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High-Level Expression of Acidic Partner–Mediated Antimicrobial Peptide from Tandem Genes in *Escherichia coli*

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Abstract

A novel strategy for constructing multiple joined genes of acidic partner–mediated antimicrobial peptide is described. This strategy allows the expression of antimicrobial peptide by *Escherichia coli* in a stable form and with high yield. Cecropin A (1–8)–melittin (1–10) (CAME) hybrid peptide was selected as a model of antimicrobial peptide. An acidic fragment from magainin intervening sequence was fused to the antimicrobial peptide as a partner to neutralize the lethal effects on the host cells. Multiple copies of the fusion peptide gene were tandemly linked and cloned into the expression vector pET21a. Multimers were expressed at high levels, reaching up to 36% of total cell proteins, and expression levels were proportional to the degree of multimerization. The fusion proteins were mainly expressed as inclusion bodies, probably owing to cysteine residues in the multimers. The target CAME peptide was obtained by cleaving the multimers with cyanogen bromide and purified by cation-exchange chromatography. Recombinant CAME peptide showed strong antimicrobial activities against both Gram-negative and -positive bacteria. These results might provide an efficient solution for high-level expression of various kinds of antimicrobial peptides that are toxic to the host.

Index Entries: Antimicrobial peptide; expression; tandem genes; fusion protein; inclusion bodies.

Introduction

Antimicrobial peptides are a class of small cationic peptides, which have been used as new antimicrobial substances in recent years, because they play an important role in the host's defense system against pathogen

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infections. The peptides exhibit potent antimicrobial activities against a broad range of microorganisms *(1)* but do not induce lyses of erythrocytes or lymphocytes at comparable concentrations *(2,3)*. Moreover, the antimicrobial peptides do not develop the same microbial resistance as conventional antibiotics, because of the different mechanism of action *(4)*. For therapeutic application, the production of small antimicrobial peptides by recombinant DNA techniques has become an intriguing challenge *(5)*.

Escherichia coli is the most commonly used host for antimicrobial peptides, because of its fast growth rate and well-established expression systems *(5–8)*. However, there are some difficulties, such as low yield *(6)*, proteolytic degradation of the fusion protein *(5)*, and toxicity of expression products to the host *(8)*. Different approaches have been attempted to overcome these barriers: using fusion expression *(5)*, constructing expression vectors containing multiple joined genes *(9)*, or adding an anionic fragment to counteract the cationic peptide portion *(10)*.

In the present study, an integrated approach of fusing an acidic partner to the cationic antimicrobial peptide as a fusion peptide and then constructing tandem expression of the fusion peptide was proposed. Because the positive charges of the antimicrobial peptide could be neutralized by the acidic partner, the toxic effects on the host cells are expected to decrease significantly. Herein we describe a novel strategy for constructing multiple joined genes of fusion peptide. This strategy allows the antimicrobial peptide to be expressed in a stable form and with high yield in *E. coli*. The cecropin A (1–8)–melittin (1–10) (CAME) hybrid peptide, which forms perfect α-helical conformation and has been proven to possess strong antimicrobial activities without hemolytic activity *(7)*, was selected as a model of antimicrobial peptide.

Materials and Methods

Strains, Vectors, and Enzymes

E. coli strain DH5α (TaKaRa, Dalian, China) and BL21 (DE3) (Novagen, Madison, WI) were used as host strains for cloning and expression, respectively. The *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C, and 50 μg/mL of ampicillin was added for plasmid-containing strains. pUC19 (TaKaRa) and pET21a (Novagen) were used to construct cloning and expression vectors, respectively. The restriction enzymes *Eco*RI and *Sal*I, *E. coli* DNA ligase and T4 polynucleotide kinase were purchased from TaKaRa and used according to the supplier's recommendations. A QIAprep Spin Miniprep Kit and a QIAquick Gel Extraction Kit were purchased from Qiagen (Valencia, CA). CM-cellulose was purchased from Sigma (St. Louis, MO).

Construction of Tandem Multimers of MIS-CAME (MC) Gene

Figure 1 presents the strategy for tandem multimerization of MIS-CAME (MC) gene. Briefly, two complementary single-stranded oligodeoxynucleotides coding for the 39 amino acid of MIS-CAME peptide,

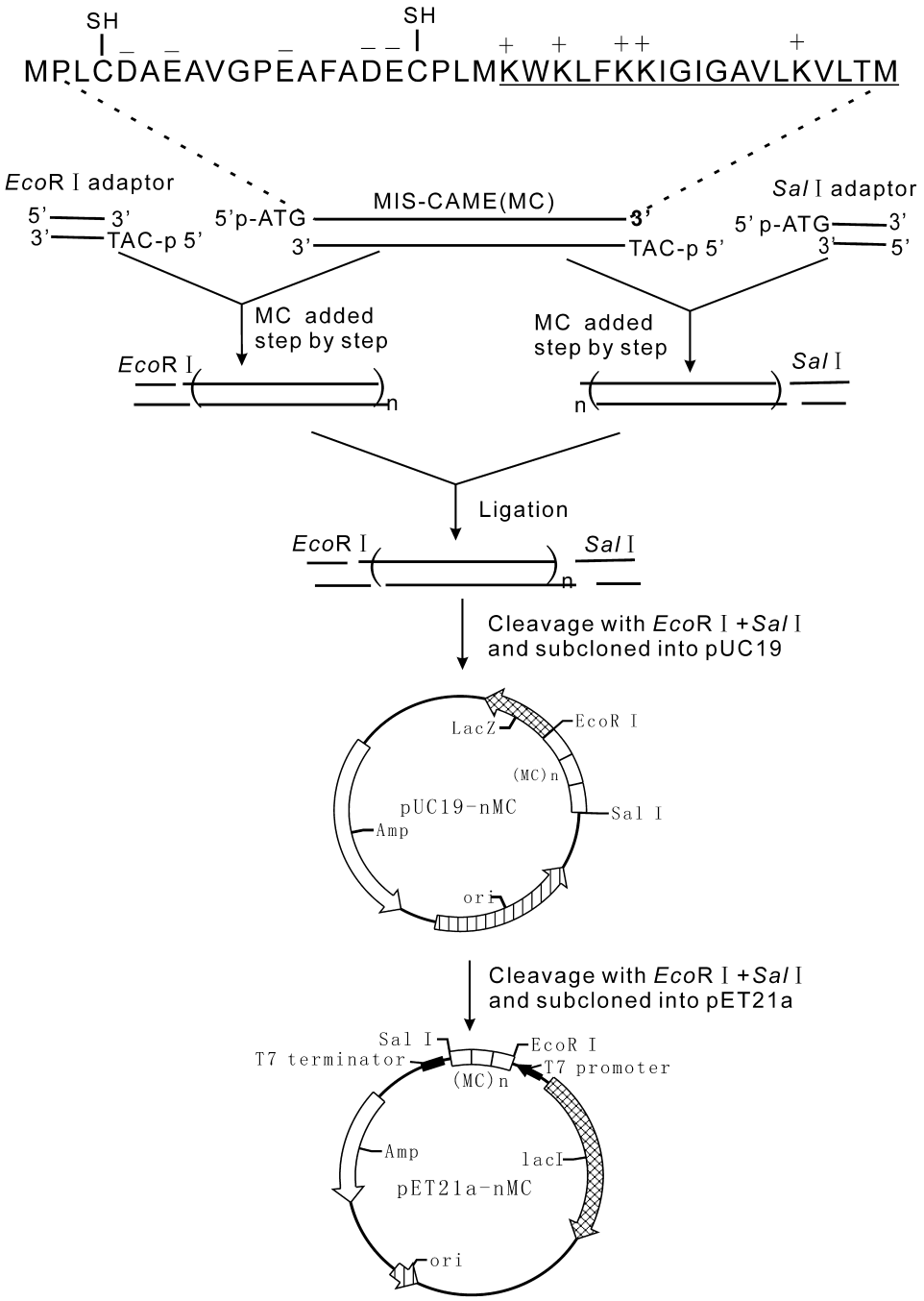

Fig. 1. Schematic representation of directional multimerization of MIS-CAME (MC) gene. *Eco*RI and *Sal*I adaptors were first ligated with MC gene at a high molar ratio (10:1) to ensure that the specified end of the gene was capped with the adaptor. Next, more fractions of MC gene were added into the ligation system step by step, and the MC genes were tandemly linked by head-to-tail ligation. At the proper time, two ligation products were mixed and continued to ligate to tandem repeats of MC gene flanked with *Eco*RI and *Sal*I restriction site. The ligation mixture was separated in agarose gel, and DNA bands containing different copies of MC gene were separately cut and recovered. Finally, the recovered multiple MC genes were digested with *Eco*RI and *Sal*I and cloned into cloning vector pUC19, then subcloned into expression vector pET21a. The CAME sequence is underlined in the fusion peptide.

in which MIS gene is fused to the 5′ side of CAME gene, were designed in accordance with preferential codon usage for *E. coli*. The two oligodeoxynucleotides were phosphorylated and then annealed to double-stranded MIS-CAME gene with three complementary oligodeoxynucleotides overhanging on the 5′ end of both strands (5′ ATG/5′ CAT). Oligo E2 (5′ CATGAATTCGAG 3′) was phosphorylated and annealed with oligo E1 (5′ CGCGAATTC 3′) to form double-stranded 5′-side *Eco*RI adaptor containing an EcoRI restriction site and a 5′-CAT sticky end. In addition, oligo S1 (5′ ATGGTCGACGCT 3′) was phosphorylated and annealed with oligo S2 (5′ AGCGTCGAC 3′) to form double-stranded 3′ side *Sal*I adaptor containing a 5′-ATG sticky end and a *Sal*I restriction site. The two adaptors were first ligated with MC gene at a high molar ratio (10:1), respectively, to ensure that the specified end of the gene was capped with the adaptor. Next, more fractions of MC gene were added into the ligation system step by step, and the MC genes were tandemly linked by head-to-tail ligation. At the proper time, two ligation products were mixed and ligated to tandem repeats of MC gene flanked with *Eco*RI and *Sal*I restriction site. The ligation mixture was separated in agarose gel, and DNA bands containing different copies of MC gene were separately cut and recovered using a QIAquick Gel Extration Kit.

Construction of Cloning Vector Containing Tandem Multimers of MC Gene

The recovered two, four, six, and eight copies of MC gene were digested with *Eco*RI and *Sal*I and ligated with linearized pUC19 vector, which was previously cleaved with *Eco*RI and *Sal*I. *E. coli* DH5α competent cells were transformed with the recombinant pUC19 and spread on LB agar plates containing 100 μg/mL of ampicillin, which were previously spread with 40 μL of 40 mg/mL X-gal on top of the agar. The white colonies harboring recombinant pUC19 plasmids were selected by white/blue screening *(11)*. The recombinant plasmids were further confirmed by restriction mapping and DNA sequencing.

Expression of Multiple MC Fusion Peptide

To construct expression vector pET21a-nMC (n = 2, 4, 6, 8) under the control of T7 promoter, the plasmids pUC19-nMC (n = 2, 4, 6, 8) were digested with *Eco*RI and *Sal*I and the smaller fragments were recovered. Then the *Eco*RI-*Sal*I fragments containing multiple MC genes were cloned into linearized pET21a vector previously digested with *Eco*RI and *Sal*I. The constructs were transformed into the *E. coli* host BL21 (DE3) and the desired clones were selected. Each clone was cultured overnight in 3 mL of LB medium with 100 μg/mL of ampicillin at 37°C and diluted to 1:100 into fresh LB medium with 100 μg/mL of ampicillin. Then 0.5 m*M* isopropyl-β-D-thiogalactoside (IPTG) was added at OD_{600} = 0.6 to induce expression.

The cells were harvested after 4 h of induction, and whole-cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) *(11)*.

E. coli cells harboring the expression vector pET21a-MC4, which has four copies of MC gene, were cultivated in a 5-L fermentor and induced with 0.5 m*M* IPTG at $OD_{600} = 0.6$. The cells were harvested by centrifuging at 6000*g* for 10 min. After lysis of the cells by sonication, the inclusion bodies were recovered by centrifuging at 10,000*g* for 30 min at 4°C and washed once with 50 m*M* Tris-HCl buffer (pH 8.0). The levels of the expressed multimers relative to total cell proteins were quantified by densitometry. The supernatant and inclusion bodies were also analyzed by SDS-PAGE to determine in which fraction the fusion protein was present.

Production, Purification, and Characterization of CAME Peptide

The inclusion bodies were denatured and solubilized in 1 *M* HCl and 6 *M* guanidinium chloride and cleaved by incubating with 1 *M* cyanogen bromide (CNBr) at 30°C for 24 h. After the cleavage reaction, the insoluble materials were removed by centrifuging at 10,000*g* for 30 min. The supernatant containing the cleaved peptides was dialyzed and applied to a CM-cellulose cation-exchange column. After equilibration with two bed volumes of 20 m*M* NaAc buffer (pH 5.0), the bound fractions mainly consisting of CAME peptide were eluted with a linear gradient of 0–1.0 *M* NaCl in 20 m*M* NaAc buffer (pH 5.0) at a flow rate of 1 mL/min. Each peak was collected and tested for antimicrobial activity after lyophilization.

The purified product was run on a tricine-SDS-PAGE gel according to the method described by Schagger and von Jagow *(12)*. Half of the gel was stained with Coomassie R-250, while the remaining gel was used for N-terminal sequence analysis. After electrophoresis, the proteins were transferred to polyvinyl difluoride (PVDF) membrane by electroblotting and stained with Coomassie R-250 for 1 min. The band of interest was excised and subjected to automated Edman degradation with an Applied Biosystems 477A sequencer.

Antimicrobial Activity of Purified CAME Peptide

Antimicrobial activity was measured by determining the minimal inhibitory concentration (MIC) following the method of Dathe et al. *(13)* with modification. *E. coli* D31 and *Staphylococcus aureus* preserved in our laboratory were used to determine the MIC. The bacterial cultures in mid-logarithmic phase were diluted to 5×10^5 colony-forming units/mL in Mueller-Hinton broth and incubated in 96-well microplates containing different concentrations of purified CAME peptide (twofold serial dilutions) in a final volume of 100 µL. After incubation at 37°C for 16 h with gentle shaking, the OD_{600} was measured. MIC is expressed as the lowest concentration that causes 100% inhibition of growth.

Results

Construction of Tandem Repeats of MIS-CAME Fusion Peptide Gene

For the design and synthesis of the gene coding for the 39 amino acid of MIS-CAME fusion peptide, DNA sequence coding for the 18 amino acid of CAME hybrid peptide was fused to the 3′ side of MIS gene. Two methionine codons (ATG) were introduced to flank CAME gene for the cleavage of tandem multimers with CNBr. In addition, two cysteine codons were introduced to flank the MIS gene in order to enhance the interaction between MIS and CAME in tandem multimers and promote the expressed protein to form inclusion bodies. Thus, following the procedure described in Materials and Methods, the MIS-CAME genes were tandemly linked to multiple genes flanked with *Eco*RI and *Sal*I restriction site. The ligation mixture was separated in agarose gel, and DNA bands containing different copies of MC gene were separately cut and recovered using a QIAquick Gel Extration Kit.

Construction of Cloning Vector Containing Multiple MC Genes

The recovered two, four, six, and eight copies of MC gene were digested with *Eco*RI and *Sal*I and cloned into pUC19 vector. The clones harboring recombinant pUC19 were conveniently selected by white/blue screening and further confirmed by restriction mapping. The recombinant pUC19 plasmids containing two, four, six, and eight copies of MC gene were digested with *Eco*RI and *Sal*I and then analyzed on 1% agarose gel (Fig. 2A). Sequencing results of pUC19-nMC (n = 2, 4, 6, 8) confirmed that tandem genes of MIS-CAME had been correctly inserted into the pUC19 plasmids.

Expression of Multiple MC Fusion Peptide

The recombinant cloning plasmids, pUC19-nMC (n = 2, 4, 6, 8), were digested with *Eco*RI and *Sal*I, and the fragments containing multiple MC genes were in-frame subcloned into the expression vector pET21a. The host cells, BL21 (DE3), harboring pET21a-nMC (n = 2, 4, 6, 8) were grown, induced, and harvested, and whole-cell lysates were analyzed by SDS-PAGE (Fig. 2B). Evident bands of target proteins were visible from two, four, six, and eight copies of the MC gene, and the expression levels were proportional to the number of copy. The expression protein from two copies was about 12% of total cell proteins, and the expression protein from six copies reached up to 36% of total cell proteins. Then the cells harboring four copies of MC gene were disrupted by sonication, and the soluble and insoluble fractions were analyzed by SDS-PAGE (Fig. 3). The results showed that the fusion protein mainly existed as inclusion bodies.

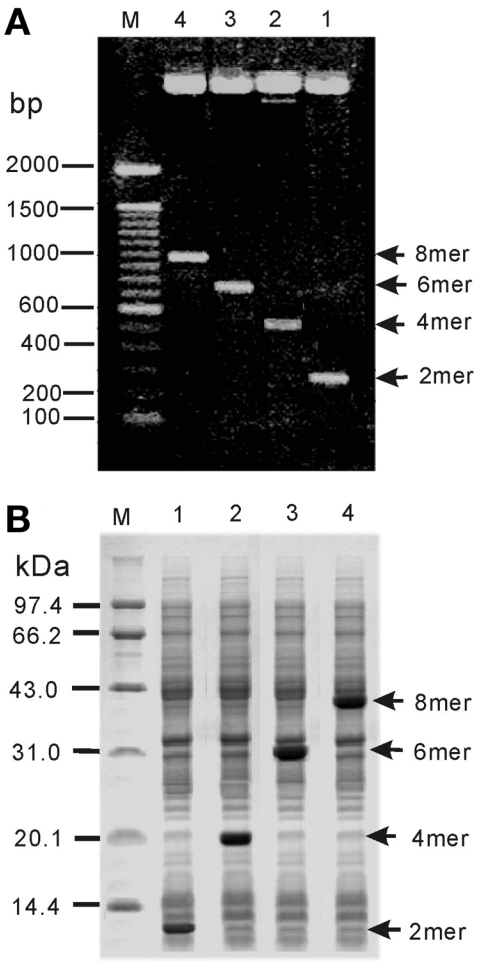

Fig. 2. Agarose gel and SDS-PAGE analysis of multiple MIS-CAME peptides. **(A)** Agarose gel analysis of multiple MIS-CAME genes. pUC19-nMC ($n = 2, 4, 6, 8$) was digested with *Eco*RI + *Sal*I and then electrophoresed in a 1.0% agarose gel. Lane M, 100-bp ladder; lanes 1–4, *Eco*RI+ *Sal*I digested pUC19-MC2, -MC4, -MC6, and -MC8, which contain two, four, six, and eight copies of the MIS-CAME gene, respectively. **(B)** Expression of multiple MIS-CAME peptides in *E. coli* BL21 (DE3). The recombinant proteins were resolved on SDS-PAGE constituting 5% stacking gel and 12% resolving gel. Lane M, molecular weight markers; lanes 1–4, BL21 (DE3) harboring pET21a-MC2, -MC4, -MC6, and -MC8, respectively. Arrows indicate the recombinant proteins.

Production, Purification, and Characterization of CAME Peptide

The inclusion bodies were treated with 1 *M* CNBr, and the samples were subjected to CM-cellulose cation-exchange chromatography, producing >90% homogeneity of CAME peptide, as shown in Fig. 4. About 89 mg of pure CAME peptide was obtained after CM-cellulose cation-exchange chromatography, with 90% purity from 1 L of *E. coli* culture. The target CAME peptide with high purity and good yield could be obtained by

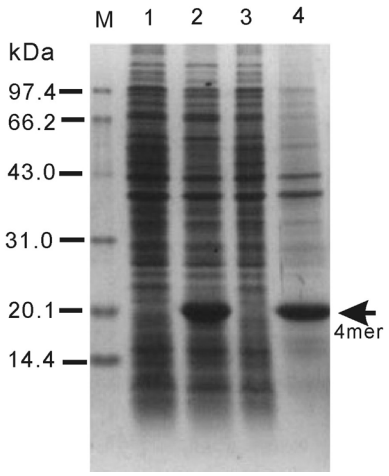

Fig. 3. Solubility analysis of 4-mer fusion peptides: lane M, molecular weight markers; lanes 1 and 2, total cell proteins before and after and after IPTG induction; lanes 3 and 4, soluble fractions and inclusion bodies isolated from total cell proteins.

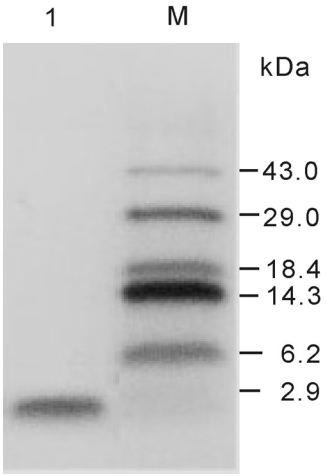

Fig. 4. Tricine-SDS-PAGE analysis of purified CAME peptide: lane M, molecular weight markers; lane 1, purified CAME peptide after CNBr cleavage and CM-cellulose cation-exchange chromatography.

combining efficient CNBr cleavage and conventional cation-exchange chromatography, since CAME peptide has a much higher isoelectric point (calculated to be about 10.60) than the acidic partner or other host proteins.

The purified CAME peptide was run on a tricine-SDS-PAGE gel and transferred to a PVDF membrane. The band of interest was excised and subjected to sequence analysis. The N-terminal sequence of the CAME peptide was KWKLFKKIGI, which was identical to the theoretical sequence. An additional homoserine at the C-terminus of the recombinant CAME

peptide were derived from a methionine residue after CNBr cleavage and did not influence the antimicrobial activity of recombinant CAME peptide.

Antimicrobial Activity of Purified CAME Peptide

The purified recombinant CAME peptide was tested for its antimicrobial activity by determining MIC against two bacteria, *E. coli* D31 and *S. aureus*, representing Gram-negative and -positive bacteria, respectively. The MIC values of recombinant CAME peptide against *E. coli* and *S. aureus* were 8 and 16 μg/mL, respectively, within the range of the antimicrobial activities of synthetic CA (1–8)–ME (1–10) hybrid peptide *(7)*.

Discussion

There are two major problems in the expression of antimicrobial peptides in *E. coli*: (1) the expressed peptides are toxic to the host, and (2) the high content of positively charged amino acids makes them very sensitive to protease. It is well known that many of the natural antimicrobial peptides, such as magainin *(2,14)* and defensin *(15,16)*, are synthesized as precursors in which anionic prosequence neutralizes the cationic peptide. One possible reason is that the anionic prosequence masks the toxicity of the cationic peptide on the host cells by neutralizing its positive charges. Furthermore, a folded structure between the cationic peptide and anionic prosequence protects the precursors from proteolytic degradation. Recently, successful expression of the antimicrobial peptide has been achieved either by expressing the precursor of the antimicrobial peptide instead of the mature peptide *(17)* or by fusing an anionic partner to the antimicrobial peptide to mimic a neutral precursor *(10,18)*. Constructing multimers of the small peptides is another practical means to enhance their expression *(9,10,19–22)*.

We have described a novel strategy to construct tandem multimers of the fusion peptide gene. This strategy does not require special amplification vector such as PBBS1 *(10,20–22)*, which could be digested to produce asymmetric cohesive ends. It also does not require special sequence for two isocaudamers *(9,19)*. This strategy may provide an alternative for constructing tandem multimers of other genes by changing adaptors containing other restriction sites or adopting suitable linker for other specific cleavage of peptide bonds.

To verify the feasibility that tandem multimers of acidic partner–mediated antimicrobial peptide could enhance its expression, we selected CAME hybrid peptide as a model peptide and magainin intervening segment as the acidic partner. Two methionine codons (ATG) are introduced to flank the CAME gene in order to cleave the multimers with CNBr to produce active peptide. On cleavage, the 19 amino acid of CAME peptide with an extra homoserine at the C-terminus is released from the fusion protein. The MIC value of recombinant CAME peptide indicates that it has activity comparable with that of synthetic CA (1–8)–ME (1–10) peptide

published previously *(7)*, and the additional homoserine residue has little effect on its biologic activity. The acidic partner was selected because it was considered that its length and opposite charge could equilibrate the CAME peptide. When tandem repeats of MIS-CAME fusion peptide were expressed in pET21a under the control of T7 promoter, the expression level was substantially improved. Expression from a single copy of fusion peptide was very low (data not shown), but that from two copies was evidently enhanced. As the copy number increased, the expression level increased significantly. In particular, the expression level from six copies reached up to 36% of total cell proteins. However, the expression from eight copies decreased slightly, suggesting that the expression levels are not always proportional to the degree of multimerization. The expression levels from more than eight copies were not studied.

Several studies have demonstrated that the formation of inclusion bodies could mask the positive charges of the antimicrobial peptide, thereby resulting in high-level expression *(10,22,23)*. Therefore, we introduced two cysteine residues to flank the acidic partner in order to enhance the interaction between MIS and CAME, which could promote the expression protein to form inclusion bodies. In the study, the effects of hydrosulfide groups on the expression of monomer and dimer fusion peptide were negligible. The possible reason is that the disulfide bonds formed in them may not form stable structures for efficient neutralization. However, with larger multimers, complex crosslinks of intra- or intermolecular disulfide bonds may lead to efficient charge neutralization for the formation of inclusion bodies. The fusion proteins in inclusion bodies could be easily purified and do not need refolding to become active. As a result, the proteins are conveniently cleaved with CNBr, and the target CAME peptide can be easily purified with >90% purity and high yield using cation-exchange chromatography in a single step.

The method described herein might be suitable for expressing other antimicrobial peptides. The acidic partner played a key role in this expression system by masking the positive charges of the cationic peptide. In addition, disulfide bonds in the multimers would prompt the expression protein to form inclusion bodies and further mask the toxicity of the peptide. The results showed that the expression levels of the multimers were enhanced with the increasing copy, since more cysteine residues in larger multimers have a stronger tendency to form stable inclusion bodies. In conclusion, the method that we have described opens a door to the mass production of antimicrobial peptides, thereby satisfying the need of biologic and clinical studies.

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