Rapid and efficient production of cecropin A antibacterial peptide in *Escherichia coli* by fusion with a self-aggregating protein

Meng Wang¹, Kaiwen Zheng¹, Jinglian Lin¹, Minhua Huang¹, Yi Ma¹,², Shan Li¹, Xiaochun Luo¹ and Jufang Wang¹,²*

**Abstract**

**Background:** Cecropin A (CeA), a natural cationic antimicrobial peptide, exerts potent antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria, making it an attractive candidate substitute for antimicrobials. However, the low production rate and cumbersome, expensive processes required for both its recombinant and chemical synthesis have seriously hindered the exploitation and application of CeA. Here, we utilized a short β-structured self-aggregating protein, ELK16, as a fusion partner of CeA, which allowed the efficient production of high-purity CeA antibacterial peptide with a simple inexpensive process.

**Results:** In this study, three different approaches to the production of CeA peptide were investigated: an affinity tag (His-tag)-fused protein expression system (AT-HIS system), a cell-free protein expression system (CF system), and a self-assembling peptide (ELK16)-fused protein expression system (SA-ELK16 system). In the AT-HIS and CF systems, the CeA peptide was obtained with purities of 92.1% and 90.4%, respectively, using one or more affinity-chromatographic purification steps. The procedures were tedious and costly, with CeA yields of only 0.41 and 0.93 μg/mg wet cell weight, respectively. Surprisingly, in the SA-ELK16 system, about 6.2 μg/mg wet cell weight of high-purity (approximately 99.8%) CeA peptide was obtained with a simple low-cost process including steps such as centrifugation and acetic acid treatment. An antimicrobial test showed that the high-purity CeA produced in this study had the same antimicrobial activity as synthetic CeA peptide.

**Conclusions:** In this study, we designed a suitable expression system (SA-ELK16 system) for the production of the antibacterial peptide CeA and compared it with two other protein expression systems. A high yield of high-purity CeA peptide was obtained with the SA-ELK16 system, which greatly reduced the cost and time required for downstream processing. This system may provide a platform for the laboratory scale production of the CeA antibacterial peptide.

**Keywords:** Self-aggregating protein, Cecropin A, Antimicrobial peptide, ELK16, *Escherichia coli*

**Background**

Antimicrobial peptides (AMPs) are small peptides of 10–50 amino acids that are effective in the innate immune systems of a wide variety of organisms, including bacteria, plants, insects, and mammals [1–3]. In general, they are cationic, amphipathic, β-folded or α-helix peptides that display rapid, strong, and broad-spectrum activities against a wide range of pathogens, including bacteria, fungi, plants, and even insects [4–6].

In recent decades, the widespread use of antimicrobials has resulted in a rapid increase in antimicrobial resistance, which has increased the worldwide morbidity [7] and economic losses [8, 9]. This has led to enormous efforts to explore and develop new antimicrobial agents, such as AMPs. Moreover, because of their broad spectrum of antibacterial properties but no hemolytic or cytotoxic activities, AMPs are considered attractive targets for the development of new antimicrobial compounds [10, 11]. Cecropin A (CeA) is a natural linear cationic α-helical AMP isolated...
from insects [12–14]. It contains a strongly cationic region at its N-terminus and a large hydrophobic tail at its C-terminus, which allows its interaction with the microbial membrane, and then induces cell lysis by pore formation [1]. It has a wide spectrum of antimicrobial activities against Gram-negative bacteria, Gram-positive bacteria, and fungal phytopathogens [15]. More importantly, this peptide does not induce the lysis of erythrocytes or lymphocytes, even at high concentrations [16]. Therefore, the CeA peptide has become a potentially useful antimicrobial substance in biochemical and pharmaceutical research [17].

Because the chemical synthesis of the CeA peptide or extraction from natural sources are extremely expensive, with low yields [6, 18–20], the development of a simple way to efficiently produce high-purity CeA peptide is urgently required. In recent years, there has been much research into the production of CeA and its derivatives [21–24]. To reduce the production costs, *Pichia pastoris* and *Bacillus subtilis* have been used as the expression hosts for the secretion of the CeA peptide [23, 25, 26]. To avoid the formation of inclusion bodies, some researchers have fused the peptide to glutathione S-transferase (GST) or ubiquitin-related modifier (SUMO) [33, 34], GST [35], and TRX [31, 36] were selected as fusion partners for the efficient expression of AMPs. Here, we incorporated a self-cleaving peptide Mxe GyrA intein [37] as the fusion carrier in the AT-HIS system to avoid the degradation of CeA by proteases to improve its expression. High-purity CeA peptide was released by the self-cleavage of Mxe GyrA intein and then purified with nickel ion affinity chromatography (fusion mode “CeA–Mxe–His”), shown in Fig. 1a). In the CF expression system, a His6 tag was fused to the CeA peptide by RF cloning to allow its downstream purification. RF cloning is a simple and restriction-free method for insertion of foreign DNA into a plasmid at any desired location [38]. To achieve the high-level expression of the CeA peptide, two different fusions were constructed: N-terminal fusion (His6–CeA) or C-terminal fusion (CeA–His6) (fusion mode “His6–CeA” or “CeA–His6”, respectively, shown in Fig. 1a). Recent studies have reported that some peptides like ELK16 can assemble into aggregates when expressed in *E. coli*. Based on the results of this study, ELK16 was selected and attached to the C-terminus of Mxe GyrA intein (CeA–Mxe–ELK16) in the SA-ELK16 system to induce and increase the formation of active fusion protein aggregates in *E. coli* (fusion mode “CeA–Mxe–ELK16”, shown in Fig. 1a). These aggregates were then separated by simple centrifugation and high-purity CeA peptide was successfully released into soluble fractions after dithiothreitol (DTT)-induced Mxe GyrA intein cleavage and acetic acid treatment (Fig. 1b).

**Expression, optimization and purification of CeA peptide with the AT-HIS system**

*E. coli* BL21(DE3) cells harboring plasmid pET21a–AM–His were cultured at 37 °C and isopropyl β-D-1-thigalactopyranoside (IPTG) was added when the optical density of the cells at a wavelength of 600 nm (OD600) reached 0.6–0.8 to initiate the expression of CeA–Mxe–His fusion. To achieve high-level expression of the soluble CeA–Mxe–His fusion, different incubation temperatures (37 °C, 25 °C, and 16 °C) were investigated. As shown in Fig. 2a, large amounts of the CeA–Mxe–His fusion were successfully expressed in *E. coli* BL21(DE3) cells at all temperatures (bands marked with red arrow). However, the fusion polypeptide was expressed and deposited primarily in inclusion bodies when the cells were incubated at 37 °C for 5 h. When the temperature was reduced to 25 °C or 16 °C, large amounts of soluble CeA–Mxe–His fusion were produced (Fig. 2a). Different concentrations of IPTG (0.1, 0.5, or 1 mM) were also tested. As shown in Fig. 2b, a sufficient amount of soluble fusion was produced after induction with 0.1 mM IPTG and there was no obvious increase in the amount of CeA–Mxe–His fusion when 0.5 or 1 mM IPTG was

**Results**

**Design and construction of three different CeA fusions**

Because AMPs are toxic to host cells and are readily degraded by proteases [32], various proteins such as small ubiquitin-related modifier (SUMO) [33, 34], GST [35], and TRX [31, 36] were selected as fusion partners for the efficient expression of AMPs. Here, we incorporated a self-cleaving peptide Mxe GyrA intein [37] as the fusion carrier in the AT-HIS system to avoid the degradation of CeA by proteases to improve its expression. High-purity CeA peptide was released by the self-cleavage of Mxe GyrA intein and then purified with nickel ion affinity chromatography (fusion mode “CeA–Mxe–His”), shown in Fig. 1a). In the CF expression system, a His6 tag was fused to the CeA peptide by RF cloning to allow its downstream purification. RF cloning is a simple and restriction-free method for insertion of foreign DNA into a plasmid at any desired location [38]. To achieve the high-level expression of the CeA peptide, two different fusions were constructed: N-terminal fusion (His6–CeA) or C-terminal fusion (CeA–His6) (fusion mode “His6–CeA” or “CeA–His6”, respectively, shown in Fig. 1a). Recent studies have reported that some peptides like ELK16 can assemble into aggregates when expressed in *E. coli*. Based on the results of this study, ELK16 was selected and attached to the C-terminus of Mxe GyrA intein (CeA–Mxe–ELK16) in the SA-ELK16 system to induce and increase the formation of active fusion protein aggregates in *E. coli* (fusion mode “CeA–Mxe–ELK16”, shown in Fig. 1a). These aggregates were then separated by simple centrifugation and high-purity CeA peptide was successfully released into soluble fractions after dithiothreitol (DTT)-induced Mxe GyrA intein cleavage and acetic acid treatment (Fig. 1b).

**Expression, optimization and purification of CeA peptide with the AT-HIS system**

*E. coli* BL21(DE3) cells harboring plasmid pET21a–AM–His were cultured at 37 °C and isopropyl β-D-1-thigalactopyranoside (IPTG) was added when the optical density of the cells at a wavelength of 600 nm (OD600) reached 0.6–0.8 to initiate the expression of CeA–Mxe–His fusion. To achieve high-level expression of the soluble CeA–Mxe–His fusion, different incubation temperatures (37 °C, 25 °C, and 16 °C) were investigated. As shown in Fig. 2a, large amounts of the CeA–Mxe–His fusion were successfully expressed in *E. coli* BL21(DE3) cells at all temperatures (bands marked with red arrow). However, the fusion polypeptide was expressed and deposited primarily in inclusion bodies when the cells were incubated at 37 °C for 5 h. When the temperature was reduced to 25 °C or 16 °C, large amounts of soluble CeA–Mxe–His fusion were produced (Fig. 2a). Different concentrations of IPTG (0.1, 0.5, or 1 mM) were also tested. As shown in Fig. 2b, a sufficient amount of soluble fusion was produced after induction with 0.1 mM IPTG and there was no obvious increase in the amount of CeA–Mxe–His fusion when 0.5 or 1 mM IPTG was
used. In summary, we selected 25 °C, 0.1 mM IPTG and 12 h as the optimal conditions for the expression of CeA–Mxe–His fusion.

The CeA–Mxe–His fusion was purified with nickel ion affinity chromatography and high-purity CeA–Mxe–His fusion was collected (Additional file 1). To obtain more CeA peptide, the efficiency of Mxe GyrA intein cleavage was optimized. Figure 2c shows that nearly 100% of the CeA–Mxe–His fusion was successfully cleaved by the addition of 40 mM DTT, which is consistent with other reports [34, 37], and the CeA peptide was successfully released from the fusion (the band at 5 kDa in line SL in Fig. 2d). Approximately 92.1% of the CeA peptide was obtained after a second round of nickel ion affinity chromatography, with a final yield estimated to be 0.41 μg/mg wet cell weight (Fig. 2d and Table 1). Unfortunately, severe intracellular self-cleavage of the CeA–Mxe–His fusion in the AT-HIS system could not be prevented, even when the low temperature (16 °C) and low concentration of IPTG (0.1 mM) were used, which greatly reduced the production of CeA peptide (two major bands in Fig. 2a and b).

**Expression and purification of CeA peptide with the CF system**

In the CF system, two different plasmids (pET21–His<sub>6</sub>–CeA and pET21–CeA–His<sub>6</sub>) were constructed to express the CeA peptide. The purified plasmid DNA was added as the template to the cell free reaction mixtures (RMs) and expressed at 30 °C for 16–18 h and then analyzed.
by tricine SDS-PAGE. However, the CeA peptide was not expressed when plasmid pET21–CeA–His6 was added to RM as the template (data not show). Fortunately, as shown in Fig. 3a, His6–CeA was successfully expressed as the soluble form, which suggests that the N-terminal fusion of the His tag improved the expression of CeA peptide. Different concentrations (12–22 mM) of Mg2+ were also evaluated for enhanced expression of CeA peptide. Results in Fig. 3a shows that the expression of the CeA peptide increased with increments in the Mg2+ concentration and the highest production of soluble CeA peptide was achieved at an Mg2+ concentration of 16 mM. A further increase in the Mg2+ concentration did not improve the expression of the soluble CeA peptide, so an optimum Mg2+ concentration of 16 mM was selected.

The CeA peptide was purified with nickel ion affinity chromatography, as reported previously [39]. As shown in Fig. 3b, the amount of CeA peptide successfully purified was about 0.93 μg/ml of reaction mixture, with a purity of approximately 90.4% (Table 1).

**High-purity CeA peptide released in the SA-ELK16 system**

Previous studies have reported that some polypeptides like ELP, 18A, and ELK16 can induce the formation of aggregates in vivo [30, 40–42]. To verify the self-assembly property of ELK16, two different fusions, GFP–Mxe–ELK16 and GFP–Mxe–His, were constructed, expressed in *E. coli*...
BL21(DE3) cells, and then analyzed with fluorescence confocal microscopy. As shown in Fig. 4, the fluorescent signal clearly localized the cellular distribution of GFP–Mxe–ELK16 (Fig. 4a), whereas the GFP–Mxe–His expressing cells displayed uniform green fluorescence throughout the cytoplasm (Fig. 4d). These data indicate that the ELK16 fusion could induce the formation of active protein aggregates in *E. coli* BL21(DE3) cells.

Based on the above results, ELK16 was chosen as the carrier protein fused to the CeA peptide (CeA–Mxe–ELK16) to induce the formation of active inclusion bodies in *E. coli* BL21(DE3) cells.

To obtain more CeA peptide, the efficiency of Mxe GyrA intein cleavage was investigated and optimized. The CeA–Mxe–ELK16 aggregates were first subjected to

| Expression systems | AT-HIS expression system | CF expression system | SA-ELK16 expression system |
|--------------------|--------------------------|----------------------|---------------------------|
| Required steps     | Step 1: Expression of fusion protein CeA-Mxe-PT-His6 | Step 1: Expression of antibacterial peptide His6-CeA | Step 1: Expression of fusion protein CeA-Mxe-ELK16 |
|                    | Step 2: Cell disruption and supernatant collection | Step 2: Ni2+ affinity chromatography | Step 2: Cell disruption and acquisition of protein aggregates |
|                    | Step 3: Ni2+ affinity chromatography | Step 4: Intein-mediated cleavage | Step 3: Intein-mediated cleavage |
|                    | Step 4: Intein-mediated cleavage | Step 5: The second Ni2+ affinity chromatography | Step 4: Acriflavine treatment and collection of CeA peptide. |
| Time consuming     | 5 days | 2 days | 2 days |
| Final yield of peptide production (µg/mg wet cell pellet) | 0.41 | 0.93 | 6.20 |
| Purity of bio-produced Cepropin A peptide (%) | 92.1 | 90.4 | 99.8 |

*Yield of cecropin A peptide after intein-mediated cleavage in LB culture: 2.87 ± 0.62 mg/ml wet cell weight in the SA-ELK16 system and 1.48 ± 0.48 mg/ml wet cell weight in the AT-HIS system, estimated with the densitometric analysis software Gel-Pro Analyzer or BCA Protein Assay Kit

*Yield of CeA peptide in CF system. 0.93 µg/ml of reaction mixture

concentrations of IPTG (0.2, 0.5, and 1 mM) were tested. As shown in Fig. 5, a major band at approximately 30 kDa was observed, indicating that the CeA–Mxe–ELK16 fusion was successfully expressed. Approximately 50.23–78.16 µg/mg wet cell weight accumulated as insoluble aggregate and there was no intracellular self-cleavage of the fusion which reduced the toxicity to the host cells (Table 2). There was a slight increase in the expression of the CeA–Mxe–ELK16 fusion at a higher IPTG concentration (0.5 or 1 mM), and ultimately 1 mM IPTG was selected as the optimal concentration.
cleavage with 40 mM DTT at 25 °C for 0–14 h. As shown in Fig. 6a, about 22.7% of the aggregates were cleaved after 3 h, and when the incubation time was extended to 12 h, nearly 46.7% of the fusion was cleaved, and this proportion remained constant with longer incubation time. DTT concentrations of 40–80 mM were also tested at 25 °C for 12 h, and 55–76.2% of the fusion aggregate was cleaved, indicating that 40 mM DTT was sufficient to induce the cleavage of the majority of CeA–Mxe–ELK16 aggregates (Fig. 6b and Table 2). Considering high temperature (25 °C) can reduce the stability and activity of the CeA peptide, different incubation temperatures (including 25 °C and 4 °C) were tested. As shown in Fig. 6c, incubation of the samples at 4 °C and 25 °C generated the same amounts of cleaved fusion aggregates. Based on these results, we performed all subsequent cleavage reactions with 40 mM DTT at 4 °C for 12 h. Then the cleaved samples were centrifuged at 12,000 g for 30 min at 4 °C. Unfortunately, the released CeA peptide remained in the insoluble fraction after centrifugation (band marked with a red star in Fig. 7-P1). According to recent studies, acetic acid treatment can denature and precipitate the majority of E. coli proteins [30, 43]. Moreover, the structure of CeA peptide is simple (only two α helices with no disulphide bond) and has a higher flexibility (it can fit its conformation to different circumstances) [44, 45]. Therefore, CeA peptide can resume a suitable structure in PBS buffer after acetic acid treatment. Based on this principle, different concentrations of acetic acid (0.5–3% [v/v]) were added to the cleaved samples to solubilize the CeA peptide. As can be seen in Fig. 7, CeA peptide of high purity (approximately 99.8%, Table 2) was successfully dissolved in the soluble fraction when the cleaved samples were incubated with acetic acid. Interestingly, increasing amounts of CeA peptide were dissolved in the soluble fraction as the increased amount addition of acetic acid added. Ultimately, the addition of 2.5% acetic acid was selected, because higher concentrations of acetic acid may affect the stability of CeA peptide. The dissolved CeA peptide was then concentrated and dialized in phosphate-buffered saline (PBS) for further study.

**Antibacterial properties of the bioproduced CeA peptide**

The in vitro antimicrobial activity against E. coli ATCC 25922 of the CeA peptide bio-produced with the three different expression systems was determined as the minimal
inhibitory concentrations (MICs) with the broth microdilution method [45]. The results are shown in Fig. 8. The MICs of the CeA peptides generated in the three systems against *E. coli* ATCC25922 were the same (9.75 ng/μl), and consistent with the MIC of the chemically synthesized CeA peptide against *E. coli* ATCC 25922. These results indicate that the CeA peptides produced by these three protein expression systems had the same antimicrobial activity as synthetic CeA, and that the N-terminal additional of histidine to the CeA peptide in the CF system had no negative effect on its antibacterial activity.

### Discussion

In this study, we investigated three different protein expression systems for the production of the CeA peptide: the AT-HIS system, CF system, and SA-ELK16 system. By comparing the results, we developed a cost-effective and efficient protein expression system for high-purity CeA peptide.

In the AT-HIS system, two rounds of nickel ion affinity chromatography were required which was both expensive and time-consuming (approximately 5 days, Table 1). More importantly, a large amount of CeA peptide was lost in the second round of Ni²⁺ affinity chromatography, which significantly limited the large-scale production of CeA peptide. Finally, CeA peptide with 92.1% purity of was obtained at a yield of 0.41 μg/mg wet cell pellet, indicating that AT-HIS system is not an ideal system for the production of the CeA peptide. Due to its open nature and membrane-free, cell free protein expression system was considered the most suitable platform for expression of toxic proteins in recent years [46]. We tested the efficacy of the CF expression system for producing the CeA peptide. Table 1 shows that about 0.93 μg/ml reaction mixture of the CeA peptide was

| Expression systems | MW (kDa) | Fusions yield a (μg/mg wet cell pellet) | Peptide yieldb (μg/mg wet cell pellet) | Cleavage efficiencyc (%) | Percent recoveryd (%) | Peptide puritye (%) |
|--------------------|---------|----------------------------------------|----------------------------------------|--------------------------|----------------------|---------------------|
| CF system          | 4.6     | —                                      | 0.93f                                | —                        | —                    | 90.4                |
| AT-HIS system      | 4.4     | 4.85                                   | 0.41                                  | 100                      | 52.14                | 92.1                |
| SA-ELK16 system    | 4.4     | 51.16                                  | 6.20                                  | 79.50                    | 78.38                | 99.9                |

*aYield of fusion proteins in the form of aggregates or soluble fractions
bYield of cecropin A peptides after intein-mediated cleavage in LB culture: 2.87 ± 0.62 mg/ml wet cell weight in the SA-ELK16 system and 1.48 ± 0.48 mg/ml wet cell weight in the AT-HIS system, estimated with the densitometric analysis software Gel-Pro Analyzer or BCA Protein Assay Kit
cCleavage efficiency was calculated by dividing the amount of cleaved protein aggregate by the amount of total aggregate before cleavage
dPercentage recovery of cecropin A peptide was calculated by dividing the mass of cecropin A peptide after cleavage by the mass that could be theoretically obtained from the fusion protein
ePurity of CeA peptide was calculated with the BandScan software
fYield of CeA peptide in the CF system was 0.93 μg/ml of reaction mixture
eventually collected, with 90.4% purity. When the CF and AT-HIS systems were compared, the CF system performed better than the AT-HIS system in that the final yield of CeA peptide was 2-fold higher than with the AT-HIS system. However, this yield is relatively low compared with the production of other AMPs, and falls far short of the yields required for large scale production [47, 48]. Moreover, the purity of the CeA peptide produced by these two expression systems was less than 95% and is inappropriate for further research. Most importantly, several cumbersome and expensive procedures like nickel affinity chromatography are required, which are not practicable for the large-scale production of the CeA peptide. Therefore, there is an urgent need to investigate and explore a high and efficient system for the production of CeA peptide.

To achieve a highly efficient low-cost production process for high-purity (> 98%) CeA peptide, the small self-assembling protein ELK16 was attempted in this study. In SA-ELK16 system, the recovery of CeA peptide was estimated to be 78.38%, higher than that in AT-HIS system (52.14%, Table 2). Finally, highly pure (~ 99.8%) CeA peptide was obtained after the cleavage of Mxe GyrA intein (Table 1), with a yield of 6.20 μg/mg wet cell pellet, which was 15-fold higher than the yield of the AT-HIS system and 6.7-fold higher than that of the CF protein expression.
system (Table 2). These results indicated that SA-ELK16 system was more efficient than AT-HIS system and CF system for production of CeA peptide. Furthermore, the CeA peptide was produced rapidly (2 days) with simple centrifugation and acetic acid treatment, eliminating expensive and tedious procedures like nickel affinity chromatography or high-performance liquid chromatography, dramatically reducing the production cost (Table 1). These factors make the SA-ELK16 system an ideal candidate for the laboratory scale production of the CeA peptide.

However, in SA-ELK16 system, much effort is still needed for the industrial production of CeA peptide. Such as the cleavage of CeA–Mxe–ELK16 fusion was induced by DTT in this study which was expensive at industry scale. Some other self-cleavage inteins like Ssp dnaB and sortase, which cleavage reactions were activated by pH and calcium ion, can be used to reduce the costs at industry production of CeA peptide [49–51]. Besides, the downstream process like dialysis also need to be further optimized to meet the needs of industrial production of CeA peptide.

Conclusions
Cecropin A is a natural linear cationic AMP, with rapid and potent activity against a broad spectrum of pathogens, including bacteria, fungi, viruses, and neoplastic cells. Therefore, it has great potential utility in various biotechnological applications [2, 19, 22]. However, the exploitation of the CeA peptide is seriously limited by the low yields and expensive purification costs of its production. Here, we report a fast, economical and efficient expression system, the SA-ELK16 system, for the laboratory scale production of high-purity CeA peptide, which circumvents the difficulties encountered by ordinary recombinant methods or expensive chemical syntheses. It completely avoids the use of affinity resins and greatly reduces the cost and time required, providing a novel platform for laboratory scale production of the CeA peptide. This study lays a solid foundation for further research into the CeA peptide.

Methods
Strains and materials
Competent E. coli DH5α and BL21(DE3) cells were purchased from Tiangen Biotech (Beijing, China). Oligonucleotides for gene manipulation were synthesized by Invitrogen (Shanghai, China) or Tianyi Huiyuan Gene Technology (Guangzhou, China). The restriction endonuclease DpnI used for restriction-free cloning (RF cloning) was obtained from Fermentas Thermo Scientific (Glen Burnie, MD, USA). The Hi Trap 5 mL pre-packed column used for protein purification was purchased from Qiagen (Dusseldorf, Germany). All chemical reagents used in this study were of analytical grade.

Construction of recombinant plasmids
All three recombinant expression vectors used in the study were constructed with RF cloning. Like fusion PCR cloning, RF cloning uses the appropriate DNA fragment as a megaprimer for the linear amplification of the vector to introduce a foreign DNA into the plasmid at a predetermined position [38]. CeA peptide, Mxe GyrA intein, and ELK16 were synthesized with the appropriate DNA sequences by codon-optimized expression in E. coli BL21(DE3) by Sangon Biotech (Shanghai, China). The DNA fragments encoding the CeA peptide, Mxe GyrA intein, and ELK16 were cloned into the vector pET30a with RF cloning to construct pET30a–AM–ELK16. To construct pET21a–AM–His, primers AMH-F and AMH-R (see Additional file 2) were used to amplify the AM sequence encoding both the CeA peptide and the Mxe GyrA intein from plasmid pET30a–AM–ELK16. The amplified DNA fragment was then integrated into the pET21a vector with RF cloning. Plasmid pET21–His6c–AMP was obtained by amplifying the CeA gene from pET30a–AM–ELK16 and inserting it into pET21a. Plasmid pET21–AMP–His6c was constructed in the same way as pET21–His6c–AMP. All the primers used in this work are listed in Additional file 2.

Expression of fusion proteins in three different systems
In the SA-ELK16 and AT-HIS systems, Luria–Bertani (LB) medium containing 50 μg/ml kanamycin or 100 μg/ml ampicillin was inoculated with E. coli BL21(DE3) cells carrying pET30a–AM–ELK16 or pET21a–AM–His, respectively, and incubated at 37 °C. Different concentrations of IPTG (0.1–1 mM) were added to initiate protein expression when the cell density (OD600) reached 0.6–0.8. The cultures were then incubated under different conditions (5 h/37 °C, 12 h/25 °C, or 24 h/16 °C) to optimize protein expression. The cells were harvested by centrifugation at 7500xg for 15 min and the pellets were stored at –80 °C for further analysis. In the CF system, pET21–His6c–AMP and its derivative were expressed with a previously described method [52]. The reaction solution, containing 60 μl of reaction mixture (RM) and 900 μl of feeding mixture (FM), was added to the wells of standard 24-well microplates. To optimize the Mg2+ concentration, regenerated cellulose membranes with a molecular-weight cut-off of 14 kDa were used. The reactions were performed in the continuous-exchange cell-free configuration and incubated for 12–16 h at 30 °C with shaking (180 rpm).

Intein-mediated cleavage and peptide purification
In the CF system, the soluble fractions were harvested by centrifuging the reaction mixture at 12,000 g for 10 min. Ten-fold volumes of cold acetone were added to the supernatants to precipitate the protein [47]. The
pellets were air dried, dissolved in 1× loading buffer (20 mM Tris–HCl, 3% [w/v] glycerol, 1.5% [w/v] SDS, 5% [w/v] β-mercaptoethanol, 0.02% [w/v] bromophenol blue, pH 8.5), and denatured by heating at 95 °C for 10 min. The samples were separated and analyzed with 16% tricine SDS-PAGE. The CeA fusion produced with the CF system was purified with nickel ion affinity chromatography, as previously reported [39].

The harvested cell pellets containing the CeA–Mxe–ELK16 or CeA–Mxe–His fusions were resuspended to 10 OD_{600} units/ml of culture with lysis buffer (20 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA, pH 8.5) and the suspended cells were lysed with a high-pressure homogenizer (Constant Systems Limited, United Kingdom). The CeA–Mxe–ELK16 aggregates were separated by centrifugation at 12,000 g for 30 min at 4 °C. The soluble fractions containing the CeA–Mxe–His fusions were isolated from the aggregates by centrifugation at 12,000 g for 30 min at 4 °C, and then purified with nickel ion affinity chromatography, as described above. For Mxe GyrA intein mediate cleavage, the CeA–Mxe–ELK16 aggregates or purified CeA–Mxe–His fusions were resuspended in cleavage buffer (20 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA, pH 8.5) containing different concentrations of DTT (40–80 mM). The cleavage reaction was assessed by incubating the samples under different conditions (4 °C or 25 °C for 0–14 h) to optimize the efficiency of cleavage. The quantity of protein was determined with a BCA Protein Assay Kit (Sangon) or the Gel-Pro Analyzer software (Media Cybernetics, Houston, USA), using aprotinin as the standard and adjusting for the loading volume.

Acetic acid treatment for high-purity CeA peptide in the SA-ELK16 system
To obtain high-purity (≥ 98%) CeA peptide, different proportions of acetic acid (0.5–3%) were added to the suspensions of the cleaved CeA–Mxe–ELK16 fusion and incubated at room temperature for 10 min. The super- natant containing high-purity CeA peptide was separated by centrifugation at 12,000 g for 30 min at 4 °C. The soluble and insoluble fractions were analyzed with tricine SDS-PAGE.

Fluorescence confocal microscopy
Escherichia coli BL21(DE3) cells carrying the pET30–GFP–Mxe–ELK16 or pET21–GFP–Mxe–His plasmid were cultured and the expression of the fusion was induced by the addition of 0.2 mM IPTG. After expression, 200 μl of cells were harvested by centrifugation at 7000 g at 4 °C for 5 min, resuspended in sterile PBS (1% w/v), spotted onto a slide, air dried, and visualized with a 63x phase-contrast objective on a Zeiss LSM 710 microscope (Germany).

MIC assay
The MICs of the CeA peptides produced in the three different expression systems against *E. coli* ATCC 25922 were assessed with the broth microdilution method, as previously reported [45], and compared with that of a synthetic CeA peptide. For the antimicrobial assays, overnight cultures of *E. coli* ATCC 25922 cells were diluted with fresh Müller–Hinton broth (MHB) to an OD_{625} of 0.08–0.13 (0.5 McFarland standards), and further diluted with fresh MHB (1:100) to a final concentration of approximately 10^7 colony-forming units (CFU) per ml. The bio-produced CeA peptides were serially diluted in MHB medium and 50 μl aliquots of the diluted CeA peptides were added to 96-well plates. The standardized bacterial suspension (50 μl) was then added to each well. Growth controls contained no peptide antimicrobial, and sterility controls contained only broth. The plates were incubated at 37 °C for 16–18 h. The OD_{600} values were monitored with a microplate reader and the lowest concentration of peptide at which the growth of *E. coli* ATCC 25922 was inhibited was determined as the MIC.

Additional files

**Additional file 1:** Purification of CeA-Mxe-His fusions in AT-HIS system. (DOCX 181 kb)

**Additional file 2:** Primers used in this study. (DOCX 145 kb)

**Abbreviations**
CeA: Cecropin A; *E. coli*: Escherichia coli; FM: feeding mixture; IPTG: Isopropyl β-D-Thiogalactoside; LB: Luria-Bertani; MHB: Müller–Hinton broth; Mxe: Mxe GyrA intein; RF cloning: Restriction Free cloning; RM: reaction mixture

**Acknowledgements**
We thank Janine Miller, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

**Funding**
This work was supported by the financial support of Natural Science Foundation of Guangdong Province, China (grant number: 2015A030310322); the Innovative Program of Department of Education of Guangdong Province, China (grant number: 2013KJCX0013). We are also grateful the important role of the funding in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

**Availability of data and materials**
All data for this study are included in this published article and its Additional files.

**Authors’ contributions**
JW and MW designed the experiments. MW and KZ performed the experiments. JL, MH and XL carried out the data analysis. MW wrote the manuscript. SL, YM and JW supervised the whole work attended the discussions and revised the manuscript. All authors have read and approved the manuscript, and ensure that this is the case.

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.
References

1. Schmitt P, Mercado L, Díaz M, Guzmán F, Arenas G, Marshall SH. Schmitt P, Mercado L, Díaz M, Guzmán F, Arenas G, Marshall SH. Characterization and functional recovery of a novel antimicrobial peptide (CEC dir-CEC ret) from inclusion bodies after expression in Escherichia coli. Peptides. 2008;29(4):512–9.

2. DeLucca AJ, Bland JM, Jacks TJ, Grimm C, Cleveland TE, Walsh TJ. Fungal activity of cecropin A. Antimicrob Agents Chemother. 1997;41(2):481–3.

3. Cavallini L, Andreu D, San Segundo B. Cepalin A—derived peptides are potent inhibitors of fungal plant pathogens. Mol Plant-Microbe Interact. 1998;11(3):218–27.

4. Andreu D, Menifield R, Steiner H, Boman H. Solid-phase synthesis of cecropin A and related peptides. Proc Natl Acad Sci. 1983;80(21):6475–60.

5. Silvestro L, Gupta K, Weiser JN, Axelsen PH. The concentration-dependent membrane activity of cecropin A. Biochimie. 1997;36(38):1154–60.

6. Zhang J, Mowahedi A, Wang X, Wu X, Yin T, Zhuge Q. Molecular structure, chemical synthesis, and antibacterial activity of ABP-dHC-cecropin A from drury (Hypanthria cunea). Peptides. 2015;68:197–204.

7. Thinkhamrop J, Hofmeyer GJ, Adetoro O, Lumbiganon P. Prophylactic activity of cecropin A—melittin hybrid peptides against a (H1N1)pdm09 heterologous challenge without vaccine associated respiratory disease. Virology. 2014;471:93–102.

8. Efimova SS, Schagina LV, Ostroumova OS. Channel-forming activity of cecropin A and related peptides is augmented by surfactants. Peptides. 2015;68:197–204.

9. Yi H-Y, Chowdhury M, Huang Y-D, Yu X-Q. Insect antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr Eye Res. 2005;30(7):505–15.

10. Xue R, Wang Q, Zheng Z, Zhao L, Shang Y, Wei X, Liao X, Zhang R. Design, chemical synthesis, and antibacterial activity of ABP-dHC-cecropin A from drury (Hypanthria cunea). Peptides. 2015;68:197–204.

11. Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides: an alternative for innovative medicines? Appl Microbiol Biotechnol. 2015;99(5):2023–40.

12. Satakami M, Curtis R. Production of recombinant cecropin peptides as fusions with SUMO. Protein Expr Purif. 2011;78(2):113–9.

13. Ma Y, Yu L, Lin J, Wu S, Li S, Wang J. High efficient expression, purification, and functional characterization of native human epidermal growth factor in Escherichia coli. Biomed Res Int. 2016;2016:37360941.

14. Xia L, Liu Z, Ma J, Sun S, Yang J, Zhang F. Expression, purification and characterization of cecropin antimicrobial peptide from Bombyx mori in Saccharomyces cerevisiae. Curr Eye Res. 2015;30(1):47–54.

15. Zhou B, Xing L, Wu W, Zhang X-E, Lin Z. Small surfactant-like peptides can drive soluble proteins into active aggregates. Microb Cell Factories. 2012;11:10.

16. Wu R, Wang Q, Zheng Z, Zhao L, Shang Y, Wei X, Liao X, Zhang R. Design, characterization and expression of a novel hybrid peptides melitin (1–13)-LL37 (17–30). Mol Biol Rep. 2014;41(7):4163–9.

17. Zhang J, Li J, Movahedi A, Sang M, Xu C, Xu J, Wei Z, Yin T, Zhuge Q. A novel inclusion complex (β-CD/ABP-dHC-cecropin A) with antibiotic properties for use as an anti-Agrobacterium additive in transgenic poplar root-feeding nematode. Enzym Microb Technol. 2015;81:72–9.

18. Efimova SS, Schagina LV, Ostroumova OS. Channel-forming activity of cecropin A in lipid bilayers: effect of agents modifying the membrane dipole potential. Langmuir. 2014;30(26):7884–92.

19. Yi H-Y, Chowdhury M, Huang Y-D, Yu X-Q. Insect antimicrobial peptides and their applications. Appl Microbiol Biotechnol. 2014;98(13):5807–22.

20. Kylsten P, Samakovlis C, Hultmark D. The cecropin locus in Drosophila, a compact gene cluster involved in the response to infection. EMBO J. 1990;9(12):17.

21. X-m L, X-b J, J-y Z, H-f M, Ma Y, F-j C, Wang Y, Li X-b. Expression of the CEC ret) from inclusion bodies after expression in Escherichia coli. Protein Expr Purif. 2016;100:48–53.

22. Andersons D, Engström Å, Josephson S, Hansson L, Steiner H. Biologically active and amidated cecropin produced in a baculovirus expression system from a fusion construct containing the antibody-binding part of protein A. Biochem J. 1991;280(1):129–139.

23. Yang K, Su Y, Li J, Sun J, Yang Y. Expression and purification of the antimicrobial peptide cecropin AD by fusion with cationic elastin-like polypeptides. Protein Expr Purif. 2012;85(2):200–3.

24. Tiléz GA, Casteño-Osorio JC. Expression and purification of an active cecropin-like recombinant protein against multidrug resistance Escherichia coli. Protein Expr Purif. 2014;100:48–53.

25. J. S. Li W, Baloch AR, Wang M, Li H, Cao B, Zhang H. Efficient biosynthesis of a CEC ret) from inclusion bodies after expression in Escherichia coli. Protein Expr Purif. 2016;100:48–53.

26. Yi H-Y, Chowdhury M, Huang Y-D, Yu X-Q. Insect antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr Eye Res. 2005;30(7):505–15.
peptide cecropin A: a nuclear magnetic resonance and dynamical simulated annealing study. Biochemistry. 1988;27(20):7620–9.
45. Lee E, Shin A, Kim Y. Anti-inflammatory activities of cecropin A and its mechanism of action. Arch Insect Biochem Physiol. 2015;88(1):31–44.
46. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163–75.
47. Ma Y, Ghoshdastider U, Wang J, Ye W, Dötsch V, Filipek S, Bernhard F, Wang X. Cell-free expression of human glucosamine 6-phosphate N-acetyltransferase (HsGNA1) for inhibitor screening. Protein Expr Purif. 2012;86(2):120–6.
48. Rao X, Li S, Jc JX, Hu X, Huang J, Chen Z, Zhu J, Hu F. A novel carrier molecule for high-level expression of peptide antibiotics in Escherichia coli. Protein Expr Purif. 2004;36(1):11–8.
49. Feng X, Liu C, Guo J, Song X, Li J, Xu W, Li Z. Recombinant expression, purification, and antimicrobial activity of a novel hybrid antimicrobial peptide LFT33. Appl Microbiol Biotechnol. 2012;95(5):1191–8.
50. Vieweg S, Ansalloni A, Wang ZM, Warner JB, Lashuel HA. An intein-based strategy for the production of tag-free huntingtin exon 1 proteins enables new insights into the Polyglutamine dependence of Httex1 aggregation and fibril formation. J Biol Chem. 2016;291(23):12074.
51. Hay ID, Du J, Reyes PR, Rehm BHA. In vivo polyester immobilized sortase for tagless protein purification. Microb Cell Factories. 2015;14(1):190.
52. Ma Y, Muench D, Schneider T, Sahi H-G, Bouhss A, Ghoshdastider U, Wang J, Dötsch V, Wang X, Bernhard F. Preparative scale cell-free production and quality optimization of MraY homologues in different expression modes. J Biol Chem. 2011;286(45):38844–53.