

PCR-based Gene Synthesis, Molecular Cloning, High Level Expression, Purification, and Characterization of Novel Antimicrobial Peptide, Brevinin-2R, in *Escherichia Coli*

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Abstract Brevinin-2R, a member of a new family of antimicrobial peptides isolated from the skin of *Rana ridibunda*, displays antimicrobial activity against bacteria and fungi. In this study, we have used an assembly PCR method for the fast and extremely accurate synthesis of the *brevinin-2R* gene. A total of six primers were assembled in a single step PCR, and the assembly was then amplified by PCR to produce the final gene. The synthetic gene was cloned into the pET32a (+) vector to allow the expression of brevinin-2R as a Trx fusion protein in *Escherichia coli*. The results indicated that the expression level of the fusion protein could reach up to 25% of the total cell proteins. The expression products could be easily purified by Ni-NTA chromatography and released from the fusion protein by factor Xa protease. The peptide displayed antimicrobial activity similar to that of the

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purified brevinin that was reported earlier. This method allows the fast synthesis of a gene that optimized the overexpression in the *E. coli* system and production of sufficiently large amounts of peptide for functional and structural characterizations.

Keywords Brevinin-2R · Antimicrobial peptide · PCR-based gene synthesis · Fusion partner · Expression · *Rana ridibunda*

Introduction

Antimicrobial peptides play an important role in the innate immunity that constitutes the first line of defense against invading pathogens for a wide range of vertebrate and invertebrate species. Antimicrobial peptides are a new class of antibiotics, called peptide antibiotics, which are encoded by the genes of various organisms [1–4]. Many species, ranging from plants and insects to lower vertebrates and mammals, can produce peptide antibiotics. More than 500 peptide antibiotics have been found so far and most of them are small cationic peptides with molecular weight <5 kDa [5–7]. Recently, anionic peptide antibiotics have also been identified, not only in amphibians, but also in mammals and human beings [8–10]. Both cationic and anionic peptide antibiotics contribute to the innate host defense against a number of microbial pathogens, even some of the bacterial and fungal pathogens resistant to conventional antibiotics. Unlike conventional antibiotics, peptide antibiotics target microbial cellular membranes that distinguish various species of microorganisms from plants and animals [11]. This significant feature effectively impedes the ability of microbes to develop resistance against them [7]. They are potentially useful in clinical applications. Extensive research has spawned considerable commercial effort to develop peptide antibiotics as new classes of antiinfective therapeutics [12–14]. Nevertheless, the preparation of peptide antibiotics at a large scale is a significant challenge to develop commercial products. The expression of heterologous proteins in bacteria is by far the simplest and most inexpensive means to produce large amounts of product of interest. However, it is hard for small peptides to be expressed in engineered bacteria at high levels and be recovered from the expression systems [15]. Brevinin-2R, which is isolated from the skin of *Rana ridibunda* [16], has antimicrobial activity against gram-negative bacteria and *Candida albicans*. Brevinin-2R contains 25 amino acid residues, and this peptide is clearly a member of the brevinin-2 family. In this study, we used an assembly PCR method for the fast and extremely accurate synthesis of a 111-bp brevinin gene encoding an antimicrobial peptide. The assembly was then further amplified by PCR to produce a synthetic gene which has been cloned and successfully expressed in *E. coli*. We purified the antimicrobial peptide and examined its antimicrobial activities.

Materials and Methods

Reagents and Bacterial Strains

E. coli DH5 α was used as the host for gene manipulation. *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) was used as the host for recombinant fusion expression. Luria–Bertani (LB) medium (*w/v*): 0.5% yeast extract, 1% tryptone, 1% NaCl; 2x YT medium (*w/v*): 1.6% tryptone, 1% yeast extract, 0.5% NaCl. The oligonucleotides, two 36-, 33-, 39-, 37-, and 23-mer, were synthesized on a 40-nmol scale with no extra purification and dissolved in

water to a final concentration of 25 μM each (Bioneer, South Korea). pET-32a (+) (Novagen) was used to construct the expression vector. All restriction enzymes, T4 DNA ligase, Taq DNA polymerase, and solutions for PCR were obtained from Fermentas, Lithuania. Ni-NTA super flow and factor Xa protease were obtained from QIAGEN, USA. Tris, acrylamide, *N,N*-methylene-bisacrylamide, ammonium persulfate, and TEMED were from Merck, Germany. The sequence of the synthetic brevinin gene was designed according to the *E. coli* codon usage [17].

Gene Assembly

Equal volumes of solutions of each oligonucleotide (25 μM each) were combined and the mixture was diluted fivefold in 25 μl of a PCR mixture [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM dNTP, 0.65 U Taq DNA polymerase, and six primers at 1 μM each]. The PCR program (I) consisted of a denaturation step cycle at 94 °C for 60 s, then 25 cycles at 94 °C for 35 s, 50 °C for 40 s, 72 °C for 180 s, and a final incubation cycle at 72 °C for 10 min. After the first PCR, the second PCR was done. A pair of primers, P1: 5' **CTGGAATTCATCGAGGGTAGGAAGCTTAAAAATTTT3'** (the *EcoRI* cleavage site is in bold), and P6: 5'**GTA****CTCGAGTC**ATTAACTGGC3' (the *XhoI* cleavage site is in bold) were designed to amplify the brevinin DNA sequence. The brevinin DNA was amplified in PCR program (II) consisted of a denaturation step cycle at 94 °C for 120 s, then 32 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 240 s, and a final incubation cycle at 72 °C for 10 min. the PCR product was desalted using a PCR purification kit (Bioneer, Korea).

Cloning Steps and DNA Sequencing

PCR products were digested with *EcoRI* and *XhoI* before being inserted into the pET-32a (+) expression vector to construct the pET-brevinin plasmid using standard molecular biology techniques [18]. The ligation products were used to transform DH5 α TM *E. coli* competent cells and selection performed on LB agar supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin. Plasmids isolated from colonies were screened for the presence of insert by restriction analysis. For sequencing inserts, primers P1 and P6 were selected from those used in the synthesis.

Expression and Cell Disruption

A fresh clone of *E. coli* BL21 (DE3), harboring the pET32 vector, was grown in LB medium containing 50 $\mu\text{g}/\text{ml}$ ampicillin. When the cells had been cultured (37 °C, 250 rpm) to an optical density (OD₆₀₀) of 0.6, they were used to inoculate 30 ml of the fermentation medium (with 50 $\mu\text{g}/\text{ml}$ ampicillin) at a ratio of 5% (v/v) for recombinant protein production at various cultivation conditions. Protein expression was induced with isopropylthio-D-galactoside (IPTG). After continued culture, cells were harvested by centrifugation at 4,000 $\times g$ for 20 min, resuspended in 20 mM Tris-HCl (pH 7.5), and lysed by sonication.

Purification of Brevinin

E. coli BL21 (DE3) cells harboring the expression vector pET32a-brevinin were cultivated in 2x YT medium containing 0.5% (v/v) glucose. When the OD₆₀₀ of the culture reached

0.6, IPTG was added to a final concentration of 0.5 mM. After 4 h induction, the harvested cells (2 g wet weight from 600 ml culture) were resuspended in 60 ml of NTA-10 buffer (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol, and 10 mM imidazole, pH 7.9). Then, the cells were lysed by sonication at 400 W for 150 cycles (45 s working, 60 s free) in ice-water bath. The supernatant of the cell lysate resulting from centrifugation at $12,000\times g$ for 15 min was applied to a Ni-NTA column packed with 10 ml of Ni-NTA resin, which had been previously equilibrated with NTA-10 buffer. After washing to baseline absorbance with NTA-10 buffer, the column was washed with 50 and 300 mM imidazole. The fractions were collected and applied to 15% SDS-PAGE.

Antimicrobial Assay

The antimicrobial activity was examined by radial diffusion assay [19]. *Klebsiella pneumoniae* was grown in 3% (v/v) trypticase soy broth (TSB) at 37 °C overnight. To obtain midlogarithmic phase microorganism, 50 μ l of the culture was transferred to 50 ml of fresh TSB broth and incubated for an additional 2.5 h at 37 °C. *K. pneumoniae* were centrifuged at $900\times g$ for 10 min at 4 °C, washed once with cold 10 mM sodium phosphate buffer pH 7.4, and resuspended in 10 ml of cold sodium phosphate buffer. The cell concentration were estimated by measuring the optical density at 620 nm and were based on the relationship of the OD_{600} , $0.2=5\times 10^7$ CFU/ml. 2×10^6 *K. pneumoniae* were added to 10 ml of underlayer agar broth (10 mM sodium phosphate, 1% (v/v), (TSB), 1% agarose, pH 7.4) and the agar was poured into a Petri dish. Samples were added directly to 3-mm diameter wells that were made on the solidified underlayer agar. After incubation for 3 h at 37 °C, the underlayer agar was covered with a nutrient-rich top agar overlay and incubated overnight at 37 °C. The antimicrobial activity was evaluated by observing the suppression of bacterial growth around the 3-mm diameter wells.

Results

Design and Assembly of Gene

The design of the oligonucleotides used for the synthesis of the 111-bp brevinin gene necessitated great attention to detail, owing to the requirement for a large number to be mixed in one PCR. The nucleotide sequence of the gene was designed according to the *E. coli* codon usage preference [17]. In addition, the panel of oligonucleotides was seriously screened and matched to meet the following topic: (1) minimization of tandem or inverted repeats, which are likely to give rise to nonspecific priming; (2) optimization of the 18 nucleotide overlap between each primer, to give a melting temperature in the range 56–60 °C to allow subsequent use of the primers for DNA sequencing. The recognition sequence Ile–Glu–Gly–Arg was incorporated in the extreme 5' oligonucleotide for highly specific cleavage of fusion proteins, and additional stop codon were introduced in extreme 3' oligonucleotide. A number of unique restriction sites were introduced at strategic positions throughout the synthetic gene to facilitate subsequent gene manipulation and mutagenesis. Oligonucleotide design was performed with the aid of the Gene Runner program. This program translates a given DNA sequence into a protein sequence and then using a user defined codon usage table, backtranslates the protein sequence with an improved codon usage. Both strands of the sequence are then divided into overlapping oligonucleotides of “between” 30 and 40 bases in length; melting temperatures are

Table 1 Oligonucleotides used in this study.

Oligonucleotides	Sequences (from 5' to 3')
P1	CTGGAATTCATCGAGGGTAGGAAGCTTAAAAAATTTT
P2	GCCAAAGGTGTCGCCAGAGTCTCCTGAACAAA
P3	CTAGCTGCAAACCTCAGTGCCAGTGTTAATGACTCGAG
P4	GGCGACACCTTTGGCAAAATTTTAAAGCTTCTTACC
P5	CACTGAGTTTGCAGCTAGCTTTGTTTCAGGAGACTCTG
P6	GTACTCGAGTCATTAACACTGGC

calculated for all the overlaps and restriction sites generated along the sequence are displayed. The resulting panel of oligonucleotides was then analyzed for the presence of undesirable repeats, inverted repeats, and stem loop structures. The final selection of six unique oligonucleotides was used for gene synthesis (Table 1).

The initial assembly reaction involved the construction of the full length gene from a stoichiometric mixture of the six oligonucleotides (Fig. 1). An aliquot of this assembly reaction mixture was then used as a template for the amplification process in which only the two outermost primers of the assembly were added at a concentration on 1 μ M each. The PCR products using Taq DNA polymerase were obtained. Analysis of the two PCRs on 1.5% agarose gel revealed the presence of the 111-bp expected product (Fig. 2). The synthetic DNA products were digested with *Eco*RI and *Xho*I and ligated into *Eco*RI/*Xho*I pET32a (+) for cloning and sequencing (Fig. 3). The resulting plasmid was transformed in to *E. coli* DH5 α , and recombinant *E. coli* cells were selected on ampicillin-containing LB plates and screened by PCR using the primers.

Fig. 1 Schematic illustration of the two-step PCR-based gene synthesis. The synthetic gene is assembled by DNA polymerization from a set of overlapping complementary oligonucleotides in a first PCR (gene assembly). The assembled product is then amplified using the two outermost primers in a second PCR (gene amplification)

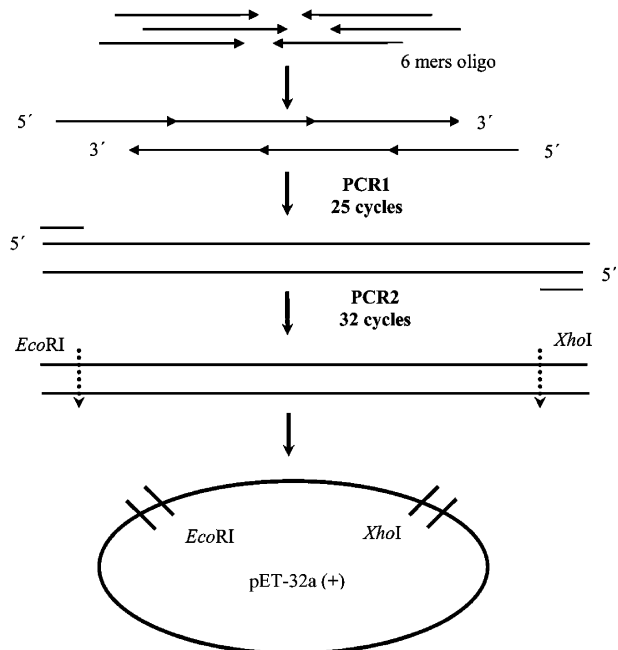
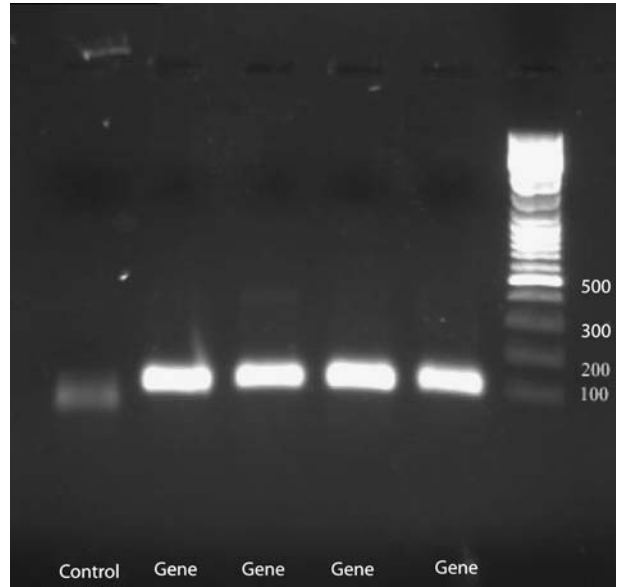


Fig. 2 Agarose gel electrophoresis of the synthetic gene product: analysis on a 1.5% gel of the PCR products from the assembly



Expression and Purification

The constructed pET32a–brevinin confirmed by DNA sequencing was transformed into the expression host *E. coli* BL21 (DE3). The expression level of Trx–brevinin, which was initially unstable and low, was increased significantly because of the addition of glucose to 2x YT culture medium to the final concentration of 0.5% (v/v) and reduction of the concentration of IPTG to 0.5 mM. The time point of induction was controlled at OD₆₀₀=

Trx-Tag

His-Tag

ATGAGC...315bp...CTGGCCTTCTGGTTCTGGCCATATGCACCATCATCATCAT

CATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAAACCGCTGCTGC

TAAATTCGAACGCCAGCACATGGACAGCCCAAGATCTGGGTACGACGACGAC

EcoRI Factor Xa protease **K L**

GACCAAGGCCATGGCTGATATCGGATCCGAATTCATCGAGGGTAGGAAGCTT

K N F A I G V A Q S L L N K A S

AAAAATTTTGCCATAGGTGTGCGCCAGAGTCTCCTGAACAAAGCTAGCT

C K L S G Q C *XhoI*

GCAAACCTCAGTGGCCAGTGTTAATGACTCGAG

Termination
codon

Fig. 3 Schematic representation of the construction of pET32a–brevinin-2R fusion gene

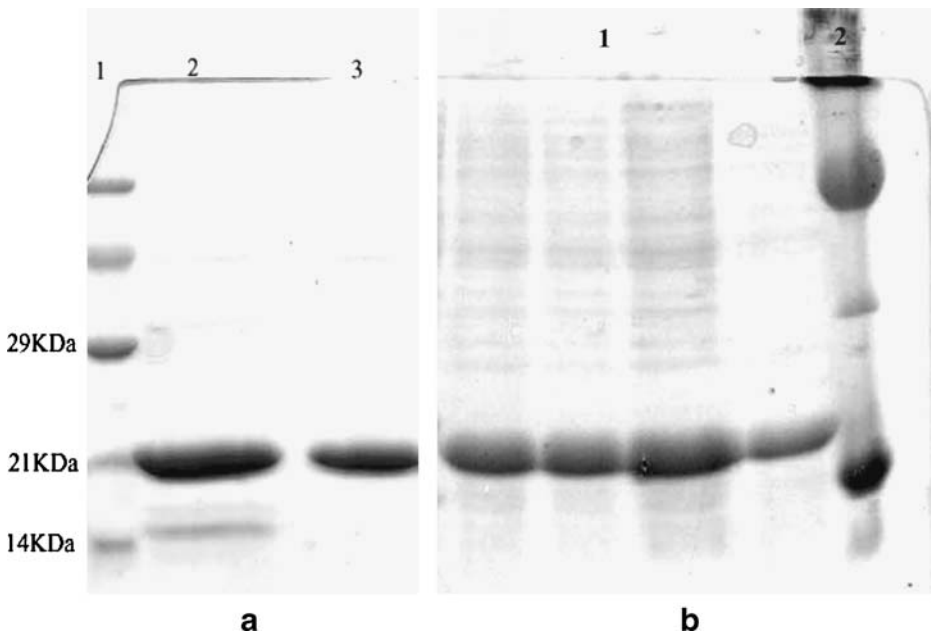


Fig. 4 SDS-PAGE analysis of expressed proteins. **a** Lane 1, molecular weight standards; lane 2, the reaction mixture of factor Xa protease digestion before SP sepharose column purification; lane 3, purified TrxA–brevinin fusion protein. **b** Lane 1, total proteins of induced cells; lane 2, molecular weight standards

0.6. As shown in Fig. 4b, the resulting expression level of Trx–brevinin could reach 15–20% of the total proteins, and more than 70% of the target proteins were in a soluble form. Moreover, the existence of the hexahistidine tag on the carrier protein provides an effective one-step purification of the fusion protein by Ni-NTA chromatography. SDS-PAGE analysis revealed the effectiveness of this purification step. The contaminating proteins were successfully removed by 50 mM imidazole wash and most of the Trx–brevinin was eluted by 300 mM imidazole. The resulting purity of the fusion protein could reach 80% or

Fig. 5 Tris–tricine SDS-PAGE analysis of purified brevinin-2R. Lane 1, purified brevinin-2R; lane 2, Ni-NTA column purified fraction of fusion protein; lane 3, molecular weight standard

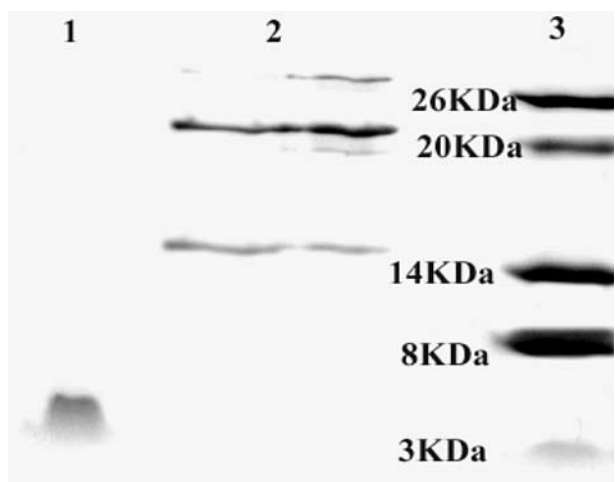
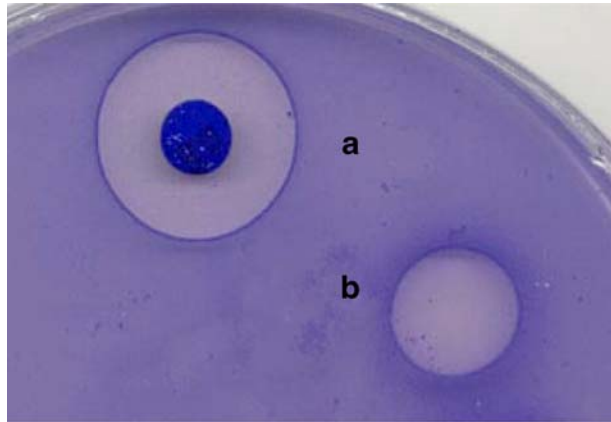


Fig. 6 Antimicrobial activities against *K. pneumoniae*. **a** Inhibition zone created by one disk 30 μg of kanamycin. **b** Shows the suppression of bacterial growth around the 3-mm diameter well containing 5 $\mu\text{g}/\text{ml}$ brevinin-2R peptide



more. The fusion protein was cleaved by factor Xa protease at 22 °C for 16 h (Fig. 4a). The cleaved fusion partner and some contaminating proteins could be removed rapidly by the Ni-NTA His Bind column (Fig. 5).

Antimicrobial Activity

The bactericidal activity of the purified brevinin-2R was quantitatively determined by using minimum inhibitory concentration (MIC) against selected microorganisms. The results indicate that the peptide have the same antimicrobial activity against bacteria of *K. pneumoniae*, similar to that reported previously [16]. Antimicrobial activity was examined by the radial diffusion assay on *K. pneumoniae*. Our results show the antimicrobial activity of isolated peptide against *K. pneumoniae* as an inhibition zone around a well-contained 5 μg peptide compared to 30 μg disk antibiotic kanamycin (Fig. 6).

Discussion

Extracts of amphibian skin have been used for centuries in folk medicine and witchcraft because of their possession of a wide spectrum of pharmacological effects [20, 21]. The source of these biologically active compounds is the dermal granular glands, and biochemically, the constituent molecules are representative of many classes including biogenic amines, peptides, proteins, alkaloids, and heterocyclics [20, 22]. The structural diversity of peptides in these amphibian defensive skin secretions probably reflects different roles, either in the regulation of physiological functions of the skin or in the defense against predators or microorganisms [20, 22]. Brevinin-2R is an amphipathic, polycationic peptide and it has antimicrobial activity against gram negative bacteria and *C. albicans*. In this study, we report the PCR-based gene synthesis, molecular cloning, sequencing, and expression of brevinin-2R in *E. coli*. We have described a PCR-based gene synthesis method for the fast and accurate construction of the *brevinin-2R* gene. The flexibility of the method in which complementary oligonucleotides are mixed together to generate a synthetic product in a single reaction, is impressive. There is no practical limit on the number of oligonucleotides, which may be mixed together, suggesting that bigger genes could be successfully synthesized [23]. PCR-based gene synthesis offers an elegant solution to the problem of producing large amounts of labeled protein for NMR when the gene

encoding the protein is unavailable or difficult to express efficiently. It is hard for brevinin-2R to be expressed alone in prokaryotic cells because of its toxic effects on hosts and high content of amino acids susceptible to proteolysis [24, 25]. Taking advantage of a commercially available vector pET32a, fusion of the small peptide to Trx could provide a high level of expression and favor intracellular accumulation of the fusion proteins in the soluble form. Several properties of the expression system of pET32a–brevinin suggest its potential in the mass production of the peptide. First, the size of the fusion partner was ten times larger than that of brevinin-2R, which facilitated the recovery of the active peptides from carrier molecules by gel filtration after cleavage by enterokinase. Second, Trx fusion partner provides an effective one-step purification of the fusion protein by Ni-NTA chromatography for the existence of the hexahistidine tag. Factor Xa protease could cleave the fusion protein with high specificity and recover the target peptide with native N terminus.

In conclusion, the described procedure might be a cost-effective method for the production of different cationic peptides that may otherwise be difficult or inconvenient to synthesize and purify using standard chemical methodologies, and can be applied in most laboratories equipped for recombinant protein expression and purification.

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