Prokaryotic Expression and Biological Characterization of Recombinant Goose β-Defensin-7 Protein Postprint

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Abstract

To investigate the biological characteristics of goose β-defensin 7 (AvBD7), the goose AvBD7 gene was subcloned into the EcoR and Xho double restriction sites of the Escherichia coli prokaryotic expression vector pProEX HTa to construct the recombinant expression plasmid pProEX-AvBD7. The recombinant plasmid was transformed into Escherichia coli Rosetta competent cells, and the bacterial culture was induced for expression with isopropyl β-D-1-thiogalactopyranoside (IPTG). Analysis by N-tris(hydroxymethyl)methylglycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) revealed that the recombinant protein was approximately 10–15 kDa, consistent with the expected size. Following purification of the recombinant goose AvBD7 protein, its in vitro antibacterial activity against Escherichia coli, Salmonella pullorum, Staphylococcus aureus, Micrococcus tetragenus, and Bacillus subtilis was determined by colony counting, as well as the effect of salt ion concentration on its antibacterial activity and its hemolytic activity against chicken erythrocytes. The results showed that the recombinant goose AvBD7 protein exhibited antibacterial activity against all five tested bacterial species, and its antibacterial activity increased with protein concentration. High salt ion concentration (150 mmol/L) significantly inhibited the antibacterial activity of the recombinant goose AvBD7 protein ($P<0.05$). The recombinant goose AvBD7 protein showed no hemolytic activity against chicken erythrocytes ($P>0.05$). These findings indicate that the recombinant goose AvBD7 protein possesses broad-spectrum antibacterial activity, that high salt ion concentration significantly reduces its antibacterial activity, and that this recombinant protein lacks hemolytic activity.

Full Text

Recombinant Expression and Biological Property Analysis of Goose Avian β-Defensin 7 Protein

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Abstract

This study investigated the biological properties of goose avian β-defensin 7 (AvBD7). The AvBD7 gene was subcloned into the EcoR and Xho sites of the prokaryotic expression vector pProEX HTa to construct the recombinant plasmid pProEX-AvBD7, which was then transformed into *Escherichia coli* Rosetta competent cells. Protein expression was induced with isopropyl-β-D-thiogalactoside (IPTG). Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) analysis revealed that the recombinant protein had a molecular weight of 10–15 kDa, consistent with the expected size. After purification, the recombinant goose AvBD7 protein was evaluated for its in vitro antimicrobial activity against *E. coli*, *Salmonella pullorum*, *Staphylococcus aureus*, *Micrococcus tetragenus*, and *Bacillus subtilis* using colony counting methods. The effects of salt ion concentration on antimicrobial activity and the hemolytic activity against chicken erythrocytes were also assessed. The results demonstrated that the recombinant goose AvBD7 protein exhibited antimicrobial activity against all five tested bacterial strains, with activity increasing in a concentration-dependent manner. High salt concentration (150 mmol/L) significantly inhibited the antimicrobial activity of the recombinant protein ($P<0.05$). Furthermore, the recombinant goose AvBD7 protein showed no hemolytic activity against chicken erythrocytes ($P>0.05$). These findings indicate that recombinant goose AvBD7 protein possesses broad-spectrum antimicrobial activity, which is significantly reduced by high salt concentrations, and that the protein lacks hemolytic activity.

Keywords: goose β-defensin 7; recombinant protein; antimicrobial activity

Introduction

Since antibiotics were widely adopted in animal husbandry, they have played a crucial role in maintaining livestock health and improving production performance. However, the extensive use of antibiotics has raised growing concerns among producers and consumers regarding their side effects. There is an urgent need for effective and environmentally friendly alternatives to control livestock diseases and improve product quality. Defensins are small molecular peptides

that participate in the initial defense mechanisms of the organism and represent a vital class of endogenous antimicrobial peptides. Their primary molecular characteristic is the presence of three disulfide bonds formed by six cysteine residues. Numerous studies have demonstrated that avian β-defensins (AvBDs) exhibit broad-spectrum antimicrobial activity, with recombinant, naturally occurring, or synthetically produced forms showing potent killing activity against bacteria, fungi, spirochetes, and enveloped viruses such as HIV. These peptides play important roles in both innate and acquired immunity. Nevertheless, natural antimicrobial peptides have extremely low abundance in vivo, making isolation and purification difficult and resulting in very limited natural yields. Chemical synthesis often fails to achieve the correct molecular conformation, making genetic engineering a practical approach for defensin production.

To date, 11 defensins have been isolated from geese, including AvBD1–AvBD7, AvBD9, AvBD10, AvBD12, and AvBD16. Among these, goose AvBD7 contains a complete open reading frame (ORF) with a cDNA composed of 201 bases encoding 66 amino acids. Building upon previous research, this study employed a histidine (His) tag prokaryotic expression system to subclone the goose AvBD7 gene into pProEX HTa, construct a recombinant expression plasmid, and induce expression with IPTG to achieve high-level expression of recombinant goose AvBD7 protein in *E. coli*. The antimicrobial and physicochemical activities of the recombinant protein were subsequently evaluated in vitro to provide a theoretical foundation for further research and application of goose defensins.

Materials and Methods

1.1.1 Strains and Plasmids

The recombinant plasmid pMD18-T-AvBD7, pMD18-T-Simple Vector, expression vector pProEX HTa, expression host *E. coli* Rosetta, *Bacillus subtilis* ATCC 9193, *Staphylococcus aureus* ATCC 29213, *Micrococcus tetragenus* ATCC 2835, *Salmonella pullorum* C79-11-S11, and *E. coli* BL21 were all maintained in our laboratory.

1.1.2 Reagents

ExTaq DNA polymerase, restriction enzymes EcoR and Xho , T4 DNA ligase, DNA markers, and IPTG were purchased from TaKaRa (Japan). Gel extraction kits were obtained from OMEGA (USA). Protein purification and refolding kits were from Novagen. All reagents used were of analytical grade.

1.2.1 Construction of Prokaryotic Expression Recombinant Plasmid

Based on the restriction sites of pProEX HTa and the recombinant plasmid pMD18-T-AvBD7, a pair of specific expression primers for goose AvBD7 was designed: P1: 5 -GAATTCATGCAGCACGTCTTCCCTAG-3 and P2: 5 -CTCGAGTCAGTGCCTCCACCCCCTC-3 . The 5 end of P1 contained

an EcoR site, while the 5 end of P2 contained an Xho site. Using the recombinant plasmid pMD18-T-AvBD7 as template, PCR amplification was performed with the expression primers. The PCR reaction mixture (25 L) contained 12.5 L Premix TaqTM (Ex TaqTM Version 2.0 plus dye), 0.5 L recombinant plasmid pMD18-T-AvBD7, 1 L each of forward and reverse primers, and sterile double-distilled water to a final volume of 25 L. The PCR program was as follows: initial denaturation at 94 °C for 5 min; 25 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C for 10 min; and holding at 4 °C for 10 min.

The amplified product was digested with EcoR and Xho and cloned into the prokaryotic expression vector pProEX HTa to construct the recombinant expression plasmid pProEX-AvBD7. Positive clones were screened by plasmid PCR and double digestion. The PCR reaction mixture (25 L) contained 12.5 L Premix TaqTM (Ex TaqTM Version 2.0 plus dye), 0.5 L recombinant expression plasmid pProEX-AvBD7, 1 L each of forward and reverse primers, and sterile double-distilled water to a final volume of 25 L. The PCR program was identical to that described above. The digestion mixture (10 L) contained 3 L recombinant expression plasmid pProEX-AvBD7, 5 L ddH_2O, 0.5 L EcoR , 0.5 L Xho , and 1 L 10×H Buffer, incubated at 37 °C for 2 h. Positive plasmids were sent to Beijing Liuhe Huada Gene Technology Co., Ltd. for sequencing.

1.2.2 Expression and Purification of Recombinant Goose AvBD7 Protein

The recombinant expression plasmid pProEX-AvBD7 was transformed into *E. coli* Rosetta expression host cells. Single colonies were selected and cultured in LB liquid medium containing ampicillin at 37 °C with shaking. When the optical density (OD_{600}) reached 0.6–0.8, 1 mL of bacterial culture was reserved as a control. IPTG was added to the remaining culture at a final concentration of 0.6 mmol/L to induce protein expression. Samples (1 mL each) were collected at 2, 4, and 6 h post-induction. Inclusion body purification, refolding, and dialysis were performed according to the Novagen protein purification and refolding kit instructions, and the recombinant protein concentration was determined. One milliliter of supernatant from sonicated cells and a small amount of pellet were reserved; the pellet was resuspended in 100 L phosphate-buffered saline (PBS, pH 7.4). Pre-induction control samples, post-induction samples, supernatant, pellet, and purified protein were analyzed by Tricine-SDS-PAGE, and results were visualized using a thin-layer scanner.

1.2.3 Determination of Antimicrobial Activity of Recombinant Goose AvBD7 Protein

Antimicrobial activity was measured using the colony counting method. Purified recombinant goose AvBD7 protein and His-tag protein were diluted with sterile PBS (pH 7.4) to final concentrations of 50, 100, 250, and 500 g/mL; 250 L aliquots were added to sterile tubes. The bacterial strains described in section

1.1.1 were cultured to logarithmic phase and diluted to 2$×10^{6}$ CFU/mL with appropriate liquid medium. Ten microliters of diluted bacterial culture were added to each tube, with PBS serving as negative control. Each group had three replicates. After incubation at 37 °C with shaking for 4 h, 100 L from each dilution was plated onto LB agar and cultured overnight at 37 °C. Colonies were counted and recorded. The average colony count from three plates of the same dilution was used as the colony number for that sample. The bacterial count in each original tube was calculated based on inoculation volume and dilution factor, and a relationship graph between bacterial survival rate and recombinant protein concentration was plotted.

Bacterial survival rate (%) = (Number of surviving bacteria with recombinant goose AvBD7 protein / Number of surviving bacteria in negative control) × 100.

1.2.4 Effect of Different Salt Ion Concentrations on Antimicrobial Activity

E. coli (Gram-negative) and *M. tetragenus* (Gram-positive) were selected as indicator strains. Sodium chloride (NaCl) concentrations were adjusted with PBS to 0, 50, 100, and 150 mmol/L. Recombinant protein was diluted with these NaCl solutions to a final concentration of 250 g/mL; 250 L aliquots were added to sterile tubes, with corresponding NaCl solutions serving as negative controls. Ten microliters of bacterial culture were added to each tube, with three replicates per group. After incubation at 37 °C with shaking for 4 h, the procedure described in section 1.2.3 was followed.

1.2.5 Determination of Hemolytic Activity Against Chicken Erythrocytes

Freshly collected anticoagulated SPF chicken blood cells were washed with sterile PBS (pH 7.4) and diluted to 2–3%. Recombinant goose AvBD7 fusion protein was diluted with PBS to 100, 250, and 500 g/mL. Twenty microliters of protein solution were added to sterile tubes, with 0.2% Triton X-100 as positive control and PBS as negative control. One hundred eighty microliters of diluted erythrocytes were added to each tube, with three replicates per group. After incubation at 37 °C for 1 h, tubes were centrifuged at 1,000 g for 10 min, and the supernatant was collected. OD_{560} was measured using a micro-UV spectrophotometer. Each sample was analyzed in triplicate, and the average value was calculated.

Hemolytic activity (%) = [(OD - OD $_{6}$) / (OD - OD $_{6}$)] × 100.

1.2.6 Statistical Analysis

Data were analyzed using SPSS 18.0 software for analysis of variance. All data are expressed as mean ± standard error.

Results

2.1 Expression and Purification of Recombinant Goose AvBD7 Protein

Using the recombinant plasmid pMD18-T-AvBD7 as template, PCR amplification was performed to construct the expression recombinant plasmid pMD18-T-S-AvBD7. Both the expression recombinant plasmid and the expression vector pProEX HTa were digested with EcoR and Xho , and the target fragments were recovered and transformed into *E. coli* Rosetta competent cells to construct the recombinant expression plasmid, which was identified by double digestion, PCR, and sequencing. Electrophoresis results showed that the size of the digested product was consistent with the band obtained by plasmid PCR amplification.

[Figure 2: see original paper]

Fig. 2 Expression and purification of recombinant goose AvBD7 protein. Lane 1: supernatant; Lane 2: inclusion body; Lane 3: without IPTG induction; Lanes 4–6: fusion protein expression at 2, 4, and 6 h after IPTG induction; Lane 7: purified protein; M: protein marker (ku).

2.2 Determination of Antimicrobial Activity of Recombinant Goose AvBD7 Protein

The antimicrobial activity of recombinant goose AvBD7 protein against five bacterial strains was measured using the colony counting method. As shown in Fig. 3 [Figure 3: see original paper], compared with the control, the recombinant goose AvBD7 protein exhibited significant antibacterial effects against all tested bacteria ($P<0.05$), with activity increasing as protein concentration increased. The protein showed the strongest antimicrobial activity against *M. tetragenus*, with highly significant effects at concentrations of 50–500 g/mL ($P<0.01$). Strong activity was also observed against *S. aureus*, with significant or highly significant antibacterial effects at 100–500 g/mL ($P<0.05$ or $P<0.01$). Lower activity was detected against *E. coli*, *S. pullorum*, and *B. subtilis*, with significant or highly significant effects only at concentrations of 250–500 g/mL ($P<0.05$ or $P<0.01$).

[Figure 3: see original paper]

Fig. 3 Antimicrobial activity of recombinant goose AvBD7 protein and His-tag protein. * indicates significant difference ($P<0.05$), ** indicates extremely significant difference ($P<0.01$). The same as below.

2.3 Effect of Different Salt Ion Concentrations on Antimicrobial Activity

The antimicrobial activity of recombinant goose AvBD7 protein at various salt concentrations was determined by colony counting. As shown in Fig. 4 [Figure 4: see original paper], for both Gram-positive (*M. tetragenus*) and Gram-negative

(*E. coli*) indicator strains, bacterial survival rates increased (i.e., antimicrobial activity decreased) as salt ion concentration increased when the recombinant protein concentration was 250 g/mL. When salt concentration reached 100 mmol/L, the antimicrobial activity against *E. coli* decreased significantly compared with the control (0 mmol/L salt) ($P<0.05$). At 150 mmol/L salt concentration, antimicrobial activity against *M. tetragenus* decreased significantly compared with the control ($P<0.05$).

[Figure 4: see original paper]

Fig. 4 Effects of NaCl concentration on the antimicrobial activity of recombinant goose AvBD7 protein.

2.4 Determination of Hemolytic Activity of Recombinant Goose AvBD7 Protein

The hemolytic activity of different concentrations of recombinant goose AvBD7 protein was evaluated. As shown in Fig. 5 [Figure 5: see original paper], the hemolytic activity of the recombinant protein at concentrations of 100, 250, and 500 g/mL was extremely low, with no significant difference compared with the PBS negative control ($P>0.05$), indicating that the recombinant protein lacks hemolytic activity.

[Figure 5: see original paper]

Fig. 5 Hemolysis activity of recombinant goose AvBD7 protein. Positive control: 0.2% Triton X-100.

Discussion

Over the past two decades, significant progress has been made in research on AvBDs from chickens, ducks, turkeys, penguins, and ostriches. However, reports on goose antimicrobial peptides remain relatively scarce. Numerous studies have demonstrated that avian defensins exhibit broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as fungicidal activity in vitro. Nevertheless, natural antimicrobial peptides are present in extremely low quantities in vivo, making isolation and purification difficult. Chemical synthesis often fails to achieve correct molecular conformation, necessitating production through genetic engineering. Due to their small molecular size and susceptibility to proteolytic degradation, as well as their strong toxicity to host *E. coli*, defensins cannot be directly expressed in this bacterium; however, fusion protein expression can overcome this limitation.

To date, glutathione S-transferase (GST) fusion and His-tag fusion expression systems are the two most commonly used prokaryotic expression systems. Many defensins have been successfully expressed at high levels using the GST fusion system with good results. However, because GST has a relatively large molecular weight compared with defensins, it can affect their activity. Therefore, this study employed the His-tag fusion system to express recombinant goose AvBD7

protein. Tricine-SDS-PAGE analysis revealed that most of the protein existed in inclusion bodies. Since proteins in inclusion bodies lack biological activity, the Novagen Protein Refolding Kit was used to refold the inclusion bodies, yielding active recombinant protein. Antimicrobial activity assays demonstrated that the recombinant protein exhibited significant antibacterial effects against *B. subtilis*, *S. aureus*, *M. tetragenus*, *S. pullorum*, and *E. coli*, consistent with previous reports and confirming that avian AvBDs possess broad-spectrum antimicrobial activity.

Studies have shown that different defensin types exhibit varying antibacterial spectra. For example, chicken AvBD1 and AvBD2 inhibit *Candida albicans*, *E. coli*, and *Salmonella enteritidis*, while recombinant chicken AvBD6 shows weaker activity against *Salmonella*. Penguin AvBD103a and AvBD103b inhibit *Bacillus subtilis*, *Aspergillus fumigatus*, and *Staphylococcus albus*, while duck AvBD10 inhibits *Pasteurella multocida* and *S. aureus*. To investigate the effect of the His-tag on antimicrobial activity, Li et al. measured the antibacterial activity of His-tag protein against the five bacterial strains used in this study and found no antimicrobial activity, indicating that the His-tag does not affect the recombinant protein' s activity. The present study further confirms these results.

Multiple studies have demonstrated that AvBDs lose antimicrobial activity under high-salt conditions, consistent with previous reports. Our results show that high salt concentration (150 mmol/L) significantly inhibited the antimicrobial activity of recombinant goose AvBD7 protein against both Gram-negative and Gram-positive bacteria. Not only NaCl concentration but also other ion concentrations or the absence of specific ions can affect antimicrobial activity. This may be because high ion concentrations disrupt the electrostatic interaction between bacterial outer membrane anions and the positive charges of antimicrobial peptides, thereby affecting defensin activity. To further investigate the properties of goose defensins, we evaluated the hemolytic activity of the recombinant protein, which was found to be very low even at the highest concentration (500 g/mL). This aligns with results for avian and human defensins, suggesting that defensins are not toxic to animal cells, possibly because animal cell membranes contain high levels of cholesterol and lack negatively charged phospholipids, which prevents interaction with antimicrobial peptides.

These findings provide a theoretical basis for the potential application of AvBD recombinant proteins as efficient peptide antibiotics and poultry feed additives.

In conclusion, this study successfully expressed recombinant goose AvBD7 protein using the prokaryotic expression vector pProEX HTa. The protein, with a molecular weight of 10–15 kDa, was expressed as inclusion bodies and exhibited antimicrobial activity against *B. subtilis*, *S. aureus*, *M. tetragenus*, *S. pullorum*, and *E. coli*. At 150 mmol/L salt concentration, the antimicrobial activity against *E. coli* and *M. tetragenus* was significantly reduced. Additionally, the recombinant protein showed no hemolytic activity against chicken erythrocytes.

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Note: Figure translations are in progress. See original paper for figures.