Expressions of Antimicrobial Peptide Gallinacin-1,-2 and-3 mRNAs in the Oviduct of Laying Hens

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The aim of this study was to determine the profiles of mRNA expressions of gallinacin (Gal)-1, -2 and -3, which are the antimicrobial peptides, in the oviduct of laying hens. The oviductal tissues of White Leghorn laying hens were collected. Expressions of Gal-1, -2 and -3 mRNA were examined by semiquantitative RT-PCR and localized in the vagina by *in situ* hybridization. The expressions of Gal-1, -2 and -3 were observed in infundibulum, magnum, isthmus, uterus and vagina by RT-PCR. The expressions of Gal-1, -2 and -3 were greater in the infundibulum and vagina, except for Gal-2 in the vagina. The signals of each Gal mRNA were identified in the basal cells of surface epithelium of vagina. These results suggested that antimicrobial peptide, Gal-1, -2 and -3 were expressed in the oviduct with a greater expression in the infundibulum and vagina, and surface epithelium were the major site for the synthesis of Gals in the vagina.

Key words : antimicrobial peptide, chicken, gallinacin, oviduct, mRNA expressions

Introduction

Defensins are endogenous peptides that contribute to the antimicrobial properties, and three defensin subfamilies (α , β and θ -defensins) exist in vertebrates (Sugiarto and Yu, 2004). In birds, only β -defensin have been found (Bensch *et al.*, 1995 ; Sugiarto and Yu, 2004 ; Xiao *et al.*, 2004). The antimicrobial activities spectra of defensins include Gram-positive and Gram-negative bacteria, protozoan, some fungi and enveloped virus (Evans *et al.*, 1994, 1995 ; Sugiarto and Yu, 2004). Until now a total of 13 different β -defensins designated as gallinacin (Gal)-1 to -13 are reported in chicken genome, whereas Gal-1, -2 and -3 were identified before the discovery of sequences of Gal-4 to -13 (Harwig *et al.*, 1994 ; Brockus *et al.*, 1998 ; Zhao *et al.*, 2001 ; Lynn *et al.*, 2004 ; Xiao *et al.*, 2004). The immunity of hen oviduct is essential to protect the oviductal tissues from infection and bacterial contamination of eggs. The presence of immunocompetent cells including antigen presenting cells, T and B cells that play in the acquired immunity has been reported (Yoshimura *et al.*, 1997 ; Zheng *et al.*, 1997, 1998). Their population in the oviduct was increased in laying hens compared with immature ones under the effect of estrogen (Zheng *et al.*, 1998). They were more

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abundant in the two ends of oviduct, namely in the infundibulum and vagina (Yoshimura *et al.*, 1997 ; Zheng *et al.*, 1997, 1998). The immunity of the vagina may be most important within the oviduct for the protection from infection because it opens to cloaca where various microorganisms exist. However, little is known about the factors responsible in the innate immunity in the oviduct.

In mammals, it is suggested that vaginal epithelium has a powerful innate immune system, and antimicrobial peptide may play important roles in vaginal immune response and in the elimination of pathogens from the female reproductive tract (Pivarcsi *et al.*, 2005). Recent studies have reported the expression of Gal-1 to -13 mRNA in the various organs of 2- or 3-month-old chickens that should be immature and before egg-laying (Zhao *et al.*, 2001 ; Xiao *et al.*, 2004). Zhao *et al.* (2001) did not find Gal-1, -2 and -3 expressions in the oviduct of 3-month-old chickens. Xiao *et al.* (2004) found mRNA expression of Gal-8 to -13, but not Gal-4 to -7, in the oviduct of 2-month-old chickens. As the population of immunocompetent cells changes in association with oviducal development during sexual maturation under an effect of estrogen (Zheng *et al.*, 1997, 1998), the expression of Gals may also be different between immature and matured birds. However, the Gal mRNA expressions in the oviduct of laying hens have not been examined.

The goal of this study was to show the evidence and profiles of mRNA expressions of Gal-1, -2 and -3 in the oviduct of laying hens. *In situ* hybridization study was performed to localize their expressions in the vagina where the immunity is most important in the oviduct to eliminate the pathogenic agents.

Materials and Methods

Birds and Tissues Collection

White Leghorn hens laying five eggs or more in a sequence were kept in individual cages under a light regimen of 14 h L : 10 h D and were provided with commercial feed and water *ad libitum*. The birds were euthanized by decapitation after 1–2 h of oviposition (n=4). The oviducts were excised, and the tubular region of infundibulum, middle part of magnum, isthmus, uterus and vagina were collected for RT-PCR analysis. A part of vagina tissues were embedded in cryo-embedding medium (OCT compound ; Sakura Co., Tokyo, Japan) and snap-frozen in isopentane and solid CO₂ mixture for *in situ* hybridization analysis. The experiments were done in agreement of the regulations of Hiroshima University for experiments on animals.

Semi-quantitative RT-PCR

1. Preparation of total RNAs

Total RNAs were extracted from the mucosal tissues of the infundibulum, magnum, isthmus, uterus and vagina using Sepasol RNA I Super (Nacalai Tesque, Inc. Kyoto, Japan) according to the manufacturer's protocol. The pellet of RNA was resuspended in TE buffer, treated with DNase 1 (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for 60 min. The concentration was measured with Gene Quant pro (Amersham Pharmacia Biotech, U.K.) and stored at –80°C until analysis.

2. RT-PCR

The semi-quantitative RT-PCR was performed as described by our previous report (Chowdhury *et al.*, 2004). The RNA samples were reverse transcribed using Rever Tra Dash (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's protocol. The reaction mixture (10 μ l) consisted of 1 μ g of total RNA sample, 1 $\times$ RT buffer, 1 mM each dNTP, 20 U RNase inhibitor, 0.5 μ g Oligo(dT)20, 50 U Rever Tra Ace. The PCR was performed using Takara TaqTM (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Primers used for PCR are shown in Table 1. The PCR mixture (50 μ l) contained cDNA corresponding to 1 μ g of the initial total RNA, 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1.25 U Takara Taq, 0.2 μ M each primer. Amplification of Gal-1, -2 and -3 cDNA was performed in a Programmable Thermal Controller PTC-100 (MJ Research Inc., Waltham, MA) under the following conditions : 94°C for 30 sec, then 35 cycles at 94°C for 30 sec to denature, 58°C for 60 sec to anneal, 72°C for 90 sec for the extension. The PCR for β -actin cDNA was carried out by a same manner except for annealing at 55°C. In the preliminary experiment, different cycles of PCR, namely 30, 35 and 40 cycles, had been performed for standardization of PCR cycles in each sample. The PCR products were separated by electrophoresis on a 3% (w/v) agarose gel containing 0.4% ethidium bromide. The density of each band on photographs was quantified using a Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD), and Gal-1, -2 or -3/ β -actin ratio was obtained. The nucleotide sequence of PCR products of Gal-1, -2 and -3 were also determined using a BigDye terminator Sequence Kit (ver. 3.1, Applied Biosystems, Foster city, CA) with an ABI 3100 automated sequencer (Applied Biosystems, Foster city, CA). Sequences were analyzed with the G-PROF and GENETYX program packages (Genetyx Co. Ltd., Tokyo, Japan). The sequences of RT-PCR products of each Gal obtained in the current study corresponded to those reported in DNA data bank (Gal-1, 89–493 of nucleotide sequence in Acc. No. AF033335 ; Gal-2, 114–350 in Acc. No. AF 033336 ; Gal-3, 113–387 in Acc. No. NM 204650).

In situ Hybridization

1. Preparation of probes

Table 1. Primers for RT-PCR analysis of Gallinacin-1, -2 and -3 and β -actin and accession number used to design the primers

Target	Primers	Accession number
Gal-1	F : 5'-AAACCATGCGGATCGTGTACCTGC-3' R : 5'-CAATGCTAAACTGCACACCTTTA-3'	AF033335
Gal-2	F : 5'-GTTCTGTAAAGGAGGGTCCTGCCAC-3' R : 5'-ACTCTACAACACAAAACATATTGC-3'	AF033336
Gal-3	F : 5'-CTGCCGCTTCCCACACATAG-3' R : 5'-GCAATGCCAAACTGCACGCCTTTA-3'	NM204650
β -actin	F : 5'-TGGATGATGATATTGCTG-3' R : 5'-TTGCTCTGGGCTTCATCAC-3'	X00182

The PCR products obtained by the RT-PCR for Gals as described above were used to prepare the probes labeled with digoxigenin. Digoxigenin labeling of PCR products was performed using a PCR DIG probe synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mixture (50 μ l) contained 2 μ l of PCR product (approximately 0.2 μ g), 2.5 U Takara Taq (Takara Bio Inc., Shiga, Japan), 1 $\times$ PCR Dig probe synthesis mix, 1 $\times$ PCR buffer, 0.2 μ M of each primer. The PCR conditions were the same as described above. The resulting DIG-labeled DNA products were separated by electrophoresis using a 3% agarose gel, and isolated using DNA Gel Extraction kit (Millipore Co., Billerica, MA, USA). Then the products were precipitated using 4 M LiCl and ethanol and dried, followed by dissolving in 30 μ l TE.

2. *In situ* hybridization

Cryostat sections (10 μ m) of vagina were air-dried on the slides pretreated with 2% (v/v) 3-aminopropyl triethoxysilane (Sigma Co., St. Louis, MO, USA) in acetone. The sections were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 min. They were then treated with proteinase K (E. Merck, Darmstadt, Germany), 2 μ g/ml in 100 mM Tris-HCl with 50 mM EDTA, pH 8.0, at 37°C for 15 min. After brief washing in PBS, the sections were fixed again in 4% PFA for 10 min, and washed in PBS for 5 min. The slides were then immersed in 0.1 M triethanolamine, pH 8.0, containing 0.25% (v/v) acetic anhydride for 20 min. Then they were washed twice in PBS (10 min each), and dehydrated through successive baths of ethanol (70 to 100%) and air-dried. The sections were rehydrated with PBS, and then incubated at 37°C for 30 min with hybridization buffer [30 μ l of 100% deionized formamide, 20 μ l of 20 $\times$ SSC, 20 μ l TE, 5 μ l of denatured salmon sperm DNA (10 mg/ml), and 10 μ l of H₂O]. For the hybridization solution, 10 μ l DIG-labeled PCR probes of Gal-1, -2 or 3 was added to 500 μ l hybridization buffer. The hybridization solution was incubated at 100°C for 3 min, then immediately cooled on ice for 10 min. Sections were applied with the hybridization solution and covered with a piece of parafilm, incubated in a humidified chamber at 37°C for 18 h. After hybridization, slides were washed with 30% (v/v) formamide/2 $\times$ SSC (20 min $\times$ 2 times) and PBS containing 0.05% Tween (20 min) at 42°C.

After equilibrating with buffer I (0.1 M Tris, 0.15 M NaCl, pH 7.5) at room temperature for 5 min, the slides were incubated with 5% (w/v) blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany) diluted in buffer 1 at room temperature for 30 min. Then the slides were incubated in a humidified chamber overnight at 4°C with anti-DIG antibody conjugated with peroxidase (Roche Diagnostics GmbH, Mannheim, Germany) diluted to 1 : 100 in PBS containing 0.5% (w/v) BSA. Finally, the immunoreactions were visualized by incubating the sections with a reaction mixture consisting of 0.02% (w/v) 3, 3'-diaminobenzidine tetrahydrochloride and 0.005% (v/v) H₂O₂ in 0.05 M Tris-HCl buffer, pH 7.6.

For the control staining, sections were incubated with hybridization solution containing no probes, followed by immunostaining using anti-DIG antibody. No signal was appeared in the control staining, suggesting the signals of Gal-1, -2 and -3 were specific (Data not shown).

Statistical Analysis

Significance of differences in the Gal-1, -2 and -3/ β -actin ratio was examined by one-way ANOVA followed by Duncan's multiple range test (Duncan, 1955). Significance was accepted when the P value was less than 0.05.

Results

The bands of PCR products for Gal-1, -2 and -3 were observed in all segments of oviducts including infundibulum, magnum, isthmus, uterus and vagina (Fig. 1 A). The expression of Gal-1 was greater in the infundibulum and vagina than magnum and isthmus, whereas Gal-2 mRNA expression was stronger only in the infundibulum than the other segments of oviduct. The Gal-3 mRNA expression was greater in the infundibulum and vagina than the other oviductal segments (Fig. 1 B). Figure 2 shows the expression of Gal-1, -2 and -3 in the vagina examined by *in situ* hybridization. The signals of each Gal mRNA were identified in many of the basal cells of surface epithelium in the mucosal folds (Fig. 2).

Discussion

We are reporting that mRNAs of antimicrobial peptide, Gal-1, -2 and -3, were expressed in the oviductal mucosal tissue, and surface epithelial cells in the vagina showed signals for the expression. The expression of each type of Gal was stronger in the infundibulum and vagina than the other segments except for Gal-2 in the vagina.

Zhao *et al.* (2001) examined expressions of Gals in various tissues of a healthy 3-

Fig. 1. RT-PCR of Gallinacin (Gal)-1, -2 and -3 in the oviduct of laying hen.

(A) RT-PCR products of Gal-1, -2 and -3. In=infundibulum, Ma=magnum, Is=isthmus, Ut=uterus, Va=vagina. (B) Semi-quantitative RT-PCR analysis of Gal-1, -2 and -3 mRNA expressions in the oviduct. The values are mean $\pm$ SEM of densitometric values of Gal-1, -2 and -3 mRNA to β -actin mRNA (n=4 each). Values with different superscripts (a, b) are significantly different (P < 0.05).

Fig. 2. *In situ* hybridization for gallinacin (Gal)-1, -2 and -3 mRNA expressions in the vagina.

(a), (b) and (c) show gallinacin -1, -2 and -3 mRNA expressions, respectively. Signals are found in the basal cells of surface epithelium (arrows). L= vaginal lumen, S=stroma. Scale bar=50 μ m.

month-old chicken. They reported that Gal-1 and -2 were expressed strongly in healthy bone marrow and a lesser extent in lung. They also showed strong expression of Gal-3 in tongue, bursa of Fabricius and trachea, moderate expression in the skin, esophagus and air sacs, and weaker expression in bone marrow, large intestine, kidney and ovary, suggesting that Gal-3 was expressed widely in nonmyeloid cells. They did not show expressions of Gal-1, -2 and -3 in the oviduct of 3-month-old chickens. However, the current study revealed that Gal-1, -2 and -3 mRNAs were expressed in all segments of oviduct. Thus, we assume that the expression of Gals occurs in the developed oviduct of laying hens, and gonadal steroids responsible in the oviductal development may be significant factors for this process.

The expressions of Gal-1, -2 and -3 mRNAs were greater in the infundibulum and vagina except for Gal-2 in the vagina. Because Gals have antimicrobial activities against Gram-positive and Gram-negative bacteria (Evans *et al.*, 1994, 1995 ; Sugiarto and Yu, 2004 ; Higgs *et al.*, 2005), they may play the role in earlier defense against pathogenic agents as innate immunity in the oviduct. Previous studies showed that the populations of antigen presenting cells, T and B cells that are responsible in adaptive immunity were also greater in these two ends of oviduct (Yoshimura *et al.*, 1997 ;

Zheng *et al.*, 1997, 1998). Thus it is likely that both innate immunity mediated by Gals and adaptive immunities are more developed in the two ends of oviduct, namely infundibulum and vagina.

In mammals, β -defensins promote adaptive immune responses by recruiting dendritic and T cells (Yang *et al.*, 1999), and enhances IgG synthesis through affecting B and T cells interactions to link innate immunity with adaptive immune system (Lillard *et al.*, 1999). Thus it is assumed that there may be a linkage between higher expression of Gal-1 and -3 and the greater population of immunocompetent cells (Yoshimura *et al.*, 1997 ; Zheng *et al.*, 1997, 1998) in the infundibulum and vagina in laying hens, although further studies are needed to confirm this theory.

The immunity in the vagina is most important for the defense system in the oviduct because it opens to cloaca where various microorganisms exist. The signal of Gal-1, -2 and -3 expressions were found in the basal cells of surface epithelium in the vagina. Out of Gal-1, -2 and -3, only expression of Gal-2 was not higher in the vagina than in the magnum and isthmus. Thus, although the localization of the three types of Gals was same, there may be some difference in the factors controlling their expressions among them. In humans, β -defensin expression was localized in the surface epithelial layers of the vagina and other regions of genital tract (Quayle *et al.*, 1998 ; Schaefer *et al.*, 2005 ; Valore *et al.*, 1998 ; Pivarcsi *et al.*, 2005), and epithelial β -defensin may prevent microorganisms from entering surfaces and prevent the attack of microorganisms that have already entered the epithelia (Schroder, 1999). The cells expressing Gals are suggested to play a significant role in defending against pathogenic agents in the oviduct. Furthermore, eggs are rich source of antimicrobial protein/peptides such as lysozyme and ovotransferrin in egg-white (Pellegrini *et al.*, 1997 ; Ibrahim *et al.*, 2000 ; Mine *et al.*, 2004). If the Gals are secreted from the epithelial cells to the vaginal lumen, they may be incorporated in the substances on the surface of egg-shell and play roles as antimicrobial factors. The expressions of Gals in the infundibulum may have some roles in elimination of foreign organisms. Although the possibility of the appearance of bacteria in this segment is unclear, sperms migrate and stay in this segments for fertilization in copulated or inseminated hens. Zheng *et al.* (1998) suggested that the infundibulum contains many immunocompetent cells, and they may play roles in the removal of not only bacteria but also sperms that did not participate in fertilization. The higher expression of Gals in the infundibulum may have roles resembled to those immunocompetent cells.

In conclusion, we suggest that expression of antimicrobial peptide, Gal-1, -2 and -3 occurred in the oviduct of laying hens with a greater expressions in the infundibulum and vagina compared with the other segments except for Gal-2 in the vagina. The surface epithelial cells of mucosa may be the major site for the synthesis of these peptides in vagina. The Gals probably play significant roles in innate immunity of the oviduct to defend against pathogenic agents.

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