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Full Length Research Paper

Prokaryotic expression of antimicrobial ovine β-defensin-1 in *Escherichia coli*

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Ovine β-defensin-1 (sBD-1) is a small, cationic peptide, with three canonical cysteine disulfide intramolecular bonds. It can inhibit the growth of Gram-positive and Gram-negative bacteria, yeast and fungi. We isolated sBD-1 by RT-PCR of small intestinal cDNA. The open reading frame of the cDNA was 192 bp, which codes 64 amino acids, and the mature peptide of the cDNA was 114bp, which encodes 38 amino acids. In order to understand their antimicrobial activities, the pre-peptide sBD-1 and the mature peptide of sBD-1 were studied. The two proteins were expressed in *Escherichia coli*. The fusion proteins were purified and digested by enterokinase. The digestion products showed that the mature peptide can inhibit the growth of *E. coli* and *P.aeruginosa.* It will be valuable to produce sBD-1 and other defensins for veterinary medical research on antibiotic treatment in ovine production.

Key words: Antimicrobial peptide, prokaryotic expression, ovine, β-defensin-1.

INTRODUCTION

Defensins comprise a major subclass of the family of antimicrobial peptides. Depending on the size and pairing of their six cysteine residues, defensins of higher vertebrates are classified as α-, β and θ-defensin (Wang et al., 2003). The β-defensins (BD) are characterized by six cysteines and have been found in many animal species such as bovine (Luenser and Ludwig, 2005), ovine (Luenser et al., 2005), porcine and humans (Maxwell et al., 2003). BD is produced principally in the epithelial cells of a number of organs including skin, lung, kidney, pancreas, uterus, eye, and nasal and oral mucosa (Klotman and Chang, 2006). In ovine, β-defensin-1 (sBD-1) is the most prevalent defensin throughout the digestive tract with the exception of the distal ileum (Huttner, 1998).

Defensins are a family of small cationic peptides with a broad range of antimicrobial activities. They can inhibit the growth of fungi, GRAM-positive, and gram-negative bacteria, such as *Staphylococcus aureus* (Hoover, 2000, 2002), a bacterium that causes infections ranging from skin abscesses to life-threatening conditions such as endocarditis and toxic shock. In recent years, several classes of antimicrobial peptides have been purified from mammalian phagocytes (Sawai, 2002). Similar molecules have also been isolated from specialized epithelia, suggesting that antimicrobial peptides may play a role in the intrinsic resistance of tissues to microbial invasion (Morrison, 2002). sBD-1, as an antimicrobial peptide, can replace the addition of antibiotics to ovine feed. Recently, the antibiotics resistance was hotter and hotter. This would be favorable to consumers because it would eliminate residual antibiotics in meat products. sBD-1 production *in vivo* shows great promise for veterinary medicine.

Over the past several years, several small cationic peptides have been synthesized successfully by recombinant gene expression methods (Wu et al., 2003). Because of its rapid growth rate and easily established protein expression system, *Escherichia coli* are used as the host cell (Hans and Kim, 2005). Many difficulties have

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Table 1. PCR primers for amplification of sBD-1, mature (msBD-1) and pre-peptide (psBD-1).

Parameter	Sequence
psBD-1	5'- CCGGAATTCCAACATGAGGCTCCATCACCTG-3' 5'- TTTGCGGCCGCTTACTTCTTTCTGCAGCAT-3'
msBD-1	5'-CTAGCCATGGGTAATCGTCTAAGCTGCCAT-3' 5'-TTTGCGGCCGCTTACTTCTTTCTGCAGCAT-3'
sBD-1	5'- GAATTCAACATGAGGCTCCATCACCTG -3' 5'- ATGCGGCCGCTTACTTCTTTCTGCAGCAT -3'

been encountered in the expression of genes encoding for antimicrobial polypeptides, often times due to their cytotoxicity and sensitivity to proteolytic degradation (Ganz, 1999, 2004). The fusion strategy (Ma et al., 2009) has been utilized in the production of small antimicrobial cationic peptides in *E. coli* to alleviate these problems, but fusion proteins tend to form inclusion bodies, which leads to inactivation of the expressed proteins (Li et al., 2004). The *E. coli* combined translation cell free system is popularly used because of its capability to directly synthesize protein from an exogenous gene. The system can be operated in batch or continuous mode. The batch-mode method is relatively simple and convenient; however, the efficiency of protein synthesis is quite low (Martemyanov et al., 1997; Maxwell et al., 2003).

As a potential therapeutic peptide, the fusion expression of sBD-1 has never been reported. In the present study, both the pre-peptide sBD-1 (psBD-1) and the mature peptide of sBD-1 (msBD-1) were synthesized and expressed using an *Escherichia coli* expression system. To improve the production of sBD-1, the conditions of cultivation and induction were systematically optimized. The fusion protein was purified and cleaved to obtain psBD-1 and msBD-1. Antibiotic potentency of the two peptides were observed.

MATERIALS AND METHODS

Strains, plasmids, and culture medium

E. coli DH5αF' (TaKaRa, Japan) was cultivated at 37°C in LB medium. *E. coli* BL21 (DE3) was used as the host for the expression of heterologous protein. Plasmid pMD19-T Simple (TaKaRa, Japan) and pET32a (Novagen, USA) were used as cloning and expression vectors, respectively. All restriction enzymes and T_4 DNA ligase were purchased from TaKaRa, Japan. Luria–Bertani (LB) medium (w/v) containing 0.5% yeast extract, 1% tryptone and 1% NaCl was used for manipulation of molecular clones and seed cultures.

The cloning of sBD-1

Total RNA of ovine small intestine was isolated using TRIzol reagent (TaKaRa), according to the manufacturer's recommendations, and then treated with RNase-free DNase I (TaKaRa). The concentration and purity of RNA were checked by absorbance at 260 and 280 nm.

The cDNA fragment encoding the sBD-1 protein was amplified by the reverse transcription polymerase chain reaction (RT-PCR) from total RNA, using *ExTaq* DNA polymerase (TaKaRa) and the synthetic oligonucleotide primers sBD-1 (Table 1). DNA amplification was conducted with 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 59°C), and elongation (2 min at 72°C). PCR products were cloned into the PMD19-T simple vector and sequenced by TAKARA company. The cloning plasmid was named PMD19-T-sBD-1. Nucleotide and deduced amino acid sequence comparisons were made using the BLAST (basic local alignment search tool) programs BLASTN (Zhang et al., 2000) and BLASTX, respectively, on non-redundant nucleotide and protein databases of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). These tools were used to deduce the amino acid sequence encoded by the gene sBD-1.

Construction of expression vectors

The psBD-1 and msBD-1 cDNAs were amplified by PCR, using PMD19-T-sBD-1 as template. Primers are reported in Table1. PCR products of psBD-1 and msBD-1 cDNAs were purified and cloned between EcoRI and NotI, and NcoI and NotI sites, respectively in pET32a vector. The two cloned vectors were transformed into *E. coli* BL21 (DE3). The transformed cells were cultured in LB Broth at 37°C overnight with shaking at 200 rpm. 500 µl from overnight culture was added into 30 ml of fresh LB containing Amp 0.1 mg/ml for 1 h until the OD reached 0.4 to 0.6. Then the cells were induced by 0.5 mM IPTG. After 5 h incubation, cells were harvested by centrifugation at 12000 rpm for 2 min at 4°C. Cells were then washed by 1×PBS. Subsequently, the first lysis (prolysis) buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4, 1 mMPMSF) was added and mixed to make a homogeneous solution. The cells were lysed by sonication at 6× pulse for 8 min on ice (20 s pulse on and followed by 10 s pulse off). The cells were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was decanted and binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) was added as the 1 ml HisTrap FF (GE Healthcare, Sweden). The sample was flowed on binding buffer at the rate of 0.5ml/min. After loading the sample, the fusion protein was eluted by elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). After collection of the fusion protein, the inherent buffer in the sample was exchanged for an enterokinase buffer (50mM Tris PH7.8, 2mMCaCl2, and 50mM NaCl) by gel chromatography using Sephadex G-25. The fusion protein was then digested by the enterokinase (NEB, UK) at room temperature for 24 h. The digested sample was desalted by

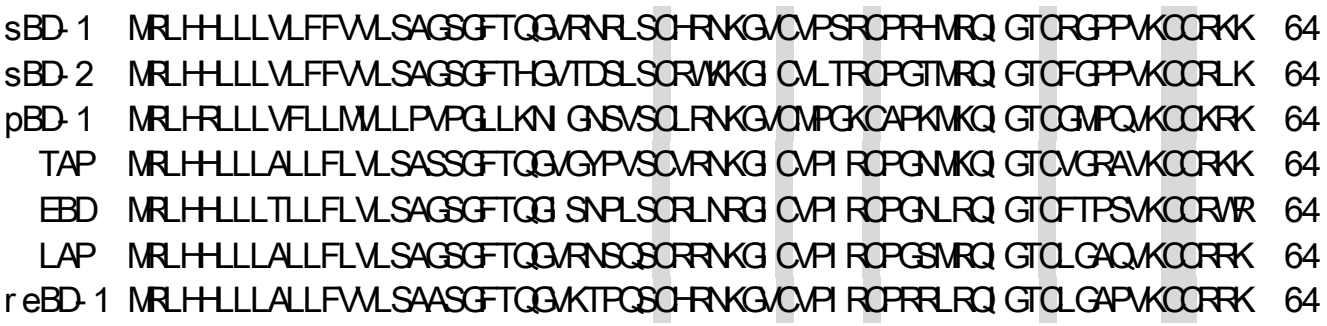

Figure 1. Comparison of deduced sBD-1 sequences with ovine BD-2, reindeer BD-1 and porcine. Gene Bank numbers are as follow: EBD, AF000362; LAP, S76279; TAP, AF014106; sBD-2, U75251; reBD-1, ABH11654; pBD-1, AF031666.

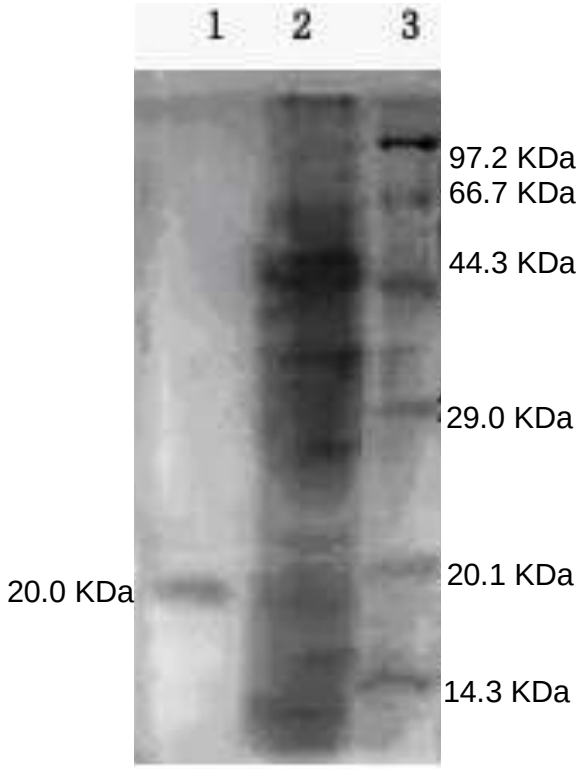

Figure 2. msBD-1 induced and purified products. Lane: 1. purified msBD-1; 2. msBD-1 induction products; 3. protein marker.

Sephadex G-10 with desalting buffer (20mM Tris PH7.8, 50mCH_3COONH_4). Finally, the sample was freeze dried.

The final digestion product was dissolved in 20 mM Tris (pH 7.8), to a final concentration of 50 µg/ml. Then, the solution was serially diluted from 1:2 to 1:32.

Antimicrobial assays

Antimicrobial properties of recombinant psBD-1 and msBD-1 were tested against sensitive strains of *E. coli* 44101 and *P. aeruginosa* 26003. About 10^6 colony forming units of *P. aeruginosa* was inoculated into 1 ml M-H medium in the log phase. To deduce the optimal concentration of antimicrobial activity, different concentrations psBD-1 and msBD-1 were tested (pH 7.8, 25 µg/ml). Controls were equivalent volumes of 20 mM Tris buffer. Bacterial cultures were shaken for 14 h and concentrations were determined by measurement of OD_{600}.

The inhibitory zone was measured to determine antimicrobial activity. 400 µl optimal concentrations of psBD-1 or msBD-1 were placed into wells on plated *E. coli*. The same concentration fusion proteins were also determined. Negative controls used 20 mM Tris buffer.

RESULTS

sBD-1 cloning

Based on RT-PCR, a 214bp sBD-1 cDNA was amplified. sBD-1 encodes a predicted 64 amino acid protein of 7.2 kDa with first 26 amino acid residues serving as a signal peptide. The mature peptide consists of 38 aa residues of about 3.8 KD (Gene Bank number U75250). A BLASTX search of the GenBank protein database showed that the amino acid sequence has homology with other known defensins characterized from different animals with six-conserved cysteine residues forming three disulfide bridges (Figure 1).

Construction of expression vectors and prokaryotic expression of psBD-1 and msBD-1 fusion proteins

The psBD-1 and msBD-1 proteins from prokaryotic expression were purified and characterized. The fusion protein yields were not largely affected by increasing or reducing the concentration of IPTG. Induction time was 6 h at 0.5 mM IPTG; and after 6 h, there was no noticeable change. Molecular weights of psBD-1 and msBD-1 were about 7.2 KDa and 3.8 Kda, respectively. Fusion protein molecular weight of psBD-1 and msBD-1 were 24 KDa and 20 Kda, respectively. The excess peptide was the tag of the pET32 vector. The fusion and purified proteins were loaded into SDS-PAGE to determine the purification effectiveness (Figures 2 and 3).

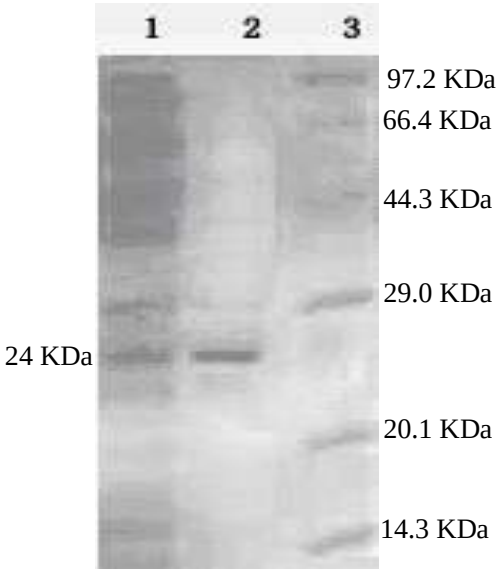

Figure 3. psBD-1 induced and purified products. Lane: 1. psBD-1 induction product; 2. purified psBD-1; 3. protein marker.

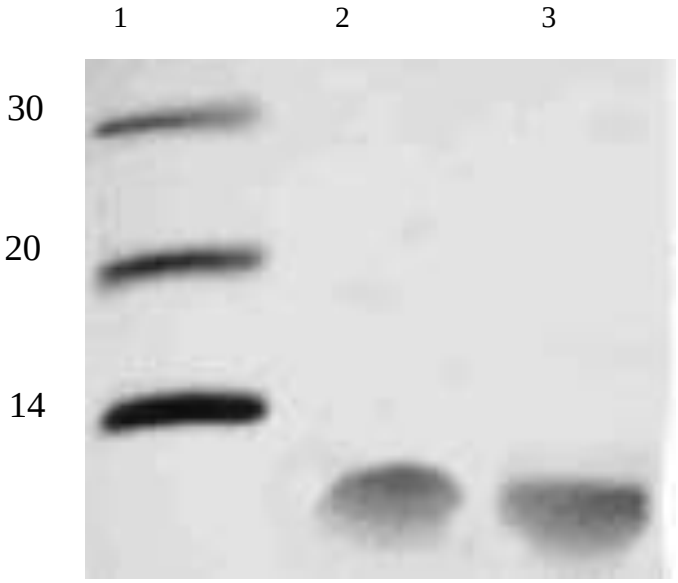

Figure 4. msBD-1 and psBD-1 enterokinase digestion products. Lane: 1. msBD-1; lane 2. psBD-1; lane 3. protein marker.

Cleaving the fusion protein

Fusion proteins were digested by enterokinase; Tricine SDS-PAGE was used to detect cleavage. Digestion products were desalted and freeze-dried (Figure 4).

Antimicrobial assays

psBD-1 did not show antimicrobial activity, even at high concentration (1 mg/ml) (the data was not shown). The optimal concentration of msBD-1 against the *P. aeruginosa* was shown by percent inhibition (Table 2). The optimal concentration was 96% at 12.5 μg/ml. Moreover, inhibition of *E. coli* by msBD-1 was demonstrated (Figure 5).

DISCUSSION

The soluble expression of heterologous proteins in *E. coli* is a good way to obtain the active form of a protein, especially proteins with multiple disulfide bridges. Several expression systems have been developed by fusing an antibiotic peptide with a partner protein or with anionic properties to avoid the toxicity of the heterologous protein to the host cells and the degradation of the products by bacterial proteases (Li et al., 2004; Xiang et al., 2002). msBD-1 is a cationic peptide with three disulfide bridges. The soluble expression of msBD-1 is an optimal method to obtain a bioactive defensin.

Defensins are positively-charged and contain both hydrophobic and hydrophilic domain peptides (Ganz, 2004). They are among the most potent antimicrobial peptides advanced by the mammalian defense system to protect against invading pathogens. The antimicrobial activity is remarkably specific, with little cytotoxicity to mammalian cells even at concentrations ten-fold or higher than those required for antimicrobial activity (Kim et al., 2003). In ovine, sBD-1 is expressed throughout the gastrointestinal tract (Huttner, 1998). This distribution is believed to be very beneficial for protecting the body invaded by microbes. sBD-1 is mainly expressed in the gastrointestinal tract, not in the blood of ovine (Huttner, 1998). sBD-1 is a key element in the bodies first line of defense for ovine.

Bohling et al. (2006) showed that an *α*-helix is often present at the N-terminus of *β*-defensins. Because N-terminal regions are often times aliphatic (Karen et al., 2008), this region is likely to direct membrane insertion and disruption. The msBD-1 showed antimicrobial activity while psBD-1 did not. The psBD-1 contains signal peptide, and the N-terminal region is likely to be inactive and not able to insert and disrupt the microbial membrane. The msBD-1 can easily insert and disrupt the microbial membrane because an *α*-helix is present of the N-terminal.

Defensins are at low concentrations active against bacteria (Kim et al., 2003). Antimicrobial activity of msBD-1 was observed at concentrations as low as 12.5 μg/ml, which strongly inhibited sensitive strains of *E. coli*. Generally, metabolically active bacteria are much more sensitive to defensins than bacteria made inactive by nutrient deprivation or metabolic inhibitors. Defensins may be the new antimicrobial compounds in the future. This strategy of producing msBD-1 opens a novel way to

Table 2. msBD-1 percent inhibition against *P. aeruginosa* at various concentrations.

msBD-1 (µg/ml)	Percent inhibition (%)
50.0	96.3
25.0	96.1
12.5	95.9
6.25	73.3
3.13	27.8
1.57	6.4

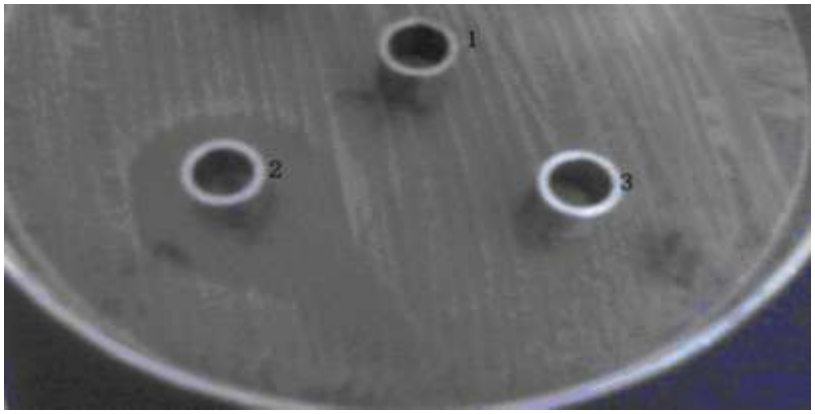

Figure 5. The bioactivity of msBD-1 against *E. coli*. Inhibitory zone test, using *E. coli*. as the sensitive strain: 1. negative control 20-mM Tris (pH 7.8); 2. 10 µg/ml msBD-1; 3. 1 mg/ml msBD-1 fusion protein.

produce functional defensins on a large scale and can also become a new method to administer antibiotics in ovine production, especially meat production. In the future, it will be helpful to produce additional defensins for the benefit of veterinary medical research.

Conclusion

In summary, the present study illustrates that msBD-1 can be successfully expressed and purified from *E. coli* BL21 while retaining their antimicrobial activity. Overall, the proposed approach may have potential benefit for the further designing of defensin-based therapeutic agents.

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