5-1-2011

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EXPRESSION, PURIFICATION, AND ANTIMICROBIAL ACTIVITY OF AVIAN BETA-DEFENSIN-2, -6, AND -12

By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

April 2011

EXPRESSION, PURIFICATION, AND ANTIMICROBIAL ACTIVITY OF AVIAN BETA-DEFENSIN-2, -6, AND -12

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Title of Study: EXPRESSION, PURIFICATION, AND ANTIMICROBIAL ACTIVITY OF AVIAN BETA-DEFENSIN-2, -6, AND -12

Pages in Study: 67

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Total RNA was extracted from chicken oviduct epithelial cells. Avian Beta-defensin (AvBD)-2, -6, and -12 cDNAs were amplified by reverse transcription-PCR and cloned into pRSET A, a protein expression vector. The hexa-histidine-tagged AvBD peptides were expressed in *Escherichia coli* (*E. coli*) BL21(DE3)plysS and affinity-purified. The antimicrobial activities of the recombinant AvBDs against *E. coli*, *Salmonella enterica* serovar *Typhimurium* (*S. typhimurium*), and *Staphylococcus aureus* (*S. aureus*) were determined. At 8, 16 and 32 µg/ml, all three rAvBDs killed and inhibited the growth of *E. coli*, *S. typhimurium*, and *S. aureus*. The killing of rAvBD-2, -6, and -12 against stationary phase *E. coli* and *S. aureus* was pH dependent in the range investigated. In addition, the killing-curves showed that rAvBDs exerted their antimicrobial function within 30 minutes of treatment, suggesting the fast killing mechanisms of rAvBDs.

DEDICATION

This thesis is dedicated to my parents who offered me unconditional love and supported me all the way since the beginning of my studies.

ACKNOWLEDGEMENTS

.First of all, I would like to sincerely thank my major professor, Dr. H. Bailey, and my committee, Dr. Shuping Zhang, Dr. M. Zhenyu Zhang, Dr. Lesya M. Pinchuk, and Dr. Todd Pharr, for their help and encouragement during my master program and dissertation process. And I also give my appreciation to all the faculty and staff at Mississippi Veterinary Research & Diagnostic Laboratory, for their invaluable technical assistance.

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CHAPTER I

INTRODUCTION

Defensins are cysteine-rich antimicrobial peptides that vary in length from 63 to approximately 104 amino acids with a characteristic β-sheet-rich fold. These peptides are also enriched in hydrophobic and cationic amino acid residues. Defensins have been found in many species, including insects, mammals, and birds and have acquired the name 'defensins' based on their association with host defence settings [Xiao et al., 2004] . Defensins, vital contributors to host immune response, are an efficient part of the innate host defense because of their ability to recognize and neutralize invading microorganisms quickly, and also contribute to adaptive immunity through their effector and regulatory functions [Tomas Ganz, 2003].

There are two main subfamilies of defensins, α-defensins, which have only been identified in mammals, and β-defensins, found throughout vertebrate species. These two subfamilies of defensins differ in disulfide bridge pairing between cysteine residues; the disulfide bridge paring is Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5 in α-defensins, whereas in β-defensins, it is Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6 (the number indicates the location of the cysteine residue in the amino acid sequence from the N-terminus) [Selsted et al., 2005]. A third type of defensin, θ-defensins, are encoded by α-defensin-like precursors and have been found only in primates. In evolutionary terms, all defensin subfamilies must have evolved from an ancestral β-defensin gene by duplication and

diversification, since α- or θ-defensins have not been found in phylogenetically much older vertebrates, such as birds and fish [Semple et al., 2003].

The chicken (*Gallus gallus domesticus*) genome encodes at least 14 different β-defensins, with no α- or θ-defensin genes being identified [Albert et al., 2008]. The β-defensins in chicken had been previously called gallinacins; however, it has now been agreed to use their gene names [i.e., avian β-defensins (AvBD)] [Lynn et al., 2007], we therefore decided to use the new terminology for this study.

Avian β-defensin genes are clustered densely within a 86.0 kb distance on the chromosome 3q3.5-q3.7 [Lynn et al., 2007], and each gene possesses the same genomic structure of four exons that are separated by three introns of various lengths with the exception of AvBD12 gene where the last two exons have fused [Xiao et al., 2004]. The 1st exon corresponds to the 5′ UTR region, and the 2nd exon encodes the signal peptide and part of the propiece, while the remaining part of the short propiece and majority of the mature peptide are encoded by the 3rd exon. The 4th exon encodes the remaining part of the mature peptide and the 3′ UTR region [Albert et al., 2008].

Although the details of mechanisms ruling the synthesis, storage and activation of avian β-defensins are lacking, they can be expected to parallel that of mammalian defensins. Myeloid β-defensins are synthesized in the bone marrow; after biosynthesis and intracellular trafficking through the Golgi apparatus, the signal peptide (about 19 amino acids), functioning to anchor the prodefensin peptide in the endoplasmic reticulum (ER) membrane, is rapidly proteolytically cleaved to generate a prodefensin peptide with little or no microbicidal activity [Satchell et al., 2003], and the prodefensins are stored into specific granules as mature peptides [Yount et al., 1999]. Non-myeloid biosynthesis of β-defensin has been described to some detail for mammalian epithelial cells [Dale et

al., 2001; Oren et al., 2003]. In chicken, non-myeloid β-defensin expression has been found for epithelial cells and tissues [Ohashi et al., 2005; Yoshimura et al., 2006; Zhao et al., 2001], but data describing their biosynthesis in these cells and tissues has not yet been reported. Due to the local repertoire of proteolytic enzymes and inhibitors, the proteolytic processing of avian β-defensin proforms is host and tissue-specific and may result in multiple forms with different properties. For instance, human epididymis β-defensin-like peptides are processed by furin-like convertases, which are the major processing enzymes of the secretory pathway located in the trans-Golgi network [Gu et al., 2001; von Horsten et al., 2002]. Human β-defensin-1 (hBD-1) produced by oral keratinocytes is processed into one major form (47 amino acids) and several minor forms (40–44 amino acids) [Diamond et al., 2001] by yet unidentified proteases, whereas multiple truncated forms of hBD-1 occurring in plasma can be explained by prodefensin cleavage by a chymotrypsinlike enzyme [Hiratsuka et al., 2000; Zucht et al., 1998]. Furthermore, details of avian β-defensin intracellular trafficking, storage and activation in myeloid and non-myeloid cells are lacking.

Differing in tissue expression, gene regulation, structural properties, and biological activities, avian β-defensins are found to be constitutively expressed in some cell populations and tissues, including respiratory tract, skin, digestive tract, urogenital tract, leukocytes, etc., which are involved in the innate immune response against microbial infections. For example, avian heterophils, like the neutrophils of some mammalian species, contain multiple β-defensins [Schneider et al., 2005; Selsted et al., 1993]. In addition, the expression of avian β-defensins also can be induced or upregulated in some tissues in response to microbial infection or by proinflammatory stimulants, such as cytokines (IL-1α, IL-1β, TNF-α, IFN-γ, and TGF-1), LPS, bacteria, yeast and other

stimulants such as PMA, isoleucine and 1, 25-dihydroxyvitamin D3 [Albert et al., 2008]. For example, AvBD-3 was significantly upregulated in tracheal tissue in response to *Haemophilus paragallinarum* infection, but not in other tissues [Zhao et al., 2001]. Small intestinal AvBD-4, -5 or -6 mRNA levels were not upregulated in response to an oral challenge with *Salmonella* serovars [Panagiota Milona et al., 2007].

Being constitutively or inducibly expressed, avian β-defensins have been shown to contribute to host innate defense via direct bactericidal activity. These peptides comprise a N-terminal signal sequence, a propiece, and a C-terminal mature peptide. Avian β-defensin peptides are highly cationic due to the presence of numerous arginine, lysine and histidine residues, and they have a three-dimensional amphipathic structure, i.e. they possess hydrophilic amino acid residues aligned along one side and hydrophobic amino acid residues aligned along the opposite side. The tertiary structure of these peptides consists of three antiparallel β-sheets, which are constrained by three intramolecular disulfide bridges formed by cysteine residues, making up the characteristic "defensin-like" fold. For most mature avian β-defensins, disulfide bridges and their connectivity appear not to be important for direct antimicrobial activity, but may play a prominent role in other functions, such as protection against proteolysis and chemotaxis [Albert et al., 2008], [Klu¨ver et al., 2005], [Selsted et al., 2005], [Wu et al., 2003]. On the other hand, the highly variable amino acid composition and positioning appear to determine the extent to which individual avian β-defensins specifically target certain types of microorganisms [Torres et al., 2004]. The amphipathic character of avian β-defensins may be responsible for their antimicrobial activities since they can favourably attach and insert into their targets, such as bacterial membranes. The initial contact between the peptide and the target organism is electrostatic, as most bacterial

surface components, such as lipopolysaccharides (LPS), lipoteichoic acid (LTA) and anionic phospholipids, are anionic. During initial electrostatic interactions, avian β-defensins also can competitively displace Ca^{2+} and Mg^{2+} ions which are important for microbial outer membrane stability, due to their higher affinity for divalent cation binding places. Their amino acid composition, amphipathicity, cationic charge and size allow avian β-defensins to attach to and insert into membrane bilayers to form pores by 'carpet wormhole' model [Ganz, 2003]. Or allow them to penetrate into the cell to bind intracellular molecules which are crucial to cell living. The intracellular binding model of avian β-defensins includes inhibition of cell wall synthesis, alteration of cytoplasmic membrane, activation of autolysin, inhibition of the synthesis of DNA, RNA, and protein, and inhibition of certain enzymes [Lehrer et al., 1989; Tang et al., 1999]. However, in many cases, the exact mechanism is not known.

Most avian β-defensins show inhibitory and killing activities against Gram-negative and Gram-positive bacteria (including strains resistant to conventional antibiotics), mycobacteria (including *Mycobacterium tuberculosis*), enveloped viruses, fungi and even transformed or cancerous cells. For example, synthetic chicken AvBD-9 peptide showed strong microbicidal activity against the Gram-negative bacterium *Campylobacter jejuni* (3.7 mM), Gram-positive bacteria, *Clostridium perfringens*, *S. aureus* (1.9–3.7 mM) and the yeasts *Candida albicans* and *Saccharomyces cerevisiae* (1.9 mM); additionally, Treatment with synthetic AvBD-9 resulted in a 3 log unit reduction in *Clostridium perfringens* survival within 60 min, indicating fast killing kinetics [Albert van Dijk et al., 2007]. Evans et al. (1995) demonstrated bactericidal and fungicidal activity of chicken heterophil AvBD1, at peptide concentrations of 0.4–3.4 mM against avian pathogens. However, these peptides were not able to kill *Pasteurella*

multocida or neutralize Infectious Bronchitis Virus, an enveloped coronavirus of chickens.

In addition to their important roles in innate host defense via direct antimicrobial activity, avian β-defensins have also been shown to contribute to adaptive immunity through effector and regulatory functions. They have been demonstrated to have a number of immunomodulatory functions involved in the clearance of infection, including the ability to alter host gene expression, act as chemokines and/or induce chemokine production, inhibit lipopolysaccharide induced pro-inflammatory cytokine production, promote wound healing and adaptive immunity by selective recruitment by chemotaxis of monocytes [Territo et al.,1989], T lymphocytes [Chertov et al., 1996], immature dendritic cells [Yang et al., 1999] and mast cells [Niyonsaba et al., 2002] to sites of inflammation. Furthermore, they are able to enhance macrophage phagocytosis [Fleischmann et al., 1985], and to induce histamine release from peritoneal mast cells [Befus et al., 1999].

Identification of these avian β-defensins will aid in the study of the innate and adaptive immune response of the chicken, an economically important species. Moreover, these novel antimicrobial peptides may be exploited for the development of new therapeutic agents for economically significant chicken diseases such as coccidiosis, which results in a loss to the world poultry industry that is estimated at $700 million annually [David et al,. 2004]. These peptides could be potentially developed as natural alternatives to the artificial antibiotics that are commonly fed to chickens and which are of growing public concern. For these peptides, there are several different potential strategies for their general therapeutic application: 1) as immunostimulatory agents that enhance natural innate immunity, 2) as single anti-infective agents, 3) in combination with conventional antibiotics or antivirals to promote any additive or synergistic effects,

and 4) as endotoxin-neutralizing agents to prevent the potentially fatal complications associated with bacterial virulence factors causing septic shock. Despite many attractive attributes, the challenges of bringing a successful avian β-defensins to market remain formidable [Gordon et al., 2005].

Focusing on the three avian β-defensins, AvBD-2, -6, and -12, differing in their charge, we report here on their *in vitro* expression, purification, and antimicrobial activities against food-borne pathogens. The hexahistidine-tagged mature AvBD peptides were expressed in *Escherichia coli* (*E. coli*) BL21(DE3)plysS and affinity-purified by immobilized-metal (Ni^{2+}) affinity column chromatography. The growth inhibitory and killing properties of recombinant AvBDs (rAvBDs), determined by colony counting assay, were tested against *E. coli*, *Salmonella enterica* serovar *Typhimurium*, and *Staphylococcus aureus*. For practical purposes, the nomenclature of chicken β-defensin and related sequences in this paper is based on the recently proposed update of the avian β-defensin nomenclature by Lynn et al [Lynn et al., 2007].

CHAPTER II

MATERIALS AND METHODS

2.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Chicken oviduct epithelial cells were cultured and infected as described previously [Li et al., 2009]. Total RNA was extracted from the infected cells using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Before reverse transcription, RNA was treated with RNase-free DNase I (Ambion) to remove DNA contamination. First-strand cDNA was synthesized by using Taqman® Reverse Transcription Reagents (Applied Biosystems) and reverse transcription was performed at 25°C for 10 min, 48°C for 45 min, followed by heat inactivation for 10 min at 95°C. The resulting cDNA was used as templates for subsequent PCR amplification of gene fragments encoding the mature peptide of AvBD-2, -6, and -12. PCR primers are listed in Table1. PCR was performed using a DNA *Taq* polymerase (Sigma, St. Louis, MO) under the following amplification conditions: 95°C denaturation for 3 min, followed by 35 cycles of 95°C denaturation for 30 sec, annealing for 20 sec, and 72°C extension for 30 sec, and a final extension at 72 °C for 5 min. The annealing temperature was optimized for each gene.

Table 2.1 Primer Sequences Used for Reverse-Transcription (RT)-PCR Analyses

Gene	T(°C)	Primer sequence (5′-3′)
AvBD-2	55	Forward---GATGGGGATCCCGGGACATGCTGTTCTGT Reverse---TCCATGGTACCTTATGCATTCCAAGGCCA
AvBD-6	52	Forward---GATGGGGATCCAGCCCTATTCATGCTTGTAG Reverse---TCCATGGTACCTCAGGCCCACCTGTTC
AvBD-12	52	Forward---GATGGGGATCCGGCCCAGACAGCTGTAAC Reverse---TCCATGGTACCTCAGGTCTTGGTGGGAG

Note: The forward primers contain a BamH I restriction site (GGATCC) and the reverse primers a Kpn I restriction site (GGTACC), which are underlined. T (°C) represents the optimum annealing temperature for PCR analyses.

2.2 Plasmids Construction

RT-PCR products, including AvBD-2, AvBD-6, and AvBD-12, were separated on a 1.5% Agarose/EtBr gel and visualized using the AlphaImager system (Alpha Innotech). DNA bands with expected sizes were excised from the gel and DNA was purified from gel by using the Wizard® PCR prep DNA purification system (Promega, Madison, WI). The purified PCR products were cloned into pCR 2.1-TOPO® (Invitrogen, Carlsbad, CA) to generate plasmids pCR2.1-AvBD-2, pCR2.1-AvBD-6, and pCR2.1-AvBD-12, respectively. The resultant plasmids were transformed into *E. coli* TOPO10 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The transformed cells were selected on LB agar containing 50µg/ml ampicillin and 30µg/ml X-gal. Clones with inserts were screened by colony-lysis PCR. *E. coli* TOPO10 strains harboring plasmids pCR2.1-AvBD-2, pCR2.1-AvBD-6, and pCR2.1-AvBD-12 were designated as ZM-50, ZM-51, and ZM-52, respectively. Subsequently, the cloned gene fragments were excised from pCR2.1-AvBD-2, pCR2.1-AvBD-6, and pCR2.1-AvBD-12 by restriction endonuclease digestion with *Bam*H I (Promega, Madison, WI) and *Kpn* I (Promega,

Madison, WI) and subcloned into the corresponding sites of pRSET A, a protein expression vector, by using T_4 DNA ligase (Invitrogen, Carlsbad, CA). The resultant plasmids, designated as pRSET A-AvBD-2, pRSET A-AvBD-6, and pRSET A-AvBD-12, respectively, were transformed into *E. coli* BL21(DE3)plysS. The transformed *E. coli* cells were selected on LB agar containing 50 µg/ml ampicillin and 35 µg/ml chloramphenicol. *E. coli* BL21(DE3)plysS strains harboring pRSET A-AvBD-2, pRSET A-AvBD-6, and pRSET A-AvBD-12, were designated as ZM-51E, ZM-52E, and ZM-53E, respectively. Plasmids were purified using the Wizard® Plus Minipreps DNA Purification System (Promega, Madison, WI) and sequenced commercially (Eurofins MWG Operon, Huntsville, AL) to confirm the correctness of the inserts.

2.3 Protein Expression

A single colony from fresh cultured LB agar plate was inoculated into 10 ml SOB containing 50 µg/ml ampicillin and 35 µg/ml chloramphenicol and incubated overnight at 37°C with vigorous shaking (220 rpm). The overnight culture was then added into 1 L SOB without antibiotics to an OD_{600} of 0.1. The inoculated culture was incubated at 37°C with vigorous shaking (220 rpm) to an OD_{600} of 0.4 - 0.6 at which time isopropyl-β-D-thiogalactopyranoside (IPTG) is added to a final concentration of 0.2 mM to induce the expression of 6xHis-tagged avian β-defensin peptides. After an additional 2-hour incubation at 37°C with shaking, induced bacteria were harvested by centrifugation at 3,000 g for 10 minutes at 4 ºC. The pellet was resuspended in 20ml buffer B (7 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0). The bacterial suspension was sonicated for 10 minutes at 20 kHz using 1 s-1 pulse and 30 - 40% acoustic power and centrifuged at

13,000 g for 10 minutes to remove cell debris. The supernatant fraction was stored at -20 ºC prior to protein purification.

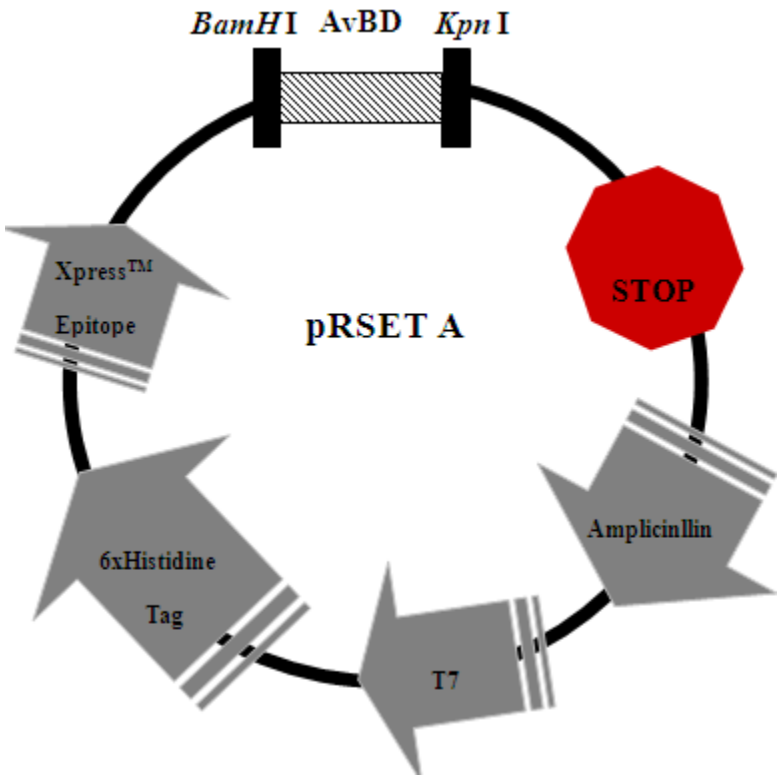


Figure 2.1 Map of pRSET A Vecto

Note: T7 promoter: bases 20-39
6xHis tag: bases 112-129
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 201-248
Ampicillin resistance gene: bases 1042-1902

2.4 Purification of Recombinant Avian β-defensins (rAvBDs)

Purification of the recombinant peptide was performed by affinity chromatography using Ni^{2+}-NTA anti-His protein superflow columns (Qiagen sciences, Germantown, MD). The supernatant fraction of bacterial lysate (10 ml) was applied to the column, which was subsequently washed with buffer C (7 M urea, 0.1 M NaH_2PO_4,

0.01 M Tris-HCl, pH 6.3). The membrane-bound 6xHis-tagged peptides were eluted with 5 ml buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 4.5). The peptide solution was added into a Slide-A-Lyzer dialysis cassette (molecular mass cutoff 2,000 Da, Fisher scientific, Pittsburgh, PA) and dialyzed sequentially against the peptide buffer (50 mM NaCl and 20 mM Tris-HCl) containing 6 M, 4 M, 2 M urea for 4 hours each time and finally the peptide buffer without urea for 12 hours at 4 ºC. Phenylmethylsulfonyl Fluoride (PMSF) (Fisher scientific, Pittsburgh, PA) was added into all buffer systems to a final concentration of 1 mM to prevent protein degradation. Following dialysis, the recombinant peptides were concentrated using a Microcon Centrifugal Filter devices (Molecular mass cutoff = 3,000 Da, Fisher Scientific, Pittsburgh, PA). The protein concentrations were determined by measuring the absorbance at 280 nm.

2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western-Blot Analysis

Recombinant 6xhis-tagged peptides were separated on 16.5% (wt/vol) polyacrylamide gel and transferred electrophoretically onto 0.2 µm nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA). The membrane was blocked in phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween 20 and 5% (wt/vol) milk powder for 1 hour at room temperature with gently agitation. Following 3 washes for 5 minutes each with PBS-Tween 20 (0.05%), the membrane was incubated in anti-Xpress primary antibody (1:2000 dilution in blocking solution) (Invitrogen, Carlsbad, California) for 1 hour at room temperature with gentle agitation. The membrane was washed 3 times for 5 minutes each with PBS-Tween 20 (0.05%) and incubated in anti-mouse IgG-HRP conjugate (1:500 dilution in blocking solution) (Promega, Madison, WI)

for 1 hour at room temperature with gentle agitation. Detection of recombinant peptides was achieved by visualization of color after development in 4-chloro-1-naphthol (Sigma-Aldrich, St. Louis, MO) and 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO).

2.6 Analysis of rAvBD Characteristics

The molecular mass and hydrophobicity of rAvBD-2, -6, and -12 were calculated using the peptide property calculator (GenScript). The net charge at pH6 and pH7 were calculated using the protein calculator v3.3 (http://www.scripps.edu/~cdputnam/protcalc.html.).

2.7 Antimicrobial activity

Colony-counting method was used to determine the antimicrobial activities of AvBDs against *Staphylococcus aureus* (*S. aureus*) ATCC 29213, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *E. coli* ATCC 25922. Bacterial cultures were maintained on LB agar plates at 37°C. To evaluate the inhibitory activities, 3 to 5 colonies from fresh culture plate (< 24 hours) were resuspended in 5 ml sterile distilled water to achieve a McFarland standard of 0.5 (~10^8 CFU/ml). Ten µl bacterial suspension was inoculated into 10 ml Mueller-Hinton broth to obtain a final concentration of approximately 10^5 CFU/ml. Equal volumes (25 µl) of bacterial suspension and rAvBD at varying concentrations were mixed in the wells of a 96-well plate. The final peptide concentrations were 8 µg/ml, 16 µg/ml, and 32 µg/ml. The bacteria-peptide mixtures were incubated at 37°C for 3 hours and serially diluted in PBS. Fifty microliters of each dilution was plated on LB agar plate and incubated at 37ºC for 24 hours. Colonies on each plate were counted after incubation. To investigate the synergistic antimicrobial activities of rAvBDs, different combinations of rAvBDs at a final concentration of 16

μg/ml were tested against *S. aureus*, *S. enterica* serovar Typhimurium, and *E. coli*. The antimicrobial assays for combined AvBDs were performed as described for individual AvBDs. For negative control, 25 μl peptide buffer (20 mM Tris-HCl, 50 mM NaCl) was used to replace rAvBD. The percentage of inhibition was calculated using the following formula: [CFU (control) - CFU (treatment)] / CFU (control) %. The experiments were repeated three times with duplicate wells in each experiment.

The killing activities of rAvBDs were determined using bacterial suspensions prepared in 100x diluted Mueller Hinton broth (1 ml broth in 99 ml distilled water). Bacterial growth in 100x diluted Mueller Hinton broth was pretested by colony-counting method. To perform killing assays, equal volumes (25 μl) of bacterial suspension containing 10^5 CFU/ml and rAvBDs at varying concentrations were mixed and incubated at 37ºC for 3 hours. Ten-fold serially diluted peptide-bacteria mixtures were plated on LB agar plates followed by colony enumeration. To investigate the effect of pH and the incubation time on the antimicrobial activities of rAvBDs, equal volumes (25 μl) of bacterial suspension in 100x diluted Mueller Hinton broth and rAvBDs at a final concentration of 16 μg/ml were coincubated for various times. The reaction mixtures were 10-fold serially diluted and plated on LB agar plates for colony counting. For negative controls, 25 μl peptide buffer (20 mM Tris-HCl, 50 mM NaCl) was used to replace rAvBD to treat bacteria. The killing activity was calculated using the following formula: [CFU (control) - CFU (treatment)] / CFU (control) %. The experiments were repeated three times with duplicate wells in each experiment.

The antimicrobial killing kinetics were determined by incubating equal volumes (25 μl) of bacterial suspension prepared in 100x diluted Mueller Hinton broth and rAvBDs at a final concentration of 16 ug/ml at 37°C for various times (30, 60, 90, 120,

150 min). After incubation, 10-fold serial dilutions were plated on LB agar plates and incubated at 37C for 24 hours followed by CFU enumeration. The effect of pH on the antimicrobial activity of rAvBDs was evaluated by conducting the killing assays at pH6 and pH 7. All experiments were repeated three times with duplicate wells in each experiment.

2.8 Statistical Analysis

The mean value of data collected from three independent experiments and the standard deviation were calculated by using SAS9.1 software. The differences in the antimicrobial activities among individual rAvBDs and rAvBD combination groups were determined using the SAS9.1 software with *t* test program.

CHAPTER III

RESULTS

3.1 Amplification of AvBD Genes

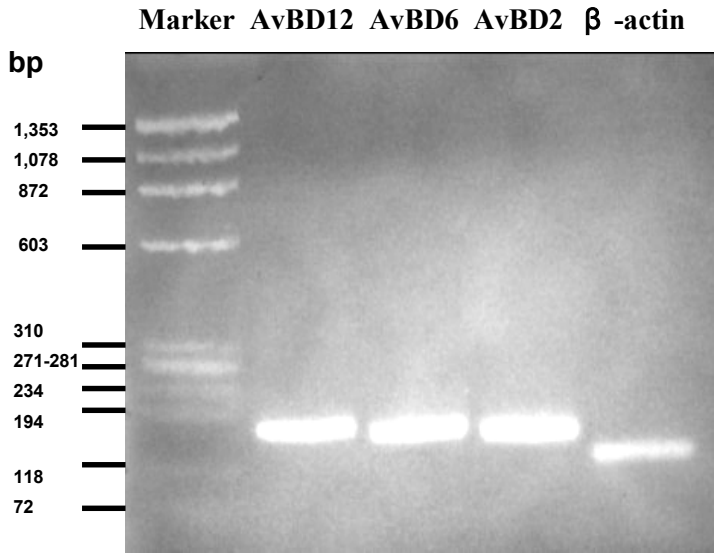


Figure 3.1 Electrophoresis of RT-PCR products.

Note: Total RNA was extracted from chicken oviduct epithelial cells infected with *Salmonella enterica* serovar Typhimurium. Two-step RT-PCR was carried out to amplify gene fragments encoding mature peptides of AvBD-2, -6, and -12. The amplified products were subjected electrophoresis on 1.5% agrose gel. The sizes of AvBD-2, -6 and -12 are 120, 129 and 138 bp, respectively. Chicken β-actin (95 bp) cDNA was used as a control.

RT-PCR was conducted to amplify the gene fragment encoding mature peptide AvBD-2, -6, or -12. Electrophoresis of RT-PCR products (Figure 2) showed DNA bands with expected sizes of 120 base pair (bp) for AvBD-2, 129 bp for AvBD-6, and 138 bp for AvBD-12. The amplified cDNA was directionally cloned into the bacterial expression

vector pREST A. The orientation and the identities of the cloned AvBD gene fragments were confirmed by sequence analysis.

3.2 Expression and Characterization of rAvBDs

AvBD-2, -6, and -12 were expressed in *E. coli* strain BL21(DE3)plysS cells as N-terminal 6xhis-tagged recombinant peptides and purified by Ni-NTA affinity chromatography. The yields of rAvBD-2, -6, and -12 were typically 0.92 mg, 1.24 mg, and 1.52 mg per liter of bacterial culture, respectively. The rAvBD-2, rAvBD-6, and rAvBD-12 contained N-terminal 6xhis, leader peptide for periplasmic targeting, and the XpressTM epitope (a total of 35 amino acid residues) and 39, 42, and 45 amino acid residues specific to AvBD-2, AvBD-6, and AvBD-12, respectively (Table 2). With or without the N-terminal tag/epitope derived from pRSET A, rAvBD-2 was most hydrophobic, followed by rAvBD-6 and then rAvBD-12 wherease rAvBD-6 exhibited the highest net charge at both pH 7 and pH 6 followed by rAvBD-2 and then AvBD-12. The predicted properties of all three rAvBDs were summarized and presented in Table 3. SDS and Western analyses of recombinant peptides revealed bands of approximately 10 kDa, consistent with the predicted sizes of 8.447 kDa for rAvBD-2, 8.867 kDa for rAvBD-6, and 9.022 kDa for rAvBD-12.

Table 3.1 Amino Acid Sequences of Recombinant Hexahistidine-tagged Mature AvBDs*

Peptide	Amino acid sequence
6xhis-tag	MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS
AvBD-2	RDMLF**C**KGGS**C**HFGG**C**PSHLIKVGS**C**FGFRS**CC**KWPWNA
AvBD-6	SPIHA**C**RYQRGV**C**IPGP**C**RWPYYRVGS**C**GSGLKS**CC**VRNRWA
AvBD-12	GPDS**C**NHDRGL**C**RVGN**C**NPGEYLAKY**C**FEPVIL**CC**KPLSPTPTKT

*The 6xHis tag, leader peptide, and the XpressTM Epitope region is underlined. Cysteine residues are bolded.

Table 3.2 The Predicted Characteristics of Recombinant AvBDs*

Characteristic	**Peptid**	**AvBD-2**	**AvBD-6**	**AvBD-12**
Length (aa)	6xhis-tagged	75.00	78.00	81.00
	Mature	39.00	42.00	45.00
Molecular mass (kDa)	6xhis-tagged	8.45	8.87	9.02
	Mature	4.32	4.74	4.90
Hydrophobicity (%)	6xhis-tagged	24.00%	23.00%	20.00%
	Mature	28.00%	26.00%	22.00%
Net charge at pH 6.0	6xhis-tagged	8.20	10.40	4.50
	Mature	5.50	7.70	1.80
Net charge at pH 7.0	6xhis-tagged	3.70	6.40	0.40
	Mature	4.20	7.00	1.00

e property calculator (GenScript). The net charge was calculated using protein calculator v3.3. *The molecular masse and hydrophobicity were calculated using the peptid

3.3 Bacterial growth inhibition by rAvBDs

Since the antimicrobial potency has been shown previously not to be significantly altered by the presence of a hexahistidine tag [S.Yenugu et al., 2003], the 6xhis-tagged recombinant peptides were used to determine the antimicrobial activities of AvBD-2, AvBD-6, and AvBD-12. At 8, 16, and 32 μg/ml, all three recombinant peptides exhibited inhibitory active against both gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*E. coli* and *Salmonella enterica* serovar Typhimurium) which, however, were

not concentration-dependent (Figure 3). At 8 and 16 µg/ml, rAvBD-6 was more effective than rAvBD-2 and rAvBD-12 in inhibiting the growth of *S. aureus* and *E. coli*. In contrast, rAvBD-2 and rAvBD-12 were more potent than rAvBD-6 against *S. enterica* serovar Typhimurium (Figure 3). In general, rAvBDs showed highest inhibitory activity against *S. aureus* and lowest against *S. enterica* serovar Typhimurium.

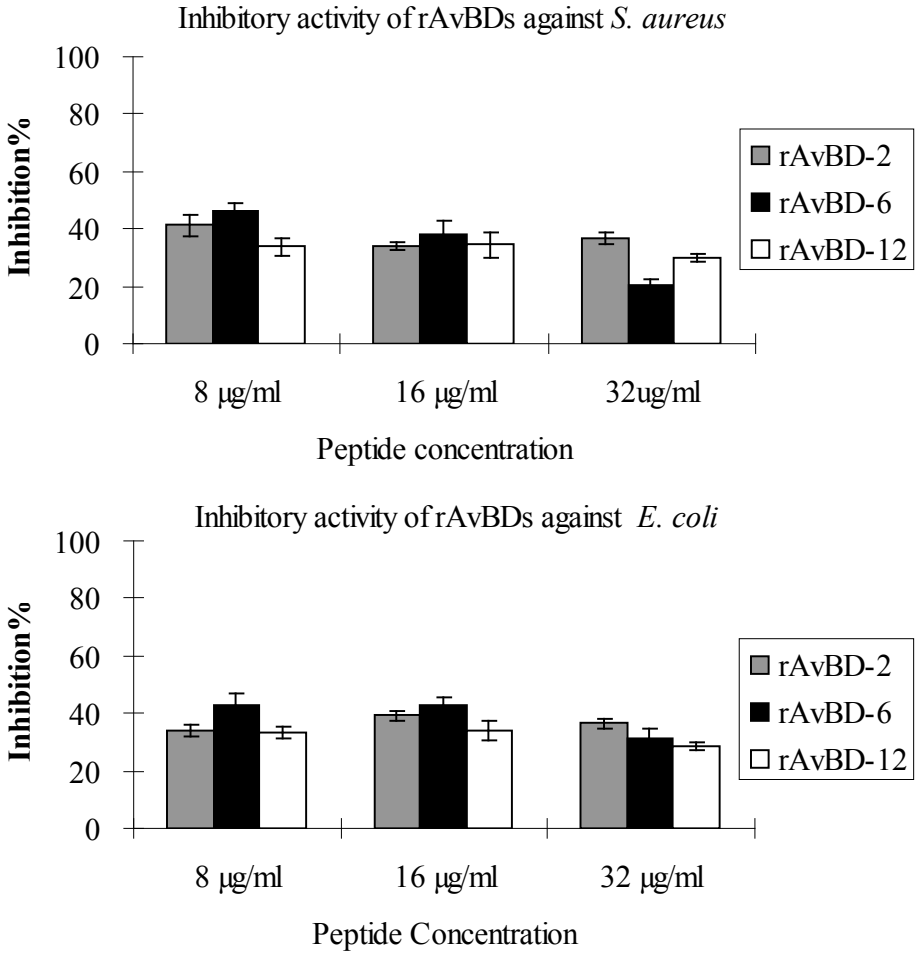


Figure 3.2 Inhibitory activities of rAvBDs against S. aureus, E. coli, and S. enterica serovar Typhimurium. Equal volumes (25 µl) of bacterial suspension (105 CFU/ml) in Mueller Hinton broth and rAvBD (105 CFU/ml) were coincubated at 37°C for 3 hours followed by colony enumerations. Data shown (bars) are geometric means of three independent experiments ± standard deviation.

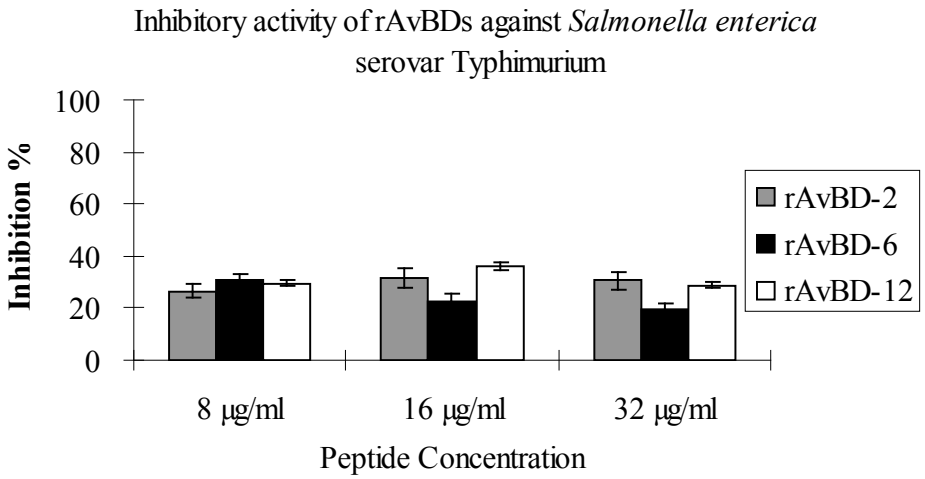


Figure 3.2 Continued

To investigate whether the three rAvBDs work synergistically or antagonistically, the combined growth inhibitory activity of different rAvBDs (final concentration 16µg/ml) against S. auereus, E. coli, and S. enterica, serovar Typhimurium were determined. No significant synergism between any rAvBDs against any bacterial species was observed. When rAvBD-2 and rAvBD-12 were combined, antagonistic activities against S. aureus were observed, compared to rAvBD-2 ($p=0.018$) and rAvBD-12 ($p=0.018$) alone, and against S. enterica serovar Typhimurium, compared to AvBD-2 ($p=0.009$) and rAvBD-12 alone ($p=0.023$). No antagonistic effect against E. coli was detected between rAvBD-2 and rAvBD-12. No antagonistic activities against the bacterial species tested were found between rAvBD-6 and rAvBD-12 or rAvBD-2 and rAvBD-6 (Figure 4).

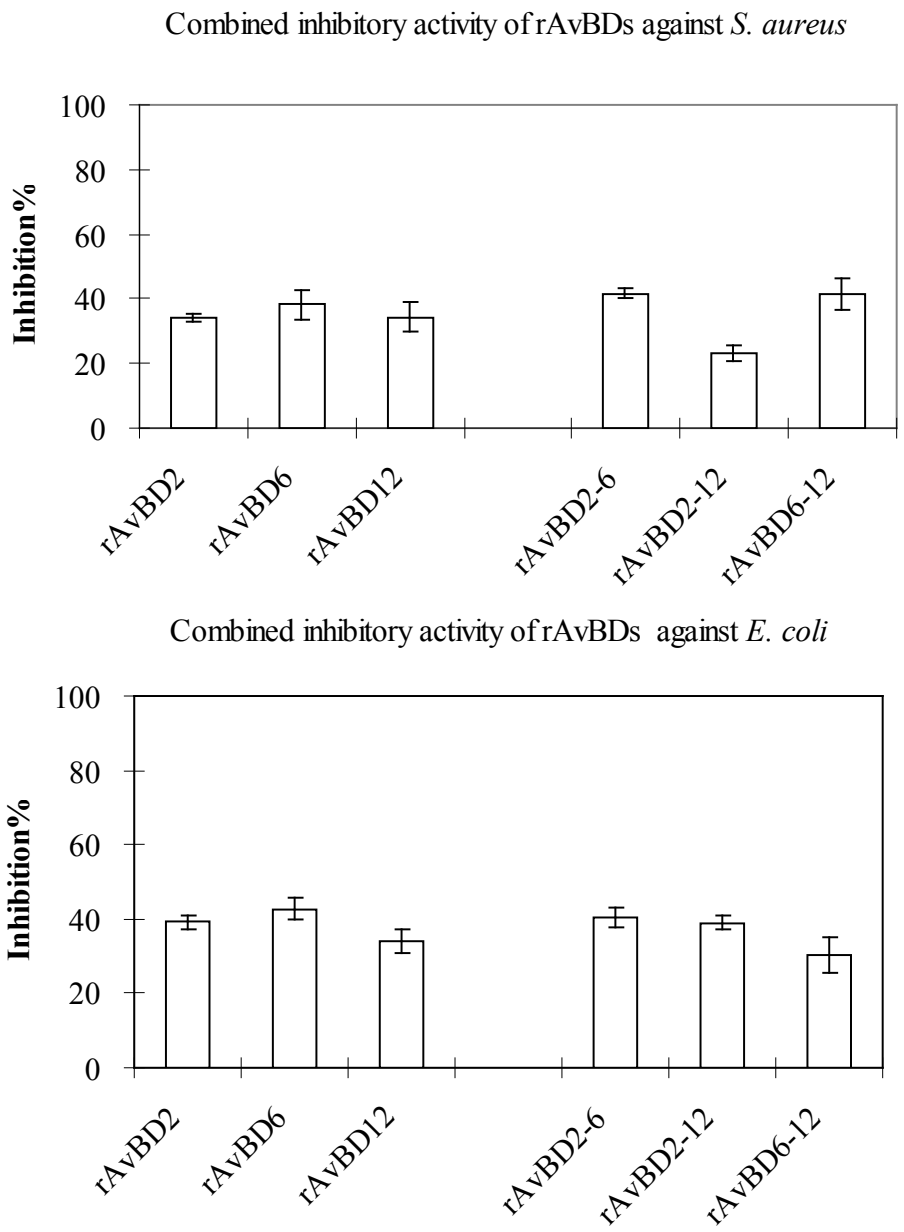


Figure 3.3 Combined inhibitory activities of rAvBDs against S. aureus, E. coli, and S. enterica serovar Typhimurium. Equal volumes (25 µl) of bacterial suspension in Mueller Hinton broth (105 CFU/ml) and rAvBD at a final concentration of 16 µg/ml were coincubated at 37°C for 3 hours followed by colony enumerations. Data shown (bars) are geometric means of three independent experiments ± standard deviation.

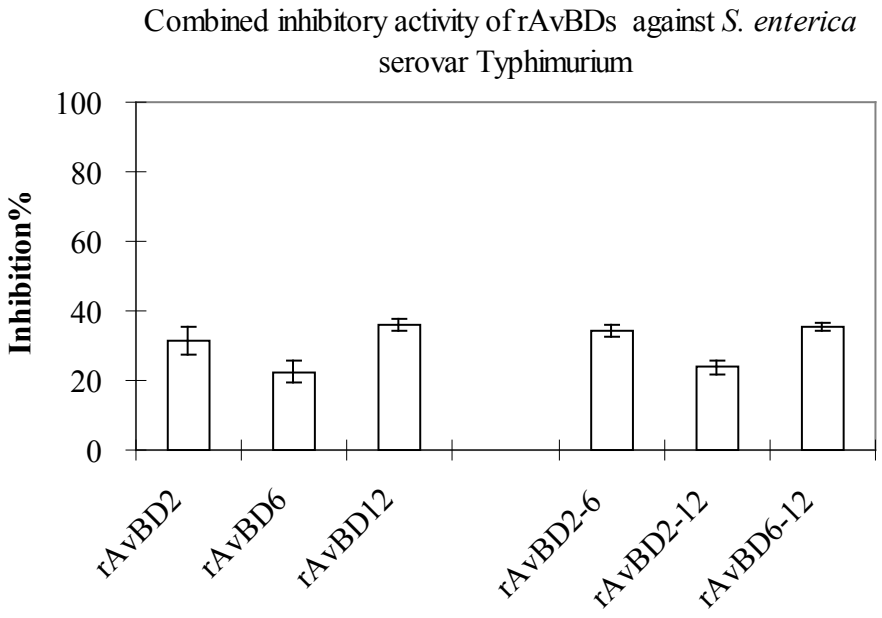


Figure 3.3 Continued

3.4 Killing activities of recombinant peptides

To determine whether rAvBDs were bactericidal, killing assays were performed by incubating the bacteria in diluted Mueller Hinton broth (at a final dilution of 200x) with or without the presence of rAvBDs. At this dilution, no significant bacterial growth or death was detected within the time period (3 hours) tested (data not shown). To determine the effect of pH on the antimicrobial activities of rAvBDS, killing assays were carried out at both pH 7.0 and pH 6.0.

At pH 7, all three recombinant peptides exhibited killing activities against the three bacterial species tested with *E. coli* being most susceptible to rAvBDs. The killing activities of rAvBD-2 and rAvBD-6 against *E. coli* and *S. aureus* were significantly higher than that of rAvBD-12 whereas rAvBD-12 was slightly more effective than rAvBD-2 and rAvBD-6 in killing *S. enterica* serovar *Typhimurium*. Therefore, the bactericidal activity of rAvBDs was bacterial species-dependent.

Stronger bactericidal activities against *S. aurues* and *E. coli* were observed for all three rAvBDs at pH 6.0 than at pH 7.0, indicating a pH-dependent killing mechanism for these peptide/bacteria combinations. However, reducing pH had no significant effect on rAvBDs-mediated killing of *S. enterica* serovar Typhimurium. In fact, rAvBDs demonstrated minimal bactericidal activity against *S. enterica* serovar Typhimurium under both neutral and acidic pH conditions (Figure 5 and 6).

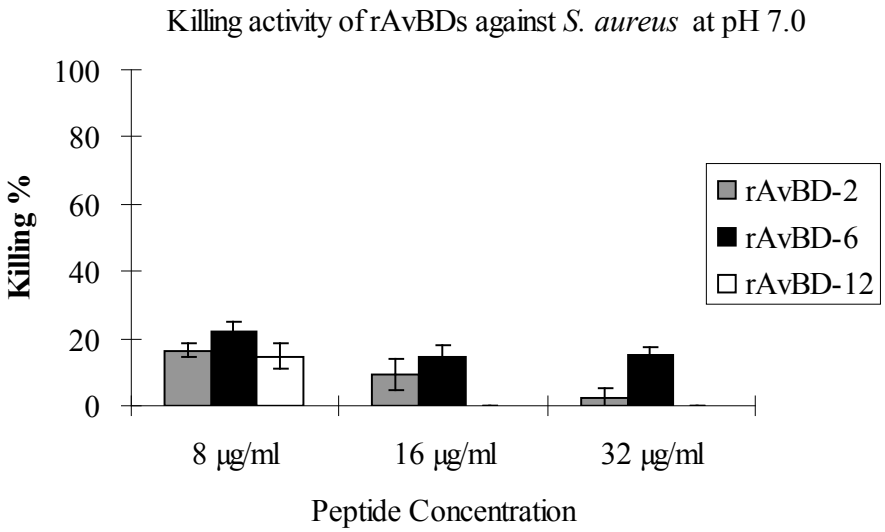


Figure 3.4 Killing activities of rAvBDs against S. aureus, E. coli, and S. enterica serovar Typhimurium at pH 7.0. Equal volumes (25 µl) of bacterial suspension (105 CFU/ml) in 100x diluted Mueller Hinton broth and rAvBD at a final concentration of 16 µg/ml were coincubated at 37°C for 3 hours followed by colony enumerations. Data shown (bars) are geometric means of three independent experiments ± standard deviation

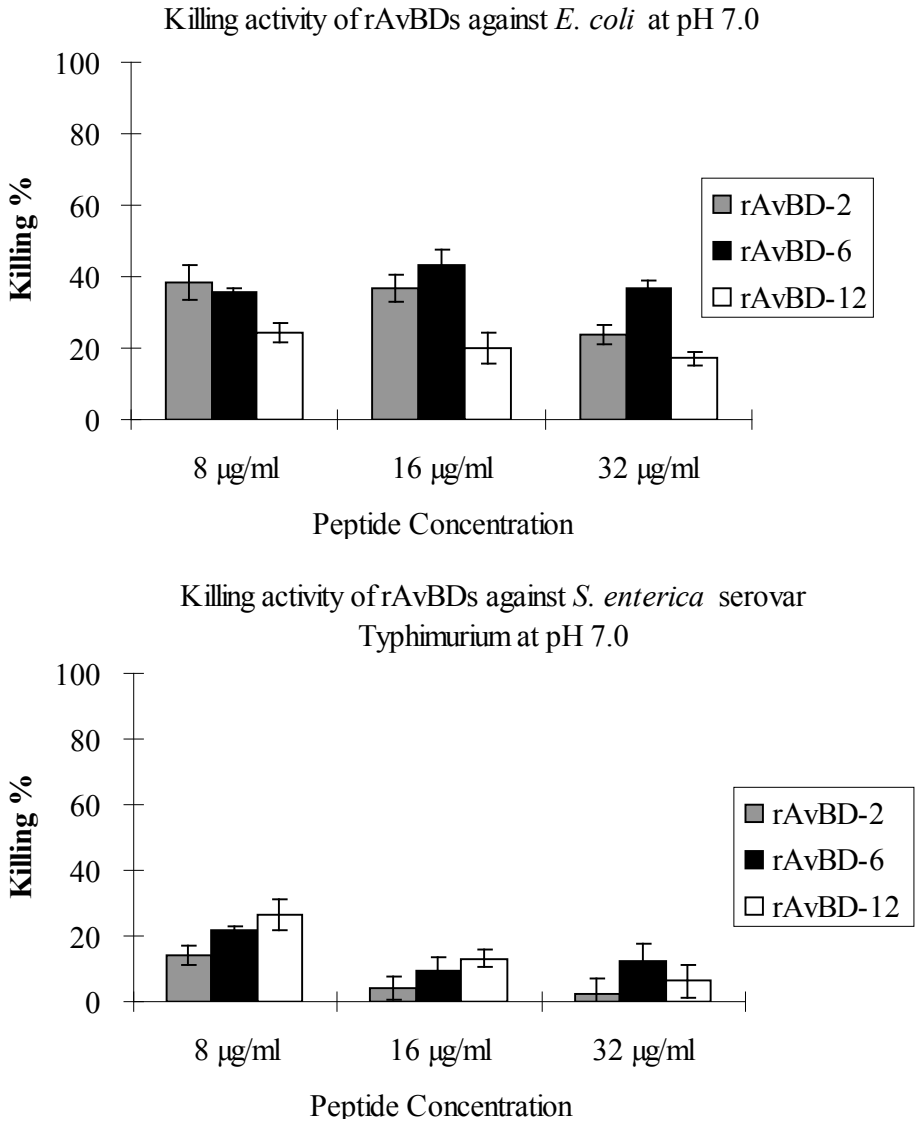


Figure 3.4 Continued

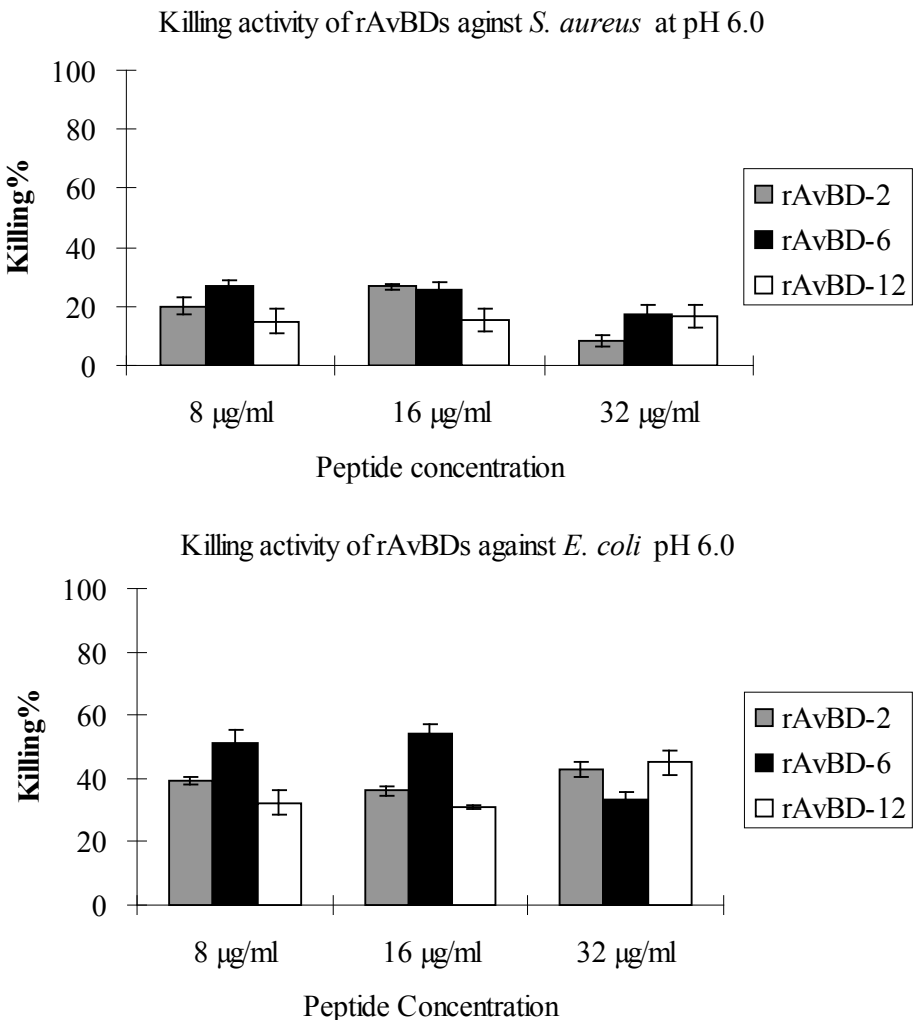


Figure 3.5 Killing activities of rAvBDs against *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium at pH 6.0. Equal volumes (25 µl) of bacterial suspension (10^5 CFU/ml) in 100x diluted Mueller Hinton broth and rAvBD at a final concentration of 16 µg/ml were coincubated at 37°C for 3 hours followed by colony enumerations. Data shown (bars) are geometric means of three independent experiments ± standard deviation.

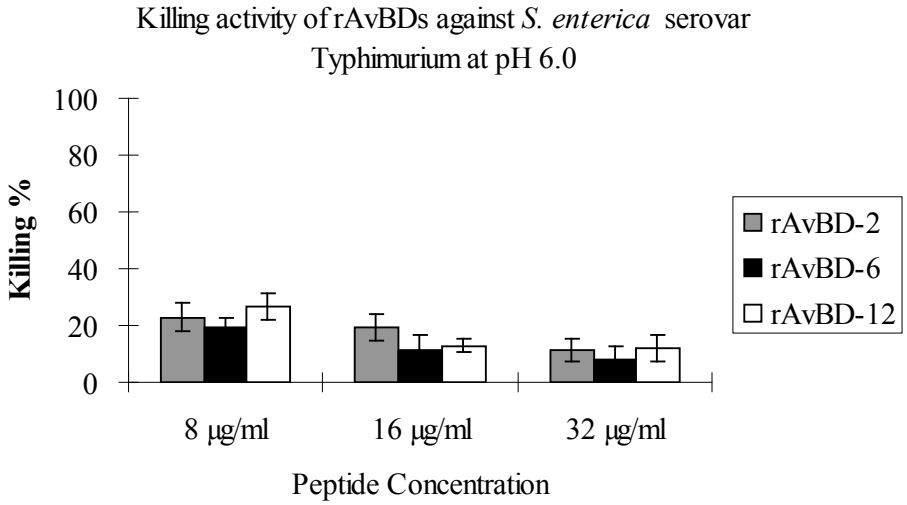


Figure 3.5 Continued

3.5 Killing kinetics of rAvBD

To determine whether the bactericidal activities were time-dependent, killing assays were performed by treating *S. auereus* and *E. coli* with rAvBDs at a final concentration of 16 µg/ml for various times at either pH 7.0 or pH 6.0. The results showed that rAvBDs exerted bactericidal effect within 30 minutes of treatment (Figure 7 and Figure 8). A higher percentage of *S. aureus* than *E. coli* was killed by rAvBDs within 120 minutes of treatment (Figure 7 and Figure 8). The killing activities of the three rAvBDs against *S. aureus* reached their peak between 60 and 120 minutes followed by a decline at 150 minutes (Figure 7). In contrast, sustained killings of *E. coli* by the three rAvBDs were observed within the time period tested (Figure 8). The bactericidal effects of the rAvBDs were significantly enhanced by decreasing pH. The pH effect was significantly greater on rAvBD-mediated killing of *S. aureus* than *E. coli* (Figure 7 and Figure 8).

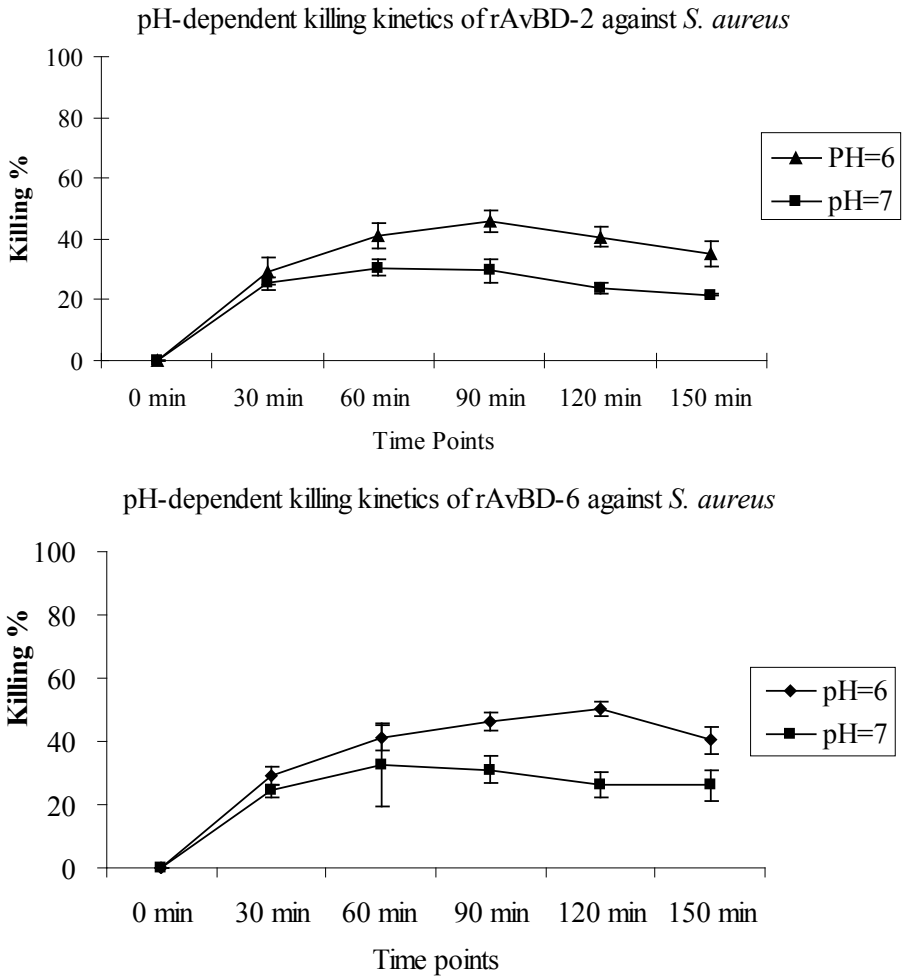
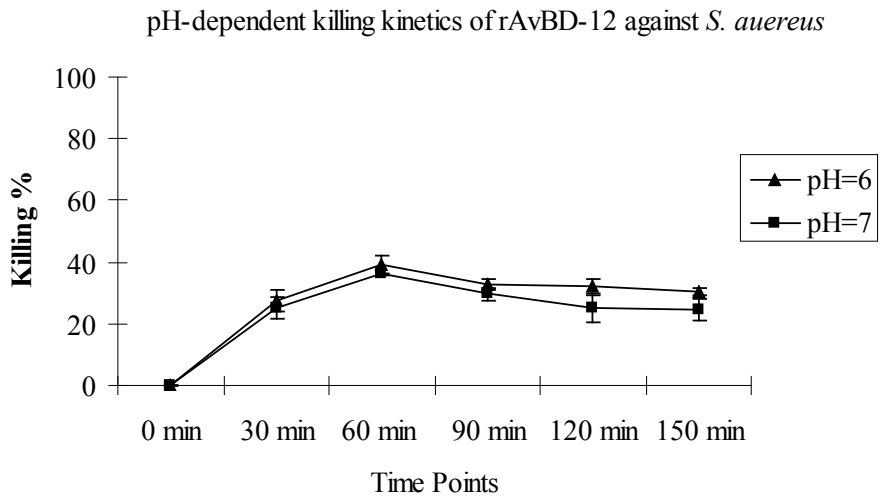


Figure 3.6 pH-dependent killing kinetics of rAvBDs against S. aureus. Equal volumes (25 µl) of bacterial suspension (105 CFU/ml) in 100x diluted Mueller Hinton broth and rAvBD at a final concentration of 16 µg/ml were coincubated at 37°C for various times followed by colony enumerations. Data shown (line graph) are geometric means of three independent experiments ± standard deviation.



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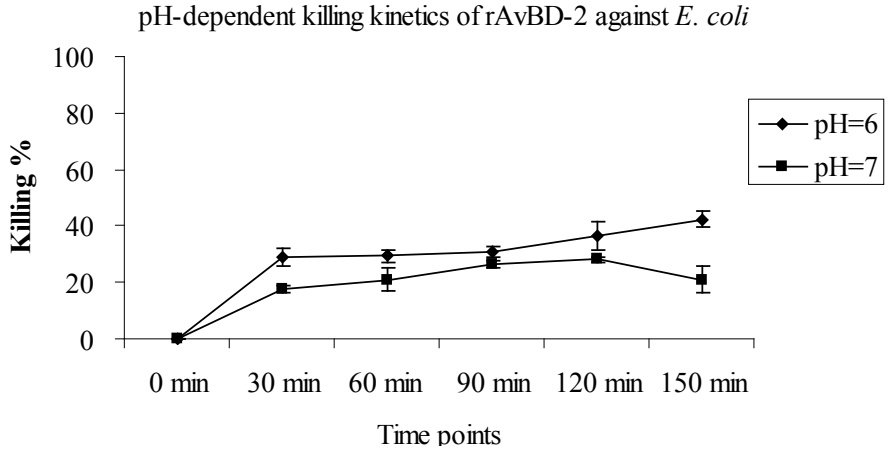

Figure 3.7 pH-dependent killing kinetics of rAvBDs against E. coli. Time-dependent killing of stationary phase E. coli by recombinant AvBDs at pH 6.0 and 7.0. Equal volumes (25 µl) of bacterial suspension (105 CFU/ml) in 100x diluted Mueller Hinton broth and rAvBD at a final concentration of 16 µg/ml were coincubated at 37°C for various times followed by colony enumerations. Data shown (line graph) are geometric means of three independent experiments ± standard deviation

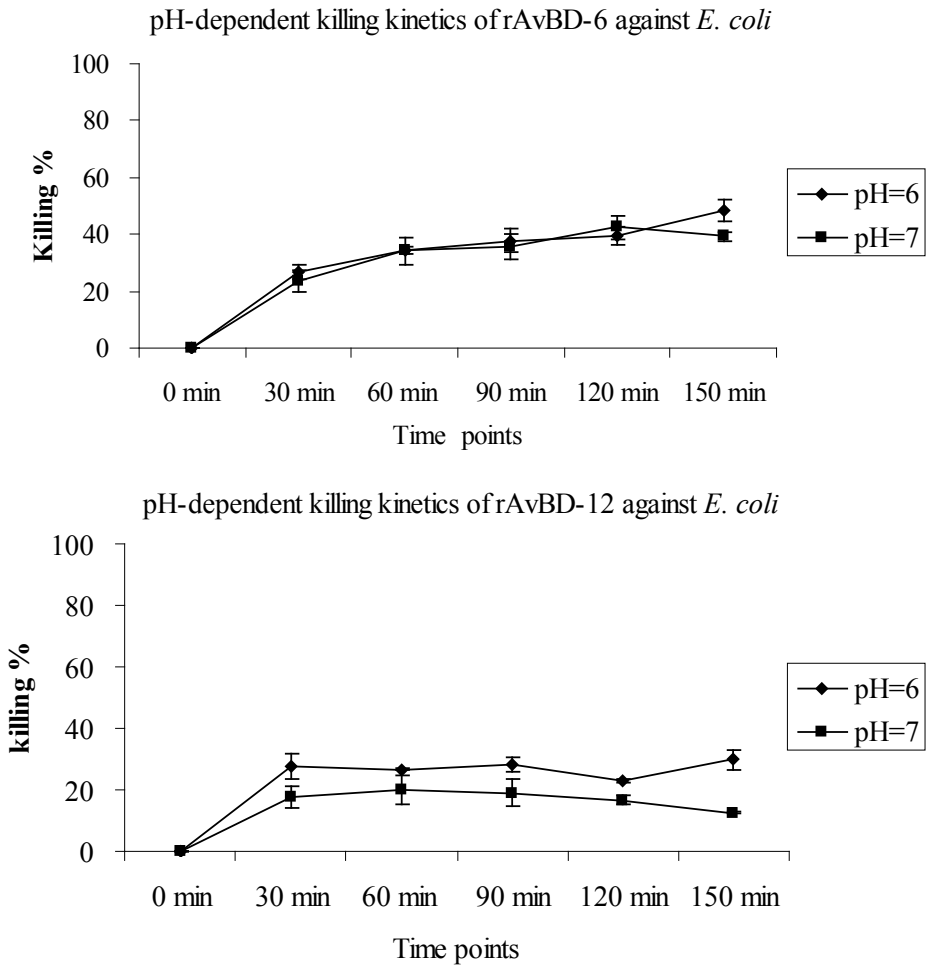

Figure 3.7 Continued

CHAPTER IV

DISCUSSION

4.1 The expression of recombinant AvBD-2, -6, and -12 in E. coli.

It has been shown that the chicken genome contains 14 genes encoding avian β-defensin [Albert et al., 2008]. Some of these defensins have been directly purified from chicken tissues and others expressed in vitro as recombinant proteins [Harwig et al., 1994, Milona et al., 2007, and Albert van Dijk et al., 2007]. To understand the local innate immunity in chicken reproductive system, we have characterized the expression profiles of all 14 β-defensin genes in chicken oviduct epithelia cells and found that AvBD-2 and AvBD-6 are induced by *Salmonella* infection and AvBD-12 is constitutively expressed [Ebers et al., 2009]. To characterize the biological roles played by these defensins, we expressed the mature peptides of AvBD-2, -6, and -12 in BL21(DE3)plysS as recombinant peptides with an N-terminal 6xhis tag. The total yields of rAvBD-2, -6, and -12 were typically 0.92 mg, 1.24 mg, and 1.52 mg, respectively, from one liter culture of BL21(DE3)plysS cells.

4.2 Inhibition of bacterial growth by recombinant AvBD-2, -6, and -12.

All three rAvBDs inhibited the growth of *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium which appeared to be peptide- and bacterial species-dependent. In general, rAvBD-2 and rAvBD-6 were more potent than rAvBD-12 in inhibiting the growth of *S. aureus* and *E. coli.* The inhibitory potency could be characterized as rAvBD-6 > rAvBD-2 > rAvBD-12 which coincided with their net charges in the order of 6.40,

3.70, and 0.40 for 6xhis tagged rAvBD-2, rAvBD-6, and rAvBD-12 or 7.70, 5.50, and 1.80 for mature AvBD-2, AvBD-6, and AvBD-12, respectively. Both rAvBD-2 and rAvBD-6 were more effective against *S. aureus* and *E. coli* than *S. enterica* serovar Typhimuirum. This observation is similar to what has been described for rAvBD-9 that inhibited the growth of *E. coli*, but not *S. enterica* serovar Typhimurium [Albert van Dijk, 2007]. In contrast, minimal difference in the inhibitory activity of rAvBD-12 against different bacterial species was observed and a slightly higher percentage of *S. enterica* serovar Typhimurium was inhibited by rAvBD-12 than that by rAvBD-6 which was in line with previous findings that rAvBD-5 with a lower positive charge (+4) than rAvBD-6 (+7) exhibited stronger antimicrobial effects against *Salmonella* species [Milona et al., 2007].

It is known that *S. enterica* has evolved multiple mechanisms to resist antimicrobial molecules, such as β-defensins [Gunn *et al*., 2000; Guo *et al*., 1998, McPhee et al., 2003]. One mechanism involves the addition of 4-aminoarabinose (Ara4N) to the phosphate group of the lipid A backbone, for which two genetic loci, *pmr*E and *pmr*HFIJKLM, are required [Gunn *et al*., 2000]. Another mechanism utelizes the *PhoP-PhoQ* regulated *pag*P gene that functions to increase acylation of lipid A [Gunn *et al*., 2000; McPhee et al., 2003]. Interruption of the *pag*P gene increases the outer membrane permeability to β-defensins [Guo *et al*., 1998]. Modifications of the lipid A reduce the net negative charge of LPS on the surface of *S. enterica* serovar Typhimurium which interferes with the initial electrostatic attraction between the bacteria and cationic peptides, thereby conferring resistance to β-defensins. Understandably, reduction of charge via modification of lipiA would play a more significant role in avoiding highly charged cationic peptides than minimally charged peptides, such as AvBD-12 (+0.4). Our

investigation also found a significant antagonistic effect of rAvbD-2 and AvBD-12 on their inhibitory activities against *S. aureus* and *S. enterica* serovar Typhimurium, but not *E. coli* which again highlights the diverse mechanisms underlying the antimicrobial function of AvBDs. It has been suggested the amphipathicity and size of β-defensins allow them insert into the membrane of microbes following initial contact [Tomas Ganz., 2003]. Since the charges of rAvBD-2 and rAvBD-6 are similar and rAvBD-6 does not work against rAvBD-12, the antagonism between rAvBD-2 and rAvBD-12 can not be attributed to an interference of charged-based peptide-bacterium attraction. This opens the possibility of competition by the two rAvBDs for other pathways involved in bacterial membrane damage or growth inhibition.

4.3 Bacterial killing by rAvBD-2, rAvBD-6, and rAvBD-12.

Data from the present study shows that all three rAvBDs are not only bacterial static, but also bactericidal. These peptides are more effective in killing *E. coli* than *S. aureus* and *S. enterica* serovar Typhimurium at pH 7.0. The bactericidal potency of the peptides against *E. coli* and *S. aureus* can be defined as rAvBD-6 > rAvBD-2 > rAvBD-12 which again correlates with the net charge of these AvBDs. Consistent with data from bacterial inhibition assays, rAvBD-12 was slightly more effective in killing *S. enterica* serovar Typhimurium. The bactericidal activities against *S. aureus* and *E. coli* were pH dependent as evidenced by the increased killing at pH 6.0, compared to pH 7.0. The increased killing activities correlated with the elevated charges of rAvBDs at pH 6.0 which favors the attachment of AvBDs to anionic components of bacteria, such as lipoteichoic acid (LTA) of *S. aureus* and lipopolysaccharides (LPS) of *E. coli*. However,

the bactericidal activity of rAvBD-12 against *S. enterica* serovar Typhimurium was not subject to pH change.

4.4 Killing kinetics of rAvBD-2, rAvBD-6, and rAvBD-12.

The killing-curves showed that rAvBDs exerted their antimicrobial function within 30 minutes of treatment. It is unknown why the killing activity of rAvBDs against *S. aureus* declined after certain period of treatment. These assays were conducted by incubating bacteria in a minimal nutrient medium (Mueller Hinton broth at a final dilution of 200x) which maintained the viability of bacteria without promoting bacterial growth within the time of assay. However, it was still possible that the replication of a resistant population of *S. aureus* occurred. Similar kinetics has been reported for synthetic human β-defensins (hBDs). A 1.5 and 1 log unit decrease in survival of *S. aureus* cells within 60 min was observed after treatment with synthetic hBD-2 and hBD-3, respectively, indicate an effective mechanism of human β-defensins [Sahl et al., 2005]. In addition, examination of synthetic AvBD-9-treated *C. perfringens* by transmission electron microscopy showed dose-dependent morphological effects, as seen for other defensins. A 30-min treatment of 10^8 CFU/ml *C. perfringens* cells with synthetic AvBD-9 concentrations ranging from 1.56 to 25µg/ml induced dose-dependent changes. At lower concentrations, clumping of intracellular material and irregular septum formation during cell division were observed. Most cells showed signs of cytoplasm retraction and detachment of the cytoplasmic membrane from the peptidoglycan layer at higher concentrations, sometimes resulting in mesosome-like structures. Prior to complete lysis, often originating at the cell poles or at the septa of dividing cells, many cells developed a ghostlike appearance [Albert van Dijk, 2007]. Similar observations were also described

by Lee et al. (2004), who observed chromatin condensation in *Haemophilus influenza* after a 30-min treatment with 10 μg/ml hBD-2 [Lee et al., 2004].

When the influence of pH on killing-curve studies was tested, the results showed that killing curves were different at two pH levels, and rAvBDs exhibited stronger killing effects at pH 6.0 at all time points investigated (30, 60, 90, 120, and 150 min). This indicated that rAvBDs' killing against stationary phase *E. coli* and *S. aureus* was pH dependent in the range investigated. Similar observations were obtained in killings assays, which suggested that at pH 6.0, stronger killing potencies of rAvBDs were observed after three hour incubation of rAvBDs and bacteria cells, *E. coli* and *S. aureus*.

4.5 Potential immune functions of AvBD-2, -6, and -12 in chicken cells and tissues.

The oviduct, consisting of the infundibulum, magnum, isthmus, uterus, and vagina, can be infected by various microorganisms, including *Salmonella enterica* serovars [Barnhart et al., 1993] and *Mycoplasma meleagridis* [Yamamoto and Herrad, 1966]. Local immunity plays essential roles in preventing infections. Although the adaptive immune responses have been well characterized [Yoshimura et al., 1997, Zheng et al., 1997, Zheng et al., 1998, Zheng et al., 1999, and Zheng et al., 2001], less is known about the components of local innate immunity. Ohashi et al. (2005) reported that all segments of chicken oviduct express AvBD-1, -2, and -3 with a higher expression in the infundibulum and vagina [Ohashi et al., 2005]. Yoshimura et al. (2008) showed that 11 out of of 14 AvBD genes, except AvBD-6, AvBD-13, and AvBD-14, are expressed in all segments of chicken oviduct [Yoshimura et al., 2008]. The expression of AvBD-1, -2, and -3 are enhanced by exposure to *S. enterica* serovar Enteritidis and the expression of AvBD-5, -10, -11, and -12 was induced by LPS stimulation [Yoshimura et al., 2006,

Yoshimura et al., 2008]. Characterization of AvBD expression profiles by Ebers et al. has shown that both AvBD-2 and AvBD-6 are induced by Salmonella and AvBD-12 is consitutively expressed [Ebers et al., 2009]. Data from the present study demonstrate that the two Salmonella-inducible cationic peptides, AvBD-2 and AvBD-6 had minimal antimicrobial activity against *S. enterica* serovar Typhimurium, suggesting that the induced expression of AvBD-2 and -6 genes is more likely a general response to conserved bacterial components. Instead, AvBD-12, a less potent AvBD against other bacterial species, has a slight advantage over AvBD-2 and AvBD-6 in inhibiting or killing *S. enterica* serovar Typhimurium. Constitutive expression of AvBD-12 may be an important innate defense mechanism against *Salmonellae* colonization in chicken oviduct.

Moderate expression of AvBD-2 in both small and/or large intestine, weak expression of AvbD-12 in large intestine, moderate to strong expression of AvBD-2 and AvBD-6 in leukocytes, moderate expression of AvBD-2 and AvBD-6 in lung, and strong expression of AvBD-12 in oviduct have been observed in chicken hosts [Harwig et al., 1994; Lynn et al., 2004; Ohashi et al., 2005; Zhao et al., 2001; Sadeyen et al., 2004, and Xiao et al., 2004]. These findings along with the antimicrobial data generated by the present work suggest that AvBD-2 and AvBD-6 are essential to clearing systemic infections and AvBD-12 is important to control *Salmonella* colonization in reproductive tract.

In summary, we have expressed and purified rAvBD-2, rAvBD-6, rAvBD-12 and determined their antimicrobial activities under different pH conditions. Comparative characterization of these AvBDs has provided new insight into the mechanisms governing AvBD-mediated innate immune defense in chickens. These data also suggest

that AvBD-2, -6, and -12 could be utilized as antimicrobial agents under both *in vivo* and *in vitro* conditions.

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APPENDIX A

BUFFER AND MEDIA

1. Media for chemical-complement cells

1.1 Luria Bertani (LB) medium

Bacto™ Tryptone	10 g/L
BBL™ Yeast Extract	5 g/L
Sodium Chloride (NaCl)	10 g/L

1.2 SOB medium

Bacto™ Tryptone	20 g/L
BBL™ Yeast Extract	5 g/L
Sodium Chloride (NaCl)	0.5 g/L
Potassium Chloride (KCl)	0.186 g/L

2. Buffers for SDS-PAGE

2.1 2mol/L Tris-HCl (pH 8.8)

Tris	242 g/L
HCl adjust pH to 8.8.	

2.2 1mol/L Tris-HCl (pH 6.8)

Tris	121 g/L
HCl adjust pH to 6.8.	

2.3 10% Lauryl Sodium Sulfate (SDS)

SDS	100 g/L

2.4 30% (w/v) Acrylamide / 0.8% (w/v) Bis-Acrylamide

Acrylamide	292 g/L
Bis-acrylamide	8 g/L

2.5	10% Ammonium persulfate	
	Ammonium persulfate	100 g/L
2.6	2.5*Separating gel Buffer	
	2mol/L Tris-HCl (pH 8.8)	470 ml/L
	10% SDS Solution	25 ml/L
	Distilled Water	505 ml/L
2.7	5*Stacking gel Buffer	
	1mol/L Tris-HCl (pH 6.8)	625 ml/L
	10% SDS Solution	50 ml/L
	Distilled Water	325 ml/L
2.8	Electrophoresis Buffer	
	Tris	3 g/L
	Glycine	14.4 g/L
	SDS	1 g/L
2.9	12% Acrylamide gel	
	30% Acrylamide / 0.8% bis-acrylamide stock solution	3300 µl
	2.5*Separating gel Buffer	3300 µl
	Distilled Water	1650 µl
	TEMED (N,N,N',N'-tetramethylethylenediamine)	7.5 µl
	10% Ammonium persulfate	75 µl

2.10 5% Stacking gel

30% Acrylamide/0.8%bis-acrylamide stock solution	420 µl
5*Stacking gel Buffer	495 µl
Distilled Water	1500 µl
TEMED (N,N,N', '-tetramethylethylenediamine)	3 µl
10% Ammonium persulfate	22.5 µl

3. Buffers for Western Blot

3.1 Transfer Buffer

Tris	3.03 g/L
Glycine	14.4 g/L
Methanol	200 ml/L

3.2 Phosphate-Buffered Saline (PBS)

NaCl	8 g/L
KCl	0.2 g/L
Na_2HPO_4	1.42 g/L
KH_2PO_4	0.25 g/L

3.3 PBST Buffer

NaCl	8 g/L
KCl	0.2 g/L
Na_2HPO_4	1.42 g/L
KH_2PO_4	0.25 g/L
Tween-20	500 µl

3.4 Blocking Buffer:

NaCl	8 g/L
KCl	0.2 g/L
Na_2HPO_4	1.42 g/L
KH_2PO_4	0.25 g/L
Tween-20	500 µl
Non-Fat Milk	50 g/L

4. Buffers for protein purification by using Ni-NTA Anti-His protein superflow columns

4.1 Buffer B

NaH_2PO_4	13.8 g/L
Tris	1.3 g/L
Urea	420 g/L
Adjust pH to 8.0 using NaOH	

4.2 Buffer C

NaH_2PO_4	13.8 g/L
Tris	1.3 g/L
Urea	420 g/L
Adjust pH to 6.3 using HCl	

4.3 Buffer E:

NaH_2PO_4	13.8 g/L
Tris	1.3 g/L

Urea	480 g/L
Adjust pH to 4.5 using HCl	

5. Dialysis buffer (pH 8.0)

5.1 6M Urea dialysis buffer

Urea	360 g/L
NaCl	30 g/L
Tris	2.5 g/L

5.2 4M Urea dialysis buffer

Urea	240 g/L
NaCl	30 g/L
Tris	2.5 g/L

5.3 2M Urea dialysis buffer

Urea	120 g/L
NaCl	30 g/L
Tris	2.5 g/L

5.4 0M Urea dialysis buffer

NaCl	30 g/L
Tris	2.5 g/L

APPENDIX B

PROCEDURES

1. Extraction of RNA from cells using TRIzol:

1.1 Discard supernatant, add 1000 µl Trizol, and incubate at room temperature for 5 minutes.

1.2 Mix cell lysate with 200 µl chloroform in a 1.5 ml eppendorf centrifuge tube and incubate at room temperature for 2 minutes.

1.3 Centrifuge at 12, 000 rpm for 15 minutes at 4°C.

1.4 Transfer upper layer into a new 1.5 ml centrifuge tube.

1.5 Add equal volume isopropanol and incubate on ice for 10 minutes.

1.6 Centrifuge at 12, 000 rpm for 15 minutes at 4°C.

1.7 Discard supernatant, add 500 µl 75% ETOH along the tube wall. Do not mix or vortex.

1.8 Centrifuge at 7, 500 rpm for 5 minutes at 4°C.

1.9 Diacard supernatant and place tubes in a fume hood, air dry for 5-10 minutes.

1.10 Resuspend RNA in 50 µl DNase and RNase free water.

2. Re-extraction RNA using phenol-chloroform.

2.1 The volume of the RNA should be over 100 µl, if not, add RNase-DNase free water to at least 100 µl.

2.2 Add phenol to the RNA solution as 1:1 (v/v), and mix well.

2.3 Centrifuge at 13,000 rpm for 5 minutes and transfer the upper layer to a new tube.

2.4 Add chloroform to the RNA solution as 1:1 (v/v), and mix well.

2.5 Centrifuge at 13,000 rpm for 5 minutes and transfer the upper layer to a new tube.

2.6 Add equal volume of Isopropanol into RNA solution and 1/10 volume of sodium acetate (2 M pH 4.0), mix well.

2.7 Centrifuge at 13,000 rpm for 15 minutes at 4°C. Discard supernatant.

2.8 Add 500 µl 70% ETOH without touching the RNA pellet. Centrifuge at 13,000 rpm for 10 minutes 4°C.

2.9 Air dry. Apply appropriate amount of RNase-DNase free water to elute the RNA.

3 Reverse transcription reaction:

3.1 Mixture 1

RNA	15 µl
10*RT buffer	5 µl
$MgCl_2$	5 µl
DNase I (2 Units)	1 µl
RNase Inhibitor	1 µl
Total volume	27 µl

Mixture 1 was incubated in room temperature for 15 minutes, and incubated at 75°C for 15 minutes to inactivate DNase I.

3.2 Mixture 2

DNase RNase free water	13.5 µl

RNase Inhibitor	1 µl
dNTPs	4 µl
Hexamer	3 µl
Reverse Transcriptase (150 Units)	1.5 µl
Total volume	23 µl

Combine Mixture 1 and 2 when mixture 1 reaches room temperature. The final volume is 50 µl.

3.3 Program:

25°C	10 minutes
48°C	45 minutes
95°C	10 minutes

4. PCR reaction systems:

4.1 Reagents

cDNA template	5 µl
10x Buffer	5 µl
$MgCl_2$	5 µl
dNTPs	3 µl
Taq Polymerase	1 µl
DNase free water	27 µl
Forward primer (100pmol/µl)	2 µl
Reverse primer (100pmol/µl)	2 µl

Final volume	50 µl

4.2 Amplification condition:

Stage	Temperature	Time (minute)	Repeat
1	95°C	3:00	1
2	95°	0:30	35
	(AvBD-2) 55°C	0:20	
	(AvBD-6) 52°C	0:20	
	(AvBD-12) 51°C	0:20	
	(AvBD-12) 56°C	0:20	
	72°C	00:30	
3	72°C	5:00	

5. PCR-screening of cloned insert

5.1 Reagents

DNA template	1 µl
10x Buffer	2 µl
$MgCl_2$	2 µl
dNTPs	0.75 µl
Taq Polymerase	0.25 µl
DNase free water	13 µl
Forward primer (100pmol/µl)	0.5 µl
Reverse primer (100pmol/µl)	0.5 µl

Final volume	20 µl

5.2 Amplification condition:

Stage	Temperature	Time (minute)	Repeat
1	95°C	5:00	1
2	95°	0:10	40
	50°C	0:20	
	72°C	0:30	
3	72°C	5:00	

6. Purification of DNA from agarose gel by using the Wizard® PCR prep DNA purification system from Promega:

6.1 Separate PCR product by gel electrophoresis.

6.2 Excise DNA band with expected size.

6.3 Place the agarose slice in a 1.5 ml eppendorf centrifuge tube containing 1 ml resin.

6.4 Incubate at 56°C until the agarose is dissolved.

6.5 Prepare a Wizard® Minicolumn by attaching the syringe barrel to a minicolumn and inserting the minicolumn/syringe barrel into a vaccum manifold.

6.6 Add resin/DNA mixture to the syringe Barrel.

6.7 Apply vaccum to pull liquid through the Minicolumn. Release vaccum when all the liquid has passed through the Minicolumn.

6.8 Add 2 ml of 80% isopropanol to the syringe Barrel. Apply vaccum to pull solution through the Minicolumn.

6.9 Dry the resin by continuing to apply the vaccum for 25 seconds.

6.10 Remove the syringe Barrel and transfer the minicolumn to a 1.5 ml microcentrifuge tube.

6.11 Centrifuge at 10,000g for 2 minutes and transfer the Minocolumn to a clean 1.5 ml microcentrifuge tube.

6.12 Add 20 µl preheated Nuclease-Free H20 to minicolumn and wait 2 minutes.

6.13 Elute the DNA by centrifuging at 10,000g for 20 seconds at room tempature.

6.14 Remove and discard minicolumn, store DNA at -20°C or use immediately.

7.0 TOPO TA cloning (Invitrogen) and chemical transformation:

7.1 Gently mix 4 µl fresh PCR product, 1 µl Salt solution from the kit, and 1 µl pCR2 TOPO vector.

7.2 Incubate at room temperature for 30 min.

7.3 Add the mixture to a vial of One Shot® E. coli Top 10.

7.4 Incubate on ice for 30 min.

7.5 Heat-shock the cells by incubating the mixture at 42°C for 30 sec.

7.6 Transfer the tube immediately onto ice and add 250 µl of room temperature S.O.C. medium followed by shaking at 37°C for one hour.

7.7 Spread 100µl culture on a pre-warmed LB plate containing ampicillin 100 µl/ml and X-gal 30 µg/ml.

7.8 Incubate the culture plate at 37°C for 16 hours.

7.9 Pick white colonies and subculture onto new plates.

7.10 PCR screening for insert.

8 Plasmid extraction by using Wizard® Plus Minipreps DNA Purification system from Promega:

8.1 Resuspend a full loop (10 µl inoculation loop) of bacterial colonies in 400 µl Cell Resuspension Solution in a 1.5 ml centrifuge tube.

8.2 Add 400 µl Cell Lysis Solution, mix by inverting the tube 4 times, and incubate at room temperature for 5 minutes.

8.3 Add 400 µl of Neutralization Solution, mix by inverting the tube 4 times, and incubate on ice for 5 minutes.

8.4 Centrifuge the bacterial lysate at 10,000 rpm for 5 minutes.

8.5 Attach a syringe barrel to a minicolumn, insert the tip of the minicolumn/syringe barrel assembly into the vacuum manifold.

8.6 Thoroughly mix the DNA purification resin and transfer 1ml into each barrel.

8.7 Transfer bacterial lystate to the barrel containing resin.

8.8 Open the stopcocks and apply a vacuum to pull the resin and lysate mix into minicolumn. When all liquid has completely passed through column, break the vacuum.

8.9 Add 2 ml of the column wash solution to the barrel and reapply the vacuum to draw the solution through the minicolumn.

8.10 Dry the resin by continuing to draw a vacuum for 25 seconds.

8.11 Remove the barrel and transfer the minicolumn to a 1.5 ml centrifuge tube.

8.12 Centrifuge at 10,000 rpm for 2 min to remove any residual column wash solution.

8.13 Transfer minicolumn to a new centrifuge tube.

8.14 Add 50 µl preheated Nuclease-Free H20 to minicolum and wait for 2 min.

8.15 Centrifuge at 10,000 rpm for 20 seconds to elute DNA.

8.16 The DNA can be used immediately or saved in -20°C for lone term storage.

9. Restriction enzyme, BamH I and Kpn I, digestion:

9.1 Reagents:

Plasmid DNA	20 µl
10* Multicore Buffer	6 µl
BSA	0.5 µl
BamH I	3 µl
Kpn I	3 µl
DNase-RNase-free water	27.5 µl
Final volume	60 µl

9.2 Incubated in a water bath 37°C for 1.5 hours.

10. Ligation using T4 ligase from Invitrogen:

10.1 Reaction system (all the buffers and enzymes are from the T4 Ligation kit)

pRSET A	10 µl

AvBDs' DNA insert	10 µl
5X Buffer	2.5 µl
T4 ligase	0.1 µl
DNase-RNase-free water	2.4 µl
Final volume	25 µl

10.2 Incubated at room temperature for 2 hours.

11. Preparation of competent cells BL21(DE3)PlysS and Top10F′:

11.1 Culture Top10F' and BL21(DE3)PlysS on LB agar containing the desired antibiotics. (Top10F′: 10 µg/ml Tetracycline, BL21(DE3)PlysS: 30 µg/ml chloramphenicol.)

11.2 Inoculate individual colonies into SOB medium (100 ml).

11.3 Incubate at 37°C with vigorous shaking (230 cycles/minute).

11.4 When OD600 reaches approximately 0.5, transfer culture into 50ml pre-chilled tubes.

11.5 Centrifuge at 4,500 rpm for 10 min at 4°C.

11.6 Discard medium and put tube up side down for 1 min.

11.7 Resuspend each bacterial pellet in 10 ml of ice-cold 50 mM CaCl2.

11.8 Keep bacterial suspension on ice for at least 30 min.

11.9 Centrifuge the CaCl2-treated cells at 4500 rpm for 10 min at 4°C.

11.10 Discard medium and gently resuspend each pellet in 2 ml of ice-cold 50 mM CaCl2.

11.11 Keep bacteria on ice.

11.12 Aliquot 100 µl of CaCl2-treated cells into pre-chilled 1.5 ml centrifuge tubes.

11.13 Store at -80°C.

12. Genomic DNA electrophoresis:

12.1 Mix genomic DNA with DNA loading buffer (Sigma).

12.2 Loaded into 1% agarose gel.

12.3 Electrophoresis at 125 V until the front dye goes to the middle of the gel.

13. RNA electrophoresis:

13.1 Mix RNA with RNA loading buffer (Sigma).

13.2 Loaded into 1.5% agarose gel.

13.3 Electrophoresis at 125 V until the front of dye goes to the middle of the gel.

14. Phenol-chloroform purification of DNA:

14.1 The volume of the DNA should be over 100 µl, if not, add RNase-DNase free water to at least 100 µl.

14.2 Add phenol to the DNA solution as 1:1 (v/v), and mix well.

14.3 Centrifuge at 13,000 rpm for 5 minutes, and transfer the upper layer to a new tube.

14.4 Add chloroform to the DNA solution as 1:1 (v/v), and mix well.

14.5 Centrifuge at 13,000 rpm for 5 minutes, and transfer the upper layer to a new tube.

14.6 Add isopropanol into DNA solution as 1:1 (v/v), and also add sodium Acetate (2 M pH 4.0) as 1:10 (v/v).

14.7 Incubate at -20°C for 10 minutes, and centrifuge at 13,000 rpm for 15 minutes. Discard the liquid.

14.8 Add 70% ETOH without touching the DNA pellet by tip. Centrifuge at 13,000 rpm for 10 minutes.

14.9 Air dry. Apply appropriate amount of RNase-DNase free water to elute the DNA.

14.10 Use DNA immediately or store at -20°C.

15. Transformation of Top10F′ with pRSET A

15.1 Mix e 3 µl plasmid DNA pRSET A (0.5 µg/µl) with of 100 µl Top10F' competent cell and add.

15.2 Incubate cell on ice for 30 min.

15.3 Heat shock cells at 42°C for 45 sec and return cell tube to ice for 2 min.

15.4 Add 250 µl SOC medium and incubate the cultures for 45 min at 37°C with vigorous shaking (235 cycles/min).

15.5 Plate 50 µl cells to LB plate containing 100 µg/ml Ampicillin for selection of plasmid pRSET A.

15.6 Incubate plate at 37°C overnight.

16. Transformation of BL21(DE3)PlysS with plasmids pRSET A-AvBD-2, -6, and -12.

16.1 Mix 3 µl plasmid DNA with 100 µl BL21(DE3)PlysS competent cell.

16.2 Incubate on ice for 30 min.

16.3 Heat shock cells at 42°C for 45 sec and place the cells on ice for 2 min.

16.4 Add 250 µl SOC medium and incubate for 45 min at 37°C with vigorous shaking (235 cycles/min).

16.5 Plate 50 µl cells onto a LB plate containing 100 µg/ml Ampicillin and 30 µl/ml Chloramphenicol for selection of plasmid pRSET A-AvBD-2, -6 and -12.

16.6 Incubate plate at 37°C overnight.

17. SDS-PAGE Gel and Western blot:

17.1 Mix Protein 1:1 with SDS-Sample Buffer and heat at approximately 100°C for 5 min.

17.2 Run on an SDS-PAGE minigel until the blue dye is at the bottom of the gel.

17.3 Cut the nitrocellulose membrane and the filter paper to the size of the gel.

17.4 Equilibrate the gel and wet the membrane, filter paper, and fiber pads in transfer buffer for at least 15 min.

17.5 Assemble the "gel sandwich" as follows: the cassette with gray side down, fiber pad, filter paper, gel, membrane, filter paper, fiber pad.

17.6 Put the "gel sandwich" in tank and make sure the protein would be transferred electrically from the negative pole to the positive pole.

17.7 Add cooling unit and transfer buffer (completely fill tank).

17.8 Transfer at 125 V, 0.35 A for 1.5 hour.

17.9 Rinse membrane three times in PBS.

17.10 Block the membrane for one hour in blocking buffer at room temperature on a shaker.

17.11 Rinse membrane three times in PBS and transfer membrane to a tray containing Anti-Xpress Antibody diluted 1:5000 to 10 ml blocking buffer.

17.12 Incubate with gentle agitation for one hour at room temperature.

17.13 Transfer membrane to a tray containing PBST and wash three times.

17.14 Transfer membrane to a tray containing Anti-mouse IgG-HRP conjugate with 1:500 dilutions and incubated one hour with gentle agitation.

17.15 Transfer membrane to a tray containing PBST and wash three times.

17.16 Mix the first solution (15 mg 4-chloro-1-naphthol in 5 ml cold methanol) with the second solution (30 µl 30% hydrogen peroxide in 25 ml PBS) and pour gently onto the membrane.

18. Recombinant peptide purification:

18.1 Thaw BL21(DE3)PlysS cell pellet on ice and resuspended in "Buffer B".

18.2 Gently shake one hour at room temperature until the lysis is complete (the solution becomes translucent).

18.3 Sonicat for 10 times, 5 sec. each time.

18.4 Centrifuge lysate at 10,000 x g for 30 minutes at room temperature to pellet cellular debris.

18.5 Collect supernatant containing solubilized hexahistidine-tagged recombinant peptides and transfer into a fresh tube.

18.6 Position the Ni-NTA superflow column on the rack.

18.7 Remove the storage buffer from above the resin either by using a pipette or by allowing it to drain through by gravity flow.

18.8 Equilibrate the column by pipetting 10 ml “Buffer B” into column, and allowing it to drain through completely by graving flow.

18.9 Transfer the cleared lysates into the equilibrated columns and allow the columns to drain by gravity flow.

18.10 Perform the first wash step by pipetting 10 ml “Buffer B” into each column. Allow the buffer to drain through completely by gravity flow.

18.11 Perform a second wash step by pipetting 10 ml “Buffer C” unto each column. Allow the buffer to drain through completely by gravity flow.

18.12 Place an elution vessel under column outlet.

18.13 To elute the hexahistidine-tagged peptides, pipette 3 ml “Buffer E” urea into each column. Allow buffer to flow through completely, and collect flow-through in the elution vessels.

18.14 Elute again with 3 ml “Buffer E”.

19. Extensive dialysis of recombinant peptides:

19.1 Remove the Slide-A-Lyzer Cassette from its pouch and immerse it in 8 M dialysis buffer. Hydrate for 10 – 15 minutes.

19.2 Remove cassette from buffer and remove excess liquid by tapping the edge of the cassette gently on paper towels.

19.3 Fill the syringe with the recombinant peptide, leaving a small amount of air in the syringe.

19.4 With the bevel sideways, insert the tip of needle through one of the syringe ports located at a top corner of the cassette.

19.5 Inject peptide slowly. Withdraw air by pulling up to the syringe piston.

19.6 Remove the syringe needle from the cassette while retaining air in the syringe.

19.7 Float the cassette in the 8 M dialysis solution.

19.8 Dialyze for at least 2 hours at room temperature.

19.9 Change the dialysis buffer to 6 M dialysis buffer and dialyze for 2 hours at room temperature.

19.10 Change the dialysis buffer to 4 M dialysis buffer and dialyze for 2 hour at room temperature.

19.11 Change the dialysis buffer to 2 M dialysis buffer and dialyze for 2 hour at room temperature.

19.12 Change the dialysis buffer to 0 M dialysis buffer and dialyze for 2 hour at room temperature.

19.13 To remove peptides, fill syringe with a volume of air at least equal to the peptide size.

19.14 Penetrate the gasket with the needle through a top, unused syringe guide port. Discharge air into cassette cavity to separate membranes, which prevents needle penetration of the membrane.

19.15 Turn the unit so that needle is on the bottom and allow the peptide to collect near the port. Withdraw the peptide into the syringe.

19.16 Inject the peptide slowly into a fresh tube, store peptide at -80℃.

Abbreviations:

CAMPs, Cationic antimicrobial peptides; CDC, Centers for disease Control; AvBD, Avian β-defensin; ATCC: American type collection; RT-PCR, reverse transcriptase polymerase chain reaction; UTR, untranslated region; aa, amino acid; KDa, Kilodalton; GI tract, Gastrointestinal tract.