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Identification of three novel avian beta-defensins from goose and their significance in the pathogenesis of *Salmonella*

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ABSTRACT

Here, we report the characterization of three avian β-defensins (AvBDs) from the goose, named *anser*_AvBD1, AvBD3, and AvBD6, respectively. All of *anser*_AvBDs exhibited broad antibacterial activity. In addition, the antibacterial activity of all of the AvBDs against *Staphylococcus aureus* and *Proteus mirabilis* decreased significantly in the presence of 100 mM NaCl ($P < 0.01$). None of the AvBDs showed hemolytic activity. In order to assess the significance of these *anser*_AvBDs in the infection of *Salmonella enteritidis*, mRNA expression of Toll-like receptor (TLR) 4 and *anser*_AvBDs in tissues of both control and infected geese was evaluated. We observed a significant up-regulation of TLR4, *anser*_AvBD1, 3, and 6 in some immune tissues evaluated, in response to *S. enteritidis* infection. These data demonstrated that TLR4 may serve a possible role in eliciting host immune responses to pathogens, and β-defensins may play a pivotal role in the host defense mechanisms of the goose.

1. Introduction

The extensive world-wide morbidity and mortality caused by microbial disease highlights the need for new insights into the host immune response, and novel treatment approaches. Antimicrobial peptides (AMPs) are structurally diverse innate immune molecules that provide protection against infection for all classes of life (Menendez and Brett Finlay, 2007). In the past two decades multiple AMPs have been identified that are produced by many different organisms, ranging from animals to plants. Most AMPs have a low molecular weight (<10 kDa), are membrane-active and display hydrophobic and/or cationic properties. Based on their structural characteristics, AMPs can be divided into several groups, mainly α-helical peptides (*e.g.* cecropin), cysteine-rich peptides (*e.g.* defensin), proline-rich peptides (*e.g.* drosocin), and glycine-rich peptides (*e.g.* hymenoptaecin) (Reddy et al., 2004). The antimicrobial activity of most of AMPs comes from the ability of these molecules to insert into the microbial membrane, resulting in membrane destabilization and microbial lysis (Radek and Gallo, 2007; Richards et al., 2012). These peptides are produced rapidly by neutrophils, macrophages and epithelial cells in response to infection or injury and can mediate inflammation and stimulate the immune system upon detection of pathogens (Menendez and Brett Finlay, 2007).

Among these naturally occurring AMPs, defensins form a unique family of cysteine-rich, small cationic peptides. Defensins act as a first line of defense against invading pathogens and their antimicrobial activity relies on non-oxidative mechanisms (Sahl et al., 2005). Some defensins are also chemoattractant for monocytes, lymphocytes and dendritic cells, and thus they act as a link between innate and adaptive immune responses (Ganz, 2003). Based on the spacing pattern of cysteines, defensins from vertebrates are divided broadly into three sub-families, namely α-, β-, and θ-defensins (Ganz, 2003). Numerous β-defensins have been identified in birds, and are named avian β-defensins (AvBDs), which are now considered to be one of the key components of innate immunity in avian species. They display a wide range of microbicidal or microbiostatic activities against Gram-negative and Gram-positive bacteria, fungi, and viruses (van Dijk et al., 2008; Ma et al., 2009a,b, 2011, 2012a,b; Wang et al., 2010). β-Defensins have been found to be expressed constitutively or inducibly by neutrophils and epithelial cells from many mammals and birds, including the goose (Selsted and Ouellette, 2005; van Dijk et al., 2008; Derache et al., 2009; Ma et al., 2012a). The interest in defensins as therapeutic drugs is growing because defensins may be an alternative to the controversial use of antibiotics. In birds, a potential use of these peptides has

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Table 1
PCR primer sequences and predicted product lengths.

Target mRNA	Sense primer (5′–3′)	Antisense primer (5′–3′)	Product size (bp)	GenBank accession no
*anser*_AvBD1 (RT-PCR)	AAACCATGCGGATCGTGTACCTGC	ATGGGGGTTGTTTCCAGGAGC	264	JQ359443
*anser*_AvBD3 (RT-PCR and real time PCR)	GAACTGCCACTCAGTGCAGAAT	ATGGGGGTTGTTTCCAGGAGC	183	–
*anser*_AvBD6 (RT-PCR)	ATGAGGATCCTTTACCTG	TCAGGCCCACCTGTTCCT	204	JQ359442
anser 18S rRNA (RT-PCR and real time PCR)	TCCCAGTAAGCGCGAGTCAT	ACGGGCGGTGTGTACAAAG	65	AB064942
*anser*_AvBD1 (real time PCR)	GAAACAAGGAGAAATGTCATCG	ATGGGGGTTGTTTCCAGGAGC	183	JQ359443
*anser*_AvBD6 (real time PCR)	GTCAGCCCTACTTTTCCAGC	GCCCACCTGTTCCTCACAC	143	JQ359442
*anser*_AvBD1 (protein expression)	GAATTCATGCGGATCGTGTACCT	GTCGACTCAACCAAATATC	210	JQ359443
*anser*_AvBD3 (protein expression)	GGATCCATGACTGCCACTCAGTG	GTCGACTCAATGGGGGTTGTTTC	198	–
*anser*_AvBD 6 (protein expression)	GAATTCATGAGGATCCTTTACCTG	GTCGACTCAGGCCCACCTGTTCCT	216	JQ359442

been proposed in particular to fight antibiotic-resistant bacteria, including *Salmonella*, a major zoonotic agent that causes food poisoning (EFSA, 2007). In previous studies, it was shown that several AvBDs from different avian species were highly expressed in the intestinal tissue of birds that are resistant to *Salmonella* colonization (Sadeyen et al., 2006; Ma et al., 2012a). In addition, most AvBDs can either be expressed constitutively or be induced in response to microbial infections, including those involving *Salmonella*, and their regulation is often dependent on the site of synthesis (van Dijk et al., 2008; Ma et al., 2011, 2012a). The complex interplay between host immune factors and bacterial defense systems during the early stages of *Salmonella* infection is still poorly understood. β-Defensins are likely to be one of the earliest-encountered components of the immune system. They protect the host against infection both directly, through potent bactericidal activity, and indirectly, by inducing chemotaxis of monocytes and neutrophils to the site of infection (Bader et al., 2005).

In a recent report from this laboratory, it was demonstrated that expression of four *anser*_AvBDs (2, 5, 9, and 10) were significantly upregulated in various immune tissues from geese after *Salmonella enteritidis* infection. In the present study, another three novel AvBDs from the goose (*Anser cygnoides*) have been isolated and characterized. These newly identified peptides kill both Gram-positive and Gram-negative bacteria effectively. Furthermore, we examined whether expression of Toll-like receptor (TLR) 4 and these three novel *anser*_AvBDs was altered in response to *Salmonella* infection. The upregulation of these antimicrobial peptides in several tissues, especially in immune tissues post-infection, suggests strongly that β-defensins may play a pivotal role in the host defense mechanisms of the goose.

2. Materials and methods

2.1. Animals

Thirty 1-day-old healthy female Chinese geese were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Harbin, China. The birds were maintained in isolators with negative pressure and food and water were provided *ad libitum* until they were 15 days old. The food was corn-soybean meal based, and formulated to meet or exceed the minimum requirements of geese based on NRC (1994). The main composition of the food is as following: ME (MJ/kg), 12.3; crude protein (%), 20.5; lysine (%), 1.05; methionine + cysteine (%), 0.62; calcium (%), 0.72; total phosphorus (%), 0.65. Both the food and water were sterilized before fed.

2.2. Ethics statement

All animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China.

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

Five healthy 15-day-old geese (all layers) mentioned above were euthanized by intravenous administration of sodium pentobarbitone. Approximately 1 g of each of 23 tissues, namely the skin, tongue, esophagus, larynx, glandular stomach, muscular stomach, trachea, lung, heart, liver, kidney, breast muscle, spleen, bone marrow, bursa of Fabricius, Harderian glands, thymus, cecal tonsil, small intestine, cecum, rectum, large intestine, and pancreatic tissue were obtained from the same locations. All the samples 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.

For RNA extraction, equal amount tissues (1 g) were excised in cold RNase-free phosphate buffered saline (PBS) to process tissue homogenate. The total cellular RNA was extracted from 100 μl aliquots of the respective tissue homogenate using TRIzol reagent (Invitrogen, Beijing, PR 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. To evaluate RNA quality, the optical density (OD) of RNA at wavelength 260 and 280 nm was examined, respectively. The ratio of OD260 to OD280 was within 1.8–2.2 (data not shown). Reverse transcription was carried out in a 40 μL reaction mixture containing 20 μL RNA using oligo-dT primers. All of the process was conducted under RNase-free condition. The AMP-specific cDNA was amplified by PCR using Ex-Taq polymerase and primers designed internally from three 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.

2.4. Sequence analysis of anser_AvBDs

Basic searches were conducted with a local alignment search tool (BLAST) using the three entire AvBDs from the geese. Sequences of the other known AvBDs were selected for sequence comparison with the three novel *anser*_AvBDs. The signal peptides of the three novel *anser*_AvBDs were analyzed using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP).

2.5. Protein expression and purification

The DNA fragments that encoded the *anser*_AvBDs were amplified by PCR from the plasmids described above, using the primers for protein expression shown in Table 1. The PCR products, which contained the coding sequence of either *anser*_AvBD1 or *anser*_AvBD6 flanked by EcoR I/Sal I, and *anser*_AvBD3 flanked by