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# High-level Expression of Cecropin X in *Escherichia coli*

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**Abstract:** Cecropin X is a short cationic peptide with a broad antibacterial and antitumor spectrum. Here, we report the production of a tumor necrosis factor (TNFα)-cecropin X fusion protein under the control of a temperature-inducible $P_R$ promoter in the bacterial expression vector pRC. During fermentation, we studied and optimized essential parameters including the type of host cells, medium, timing of induction, post-induction time and dissolved oxygen level. Using the suitable conditions in the fermentation, up to 20 % ~ 23 % of the total cellular proteins is produced as the fusion protein, mostly in the form of inclusion bodies. After washing, on average about 5.27 g dried inclusion bodies could be collected from 1 L broth and the purity of inclusion bodies reached 80 %. Cecropin X obtained by cleaving the fusion protein with cyanogen bromide showed remarkable tumorcidal activity against mouse Lewis lung carcinoma 3LL *in vivo*.

**Keywords:** Cecropin X; *Escherichia coli*; Inclusion body; Fermentation; Tumorcidal activities

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## 1. Introduction

Cecropins are antibacterial peptides produced by insects in response to bacterial infection [1,2]. Cecropin CMIV, which was isolated from Chinese silkworm moth, is an amphipathic peptide of 35 amino acid residues with an amidated C-terminus and is absent of methionine and cysteine [3]. It was initially discovered due to its role in innate immunity and, in particular, its ability to lyse bacteria. However, like other antibacterial peptides, it displays a wide range of biological activities. Zhang's group had showed that cecropin CMIV could kill bacteria, tumors and fungi without being toxic to mammalian cells [4,5].

Although cecropin CMIV can be separated from silkworm moth, its native concentration is very low. To elucidate the biological function(s) of cecropin CMIV further, it is necessary to produce enough cecropin CMIV through DNA recombinant techniques. The expression of heterologous proteins in *Escherichia coli* is by far the simplest and most inexpensive means to produce large amounts of such products of interest.

Considering the importance of the amidated C-terminus for the antibacterial activity and the lack of amidation in *E. coli* [6,7], the cDNA encoding cecropin X - a mutant CMIV, with an asparigine residue located at the C-terminus of the peptide, was synthesized in our laboratory [8]. Then, two methods were described for the production of cecropin X. In the first method, the cDNA was cloned into the fusion expression vector pEZZ318 and the fusion protein was expressed in *E. coli* HB101 [9]. As expected, the purified cecropin X exhibited wide spectral antibacterial and tumorcidal activities without killing normal eukaryotic cells *in vitro* [10] as cecropin CMIV did. The results made the production of cecropin X using prokaryotic system possible. However, the yield of cecropin X from this system was too low to meet the demands of further research.

In the second method, the cDNA of cecropin X was fused to the 3'-terminus of the mutated tumor necrosis factor b (TNFb) gene, which was directly under the control of an inducible T7 promoter in pET-11d vector. A high expression level of the fusion protein was achieved in flask [11]. Unfortunately, when the system was scaled up, the recombinant pET plasmid exhibited apparent segregational instability and the productivity in a bioreactor was significantly affected. This problem may stem from the generation and accumulation of plasmid-free cells from the plasmid-harboring bacteria [12,13].

Therefore, a stable and high-yield system became an urgent need to provide large quantities of cecropin X for preclinical and clinical trials. In this report, we constructed the pRC/ TNFα-cecropin X bacterial expression vector, studied and optimized critical parameters during the fermentation. Using this new expression system, the TNFα-cecropin X fusion protein was overexpressed stably in the form of inclusion body and the cecropin X was obtained by cleaving the fusion protein with cyanogens bromide. The *in vivo* tumorcidal activities of cecropin X were evaluated.

## 2. Results and Discussion

### *2.1 Plasmid construction*

The expression vector pRC/ TNFα-cecropin X was constructed as shown in Figure 1. When small cationic peptides with intrinsic antibacterial activity were expressed in bacteria, the fusion protein expression system was chosen as a method to minimize toxicity to the host bacteria and susceptibility to endogenous proteolytic digestion and degradation [14-20]. Here, the plasmid pRC, with the bacteriophage λ promoter $P_R$ - a temperature-inducible promoter, was used. As previous reports [21,22] indicate, the fusion protein's expression is controlled easily and the expression level is guaranteed. The native tumor necrosis factor (TNFα) was selected because of several advantages: (1) TNFα is a low molecular weight protein (153 amino acid residues). When used as a fusion partner, the fused protein comprises relatively large amounts of target protein; (2) Because there is no Met residue in TNFα, it allows for flexibility in the design of a specific protease digestion as well as for CNBr cleavage; (3) TNFα (PI: 6.0) has a relatively low PI than TNFb (PI: 8.25), which may be favor in the purification of cecropin X.

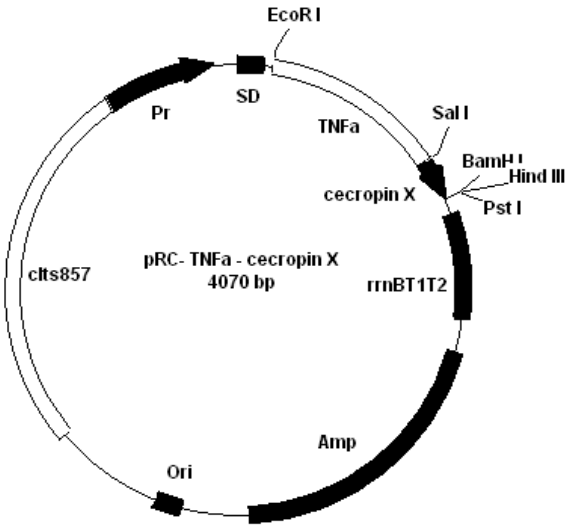


**Figure 1.** Schematic representation of expression vector, pRC/ TNFα-cecropin X, with the temperature-inducible $P_R$ promoter.

### *2.2. Expression of TNFα–cecropin X fusion protein in the E. coli strain TG1*

To assess the expression of the fusion protein, LB medium was used in culturing TG1 transformed by pRC/TNFα–cecropin X. A foreign protein was expressed, mostly in the insoluble fractions, with an apparent molecular weight of 21.0 kDa. CNBr digested a small portion of foreign protein and the concentrated supernatant fraction after dialysis showed obvious antibacterial activity against *E. coli* K12D31. To maximize the yield of cecropin X, there was a need to optimize the conditions of cell growth and the expression of fusion protein in batch fermentation.

### *2.3. Optimization of culture conditions for the overexpression of TNFα–cecropin X fusion protein in E. coli*

#### 2.3.1 Effect of host cells on fusion protein formation

It is well known that gene expression levels can vary significantly in different E. coli strains, even though the same expression vector is used [23,24]. A wide variety of E. coli host strains had been used for different target genes with the pRC vector [21]. Besides TG1, we tried five other E. coli strains, DH5α, 7118, BL21, MZ-1 and BL21 (DE3), to evaluate the expression level of full-length fusion protein. The results (Figure 2) indicated that the expression level of TNFα-cecropin X displayed great variations in different host strains. In the E. coli strain BL21, up to 21.8 % of the total cellular proteins was the fusion protein, TNFα-cecropin X. Thus, BL21 was chosen as the host cell for the pRC/ TNFα-cecropin X vector in subsequent experiments.

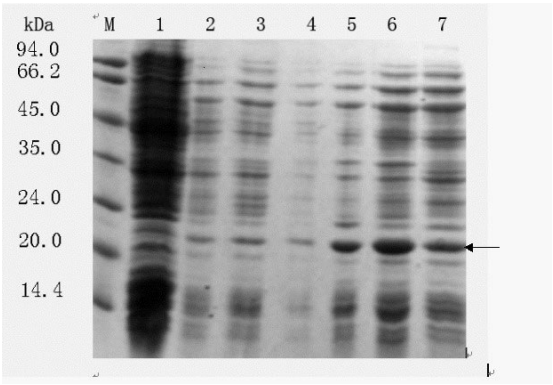


**Figure 2.** Comparison of fusion protein formation in six recombinant E. coli strains harboring pRC/TNFα–cecropin X; Lane 1: total bacterial extracts of non-induced TG1 (pRC/ TNFα–cecropin X) as the control; Lane 2 - Lane 7: total bacterial extracts of induced TG1, DH5α, 7118, BL21 (DE3), BL21 and MZ-1 harboring pRC/ TNFα–cecropin X, respectively.

#### 2.3.2 Fermentation medium

Initially, we used LB as the growth medium for TNFα-cecropin X expression. To improve the growth characteristics of the cells in scaled-up fermentation, different media including TB, SOB and SOC (Table 1) were examined, considering the ratio of tryptone to yeast extract, salts and source of carbon.

**Table 1.** Media compositions.

| **Per liter** | **LB** | **TB** | **SOB** | **SOC** |
|---|---|---|---|---|
| **Tryptone** | 10 g | 12 g | 20 g | 20 g |
| **Yeast extract** | 5 g | 24 g | 5 g | 5 g |
| **Salt** | NaCl 10 g | 2.31 g $KH_2PO_4$<br>12.54 g $K_2HPO_4$ | NaCl 0.5 g<br>KCl 0.186 g<br>MgCl2 0.95 g | NaCl 0.5 g<br>KCl 0.186 g<br>MgCl2 0.95 g |
| **Carbon** | | Glycerol 4 ml | | Glucose 3.6 g |

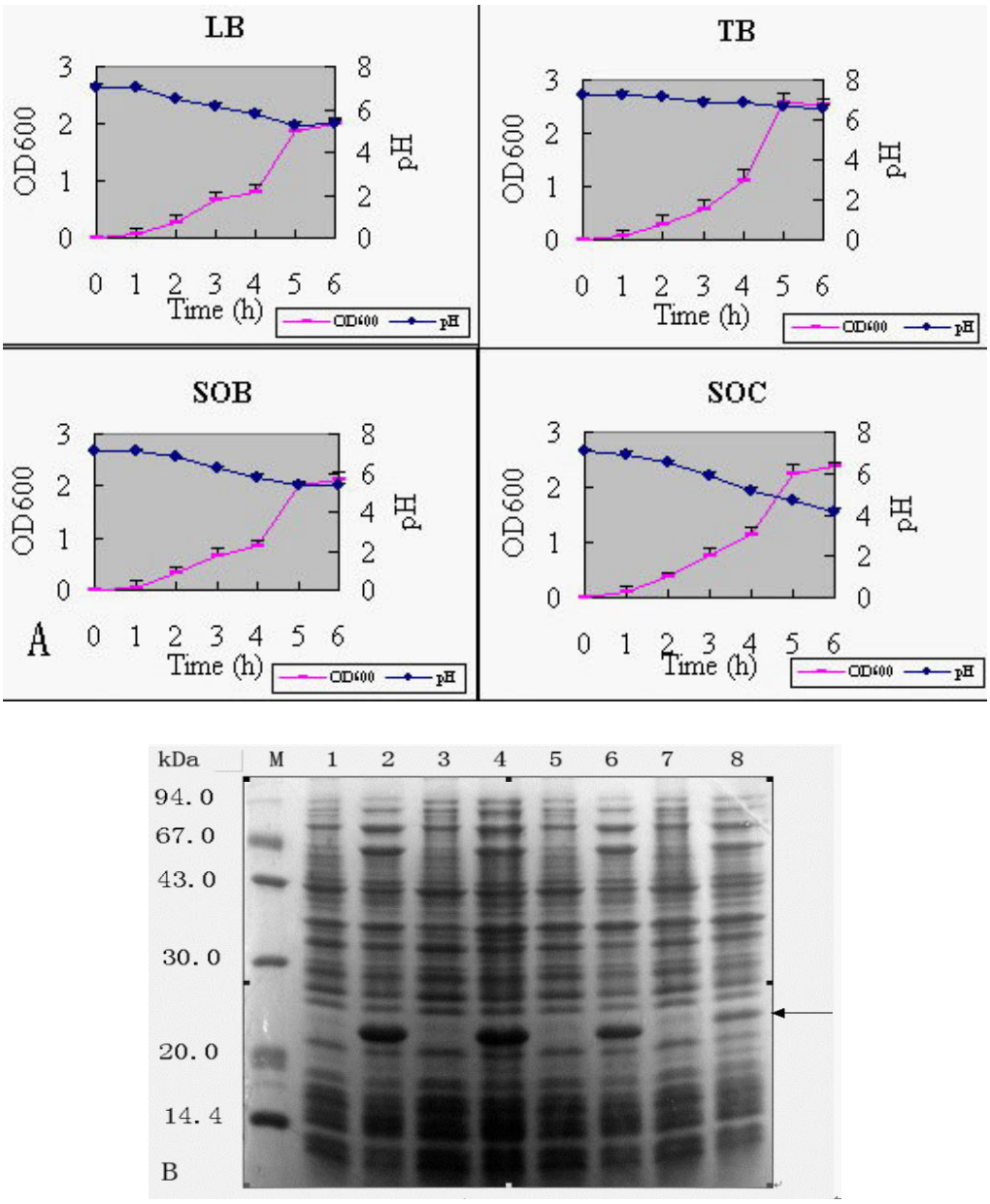


**Figure 3.** Medium effect on cell growth and expression level A: Growth profiles of BL21 (pRC/ TNFα–cecropin X) in 200 ml LB, TB, SOB and SOC medium in 500 ml shake flasks. Data are reported as an average of three runs with the standard deviation; B: SDS-PAGE of fusion protein, TNFα–cecropin X at various media. Lane 1, 3, 5 and 7: total bacterial extracts of non-induced BL21 (pRC/ TNFα–cecropin X) in LB, TB, SOB and SOC medium. Lane 2, 4, 6 and 8: total bacterial extracts of induced BL21 (pRC/ TNFα–cecropin X) in LB, TB, SOB and SOC medium.

Cell growth profiles and expression levels in these media are compared in Figure 3, which shows that an additional carbon source, whether glycerol or glucose, was beneficial for cell growth. However, in SOC medium, the expression level of the fusion protein was greatly reduced, compared to that in other media, although the cell density was higher than in other media without an additional carbon source. An evident decrease of pH values has been observed in SOC medium. The accumulation of

acetic acid from the consumption of glucose might be the major reason that affected the pH of the medium and hence the expression of the fusion protein. Contrarily, the TB medium could maintain neutral pH values. Thus, for TNFα-cecropin X expression, the medium should be well buffered and provided with glycerol as additional carbon source, whereas the ratio of tryptone to yeast extract was not important.

2.3.3 Induction timing

In the BL21 (pRC/ TNFα–cecropin X) system, a temperature shift initiates the transcription of the fusion gene on the plasmid and represents the turning point between cell growth and fusion protein biosynthesis. To evaluate the effect of induction timing, heat-induction had been done at different stages of growth. Cell densities at the end of induction and expression level of fusion protein were compared (Supplemental Table 1). The expression level of fusion protein reached the highest when bacterial were induced at the $4^{th}$ hour, in the middle stage of exponential growth. Hence, induction at the middle stage of exponential growth was adopted.

2.3.4 Post-induction time

The optimal induction time was determined by analyzing samples taken at regular time after induction. The cell densities at the end of induction and expression level of fusion protein were compared (Supplemental Table 1). The expression level of fusion protein reached the highest after 3 hours of induction, where the highest cell density reached after 5 hours of induction. Thus, 5 hours induction was chosen for sequent experiments.

2.3.5 Effect of aeration

During fermentation, the dissolved oxygen level usually affects cell growth and expression of foreign proteins. For flask cultures, medium volume could affect dissolved oxygen level [25,26]. To evaluate the effect of aeration on fermentation in flask, culture was carried out in 500 ml flasks and the media volume was chosen as 50, 100, 150, 200, 250 and 300 ml, respectively. Cell densities at the end of induction and expression level of the fusion protein were compared (Supplemental Table 1). It is clear that the cell density decreased gradually with the increase of culture volume, whereas the expression level changed less. Based on almost identical expression levels, the results indicated that the higher aeration, the higher yield of fusion protein during fermentation.

2.3.6 Batch fermentation in a 30 l fermentor

To confirm our results from the above experiments and to obtain large quantities of fusion protein, we performed fermentation in a 30 l fermentor with a 20 l working volume. During the batch fermentation, *E. coli* BL21 was chosen as the host cell, TB was the culture medium, induction was done at the middle stage of exponential growth and 5 hours was selected as the post-induction time. After testing, we found that the effect of aeration in batch fermentation was consistent with that in flask. It led us to choose the rate of air supply and agitation as high as possible. Therefore, air was

supplied at a rate of 12 l/min and the agitation rate was set at 400 rpm under this scale. The profiles of cell growth, pH and expression level of fusion protein are shown in Figure 4 and Table 2.

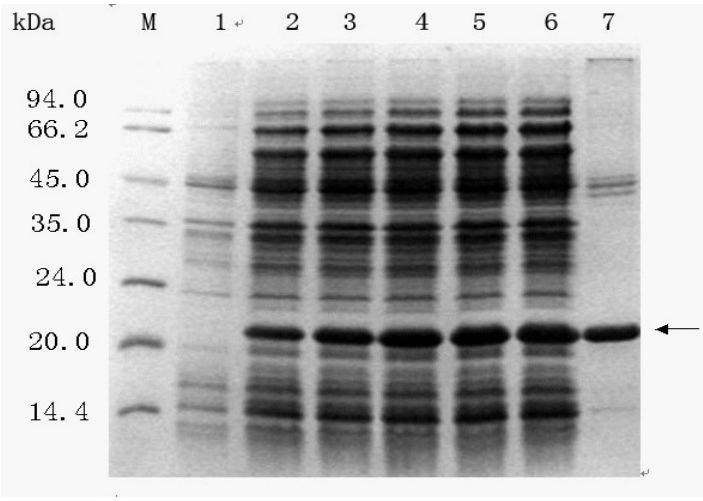


**Figure 4.** SDS-PAGE analysis of TNFα–cecropin X at regular time during the batch fermentation Lane 1: total protein before induction. Lane 2, 3, 4, 5 and 6: total proteins at 1, 2, 3, 4 and 5 hours after induction. Lane 7: inclusion bodies after washing.

**Table 2.** Summary of batch fermentation of BL21 (pRC/ TNFα–cecropin X).

| **Sample (h)** [A] | **$OD_{600}$** | **pH** | **Total protein (g /l)** [B] | **Fusion protein (%)** [C] | **Cecropin X (g /l)**[D] |
|---|---|---|---|---|---|
| 0 | 8.128 | 7.15 | 9.255 | 0 | 0 |
| 1 | 12.972 | 6.92 | 12.438 | 15.08 | 0.357 |
| 2 | 16.293 | 6.87 | 17.238 | 19.92 | 0.654 |
| 3 | 18.621 | 6.77 | 19.347 | 22.32 | 0.823 |
| 4 | 20.138 | 6.68 | 21.973 | 22.43 | 0.939 |
| 5 | 21.453 | 6.70 | 22.778 | 22.51 | 0.977 |

[A] Sample analyzed every hour after induction

[B] Estimated by BCA protein assay

[C] Estimated by densitometry analysis of SDS-PAGE gels

[D] Estimated from fusion protein ($D=B\times C\times Mw_{Cecropin\ X}/Mw_{fusion\ protein}$, the molecular weight of cecropin X is about 4000Da and the fusion protein's about 21000Da)

After the cell pellet was collected, inclusion bodies were recovered and cecropin X was purified according to the method described in section 4.5. At the end of washing, about 5.27 g dried inclusion bodies could be collected from one liter broth and the purity reached 80 % (Figure 4, Lane 7). The purification process of cecropin X was summarized in Table 3. On average, about 100 mg cecropin X was recovered from one liter broth and the purity was 98 %. We could obtain adequate amount of cecropin X from this scale of fermentation to meet the demand of future preclinical experiments,

despite that the recovery yield was only 13 %. This represents a significant improvement in the expression and volumetric productivity of cecropin X in *E. coli*.

**Table 3.** Purification summary of cecropin X.

| **Purification steps** | **cecropin X (mg)** | **Yield (%)** |
|---|---|---|
| **Inclusion bodies 5.2 g** [A] | 812 [B] | 100 |
| **Supernatant liquid** [C] | 143 [D] | 17.6 |
| **Purified cecropin X** | 106 [D] | 13.1 |

[A] Weight of the dried inclusion bodies from one liter broth

[B] Estimated from fusion protein as Table 2.

[C] After cleavage, dialysis and centrifugation, cecropin X was concentrated in supernatant fraction

[D] Determined by the linear regression equation prepared by serial dilution of a peptide standard of known concentration (determined by amino acid composition analysis)

*2.4 RP-HPLC analysis of cecropin X*

Purified cecropin X was analyzed by RP-HPLC (Figure 5). It was eluted as a single peak with 98 % purity (estimated from percentage of total peak area). The retention time was about 15 min. Mass spectrometry (3876 Da), isoelectric focusing (10.5-11.0), C- (I-N) and N- (R-W-K-I-F-K-K-I-E-K) terminal sequencing, and amino acid composition analysis (data not shown) have analyzed the cecropin X with this purity, the measured results were all coincident with the theoretical values.

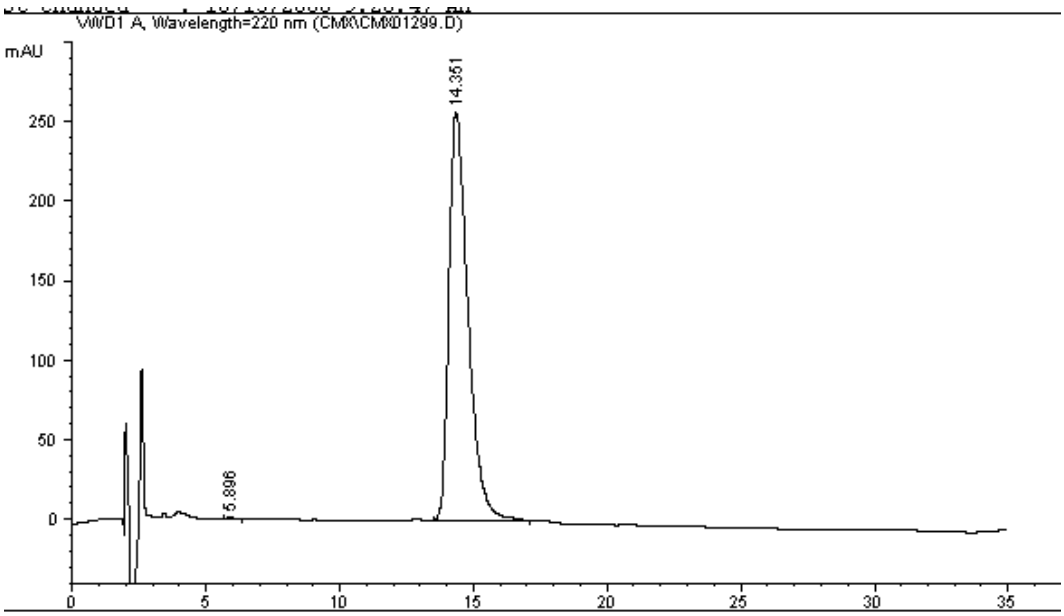


**Figure 5.** RP-HPLC analysis of purified cecropin X from batch fermentation.

*2.5 Assay of biological activity*

As cecropin X had been shown to inhibit the growth of almost all kinds of cancer cells that we had screened *in vitro* (data not shown), the next step is to investigate its biological activity *in vivo*. We used Mouse Lewis lung carcinoma 3LL model to determine the efficacy of cecropin X purified from batch fermentation. During the test, normal saline was used as the negative control; a commonly used chemotherapy drug - Cyclophosphamide (CTX [27], which belongs to a class of cancer drugs known as alkylating agents) was chosen as the positive control. The results revealed that cecropin X (i.v.) significantly inhibited Lewis lung carcinoma 3LL ($P < 0.01$) in mice. At the dose range of 0.5 to 32 mg/ kg day, the inhibition rates ranged from 27.17 % to 54.34 %. No significant gross side effect was observed (Table 4).

**Table 4.** Effect of cecropin X on tumor weight in mice ($P < 0.01$).

| Group | Dose (mg/kg day) | Given pathway | Animal number Start/End | Weight (g) Start/End | Tumor weight (g) X±SD | Inhibitory rate (%) |
|---|---|---|---|---|---|---|
| Cecropin X | 32 | i.v. | 10/10 | 20.1/25.1 | 1.21±0.14 | 54.34 |
| Cecropin X | 8 | i.v. | 10/10 | 20.7/25.9 | 1.39±0.11 | 47.55 |
| Cecropin X | 2 | i.v. | 10/10 | 20.5/25.3 | 1.69±0.13 | 36.23 |
| Cecropin X | 0.5 | i.v. | 10/10 | 20.4/25.5 | 1.93±0.11 | 27.17 |
| CTX | 30 | i.p. | 10/10 | 20.3/23.4 | 0.27±0.12 | 89.74 |
| NS | — | i.v. | 20/20 | 20.4/25.9 | 2.65±0.23 | — |

## 3. Conclusions

As the biochemical mechanisms for antibacterial peptide-induced effect are gradually revealed [28-30], scaled-up production of antibacterial peptides has become more urgently needed for further studies and clinical trials. In this paper, we constructed the expression vector pRC/ TNFα–cecropin X to effectively express the TNFα–cecropin X fusion protein by heat induction. Because this system requires no inducing agents such as IPTG, it is more economical and more environmental friendly. These merits will become more prominent in scaled-up operations. At the same time, our result sheds light on large-scale production of many promising families of antibacterial peptides.

The purity of cecropin X from batch fermentation was examined by RP-HPLC and the biological activity was estimated in the mouse Lewis lung carcinoma 3LL model. The major drawback is the relatively low recovery yield; only 100 mg cecropin X was purified from one-liter broth on average. More efficient purification techniques for cecropin X are under study to meet the demand of further clinical trials.

## 4. Materials and methods

### *4.1 Plasmid construction*

Isolation and transformation of plasmid DNA were carried out as described before [31]. Restriction endonucleases and T4 DNA ligase were used according to the directions as recommended by the manufacturer (NEB). The plasmid pUC19/ cecropin X and pET11d/ TNFb were constructed previously [11] and used as template DNAs for PCR. The primers used to amplify the coding region of cecropin X were primer 1 (forward): 5'-CGGTCGAC*ATG*AGATGGAAAATCTTCAAG -3' (the *Sal*I site was underlined and the additional start codon ATG were italicized) and primer 2 (reverse): 5'-CCGGATCCA*CTA*GTTGATGGTAGC-3' (the *BamH*I site was underlined and the complementary sequences of stop codon were italicized). The primers for TNFα were primer 3 (forward): 5'-GCGAATTCATGGTTCGTTCTTCT -3' (the *EcoR*I site was underlined) and primer4 (reverse): 5'-CCGTCGACAGAGCGATAATACCG-3' (the *Sal*I site was underlined). The recombinant plasmid, pRC/ TNFα–cecropin X was constructed by inserting the fragments of TNFα and cecropin X (amplified by PCR using above primers and digested with corresponding restriction endonucleases) between the *EcoR*I and *BamH*I sites on pRC vector [21] and transformed into *E. coli* Top 10. The transformants were confirmed by DNA sequencing.

### *4.2 Medium [31]*

Isolatio Terrific Broth (TB medium), Luria Bertani medium (LB medium), SOB medium, and SOC medium were prepared according to the Table 1.

### *4.3 Strains [31]*

*E. coli* strain Top 10 was used for subcloning and plasmid amplification. *E. coli* strains TG1, DH5α, 7118, BL21, BL21 (DE3) and MZ-1 were used as the expression hosts.

### *4.4 Expression of fusion protein*

The pRC/ TNFα–cecropin X plasmid was transformed into *E. coli* cells. Transformants were grown in LB medium containing 50 μg/ml ampicillin (same concentration for all subsequent experiments) overnight at 200 rpm and 30 °C. The overnight culture was inoculated into fermentation medium at the ratio of 1 % under various culture conditions. Shifting the temperature to 42 °C induced protein expression. After induction for several hours, bacterial pellet was collected by centrifugation at 5000 g for 10 min at 4 °C. The drained pellet was stored at -80 °C or subjected to cell disruption. Harvested cellular pellet was re-suspended in 20 mM PBS buffer (pH 6.0) and lysed by sonication. The mixture was centrifuged at 9000 g at 4 °C for 30 min; soluble and insoluble fractions were collected respectively and analyzed by SDS-PAGE.

*4.5 Expression of fusion protein in a 30 l fermentor and purification of cecropin X*

Eight hundred milliliters of overnight seed culture was inoculated to 20 L TB medium in a 30 l fermentor (BIOF-2000, Shanghai Gaoji Co., China). During fermentation, dissolved oxygen concentration and pH value changed spontaneously. Protein expression was induced by shifting the temperature to 42 °C at the mid-phase of exponential growth. The broth was sampled at regular interval for various analyses. Fermentation broth was centrifuged at 5000 g for 10 min at 4 °C. Harvested cellular pellet was re-suspended in 20 mM PBS buffer (pH 6.0) and disrupted by two passages in a homogenizer (APV 1000, Denmark). Inclusion bodies were recovered by centrifugation (9000 g, 4 °C, 30 min) and washed sequentially with five different solutions: 20 mM PBS buffer (pH 6.0), 0.5 % NaCl, 2 % Triton, TE buffer (pH 8.0), 2 M Urea and deionized water. Inclusion bodies from 1 l broth were subsequently dissolved in solution A (6 M Urea in 1.0 M HCl) and treated with 100:1 molar excess of CNBr (cyanogen bromide, from Sigma) with respect to the fusion protein for 24 hours at room temperature in the dark. The reaction products were dialyzed against 80-fold 50 mM ammonium acetate ($NH_4Ac$), pH 5.5 (solution B). The dialyzed sample was centrifuged at 10,000 g for 30 min, and the supernatant fraction was applied to a CM-cellulose column (3.6 × 20 ) equilibrated with solution B, at a flow rate of 1.0 ml/min. The column was washed with three bed volumes of solution B and bound proteins were eluted with a linear gradient of $NH_4Ac$ (0.05-0.75 M $NH_4Ac$) at a flow rate of 2.0 ml/min. The absorbance was measured online with a UV monitor at 220 nm. The peak fractions were collected, analyzed by reverse-phase HPLC, and twice dialyzed against 40 volumes of Milli-Q water for 6 hrs. The dialyzed sample was then lyophilized and stored at 4 °C for further use.

*4.6 SDS-PAGE and reverse-phase HPLC analysis*

SDS-PAGE was performed under reducing conditions using 15 % polyacrylamide gels, and resolved proteins were visualized by staining with Coomassie Brilliant Blue R250. The images of gels were scanned with a UVP white/ultraviolet transilluminator and protein bands were quantified with Grab-it 2.5 and Gelwork software. An Integrated Agilent 1100 system (Agilent, USA) was used to evaluate the purity of cecropin X. Samples were loaded onto a Agilent Zorbax 300 Extend-C18 reverse HPLC column (4.6 × 150 mm, 5 μm particles) equilibrated with 0.1 % TFA in water and were eluted with a linear gradient of acetonitrile (20-60 %) in 0.1 % TFA within 35 min. The chromatograms were recorded by measuring light absorbance at 220 nm.

*4.7 Assay of biological activity of cecropin X [32]*

Mouse Lewis lung carcinoma 3LL model was established by injecting $2.4 \times 10^6$ 3LL carcinoma cell suspension into virgin female mice (18-22 g). One day after injection, the mice were separated blindly into two NS (normal saline) groups, four cecropin X application groups (i.v.) and one positive control group (i.p.CTX, Cyclophosphamide). The dose of cecropin X was from 0.5 to 32 mg/ kg day for 7 days. The mice were sacrificed and the tumors were weighed. Inhibitory rate was calculated by using the following formula: IR (%) = (average tumor weight of negative controls-average tumor weight of test mice) / average tumor weight of negative controls ×100

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**References and Notes**

1. Steiner, H.; Hultmark, D.; Engstrom, A.; Bennich, H.; Boman, H.G. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **1981**, *292*, 246-248.
2. Boman, H.G.; Hultmark, D. Cell-free immunity in insects. *Ann. Rev. Microbiol.* **1987**, *41*, 103-126.
3. Zhang, S.Q.; Qu, X.M.; Qi, Z.W. Insect immunity and the applied prospect of cecropins. *J. Biochem.* **1987**, *3*, 11-18.
4. Zhang, S.Q.; Jia, H.W.; Zhu, Y.D. Ultrastructure observation of K562 leukemia cells treated with antibacterial peptide CM4 component. *Prog. Biochem. Biophys.* **1997**, *24*, 159-163.
5. Xu, J.; Zhang, S.Q. The mechanism of antibacterial of antibacterial peptide CMIV component was observed by confocal laser scanning microscope. *Prog. Nat. Sci.* **2001**, *10*, 1105-1109.
6. Dou, F.; Xie, W.; Dong, X.Y.; Xu, X.X. The terminal structure plays an important role in the biological activity of cecropin CMIV. *Sci. China (Ser. C)* **1999**, *42*, 494-500.
7. Nakajima, Y.K.; Qu, X.M.; Natori, S.J. Interaction between liposomes and Sarcotoxin IA, a potent antibacterial protein of *Sarcophaga Peregrina* (Flesh Fly). *J. Bio. Chem.* **1987**, *262*, 1665-1669.
8. Xie, W.; Qiu, Q.F.; Chen, J.N.; Ma, Z.; Xu, X.X. Chemical synthesis and cloning of cecropin CMIV gene from Chinese silkworm. *J. Nanjing Univ. (Nat. Sci.)* **1996**, *32*, 474-478.
9. Xie, W.; Qiu, Q.F.; Dong, X.Y.; Hua, Z.C.; Xu, X.X. Fusion expression of mutated cecropin CM IV in *E. coli*. *Sci. China (Ser. C)* **1997**, *40*, 225-231.
10. Xie, W.; Dou, F.; Qiu, Q.F.; Zhang, J.; Zhang, X.; Xu, X.X. Production and activities against clinical pathogenic bacteria of recombinant CMIV mutant. *Prog. Microbiol. Immunol.* **1997**, *25*, 46-49.
11. Wang, L.; Wu, H.H.; Dou, F.; Xie, W.; Xu, X.X. High-level expression of cecropin CMIV in *E. coli* from a fusion construct containing the human tumor necrosis factor. *Biochem. Mol. Biol. Int.* **1997**, *41*, 1051-1056.
12. Seegers, Jos. F.M.L.; Franke, C.M.; Kieweit, R.; Venema, G.; Bron, S. Use of continuous culture for the selection of plasmids with improved segregational stability. *Plasmid* **1995**, *33*, 71–77.
13. Nayak, D.P.; Vyas, V.V. Improved stability and expression of a recombinant shuttle plasmid in *Escherichia coli* during fedbatch cultivation. *World. J. Microbiol. Biotechnol.* **1999**, *15*, 65-71.
14. Andersons, D.; Engstrom, A.; Josephson, S.; Hansson, L.; Steiner, H. Biologically active and amidated cecropin produced in a baclovirus expression system from a fusion construct containing the antibody-binding part of protein A. *Biochem. J.* **1991**, *280*, 219-224.
15. Hara, S.; Yamakawa, M. Production in Escherichia coli of moricin, a novel type antibacterial peptide from the silkworm, Bombyx mori. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 664–669.
16. Harrison, S.J.; McManus, A.M.; Marcus, J.P.; Goulter, K.C.; Green, J.L.; Nielsen, K.J.; Craik, D.J.; Maclean, D.J.; Manners, J.M. Purification and characterization of a plant antimicrobial peptide expressed in Escherichia coli. *Protein. Expr. Purif.* **1999**, *15*, 171–177.

17. Lee, J.H.; Kim, J.H.; Hwang, S.W.; Lee, W.J.; Yoon, H.K.; Lee, H.S.; Hong, S.S. High-level expression of antimicrobial peptide mediated by a fusion partner reinforcing formation of inclusion bodies. *Biochem. Biophys. Res. Commun.* **2000**, *277*, 575–580.
18. Hwang, S.W.; Lee, J.H.; Park, H.B.; Pyo, S.H.; So, J.E.; Lee, H.S.; Hong, S.S.; Kim, J.H. A simple method for the purification of an antimicrobial peptide in recombinant Escherichia coli. *Mol. Biotechnol.* **2001**, *18*, 193–198.
19. Skosyrev, V.S.; Kulesskiy, E.A.; Yakhnin, A.V.; Temirov, Y.V.; Vinokurov, L.M. Expression of the recombinant antibacterial peptide sarcotoxin IA in *Escherichia coli* cells. *Protein. Expr. Purif.* **2003**, *28*, 350–356.
20. Xu, X.X.; Jin, F.L.; Yu, X.Q.; Ji, S.X.; Wang, J.; Cheng, H.X.; Wang, C.; Zhang, W.Q. Expression and purification of a recombinant antibacterial peptide, cecropin, from *Escherichia coli*. *Protein. Expr. Purif.* **2007**, *53*, 293-301.
21. Chen, J.J.; Sun, M.; Chen, C.Q.; Lu, F.; Wang, D.B. Construction and application of a high level expression vector containing $P_R$ promoter. *Prog. Biochem. Biophys.* **1996**, *26*, 495-499.
22. Palomares, A.J.; Vazquez, M.E.; Rodriguez-Llorente, I.D.; Dary, M.; Caviedes, M.A. Plasmid transfer detection in soil using the inducible Lambda $P_R$ system fused to eukaryotic luciferase genes. *Microbial. Ecology.* **2001**, *41*, 352-359.
23. Lan, P.; Tseng, C.; Lin, M.; Chang, C. Expression and purification of human placenta lactogen in *Escherichia coli*. *Protein. Expr. Purif.* **2006**, *46*, 285-293.
24. Jeong, K.; Choi, J.; Yoo, W.; Keum, K.; Yoo, N.; Lee, S.; Sung, M. Constitutive production of human leptin by fed-batch culture of recombinant rpoS- *Escherichia coli*. *Protein. Expr. Purif.* **2004**, *36*, 150-156.
25. Zhang, X.; Sun, T.; Liu, X.; Gu, D.; Tang, Z. Production of granulocyte-macrophage colony stimulating factor (GM-CSF) by high cell density fermentation of secretory recombination *Escherichia coli*. *Process. Biochem.* **1999**, *34*, 55-58.
26. Peng, L.; Xu, Z.; Fang, X.; Wang, F.; Cen, P. High-level expression of soluble human β-defensin-2 in *Escherichia coli*. *Process. Biochem.* **2004**, *39*, 2199-2205.
27. Datta, A.; Kitson, R.P.; Xue, Y.M.; Al-Atrash, G.; Mazar, A.P.; Jones, T.R.; Goldfarb, R.H. Combined chemoanti-angiogenic cancer therapy against Lewis lung carcinoma (3LL) pulmonary metastases. *In vivo.* **2002**, *16*, 451-457.
28. Silvestro, L.; Gupta, K.; Weiser, J.N.; Axelsen, P.H. The concentration-dependent membrane activity of Cecropin A. *Biochem* **1997**, *36*, 11452-11460.
29. Kaspar, A.A.; Okada, S.; Kumar, J.; Poulain, F.R.; Drouvalakis, K.A.; Kelekar, A.; Hanson, D.A.; Kluck, R.M.; Hitoshi, Y.; Johnson, D.E.; Froelich, C.J.; Thompson, C.B.; Newmeyer, D.D.; Anel, A.; Clayberger, C.; Krensky, A.M.A distinct pathway of cell-mediated apoptosis initiated by granulysin. *J. Immunol.* **2001**, *167*, 350-356.
30. Chen, J.; Xu, X.; Underhill, C.B.; Yang, S.M.; Wang, L.P.; Chen, Y.X.; Hong, S.G.; Creswell, K.; Zhang, L.R. Tachyplesin activates the classic complement pathway to kill tumor cells. *Cancer. Res.* **2005**, *65*, 4614-4622.
31. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbour Laboratory Press: New York, **1989**; pp. 16-69.

32. Ge, B.S.; Tang, Z.H.; Zhao, F.Q.; Ren, Y.H.; Yang, Y.; Qin, S. Scale-up of fermentation and purification of recombinant allophycocyanin over-expressed in *Escherichia coli*. *Process Biochem.* **2005**, *40*, 3190-3195.