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High-level expression of housefly cecropin A in *Escherichia coli* using a fusion protein

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ABSTRACT

Objective: To investigate the effect of utilizing a molecular partner on high-level expression of *Musca domestica* (*M. domestica*) cecropin in *Escherichia coli* (*E. coli*) and to identify the expressed products. **Methods:** The genomic sequence of *M. domestica* cecropin A (MC) and *M. domestica* ubiquitin (UBI) were searched from Genbank and amplified by reverse transcriptase polymerase chain reaction (RT-PCR). Two expression plasmids, pET32a-MC and pET32a-UBI-MC, were constructed and transferred into *E. coli* and were then induced by Isopropyl β-D-1-Thiogalactopyranoside (IPTG). The expression of the fusion proteins Trx-MC and Trx-UBI-MC was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fusion protein Trx-MC was verified by Western blot analysis. The bactericidal activity of the purified MC was quantitatively determined using *E. coli* BL21(DE3). **Results:** The result showed that the fusion proteins were successively expressed in *E. coli* BL21 cells. A band at the expected position of 24 kDa representing the Trx-MC target protein was positivelystained, and the band at 4 kDa representing the hydrolysis of mature MC protein was also observed at the expected position. The expression levels of Trx-UBI-MC were higher than that of Trx-MC in *E. coli*. MC exhibited antimicrobial activity. **Conclusions:** With high-level expression of housefly cecropin A in *E. coli* using a fusion protein, MC exhibited antimicrobial activity.

1. Introduction

Antibacterial peptides are widely distributed in nature and play an important role in primary host defense against pathogenic microorganisms[1]. Cecropins are antimicrobial peptides(AMPs) with a simple structure and have been found to provide the host with innate protection from bacteria, fungi, and viruses. Even at high concentrations, they do not induce lysis of erythrocytes or lymphocytes. More importantly, microorganisms do not appear to develop resistance to antibacterial peptides. Therefore, cecropin has received increasing attention as a potential antimicrobial agent and has been exploited as a drug[2]. However, isolation and purification of cecropin from natural sources is less efficient and time-consuming, while chemical synthesis of peptides is costly. For pharmaceutical application, a method to economically produce a large quantity of antimicrobial peptides is needed. Bacterial expression of heterologous proteins is an easy and inexpensive tool for producing large amounts of recombinant proteins. Cecropin from housefly cannot be expressed in the *Escherichia coli* (*E. coli*) system directly because of its strong toxicity to host cells. In the past decade, several biological expression systems have been developed by fusing the antibiotic peptide with a partner protein, possessing anionic properties, to prevent toxicity in the host cells from the resulting products. The presence of an anionic part in the fusion partner was considered essential because of its ability to neutralize the positive charge of antibiotic peptides and to result in the efficient expression of proteins of interest. Although certain carrier molecules could greatly improve the stability of passenger proteins in the expression host, the relative low yield and the subsequent isolation of the target products are difficult to overcome. The identification of a versatile carrier molecule to fit the expression of both cationic and anionic peptides at high levels is a challenge [3-8].

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Ubiquitin (UBI) has been used as a fusion partner in expression and purification of fusion proteins in plants, yeast, and bacteria. UBI is a highly conserved 76-amino-acid protein, with functions as a chaperone in ribosome biogenesis and as a marker for targeting proteins to proteasomes. UBI hydrolases remove the UBI moiety from UBI fusion proteins to produce authentic proteins *in vivo* in yeast or higher eukaryotic organisms. The plasmid pET32a(+) with the thioredoxin (Trx) gene was employed as a fusion partner of the cecropin gene from housefly. The carrier protein Trx can alleviate the toxic effects of recombinant protein on host cells and prevent proteolytic degradation of the target peptide. Moreover, Trx can also accelerate the soluble expression of the recombinant protein[9-11].

The natural structures of cecropin A from housefly (MC) and Trx-MC were simulated using bioinformatics techniques. The feasibility of MC expression with fusion proteins in *E. coli* was analyzed. The influence of UBI on the amount of fusion proteins expressed in *E. coli* was also determined. These were also verified with experimental data, and the amounts of Trx-UBI-MC and Trx-MC fusion proteins expressed in *E. coli* were compared. Trx-MC fusion protein was verified by Western blot analysis.

2. Materials and methods

2.1. Materials

Restriction enzymes, EcoRI, XhoI, HindIII, Taq DNA polymerase, pMD-18-T vector, T4DNA ligase, and RT-PCR kit were purchased from TakaRa Biotechnology (Dalians, China). The host strain *E. coli* B21(DE3) and plasmid pET32a(+) were obtained from Qiagen (Valencia, CA) .

2.2. Housefly and culture

The housefly *Musca domestica* (*M. domestica*) was provided Guangdong provincial Center for Disease Control and Prevention (CDC) and cultured in our laboratory. The components of the feeding stuff of the larvae were as follows: oaten 600 g, milk powder 40 g, yeast powder 8 g, peptone 15g, rice flour 10 g, and water 1 500 mL. After the components were mixed and filled in a feeding bottle, they were treated with 120 ℃ high-handed killing bacterial and kept in a refrigerator at 4 ℃ as a stand-by[12].

The third instar housefly larvae were treated with *E. coli* by needle pricking and then the larvae were cultured in a beaker for 48 h.

2.3. Total RNA extraction and cloning of the MC and UBI genes

Total RNA was prepared from housefly and reverse transcribed with random hexamer using SuperScriptTM (Invitrogen). Specific primers were designed based on the nucleotide sequence of *M. domestica* ceropin (GenBank Accession No. AF416602) and *M. domestica* UBI (GenBank Accession No. DQ115796).

MC primer pairs: 5′-ACGAAGTTGTTGGGGATGGTTGAAAAA AATCGGCA-3′/5′-CACTCGACTTAACCCTTTAATGTGCGG-3′. In these primer pairs, two restriction enzyme sites, HindIII (A/AGCTT) and XhoI (C/TGGAG), were added to the 5′- and 3′-termini, respectively, of F/R and three basic groups were also added to 5′-terminus of MC primers F/R for protection. UBI primer pairs: 5′-GCGGAATTATGCAGATTTTCGTGAAAAC CTTGAC-3′/5′-TAAAAGCTTGCCACCGCGCAGGCGAAGGACC-3′. In these primer pairs, two restriction enzyme sites, EcoRI (G/AATC) and HindIII, were added to 5′- and 3′- termini, respectively of F/R and three basic groups were also added to the 5′-terminus of UBI primer F/R for protection. The DNA fragments were purified by agarose gel electrophoresis. MC and UBI fragments were cloned into pMD18-T vector. The recombinant plasmids were transformed into competent *E. coli* DH5 α . All positive recombinant clones were confirmed by PCR amplification and DNA sequencing.

2.4. Analysis of the MC gene using bioinformatics

Helix and corner sites of MC were prognosticated using ProtScale (http://us.expasy.org/) software. The structural dimensions of MC and Trx-MC were prognosticated using CPH model software. The 5′-terminal structures of the mRNA of the fusion proteins Trx-MC and Trx-UBI-MC were prognosticated using the RNA structure 4.5 and RNA draw 1.6 software[13]. The protease hydroxylamine site of MC was prognosticated using peptide cutter software to cleave polymers into MC monomers.

2.5. Construction of the fusion expression plasmids pET32a-MC and pET32a-UBI-MC

A tryptohane (Trp) residue was added to the N-terminus of MC for the construction of a hydrolysis site of chymotrypsin. Using pMD-18T-MC plasmid as a template, the MC gene was amplified by PCR. The resulting PCR fragment was separated by 1.2% agarose gel electrophoresis and purified with a DNA gel extraction kit, The MC fragments and pET32a(+) plasmid were digested with XhoI/HindIII and ligated into the pET32a(+) plasmid by the addition of T4 ligase, and incubated at 16 ℃ for 18 h to produce a recombinant pET-32-MC. The presence of MC and its open reading frame (ORF) were verified by plasmid PCR amplification and DNA sequencing.

And pET-32a-UBI-MC was established as the forementioned methods.

2.6. Comparison of the amount of protein expressed in pET-32-MC and pET-32-UBI-MC

The expression plasmids pET32a-MC and pET-32-UBI-

MC were transformed into *E. coli* strain BL (DE3). A single bacterial colony was inoculated in rich liquid LB medium supplemented with 100 μg/mL ampicillin at 37 ℃. Overnight cultures were diluted 1∶100, grown to OD600=0.8, and then protein expression from the plasmid was induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 1 mmol/L IPTG. Expression was carried out for 4 h at 37 ℃. When the induced culture of bacteria reached OD_{600}=0.802, cells were harvested by centrifugation at 7 000 μg for 20 min at 4 ℃. The amount of protein expressed from pET32a-MC and pET32a-UBI-MC was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue.

The expression efficiency was assessed by analysis of the target protein band on SDS-PAGE with Image Pro plus 6.0 software in the CN-UV/WL Gel Imaging Formation System. The integrated optical density (IOD) value of single lanes was first determined followed by the IOD value of fusion protein bands. Specific IOD values of fusion proteins from pET32a-MC and pET32a-UBI-MC were compared.

2.7. Statistical analysis

Statistical analysis, including IOD ratio of normality test, 99% confidence interval of total average test, and *t*-test of the total average, was performed using SPSS13.0 statistical software.

2.8. Purification of recombinant proteins

The optimal engineered bacteria *E. coli* BL21 (DE3) were grown in LB medium containing 100 μg/mL ampicillin at 37 ℃. Protein expression was induced when OD_{600} reached 1.00 by the addition of 1 mmol/L IPTG. The culture was grown for another 4 h and then harvested by centrifugation (7 000 μg, 30 min, 4 ℃). The cell pellets were thawed on ice and resuspended in 50 mM Tris-HCl (pH 7.0) containing 500 mM NaCl. Resuspended cells were lysed by sonication on ice for 20 s four times.

Cell debris was removed by centrifugation at 12 000 μg for 30 min at 4 ℃ and the supernatant was applied directly to a His Trap HP (5 mL) column (Amersham Biosciences, Sweden) preequilibrated with a binding buffer (20 mM Na_3PO_4, 500 mM NaCl, and 5 mM imidazole; pH 7.4). The column was washed with binding buffer and the bound protein was eluted by a linear gradient of 5-500 mM imidazole in a buffer (20 mM Na_3PO_4 and 500 mM NaCl; pH 7.4) at 1 mL/min. The eluted fractions were collected and analyzed by 15% SDS-PAGE and Western blot. The PAGE gel containing Trx-MC target bands were "smashed" and placed into dialysis bags. They were then dialyzed in an electroosmosis bucket containing SDS-PAGE buffer at 300 V for 15 min and the target protein was dialyzed into dialysis bags. The fractions containing Trx-MC were collected, concentrated with PEG2000, and dialyzed in water for desalination.

2.9. Production of antibodies against the hemolymph from housefly larvae

The hemolymph from housefly larvae was collected following the procedure of Zheng *et al*[12]. and was injected into two New Zealand white rabbits. The polyclonal antibody was generated according to Wang *et al*[14], and the antibody titer was determined by agar diffusion test.

2.10. Identification of Trx-MC fusion protein by Western blot

The purified recombinant Trx-MC was run in a 15% Tris-Tricine electrophoresis column, and then transferred into a polyvinylidene difluoride (PVDF) membrane. Housefly larva hemolymph was first added into the center holes of the agar diffusion plates, followed by the addition of 1∶2, 1∶4, 1∶8, and 1∶16 diluted rabbit serum antibodies into holes around the center hole on the agar diffusion plates. This was then probed with rabbit anti-MC sera as described in Section 2.7.

2.11. Antimicrobial activity of the recombinant MC

The antimicrobial activity of recombinant MC was tested against *E. coli* BL21 (DE3) bacteria using a liquid growth inhibition assay. Only single *E. coli* BL21 (DE3) cell was inoculated into 10 mL LB (Amp-) liquid medium with an inoculating loop and grown overnight on shock tabel at 250 rpm and 37 ℃. A fresh culture was incubated with 1∶20 (overnight culture containing bacterial: fresh culture) at 37 ℃ until the optical density reached the logarithmic stage. The cells were then treated with 0.035 mg/mL MC. The control group cells were treated with 0.035 mg/mL pepsin liquid, Trx-MC, and 50 μg/mL ampicillin. The antibacterial effect of the culture was evaluated by measuring OD_{600} hourly using a microplate reader.

3. Results

3.1. Amplifying MC and UBI fragments by RT-PCR

The results showed that the size of the amplified MC and UBI fragments were consistent with the expected fragment size (Figure 1). And the cloned MC sequence was 100% identical with the No. AF416602 sequence in GenBank. The cloned UBI sequence was 99.93% identical with No. DQ115796 sequence in GenBank, whereas the cloned UBI amino acid sequence was 100% identical with No. DQ115796 sequence in the GenBank.

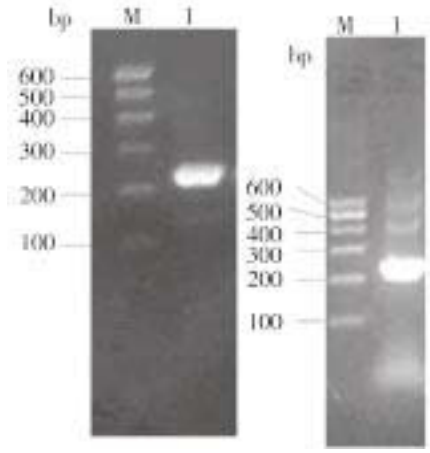

Figure 1. The RT-PCR products of cecropin A and ubiquitin.
A1: The RT-PCR products of cecropin A; AM: 100 bp DNA Ladder;
B1: The RT-PCR products of ubiquitin; BM: 100 bp DNA Ladder.

3.2. Results of forecast analysis using bioinformatics

The structural dimensions of MC and Trx-MC were predicted using CPHmodels software. The results showed that the structure of MC was an α-helix with a β-corner at the 11th to 14th amino acid residue of MC (Figures 2 and 3). The structure of MC determined by CPHmodes software matched with the experimental structure.

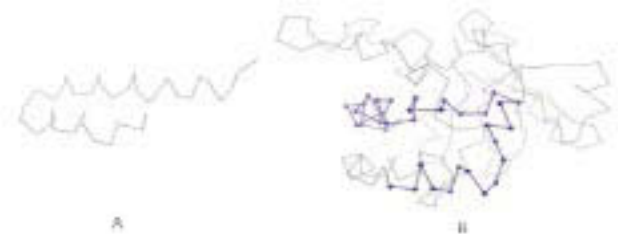

Figure 2. Predicted tertiary structure.
A: The tertiary structure of MC; B: The tertiary structure of Trx & MC.

Figure 3. Result of RNA structure 4.5.
A: The mRNA structure of Trx & Ubiquitin & MC; B: The mRNA structure of Trx & MC; The arrow indicates the mRNA 5′-terminal.

The results of the structural dimensions of Trx-MC showed that the structure of Trx-MC fusion protein was globe-shaped. The amino acid residues of MC were found to be enwrapped in the protein molecules (Figure 2). The results also showed that the expression of MC in *E. coli* using a fusion protein was feasible. mRNA 5′-termini of Trx-MC and Trx-UBI-MC had no folding and the terminal spatial structures for Trx-MC and Trx-UBI-MC were similar (Figure 3). Molecular weight (MW) of was 24 kDa. The analysis of MC peptide chain showed that there was a lack of chymotrypsin hydroxylamine site was added into Trx and MC link place.

3.3. Construction of Trx-MC and Trx-UBI-MC fusion gene expression vector

The results showed that the anticipated band at 350 bp was amplified by DNA sequencing and the sequence of MC was 100% identical with No. AF416602 MC of the housefly in GenBank. The ORF of the predicted MC amino acid sequence was found to be correct (data not shown).

3.4. Quantification analysis of the expression of pET32a-MC and pET32a-UBI-MC fusion proteins

The expression of fusion protein from the recombinant plasmids pET32a-MC and pET32a-UBI-MC induced by IPTG was analyzed by SDS-PAGE. The results showed that the molecular weights of Trx-MC and Trx-UBI-MC were consistent with theoretical calculations (Figure 4). The IOD ratio of the two fusion proteins was in line with normal distribution, and the total mean difference of ratio was statistically significant ($P<0.01$) . It is inferred that the UBI partner increased the proportion of fusion protein in total bacterial protein.

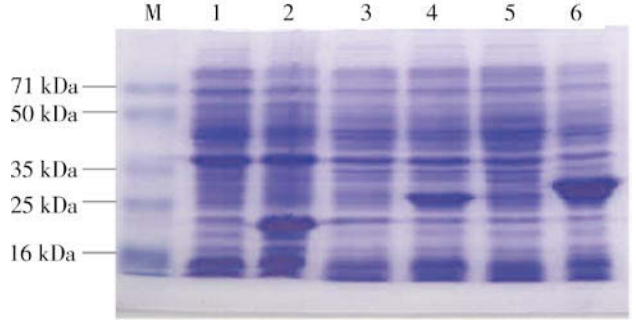

Figure 4. SDS-PAGE analysis of protein expression of pET32a-MC and pET32a-UBI-MC.
M: Protein marker;
Lane 1: Protein expression of pET32a/BL21 without induction by IPTG;
Lane 2: Protein expression of pET32a/BL21 with induction by IPTG;
Lane 3: Protein expression of pET32a-mc/BL21 without induction by IPTG;
Lane4: Protein expression of pET32a-mc/BL21 with induction by IPTG;
Lane5: Protein expression of pET32a-ubiquitin&mc/BL21 without induction by IPTG;
Lane6: Protein expression of pET32a-ubiquitin&mc/BL21 with induction by IPTG.

3.5. Western blot assay of the fusion protein Trx-MC

After 24 h, precipitation lines appeared in the 1∶2, 1∶4, and 1∶8 holes, but did not appear in the 1∶16 hole. This result showed that the antibody titer was 1∶8 (Figure 5).

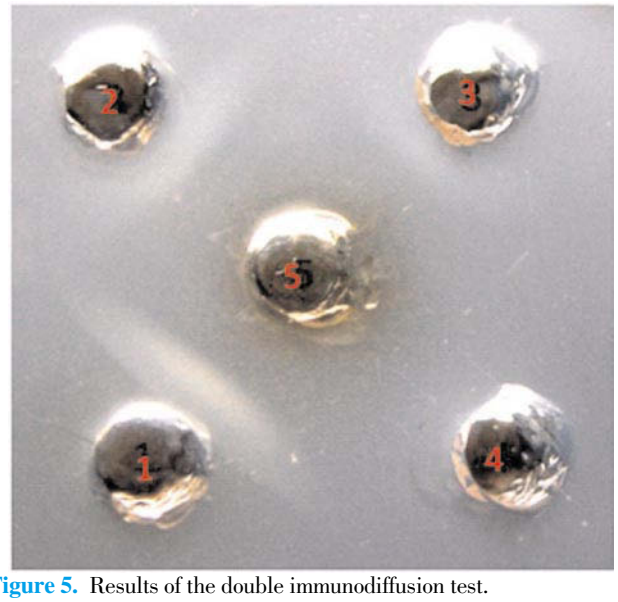

Figure 5. Results of the double immunodiffusion test.
One, two, three and four are the sera of the immunized rabbits, Five is the homogenate solution of *M. domestica* larvae.

The recombinant expression products revealed a 24 kDa Trx-MC target band at the expected position after staining with Coomassie Brilliant Blue G-250. In addition, the fusion protein expression was further confirmed by Western blot analysis with rabbit anti-housefly larva hemolymph antibody. A band with molecular weight of about 24 kDa was positively-stained (Figure 6).

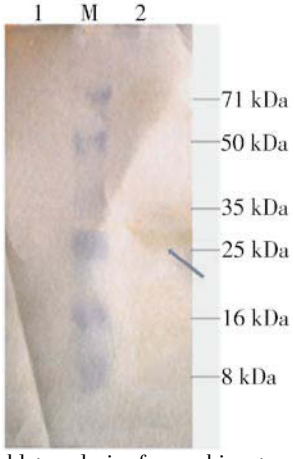

Figure 6. Western blot analysis of recombinant protein.
M: Prestained protein molecular marker;
1: Detection of Trx with sera of immunized rabbits;
2: Detection of Trx&MC fusion protein with sera of immunized rabbits.

3.6. Cleavage of recombinant protein Trx-MC and antibacterial activity assay of MC

The result showed that the hydrolysis protein was a 4 kDa protein at the expected position of MC (Figure 7), and MC exhibited substantial antimicrobial activity (Figure 8).

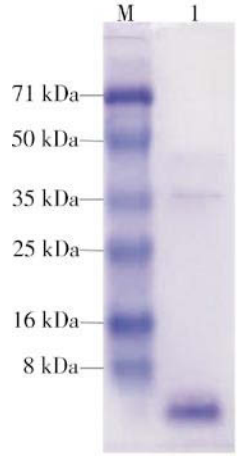

Figure 7. Tricine-SDS-PAG analysis of hydrolyzate.
M: marker; 1: hydrolyzate.

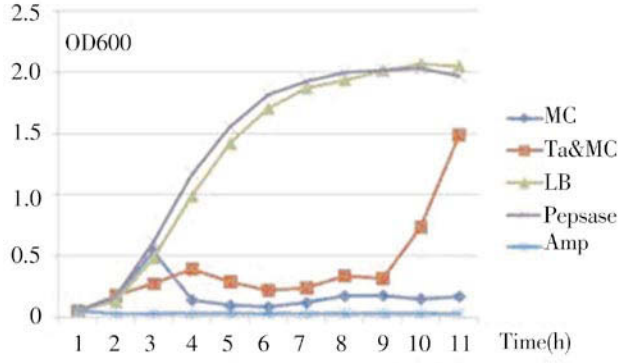

Figure 8. Inhibition growth experiment in liquid.

4. Discussion

Cecropins have been isolated from many insects. Their molecular weights have been found to be low, and they also have a simple structure. Cecropins show a wide spectrum of activity against both Gram-positive and Gram-negative bacteria by destroying the ionic balance of the bacterial membrane through formation of ionic pores[15]. Cecropin holds great promise as a drug. Based on the principles of structure-determining functions of antimicrobial peptides[16], target protein structures were constructed using a carrier molecule with the target protein for efficient protein expression in the *E. coli* system. The structure of Trx-MC fusion protein was analyzed using bioinformatic techniques. Trx was found to enwrap MC within the molecule, so as to nullify the lethality of MC in the prokaryotic host bacteria. Based on protein structure-function relationships, we analyzed the structure of MC using bioinformatic techniques by adopting different strategies to alter the target protein structure in bacteria, so as to transform its biological activity. Our aim was to avoid toxicity of the expression protein in the host cell and make feasible expression of antibiotic peptides in prokaryotic systems.

Gene transcription level, protein translation coefficient, and protein half-life data determine protein quantity in cell. The pET32a expression vector is a fusion expression vector

and its polyclonal site is located in the Trx posterior. We constructed Trx-MC and Trx-UBI-MC fusion genes, withthe Trx gene transcription regulation element and common induction conditions. The transcription levels of the two fusion genes were found to be equal. The structures of the Trx-MC and Trx-UBI-MC fusion protein mRNAs were analyzed using RNA structure 4.5 and RNA draw 1.1 software[17]. The results showed that the mRNA 5′-terminal ribosome structures in the combination region in both fusion proteins were similar. Their translation initiation factor-binding efficiency was also similar and therefore the mRNA protein translation of both Trx-MC and Trx-UBI-MC proteins was considered to be the same under similar conditions .

The present results showed that the proportion of Trx-UBI-MC fusion protein in whole bacterial protein was substantially higher than that of Trx-MC fusion protein. The former had a rasing range 1.3 times that of the latter. The molecular weight of Trx-UBI-MC and its amino acid residue quantity was 0.39 and 0.36 times, respectively more than Trx-MC. The amount of fusion protein in Trx-UBI-MC was far higher than in Trx-MC. The protease hydrolysis site of fusion protein Trx-MC was also analyzed using bioinformatics software. Because the pepsase hydrolysis site was added to the Trx and MC linking site, purified Trx-MC protein was hydrolyzed by using pepsase for releasing MC. The amino acid chain of Trx-MC fusion protein was analyzed using peptide cutter software, and the results showed that MC′s molecular weight was about 4 kDa. The molecular weight of other fragments was less than 1.5 kDa and that of pepsase was 11.7 kDa. Among the pepsase hydrolysis products, using dialysis bags with a retention molecular weight of between 2 and 10 kDa, MC was isolated from the pepsase hydrolysis products.

Trx-MC fusion protein was identified by Western blot using rabbit anti-sera against housefly larvae hemolymph. The results suggest that the expression fusion protein Trx-MC is from the housefly cecropin and therefore that the MC is natural the study showed that the housefly cecropin mature peptide was successfully expressed in *E. coli* and that the purified MC had a good antimicrobial activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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