Discovery and characterization of *Coturnix chinensis* avian β-defensin 10, with broad antibacterial activity

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A novel avian β-defensin (AvBD), AvBD10, was discovered in the liver and bone marrow tissues from Chinese painted quail (*Coturnix chinensis*) in the present study. The complete nucleotide sequence of quail AvBD10 contains a 207-bp open reading frame that encodes 68 amino acids. The quail AvBD10 was expressed widely in all the tissues from quails except the tongue, crop, breast muscle, and thymus and was highly expressed in the bone marrow. In contrast to the expression pattern of AvBD10 in tissues from quail, the chicken AvBD10 was expressed in all 21 tissues from the layer hens investigated, with a high level of expression in the kidney, lung, liver, bone marrow, and Harderian glands. Recombinant glutathione S-transferase (GST)-tagged AvBD10s of both quail and chicken were produced and purified by expression of the two cDNAs in *Escherichia coli*, respectively. In addition, peptide according to the respective AvBD10s sequence was synthesized, named synthetic AvBD10s. As expected, both recombinant GST-tagged AvBD10s and synthetic AvBD10s of quail and chicken exhibited similar bactericidal properties against most bacteria, including Gram-positive and Gram-negative forms. However, no significant bactericidal activity was found for quail recombinant GST-tagged AvBD10 against *Salmonella choleraesuis* or for chicken recombinant GST-tagged AvBD10 against *Proteus mirabilis*. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: avian β-defensin 10; quail; expression; synthetic peptide; recombinant peptide; antimicrobial activity

Introduction

Innate immunity is the primary line of active defense against invading pathogens in vertebrates and invertebrates. Part of the innate immune response is the secretion of antimicrobial peptides (AMPs). The AMPs are a crucial component of the nonspecific defenses [1]. In general, these molecules show common features such as small size (less than 100 amino acids), positive charge, and a broad specificity against pathogens; they can be active against bacteria, fungi, enveloped viruses, and even some parasites [2]. Besides the classic bactericidal role of AMPs, several additional roles in innate immunity have recently been assigned to AMPs. These include chemotactic activities for immature dendritic cells, memory T cells, and monocytes [3,4]; stimulation of angiogenesis [5]; and mast cell activation to induce chemotaxis, histamine release, and prostaglandin production [6]. To date, no resistance against AMPs has been found, and this characteristic makes them a potential alternative to antibiotics for medical and veterinary use, especially where microbial antibiotic resistance is an issue [7]. Several major families of AMP occur throughout the vertebrates. These include the defensins [8], cathelicidins [1,9], and liver-expressed AMPs [10], each with a unique signature motif and specific range of actions.

Defensins, a subset of AMPs, are cysteine-rich peptides that contain cationic amino acid residues [11]. In vertebrates, three subfamilies (α, β, and θ) can be distinguished on the basis of how the six cysteines in the mature peptide form intramolecular disulfide bridges. The α-defensins and θ-defensins have only been found in mammals to date [1,12]. The β-defensins, which have bonds formed between C1–C5, C2–C4, and C3–C6, are known to be present in avian species. To date, more than 30 avian β-defensins (AvBDs) have been identified, from chicken [13–17], turkey [14], king penguin [18], ostrich [19,20], and duck [21–23]. Most of AvBDs exhibit antimicrobial activity against a wide range of pathogens, including bacteria and fungi [13,16–27]. A more recent study in our laboratory has identified quail AvBD9 in Chinese painted quail (*Coturnix chinensis*). The complete nucleotide sequence of quail AvBD9 contains a 204-bp open reading frame (ORF) that encodes 67 amino acids. Quail AvBD9 has been shown to be expressed widely in quail tissues and has a wide spectrum of antibacterial activity [28]. In the present study, we extend these findings in quail and show that another AvBD gene, quail AvBD10, is present in quail. We also show that quail AvBD10 differs in the level of tissue expression from chicken AvBD10. In addition, we found that both recombinant and synthetic quail AvBD10s exhibited similar bactericidal properties to recombinant and synthetic chicken AvBD10s.

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Materials and Methods

Animals

Three healthy adult Chinese painted quails (*C. chinensis*) were purchased at 80 days old from a commercial supplier. Three healthy Hyline layer hens (50 weeks of age, with a laying rate of 80%) were obtained from a commercial layer farm. All of the birds were raised under standard conditions, and feed and water were provided *ad libitum*.

RNA Extraction, Reverse Transcriptase Polymerase Chain Reaction Amplification, and Sequencing

Approximately 1 g of each of liver and bone marrow tissue obtained from three adult quail and three layer hens was used to process tissue fluid, and the total cellular RNAs were extracted from 200-µl aliquots of the respective tissue fluid using TRIzol reagent (Invitrogen, Beijing, China) according to the manufacturer's instructions. The RNAs were air dried for 2–10 min, redissolved in 20-µl RNase-free water, and stored at −70 °C until use. To evaluate the quality of the RNA, the optical density (OD) of the RNA was examined at wavelengths of 260 and 280 nm, respectively. The ratio of OD260 to OD280 was within 1.8 to 2.2 (data not shown). Reverse transcriptions (RTs) were performed using oligo-dT primer in a 40-µl reaction mixture containing 20-µl RNA. The specific cDNAs obtained were amplified by polymerase chain reaction (PCR) using Ex-Taq polymerase (TAKARA Bio Inc., Otsu, Shiga, Japan) with respective primers designed according to the known sequences of chicken AvBD10 (Table 1), previously known as gallinacins (Gals) [16,26]. 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 s, annealing at 50 °C for 30 s, and polymerization at 72 °C for 1 min. The final polymerization step was performed at 72 °C for 10 min. The PCR products from both quail and layer hens were cloned into the pMD18-T vector (TAKARA Bio) to confirm amplification, followed by sequencing of the recombinant plasmids.

Sequence Analysis of the Quail AvBD Gene

Basic searches were conducted with a local alignment search tool (BLAST) using the entire quail AvBD gene. Sequences of known AvBDs, some mammalian β-defensins, including goat (*Capra hircus*) β-defensins, cattle (*Bos taurus*) β-defensins, and porcine (*Sus scrofa*) β-defensins, and some mammalian β-defensin-10s were selected for sequence comparison with quail AvBD10 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, Madison, WI, USA) [29]. The signal peptide of the quail AvBD was analyzed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/).

Expression of AvBD10 mRNA in Different Tissues from Quails and Layer Hens

All of the birds listed above were euthanized by intravenous administration of sodium pentobarbitone. Twenty-one tissues, including the tongue, muscular stomach, glandular stomach, skin, crop, trachea, breast muscle, kidney, lung, liver, heart, cecal tonsil, bone marrow, bursa of Fabricius, Harderian glands, spleen, thymus, esophagus, rectum, small intestine, and ovary, were rinsed, dissected immediately, and squeezed between Whatman filter papers 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. The levels of AvBD10 mRNA in tissues from both quails and layer hens were measured with the real-time RT-PCR method using SYBR Premix Ex TaqTM (TAKARA Bio). Equal amounts of tissues (1 g) were used to process tissue fluid. The total cellular RNAs were extracted and RT was performed as described above. Real-time PCR was performed with an iQ5 real-time PCR system (Bio-Rad, Hercules, CA, USA). Serial 10-fold dilutions of plasmids containing quail AvBD10, quail 18S ribosomal RNA (18S rRNA), chicken AvBD10, or chicken β-actin DNA were used as standard controls, respectively, as described by Sadeyen *et al.* [30]. Plasmids containing quail 18S rRNA or chicken β-actin DNA were stored in our laboratory, which were selected as the most stable reference genes across all samples from a panel of potential genes, including 18S rRNA, β-actin, and glyceraldehyde 3-phosphate dehydrogenase from quails and layer hens (data not shown). The initial DNA concentrations were quantified using a spectrophotometer (NanoDrop Technologies, Newcastle, Delaware, USA). In order to calibrate the gene copy number for a known amount of PCR product, we used the following equation: gene copy number = {[(0.978 × 10^9) × DNA content (pg)]/PCR product size (bp)} [31]. The optimal concentrations of forward and reverse primers were determined by titrating 1, 5, and 10 µmol concentrations (data not shown). The real-time PCR reactions (25 µl) contained 2-µl template cDNA sample or

Table 1. Polymerase chain reaction primer sequences and predicted product lengths

Target mRNA	Sense primer	Antisense primer	Product size (bp)	GenBank accession no.
Quail AvBD10 (RT-PCR)	5'-ATGAAGATCCTCTGCCTGCTCTTC-3'	5'-CTGCGCCGGAATCTTGGCACAGC-3'	207	HM452158
Chicken AvBD10 (real-time PCR)	5'-GGCTCAGCAGACCCACTTTTCC-3'	5'-CTGCGCCGGAATCTTGGCAC-3'	146	NM_001001609
Quail AvBD10 (real-time PCR)	5'-CTGTTCTCCTCTTCCTCTTCCAG-3'	5'-AATCTTGGCACAGCAGTTTAACA-3'	169	HM452158
Chicken β-actin (real-time PCR)	5'-GCGCTCGTTGTTGACA-3'	5'-TCATCCCAGTTGGTGACA-3'	224	L08165
Quail 18S rRNA (real-time PCR)	5'-TGATTAAGTCCCTGCCCTTT-3'	5'-CCGAGGACCTCACTAAACCA-3'	72	AF173611
Chicken (quail) AvBD10 (protein expression)	5'-GGATCCATG TTCCTCTTCCTC-3'	5'-GCGGCCGCTACTGCGCCGGAATCTTGGC-3'	189	NM_001001609/ HM452158

a known concentration of standard plasmid, 12.5 µl of 2× SYBRIIGreen, 8.5-µl nuclease-free water, and 1 µl (5 µmol) of each primer. The primers are shown in Table 1. The reaction was performed at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The PCR amplification was followed by melting curve analysis in which the temperature was decreased from 95 to 65 °C at a rate of 0.2 °C/s, with continuous monitoring of the decline in fluorescence. All gene amplifications were performed in triplicate. Only PCR products that showed the unique melting temperature, which confirmed a unique PCR product, were retained for further quantitative automated analysis with the Opticon Monitor 3 software (Bio-Rad). Standard curves were created by plotting the threshold cycle (Ct) *versus* copy number, with the DNA serially diluted from 4.0×10^3 to 10^{11} copies per microliter. The quantification of the PCR products in the samples was performed from the respective standard curves. The results were finally expressed for each sample as the copy number of each target cDNA normalized to 10^8 times the copy number of the control gene, 18S rRNA for quails and β-actin for layer hens, using the formula (target gene cDNA copy number/control gene cDNA copy number) $\times 10^8$, and analyzed using GeNORM [32]. Furthermore, the specificity of the reaction was checked by cloning and sequencing of three independent PCR products along with the melting curve analysis.

Protein Expression and Purification

The DNA fragments that encoded the quail and chicken AvBD10 genes, respectively, were amplified by PCR from the plasmids described above using the primers for protein expressions showed in Table 1. The PCR products, which contained the quail or layer hen AvBD10 partial signal sequence (11 amino acids) and full mature AvBD gene coding sequences flanked by *Bam*HI and *Not*I restriction sites, were inserted into the pGEX-6p-1 vector (Amersham, South San Francisco, CA 94080, USA) at the *Bam*HI and *Not*I sites, and the resultant plasmid was designated quail or layer hen rAvBD-pGEX and sequenced again. The constructs confirmed to contain the quail or layer hen AvBD gene were transformed into competent *E. coli* BL21 (DE3) cells. Expression of the fusion proteins was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG), and the proteins were purified using a purification and refolding kit (no. 70123-3; Novagen, Darmstadt, Germany), according to the manufacturer's instructions. The fusion proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V, using the Mini-protean III system (Bio-Rad), and stained with Coomassie brilliant blue R-250 [33]. The concentration of purified protein was determined by the Bradford method using bovine serum albumin as the standard [34]. In addition, both recombinant proteins were subjected to matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry analysis for sequence confirmation.

Peptide Synthesis

A linear N-terminal acetylated form of the predicted mature quail and chicken AvBD10 peptides were custom synthesized and purified with high-performance liquid chromatography at GL Biochem Ltd, Shanghai, China (http://www.glschina.com/en/profile.htm).

Antimicrobial Activity

Colony counting assays were performed to investigate the antimicrobial activities of glutathione S-transferase (GST), recombinant GST-tagged AvBD10s and synthetic AvBD10s of quail and chicken, against three strains of Gram-positive bacteria [*Micrococcus tetragenus* (ATCC 2835), *Lactobacillus* sp. (ATCC 33222), and *Staphylococcus aureus* (ATCC 29213)] and eight strains of Gram-negative bacteria [*Bordetella bronchiseptica* (S80103), *Proteus mirabilis* (ATCC 29245), *Pseudomonas aeruginosa* (ATCC 9027), *Pasteurella multocida* (ATCC 6529), *E. coli* BL21(DE3), *Salmonella pullorum* (C79-11-S11), *Salmonella choleraesuis* (CVCC 2140), and *Salmonella enterica* (ATCC 3021)] according to previous reports [28]. Briefly, the bacterial strains were cultured to the mid-logarithmic growth phase. Final dilutions were prepared in minimal media (diluted 1000-fold in distilled water) to reach a cell density of 2.5×10^6 colony-forming units (CFU)/ml. The antimicrobial activities of both recombinant and synthetic AvBDs were determined by the colony counting assay. A diluted bacterial culture (10 µl) was treated with 250 µl of AvBD10s (0 to 100 µg/ml in phosphate buffered saline) in polypropylene microtiter plates and preincubated for 3 h at conditions suited to the strain investigated; 100 µl of minimal medium was added after 3 h, and the plates were incubated for a further 12–18 h. The sample was rediluted 100-fold to 10^6-fold in a minimal medium and transferred to Tryptose Soya Agar plates, and the colonies were counted after 24 h of incubation. Bactericidal activity was calculated as a percentage of the CFU of bacteria not exposed to AMPs but subjected to the same experimental conditions. Results from the antimicrobial assay represent the mean of the three independent experiments with three replicates per experiment.

Statistics

Data are expressed as the mean ± SD. Statistical analyses, where appropriate, were performed using one-way analysis of variance using the General Linear Models procedure of SAS software [35]. A difference with $P < 0.05$ was considered to be statistically significant.

The nucleotide sequence obtained in this study is available from GenBank under the accession number HM452158.

Results

Cloning and Identifying of a Novel AvBD in Quail

A novel AvBD was cloned and sequenced from liver and bone marrow tissues of quail and compared with the published AvBDs. The complete nucleotide sequence of the cDNA contained a 207-bp ORF that encoded 68 amino acids, with a high isoelectric point (pI) value (+7.95) (Figure 1). Multiple alignment of the novel AvBD with chicken and duck AvBDs (Figure 1) showed that all the proteins have a signal peptide of 22 amino acids, a signature motif of six conserved cysteines, and two GXC motifs that are conserved across all β-defensins (Figure 1). Using the Clustal V routine of the MEGALIGN program, we determined the identity and homology between the novel AvBD and other AvBDs. The highest percentage similarities were shown between the novel AvBD and chicken AvBD10 (83.8%), with 57 identical amino acid residues, and between the novel AvBD and duck AvBD10 (80%), with 47 identical amino acid residues. Based on these analyses, the novel AvBD was identified conclusively as a β-defensin ortholog in quails and was named quail AvBD10,
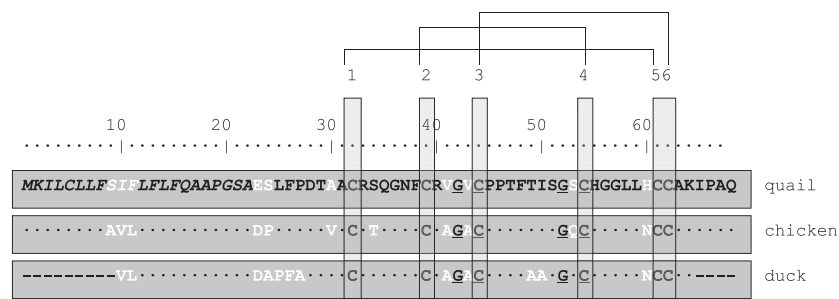
Figure 1. Deduced amino acid sequence alignment of the quail AvBD10 with related AvBD10s from chicken and duck. Signal sequences of quail AvBD10 are italic. The conserved six cysteines (C) are framed and linked to show intramolecular disulfide bond connectivity. The conserved Gly–Xaa–Cys triads, which define the β-defensin structure, are underlined. The amino acids that differ among quail, chicken, and duck AvBD10 are in white. *Dots* indicate identical residues. *Dashes* indicate no identical or conserved residues observed.

considering its similarity with chicken and duck AvBD10, according to the nomenclature suggested for AvBDs [36]. However, the novel AvBD showed a low percentage similarity (35.8%) with the recently reported quail AvBD9, with 27 identical amino acid residues only.

Phylogenetic analysis was carried out, and the results are shown as a dendrogram in Figure 2 to illustrate the evolutionary relationships between quail AvBD10 and other β-defensins at the amino acid level. Quail AvBD10 is grouped together in a single subcluster with AvBD10s from chicken and duck, and with zebra finch (*Taeniopygia guttata*) β-defensin 10.

Tissue Distribution

Constitutive expression of quail AvBD10 was detected by real-time RT-PCR in various tissues from three healthy quail (Figure 3). It was expressed widely in all tissues of the quail except the tongue, crop,
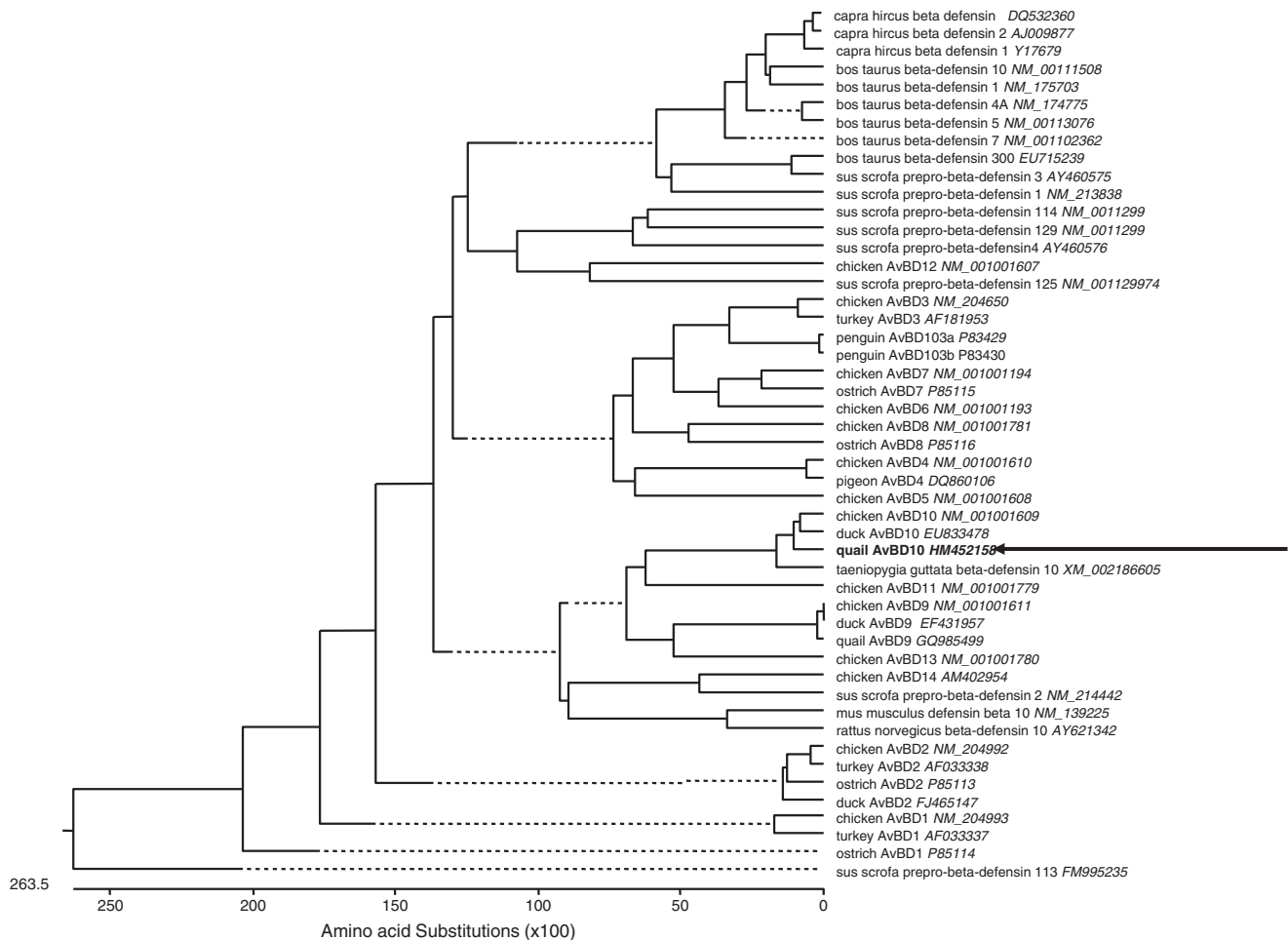
Figure 2. Phylogenetic relationships based on the sequences of the quail AvBD10, other AvBDs, and β-defensins from some mammalian species using the MEGALIGN program DNAStar with Clustal V [29]. The sequence identified in the current study is indicated by an arrow. Accession numbers of the sequences are shown in the figure in italic.
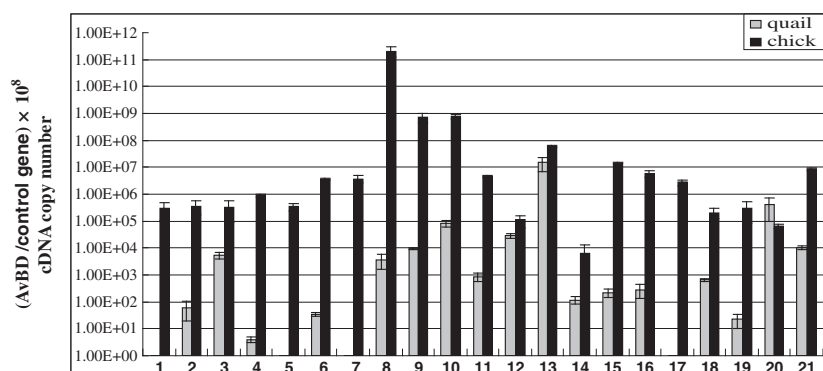
Figure 3. Expression of AvBD10 mRNA in various tissues from 80-day-old quails and 50-week-old layer hens. The AvBD cDNA copy number was measured by real-time RT-PCR in three samples. 1, tongue; 2, muscular stomach; 3, glandular stomach; 4, skin; 5, crop; 6, trachea; 7, breast muscle; 8, kidney; 9, lung; 10, liver; 11, heart; 12, cecal tonsil; 13, bone marrow; 14, bursa of Fabricius; 15, Harderian gland; 16, spleen; 17, thymus; 18, esophagus; 19, rectum, 20, small intestine; 21, ovary. Only positive bars were presented. Three independent experiments were performed with three replicates per experiment, and each bar is the mean $\pm$ SD. The data were analyzed using SAS [35].

breast muscle, and thymus. The highest level expression was seen in the bone marrow. A moderate level of expression was seen in the lung, liver, cecal tonsil, small intestine, and ovary. A low level of expression, but consistently detectable, was seen in the other tissues investigated. To compare the pattern of expression of AvBD10 in tissues between quails and chickens, tissues from three healthy layer hens were investigated by real-time RT-PCR. The sequences of AvBD10 cloned from the tissues of layer hens in this study were identical with the chicken AvBD10 sequence in the GenBank database (data not shown). In contrast to the pattern of expression of AvBD10 in tissues from quails, the chicken AvBD10 was expressed in all of the 21 tissues from the layer hens investigated, with a high level of expression in the kidney, lung, liver, bone marrow, and Harderian glands; a moderate level of expression in the tongue, muscular stomach, glandular stomach, skin, crop, trachea, breast muscle, heart, cecal tonsil, spleen, thymus, esophagus, rectum, small intestine, and ovary; and a low level of expression in the bursa of Fabricus of layer hens. A higher level of expression of AvBD10 was found in tissues from layer hens in comparison with those from quails, except for the small intestine ($P < 0.05$ or $P < 0.01$).

## Expression and Purification of Quail and Chicken Recombinant glutathione S-transferase-tagged AvBD10s

High levels of expression of both recombinant GST-tagged AvBD10s were noted in *E. coli* after induction with 0.6 mmol/l IPTG (Figure 4). Both recombinant GST-tagged AvBD10s (molecular weight, 32 kDa) were produced as inclusion bodies (Figure 4). The GST-tagged AvBD10s were purified and visualized as a pronounced band on SDS-PAGE gels (Figure 4). The concentrations of purified GST-tagged AvBD10s of quails and layer hens were 1600 and 1530 μg/ml, respectively.

Antibacterial Activity

The survival of a wide range of bacteria treated with GST, quail or chicken recombinant GST-tagged AvBD10, and quail or chicken synthetic AvBD10 peptides was determined using colony counting. The data are depicted in Figure 5 and Table 2. It was shown that GST showed no bactericidal activity against all of the bacterial strains investigated. However, both recombinant GST-tagged
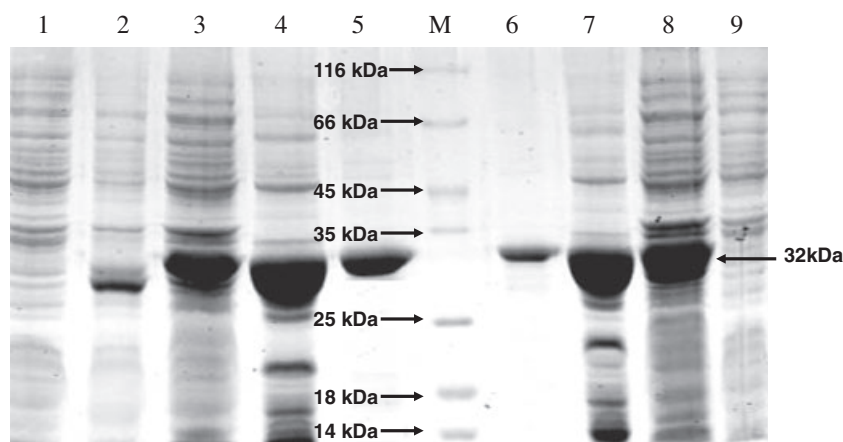
Figure 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of recombinant GST-tagged quail and chicken AvBD10 proteins expressed in *E. coli* BL21(DE3) cells. Lane M, protein molecular weight marker; lane 1, total protein of BL21 containing chicken AvBD10 without IPTG induction; lane 2, total protein of BL21 containing GST after induction with IPTG; lane 3, total protein of BL21 containing chicken recombinant GST-tagged AvBD10 after induction with IPTG; lane 4, inclusion bodies with chicken recombinant GST-tagged AvBD10; lane 5, purified chicken GST-tagged AvBD10 protein after induction with IPTG; lane 6, purified protein of quail recombinant GST-tagged AvBD10 after induction with IPTG; lane 7, inclusion bodies with quail recombinant GST-tagged AvBD10; lane 8, total protein of BL21 containing quail recombinant GST-tagged AvBD10 protein after induction with IPTG; lane 9, total protein of BL21 containing quail recombinant GST-tagged AvBD10 without IPTG induction; IPTG, isopropyl-beta-D-thiogalactoside.
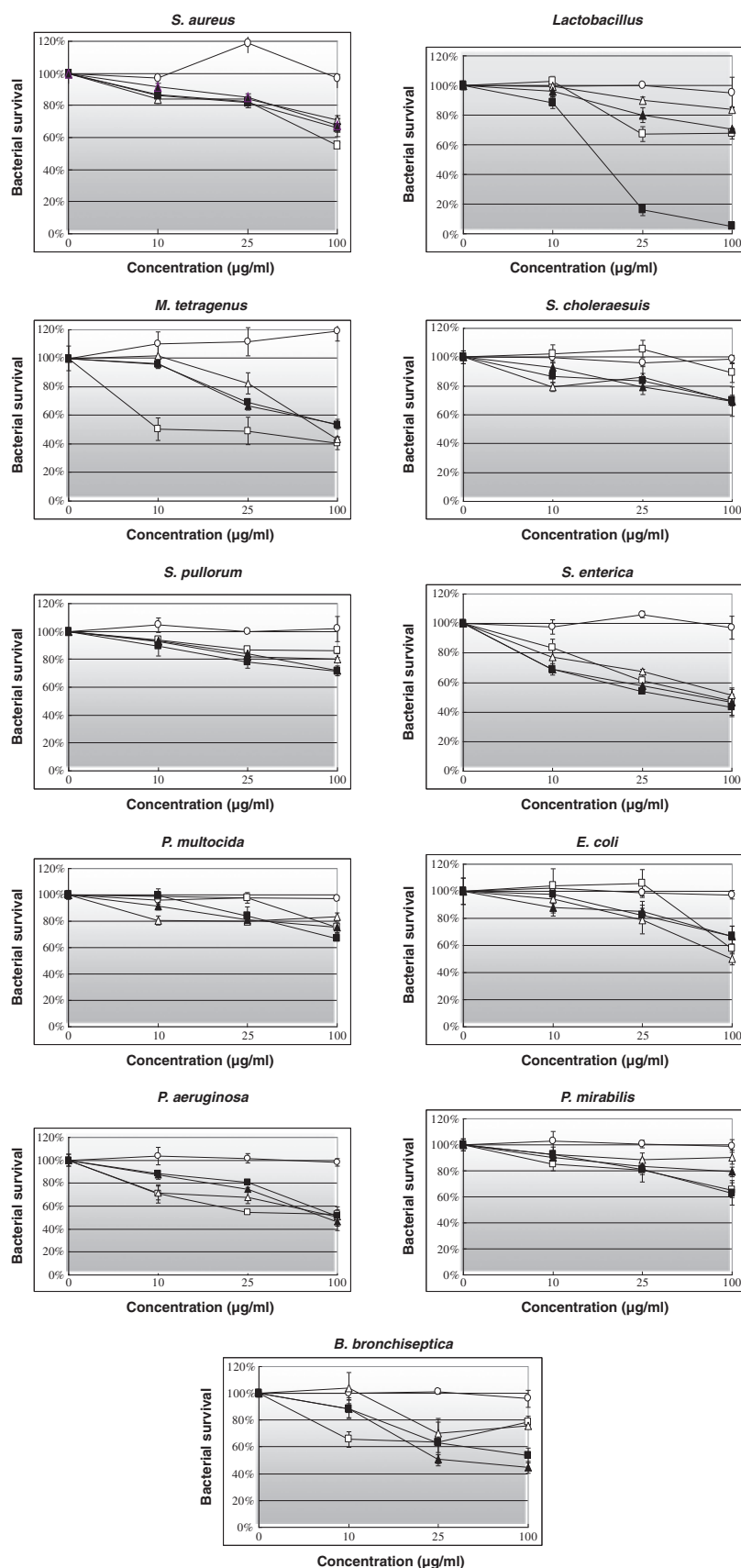
Figure 5. Antimicrobial activities of GST (○), quail recombinant GST-tagged AvBD10 (rAvBD10) (□), chicken rAvBD10 ( ), quail synthetic AvBD10 (sAvBD10) (■), and chicken sAvBD10 (▲). Bactericidal activity was calculated as the percentage of colony counts of bacteria not exposed to AMPs but subjected to the same experimental conditions. All kill–curve studies were performed in three independent experiments with three replicates per experiment. Each point is the mean ± SD. The data were analyzed using SAS [35].

Table 2. Antimicrobial activities (bacterial survival) of 100 µg/ml GST, quail recombinant GST-tagged AvBD10, chicken rAvBD10, quail synthetic AvBD10, and chicken sAvBD10

Bacterial strains	GST	Quail rAvBD10	Chicken rAvBD10	Quail sAvBD10	Chicken sAvBD10
S. aureus	97.23% ± 2.11%	54.85% ± 2.00%	71.16% ± 2.50%	65.90% ± 5.36%	67.65% ± 4.40%
Lactobacillus	95.16% ± 10.40%	67.56% ± 3.75%	84.14% ± 1.56%	49.57% ± 0.45%	70.73% ± 5.15%
M. tetragenus	118.78% ± 6.49%	40.61% ± 4.35%	44.00% ± 1.14%	53.49% ± 1.96%	53.74% ± 3.62%
S. choleraesuis	98.51% ± 2.71%	89.04% ± 6.60%	69.31% ± 10.30%	69.93% ± 3.77%	69.15% ± 2.43%
S. pullorum	101.81% ± 8.99%	86.03% ± 2.22%	80% ± 1.30%	71.72% ± 3.58%	72.09% ± 1.33%
S. enterica	97.07% ± 7.85%	47.37% ± 4.72%	51.41% ± 4.13%	43.24% ± 5.20%	46.50% ± 9.70%
P. multocida	97.34% ± 1.56%	75.40% ± 2.52%	84% ± 2.89%	66.92% ± 3.83%	75.44% ± 4.07%
E. coli	97.36% ± 3.21%	57.55% ± 3.56%	50.35% ± 4.70%	66.69% ± 7.30%	66.69% ± 7.30%
P. aeruginosa	98.15% ± 3.26%	52.88% ± 3.63%	50.48% ± 5.77%	51.17% ± 8.39%	46.42% ± 7.84%
P. mirabilis	99.05% ± 4.95%	65.00% ± 5.74%	90.43% ± 5.74%	63.13% ± 9.64%	79.24% ± 3.64%
B. bronchiseptica	96.09% ± 6.31%	78.76% ± 4.05%	76.00% ± 0.21%	53.56% ± 5.41%	44.92% ± 4.45%

rAvBD10, recombinant GST-tagged AvBD10; sAvBD10, synthetic AvBD10.

AvBD10s showed similar bactericidal activity to respective synthetic AvBD10s against most of the bacterial strains investigated. Furthermore, all of AvBD10s showed similar antibacterial activity against *S. aureus*, *S. enterica*, *E. coli*, *P. aeruginosa*, and *B. bronchiseptica*. However, the quail synthetic AvBD10 showed higher bactericidal activity against *Lactobacillus* sp. than others ($P < 0.01$). In order to verify the results, several time experiments were repeated. The quail GST-tagged AvBD10 showed higher bactericidal activity against *M. tetragenus* than others ($P < 0.05$). Low bactericidal activity of all of AvBD10s against *P. multocida*, *P. mirabilis*, and *S. pullorum* was found. In addition, no significant bactericidal activities were found for quail GST-tagged AvBD10 against *S. choleraesuis* and for chicken GST-tagged AvBD10 against *P. mirabilis* ($P > 0.05$).

Discussion

The present study has shown that quail possesses multiple β-defensin genes, as is known to occur in other animals. A single β-defensin gene has been reported previously in quail, referred to as quail AvBD9, and this is clearly related to the other AvBD9 and mammalian β-defensin-9 genes identified to date [28]. Although the exact relationship between quail AvBD9 and AvBD10 is unclear, they both possess two apparent characteristics of β-defensin molecules, a signature motif of six conserved cysteines, and two GXC motifs. Identical with other AvBDs, quail AvBD10 has a clear signal peptide (22 amino acid residues), which suggests that it is secreted, and a positive charge typical of AMPs.

Phylogenetic analysis of the amino acid sequences of β-defensins revealed that there are three major clusters, and the avian and mammalian defensins were distributed uniformly in these clusters. Quail AvBD10 grouped together in a single subcluster with AvBD10s from chicken and duck, and with zebra finch (*T. guttata*) β-defensin 10, pointing to a very close evolutionary relationship and a common ancestry of these avian genes. Moreover, the clustering of certain AvBD genes, namely, that of chicken AvBD12, with the mammalian β-defensins, including some goat, cattle, and porcine β-defensins, suggested that β-defensins might be originated from a common β-defensin-like gene. This may have existed before the earliest common ancestor of birds and mammals, which was estimated to be approximately 310 million years ago [26,37].

Analysis of the sites of expression of AvBD10s revealed different expression pattern between quail and chicken in the current study. Quail AvBD10 was expressed in all tissues of the quail except the tongue, crop, breast muscle, and thymus. However, the chicken AvBD10 was expressed in all 21 of the tissues from the layer hens investigated. The difference could be due to variability in β-defensin expression between individual animals or choice of breed. Although a trace amount of quail AvBD10 mRNA was detectable in other nonepithelial quail tissues, very strong mRNA expression was noted in the bone marrow. A number of AvBDs, including chicken and duck AvBD10, have been reported to show strong expression in bone marrow [21,26]. The present findings with respect to chicken AvBD10 confirm these reports. The myeloid defensins, also classified as avian heterophil β-defensins in birds, are crucial to combat invading pathogens and to protect epithelial surfaces. Avian heterophils are deficient in oxidative mechanisms for pathogen destruction [38]. Hence, the nonoxidative mechanisms employed by these peptides [39] may play a major role in the innate immune responses of birds. In addition to high-level expression in bone marrow, the extensive expression of the AvBD10s of both quail and chicken in tissues of immunological importance, such as the cecal tonsil, bursa of Fabricius, Harderian gland, and spleen, implies that apart from direct antimicrobial activity, they may play a role in recruiting immune cells to the sites of pathogen entry, thereby facilitating the development of the adaptive immune response. Defensins have been studied extensively for their chemotactic ability for immature dendritic cells and memory T cells [40]. Among avian defensins, only duck AvBD2 was found to exhibit chemotactic activity to the DT-40 cells [23]. Thus, it is possible that AvBD10s may play a similar role in both quail and layer hens. Apart from tissues of immunological importance, quail AvBD10 was expressed widely in all the tissues of quail except the tongue, crop, breast muscle, and thymus. In contrast, the chicken AvBD10 was expressed widely in all the tissues from layer hens, which is in agreement with the results reported previously for chickens [16,26]. The wide expression of AvBD10s in the tissues of both quail and layer hens indicates that AvBD10s may contribute to the innate immune defense of these tissues in both species. However, quail AvBD10 may lack such roles in the tongue, crop, breast muscle, and thymus of quail.

The functional consequences of the differences in the amino acid sequence of quail AvBD10 compared with chicken AvBD10

(and other AvBDs) are hard to predict. Up to date, besides being presented in both chicken and quail, AvBD10 has been identified in duck [21]. Although differences in the amino acid sequence of quail AvBD10 compared with AvBD10s from both chicken and duck exist (Figure 1), both of quail and chicken AvBD10s exhibited similar antimicrobial activities against most of the bacteria investigated (Figure 5), which is consistent with the results reported recently for quail AvBD9 [28] and for other AvBDs including duck AvBD10 [19–23,27]. These results suggested that differences in the amino acid sequence of AvBD10s from quail, chicken, and duck have little influence on their antimicrobial activity. It is to be mentioned that partial signal peptide was included in both recombinant AvBD proteins. Initially, we used the sequence that had no amino acids in signal peptides to prepare recombination and found that the level of expression is considerably low (data not shown). Subsequently, when we added a part of the signal peptide, the level of expression became sufficiently high to allow purification, though the reason needed to be further investigated. Interestingly, both recombinant AvBD proteins containing partial signal peptide displayed similar antimicrobial activity with respect to synthetic proteins based on mature peptide. Similar studies have been found on expression of duck AvBD2 by Soman *et al.* [23] and quail AvBD9 [28]. These results suggested that recombinant AvBD proteins containing partial signal peptide have little effect on their antimicrobial activity. It is interesting that quail synthetic AvBD10 showed higher antimicrobial activity against *Lactobacillus* sp. than other peptides investigated in the present study. We repeated these experiments several times to verify the results. The reason remains to be studied further. In addition, the antimicrobial activities of AvBD10s in the present study were lower than quail AvBD9 in our previous study [28]. The difference may be partly due to the high pI value of quail AvBD9. Sequence analysis revealed that the quail AvBD9 was cationic with a higher pI value (+8.29) than quail AvBD10 (pI value, +7.95), which has been hypothesized to be the major factor that defines the antimicrobial activity of AvBDs [41]. These molecules kill microorganisms by means of permeabilization of the microbial membrane, which is composed of negatively charged components such as phospholipids, teichoic acids, and lipopolysaccharides [42,43]. It is believed that electrostatic interactions dictate not only the uptake of cationic defensins across the bacterial cell wall but also their ability to permeabilize the cytoplasmic membrane and to induce leakage of cellular contents [44].

Although the reports of the activity of other AvBDs cannot be compared directly with our own findings owing to different experimental set-ups and the use of different activity assignments, it is clear that the antimicrobial activity of these orthologs is comparable. Furthermore, the observed antimicrobial activity of quail AvBD10 is in a similar range to that of its ortholog in layer hens, and defensins from other avian species [19–23,27]. In analogy with these, usually better-studied peptides, it is likely that quail AvBD10 fulfills an important antimicrobial role in the quail.

In conclusion, we identified, for the first time, a novel AvBD from quail, referred to as quail AvBD10. Quail AvBD10 has been shown to be expressed widely in quail tissues. In addition, the peptide exhibits antimicrobial activity against a broad range of bacteria. These results indicate that quail AvBD10 could play an important role in the innate immune response. Stimulation of the production of quail AvBD10 in the tissues through, for example, dietary modulation could provide a means to increase the health of quail, thereby reducing the occurrence of infections in quail and the subsequent use of antibiotic treatment.

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