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Recombinant expression of novel protegrin-1 dimer and LL-37-linker–histatin-5 hybrid peptide mediated biotin carboxyl carrier protein fusion partner

Santhasiri Orrapin, Sorasak Intorasoot *

Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand

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ABSTRACT

Antimicrobial peptides (AMPs) hold great promise as potential therapeutic approach for curing of infectious diseases. Prokaryotic protein expression renders high scalability with an effective purification of several heterogeneous proteins. However, it might be inappropriate for recombinant AMPs expression thereby its antimicrobial activity against the host cells. Several fusion partners demonstrated antimicrobial activity neutralization of AMPs expression and purification in *Escherichia coli*. In order to improve the antimicrobial effect, several hybrid AMPs have been designed and developed. As expected to increase the antimicrobial activity, a dimeric form of porcine protegrin-1 (PG-1) and human LL-37-linker–histatin-5 (LL-37-linker–Hst-5) hybrid peptide were alternatively constructed in this study. Hydroxylamine hydrochloride and thrombin cleavage sites were designed for releasing of hybrid peptide and PG-1 dimer from biotin carboxyl carrier protein (BCCP) fusion partner. The full-length AMPs gene was connected down-stream of BCCP gene using the overlap extension-PCR, cloned into pET-28a vector and expressed in *E. coli* BL21(DE3)pLysS. After IPTG induction, approximately 20% of BCCP-AMPs was expressed as intracytoplasmic inclusion bodies with an expected molecular weight of 24.5 kDa. The mean of purified and refolded BCCP-AMPs was 1.5 mg/L with 76% purity. The presence of expressed protein was subsequently determined by Western blotting analysis. Finally, radial diffusion assay supported that these peptides displayed functional antimicrobial activity against *E. coli* and *Staphylococcus aureus* standard strains. Two novel AMPs established in this study would be potentially developed as extensive intervention for treating of infectious diseases.

Introduction

Infectious diseases caused by microorganisms are involved in life-threatening worldwide. During the decade, an annually increasing of the prevalence of antibiotic resistance pathogens has been published [1,2]. Over-used drugs, inappropriate treatment and prolonged therapy of infected patients are associated with the development of drug-resistant strain [3]. Additionally, the biofilm formation was recently described and potentially involved in an immune evasion. This virulence factor leaded to complicated treatment and chronic illness [4]. Antimicrobial peptides (AMPs)[1] are the small cationic peptides that produced by a broad range of organisms including invertebrate and vertebrate [5,6]. According to the potent antimicrobial activity, AMPs have been considered as a new generation antimicrobial agent for treating of infectious microorganisms.

Protegrin 1 (PG-1), an 18 amino acid peptide constitutively produced by porcine leukocytes, has been established to be a potent antimicrobial peptide in several studies [7–9]. Mode of action of PG-1 has been previously defined as the barrel stave model and pore formations caused microbial membrane leakages are typically correlated with 4–5 parallel PG-1 dimer [8,9]. It exhibited an excellent activity with multi-drugs resistant bacteria and preferred long term treatment without resistant [10]. Interestingly, an early step inhibition of HIV-1 virus replication has been reported by this peptide [11].

LL-37 and histatin-5 (Hst-5) were human short polypeptide that mostly found in lysosomes of polymorphonuclear leukocytes and saliva, respectively. LL-37 was classified into a cathelicidin family and expressed in a variety of cells, tissues and body fluids. The mature peptide was 37 amino acids containing leucine and lysine rich residues and defined to be α-helical structure [12]. Antibacterial and antifungal activity has been reported in several kinds of

* Corresponding author. Tel.: +66 5394 5086x19; fax: +66 5394 6042.
E-mail address: sorasak058@yahoo.com (S. Intorasoot).

[1] *Abbreviations used:* AMPs, antimicrobial peptides; PG-1, protegrin 1; BCCP, biotin carboxyl carrier protein; BPL, biotin protein ligase; GST, glutathione S-transferase; CBM, carbohydrate-binding module; SUMO, small ubiquitin-related modifier; NC-CN, N- to C-terminal and C- to N-terminal; HHCl, hydroxylamine hydrochloride; VRE, vancomycin resistant Enterococci.

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microorganisms. Selective microbial membrane attachment and membrane permeabilization relied on their positive charged amino acids on the peptide [13]. Currently, the abolition of microbial biofilm formation was observed in this peptide [14]. Hst-5 was a 24 amino acids histidine-rich peptides that predominantly action in fungicidal activity [15,16]. The mechanism was involved in cell surface binding, penetration and targeting to the subcellular organelle, mitochondria [17].

Biotin carboxyl carrier protein (BCCP) was a subunit of bacterial acetyl-CoA carboxylase that involved in an initial step of fatty acid biosynthesis [18]. The C-terminal 87 amino acid residues of BCCP were revealed to be the natural biotinylated sequence that utilized for biotin binding. Biotin could covalently attach to a specific lysine residue of the BCCP through the catalysis of biotin protein ligase (BPL) enzyme or *Escherichia coli* BirA protein [19]. According to the specific binding with biotin, BCCP is usually applied for fusion with target proteins in many studies [20,21]. Hence, this biotinylated ligand could facilitate protein detection and separation using the high affinity binding between biotin and avidin/streptavidin complex [22].

Due to the costly of chemical peptide synthesis, large scale production of AMPs is unable in pharmaceutical applications. Recombinant protein expression in bacteria or yeast prefers an alternative strategy for cost-effective and easily established scale-up. However, AMPs expression in microorganisms was limited in their toxicity to the producing host [23]. Fusion partners have been employed for antimicrobial activity neutralization and isolation of antibiotic peptides from microorganisms. In addition, the carrier proteins allowed for protection of proteolytic degradation and increasing their expression efficiency [24,25]. Many fusion partners were applied for recombinant protein expression. The glutathione S-transferase (GST), the family III carbohydrate-binding module (CBM) and small ubiquitin-related modifier (SUMO) were facilitated for protein purification, while the thioredoxin and polypeptide F4 enhanced the high yield of protein expression in *E. coli*.

The objective of this study was to express three antimicrobial peptides, PG-1, LL-37 and Hst-5 in bacteria. *E. coli* BL21(DE3)pLysS host strain was selected for peptide expression in this study. The BCCP was firstly applied as fusion partner for antimicrobial activity neutralization, protection of peptide degradation and rapid purification. The codon usage sequence of PG-1 dimer in N- to C-terminal and C- to N-terminal (NC-CN) format connected with LL-37-linker–Hst-5 was commercially constructed and inserted downstream to BCCP gene. Thrombin and hydroxylamine hydrochloride (HHCl) were utilized for separation of individual peptides from fusion partner. The antimicrobial activity of expressed peptides was characterized with bacterial pathogens.

Materials and methods

Bacterial strains and vector

E. coli TOP10F and BL21(DE3)pLysS strains were used as host cells for recombinant plasmids propagation and protein expression, respectively. The pET-28a expression vector utilized for expression of protein construct was commercially supplied from Novagen, USA.

The AMPs and BCCP gene amplification by PCR

The pUC-57 plasmid construct consisting of proper bacterial codon usage of PG-1 dimer (N-to C-terminus connected with C-to N-terminus) and LL-37-$(Gly_4Ser)_3$-Hst-5 hybrid DNA fragment (Biobasic Inc., Canada) was used as template for PCR amplification using AMP primers (Table 1). The pAK400cB-single chain Fv-Survivin, kindly provided by Assoc. Prof. Chatchai Tayapiwatana, Department of Medical Technology, Faculty of Associated Medical Sciences, was used as the template for BCCP gene amplification using BCCP primers (Table 1). PCR reaction was performed in a total reaction of 50 μl containing 5 μl of 10× buffer, 2.5 mM $MgSO_4$, 2 mM dNTP mixed, 10 μM of forward (F) and reverse (R) primer, 1 μl of plasmid DNA and approximately 1 unit of KOD DNA polymerase (TOYOBO CO., LTD, Japan). The PCR profiles for AMPs amplification were as follows: pre-heat at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 5 min. The PCR conditions utilized for BCCP gene amplification were similar to the peptide gene amplification whereas the annealing temperature was 52 °C. The amplified products were analyzed by 2% agarose gel electrophoresis. The expected size of AMPs and BCCP gene was 411 and 296 bp, respectively. Both amplified products were gel purified by using the NucleoSpin® Gel and PCR clean-up kit (Macherey–Nagel GmbH & Co., Germany) and stored at −20 °C until use.

The BCCP-AMPs gene fusion using splicing by overlap extension (SOE)-PCR

The purified fragment of triple antimicrobial peptides and BCCP gene were used as template for gene fusion by SOE-PCR. The PCR reaction and profiles were similar to as previously described. The reaction in 50 μl containing 5 μl of 10× buffer, 2.5 mM $MgSO_4$, 2 mM dNTP mixed, 10 μM of BCCP-F forward and AMP-R reverse primers, 1 μl of each DNA template and approximately 1 unit of KOD DNA polymerase enzyme. The PCR conditions were as follows: pre-heat at 94 °C for 1 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s and final extension at 72 °C for 5 min. The amplified fragment was predicted to be 683 bp. After agarose gel electrophoresis, the DNA fragment was excised from the gel, purified using the kit and stored at 4 °C until cloning.

The BCCP-AMPs gene cloning and expression

The BCCP-AMPs gene was digested with *Nco*I and *Bam*HI restriction enzyme and cloned into linearized expression vector, pET-28a. The ligation mixture was performed in a total volume of 20 μl containing 2 μl of 10× T4 DNA ligase buffer, 100 ng digested pET28a vector, 55 ng of BCCP-AMPs DNA fragment, 1 unit of T4 DNA ligase and nuclease-free water. The mixture was incubated at 16 °C for 18 h. The ligation product was transformed into *E. coli* BL21(DE3)-pLysS competent cells using heat shock technique [26]. The positive transformants containing target gene of insert with the corrected orientation was selected by PCR amplification using T7 promoter and BCCP-R primer. Directed DNA sequencing was performed for confirming of the integrity of gene encoding sequence.

E. coli BL21(DE3)pLysS carried BCCP-AMPs gene inserted into pET-28a vector was grown in LB medium supplemented with 30 μg/ml of kanamycin at 37 °C, 200 rpm for overnight. Ten millilitre of overnight culture (1:100) was subculture in 1 L of fresh LB medium supplemented with 30 μg/ml of kanamycin and grown at 37 °C with shaking until the mid-exponential phase (OD_{600} was 0.6–0.8) was reach. Protein expression was induced by the addition of isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. After 4 h induction, bacteria cells were pelleted by centrifugation at 4 °C, 4500 rpm for 30 min. Bacterial pellets were washed once with phosphate buffer saline (PBS) and cells were collected for further purification and SDS–PAGE analysis.

Western blotting analysis

The expression of target BCCP-AMPs protein was analyzed by 12.5% SDS–PAGE gel with coomasie brilliant blue staining.