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Eggshell Matrix Proteins as Defense Mechanism of Avian Eggs

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This study focused on the role of eggshell matrix proteins as a function of potential natural antimicrobial defenses of avian eggs. The electrophoretic profile of SDS-PAGE showed that the soluble eggshell matrix proteins had three major bands of 15 000, 36 000, and 66 000 and several minor bands comprising 17 000, 25 000, 30 000, and 75 000, while insoluble matrix proteins were consisting of various bands comprising at least 16 distinct migration bands between 10 000 and 200 000. Three bacteria species, *Pseudomonas aureginosa*, *Bacillus cereus*, and *Staphylococcus aureus*, were found to be inhibited in the presence of soluble eggshell matrix proteins (100 μg/mL). On the other hand, *Escherichia coli* and *Salmonella enteritidis* were weakly inhibited at only an early stage of incubation time (up to 4 h). Scanning electron microscopy revealed that eggshell matrix proteins might interact and disrupt the membrane integrity of bacteria. The present study clearly indicated that avian eggshell matrix proteins possess a potential of novel antimicrobial defensin mechanism.

KEYWORDS: Avian egg; eggshell matrix proteins; antimicrobial defense; ovocleidin; lysozyme; gram negative; gram positive; SEM

INTRODUCTION

The eggshell is a highly specialized mineralized structure that is the only rigid part of an avian egg. Together with its membranes, the avian eggshell provides protection against physical damage and against microorganisms and small predators. The shell is especially important during embryogenesis and is a source of calcium to the embryo. The true shell consists of four morphologically distinct regions which, proceeding outward from and including the mammillary knob layer, are designated the cone, palisade, vertical crystal layer, and cuticle (*1*, *2*). The cuticle thickness on the eggs of domestic hens varies from 0.5 to 12.8 μm (*3*) over the surface of the same egg and has an effective life span of 96 h after oviposition (*4*). Theoretically the cuticle subserves a number of diverse functions, varying from reducing water loss to the first lines of defense against bacterial penetration by blocking the external surface of pores. The cuticle consists of 85−87% protein, 3.5−4.4% carbohydrates, 2.5−3.5% fat, and 3.5% ash (*5*). The shell is approximately 95% calcium carbonate by weight, and the remaining material, 3.5%, is an organic matrix consisting mainly of glycoprotein and proteoglycans (*6*−*8*).

The vertical crystal layer is limited to a thin monolayer of crystals on top of the palisade layer. There are two preferred orientations in the palisade layer, maximum toward the shell surface. The matrix, a protein/polysaccharide complex with calcium binding properties, is formed from peptides synthesized in the uterine tissue. Certain of the identified matrix proteins are eggshell-specific (Ovocleidin-17, Ovocleidin-23, a more glycosylated form of Ovocleidin 17, and Ovocleidin-116 (*6*−*8*), while others are also found in the egg white (ovalbumin, lysozyme) (*6*, *9*, *10*) or in bone (Osteopontin) (*11*). The eggshell is nature's way of protecting the developing avian embryo outside the hen. It is about 94% calcite, 4% organic components (proteins), and a variety of other elements of which magnesium and phosphorus are the most elevated (*12*).

Any form of damage or defect in the shell greatly increases the risk of penetration by microorganisms (*13*). Since the pioneering study of egg microbiology, a great many investigations have shown that the hen's egg is endowed with chemical and physical defenses against microorganisms (*14*).

Infection of the outer surface of an eggshell is potently the first step in transshell infection. Many studies have shown that the shell acquires a broad range of contaminants through contact with nest material (*14*). The shell may be considered as a series of resistances, an important one of which is the chemical compounds, in particular the proteins of the matrix. Hence, the matrix proteins were under investigation in this study as potential antimicrobial activity against the various gram positive bacteria that dominates the flora on the shell surface and the gram negative bacteria that contaminates the contents of the avian eggs.

MATERIALS AND METHODS

Extraction of Eggshell Matrix Proteins. Eggs laid by 24−30 weeks old White Leghorn hens were supplied from the research unit at Arkell Poultry Research, University of Guelph, Ontario. Eggs were cracked and eggshells soaked in warm water overnight and then washed well to remove any traces of albumin. The collected shells were dried in the incubator at 37 °C after separating them from the membranes and ground to a fine powder using a mill. The extraction method of eggshell matrix proteins was adapted from Mann and Sieldler (*8*, *15*) with a

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slight modification. The eggshell powder was demineralized with 20−45% acetic acid (Fisher Scientific, Fair Lawn, NJ) with constant stirring at 4 °C for overnight (100 g of powder per 1250 mL of acetic acid solution). The sample was placed in dialysis tubing (1000 molecular weight cutoff) and dialyzed against Milli Q water for 4 nights at 4 °C. A precipitate which formed during dialysis was collected by centrifugation (8000*g* × 20 min), and both supernatant and pellet were lyophilized.

Protein Analysis. The concentration of protein in eggshell and extracted eggshell matrix proteins was analyzed using a nitrogen analyzer (Leco FP-528, Leco Instruments Ltd., Mississauga, ON).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (*16*) in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). Samples were run on 12.5% homogeneous gels. Proteins were dissolved in sample buffer (1 M Tris-HCl, pH 6.8, containing 10% SDS) in the presence of 5% (w/v) β-mercaptoethanol, heated for 5 min at 95 °C in an Eppendorf Thermomixer 5436, and loaded onto the gel at a concentration of 15 μg/well. Gels were run at a constant current (18 mA/ slab gel), after which they were stained in coomassie brilliant blue.

Testing for Antimicrobial Activity. The following bacterial strains were used in this study. All isolates were kindly supplied by Dr. Poppe, C. C. (Health of Animal Lab., Health Canada, Guelph, ON). Gram negative: *Salmonella enteritidis* (*Sa. enteritidis*) PT4 SA 992322, *Pseudomonas aureginosa* (*P. aureginosa*) B1092-1987, and *Escherichia coli* (*E. coli*) B00627-19. Gram positive: *Staphylococcus aureus* (*St. aureus*) 23-394 and *Bacillus cereus* (*B. cereus*) F036. Bacterial strains were inoculated from the frozen stock into 9 mL of TSB (tryptic soy broth, Difco, Sparks, MI), incubated at 37 °C in a shaking incubator overnight. A loopful was streaked onto TSA (tryptic soy agar, Difco) and incubated at 37 °C overnight to get isolated colonies.

To establish a correlation between the colony forming unit (CFU)/mL and absorbance at 660 nm, a standard curve was formed for the strains of bacteria. A 9 mL aliquot of TSB was inoculated with one colony of bacteria and incubated overnight in the 37 °C shaking incubator at 250 rpm. A 50 μL aliquot of overnight culture was added to 5 mL of peptone water and incubated in the 37 °C shaking incubator for 3 h. Five serial duplicate dilutions were made per inoculum, and the absorbance at 660 nm was measured using the Novospec 2 Pharmacia Stectrophometer (Amersham Pharmacia Biotech. Piscataway, NJ). A 100 μL aliquot of each dilution was inoculated on TSA plates and incubated at 37 °C for 24 h. Plates were counted to determine the CFU/mL corresponding to the absorbance of that same dilution.

The extracted eggshell proteins prepared by acetic acid extraction were tested for antimicrobial activity at a final constant concentration, 0.1 mg/mL. A 1.0 mg/mL concentration of protein in PBS (10mM phosphate buffer, pH 7.2, containing 0.15 M NaCl) was prepared as stock solution and filter sterilized (0.22 μm). A 50 μL aliquot of overnight bacterial culture was added to 5 mL of peptone water (PW; 10 g of peptone and 5 g of NaCl in 1000 mL of MilliQ water) and incubated in the 37 °C, 250 rpm shaking incubator for 2 h to reach the exponential phase of growth. Absorbance was measured at 660 nm (PW as blank), the corresponding CFU/mL was determined by the plotted standard curve, and the necessary dilutions were made in peptone water to get a ca. 10^8 CFU/mL. A 300 μL aliquot of the above inoculum was transferred to 29.7 mL of peptone water (1:100) dilution.A 0.8 mL aliquot of the eggshell protein and 0.8 mL of PBS as the control were added to 7.2 mL of the above bacterial inoculum to obtain a 0.1 mg/mL of protein final concentration. Assays were performed in duplicates and tubes incubated in the 37 °C shaking incubator. Absorbances were read at time intervals of 0,1, 2, 4, 6, and 8 h. At time intervals 0, 4, and 8 h, dilutions were made and 100 μL of each was streaked on TSA plates and incubated at 37 °C for 24 h. Counts in CFU/mL were correlated with absorbances, and the growth rate was analyzed. For *Bacillus cereus*, the same procedure except that BHI (brain heart infusion, Difco) broth and agar were used instead of TSB and TSA and tubes and plates were incubated at 30 °C.

Only *St. aureus*, and *P. aureginosa* were tested as a function of eggshell protein concentration. Bacterial inoculum was prepared the same as above. Four different concentrations of the eggshell protein samples were made by adding 0.25, 0.5, 1, and 2 mg/mL of PBS. A 0.2 mL aliquot of each protein concentration and 0.2 mL of PBS as control were added to 1.8 mL of each of the bacterial inoculum prepared in advance. The final protein concentrations attained were 0, 0.025, 0.05, and 0.2 mg/mL. Assays were performed in duplicates and tubes incubated in the 37 °C shaking incubator. Absorbances were read at 660 nm at time 0 and after 6 h of incubation; then dilutions were made, and 100 μL of each was streaked on TSA plates and incubated at 37 °C for 24 h. CFU/mL was determined for each dilution corresponding with the read absorbances, and then the growth rate and inhibition were analyzed accordingly.

Table 1. Extraction of Eggshell Matrix Proteins by Acetic Acid Demineralization Method

acetic acid[a] concn (%, w/v)	yield[b] (mg)	
	soluble form	insoluble form
20	28.8	38.0
40	54.4	67.2
45	44.0	51.6

[a] The extraction was performed at room temperature. [b] The values are expressed as extracted eggshell matrix protein from 100 g of eggshell powder each and an average of two sets of experiments.

Scanning Electron Microscopy (SEM). Suspension of bacteria of *St. aureus* at midlog phase (10^8 cells/mL) made in TSB were incubated with 50 μg/mL of eggshell matrix proteins at 37 °C for 2 and 6 h. The bacteria were washed with 0.9% NaCl. After fixing by the addition of glutaraldehyde (Sigma) (final concentration, 2.5% (w/v)), cell pellets obtained by centrifugation were washed in 20 mM phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde. The fixed cells were carefully spotted onto the slide and then dehydrated in ethanol gradients. Dried cells were coated with gold and examined by scanning electron microscopy (Hitachi Model S-570, Hitachi, Tokyo).

Statistical Analysis. Data were analyzed by ANOVA (SPSS version 8.0 for Microsoft Windows; SPSS, Chicago, IL), and means were separated by Duncan's multiple range test. Significance was defined at $P < 0.05$.

RESULTS AND DISCUSSION

Extraction of Egg Shell Matrix Proteins. In this study, analysis of the eggshell matrix protein determined a 0.89−1.18% (w/w) content, depending on individual eggs, by means of a nitrogen analyzer (data are not shown). This value was much lower than that reported previously by Romanoff and Romanoff (*12*). This may be due to overestimating of contaminant from albumen into the eggshell membrane in early work. Recent studies have demonstrated that the eggshell matrix, which is about 2% of the total eggshell by weight, is made up of proteins (70%) and polysaccharides (11%) (*17*). In a 100 g eggshell powder, an average of 28.8 mg (20% acetic acid) and 54.4 mg (40% acetic acid) soluble proteins were obtained by acetic acid extraction method (**Table 1**). A fair amount of proteins were precipitated during dialysis against MilliQ water. The temperature did not significantly affect the yield of shell matrix proteins (data are not shown). It was demonstrated that a 40% acetic acid demineralization method was the most effective in extracting eggshell matrix proteins currently tested.

Figure 1 showed the SDS-electrophoretic pattern of the eggshell matrix soluble and insoluble proteins. Many differences were observed in the SDS-PAGE of the various bands with two compounds. SDS-PAGE revealed that precipitate during dialysis consists of various bands comprising 16 distinct migration bands between 10 000 and 200 000. Two of the major bands of insoluble fraction were assumed to be a 17 000 band, which was identified as Ovocleidin-17, and 23 000 (Ovocleidin-23)

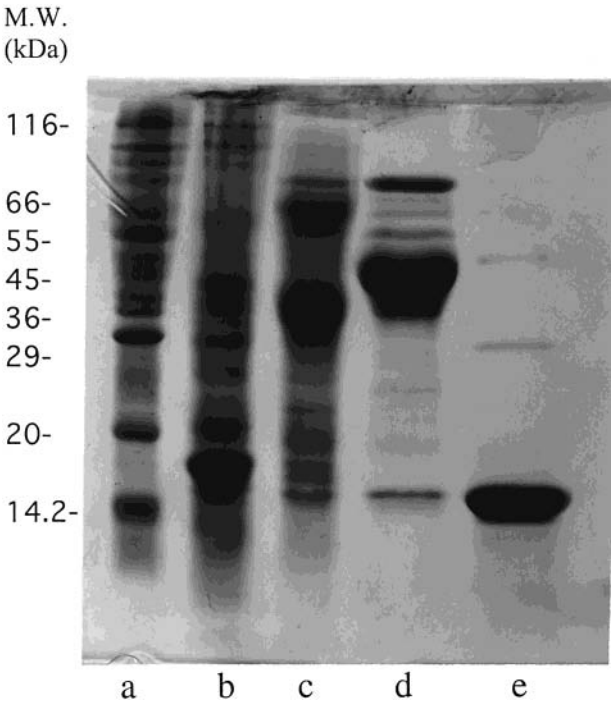

Figure 1. SDS-PAGE of eggshell matrix proteins extracted by acetic acid in 12.5% gels, respectively. Lane a, molecular marker; lane b, insoluble extracted sample; lane c, soluble extracted sample; lane d, whole egg white proteins; lane e, lysozyme. Gels were stained with Coomassie blue.

previously (*8*, *18*). The soluble fraction showed a different profile with the presence of three major bands of 15 000, 36 000, and 66 000 and several minor bands comprising between 15 000 and 76 000 (15 000, 17 000, 25 000, 30 000, and 75 000). In the past decade, biochemical characterization of avian eggshell matrix proteins was well-documented with an effort of various groups (*19*, *20*). It has been reported that some of the eggshell matrix proteins were identified as lysozyme (14 000), ovalbumin (45 000), and ovotransferrin (76 000), which are major proteins in egg white, representing 3.4, 54, and 12% of egg white, respectively (*6*, *10*, *20*). Ovocleidin-17 (17 000) was the first matrix protein to be identified as a uterus protein specific to the eggshell (*18*). This protein is 142 amino acids in length with a C type lectin domain (*8*). Ovocleidin-116 (116 000) is most abundant in the uterine fluid during the intense eggshell calcification phase and is secreted by the granular cells of the surface epithelium (*7*). This protein is the first matrix protein to be cloned. Additional uterine proteins have been identified by using antibodies against the 36 000 and 32 000 electrophoretic bands of the uterine fluid (*21*). Ovocalyxin-32 (32 000) is present during the growth phase and is predominant during the terminal phase of calcification. Ovocalyxin-36 (36 000) is also observed in the other part of the oviduct (*22*). Osteopontin, a noncollagenous phosphorylated glycoprotein, is present in the eggshell (*23*).

Microbial contamination of eggs is a well-established phenomenon and has important economic implications to the poultry industry. There are two ways in which eggs can become contaminated, namely, by transovarian and transshell routes. In practice problems associated with microbial penetration of the shell are normally manifested in some observable way, e.g., spoilage or reduced hatchability. However, it is important to appreciate that the egg presents to the invading organisms a complex series of defensive barriers (*13*), and although microbes may successfully penetrate the shell of the egg, further development of the organism may be arrested or delayed. Furthermore,

Table 2. Antibacterial Activity of Eggshell Matrix Proteins[a]

strains	CFU[b] ($\times 10^6$ cells/mL after incubation) 0 h	4 h	8 h
P. aureginosa			
control	0.68 ± 0.1^a	12.0 ± 2.1^a	29 ± 2.8^a
EMP[c]	0.69 ± 0.2^a	0.89 ± 0.1^b	1.05 ± 0.2^b
E. coli			
control	330 ± 11.9^a	760 ± 23.0^a	1100 ± 55.0^a
EMP	330 ± 20.7^a	440 ± 22.0^b	1600 ± 89.0^b
Sa. enteritidis			
control	1.75 ± 0.2^a	34.5 ± 3.4^a	1660 ± 132^a
EMP	1.70 ± 0.1^a	27.4 ± 1.7^b	2370 ± 224^b
St. aureus			
control	1.23 ± 0.3^a	46.7 ± 2.9^a	98.0 ± 9.9^a
EMP	1.19 ± 0.1^a	28.0 ± 3.7^b	55.0 ± 11.0^b
B. cereus			
control	0.04 ± 0.005^a	0.39 ± 0.01^a	12 ± 1.9^a
EMP	0.03 ± 0.002^a	0.09 ± 0.007^b	0.12 ± 0.03^b

[a] Means in the same column with same bacteria with different letters are significantly different ($P < 0.05$). [b] CFU is expressed as an average ± SD of three sets of experiments each. [c] EMP: eggshell matrix proteins (0.1 mg/mL).

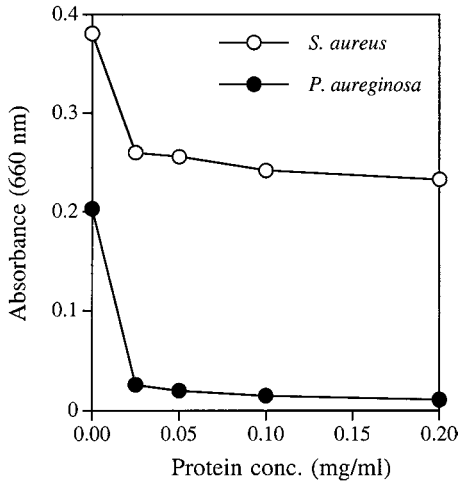

Figure 2. Antimicrobial activity of eggshell matrix proteins at different protein concentrations against *St. aureus*, and *P. aureginosa* after 8 h of incubation. Each value is expressed as an average of three sets of experiments.

it is well-established that different microorganisms differ in their ability to overcome the defense mechanism of the egg and grow in the egg contents (*24*). Hence, studying and revealing the many physical and chemical defenses of the egg against microorganisms' invasion and penetration have been the focus of many researchers throughout the years. More has been known about the physical defense, yet there is more to be discovered about the chemical defense. The focus of this study was on the chemical defenses, mainly proteins of the eggshell matrix proteins, as they are the first barrier and the first layer exposed to contamination and penetration. Some proteins were identified as either specific to the shell or present in the other egg contents, while others are yet to be discovered and defined, and this was another focus of our study. In this study, only soluble eggshell matrix proteins derived from acetic acid extraction were used for antimicrobial tests since the precipitates were difficult to solve in media or buffers used in this study. We investigated the antimicrobial activities against gram negative *(E. coli, P. auregenosa*, and *Sa. enteritidis*) and gram positive (*St. aureus* and *B. cereus*). Initially, we determined the growth inhibition effect of eggshell soluble matrix proteins in media (**Table 2**). Three bacterial species of five examined, *P. aureginosa, B.*

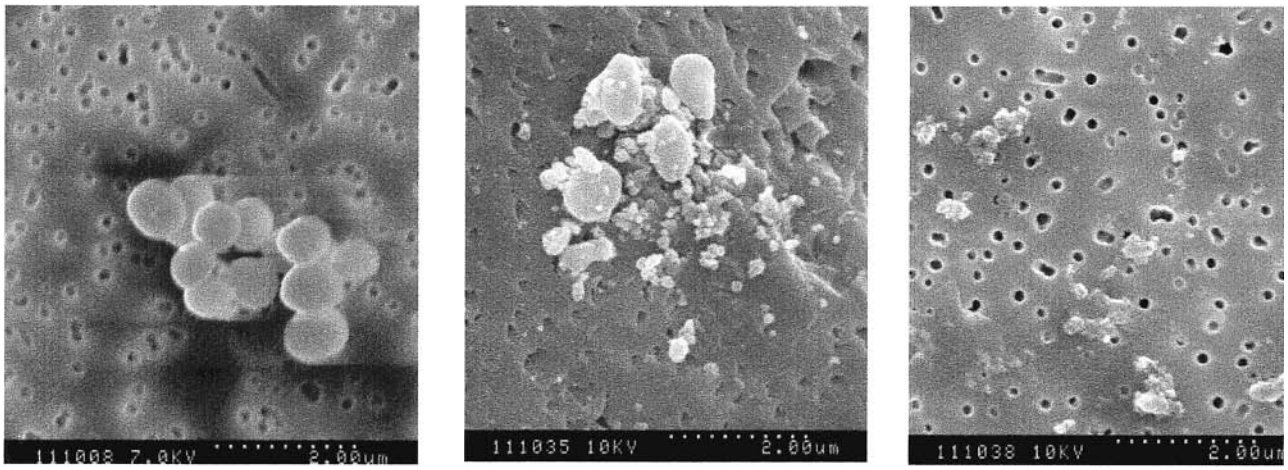

Figure 3. Scanning electron micrographs of *St. aureus* (10^8 cells/mL) incubated without (A) and with 50 μg/mL eggshell matrix proteins for 2 (B) and 6 h (C) at 37 °C. Magnification, 20000 × (reproduced at 85% of original size).

cereus, and *St. aureus* were found to be strongly inhibited. It is worth stating that a reduction and inhibition of growth was evident with *P. aureginosa* and *B. cereus* throughout the 8 h incubation period and in the first 4 h with *St. aureus*. This indicates clearly a potential antimicrobial activity of soluble eggshell matrix proteins. On the other hand, *E. coli and Sa. enteritidis* were weakly inhibited at an early stage of incubation time (up to 4 h); however, it increased substantially higher than that of the control during the late incubation time. This could be attributed to the other proteins in the extracts that could be used as nutrients by both organisms. Hence, in the initial stages the protein extracts might have slightly inhibited the growth until those nutrient components became available by the bacteria through several extracellular and/or intracellular activities.

To further verify the antimicrobial potency of eggshell matrix proteins, the antimicrobial activity against *St. aureus* and *P. aureginosa* was evaluated as a function of protein concentration. As shown in **Figure 2**, the eggshell matrix proteins caused a dramatic decrease in the CFU of *P. aureginosa* with a low concentration of 0.025 mg/mL, where it became constant. *St. aureus* showed approximately similar activity, but the decrease of CFU was weaker than that of *P. aureginosa*. The antimicrobial activity of lysozyme which was observed in eggshell soluble matrix fraction was well-known as to lyse the cell walls of certain gram positive bacteria (*25*). It has been reported that eggshell matrix and eggshell membranes consist of lysozyme, and it could add to the protective function of the eggshell during embryonic development (*10*). Some of the antimicrobial action of eggshell matix proteins could be attributed to the presence of lysozyme; however, it was reported that the minimum inhibition concentration of lysozyme which causes a dramatic decrease in the CFU of *St. aureus*, was 50 μg/mL (*26*). The lysozyme concentration in the extracted soluble fraction was estimated to be less than 5% of the total proteins accounted for by enzyme activity test (data not shown) (*27*), while eggshell matrix proteins exhibited a strong antimicrobial activity in the concentration of 25 μg/mL (the assumed lysozyme concentration with less than 1.25 μg/mL). The present results strongly suggest that eggshell matrix proteins pose novel bactericidal potential in addition to existing lysozyme activity. Further evidence to support potential antimicrobial activity of eggshell proteins was demonstrated by observing morphological changes associated with the susceptibility of gram positive *St. aureus* (**Figure 3**). The SEM micrographs showed clearly that eggshell matrix proteins exhibited direct damage of the bacterial membrane at 50 μg/mL proteins for 2 h incubation. Furthermore, prolonged incubation (6 h) produced a completely collapsed morphology of the bacteria with the membrane debris. This result suggests a possible way in which eggshell matrix proteins might interact and disrupt the membrane integrity.

In summary, the eggshell matrix soluble protein extracts have an antimicrobial activity against *P. aureginosa*, *St. aureus*, and *B. cereus*, at low concentrations (25−100 μg/mL). Antimicrobial activity of insoluble fractions is another interest; however, a different method will be required to identify its biological functions. Avians must possess protective mechanisms to prevent microbial contamination of the egg contents, the embryo, before the mineralizing shell becomes a physical barrier. Further studies on biochemical characterization of eggshell matrix proteins as bactericidal functions would provide better insight into novel natural defense mechanisms of avian eggs. Identification and isolation of antimicrobial components in eggshell matrix is ongoing in our laboratory.

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