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Identification and Characterization of a Novel Antibacterial Peptide, Avian β-Defensin 2 from Ducks

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In this study, a novel avian β-defensin (AvBD) was isolated from duck pancreas. The complete nucleotide sequence of the gene contained an 195 bp open reading frame encoding 64 amino acids. Homology, characterization and comparison of the gene with AvBD from other avian species confirmed that it was duck AvBD2. The mRNA expression of the gene was analyzed in 17 tissues from 21-day-old ducks. AvBD2 was highly expressed in the trachea, crop, heart, bone marrow, and pancreas; moderately expressed in the muscular stomach, small intestine, kidney, spleen, thymus, and bursa of Fabricius; and weakly expressed in skin. We produced and purified recombinant AvBD2 by expressing the gene in *Escherichia coli*. As expected, the recombinant peptide exhibited strong bactericidal properties against *Bacillus cereus*, *Staphylococcus aureus*, and *Pasteurella multocida*, and weak bactericidal properties against *E. coli* and *Salmonella choleraesuis*. In addition, the recombinant protein retained antimicrobial activity against *S. aureus* under different temperatures (range, -20°C to 100°C) and pH values (range, 3 to 12).

***Keywords*:** duck, AvBD, recombinant protein, antimicrobial activity

Birds are universally recognized as a major reservoir of human enteropathogens including bacteria and virus, but are themselves often asymptomatic (Milona *et al*., 2007). The general health of birds would greatly be improved if their innate immune system was boosted and was able to restrict or even prevent the colonization and dissemination of zoonotic pathogens. Defensins are small cationic antimicrobial peptides that have a triple-stranded β-sheet structure with three distinctive intramolecular disulfide bridges formed by six cysteine residues. They have been widely isolated from insects, animals, plants, and humans as part of their innate immune systems (Martin *et al*., 1995; Hancock, 1997; Oppenheim *et al*., 2003). Defensins can be subdivided into three subfamilies, according to their disulfide bonding pattern, namely α-, β-, and θ-defensins. β-Defensins have six conserved cysteines that form three disulfide bonds in a C1-C5, C2-C4, and C3-C6 conformation (Lehrer and Ganz, 2002; Ganz, 2003). All defensins show a wide range of antimicrobial properties against pathogens, including various bacteria, fungi, and certain enveloped viruses (Harwig *et al*., 1994; Evans *et al*., 1995; Schutte and McCray, 2002; Thomma *et al*., 2002; Froy and Gurevita, 2003). In addition to their direct antimicrobial activities, immunomodulatory properties have also been demonstrated for defensins. Defensins can promote adaptive immunity by selective chemotaxtic recruitment 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 induce histamine release from peritoneal mast cells (Befus *et al*., 1999), and to enhance macrophage phagocytosis (Fleischmann *et al*., 1985; Ichinose *et al*., 1996; Satchell *et al*., 2003).

Of the three defensin subfamilies found in humans and mammals, only β-defensins have been found in birds (Sugiarto and Yu, 2004). Recently, a new standardized nomenclature for avian β-defensins has been proposed (Lynn *et al*., 2007). All avian β-defensins have now been assigned gene names as avian β-defensin (AvBD). We have used this nomenclature throughout this paper. To date, approximately 30 AvBDs have been identified from chicken (Evans *et al*., 1994, 1995; Harwig *et al.*, 1994; Lynn *et al*., 2004; Higgs *et al*., 2005), turkey (Evans *et al*., 1995), king penguin (Thouzeau *et al*., 2003), ostrich (Yu *et al*., 2001; Sugiarto and Yu, 2006), and other avian species (Lynn *et al*., 2007). Most of these β-defensins exhibit antimicrobial activity against a wide range of pathogens, including bacteria and fungi (Evans *et al*., 1994; Yu *et al*., 2001; Lehrer and Ganz, 2002; Ganz, 2003; Thouzeau *et al*., 2003; Lynn *et al*., 2004; Xiao *et al*., 2004; Higgs *et al*., 2005; Sugiarto and Yu, 2006; Ma *et al*., 2008).

Duck antimicrobial peptides are believed to be composed mainly of AvBDs, defined by comparison to other avian species. The objective of this work was to identify and partially characterize an AvBD from duck tissues and to analyze the relationship between the duck β-defensin and other known β-defensins.

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Materials and Methods

Specific-pathogen-free ducks

Specific-pathogen-free (SPF) Peking ducks were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, China. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum.*

RNA extraction, reverse transcriptase-polymerase chain reaction amplification (RT-PCR) and sequencing

We extracted total cellular RNA from 200 μl of pancreatic tissue fluid obtained from a 21-day-old SPF Peking duck using TRIzol reagent (Invitrogen, China) according to the manufacturer's instructions. The RNA was air-dried for 2∼10 min, redissolved in 20 μl RNase-free water, and stored at -70°C until use. Reverse transcription was performed using oligo-dT primer in a 40 μl reaction mixture containing 20 μl RNA. The specific cDNA obtained was amplified by polymerase chain reaction (PCR) using Ex *Taq* polymerase (TaKaRa, Japan) with internal primers designed according to the known sequences of chicken AvBDs [previously known as gallinacins (Gals)] (Brockus *et al*., 1998; Xiao *et al*., 2004). The primers used were: 5'-TGGCTCAGCAGATCTGCA-3' (forward) and 5'-GAATAAATTGCCATTGCG-3' (reverse). Primers for duck β-actin were 5'-CATCGCTGACAGGATG CAGAAGGAG-3' (forward) and 5'-TGATCCACATCTGCT GCTGGTAG-3' (reverse). The PCR protocol was as follows: an initial denaturation for 5 min at 95°C followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and polymerization at 72°C for 1 min. The final polymerization step was performed at 72°C for 10 min. The PCR product was cloned into the pMD18-T vector (TaKaRa) to confirm amplification, followed by sequencing of the recombinant plasmids.

Sequence analysis of the duck AvBD gene

The basic local alignment search tool (BLAST) searches were conducted using the entire duck AvBD gene. Sequences of known AvBDs and some mammalian β-defensin-2s were selected for sequence comparison with the duck AvBD gene. Multiple alignment and phylogenetic analyses were performed using the CLUSTAL V routine of the MegAlign program provided in the DNAStar package (Windows 4.05, DNAStar, USA) (Higgins and Sharp, 1988).

We submitted the duck AvBD2 sequence to the GenBank database, and it has been assigned the accession number, **FJ465147.**

Expression of duck AvBD2 mRNA in different tissues

Five twenty-one-day SPF Peking ducks were used in this study. Ducks were sacrificed by intravenous sodium pentobarbitone administration. Seventeen tissues, including the skin, tongue, esophagus, trachea, crop, muscular stomach, breast muscle, small intestine, lung, liver, kidney, heart, spleen, bone marrow, thymus, bursa of Fabricius, and pancreas were rinsed, immediately dissected, and squeezed between a Whatman filter to remove excess blood. They were then rinsed in cold sterile saline, snap-frozen in liquid nitrogen, and stored at -70°C until further use. Total RNA extraction, cDNA synthesis, and RT-PCR were performed as described above. A 7 μl aliquot of the PCR product was analyzed on a 2.0% agarose gel. All assays were performed in duplicate.

Protein expression and purification

A DNA fragment encoding the duck AvBD2 gene was amplified by PCR from plasmid described above using the following primers: 5'-CGCGAATTCATGAGGATCCTTTAC-3' (forward), and 5'-GCGGTCGACTGGAAGAAATTTTCA-3' (reverse). The PCR product, containing the duck AvBD2 coding sequence (including the total coding gene of AvBD2) flanked by *Eco*RI and *Sal*I restriction sites, was inserted into the pGEX-6p-1 vector (Amersham, China) at the *Eco*RI and *Sal*I sites, and the resultant plasmid was designated duck rAvBD2-pGEX and sequenced again.

The construct confirmed as containing the AvBD2 gene was transformed into competent *E. coli* BL21(DE3) cells. Expression of the fusion protein was induced with isopropyl beta-D-1-thiogalactoside (IPTG) and purified as described previously (Ma *et al*., 2008) using a protein purification and refolding kit (No. 70123-3; Novagen), according to the manufacturer's instructions. A cell sample of 1 ml was taken at 2 h, 3 h, 4 h, 5 h, 6 h, and 7 h after IPTG was added. Briefly, the induced culture was harvested by centrifugation at 6,500×g for 15 min at 4°C, and the supernatant was removed and discarded. The cell pellet was resuspended in 1× Inclusion Body (IB) Wash Buffer (20 mM Tris-HCl; pH 7.5, 10 mM EDTA, 1% Triton X-100) and sonicated after a 15-min incubation at 30°C with lysozyme. The inclusion bodies were collected by centrifugation at 10,000×g for 10 min and resuspended in 1× IB solubilization buffer supplemented with 0.3% N-lauroylsarcosine. The supernatant containing the fusion proteins was filtered through a cellulose acetate filtration membrane with a pore size of 0.45 μm and passed through an affinity chromatography column of glutathione Sepharose 4B (Amersham) equilibrated with PBST (PBS + 1% Triton 100). The column was washed with six bed volumes of PBS to remove contaminating proteins. The recombinant fusion proteins were eluted with 10 ml of 50 mM of Tris-HCl buffer containing 10 mM reduced glutathione, pH 8.0. The fusion protein was concentrated using Centricon Microconcentrators (Millipore, China) with a molecular weight cutoff of 10 kDa. The fusion protein was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V, using the Mini-protean III system (Bio-Rad, China), and stained with Coomassie brilliant blue R-250 (Schagger and Von Jagow, 1987). The protein concentration was determined by analyzing the SDS-PAGE gel image with Quantity-One 4.4.0 (Bio-Rad) and by the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

Antimicrobial activity by colony-counting assay and minimal inhibitory concentration (MIC)

Colony-counting assays (van Dijk *et al*., 2007) were performed to investigate the antimicrobial activity of recombinant duck AvBD2 against the following bacterial strains: *E. coli* BL21(DE3), *Bacillus cereus* (ATCC 9193), *Staphylococcus*

aureus (ATCC 29213), *Pasteurella multocida* (ATCC 6529), and *Salmonella choleraesuis* (CVCC 2140). All bacterial strains were maintained in Luria-Bertani (LB) medium at 37°C. Bacterial strains were cultured to mid-logarithmic phase by transferring 100 μl of a stationary-phase suspension into LB medium followed by incubation and shaking for 4 h at 37°C. Mid-logarithmic phase cultures were centrifuged for 10 min at 4°C at 900×g, and bacterial pellets were diluted in

(A)

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MRILYLLF---SLLFLALQVSPGLSSPR---RDMLFC--KGGSCHFGGCPSHLIKVGSCFGFRS-CCKWPWNA          chicken AvBD2 NM_204992
MRILYLLF---SVLFLVLQVSPGLSLPQ---RDMFLC--RKGSCHFGRCPIHLIRVGSCFGFRS-CCKSPWDV            duck AvBD2 FJ465147
MRILYLLF---SVLFLVLQVSPGLSLPQ---RDMFLC--RIGSCHFGRCPIHLVRVGSCFGFRS-CCKSPWDV            duck AvBD2 AY641439
MRILYLLF---SLLFLALQVSPGLSSPK---RDMLFC--KRGTCHFGRCPSHLIKVGSCFGFRS-CCKWPWDA           turkey AvBD2 AF033338
---------------------------------LFC--RKGTCHFGGCPAHLVKVGSCFGFRA-CCKWPWDV            ostrich AvBD2 P85113
MRALCLLLLTVCLLSSQLAAGINLLTGLGQRSDHYICAKKGGTCNFSPCPLFNRIEGTCYSGKAKCC IR       pig beta defensin-2 AY506573
MRLHHLLL---ALFFLVLSAGSGFTQGI---INHRSCYRNKGVCAPARCPRNMRQIGTCHGPPVKCCRKK       goat beta defensin-2 AJ009877
MRVLYLLF---SFLFIFLMPLPGVFGGI---GDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP       human beta defensin-2 AF040153
MRTLCSLLLICCLLFSYTTPAVGSLKSIGYEAELDHCHTNGGYCVRAICPPSARRPGSCFPEKNPCCKYMK     mouse beta defensin-2 NM_010030
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(B)

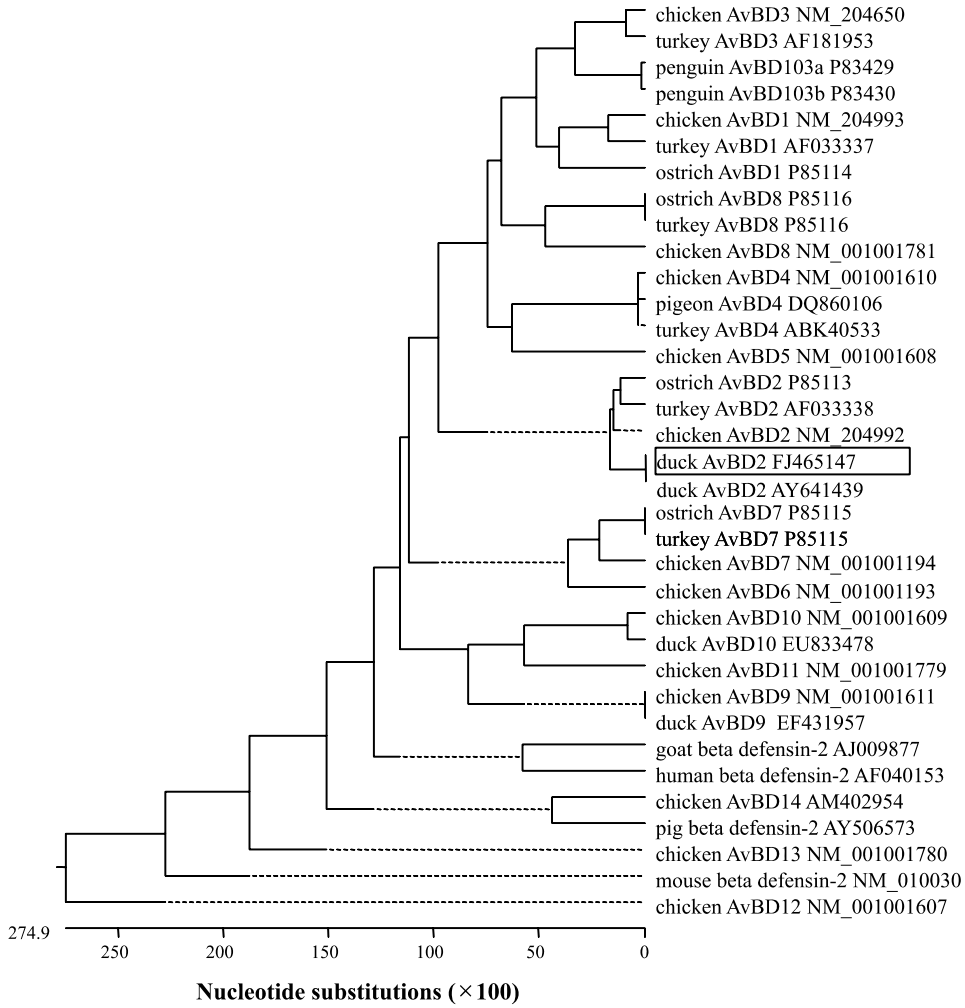

Fig. 1. Duck AvBD2 deduced amino acid sequence and phylogenetic relationships. (A) Alignment of amino acid sequences of duck AvBD2 with AvBD2 from chicken, turkey, and ostrich; and β-defensin-2 from human, mouse, goat, and pig. The six cysteines, and the gly, and pro of the 'β-defensin core motif' are underlined. "-" indicates no identical or conserved residues observed. (B) Phylogenetic relationships based on the sequences of duck AvBD2, other AvBDs, and β-defensin-2 from human, mouse, goat and pig, using the MEGALIGN program DNAStar with CLUSTAL V method (Higgins and Sharp, 1988). Note: The duck AvBD2, sequenced in this present study, is boxed. AvBD2, avian β-defensin 2.

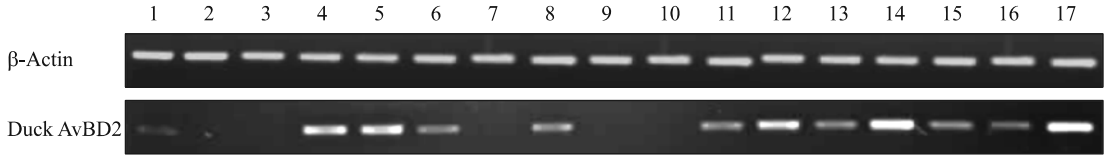

Fig. 2. Expression of duck AvBD2 mRNA in a panel of tissues from five SPF ducks. Lanes: 1, skin; 2, tongue; 3, oesophagus; 4, trachea; 5, crop; 6, muscular stomach; 7, breast muscle; 8, small intestine; 9, lung; 10, liver; 11, kidney; 12, heart; 13, spleen; 14, bone marrow; 15, thymus; 16, bursa of Fabricius; 17, pancreas. AvBD2, avian β-defensin 2; SPF, specific-pathogen-free. All assays were performed in duplicate.

minimal medium (LB medium diluted 1000-fold in distilled water for bacterial pellets). Initial concentrations of bacteria were determined by measuring optical density at 620 nm. To determine cell viability, 100 μl of 10-fold serial dilutions in PBS (pH 7.4) were transferred to Trypticase Soy agar (TSA; Oxoid Limited) plates, and colonies were counted after 24 h of incubation. Final dilutions were prepared in minimal LB media to reach a cell density of 2.5×10^8 colony forming units (CFU)/ml. The antimicrobial activity of recombinant duck AvBD2 was determined by colony-counting assay.

Kill-curve studies were performed to determine the incubation period. An 100 μl aliquot of diluted bacterial culture (mid-logarithmic phase, diluted 1000-fold in LB medium) was mixed with 100 μl of 400 μg/ml rAvBD2 (final concentration 200 μg/ml) and anaerobically incubated at 37°C. At various time points (0, 1, 2, 2.5, and 3 h), 50 μl of a bacterial suspension was diluted 10 to 1000-fold in LB medium, and 100 μl was plated on TSA medium. The number of CFU was counted after overnight incubation at 37°C. As a negative control, the bacterial suspension was incubated with 50 μl of minimal LB medium. On the basis of kill-curve results, 100 μl of diluted bacterial culture (diluted as above) was mixed with 100 μl of 0 to 1,000 μg/ml recombinant duck AvBD2 or GST (final concentration, 0, 25, 50, 100, 200, and 500 μg/ml) in polypropylene microtiter plates and preincubated for 2 h at conditions suited to the investigated strain. After 2 h, the sample was further diluted 100-to-10^6 fold in minimal medium, transferred to TSA plates, and colonies were counted after 24 h of incubation. For each antimicrobial activity assay, PBS (pH 7.4) was used as the negative control. All kill-curve studies were performed in duplicate.

The MIC of the recombinant protein was determined by a liquid growth inhibition assay (Bulet *et al*., 1993; Ma *et al*., 2008). A stock solution of recombinant protein or GST was serially diluted two-fold with PBS (pH 7.4) and 0.2% BSA as the negative control (0∼100 μg/ml). Aliquots (10 μl) from each dilution were transferred to a 96-well polypropylene microtiter plate, and each well was inoculated with 100 μl of a suspension of mid-log bacteria (10^6 CFU/ml) in poor broth [1% tryptone, 0.5% NaCl (w/v), pH 7.5]. The culture was grown for 24 h with vigorous shaking at 37°C, and bacterial growth was evaluated by measuring culture absorbance at 490 nm using a microplate reader. Growth inhibition was defined as the lowest concentration of peptide that reduced growth by more than 90%. All assays were performed in triplicate.

Temperature and pH stability

Stabilities of the recombinant peptide were conducted according to Lee *et al*. (1999). Briefly, 100 μl of 400 μg/ml recombinant duck AvBD2 or GST were incubated at -20°C, 4°C, 20°C, 40°C, 60°C, and 100°C for temperature stability measurements or at pH 3, 4, 5, 7, 10, and 12 for pH range analysis, for 30 min and used for the antimicrobial activity assay immediately thereafter. One hundred microliters of *S. aureus* culture, grown, and diluted as descsribed above, were mixed with 100 μl of AvBD2 or GST samples at a final concentration of recombinant duck AvBD2 or GST of 200 μg/ml, in polypropylene microtiter plates and preincubated for 2 h at conditions suited to the investigated strain. After incubation, samples were further diluted 100- to 10^6-fold in minimal medium, transferred onto TSA plates, and colonies were counted after 24 h. For the pH stability assay, PBS at pH 3, 5, 7, 10, and 12 was used as the negative control. All assays were performed in duplicate.

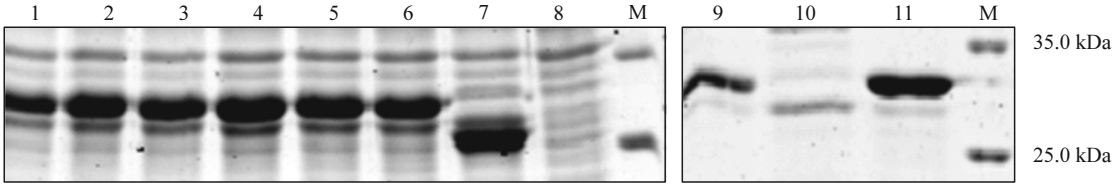

Fig. 3. SDS-PAGE analysis of recombinant duck AvBD2 fusion protein expressed in *E. coli* BL21 (DE3) cells. Lane M, protein molecular weight marker; Lanes: 1∼6, total protein of BL21 containing duck AvBD2 at 2 h, 3 h, 4 h, 5 h, 6 h, and 7 h after induction with IPTG; 7, total protein of BL21 containing pGEX after induction with IPTG; 8, total protein of BL21 containing duck AvBD2, without IPTG induction; 9, purified protein of duck AvBD2 after induction with IPTG; 10, supernatant; 11, inclusion bodies with duck AvBD2. AvBD2, avian β-defensin2; IPTG, isopropyl-beta-D-thiogalactoside.

Results

Confirmation of the duck AvBD gene

We cloned and sequenced a putative AvBD gene from duck pancreas tissue and compared it with the published AvBDs. The complete nucleotide sequence of the gene contained a 195 bp open reading frame (ORF) encoding 64 amino acids (Fig. 1A), which was identical with a duck AvBD2 sequence in the GenBank database (no related functional data have been published). However, two residue substitutions were found between duck AvBD2 isolated in this study and the published duck AvBD2 sequence (Fig. 1A). The sequence of the duck AvBD2 identified shared 78.1%, 79.7%, and 75% amino acid homology with chicken AvBD2, turkey AvBD2, and ostrich AvBD2, respectively. Furthermore, when we aligned the duck AvBD2 sequence with those of chicken,

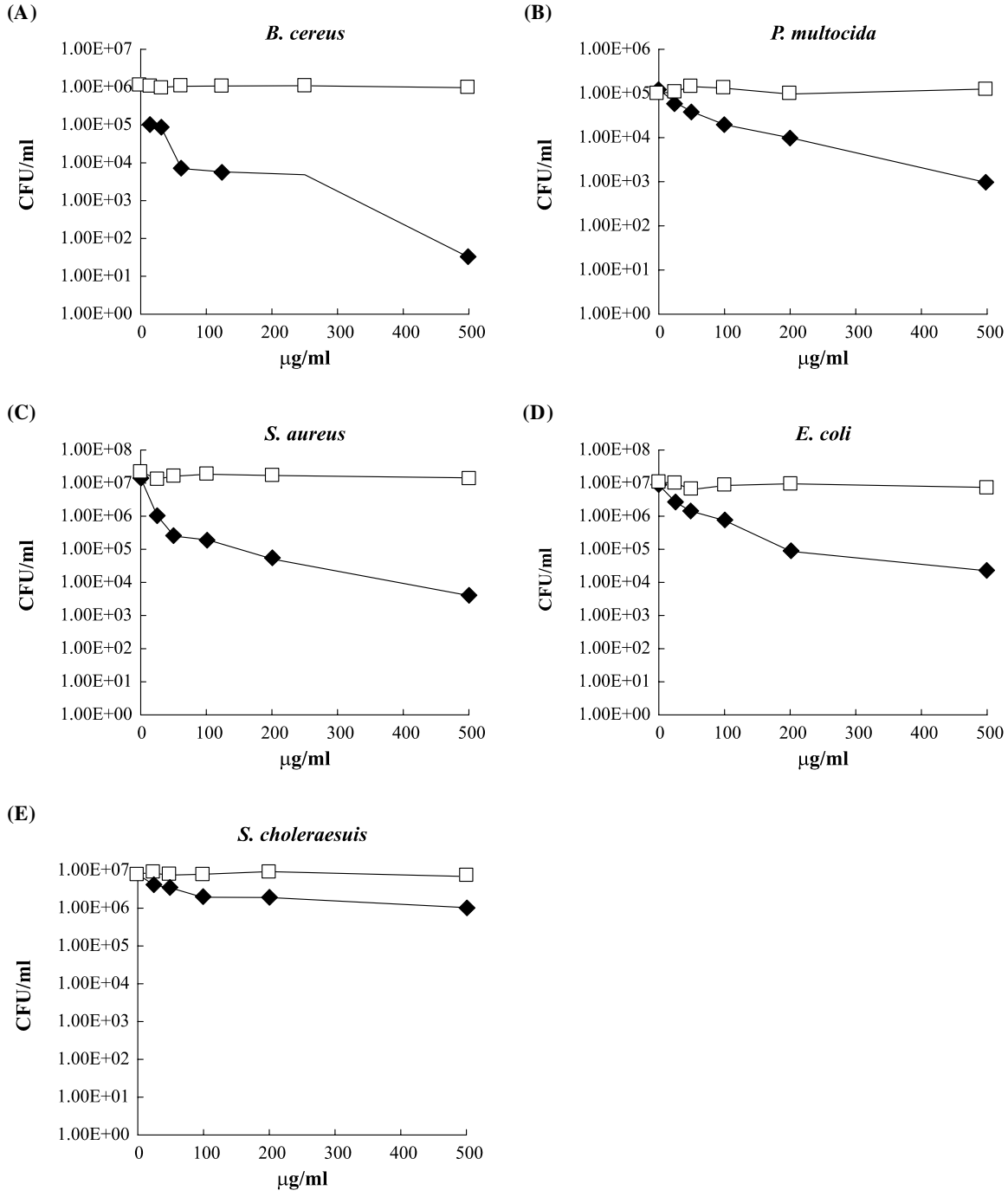

Fig. 4. Antimicrobial activity of recombinant duck AvBD2 against *S. aureus*, *B. cereus*, *P. multocida*, *E. coli*, and *S. choleraesuis* bacteria by colony-counting assay. AvBD2 (◆), GST (□). All kill-curve studies were performed in duplicate. AvBD2, avian β-defensin 2.

Table 1. Minimal growth inhibitory concentration (MIC) of recombinant duck AvBD2 (Means±SD)

Microorganism	MIC[a] (μg/ml)	
	Recombinant duck AvBD2	GST
B. subtilis	15.6±1.89	>500
S. aureus	31.3±2.33	>500
P. multocida	31.3±2.45	>500
E. coli	125.0±9.15	>500
S. choleraesuis	>400	>500

[a] All assays were performed in triplicate.
AvBD2, avian β-defensin 2

turkey and ostrich AvBD2; and with human, mouse, goat, and pig β-defensin-2, eight invariant residues (six cysteines, glycine, and proline) common to all sequences were revealed (Fig. 1A). The six cysteine residues are known to form three intramolecular disulfide bridges. These eight conserved residues have been designed as the 'β-defensin core motif', which is an essential structural element of β-defensins (Harwig *et al.*, 1994). In addition, a phylogenetic tree was constructed based on the amino acid sequences of duck AvBD we isolated from pancreas, other AvBDs, and β-defensin-2 from human, mouse, goat, and pig. The data indicated that the duck AvBD gene isolated here clustered into the same groups with chicken, turkey, and ostrich AvBD2 (Fig. 1B). Hence, we designated the novel AvBD as duck AvBD2.

Differential expression of duck AvBD2 in different tissues

To analyze the expression pattern of AvBD2 in duck tissues, RT-PCR was performed using mRNA isolated from 17 different tissues from 5 healthy SPF ducks at age 21 days (Fig. 2). These tissues included those from the digestive, respiratory, genitourinary, and immune system, together with several other organ systems. As shown in Fig. 2, AvBD2 expression was high in the trachea, crop, heart, bone marrow, and pancreas, moderate in the muscular stomach, small intestine, kidney, spleen, thymus, and bursa of Fabricius, and low in the skin.

Recombinant duck AvBD2 showed antibacterial activity against several bacterial strains

A high expression level of the recombinant duck AvBD2 was noted in *E. coli* after induction with 0.6 mM IPTG for 2~7 h (Fig. 3), and the production of the recombinant duck AvBD2-pGEX (molecular weight, 32 kDa) accounted for approximately 38% of the total protein. The recombinant protein was produced in inclusion bodies (Fig. 3). The recombinant protein was purified using a protein purification and refolding kit and visualized as a pronounced band on SDS-PAGE gels (Fig. 3).

Five pathogenic bacterial strains, *E. coli*, *B. cereus*, *S. aureus*, *P. multocida*, and *S. choleraesuis*, were used to investigate the antibacterial activity of the recombinant protein. The results of kill-curve studies showed that CFU/ml of the five bacteria achieved stability after 2 h of incubation with the recombinant protein (data not shown). The dose-dependent survival of the recombinant protein-treated bacteria was tested by colony-counting assays. The recombinant protein showed antibacterial activity compared to GST or control (PBS) (Fig. 4). A fast decline in surviving cells was observed for *B. cereus*, and moderate decline was observed for *S. aureus* and *P. multocida.* A slower decline was observed in the assays performed with *E. coli* and *S. choleraesuis.* Only at a concentration of 200 μg/ml, were *E. coli* and *S. choleraesuis* cells partially eradicated. The MIC of the recombinant protein was determined by a liquid growth inhibition assay (Table 1). The results showed that the recombinant protein inhibited the growth of *B. cereus* at low concentrations (MIC=15.6 μg/ml), and of *S. aureus* and *P. multocida* at medium concentrations (MIC=31.3 μg/ml). It was less effective against *E. coli* (MIC=125 μg/ml) and *S. choleraesuis* (MIC>400 μg/ml).

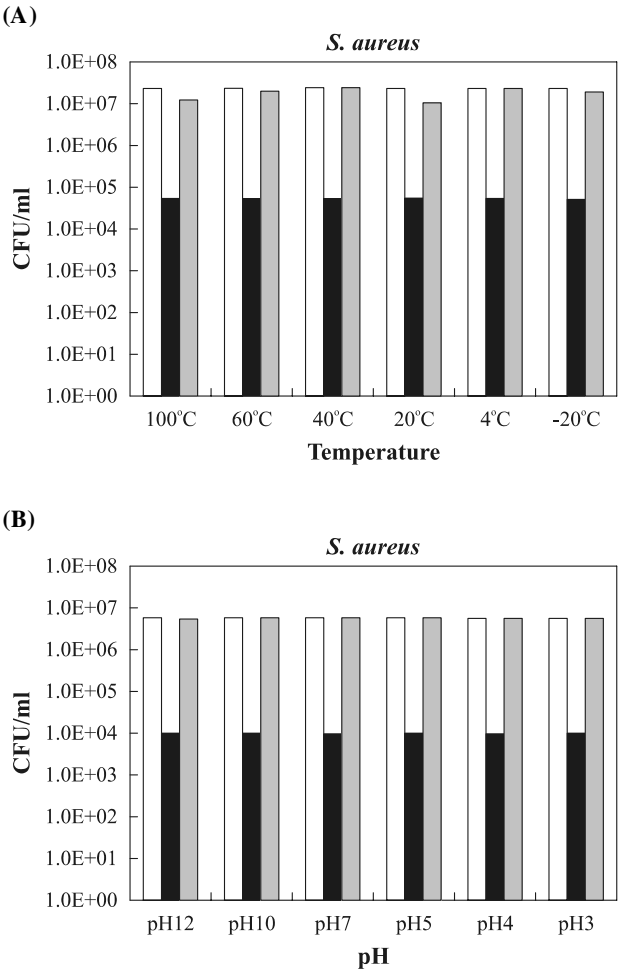

Fig. 5. Antimicrobial activity of recombinant duck AvBD2 against *S. aureus* cells at different temperatures and pH values. (A) Incubation of recombinant duck AvBD2 (black bars) or GST (gray bars) at -20°C, 4°C, 20°C, 40°C, 60°C, and 100°C for 30 min. Control: Control treated with PBS (white bars). (B) Incubation of the recombinant duck AvBD2 (black bars) or GST (gray bars) at pH 3, 4, 5, 7, 10, and 12 for 30 min. Control: Control treated with PBS (white bars) at pH 3, 4, 5, 7, 10, and 12. All kill-curve studies were performed in duplicate. AvBD2, avian β-defensin 2.

Recombinant duck AvBD2 showed antibacterial activity at different temperatures and pH values

Compared to GST or control (PBS), the recombinant protein (final concentration, 200 μg/ml) retained its antibacterial activity against *S. aureus* cells following incubation at different temperatures ranging from -20°C to 100°C (Fig. 5A), or following acid-alkali treatment at pH values ranging from 3 to 12 (Fig. 5B), indicating that the recombinant protein activity was resistant to temperatures or pHs in these ranges.

Discussion

This report characterized a novel β-defensin, duck AvBD2. Similar to the known AvBDs, the duck AvBD2 comprises an N-terminal signal sequence indicative of its secretion, and a C-terminal mature antimicrobial peptide, the latter containing discrete groups of cationic and hydrophobic amino acids. This structural conformation appears to be key to the antimicrobial activity of AvBDs, as these peptides are proposed to function by binding to the negatively charged surface components of bacterial membranes via electrostatic charge interactions, and integrating into the membranes to cause depolarization and microbial death (Brogden, 2005; Milona *et al*., 2007; Sugiarto and Yu, 2007). Alignment of the amino acid sequences of the duck peptide with other known AvBD2s, and β-defensin-2 from human and other mammals revealed that duck AvBD2 shared high amino acid homology with other known AvBD2s, and low amino acid homology with the mammalian proteins (Fig. 1B). However, duck AvBD2 shared conserved regions, including the three paired cysteine disulfide bridges and glycine and proline residues that have been designated as the 'β-defensin core motif' and are the essential structural element of β-defensins (Harwig *et al*., 1994; Xiao *et al*., 2004). Relatively high amino acid homology was observed between the AvBD homolog and AvBDs from other avian species such as the AvBD1, AvBD2, AvDB3, and AvDB4 from chicken and turkey, and the chicken and duck AvBD10 (Fig. 1B). This was thought to be due to positive Darwinian selection, such as the pressures of pathogenic microbial flora in each animal species (Hughes and Yeager, 1997; Hughes, 1999; Xiao *et al*., 2004; Ganz, 2005). Since β-defensin sequences are very short, positive Darwinian selection causes significant diversification between species at the amino acid level, over the course of evolution (Hughes and Yeager, 1997).

Similar to most chicken AvBDs (Lynn *et al*., 2004; Xiao *et al*., 2004; Milona *et al*., 2007; Ma *et al*., 2008), duck AvBD2 is constitutively expressed in multiple tissues, with predominant expression in the crop, pancreas, trachea, heart, and bone marrow, moderate expression in the muscular stomach, small intestine, kidney, spleen, thymus, and bursa of Fabricius, and weak expression in the skin. The expression of duck AvBD2 in the digestive tract and respiratory system indicates that its primary role may be to protect the digestive tract and respiratory system from bacterial infections. It is worth noting that duck AvBD2 was expressed in all immune tissues investigated, including bone marrow, spleen, thymus, and bursa of Fabricius. This suggests that this cationic peptide may have roles apart from antimicrobial activity in the innate defense responses of ducks. Several human β-defensins such as hβD-3 exhibit chemoattractant activity for immature dendritic cells, monocytes, and macrophages (Yang *et al*., 1999); thus, it is possible that AvBD2 may play a comparable role in the duck. The expression of duck AvBD in tissues ranging from bone marrow to bursa of Fabricius to kidney illustrates the important role of the AvBD gene as a bridge between the innate and adaptive immune response in ducks.

To investigate the antimicrobial activity of duck AvBD2, we produced it as a GST-tagged recombinant peptide. SDS-PAGE revealed a band of approximately 32 kDa, consistent with the expected size from AvBD2-pGEX. The antimicrobial potency of chicken AvBDs was shown previously not to be altered by the presence of a GST-tag (Ma *et al*., 2008), so the antimicrobial activity of the GST-tagged recombinant duck peptide was tested against five bacterial strains including *E. coli*, *B. cereus*, *S. aureus*, *P. multocida*, and *S. choleraesuis.* As predicted, the recombinant peptide exhibited strong bactericidal properties against *B. cereus*, *S. aureus*, and *P. multocid*, and weak bactericidal properties against *E. coli* and *S. choleraesuis*. The present results are consistent with those from most avian defensins, either those that occur naturally (Evans *et al*., 1994; Harwig *et al*., 1994; Yu *et al*., 2001; Thouzeau *et al*., 2003; Sugiarto and Yu, 2006), were chemically synthesized (Higgs *et al*., 2005; van Dijk *et al*., 2007) or were produced by recombinant expression (Milona *et al*., 2007; van Dijk *et al*., 2007; Ma *et al*., 2008). AvBDs may play an internal role in the innate immune response to bacteria. Because of the lack of a superoxide ion and myleoperoxidase in avian heterophils, birds rely more heavily upon nonoxidative defense molecules that include lysozymes, cationic proteins, and peptides such as AvBDs (Harmon, 1998). In agreement to the current results, turkey AvBD2, which shared the highest amino acid homology with duck AvBD2, inhibited the growth of *S. aureus*, but not of *E. coli* (Evans *et al*., 1994). Synthetic AvBD9 peptide was found to have strong microbicidal activity against *C. jejuni* and *S. aureus*, but was less potent against *E. coli* and not bactericidal against *S. typhimurium* (van Dijk *et al*., 2007). Differences in antimicrobial activity of these AvBDs among bacterial strains may be related to structure of the peptide and bacterial strains. Although, the actual mechanisms of how these β-defensins kill microorganisms have not been fully understood. However, many researchers believe that antimicrobial actions of these peptides are based on the two main features of antimicrobial peptides, cationic, and amphipathic (Evans *et al*., 1995). Three known models, 'barrel stave', 'micellar aggregate', and 'carpet model' have been developed based on the two main features (Powers and Hancock, 2003; Sugiarto and Yu, 2004). Antimicrobial action is initiated, in principle, by the binding of the peptide to the bacterial membrane through electrostatic interactions (Sugiarto and Yu, 2004). Upon release, antimicrobial peptides such as AvBDs permeate the membrane of bacteria, coinciding with the inhibition of RNA, DNA, and protein synthesis (Froy and Gurevita, 2003). The actual killing mechanisms remain unknown, although the hydrophobic nature of the peptides is indicative of a mechanism involving membrane permeabilisation and is probably comparable to those reported for the ostrich AvBD1 and 2 (Sugiarto and Yu, 2007). Additionally,

the current results showed that the recombinant protein retained its antimicrobial activity against *S. aureus* under different temperatures (range, -20℃ to 100℃) and pH values (range, 3 to 12). In agreement to the present results, it was reported that the antimicrobial activity of bacteriocin, an antimicrobial peptides produced by bacteria was stable from pH 2.0∼11.0 and up to 10 min heating at 100℃ (Lee *et al*., 1999), or autoclaved at 121℃ for 15 min at 103 kPa (Jung *et al*., 2008). To further verify the stability of antimicrobial peptides, we incubated the duck AvBD2 under different temperature or pH for 30 min. Similar to these results, van Dijk *et al.* (2007) found that the antimicrobial activity of chemically synthesized chick AvBD9 against *E. coli* was pH independent (range, 5.5∼7.0). These results explain a previous report that synthetic king penguin AvBD103b retains its microbicidal activity within the stomach environment *in vivo*, thus lending the bird protection against microorganisms involved in the degradation of food (Thouzeau *et al*., 2003).

In conclusion, a novel antimicrobial peptide, known as duck AvBD2, was identified and characterized from duck tissues. The antimicrobial peptide is classified as a β-defensin, characterized by the six cysteine residues and their pairings. The gene has been shown to be expressed widely in duck tissues. The recombinant peptide was effective against bacterial cells and retained stability under different temperatures (range, -20℃ to 100℃) and pH values (range, 3 to 12). Future work is required to study other roles of this AvBD in innate and adaptive immunity, such as the chemotactic function shown by its mammalian and human counterparts.

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