Microbiological Research 165 (2010) 713–718

Available online at www.sciencedirect.com
ScienceDirect

Microbiological Research

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Expression and purification of moricin CM4 and human β-defensins 4 in *Escherichia coli* using a new technology

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Received 11 September 2009; received in revised form 22 December 2009; accepted 3 January 2010

KEYWORDS
CM4;
HβD4;
Elastin-like peptide (ELP);
Intein;
Inverse transition cycling (ITC);
Self-cleavage

Abstract

Different strategies have been developed to produce small antimicrobial peptides using recombinant techniques. Here we report a new technology of biosynthesis of moricin CM4 and human β-defensins 4 (HβD4) in the *Escherichia coli*. The CM4 and HβD4 gene were cloned into a vector containing the tags elastin-like peptide (ELP) and intein to construct the expression vector pET-EI-CM4 and pET-EI-HβD4. All the peptides, expressed as soluble fusions, were isolated from the protein debris by the method called inverse transition cycling (ITC) rather than traditional immobilized metal affinity chromatography (IMAC) and separated from the fusion leader by self-cleavage. Fully reduced peptides that were purified exhibited expected antimicrobial activity. The approach described here is a low-cost, convenient and potential way for generating small antimicrobial peptide.
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Introduction

CM4 (Tu et al., 1989) and HβD4 are all cationic antimicrobial peptides with an amphipathic structure, which allows them to disrupt the cell membrane of the invading pathogen, resulting in lysis and death but atoxic to normal mammalian cells (Zhang et al., 1997) and do not acquire any microbial resistance. This new mechanism of action makes antimicrobial peptides the novel potential pharmaceutical agents to solve the problem of

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doi:10.1016/j.micres.2010.01.002

increasing antibiotic-resistance caused by the abuse of antibiotics.

The *in vivo* biosynthesis in *Escherichia coli* is a popular technique for producing antimicrobial peptide. The affinity tag is often fused with target protein to neutralize its innate toxic activity to the host and be convenient to purification during chromatographic separation. However, there are some problems associated with the use of this method. It requires costly buffer, easily consumed affinity resins and expensive apparatus. And expensive protease enzyme is required to cleave the target protein from the affinity tag, which adds additional steps to the purification process in terms of the separation of protease and the target protein. Besides, it is likely that protease attacks the target protein due to its limited specificity. Therefore affinity chromatography is not an economical method for producing recombinant protein in the long run.

The combination of elastin-like polypeptides (ELPs) with intein provides a general solution to these problems (Ge et al., 2005). ELP is a polypeptide composed of repetitive motifs of VPGXG (X is any amino acid with the exception of proline) (Urry, 1992), ELPs are characterized by phase transition behavior that they are soluble below the transition temperature (T_t) but will aggregate (not salting-out) immediately when the temperature is higher than T_t (Urry et al., 1978). This process is reversible, is called inverse transition cycling (ITC). When fused to other proteins, it retains this behavior (Meyer and Chilkoti, 1999; Meyer et al., 2001; Trabbic-Carlson et al., 2004). T_t is dependent on the composition of guest residues, chain length of the repetitive segment and the concentration of salt (Urry et al., 1991; Meyer and Chilkoti, 2004). Gently heating or adding of NaCl can trigger the specific aggregation of fusion protein from the cell lysate without irreversible precipitation of other proteins. And the fusion protein can be purified by simple centrifugation or microfiltration. The △I-CM intein is a modified protein splicing element, which allows controllable C-terminal peptide bond cleavage triggered by low pH and high temperature (Wood et al., 1999). The fusion protein containing this tag can self-cleave in the cleaving buffer easily and efficiently. Briefly the self-cleaving ELP system is a chromatography-free and protease-free system.

This system enlightened us in producing antimicrobial peptide. CM4 and HβD4 were fused downstream of ELP-intein to create tripartite fusion EI-CM4 and EI-HβD4. The ELP used in the fusion consists of 110 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly where Xaa is Val, Ala and Gly in the ratio of 5:2:3, which has a T_t below 30 °C in high-salt solution. The paper describes the details of expression and purification.

Materials and methods

Bacterial strains, vectors and enzymes

E. coli DH5α (maintained in our laboratory) was used for subcloning and plasmid amplification. *E. coli* BLR (DE3) (Novagen, USA) was used as the expression host. *E. coli* $K_{12}D_{31}$ and *P. aeruginosa* ATCC27853 conserved by our laboratory were used for antimicrobial assays. The plasmid pET-EI-GFP used to construct the expression vector was donated by Professor David W. Wood (Princeton University, USA). Restriction enzymes BsrG I and Hind III were purchased from New England BioLabs (USA), and T4 DNA ligase and DNA polymerase were from Takara Biotech Co. Ltd. (Dalian, China). All other chemical reagents were made in China and of analytical grade.

Construction of expression vectors

The translationally silent restriction site at the end of the intein (BsrG I) was also designed involved in the up primer to avoid adding any additional residues to the product protein (Wu et al., 2006). The CM4 gene was amplified from plasmid pET32a(+)-CM4 constructed previously in Zhang's laboratory (Zhou et al., 2009), the HβD4 gene was amplified from plasmid pSUMO-HβD4 also constructed in our lab. The PCR fragments were separated by 1.5% gel electrophoresis and purified with a DNA gel extraction kit (AxyGEN Union, USA). Afterward the PCR products were digested with BsrG I and Hind III and ligated into the digested plasmid pET-EI-GFP (also by BsrG I and Hind III) at the corresponding restriction sites to construct the expression vector pET-EI-CM4 and pET-EI-HβD4. The ligation mixture was transformed into *E. coli* DH5α cells for verification by sequencing.

Fusion protein expression

E. coli BLR (DE3) cells harboring constructed expression vector were cultured (1:100) in fresh LB medium supplemented with 100 μg/ml ampicillin and grown at 37 °C on a rotary shaker overnight. 10 ml overnight starter cultures were centrifuged at 2000 g for 15 min at 4 °C to remove β-lactamase, and resuspended in fresh medium of the same volume. 1 l terrific broth (tryptone 16 g/l, yeast

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