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High-level expression of the recombinant hybrid peptide cecropinA(1-8)–magainin2(1-12) with an ubiquitin fusion partner in *Escherichia coli*

Xiaoxia Xu [a,1], Fengliang Jin [b,1], Xiaoqiang Yu [c], Shunxiang Ren [b], Jian Hu [a], Wenqing Zhang [a,*]

[a] *State Key Laboratory of Biocontrol, College of Life Sciences, Sun Yat-sen (Zhongshan) University, Guangzhou 510275, PR China*
[b] *Department of Entomology, College of Natural Resource and Environment, South China Agricultural University, Guangzhou 510642, PR China*
[c] *School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110, USA*

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Abstract

The hybrid antibacterial peptide CA–MA [cecropinA(1-8)–magainin2(1-12)] with potent antimicrobial properties but no hemolytic activity is a potential alternative antibiotic. To explore a new approach for high-level expression of the hybrid peptide CA–MA in *Escherichia coli*, the sequence of ubiquitin (UBI) from housefly was inserted into the plasmid pQE30 to construct the vector pQEUBI. The cDNA fragment encoding CA–MA with preferred codons of *E. coli* was obtained by recursive PCR (rPCR) and cloned into the vector pQEUBI to express the fusion protein $(His)_6$-UBI-CA–MA. The fusion protein was expressed in soluble form under the optimized conditions at high level (more than 36% of the total proteins). With $(His)_6$-tag, the fusion protein was easily purified by Ni–NTA chromatography and 36 mg of fusion protein was purified from 1 L of culture medium. The fusion protein was efficiently cleaved by ubiquitin C-terminal hydrolase (UCH), yielding recombinant CA–MA with high antimicrobial activity. After removing the contaminants by Ni–NTA chromatography, recombinant CA–MA was purified to homogeneity by reversed-phase HPLC and 6.8 mg of pure active CA–MA was obtained from 1 L culture medium. Analysis of recombinant CA–MA by MALDI-TOF-MS showed that the molecular weight of the purified recombinant CA–MA was 2559 Da, which perfectly matches the mass (2559 Da) calculated from the amino acid sequence. Analysis of CA–MA by circular dichroism (CD) revealed that the secondary structures of CA–MA in water solution were 17.4% α-helix and 82.6% random coil but no β-sheet. Our results demonstrated that functional CA–MA can be produced in sufficient quantities using the ubiquitin fusion technique. This is the first report on the heterologous expression of a hybrid antibacterial peptide fused to ubiquitin in *E. coli*.

Keywords: Hybrid peptide CA–MA; Ubiquitin; Fusion expression; Purification; Anti-microbial activity

Antibacterial peptides are widely distributed in nature and play an important role in primary host defense against pathogenic microorganisms [1]. In recent years, a great variety of antibacterial peptides has been discovered from various sources [2,3]. Antibacterial peptides have potent activity against a broad range of microorganisms and they do not induce lysis of erythrocytes or lymphocytes even at high concentrations [4]. More importantly, microorganisms do not appear to develop resistance to antibacterial peptides. Therefore, antibacterial peptides have received increasing attentions as potential new antimicrobial substances [5].

Isolation of antibacterial peptides from natural sources is inefficient and time-consuming, while chemical synthesis of peptides is costly [6]. For pharmaceutical applications, a method to produce a large quantity of antimicrobial

* Corresponding author. Fax: +86 20 84112297.
E-mail address: wenqingzhang818@yahoo.com.cn (W. Zhang).
[1] These authors contributed equally to this work.

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peptides economically is needed. Bacterial expression of heterologous proteins is an easy and inexpensive tool for producing large amounts of recombinant proteins for structural investigations. However, antibacterial peptides cannot be expressed in *Escherichia coli* system directly because of their toxicity to host cells. To overcome this obstacle, several biological expression systems have been developed by fusing the antibacterial peptide with a partner protein that has anionic properties [7]. The presence of an anionic segment was considered essential in the fusion partner for its ability to neutralize the positive charge of antibacterial peptides and result in efficient expression of the target protein. Such fusion partners include glutathione *S*-transferase (GST) [8,9], maltose-binding protein (MBP) [10], and thioredoxin (Trx) [11,12]. Although previous studies showed that certain fusion molecules could greatly improve the stability of the target proteins in host cells, difficulties related to expression and purification were often encountered, such as low yield and subsequent purification of the target protein. Therefore, a biological expression system with high expression level and easy purification procedures for antimicrobial peptides should be developed.

Ubiquitin (UBI) has been used as a fusion partner [13,14] in expression and purification of fusion proteins in plants [15,16], yeast [17–20], and bacteria [21–26]. UBI is a highly conserved 76-amino acid protein, which functions as a chaperone in ribosome biogenesis [27–29] and as a marker for targeting proteins to proteasomes [30–32]. A few mammalian proteins have also been successfully expressed as UBI fusion proteins in bacteria [33,34]. UBI hydrolases remove the UBI moiety from UBI fusion proteins to produce authentic proteins in vivo in yeast or higher eukaryotic organisms [35,36].

The hybrid peptide cecropinA (1-8)–magainin2 (1-12) (CA–MA)[2], which has the amino acid sequence of MKWKIGKKIGIGKFLHSAKKFN, was found to display high antibacterial and antitumor activities but no hemolytic activity [37,38]. In the present study, the UBI system was explored by combining the stabilizing effect of UBI and $(His)_6$-tag for expression and purification of hybrid peptide CA–MA in bacteria. Expression, purification and characterization of CA–MA have been carried out, and antimicrobial activity of the purified recombinant CA–MA was tested.

Materials and methods

Materials

All restriction enzymes, expression plasmid pQE30, *Escherichia coli* M15 (prep4) and immobilized metal ion affinity chromatography (IMAC) (Ni–NTA) resin were purchased from Qiagen (Germany), Ni–NTA conjugate was obtained from Qiagen (Germany) for detect His-tag. C18 column (250×4.6 mm, 5 μm, 300 Å) was obtained from Pharmacia-LKB (Germany). *E. coli* strains TG1 and Top10F′ were used for routine plasmid amplification. T4 DNA ligase and *Taq* DNA polymerase were from TaKaRa Biotechnology (Dalian, China). UBI C-terminal Hydrolase (UCH) was obtained from Biomel company (USA).

Construction of expression vector with UBI

The strategy adapted for recombinant plasmid vector expressing $(His)_6$-UBI-CA–MA is shown in Fig. 1. A DNA fragment encoding housefly UBI (GenBank Accession No. DQ115796) was amplified by reverse-transcription polymerase chain reaction (RT-PCR) using cDNA of housefly as a template, and two primers: UBIF (5′-GCG *GGA TCC* ATG CAG ATT TTC GTG AAA ACC-3′) and UBIR (5′-G*AA GCT T*TT AGC CA CCGCGG A GGC GAA GGA CC-3′). The cDNA fragment, purified by agarose gel electrophoresis, was digested with BamHI and HindIII enzymes, and the digested fragment was ligated into the BamHI/HindIII digested pQE30. This recombinant plasmid (pQEUBI) was transformed into competent *E. coli* Top10F′ and the insert was sequenced to ensure the correct orientation of the coding sequence. Using this strategy, the UBI gene was fused in-frame with pQE30 plasmid to the $(His)_6$-tag, and the fusion gene was under the control of T5 promoter.

Two primers with 20 bases complementary at their 3′ ends were designed based on the CA–MA amino acid sequence (GenBank Accession No. DQ645627). The primers consisted of the preferred codons of *E. coli*, a SacII enzyme restriction site and a fragment of UBI sequence encoding the last six amino acids of UBI on the 5′ end, and a stop codon and HindIII site on the 3′ end. The sequence introduced at the 5′ end allows cloning into the SacII site of the UBI gene by placing the first amino acid of CA–MA immediately after the last amino acid Gly^{76} of UBI. The primers used for PCR were: P1 (5′-AGA CCG CGG TGG CAT GAA ATG GAA GAT TGG CAA AAA AAT CGG CAT TGG TAA GTT CTT ACA TTC TGC-3′) and P2 (5′-GTT AAG CTT TTA ATG ATG ATG ATG ATG ATG GTT GAA TTT CTT AGC AGA ATG TAA GAA CTT ACC-3′). The sequence encoding CA–MA was obtained by recursive PCR, the PCR product was purified, digested with SacII and HindIII, and cloned into the corresponding sites of vector pQEUBI. The new constructed vector pQEUBI-CA–MA was transformed into competent *E. coli* Top10F′ and the insert was sequenced to ensure that the coding sequence of CA–MA was correct and in-frame with the UBI gene (Fig. 1).

[2] *Abbreviations used:* CA–MA, cecropinA(1-8)–magainin2(1-12); RP-HPLC, reversed-phase high performance liquid chromatography; UCH, ubiquitin C-terminal hydrolase; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; IMAC, immobilized metal-ion affinity chromatography.