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# Multimeric Expression of the Antimicrobial Peptide Buforin II in *Escherichia coli* by Fusion to a Cysteine-Rich Acidic Peptide

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**Abstract** A cost-effective mass production method for a strong antimicrobial peptide, buforin II, which was isolated from the stomach of *Bufo bufo gargarizans*, has been developed. This method is based on the neutralization of the positive charge of buforin II by fusion with a cysteine-rich acidic peptide (CAP) to avoid any lethal effect on the host. The neutralized fusion peptide was multimerized and expressed in *Escherichia coli* as tandem repeats to increase the production yield. Multimers of the CAP-buforin II fusion peptide were successfully expressed at high levels in *E. coli* as inclusion bodies. More than 100 mg of pure buforin II was obtained per 1 l of *E. coli* culture after cleaving the multimeric polypeptide with CNBr. The buforin II obtained from the recombinant *E. coli* had antimicrobial activity identical to that of natural buforin II. The proposed expression system can provide a cost-effective mass production method for both antimicrobial peptides and other host-lethal basic proteins.

**Key words:** Antimicrobial peptide, expression, acidic peptide, tandem multimer, *Escherichia coli*

Antimicrobial peptides are part of the innate immune system widely distributed in nature [8]. A large number of antimicrobial peptides have been identified from various sources such as amphibians, insects, mammalians, plants, invertebrates, and prokaryotes [3, 4, 11, 13, 22, 29]. Active antimicrobial peptides share common structural features such as a high content of basic amino acid residues and a global distribution of hydrophobic and hydrophilic residues leading to amphipathic α-helical conformation under hydrophobic conditions, or β-sheet conformation [36]. These peptides exhibit potent antimicrobial activities against a broad range of microorganisms, including bacteria, protozoa, fungi, and viruses [22]. They have been shown to exert their activities directly through the lipid bilayer of the cell membrane by the formation of multimeric pores [25, 41]. Because of the antimicrobial mechanism of these molecules being quite different from that of conventional antibiotics, it is suggested that these peptides might offer an answer to the currently problematic multidrug-resistant strain development [15]. The fact that there has been no report on the induction of bacterial resistance against antimicrobial peptides supports this notion. Moreover, some of the antimicrobial peptides do not induce lysis of erythrocytes or lymphocytes at comparable concentrations in spite of their activities on prokaryotes [7, 42, 43]. Therefore, in view of drug development, these antimicrobial peptides may provide a new class of powerful antimicrobial agents.

The need for a large quantity of antimicrobial peptides for pharmaceutical applications has prompted us to investigate and develop cost-effective production methods. Recently, several researchers have attempted to develop biological expression systems for the production of antimicrobial peptides. Cecropin A has been expressed in baculovirus expression systems [1, 10] and *E. coli* [5], an insect defensin from *Phormia terranovae* in yeast [35], human defensin HNP-1 and CEME [40] in various bacterial expression systems [33], magainin from the skin of *Xenopus laevis* in the erythrocytes of transgenic mice [38] and *E. coli* [18], moricin from *Bombyx mori* in *E. coli* [9], gaegurin 4 from the skin of *Rana rugosa* in *E. coli* [16], and polyphemusin from horseshoe crab *Limulus polyphemus* in *E. coli* [34]. Each of the above systems, however, has its own limitations and is far from satisfactory for mass production: the yields were rather low [1, 5, 9, 16, 18, 34], proteolysis of the fusion protein occurred [33], or the product was toxic to the host [6]. To overcome current biological expression problems, we have developed a novel expression method in which a negatively charged acidic peptide is fused to a positively charged antimicrobial

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peptide to form a neutrally charged peptide, mimicking the natural precursor of an antimicrobial peptide [19]. In this study, the effectiveness of our approach was further demonstrated by the high level expression of the antimicrobial peptide, buforin II, in *E. coli* as inclusion bodies by fusion to an acidic peptide containing 10 cysteine residues.

## MATERIALS AND METHODS

### Strains, Vectors, and Enzymes

*E. coli* strain XL1-Blue (Stratagene, La Jolla, U.S.A.) was used as a host for subcloning and *E. coli* strain BL21 (DE3) (Novagen, Madison, U.S.A.) for gene expression. *E. coli* strains were grown in an LB medium at 37°C and ampicillin (50 μg/ml) was added for the selection of recombinant strains. pUC19 (New England Biolabs, Beverly, U.S.A.) and pBBS1 [20] were used as vectors for subcloning and multimerization of peptide genes, respectively, and pET21c (Novagen) for gene expression. pUC-CAP (kindly provided by Dr. K. Kang, KAIST) containing a sequence encoding an acidic elastase inhibitor [14], which consists of 54 amino acids with 10 cysteines, was used as a source for a cysteine-rich acidic peptide (CAP) gene. Restriction enzymes and modifying enzymes were purchased from New England Biolabs and used according to the recommendations of the supplier. A mini-scale preparation of vector DNA was carried out using the alkaline lysis method [24] and large quantities of vector DNA were prepared by the PEG precipitation method [37]. Other recombinant DNA techniques were exploited as described by Maniatis *et al.* [24] and Sambrook *et al.* [37].

### Construction of Genes Coding for Buforin II and CAP-Buforin II Fusion

For the construction of the gene coding for buforin II [31], two complementary 75-nt synthetic deoxyoligonucleotides (oligos) A (5' CCCCTGATGACCCGTAGCAGCCGTG-CGGGCCTGCAGTTTCCGGTGGGCCGTGTGCATCG-TCTGCTGCGTAAAATG 3') and B (5' GGGGCATTT-TACGCAGCAGACGATGCACACGGCCCACCGGAA-ACTGCAGGCCCGCACGGCTGCTACGGGTCATCA 3') were synthesized, based on the amino acid sequence of buforin II according to the codon usage of *E. coli*. Oligos A and B were designed to form a 71-bp DNA fragment with asymmetric cohesive ends, 5'-CCCC/5'-GGGG, which is compatible with the 5'-GGGG/5'-CCCC cohesive ends of *Bbs*I-digested pBBS1 vector. The synthetic oligos A and B were annealed to each other and phosphorylated by treatment with T4 polynucleotide kinase. The oligo hybrids were ligated into *Bbs*I-digested pBBS1 which has 5'CCCC/5'GGGG cohesive ends, resulting in pBBS1-B1. pBBS1 had been constructed previously for the gene amplification vector using the property of the class-IIS restriction enzyme [20]. For the construction of the gene for a CAP-buforin II fusion protein (Fig. 1), an in-frame fusion of the CAP and buforin II genes was carried out by two sequential polymerase chain reactions (PCR) using four oligos as primers, C (5' A-AAGAAGACGGCCCCCGGT-CGACGAGAATGCG 3'), D (5' GCTGCTACGGGTCA-TGATCCCCGCGCAGGT 3'), E (5' ACCTGCGCGGG-GATCATGACCCGTAGCAGC 3'), and F (5' TGCAT-GCCTGCAGGTCGA 3'). The 3' end (15 nt) of primer C is complementary to the 5' end of CAP gene, and contains a *Bbs*I site at its 5' end for the generation of 5' CCCC cohesive ends after *Bbs*I digestion of the PCR product. Sequences of primers D and E are complementary to each other, and the 3' ends (15 nt) of primers D and E are specific to the 3' end of the CAP gene and the 5' end of the buforin II gene as shown in Fig. 1, respectively. Primer F is complementary to the sequences outside the buforin II gene and the *Bbs*I site in pBBS1-B1 (Fig. 1). The DNA sequences coding for each of CAP and buforin II were amplified from pUC-CAP with primers C and D and from pBBS1-B1 with primers E and F, respectively. The two PCR products were mixed after purification from a 2% agarose gel and fused by recombinant PCR using primers C and F (Fig. 1). The fused PCR product was purified, *Bbs*I-digested, and cloned into *Bbs*I-cut pBBS1, producing pBBS1-CB1 in which the CAP gene is fused to the buforin II gene, as shown in Fig. 1.

### Multimerization of Genes as Tandem Repeats

Genes encoding buforin II or the CAP-buforin II fusion were tandemly multimerized using the vector pBBS1 (Fig. 2). Vectors pBBS1-B1 and pBBS1-CB1, which contain the buforin II gene and CAP-buforin II fusion gene, respectively, were digested with *Bbs*I, and monomeric DNA fragments with asymmetric cohesive ends of 5'-CCCC/5'-GGGG were purified from a 2% agarose gel. The purified monomeric fragments were self-ligated for 2 h at 16°C and cloned into *Bbs*I-digested pBBS1. The number of monomers in the vector in the XL1-Blue transformants was determined by cleaving the vector with *Bam*HI and *Xba*I, whose sites flank the multimer. The orientations of individual monomers in the vector were determined by digesting with *Pst*I which cuts a site in both the vector and each monomeric fragment.

### Expression of Multimeric Peptide Genes in *E. coli*

For the expression of multimeric peptide genes, *Bam*HI-*Hin*dIII fragments containing the multimeric peptide genes were isolated from the multimer-containing pBBS1 constructed above, and cloned into pET21c digested with *Bam*HI and *Hin*dIII. Desired clones were selected after transformation into the *E. coli* host BL21(DE3). Each

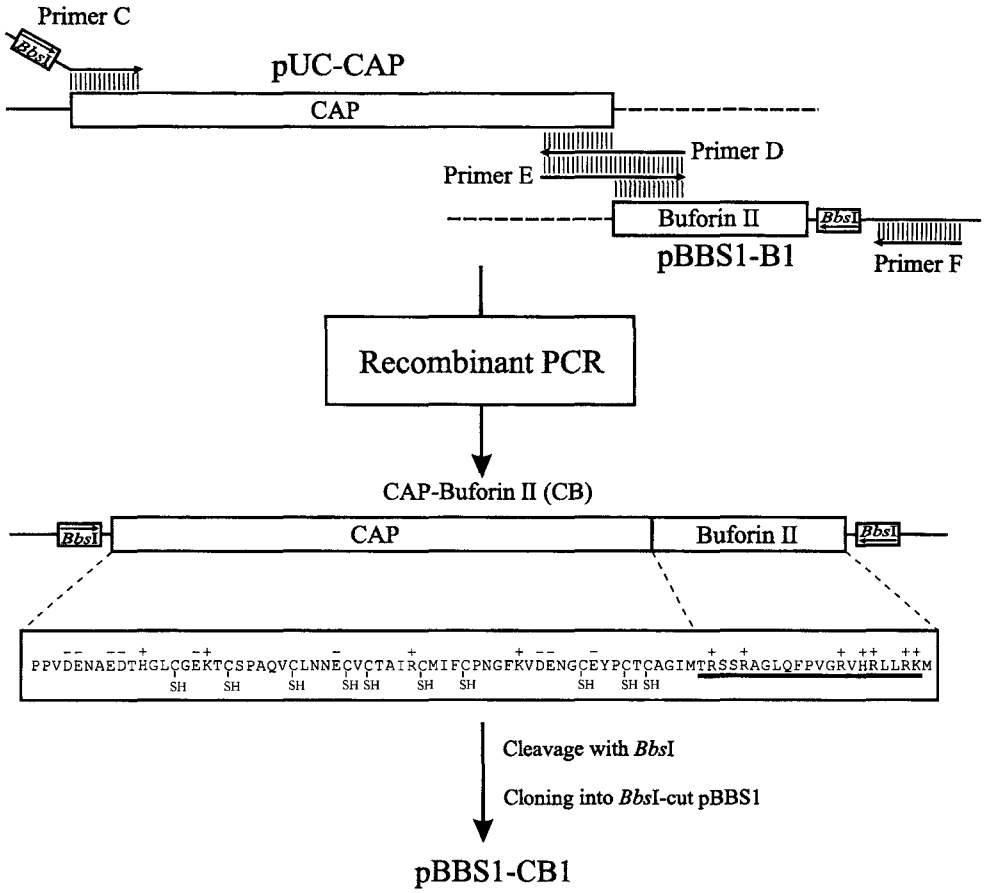


**Fig. 1.** Schematic representation of the construction of the CAP-buforin II fusion gene.
The CAP (cysteine-rich acid peptide) gene was fused to the buforin II gene by recombinant PCR, as described in the Materials and Methods section. Charged residues are indicated by + or - above the amino acid sequences. The cysteine residues are represented by C-SH. The buforin II sequence is underlined in the fusion peptide.

selected transformant was inoculated into a 3 ml LB medium supplemented with ampicillin (50 μg/ml) and grown at 37°C with shaking for 9 to 12 h. Each culture was then diluted 1:100 into a fresh LB medium containing ampicillin (50 μg/ml) and grown at 37°C. When the $OD_{600}$ reached 0.6, IPTG was added to a final concentration of 0.6 mM. The cells were harvested 3 h after induction and whole-cell lysates from the induced cultures (equivalent $OD_{600}$) were analyzed by SDS-PAGE [17]. An *in vitro* coupled transcription-translation experiment was performed with an *E. coli* T7 S30 Extract System for Circular DNA (Promega, Madison, U.S.A.) [18].

### Production of Active Buforin II

*E. coli* cells harboring the expression vector pET21c-CB4, which has four copies of the CAP-buforin II fusion gene in a tandem repeat, were cultivated in a 30-l fermentor. The IPTG-induced cells were harvested by centrifugation at 6000 ×g for 10 min. After lysis of cells by sonication, the fusion peptide expressed as inclusion bodies was recovered by centrifugation at 10,000 ×g for 30 min at 4°C and washed with a 50 mM Tris/HCl buffer (pH 8.0) containing 2 mM EDTA. The recovered inclusion bodies were denatured and solubilized in 1 N HCl containing 6 M guanidine–HCl, and cleaved by incubation with 1 M CNBr at 30°C for 20 h. The insoluble materials were removed by centrifugation at 10,000 ×g for 30 min. The supernatant containing the cleaved peptides was dialyzed against 50 mM glycine-NaOH buffer (pH 10.0) and concentrated by lyophilization. The lyophilized samples, after being dissolved in water, were applied to a 3.9×300-mm Delta Pak C18 column (Waters Associates, Milford, U.S.A.) with a 0% to 50% linear gradient of buffer A [acetonitrile containing 0.1% (v/v) trifluoroacetic acid] at 1 ml/min for 1 h. Each peak was collected and examined for antimicrobial activity after lyophilization. An electrophoretic analysis of the recombinant buforin II was carried out on tricine-SDS-PAGE.

### Characterization of Recombinant Buforin II

The molecular weight and homogeneity of the recombinant buforin II were analyzed using a matrix-assisted laser desorption/ionization (MALDI) mass spectrometer (Kartos

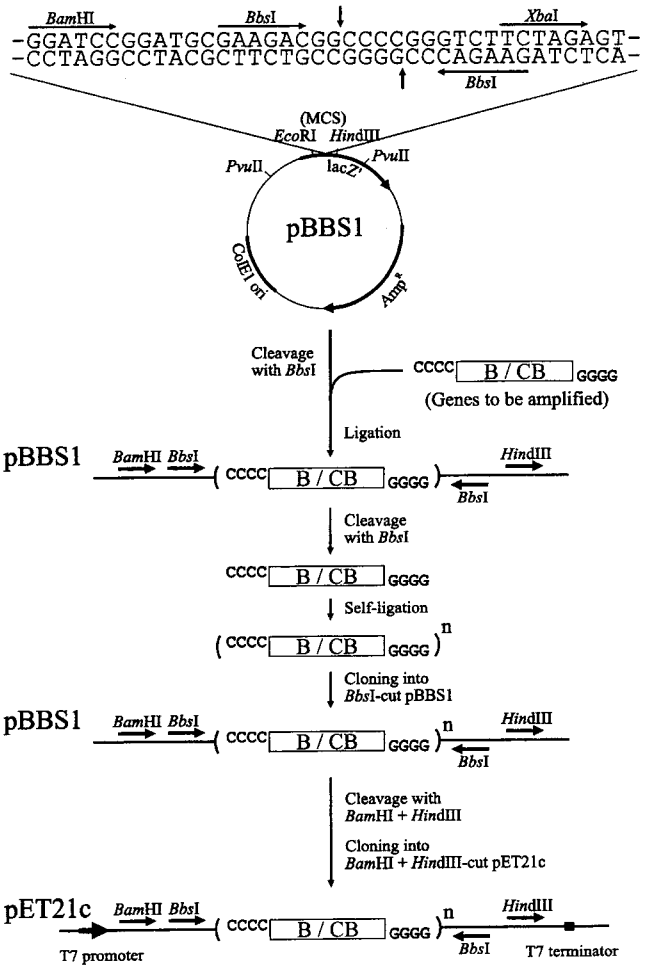


**Fig. 2.** Schematic representation of the multimerization of the target genes using the gene amplification vector pBBS1.
The gene amplification cassette contains two inversely oriented *Bbs*I sites and the same cleavage sequences. The monomeric peptide gene cloned into pBBS1 could be amplified by: (i) excision of the monomeric insert by digestion with *Bbs*I; (ii) isolation of the fragments; (iii) self-ligation of the fragments; and (iv) cloning into the original pBBS1 vector digested with *Bbs*I. B and CB indicate for genes coding for buforin II and CAP-buforin II, respectively, which were constructed as described in Fig. 1.

Kompact MALDI, Manchester, U.K.). Amino acid sequences of the recombinant buforin II were determined by automated Edman degradation performed on an Applied Biosystems (Foster City, U.S.A.) gas-phase sequencer (model 447). Antimicrobial activity was examined by a radial diffusion assay on a *Bacillus subtilis* lawn as described by Lehrer *et al.* [21]. A 20 ml of *B. subtilis* culture in a mid-logarithmic phase was washed with a cold 10 mM sodium phosphate buffer (NAPB), pH 7.4, and resuspended in 10 ml of cold NAPB. A volume containing $1\times10^6$ bacterial CFU was added to 6 ml of underlay agar [10 mM sodium phosphate, 1% (v/v) trypticase soy broth, and 1% (w/v) agarose, pH 6.5] and the mixture was poured into a Petri dish. Samples were then added directly to 3-mm wells made on the solidified underlayer agar. The underlayer agar was covered with a nutrient-rich top agar overlay and antimicrobial activity was determined by observing the zone of suppressed bacterial growth around the 3-mm wells after incubation overnight at 37°C. Water and natural buforin II were used as negative and positive controls, respectively. The minimal inhibitory concentrations (MIC) of the peptide against several gram-positive and Gram-negative bacteria and fungi were determined by incubating approximately $10^4$- $10^5$ CFU/ml of cells with serial dilutions of peptides in a 96-well microtiter plate (Nunc F96 microtiter plates, Denmark) as described by Moore *et al.* [28].

## Results and Discussion

### Expression of the Buforin II Gene as Tandem Repeats

To develop a cost-effective mass production method for antimicrobial peptide buforin II, the expression of buforin II as a concatemeric multimer was explored. The idea was that the recombinant concatemer could avoid a suicide situation by masking the intrinsic antimicrobial activity and susceptibility of the peptides from proteolytic degradation [39]. The synthetic buforin II gene codes for the buforin II and contains two methionine codons flanking the buforin II gene as shown in Fig. 1. Methionine residues were introduced to cleave the expressed buforin II multimers with CNBr to produce active buforin II. The hybridized synthetic gene was ligated into the *Bbs*I site of the gene amplification vector pBBS1 [20], resulting in pBBS1-B1. For the construction of concatemeric multimers, the DNA fragments containing the sequence encoding buforin II were isolated from pBBS1-B1 digested with *Bbs*I, self-ligated, and then recloned into the *Bbs*I site of pBBS1. Clones containing 1, 2, 4, and 6 copies of buforin II were selected (Fig. 3A, lanes 2-5) and named pBBS1-B1, -B2, -B4, and -B6, respectively. The *Bam*HI-*Hin*dIII fragments of these vectors were cloned into pET21c for the expression of the multimeric buforin II gene, and the expression level was examined by SDS-PAGE. However, the attempt to produce buforin II as the concatemeric multimers was unsuccessful. Both monomers and multimers were poorly expressed in *E. coli*, as shown in Fig. 3B (lanes 4-6). The monomers of buforin II were not shown on the gel because they ran out of the gel (Fig. 3B, lane 3). A similar result was also observed with the multimeric expression of magainin gene [18, 20]. To find the reason for this decrease in the expression levels, an *in vitro* transcription-translation was performed, which showed that the expression level decreased as the size of the multimers increased. Also, the strong interaction of buforin II with the DNAs was confirmed by a gel retardation experiment [32]. These would seem to be related to the basic nature of antimicrobial peptides, which can inhibit transcription and translation through the interaction

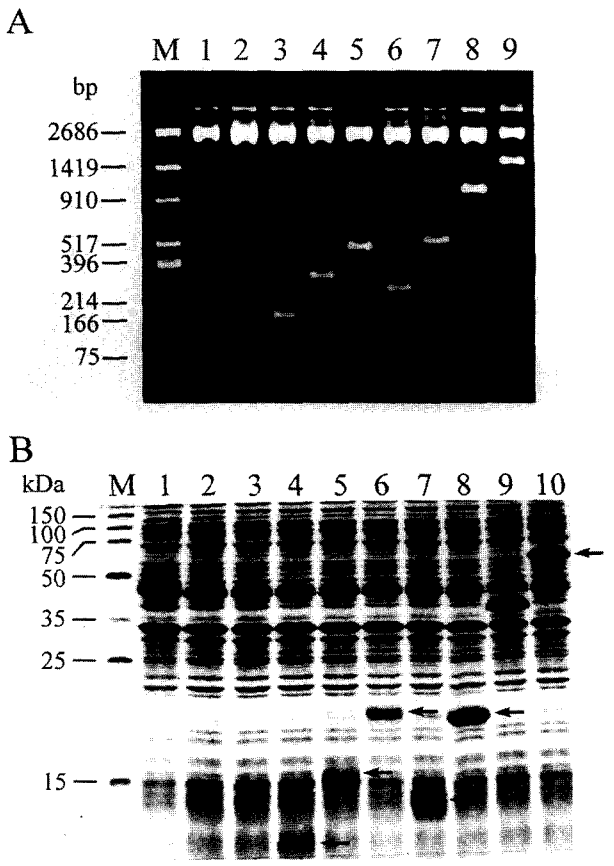


**Fig. 3.** Electrophoretic analysis and expression of multimeric peptide genes.

(A) Electrophoretic analysis of multimeric peptide genes. The number of peptide genes cloned in the gene amplification vector pBBS1 was determined by cleaving the vectors with *Bam*HI+*Xba*I, whose sites flank the multimer. The digests were electrophoresed on a 2% agarose gel in TBE buffer for 2 h at 10 V/cm, and DNA bands were stained with ethidium bromide. Lane M represents size markers. Lane 1 shows the pBBS1 vector digested with *Bam*HI+*Xba*I. Lanes 2-5 represent *Bam*HI+*Xba*I-digested pBBS1-B1, -B2, -B4, and -B6, which contain 1, 2, 4, and 6 copies of the buforin II monomer, respectively. Lanes 6-9 show *Bam*HI+*Xba*I-digested pBBS1-CB1, -CB2, -CB4, and -CB6, which contain 1, 2, 4, and 6 copies of the CAP-buforin II fusion monomer, respectively. A detailed explanation can be found in the Results and Discussion section. (B) Expression of multimers of the buforin II and CAP-buforin II fusion genes. Lane M represents size markers. Lanes 1 and 2 show the uninduced and induced BL21(DE3) containing pET21c, respectively. Lanes 3-6 represent BL21 (DE3) harboring pET21c-B1, -B2, -B4, and -B6, respectively, and lanes 7-10, BL21(DE3) harboring pET21c-CB1, -CB2, -CB4, and -CB6, respectively. Total cell proteins were analyzed by SDS-PAGE. Samples were applied to a gel after being boiled for 5 min. Proteins were stained with Coomassie brilliant blue. The arrows indicate the expressed fusion proteins from each clone.

with DNAs or RNAs [27]. Since buforin II is a highly positively charged peptide, it has an increased chance of interacting with nucleic acids in *E. coli* when expressed in multimeric forms. However, this interaction can be avoided by fusing an acidic peptide to the basic buforin II, thereby neutralizing the positive charge of buforin II through the negative charge of the acidic peptide. Many naturally occurring basic peptides and proteins, including antimicrobial peptides [12, 23, 26, 30, 42] and major basic proteins of the human eosinophil granule [2], are synthesized as precursors in which the basic peptides are electrically neutralized by the acidic pro-parts. One of the proposed roles of these acidic peptides is to protect the host cell through masking the toxicity of the mature basic peptides in a charge-dependent manner by counterbalancing the overall positive charge [6]. Based on these observations, a fusion of an acidic peptide CAP to buforin II was explored for the high level expression of buforin II in *E. coli*.

## Expression of the CAP-Buforin II Fusion as Tandem Repeats

To examine the effect of an acidic peptide on the expression of positively charged buforin II, the acidic peptide CAP was selected as the fusion partner. CAP, which contains 10 cysteines, was chosen because a previous study [19] showed that cysteine residues in an acidic fusion partner was critical for the efficient neutralization of a basic peptide in a fusion protein. Recombinant PCR was used to construct the fusion of CAP and buforin II genes as shown in Fig. 1. The fusion was cloned into the *Bbs*I site of pBBS1, resulting in pBBS1-CB1. The 248-bp DNA fragments with asymmetric cohesive ends, 5'-CCCC/5'-GGGG, which encode the fused peptide, were isolated from pBBS1-CB1 after *Bbs*I digestion, and tandemly multimerized into *Bbs*I-cut pBBS1, as described in Fig. 2. The clones, pBBS1-CB1, -CB2, -CB4, and -CB6, each containing 1, 2, 4, and 6 copies of the monomer, respectively, were selected (Fig. 3A, lanes 6-9). The tandem multimers of the fused gene were expressed in *E. coli* using the pET21c vector. The relative expression levels from each clone were measured by a densitometer (data not shown). The expression levels of the fusion peptide multimers were much higher than those of the buforin II multimers (Fig. 3B, lanes 7-10). The expression level of the fusion multimers reached the maximum at tetramer and started to decrease as the size of multimers increased further. The decrease in the expression might be ascribed to the presence of too many cysteine residues in CAP, which can inhibit the efficient charge interaction between CAP and buforin II in large multimers. In addition, CAP, which consists of 59 amino acids [14], may be too long to efficiently counterbalance the positive charge of buforin II. Therefore, it would seem that the number of free sulfhydryl groups, along with an adequate distribution of negative charges in an acidic peptide, are both important in neutralizing positive charges of an antimicrobial peptide for its efficient expression as tandem repeats.

## Purification and Characterization of Recombinant Buforin II

*E. coli* BL21(DE3) harboring pET21c-CB4 was chosen for the large-scale production of buforin II because pET21c-CB4 showed the highest expression level in *E. coli*.

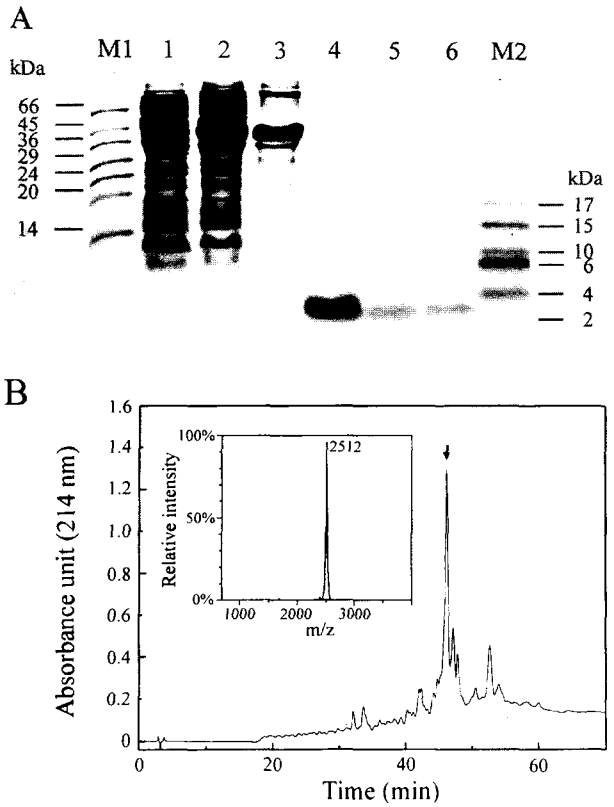


**Fig. 4.** Purification of recombinant buforin II from the tetramer of CAP-buforin II fusion expressed by BL21(DE3) harboring pET21c-CB4.

(A) SDS-PAGE analysis of purified recombinant buforin II. Lanes M1 and M2 represent size markers. Lanes 1 and 2 show total cell proteins before and after induction, respectively. Lanes 3 and 4 represent inclusion bodies isolated from the total cell proteins and solubilized inclusion bodies cleaved with CNBr, respectively. Lane 5 shows the recombinant buforin II purified by HPLC. Lane 6 is the natural buforin II. (B) Purification of the recombinant buforin II from the CNBr-cleaved mixture by reverse-phase HPLC and the determination of purity by MALDI-MS (inset).

Inclusion bodies of the CAP-buforin II fusion tetramer were solubilized and cleaved by CNBr as described in the Materials and Methods section. After the CNBr cleavage, the CNBr-cleaved multimers were subjected to reverse-phase HPLC, producing homogeneously pure buforin II (Fig. 4A). More than 100 mg of pure buforin II was obtained from 1 l of *E. coli* culture with over 90% purity, which was estimated based on the results of MALDI-MS, as shown in Fig. 4B (inset). The molecular mass of recombinant buforin II was confirmed to be 2512 Da by MALDI-MS analysis (Fig. 4B). The amino acid sequence of recombinant buforin II was determined to be identical to that of natural buforin II, except for an additional homoserine residue at the C-terminus. The purified recombinant buforin II was examined for its antimicrobial activity by determining its MIC against selected microorganisms. Recombinant buforin II had an identical antimicrobial activity to those of the natural and synthetic buforin II (Table 1). The additional homoserine residue derived from the methionine residue after the CNBr cleavage does not interfere with the antimicrobial activity of recombinant buforin II.

In conclusion, the antimicrobial peptide buforin II was successfully expressed at high levels by fusion to a cysteine-rich acidic peptide. The fusion peptide was expressed as inclusion bodies, which is beneficial to buforin II production because buforin II need not to be refolded to be active. Our approach in this study may lead to a cost-effective solution to the mass production of antimicrobial peptides and other basic peptides which are lethal to the host strain.

## Acknowledgments

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**Table 1.** Comparison of the antimicrobial activities of recombinant buforin II, natural buforin II, synthetic buforin II, and magainin II.

| Microorganism | Minimal inhibitory concentration (μg/ml)[a] | | | |
|---|---|---|---|---|
| | Recombinant buforin II | Natural buforin II | Synthetic buforin II | Magainin II |
| Gram-positive | | | | |
| *Bacillus subtilis* | 2 | 2 | 2 | 50 |
| *Staphylococcus aureus* | 4 | 4 | 4 | 50 |
| *Streptococcus mutans* | 2 | 2 | 2 | 100 |
| *Streptococcus pneumoniae* | 4 | 4 | 4 | 50 |
| Gram-negative | | | | |
| *Escherichia coli* | 4 | 4 | 4 | 100 |
| *Salmonella typhimurium* | 1 | 1 | 1 | 25 |
| *Serratia sp.* | 4 | 4 | 4 | 50 |
| *Pseudomonas putida* | 2 | 2 | 2 | 50 |
| Fungi | | | | |
| *Candida albicans* | 1 | 1 | 1 | 25 |
| *Cryptococcus neoformans* | 1 | 1 | 1 | 12 |
| *Saccharomyces cerevisiae* | 1 | 1 | 1 | 25 |

[a]Each MIC was determined from two independent experiments performed in duplicate.

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