a bacmid-
144 injected and mock (control) silkworm larva and pupa. All samples were washed three
145 times with PBS and cells were permeabilized using 0.1% Triton-X100 in PBS for 20
146 min. The cells were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence
147 was detected using a confocal laser scanning microscope (LSM 700, Zeiss, Jena,
148 Germany) and images were analyzed by Zen 2010 software.

149 SDS-PAGE and western blot analysis

150 SDS-PAGE and Western blot were carried out according to previously published
151 methods (Palaniyandi et al., 2013). For Western blot, mouse anti-FLAG M2 antibody
152 (Sigma-Aldrich Japan, Tokyo, Japan) was used as the primary antibody to detect the
153 EGFP-Cit1a fusion protein at 1:10,000 dilution. Sheep anti-mouse IgG antibody (GE
154 healthcare Japan, Tokyo, Japan) was used as the secondary antibody at a 1:10,000
155 dilution.

156 Protein concentration was measured using the BCA protein assay kit (Thermo
157 Fisher Scientific, Rockford, IL, USA).

158

159 Purification of EGFP-Cit1a fusion protein from silkworm larvae and pupae

160 The fat bodies collected from 10 silkworm larvae were suspended in 25 ml of ice-cold
161 TBS buffer (pH 7.4) and lysed by sonication 3 times for 30 s each time with 1 min
162 intervals. For silkworm pupae, 10 pupae were homogenized with TBS-100X Triton
163 (0.1%) followed by sonication. The sample was then centrifuged at 20,000 *g* for 20
164 min and the supernatant was filtered using a 0.45 μm filter. The collected filtrate was
165 used for affinity purification using anti-DDDDK tagged protein purification gel
166 (Medical and Biological Laboratories Co., LTD, Nagoya, Japan). The anti-DDDDK
167 tagged protein purification gel was equilibrated with TBS buffer prior to use. The
168 collected supernatant was mixed with 1 ml of gel and gently stirred at 4°C for 1 h.
169 This mixture was centrifuged at 2500 *g* for 5 min and the precipitated resin was
170 washed with 36 ml of TBS buffer. Proteins bound to the resin were eluted with elution
171 buffer (0.1 M glycine, pH 3.5). The purified protein was detected and confirmed using
172 CBB staining and Western blot analysis. The EGFP was removed from the EGFP-
173 Cit1a fusion protein using recombinant entrokinase (rEK; Novagen, Darmstadt,
174 Germany) according to the manufacturer's instructions. Fifty micrograms of purified
175 fusion protein sample was digested with 1 unit of rEK at room temperature for 16 h.
176 The product was analyzed by SDS-PAGE.

177 Mass spectrometry analysis

178 The molecular mass of the EGFP-FLAG-tagged Cit1ait1a was determined by SDS-
179 PAGE and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)
180 mass spectroscopy. The MALDI-TOF mass spectrum was acquired on an AutoFlex

(Bruker Daltonics, Germany) and measured in linear mode using 20-kV ion acceleration without postacceleration. The spectrum was recorded at a detector voltage of 1.65 kV and was the averaged result of at least 300 laser shots. The matrix was 2-hydroxy-5-methoxybenzoic acid (sDHB). The sample was dissolved in 0.1% Trifluoroacetic acid (TFA): acetonitrile (2:1 v/v) and mixed with the matrix solution (1:4 v/v). The mixture (1 μl) was put on a stainless target and crystallized at room temperature. A mass calibration procedure was employed prior to the analysis of a sample using protein calibration standards II (Bruker Daltonics, Germany).

Antimicrobial assays

The antimicrobial effect of EGFP-Cit1a was investigated using disk diffusion and broth microdilution methods, which are standard methods recommended by the Clinical and Laboratories Standards Institute (CLSI) for measuring *in vitro* susceptibility of bacteria to antimicrobial agents used in clinical settings (CLSI 2009). Although disk diffusion is the most popular method used to examine the antimicrobial activity of natural antimicrobials agents (Kim and Kim 2007; Mayachiew et al. 2010), the foremost disadvantages of this method are the inability to measure the minimal inhibitory concentration (MIC) value and the difficulty in examining the susceptibility of fastidious and slow-growing bacteria (Wilkins and Thiel 1973; Dickert et al. 1981). Moreover, unlike antimicrobial agents used in clinical settings, there are currently no standard CLSI interpretive criteria of disk diffusion results to support natural antimicrobial susceptibility testing. Thus, disk diffusion is unable to explain the zone diameter that it generates for natural antimicrobials (Jiang et al. 2011). For these reasons, we used two standard methods in this study: the microdilution method for measuring the MIC values and the disk diffusion method for visualization of the

inhibitory effects of EGFP-Cit1a against bacteria.

For the disk diffusion method, the bacterial inoculum was adjusted to ~10^5 colony-forming units (CFU)/ml and inoculated onto the entire surface of a Luria-Bertani (LB) agar plate. The paper disks (BD Diagnostic Systems, New Jersey, USA) were impregnated in 6 mm diameter circles with 12 µl diluted EGFP-Cit1a solutions and placed on the LB agar plate. The plates were then incubated aerobically overnight at 37°C and subsequently the inhibition zone was observed. A series of diluted EGFP-Cit1a solution in PBS was used, including a positive control using ampicillin for Gram-negative and chloramphenicol for Gram-positive bacteria. *Bacillus subtilis* (NBRC13719, NITE, Kisarazu-shi, Chiba, Japan) and *Staphylococcus aureus* (NBRC 100910, NITE) as Gram-positive and *Pseudomonas aeruginosa* (NBRC12689, NITE) and *Escherichia coli* W3110 (NBRC12713) as Gram-negative were kind gifts from Professor Shinya Kotani.

MIC determination of EGFP-Cit1a was performed using a microtiter broth dilution assay as described by Vassilevski et al. (2008). In this method, antimicrobial activity was conducted with a bacterial strain in sterilized 96-well plates in a final volume of 100 µl composed of 50 µl of suspension containing 10^5 bacteria/ml in LB culture medium and 50 µl of the peptide in serial two-fold dilutions in PBS. Mid-exponential phase cultures were diluted to a final concentration of 10^5 CFU/ml. Fifty microliters of purified rEGFP-Cit1a was added to 50 µl of the diluted bacterial suspension (~10^5 CFU/ml). The peptides, a non-treated control with PBS, a positive control with ampicillin or chloramphenicol and a negative control with BSA were tested in triplicate. The microtiter plates were incubated overnight at 37°C and the inhibition of growth was determined by measuring the absorbance at 595 nm. MIC is expressed as the lowest concentration of peptide that causes 100% growth inhibition

(Vassilevski et al. 2008).

Results

Construction of an expression recombinant BmNPV bacmid

To express the *Cit1a* from *Lachesana tarabaevi* in silkworms, EGFP-Cit1a fusion protein was expressed according to Figure 1A. *Cit1a* was fused to *egfp* as a reporter gene by PCR through the FLAG tag sequence, which was checked by agarose gel electrophoresis (Figure 1B). The fusion gene was successfully cloned into the transfer vector (pFastBac1) (Fig. 1A). The generated recombinant pFastBac1-Cit1a was verified by amplifying the target region using PCR and sequencing (data not shown). The recombinant pFastBac1-Cit1a was transformed into an *E. coli* BmDH10Bac competent cell, and finally recombinant BmNPV CP^-/EGFP-Cit1a bacmid was constructed.

Expression of EGFP-Cit1a fusion protein from silkworm larvae and pupae

For the expression and purification of the fusion protein, recombinant BmNPV CP^-/EGFP-Cit1a bacmid was prepared and injected into silkworm larvae and pupae. After 4 to 6 days, fat bodies collected from the infected larvae were suspended in TBS and sonicated to extract the expressed EGFP-Cit1a fusion protein. BmNPV CP^-/EGFP-Cit1a bacmid-injected pupae were also homogenized with TBS. The specific EGFP fluorescent band on SDS-PAGE was observed in the homogenate of the BmNPV CP^-/EGFP-Cit1a bacmid-injected pupae and larvae's fat body, but not in the larval hemolymph and mock-injected fat body (Fig. 1C). In addition, the expressed EGFP-Cit1a fusion protein was confirmed in Western blot analysis (Fig. 1D). The theoretical molecular weight of the GFP-Cit1a fusion protein was ~36 kDa, which is similar to

253 the detected molecular weight of the fusion protein and no band was observed from
254 the mock-injected silkworm (Fig. 1D).
255 Confocal laser scanning microscopy was also used to further confirm the
256 expressions of the EGFP-Cit1a fusion protein in silkworm larvae and pupae. EGFP
257 fluorescence was observed in the larval (Fig. 2A) and pupal fat body (Fig. 2C) of the
258 silkworm. Mock-infected silkworm larvae and pupae did not show any EGFP
259 fluorescence (Fig. 2B and D).
260
261 Purification of EGFP-Cit1a fusion protein from silkworm larvae and pupae
262 The expressed EGFP-Cit1a fusion protein was purified from the fat bodies of
263 the silkworm larvae and pupae using DDDDK tagged purification gel. This
264 purification gel facilitates the purification of FLAG-tagged proteins equally to anti-
265 FLAG M2 agarose gel.Several proteins tagged with FLAG have been shown to be
266 successfully purified using this gel (Deo et al. in press). A single band was detected
267 by CBB staining and Western blot (Fig. 3A) in the eluted fraction of the BmNPV CP^-
268 /EGFP-Cit1a bacmid-injected larvae's fat body. In the same manner, purified samples
269 (elution 1~3 of BmNPV CP^-/EGFP-Cit1a bacmid-injected pupa's homogenate)
270 showed a single band in CBB staining and Western blot (Fig. 3B). In SDS-PAGE
271 analysis, the band of EGFP-Cit1a was detected below 37 kDa (Fig. 3). The molecular
272 weight of the EGFP-Cit1a fusion protein, calculated from its amino acid sequence, is
273 36.067 kDa. In a previous paper, Cit1a has a 60% alpha-helix structure in 25 mM
274 SDS solution (Vassilevski et al., 2008), suggesting that Cit1a has its native
275 conformation in the sample buffer of SDS-PAGE to some extent and its structure may
276 cause the difference between the molecular weight estimated from its amino acid
277 sequence and that detected by SDS-PAGE. In addition, this protein purified from

BmNPV CP$^-$/EGFP-Cit1a bacmid-injected larvae's fat body was investigated by MALDI-TOF mass analysis. The MALDI-TOF mass spectrum demonstrated a main peak at m/z 37338 (Fig. 4). Another peak was detected at m/z 28622. This low molecular weight corresponded to that of EGFP tagged with the FLAG sequence estimated from its amino acid sequence (28197). However, no band was observed in the SDS-PAGE or Western blot. These data suggest that this low molecular weight peak might be caused during the MALDI-TOFMS experiment or it may be possible that the purified protein still contained a significant amount of contaminated proteins. Around the peak at m/z 37338, several peaks were also detected which formed a broad peak. These data also suggest that the purified EGFP-Cit1a fusion protein had several variants. Spider peptide toxins are sometimes post-translationally modified by palmitoylation, C-terminal trimming and C-terminal amidation (Windley et al., 2012). C-terminal amidation was not detected in the native Cit1a (Vassilevski et al., 2008), therefore, it is most reasonable that the EGFP-Cit1a heterogeneity may be caused by C-terminal trimming.

To confirm the fusion of Cit1a with EGFP via the FLAG tag sequence, the purified fusion protein was treated with rEK and the difference between the molecular weights of the rEK-treated and non-treated samples were investigated in SDS-PAGE. rEK recognizes the DDDDK sequence in the FLAG tag sequence and can cleave the EGFP-Cit1a fusion protein into EGFP-FLAG and Cit1a. The rEK-treated fusion protein showed two bands (~27 and ~8 kDa) (Fig. 5). The rEK digestive experiment confirmed that Cit1a was expressed fused with EGFP in the silkworm and could be separated from EGFP. The expression level between the silkworm larval fat body and pupa was compared in Western blot analysis. The amount of purified EGFP-Cit1a fusion protein was 10 μg/pupa from pupa and 7 μg/larva from the larval fat body. In

this study, EGFP was adopted as a fusion partner of Cit1a and the functional analysis of EGFP-Cit1a purified from silkworm fat body was performed in the next section.

Antimicrobial activity of Cit1a

Extensive biological studies were performed only for the synthetic Cit1a, which was tested on a number of Gram-positive and Gram-negative bacteria, and approximate MIC (low micromolar against *E. coli*) values were determined for the peptide (Lazarev et al. 2011; Lazarev et al. 2013; Polina et al. 2012; Vassilevski et al. 2008). The antimicrobial activity of Cit1a was evaluated using purified EGFP-Cit1a fusion protein, based on the clear inhibition zone surrounding the paper disks. A clear inhibition zone was observed in *E. coli* W3110*, Bacillus subtilis*, and *Pseudomonas aeruginosa* bacterial growth (Fig. 6A, C and D). However, no inhibition zone was found in *Staphylococcus aureus* (Fig. 6B). The MIC values were determined by a micro-dilution method. The MIC results indicated that *E. coli* W3110*, Bacillus subtilis*, and *Pseudomonas aeruginosa* was inhibited by the recombinant *Cit1a* at low concentrations (0.75–2.00 μM) (Table 2). The MIC value of *E. coli* W3110 was 0.75 μM. Below 0.75 μM, the growth inhibition was decreased (data not shown).

Discussion

Spider venoms represent an attractive source of peptides with a variety of different types of bioactivity, representing vast natural resources (Kuhn-Nentwig et al. 2011; Liang 2008; Vassilevski et al. 2009). Cyto-insectotoxin (cit1a) identified from spider venom having equally potent antimicrobial and insecticidal effects (Vessilevski et al. 2008) was expressed and produced using silkworm.

In this study, *egfp* was fused with cit1a to mask Cit1a activity. In a previous

report, when GFP_{uv} fusion protein was expressed in silkworm larvae, several degraded fusion proteins appeared (Park et al. 2007). EGFP-Cit1a was not significantly degraded in silkworms even if Cit1a was fused with EGFP. This indicated that the EGFP-Cit1a fusion protein was not vulnerable to proteases in silkworms. In addition, the EGFP-Cit1a fusion protein was not observed in the hemolymph (Fig. 1C, D), because EGFP-Cit1a does not have any signal sequence at its N-terminus. Cit1a does natively possess a signal sequence and pro-domain, however, in this study these sequences were removed to fuse with EGFP at the N-terminus of Cit1a.

The production of recombinant proteins using the silkworm depends on the properties of protein. The expression level of Cit1a was lower, compared to other proteins (Kato et al. 2010), but this system provides rapid production of recombinant protein. AMPs have often been produced in various expression systems as fusion proteins with carrier proteins, such as glutathione-S-transferase (GST), protein A, maltose-binding protein (MBP) and so on, to increase the AMP solubility and mask the antimicrobial activity for expression (Kozlov 2008). Originally, this Cit1a peptide possesses insecticidal activity (Vassilevski et al., 2008), but active Cit1a was expressed in silkworms. In a previous report, this Cit1a did not have cytotoxic effects on the host cell when expressed intracellularly in HEK293 cells (Lazarev et al., 2011). We presumed that this peptide also may not be toxic to insect cells when expressed intracellularly. Silkworm expression system can be used for the large-scale production of Cit1a and other linear peptide toxins through intracellular expression.

Cit1a obtained from this study showed antimicrobial effect on *E. coli* W3110, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Fig. 6A, C and D) but there was no effect on *Staphylococcus aureus* (Fig. 6B). A previous study reported that synthetic

Cit1a showed no inhibitory effect on *S. aureus* (Kozlov et al. 2008), which is similar to the results obtained in our study. These approaches demonstrate that the recombinant protein produced in silkworm is active against bacteria, as reported previously (Chen et al. 2009; Kozlov et al. 2008) and the MIC value (Table 2) falls within the MIC values of other peptides (Kozlov et al. 2008). Moreover, the MIC values of EGFP-Cit1a against *E. coli*, *P. aeruginosa*, and *B. subtilis* were comparable with those in a previous report (Vassilevski et al. 2008), indicating that the EGFP and FLAG tag do not have any negative influence on the properties of Cit1a. Also these data suggest that the EGFP-Cit1a fusion protein can be used directly without cleavage by EK and silkworm larvae can produce active Cit1a in its fat body. Cit1a has cytotoxicity to Sf-9 cells and has been known as an insecticidal peptide (Vassilevski et al. 2008). These results show the contradiction that an active insecticidal peptide can be expressed and purified in insects. However, Cit1a can be expressed in HEK293 cells as an active form to suppress the infection of a parasitic bacterium, *Chlamydia* (Lazarev et al. 2011; Lazarev et al. 2013; Polina et al. 2012). These data suggest that Cit1a can be expressed as an active form intracellularly without cytotoxicity to host cells. Moreover, EGFP fusion proteins have been utilized for the intracellular trafficking and functional analysis of expressed proteins *in vivo* (Avilov et al. 2013; Sammons and Gross 2013). EGFP-Cit1a fusion protein allows us to analyze the intracellular trafficking of Cit1a in *Chlamydia* and its suppression mechanism.

Although we used the EGFP-Cit1a fusion protein to test for biological activity against bacteria, it was confirmed that the growth inhibition of bacteria happened due only to the action of the *Cit1a* gene because the *egfp* gene has no toxic effects on the cell (Chalfie et al. 1994). Cit1a is active at low micromolar concentrations, although a certain specificity of action was shown, with some bacteria essentially resistant to the

peptide (Kozlov et al. 2008). The properties, wide spectrum of activity at micromolar concentration and membrane specificity are common to most other AMPs. These phenomena are described by the approved universal mechanism of AMP action with the plasma membrane serving as the target (Kozlov et al. 2008). Biologically active recombinant fusion protein could be obtained from both silkworm larvae and pupae, indicating that silkworm can produce soluble Cit1a to characterize it. The development of cost-effective systems for peptide production with recombinant DNA technology is of great interest due to the increasing use of peptides as pharmaceutical agents. AMPs have also been shown to repress mycoplasma and *Chlamydia* development *in vitro* (Fehri et al. 2007; Yasin et al. 1996). However, active peptide concentrations are usually 0.1 to 10 μM, corresponding to rather high therapeutic doses.

In the present paper, we expressed and produced Cit1a as an EGFP-Cit1a fusion protein using silkworm and investigated the antimicrobial activity of Cit1a, a cytolytic peptide produced by *L. tarabaevi* which represents a unique class of spider venom constituents. Antimicrobial peptides have been studied extensively because of their potential clinical applications as pharmaceutical agents (Fan et al. 2010).

In conclusion, our study developed a new strategy for the expression and production of Cit1a using silkworm fused with the EGFP. For large-scale preparation of recombinant proteins, the BmNPV bacmid system-using silkworm could be used due to its low cost, ease of treatment and high biohazard safety. The recombinant Cit1a showed high antimicrobial activity as previously reported, which makes Cit1a a promising candidate as a therapeutic.

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Figure Legends

Fig. 1 Construction of EGFP-Cit1a fusion gene and expression of EGFP-Cit1a fusion protein in silkworm. (A) Schematic representation of EGFP-Cit1a fusion gene obtained by PCR and description of EGFP-Cit1a fusion protein. Details of primer 1-5 are shown in Table.1 (B) Agarose gel electrophoresis of PCR products in PCR steps (PCR 1-3). Lane 1: PCR 1; lane 2: PCR 2;; lane 3: PCR 3; (C) EGFP fluorescence analysis of the EGFP-Cit1a fusion protein expressed in silkworm on a SDS-PAGE gel. Lanes 1, 3 and 5: homogenates of BmNPV CP^-/EGFP-Cit1a bacmid-injected pupa, larval hemolymph, and fat body, respectively; lanes 2 and 4: homogenates of mock-injected pupa and larval hemolymph, respectively; lane 6: mock-injected larval fat body. Fluorescent bands were detected using Molecular Imager FX (Bio-Rad) indicated by arrows. (D) Western blot analysis of EGFP-Cit1a fusion protein cross-reacted with antibodies is indicated by arrows. Lane 1: mock pupa homogenate; lanes 2, 4, and 6: BmNPV CP^-/EGFP-Cit1a bacmid-injected larval fat body, hemolymph, and pupa homogenate, respectively; lanes 3 and 5: mock larval hemolymph and fat body, respectively.

Fig. 2 Fluorescence detection of EGFP in silkworm larval fat body and pupa: (A) and (C), BmNPV CP^-/EGFP-Cit1a bacmid-injected larval fat body and pupa, respectively; (B) and (D), mock-injected larval fat body and pupa, respectively. Cells were stained with DAPI (blue).

Fig. 3 SDS-PAGE and Western blot analysis of purified EGFP-cit1a fusion protein. (A) SDS-PAGE and Western blot of EGFP-Cit1a purified from BmNPV CP^-/EGFP-Cit1a bacmid-injected silkworm fat body. An SDS-PAGE gel was stained with CBB. (B) SDS-PAGE and Western blot of EGFP-Cit1a purified from BmNPV CP^-/EGFP-

Cit1a bacmid-injected silkworm pupae. The arrows indicate purified EGFP-Cit1a fusion protein.

Fig. 4 MALDI-TOF mass spectrometry of recombinant EGFP FLAG-tagged cyto-insectotoxin. The sample was dissolved in 0.1% TFA: acetonitrile (2:1 v/v) and mixed with the matrix solution (1:4 v/v). The mixture (1 μl) was put on a stainless target and crystallized at room temperature. A mass calibration procedure was employed prior to the analysis of a sample using protein calibration standards I (Bruker Daltonics, Germany). The MALDI-TOF mass spectrum was acquired on an AutoFlex (Bruker Daltonics, Germany) and measured in linear mode using 20-kV ion acceleration without post-acceleration. The spectrum was recorded at a detector voltage of 1.65 kV and was the averaged results of at least 300 laser shots. SDHB was used as the matrix.

Fig. 5 rEK digestion of EGFP-Cit1a fusion protein. After the digestion of EGFP-Cit1a fusion protein by rEK, SDS-PAGE was performed followed by CBB staining. Lane 1: EGFP-Cit1a fusion protein; lane 2: rEK-treated EGFP-Cit1a fusion protein.

Fig. 6 Growth inhibitory effect of EGFP-Cit1a fusion protein on bacterial strains. (A) *E. coli* W3110. (B) *Staphylococcus aureus.* For A and B, 1: 6 μM; 2: 3 μM; 3: 1.5 μM; 4: 0.75 μM; 5: 0.385 μM; 6: 0.187 μM. (C) *Pseudomonas aeruginosa.* (D) *Bacillus subtilis*, 1: 4 μM; 2: 2 μM; 3: 1.0 μM; 4: 0.5 μM; 5: 0.25 μM; 6: 0.125 μM; 8: 100 μg/ml ampicillin for gram-negative bacteria or 100 μg/ml chloramphenicol for gram-positive bacteria.

Table 1 Primers used in this study

No.	Name	Sequence (5' to 3')	Tm (°C)	PCR product Length (bp)
1	Eco-EGFP-F	gcgaattcatggtgagcaagggcgaggag	81.1	750, 970[a]
2	EGFP-FLAG-R	cttgtcaatcgtcatccttgtagtc cttgtacagctcgtccatgcc	84.2	750
3	FLAG-Cit1a-F	gactacaaggatgacgatgacaagggtttcttcgggaatacgt ggaagaaaataaagggcaaagctgataagattatgctaaagaa agcagtaaagataatggtaaagaaagaaggaatatctaaagaa gaggcg	88.4	242
4	Cit1a-xba-R	gctctagatcacaatttttcggacgctttttgaagagctttttttcca taatacttgagtagatagagtcttatttgtttctttgacattgcatcta cttttgcctg cgcctcttctttagatattcc	87.8	242
5	EGFP-FLAG-stop-R	gctctagattacttgtcatcgtcatccttgtagtccttgtacagctc gtccatgcc	84.5	970[a]

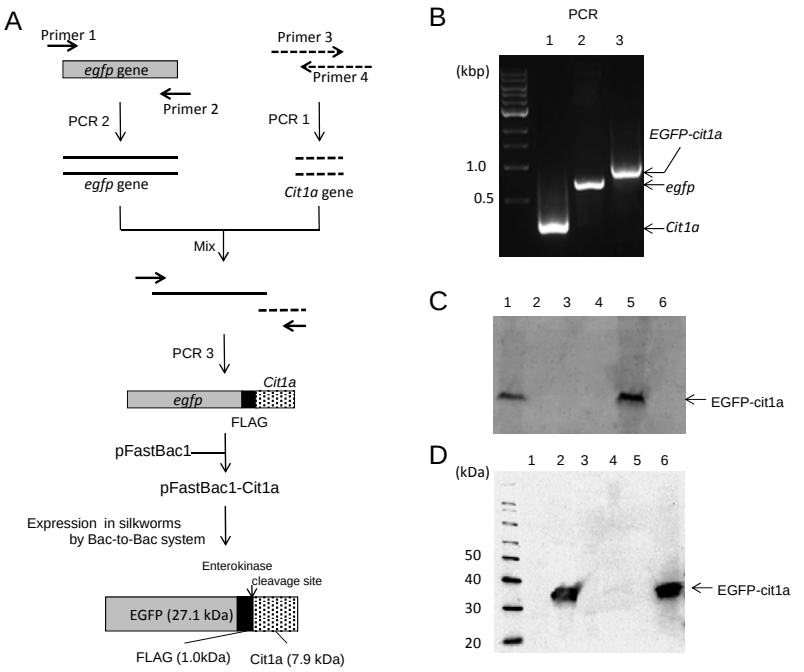
[a] PCR product was amplified using No. 1 and 5 primers.

Table 2 Antimicrobial activity of EGFP-Cit1a against several bacteria

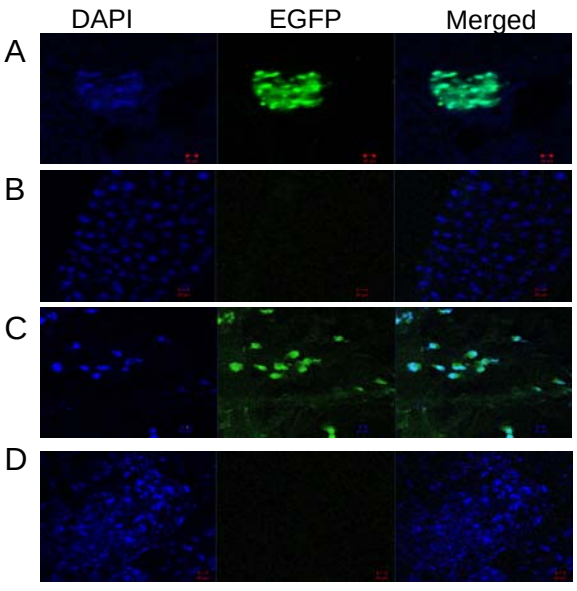
Target bacteria	MIC (μM)
Gram-positive	
Bacillus subtilis (NBRC13719)	1.5
Staphylococcus aureus (NBRC100910)	>10
Gram-negative	
Pseudomonas aeruginosa (NBRC12689)	2
Escherichia coli W3110 (NBRC12713)	0.75

MIC: Minimum Inhibitory Concentration

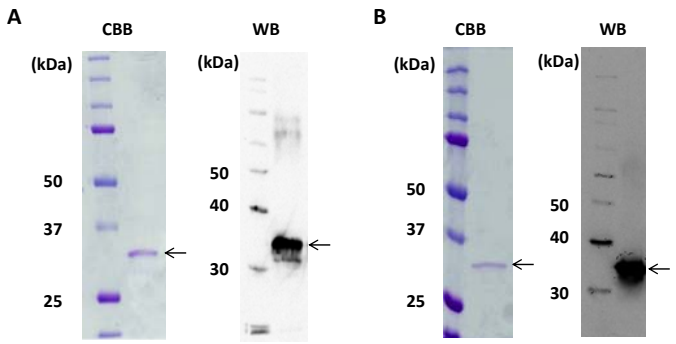
Ali et al, Fig. 1



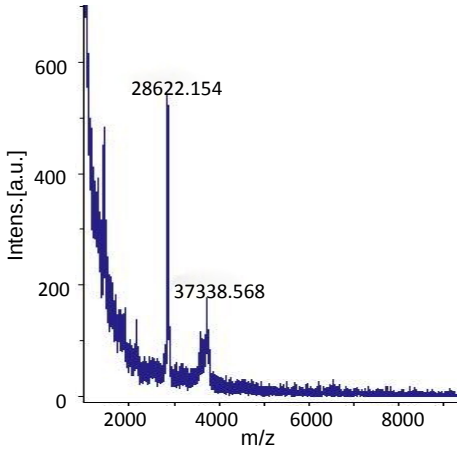
Ali et al, Fig. 2



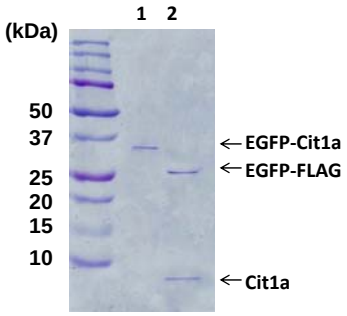
Ali et al, Fig. 3



Ali et al, Fig. 4



Ali et al, Fig. 5



Ali et al, Fig. 6