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Functional analysis and induction of four novel goose (*Anser cygnoides*) avian β-defensins in response to *salmonella enteritidis* infection

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ABSTRACT

In the current study, four novel avian β-defensins (AvBDs) (AvBD2, 5, 9, and 10) were identified in tissues from the Chinese goose (*Anser cygnoides*). The antibacterial activity of the AvBDs showed that all of these AvBDs exhibited antibacterial activity against most of the bacteria investigated ($P < 0.01$). In addition, antibacterial activity of all of the AvBDs against *Staphylococcus aureus* and *Pasteurella multocida* decreased significantly or was completely abolished at 150 mM NaCl ($P < 0.01$). None of the AvBDs showed hemolytic activity. AvBD2 and AvBD10 were expressed widely, whereas AvBD5 and AvBD9 mRNAs were expressed in a limited number of geese tissues. AvBD9 was significantly induced in some immune tissues from geese after *Salmonella enteritidis* infection. The others were significantly upregulated in small intestine and some immune tissues of the geese ($P < 0.01$). The present results suggest that the AvBDs are part of the host defense mechanism of the goose.

1. Introduction

Antimicrobial peptides (AMPs) have been recognized as key mediators of the innate immune response in many animal species, and they provide the first line of defense against potential pathogens. These peptides are capable of killing a wide variety of bacterial and viral pathogens directly. Despite broad divergence in sequence and taxonomy, most AMPs share a common mechanism of action that involves permeabilization of the pathogen cell membrane [1]. One major subclass of AMPs is the group of defensins. Defensins are cysteine-rich antimicrobial peptides that vary in length from 18 to approximately 100 amino acids and are enriched in hydrophobic and cationic amino acid residues [1]. In addition to their direct antimicrobial activities, immunomodulatory properties have also been demonstrated. Defensins can promote adaptive immunity by the selective recruitment by chemotaxis of monocytes [2], T lymphocytes [3], immature dendritic cells [4] and mast cells [5] to sites of inflammation. In vertebrates, three different defensin subfamilies (α, β and θ) exist, which differ in the disulfide bridge [6]. In poultry, only the β-defensins, which have bonds between C1—C5, C2—C4, and C3—C6, have been reported to exist [7].

Avian β-defensins (AvBDs) have been studied extensively in birds, and so far over 30 AvBDs have been identified in several avian species [7–11]. AvBDs attack a wide range of microorganisms including Gram-positive and Gram-negative bacteria, fungi and yeasts [12]. However, to date, there is no report of the characterization of AvBDs from geese.

The present study describes the functional characterization of four novel AvBDs derived from the Chinese goose (*Anser cygnoides*). These molecules exhibited antibacterial activity against both Gram-positive and Gram-negative

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bacteria. In addition, the influence of ionic strength on the antibacterial activity and hemolytic activity of these *anser_AvBD*s is described. Finally, the effect of *Salmonella* infection on mRNA expression of these four AvBDs in the tissues of geese was studied.

2. Materials and methods

2.1. Animals

Thirty-five 1-day-old healthy female Chinese geese 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.

2.2. RNA extraction, reverse transcriptase polymerase chain reaction amplification, and sequencing

Approximately 1 g of spleen, bone marrow, and kidney tissue obtained from five healthy 15-day-old geese was used to process tissue fluid, and the total cellular RNA was extracted from 100 μl aliquots of the respective tissue fluid using TRIzol reagent (Invitrogen, Beijing, PR China) according to the manufacturer's instructions. Reverse transcriptions (RT) - PCR were performed according to previous study [10] with four sets of primers (Table 1), respectively. 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 were cloned into the pMD18-T vector (TAKARA) to confirm amplification, followed by sequencing of the recombinant plasmids.

2.3. Sequence analysis of anser_AvBDs

Basic searches were conducted with a local alignment search tool (BLAST) using the four entire AvBDs from the geese. Sequences of the other known AvBDs, and some mammalian β-defensins were selected for sequence comparison with the four novel *anser_AvBD*s. 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) [13]. The signal peptides of the four novel *anser_AvBD*s were analyzed using the SignalP 3.0 server (http://www.cbs.dtu.dk/Services/signalP).

2.4. Protein expression and purification

The DNA fragments, which encoded these *anser_AvBD*s were amplified by PCR from the plasmids described above using the primers for protein expression shown in Table 1. The PCR products were inserted into the pGEX-6p-1 vector (Amersham), transformed into competent *Escherichia coli* BL21 (DE3) cells, and then induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG). The proteins were purified using a purification and refolding kit (no. 70123-3; Novagen, Darmstadt, Germany), according to

Table 1
PCR primer sequences and predicted product lengths.

Target mRNA	Sense primer	Antisense primer	Product size (bp)	GenBank accession no
Anser AvBD2 (RT-PCR)	5′-TGGCTCAGCAGATCTGCA-3′	5′-GAATAAATTGCCATTGCG-3′	420	HQ909024
Anser AvBD5 (RT-PCR)	5′-ATGCAGATCCTGCCTCTCCTCTTTGCT-3′	5′-TCAGGAATACCATCGGCTCCGGCAGCAGAA-3′	201	HM452158
Anser AvBD9 (RT-PCR)	5′-ATGAGAATCCTTTTCTTCCTTGTTGC-3′	5′-TTAGGAGCTAGGTGCCCATTTGCAGC-3′	204	HQ909023
Anser AvBD10 (RT-PCR)	5′-ATGAAGATCCTCTGCCTGCTCTTC-3′	5′-CTGCGCCGGAATCTTGGCACAGC-3′	204	HQ909025
Anser AvBD2 (real time PCR)	5′-GATTGTCTTCGCCCCACGCGGGA-3′	5′-TTATACATCCCATGGCGATTTG-3′	137	HQ909024
Anser AvBD5 (real time PCR)	5′-GCTGTCCCTTCCTCGACGATC-3′	5′-GCAATACCATCCGCTCCGGC-3′	139	HM452158
Anser AvBD9 (real time PCR)	5′-GCTTACAGCCAAGGAGATGCT-3′	5′-GGAGCTAGGTGCCCATTTGCA-3′	145	HQ909023
Anser AvBD10(real time PCR)	5′-GGTTCTGCCGATGCGCCTTTTG-3′	5′-CTGCCGCCGGAATCTTGGCAC-3′	146	HQ909025
Anser 18S rRNA (real time PCR)	5′-TCCCAGTAAGCGCGAGTCAT-3′	5′-ACGGGCGGTGTGTACAAAG-3′	65	AB064942
Anser AvBD2 (protein expression)	5′-CGCGAATTCATGAGGATCCTTTAC-3′	5′-GCGGTCGACTGGAAGAAATTTTCA-3′	247	HQ909024
Anser AvBD5 (protein expression)	5′-GGATCCCCGAATTCATGCAGATCC TGCC T CTC-3′	5′-GTCGACTCAGGAATACCATCCGCTCCGGCA-3′	201	HM452158
Anser AvBD9 (protein expression)	5′-GAATTCATGGCTGTTCTCTTCTTCCTC-3′	5′-GTCGACTTAGGAGCTAGGTGCCCATTTGCAGC-3′	201	HQ909023
Anser AvBD10 (protein expression)	5′-GGATCCATGGCTGTTCTCCTCTTCCTC-3′	5′-GCGGCCGCTACTGCGCCGGAATCTTGGC-3′	182	HQ909025