Changes in the antimicrobial potential of egg albumen during the early stages of incubation and its impact on the growth and virulence response of *Salmonella Enteritidis*

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Abstract

The antimicrobial activity of egg albumen has been widely documented. However, the changes in the chemical nature and antimicrobial activity of egg albumen and the way it controls bacteria during the incubation period have not yet to now been understood. The purpose of the present investigation was to analyze the antimicrobial potential of egg albumen during early incubation stages (0-5 days). The changes in ovotransferrin and lysozyme content in egg albumen were quantified via high performance liquid chromatography (HPLC). The results showed that the increase in concentration of ovotransferrin in egg albumen was closely related to the time of incubation, but that of lysozyme remained substantially unchanged and was very similar to that of the non-incubated eggs. The iron ion binding capacity and specific lysozyme activity of the egg albumen during early stages of incubation was also investigated. The changes occurring in egg albumen during early incubation could influence the growth of *Salmonella Enteritidis*. Very little difference was observed in the virulence response of *Salmonella Enteritidis* to egg albumen collected at different incubation periods by using the expression of *hilA* gene as an indicator.

Introduction

The mechanism of egg albumen antimicrobial activity involves specific molecules and environmental factors (Alabdeh et al., 2011; Stevens, 1996; Tranter and Board, 1984). Research data have shown that the viscous and heterogeneous structure and alkaline pH play important roles in the maintenance of antimicrobial activity of egg albumen (Kang et al., 2006; Tranter and Board, 1984; Yadav and Vadehra, 1977). In a fertile egg, as incubation begins, the egg contents go through dramatic changes. Tona et al. (2010) reported that the pH of albumen increased between egg setting time and second day of incubation, and then turned towards neutrality. The study of Benton et al. (2001) definitely shows that the avian embryo affects the physical and chemical properties of albumen prior to incubation and during the initial hours of incubation. Araki et al. (2000) reported that the proteins in thin egg albumen were degraded at the early stage (4 days) of embryonic development. The dynamic changes in egg albumen during egg incubation would result in changes in immune efficiency of egg albumen. However, up to now, little information is available concerning the changes in the antimicrobial activity of egg albumen during the avian embryonic developmental stages.

The main aim of the present study was to analyze the antimicrobial potential of egg albumen during early incubation stages. The lysozyme and ovotransferrin content in egg albumen was quantified by HPLC. The iron ion binding capacity and specific lysozyme activity of the incubated egg albumen was also investigated. Since *Salmonella Enteritidis* is much more capable than other serotypes of persistently colonizing the laying hen reproductive tract and to survive in the hostile egg white, we also assessed whether changes in egg albumen during the early incubation stages could influence *Salmonella Enteritidis* growth and virulence response by using expression of *hilA* as an indicator.

Materials and methods

Materials

This study used fresh fertilized eggs from Single Comb White Leghorn (55-60 weeks old) laid within 24 h were collected in the morning from the poultry research center farm of Huazhong Agricultural University. The Grace Vydac C4 (214TP, 5μm, 250×4.6 mm) column was purchased from Vydac (Separation Group, Hesperia, CA, USA). HPLC was carried out on a Waters 2690 alliance equipped with a PDA 2695 photodiode array detector (Waters Corporation, Milford, MA, USA). Lysozyme (Aldrich-Sigma Chemical Corporation, cat. n. 62970) and ovotransferrin (Aldrich-Sigma Chemical Corporation, cat. n. C0755) standards from chicken egg white were purchased from Sigma (Sigma Aldrich, Shanghai, China). We used 0.45 μm cellulose acetate filter (Haibo, Qindao, China) for filtration and sterile homogeneous bags (Haibo) as sterile containers for egg albumen samples. Micrococcus lysodeikticus (CGMCC 10634) were used for specific lysozyme activity determination. Luria-Bertani (LB) agar (Haibo) were used for bacteria counting. Other analytical grade inorganic and organic chemicals were from commercial sources in China (Sinopharm Chemical Company, Beijing, China).

Sample preparation

Seventy fertilized chick eggs were incubated at 38°C and 65-75% relative humidity in a forced air incubator for up to five days in each experiment. Investigations were carried out at daily intervals. At each experimental time point post laying, 10 eggs were removed and opened for egg albumen sampling. Ten egg albumen sam-
In vitro study the growth of Salmonella Enteritidis in egg albumen collected from different incubation stages

Within 0, 1, 2, 3, 4 and 5 days of incubation, 10 eggs were disinfected by immersion in 70% ethanol, and under aseptic conditions, they were dried, cracked, and the egg albumen was collected into a sterile container and thoroughly mixed. We dispensed 100 mL albumen in a sterile homogeneous bag and this was incubated at 37°C for 1 h for temperature equilibration before inoculation. Overnight culture of Salmonella Enteritidis (ATCC 10376) was washed by PBS (pH 7.4) and resuspended in PBS buffer. Salmonella Enteritidis was inoculated into albumen samples to give a final concentration of approximately 7 log cfu/mL. Bacteria-egg albumen mixtures were incubated at 37°C for different periods of time and plated on LB agar plates to determine the concentration of surviving bacteria. Unincubated albumen samples from fertilized eggs were collected as negative controls. Since different batches of eggs vary in their bactericidal activity, the time-course of survival of bacteria in egg albumen varies slightly in different assays. All assays were repeated at least three times, and data from one representative assay were presented.

Reverse transcriptase real-time PCR assay

Overnight culture of Salmonella Enteritidis was washed by PBS (pH 7.4) and resuspended in PBS buffer. The egg albumen within 0, 1, 2, 3, 4, 5 days of incubation (approximately 100 mL, collected from 10 eggs) were inoculated with approximately 7 log cfu/mL bacterial cells in a sterile homogeneous bag and incubated for 4 h at incubation temperature (38°C) before reverse-transcriptase real-time PCR analysis. To study the effect of incubation temperature on hila expression of Salmonella Enteritidis in egg albumen, fresh fertilized egg albumen was inoculated with approximately 7 log cfu/mL bacterial cells and then incubated separately at 4°C, 25°C and 38°C for 4 h. Egg albumen samples which had been treated with Salmonella Enteritidis were resolved in 1 mL trizol reagent (Invitrogen, Gaithersburg, MD, USA). RNA extraction was performed according to the procedures recommended by the manufacturer. Reverse transcription was performed to convert mRNA to cDNA according to the procedures for the first-strand cDNA. All reverse transcription and DNase reagents were purchased from Invitrogen Life Technologies. Total RNA was treated with DNase to remove any contaminating DNA and to ensure that only RNA was being amplified. Forward and reverse PCR primers (Table 2) were designed using Primer 3 software. Quantitative real-time PCR was performed according to a standard protocol using the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and SLAN real-time PCR detection system (Hongshi, Shanghai, China). Cycling conditions were incubation at 50°C for 2 min, 95°C for 1 min, and 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 45 sec. Each gene was analyzed in replicate three times for each sample tested. For gene expression analysis, samples were normalized using the 16S rRNA gene as an internal standard. The relative changes (n-fold) in hila transcription between the incubated and unincubated egg albumen samples were calculated using the 2-△△Ct method as described by Livak and Schmittgen (2001). When it comes to the study of the effect of incubation temperature on hila expression of Salmonella Enteritidis in egg albumen, the sample incubated under 25°C was used as the calibrator (assigned an expression level of 1).

Statistical analysis

Data was analyzed using the SPSS 11.5 statistical package. Mean and standard deviation were calculated for quantitative data analysis. Mean and the standard deviation were calculated using analysis of variance (ANOVA) Fisher’s F-test and then the results were subjected to Tukey’s HSD (P<0.05) method for multiple pair-wise comparisons.
Results and discussion

Changes in the concentration of antimicrobial proteins in egg albumen during the early stages of incubation

The most abundant and well characterized antimicrobials in egg albumen are ovotransferrin and lysozyme, accounting for approximately 12% and 3.4% of the total proteins in newly deposited eggs (Shawkey et al., 2008). Although the precise mode of utilization of these egg albumen proteins by the embryo is still not known, the composition of egg albumen is not static but changes during embryonic development by interactions between the external environment and developing embryo (Sugimoto et al., 1999). In this study, the albumen proteins lysozyme and ovotransferrin were identified and quantified by HPLC on a C4-column. The following retention times were determined: lysozyme 10.4 min, ovotransferrin 10.8 min (Figure 1). The concentrations of ovotransferrin and lysozyme in egg albumen during the early stage of incubation were determined. The result showed that the concentration of ovotransferrin in fresh fertilized eggs assessed via HPLC was 10.4±0.84 mg/mL, and this increased in close relationship to the time of incubation (Figure 2). After 4 days of incubation, a significant increase was observed (4.1±0.10 mg/mL). The reason for this variation may be mainly due to the shifts of water among the egg components during incubation (Holub et al., 1994). However, the concentration of lysozyme in egg albumen remained substantially unchanged and was very similar to that of the non-incubated eggs (3.0±0.32 mg/mL) during the early stages of incubation. As it is known that the egg albumen is consumed as a supplementary nutrient for the developing embryo. Research has shown that the major components of egg albumen were digested in the yolk in the later stages of embryonic development (Yoshizaki et al., 2008). Although the proteins in egg albumen showed degradation at the early stages of embryonic development (Gerhartz et al., 1999). We supposed that egg incubation conditions may result in the degradation of lysozyme content. In contrast to lysozyme, ovotransferrin content has shown higher stability during the early incubation periods.

Changes in potential antimicrobial activity of egg albumen during the early incubation stages

The protective mechanisms of egg albumen proteins intervene either by means of direct bactericidal actions or by creating an environment unsuitable to bacterial growth (Mine and Kovacs-Nolan, 2004; Palmer and Guillette 1991; Wellman-Labadie et al., 2007). As mentioned previously, ovotransferrin and lysozyme are major antimicrobial molecules of the egg albumen. By binding free iron, an essential nutrient for bacterial growth (Skarr, 2010), ovotransferrin limits infection by both gram-positive and gram-negative bacteria (Horrocks et al., 2011). However, lysozyme acts on bacteria mainly by hydrolyzing the beta-1,4 glycosidic bonds between N-acetylmuramic acid and N-acetylgalcosamine, resulting in degradation of peptidoglycan, and subsequent cell lysis (Bera et al., 2005; Burley and Vadehra, 1989).

In this present study, we determined the iron binding capacity and specific lysozyme activity of the egg albumen collected from the early incubation periods to evaluate the antimicrobial defence of the egg albumen to the developing embryo.

The changes in iron-binding capacity of egg albumen during the early stages of incubation were determined by the total iron binding capacity assay (Yamanishi et al., 2002). The concentration of ovotransferrin determined on the iron-binding capacity was increased gradually and reached nearly 23.78±0.65 mg/mL after five days of incubation, which implied an increased iron binding capacity (Figure 3). Studies have shown that iron deficiency due to the iron binding activity of ovotransferrin was one of the mechanisms implicated in the inhibition of the growth of microbes in egg albumen (Wellman-Labadie et al., 2007). And the antimicrobial activity of ovotransferrin has been proved to be higher at incubation temperatures (34-36°C) than at ambient temperatures (Tranter and Board, 1984) and to increase with protein concentration (Yamanishi et al., 2002). We, therefore, concluded that that ovotransferrin in egg albumen...
would play a crucial role in the antimicrobial defense of the embryo during the early incubation stage. The specific lysozyme activity in egg albumen decreased from 30,397 to 15,035 U/mg during the first two days of incubation. However, in the later stages of incubation, an increase in enzymatic activity was found (Figure 4). Previous research has shown that the activity of the lysozyme was influenced by pH, and the optimum range for hen egg white lysozyme was pH 5.0-7.0 (Maidment et al., 2009). We suggest that the possible reason for the changes in lysozyme activity in egg albumen during incubation may be due to the dynamic changes of pH in egg albumen. In this study, we investigated the changes of pH in egg albumen during the early stages of incubation. The results showed that the pH of albumen increased to values higher than 9.5 on the second day of incubation and then turned toward neutrality, reached the original pH value after approximately five days of incubation (data not shown). In addition, degradation of SS-bonds of lysozyme in an alkaline pH environment or the formation of a dimer, which has no lytic activity, would also contribute to these results.

**Growth of Salmonella Enteritidis in incubated egg albumen**

Egg albumen is considered to be an unfavorable medium for microbial growth. The antimicrobial nature of the albumen of avian egg has been recognized for a long time (Board and Fuller, 1974). Antimicrobial proteins, temperature and pH are growth limiting factors in the albumen (Alabdeh et al., 2011; Baron et al., 1997; Tranter and Board, 1984; Yadav and Vadehra, 1977). In the course of early embryonic development, some crucial biological characteristics that are associated with the immunological defense role of albumen go through definite changes, such as increasing antimicrobial activity of albumen proteins and rising pH. Since Salmonella Enteritidis is much more capable of surviving in egg albumen than in other serotypes, we also assessed whether the changes in egg albumen during the early incubation stages could influence the growth of Salmonella Enteritidis. Bacterial growth curves were performed in egg albumen samples collected from different incubation periods (Figure 5). The result showed that the antimicrobial effect of albumen within 0, 1, 2, 3 and 4 days of incubation retarded growth of Salmonella Enteritidis. However, within two days of incubation albumen showed considerable growth inhibition for Salmonella Enteritidis, and the effect was maintained after 24 h, suggesting a bactericidal effect.

According to Kang et al. (2006), iron restriction was a major controlling factor of Salmonella Enteritidis growth in egg albumen. We, therefore, suppose the increase of anti-Salmonella Enteritidis activity in the incubated egg albumen may be due to the increase in iron-binding activity in albumen. It is worth noting that the rise in the pH in egg albumen during the first two days of incubation would reduce the chances of survival of bacteria, because the alkaline pH (9.5) is inimical to most micro-organisms and accentuates Fe3+ chelation by ovotransferrin (Tranter and Board, 1984). In addition, structure and function studies of many egg white proteins have shown that specific domains within these native proteins retain or even exhibit enhanced biological activities (Kovacs-Nolan et al., 2005). For instance, it has been suggested that lysozyme has a bactericidal effect, but it is largely ineffective against gram-negative bacteria such as Salmonella Enteritidis. However, the activity of lysozyme has been shown to be enhanced following proteolytic digestion (Mine et al., 2004). Thus, proteolytic degradation during early incubation (especially at the second day of incubation) would result in a significant improvement in anti-Salmonella Enteritidis activity of egg albumen.

**HiIA gene response of Salmonella Enteritidis in egg albumen collected from different stages of incubation**

The hiia gene is a transcriptional activator encoded on Salmonella pathogenicity island 1 and plays an important role in its pathogenesis (Bajaj et al., 1995; Jones, 2005). The hiia gene is also thought to be a requirement for Salmonella invasion due to its transcriptional properties. hiia is known to be activated when the bacteria encounter stress-inducing conditions. Environment factors such as pH, temperature, osmolarity, and oxygen tension have been found to affect hiia expression (Bajaj et al., 1996). During early incubation stages of avian eggs, changes in egg albumen could influence Salmonella growth and virulence response. Therefore, we determined the Salmonella Enteritidis hiia expression in egg albumen during the early incubation stages by a real time polymerase chain reaction method. Results have shown that relative expression of Salmonella hiia in egg albumen within two days of incubation was higher than that in egg albumen within 0, 1, 3, 4 and 5 days of incubation (Figure 6). However, there were no significant differences (P>0.05) in hiia expression between egg albumen samples collected from different stages of incubation and the
control groups. As temperature is another stressor that is encountered by Salmonella during infection of hatching eggs, the effect of incubation temperature on the expression of hilA in Salmonella Enteritidis was also investigated in this study. Results have shown that the expression of hilA was greater at the incubation temperature 38°C than at 4°C and 25°C. However, the difference was not significant (Figure 7). We supposed that the virulence of Salmonella Enteritidis seemed to be inhibited in the albumen in the early incubation stages by using expression of hilA as an indicator. This may be important for the survival of Salmonella Enteritidis in an incubated egg and important for the transmission of it from the parent to offspring. It is proposed that stress-induced survival mechanisms enable the serotype Enteritidis to cope with the antimicrobial compounds and thus survive in egg albumen (Van Immerseel, 2010). Therefore, identification of mechanisms of virulence regulation unique to Salmonella Enteritidis should help to explain its ability to survive in hatching eggs.

Conclusions

The eggs of avian species must support life independently in an environment full of the threat of infection during the period of incubation (Cook et al., 2005). In the present work, we determined the changes in concentration and activity of the antimicrobial proteins in egg albumen during the early incubation stages. We found the antimicrobial system of the egg albumen would go through these changes presumably due to the natural metabolic processes occurring within the hatching egg. The changes in antimicrobial activity of albumen during the early incubation stages suggest an evolutionary adaptation mechanism for avian reproduction. However, there is still much left to learn regarding the immunobiology of avian egg contents and of the developing embryos they contain.

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