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ARTICLE

Antimicrobial Activity of Lipophilic Avian Eggshell Surface Extracts

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The avian eggshell cuticle is the waxy outermost layer of the mineralized eggshell in direct contact with the environment. In this study, lipophilic eggshell surface extracts from three domestic species were evaluated for their antimicrobial activity. Chicken and goose extracts demonstrated potent bactericidal activity against both Gram-positive and Gram-negative bacteria, while activity could not be detected for duck eggshell surface extracts. Using the chicken as a model species, evaluation of albumen, fecal material, and uropygial gland extracts eliminated these as a potential source of the observed activity. Results suggest that lipophilic components are incorporated into the egg during its formation and play a role in antimicrobial defense. This study represents the first successful extraction and evaluation of lipophilic antimicrobial components from the avian egg.

KEYWORDS: Eggshell; cuticle; antimicrobial; avian; lipophilic; bacteria

INTRODUCTION

The eggshell cuticle is the waxy outermost layer of the mineralized eggshell in direct contact with the environment (*1*). The cuticle is a thin noncalcified organic layer of variable thickness (0.5−12.8 μm) composed of hydroxyapatite crystals, polysaccharides, lipids, and glycoprotein (*2*). The cuticle is deposited on the mineral surface during the last 1.5 h prior oviposition (*3*). This layer is thought to regulate water/gas exchanges as well as the entry of micro-organisms, through water repellent obstruction of the eggshell pores and/or by limiting microbial colonization of the eggshell surface (*4*−*8*).

Previous studies have investigated some of the constituents of the eggshell cuticle as well as their possible contribution to the chemical aspect of antimicrobial defense (*1*, *9*). Lysozyme and ovotransferrin, two egg white proteins known for their antimicrobial activity, have been localized within the avian eggshell (*1*, *9*−*15*). In addition, aqueous extracts of eggshell and cuticle components, in Anseriformes and Galliformes, have demonstrated antimicrobial activity as well as the presence of antimicrobial proteins including lysozyme, ovotransferrin, ovocalyxin-32, and ovocleidin-17 (*1*, *9*, *16*−*18*). Few studies have investigated the composition of insoluble components within the avian eggshell. Analysis of EDTA-insoluble eggshell extracts revealed a complex profile of insoluble proteins/peptides which were concentrated within 3 locations, namely, the mammillary layer, palisades, and cuticle (*19*−*22*). To date, no studies have investigated the contribution of insoluble or lipophilic eggshell components to the antimicrobial defenses of the avian egg. Lipophilicity is closely associated with the permeation of bacterial membranes and is an important parameter in the development of antimicrobial agents (*23*).

In this study, the antimicrobial activity of eggshell surface lipophilic extracts was evaluated in three domestic avian species. Our results demonstrate potent antimicrobial activity against Gram-positive and Gram-negative bacteria. The observed activity could not be attributed to simple eggshell surface contamination but rather suggested the specific incorporation of lipophilic antimicrobial components, within the eggshell cuticle, during the formation of the egg. This is the first article describing lipophilic antimicrobial components from the avian eggshell surface.

MATERIALS AND METHODS

Ethyl Acetate Eggshell Surface Extracts. Domestic chicken, *Gallus gallus*, domestic duck, *Anas platyrhyncos*, and domestic goose, *Anser anser*, eggs (25, 25, and 16, respectively) were obtained from a local farm in Perth (Ontario, Canada). Unwashed eggs were immersed in ethyl acetate (25 mL/egg) for 1 h at room temperature. Extracts were filtered using Whatman No. 3 filter paper and concentrated by rotary evaporation at 25 °C until dry and stored at −80 °C in the dark until further use. The mass of the dry extract recovered was also recorded.

Antimicrobial Activity of Ethyl Acetate Extracts. Antimicrobial activity of ethyl acetate extracts was evaluated using an adapted version of the radial diffusion assay (*24*). *Bacillus subtilis* ATCC 19659, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, or *Escherichia coli* D31 were grown to log phase (optical density of 0.2 at 600 nm) in Luria−Burtani broth (Bioshop, Burlington, Canada). Bacterial cultures were centrifuged, washed, and resuspended in 10 mM sodium phosphate buffer (pH 7.3). Solid culture medium (1.5% low EEO agarose,

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1% biotryptone, and 0.5% yeast extract; Bioshop, Burlington, Canada) was prepared in 10 mM sodium phosphate buffer. After cooling to 42 °C, molten medium was inoculated with approximately 1×10^5 CFUs/mL. Plates were left to solidify at room temperature.

Dry ethyl acetate extracts were resuspended in 1 mL of ethyl acetate. A 10 μL sample of each extract was applied to Whatman No. 1 hole punch paper discs (6 mm diameter). Prior to application onto bacterial plates, sample discs were incubated ~1 h at room temperature to allow for the evaporation of the residual solvent. Discs treated with ethyl acetate or 10 mg/mL kanamycin in 70% ethanol were used as negative and positive controls, respectively. Bacterial plates, with applied sample discs, were incubated at 37 °C for 6−18 h. Plates were stained using 0.02 mg/mL Coomassie blue in 27% methanol and 15% formaldehyde. Plates were photographed and the diameter of clear zones measured.

Origin of Active Fraction within the Chicken Eggshell Surface Extract. An additional sample of 350 chicken eggs was treated with ethyl acetate as described. Fresh egg white from a dozen chicken eggs was also treated with an equal volume of ethyl acetate for 1 h at room temperature with gentle mixing. The resulting solution was centrifuged (22 000*g*, 4 °C, 30 min), and the liquid phase was concentrated by rotary evaporation until dry. Additionally, preen oil was collected from 12 laying hens by gentle massaging of the uropygial gland using a cotton swab as described by Reneerkens et al. (*25*). Preen oil was then extracted by immersing the cut cotton swab-stick heads in ethyl acetate for 1 h with shaking. Finally, a 50 g sample of fresh fecal material was suspended in 500 mL of ethyl acetate for 1 h at room temperature. The liquid phase was filtered and concentrated by rotary evaporation until dry. The mass of individual extracts was recorded. Dry extracts were resuspended (in 1 mL of ethyl acetate for each 625 mL of unevaporated extract; the same dilution factor as that for the small scale chicken eggshell surface extract) and evaluated for antimicrobial activity. A sample of each crude extract was suspended in methanol (1:10 dilution) and further diluted (1:10) with 15% acetonitrile and 0.1% trifluoroacetic acid. These were fractionated on a C_{18} μ-Bondapak column (Waters, Milford, USA) by reverse phase high pressure liquid chromatography (HPLC) using an acetonitrile gradient. Elution was monitored spectrophotometrically at 240 nm.

Identification of Active Components from the Purified Chicken Ethyl Acetate Fraction. The remaining chicken eggshell surface ethyl acetate extract was fractionated by HPLC, and the eluate (4 mL/min) was individually collected. Fractions were dried at 25 °C using an Eppendorf vacufuge concentrator (Fisher Scientific, Mississauga, Canada) and evaluated for antimicrobial activity. Samples of the purified and crude extracts were individually suspended in 4% sodium dodecyl sulfate, 25% glycerol, and 1.5% Tris-HCl, pH 6.8, for SDS−PAGE analysis. Bromophenol blue (0.125 mg/mL) and 1,4-dithiothreitol (7.7 mg/mL) were added prior to heating (5 min at 90 °C) and gel loading. A molecular weight marker (MBI Fermentas, Burlington, Canada) was also loaded. SDS−PAGE was carried out on a 20% polyacrylamide gel and visualized by Coomassie Blue staining. Bands of interest were excised and digested in-gel with trypsin (*26*). The extracted peptides were purified with Stage Tips (*27*) and analyzed using LC/MS/MS on an LTQ-FT mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as previously described (*28*). The resulting raw-files were evaluated with MaxQuant (*29*, *30*) version 1.1.0.44 using the ipiChick database v3.65 (http://www.ebi.ac.uk/IPI/IPIchicken.html) supplemented with the reversed database and common contaminants, such as human keratins, for protein identification.

RESULTS AND DISCUSSION

Antimicrobial Activity of Avian Eggshell Surface Ethyl Acetate Extracts. Treatment of whole chicken eggs with ethyl acetate allowed the extraction of surface components which presumably are constituents of the eggshell cuticle. Approximately 10 mg of concentrated oily extract was obtained from the initial sample of 25 chicken eggs. This extract was found to demonstrate antimicrobial activity against both *B. subtilis* and *E. coli* D31 (**Figure 1** and **Table 1**). The crude extract was more potent against the Gram-positive *B. subtilis* than the Gram-negative *E. coli* D31. Gram-positive bacteria have been reported as major microbial contaminants, accounting for up to 92% of eggshell surface

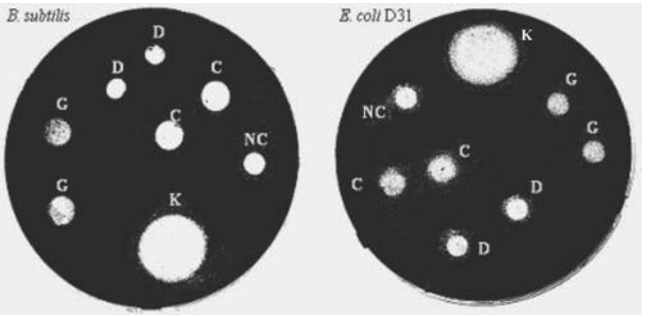

Figure 1. Antimicrobial activity of crude avian eggshell surface ethyl acetate extracts against *Bacillus subtilis* and *Escherichia coli* D31. Samples (K, kanamycin; C, chicken extract; D, duck extract; G, goose extract; NC, negative control) were applied to paper discs and placed onto an agarose plate inoculated with bacteria. After overnight incubation at 37 °C, plates were stained and the discs removed. Plates were photographed, and clear zone diameters were measured. Experiments were conducted in triplicate.

Table 1. Antimicrobial Activity of Avian Eggshell Extracts against *Bacillus subtilis* and *Escherichia coli* D31[a]

		clear zone diameter	
sample	symbol	*B. subtilis*	*E. coli D31*
kanamycin (10 mg/mL) in 70% ethanol	K	19 ± 0 mm	21 ± 0 mm
ethyl acetate negative control	NC	6 ± 0 mm	6 ± 0 mm
chicken ethyl acetate extract	C	12 ± 0 mm	10 ± 0 mm
duck ethyl acetate extract	D	6 ± 0 mm	6 ± 0 mm
goose ethyl acetate extract	G	12 ± 0 mm	10 ± 0 mm

[a] Clear zone diameter (mm) ± standard deviation is indicated for each sample depicted in **Figure 1**. Experiments were performed in triplicate.

contaminants, in duck hatcheries (*31*). Egg and eggshell components, including lysozymes, ovotransferrin, and avian c-type lectin-like proteins, extracted using aqueous solutions, have been reported to be the most active against Gram-positive bacteria (*1*, *9*, *16*−*18*). However, these individual agents were inactive when evaluated using the antimicrobial assay described in this study (data not shown). Lipophilic components of the eggshell surface may therefore provide additional antimicrobial defense and, through their strategic localization on the outer eggshell surface, represent the first line of defense of the avian egg against contamination of egg contents by Gram-positive bacteria, in particular.

The effect of dose variation on the antimicrobial activity of the chicken crude eggshell surface extract was evaluated against *B. subtilis*. A dose-dependent relationship was obtained within the 0.04−2.5 mg/mL range of the 2-fold serial dilution of the 10 mg/mL crude eggshell surface extract (**Figure 2**). On average, approximately 400 μg of material was obtained from the extraction of a single egg. Since the material obtained from a single egg (maximal concentration of 80 mg/mL when resuspended in 5 μL and applied directly) could easily surpass the minimal detection range, it is conceivable that hydrophobic components present on the surface of individual chicken eggs would enhance the antimicrobial defenses of the egg in vivo. Additionally, these antimicrobial components may enhance the food safety of eggs assuming that the integrity of the eggshell surface and cuticle is maintained during egg processing.

Eggs from domestic ducks and domestic geese were also evaluated. The domestic goose eggshell surface extract was found to demonstrate activity against both *B. subtilis* and *E. coli* D31 equal in magnitude to that observed for the chicken extract suggesting the possible conservation of the active constituents

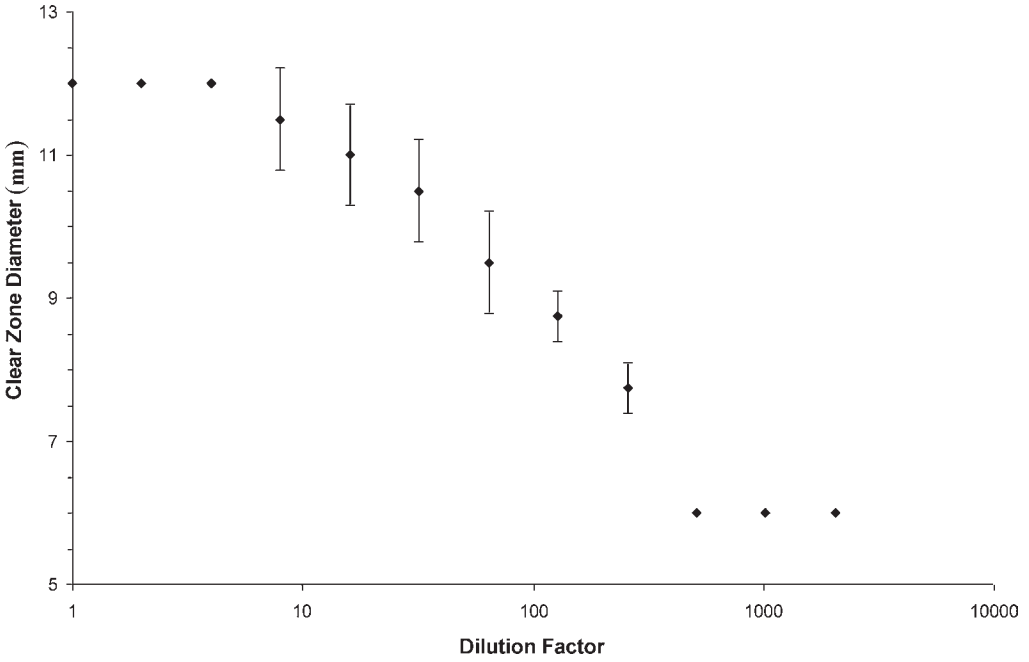

Figure 2. Dose−response curve of crude chicken eggshell surface ethyl acetate extract against *B. subtilis*. A sample (5 μL) of serially diluted (2-fold dilution) crude chicken ethyl acetate extract (10 mg/mL) was applied to Whatman No. 1 paper discs (6 mm diameter) and placed onto a plate inoculated with *B. subtilis*. The diameter of the clear zones was measured after overnight incubation. Experiments were performed in triplicate. Average clear zone diameter (mm) ± standard deviation is presented.

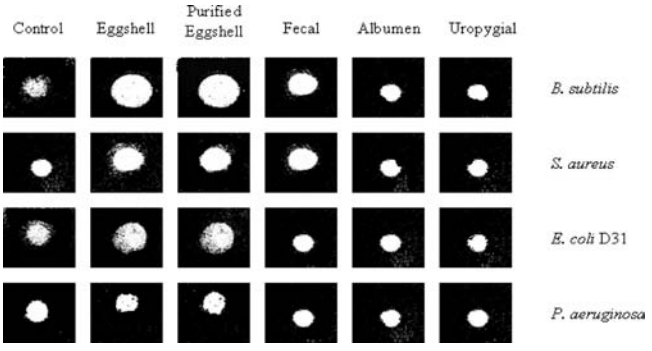

Figure 3. Comparative antimicrobial spectrum of chicken albumen, fecal material, uropygial gland material, eggshell surface, and purified eggshell surface ethyl acetate extracts. Samples of each extract were applied to paper discs and placed onto agarose plates inoculated with Gram-positive or Gram-negative bacteria. Plates were photographed after staining and disk removal. Experiments were conducted in triplicate.

across avian orders (**Figure 1** and **Table 1**). However, eggshell surface extracts from domestic duck eggs were inactive against both *B. subtilis* and *E. coli* D31 (**Figure 1** and **Table 1**). Presumably, this difference is due to a species-specific unidentified biological and/or behavioral characteristic of the domestic duck. The antimicrobial activity of eggshell surface extracts is therefore not restricted to a single gallinaceous bird species but is also observable in at least one member of the Anseriformes order. In agreement with this claim, conserved expression and activity of aqueous antimicrobial proteins across avian orders, and especially across species within orders, has been previously demonstrated (*1*, *9*, *13*).

Potential Source of Active Extract. Extracts from various sources, namely, albumen, fecal material, and preen oil, were evaluated for their activity. Egg white components are known to possess antimicrobial activity and are present within the eggshell (*1*, *9*−*15*).

Table 2. Antimicrobial Activity of Egg White, Fecal Material, Uropygial Gland Material, Crude Eggshell, and HPLC Purified Eggshell Extracts from Chicken[a]

bacteria	sample	clear zone diameter
B. subtilis	crude eggshell extract	12 ± 0 mm
	purified eggshell extract	12 ± 0 mm
	negative control	6 ± 0 mm
	fecal extract	9 ± 0 mm
	egg white extract	6 ± 0 mm
	uropygial extract	6 ± 0 mm
S. aureus	crude eggshell extract	8 ± 0 mm
	purified eggshell extract	8 ± 0 mm
	negative control	6 ± 0 mm
	fecal extract	9 ± 0 mm
	egg white extract	6 ± 0 mm
	uropygial extract	6 ± 0 mm
E. coli D31	crude eggshell extract	10 ± 0 mm
	purified eggshell extract	10 ± 0 mm
	negative control	6 ± 0 mm
	fecal extract	6 ± 0 mm
	egg white extract	6 ± 0 mm
	uropygial extract	6 ± 0 mm
P. aeruginosa	crude eggshell extract	6 ± 0 mm
	purified eggshell extract	6 ± 0 mm
	negative control	6 ± 0 mm
	fecal extract	6 ± 0 mm
	uropygial extract	6 ± 0 mm

[a] The antimicrobial activity of samples was evaluated against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* D31. Clear zone diameter (mm) ± standard deviation is indicated for each sample depicted in **Figure 3**. Experiments were performed in triplicate.

Albumen components therefore represent a potential source of the extract either through seepage across the egg membranes/shell, through elution from the eggshell, or as contaminants deposited during oviposition. Inspection of treated eggs revealed that ethyl acetate had not penetrated or compromised the contents. Additionally, in contrast to the eggshell surface extract,

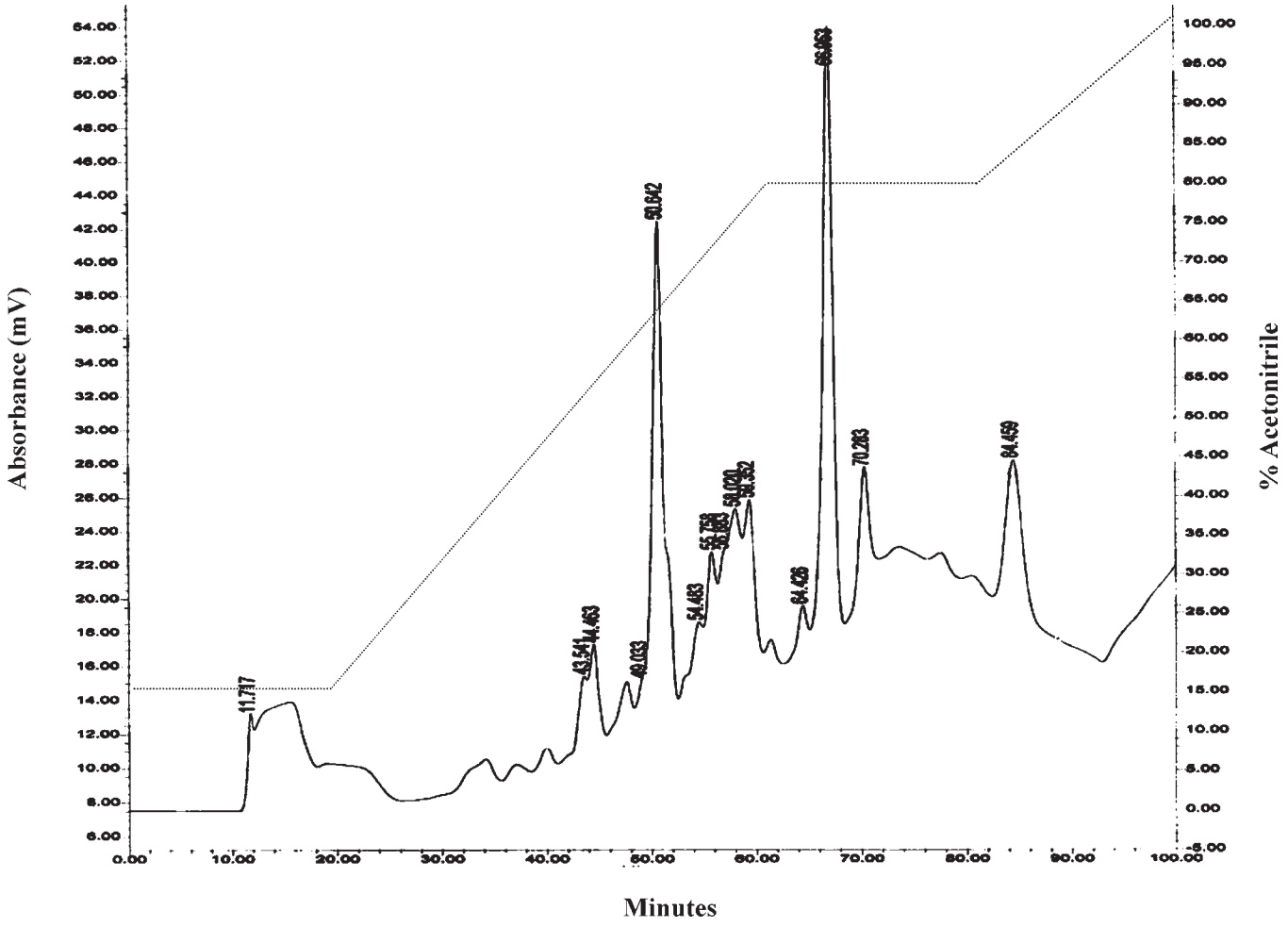

Figure 4. Analysis of crude chicken eggshell surface ethyl acetate extract by reverse phase high pressure liquid chromatography using an acetonitrile gradient on C_{18} column. Elution at 4 mL/min was monitored at 240 nm and fractions individually collected every minute. The *x*-axis describes the chromatography progression (minutes), the right *y*-axis describes the acetonitrile gradient, and the left *y*-axis indicates the absorbance (mV). A major peak demonstrating antimicrobial activity was eluted at 66.953 min.

egg white extracts failed to demonstrate activity against *B. subtilis*, *E. coli* D31, or *Staphylococcus aureus* (**Figure 3** and **Table 2**).

Bacteria present within the avian intestinal environment, including *Enterococcus gallinarum*, have been reported to secrete antimicrobials (*32*). These agents may contaminate the eggshell surface during brooding. Evaluation of fecal extracts revealed activity against both *B. subtilis* and *S. aureus* but not against *E. coli* D31 (**Figure 3** and **Table 2**). While the fecal and eggshell surface extracts were approximately equally active, against both *S. aureus* and *P. aeruginosa*, the activity of the postulated source (fecal material) was less pronounced, against *B. subtilis* and *E. coli* D31, than observed with the presumably contaminated eggshell surface extract. Furthermore, the HPLC elution profile of both eggshell surface and fecal extracts did not correspond (data not shown).

Finally, uropygial gland extracts were evaluated for their activity. Preen oil is a holocrine substance, secreted by the uropygial gland, which is spread over plumage in order to maintain feather condition (*33*). Reports suggest that preen oil promotes feather conditions through its antimicrobial and antifungal properties (*33*−*35*). Preen oil may also contaminate the egg surface and account for the observed activity of the eggshell surface extract. However, upon evaluation, no antimicrobial activity was detected with the uropygial gland extract (**Figure 3** and **Table 2**).

Preliminary Identification of Active Eggshell Surface Fraction. The remaining chicken eggshell surface extract was fractionated by HPLC. Two major peaks as well as several minor peaks were visible (**Figure 4**). Evaluation of individual fractions revealed that fractions 66 and 67, eluted between the 66 and 68 min time points, were active and corresponded to the major peak obtained at 66.953 min (**Figures 4** and **5**). SDS−PAGE analysis of this purified fraction and the crude extract revealed bands positive for Coomassie Blue staining of low molecular weight (**Figure 5**). Relative to the crude extract, the purified fraction was enriched in a band with an estimated molecular weight of approximately 17 kDa. LC/MS/MS analysis of peptides eluted from gel slices after in-gel digestion with trypsin permitted the identification of several chicken proteins (see Supporting Information, file 1). However, since the proteinaceous material extracted with ethyl acetate was insufficient for in-depth analysis, the results should be considered as preliminary. Most interesting among the tentatively identified proteins was histone H4, which was detected with two different peptides (see Supporting Information, files 2 and 3). Numerous histones and histone-derived peptides were previously reported to play a role in the innate defenses of multiple organisms including Pacific white shrimp, green tree frog, Atlantic cod, and chicken (*36*−*39*). While histones have been successfully isolated from the epithelial surfaces of a number of animals (*36*−*39*), this investigation represents the first study to detect a histone within eggshell surface extracts. Histones, like cuticle

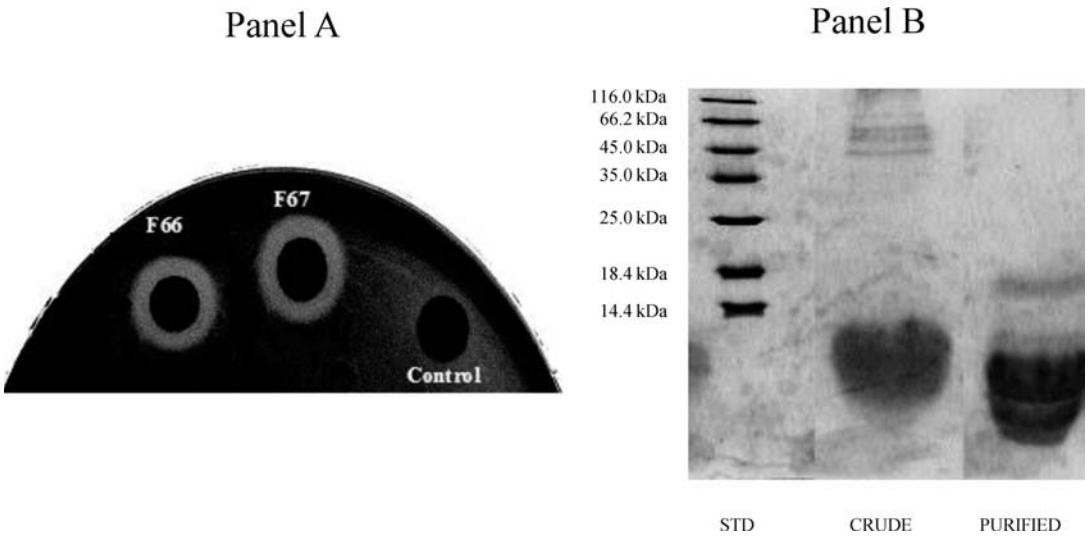

Figure 5. Antimicrobial activity (panel **A**) and SDS−PAGE analysis (panel **B**) of the purified fraction (F66 and F67) corresponding to the peak eluted at 66.953 min during reverse phase high pressure liquid chromatography of crude chicken eggshell surface ethyl acetate extract. Antimicrobial activity was evaluated against *Bacillus subtilis*. The crude extract and purified fraction were run on a 20% SDS−PAGE gel and visualized by Coomassie blue staining.

matrix protein (*40*), may be secreted by ciliated cells of the chicken mucosal epithelium and later incorporated into the eggshell cuticle. Alternatively, cuticle histones may arise from nuclei of decaying/sloughed-off oviduct epithelial cells that are present in uterine fluid during eggshell formation (*28*). The presence of antimicrobial components in eggshell surface extracts suggests a strategic localization within the eggshell cuticle; the waxy outer eggshell layer is believed to play a role in both water/gas exchanges and antimicrobial defense. Upon microbial contamination, lipophilic eggshell surface proteins may encounter the bacterial lipid bilayer and dissociate from the cuticle, thereby adopting an active conformation and accounting for the simultaneous inactivity of the unaltered eggshell and the impressive microbial resistance of eggs.

Contributions and Future Research Directions. In this study, chicken and goose outer eggshell surface lipophilic extracts were found to demonstrate antimicrobial activity against *B. subtilis*, *S. aureus*, and *E. coli*. Dose−response analysis revealed highly potent activity suggesting an in vivo role in the antimicrobial defense of the avian egg. Evaluation of the activity of albumen, fecal material, and preen oil extracts eliminated these agents as a source of extract activity. HPLC and mass-spec analysis permitted preliminary identification of some proteins within the active fraction, including histone H4. This represents the first successful detection of a histone within antimicrobial eggshell surface extracts from the avian egg. Future studies will be necessary to confirm the identity of the lipophilic antimicrobial component, to compare the antimicrobial properties of lipophilic extracts from diverse avian species that are subject to a variety of microbial environments and to confirm the localized expression of lipophilic antimicrobials within the avian reproductive tract and eggshell. Our results further indicate that proteins of pharmaceutical interest can be obtained from the avian eggshell, an inexpensive and readily available source of bioactive molecules. In addition, this study emphasizes the importance of the eggshell cuticle in food safety and antimicrobial defense of the avian egg.

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Supporting Information Available: List of tentatively identified proteins, their accession numbers, number of identified peptides, sequence coverage, slice distribution, and scores; all peptides, their sequences, the accession number of the protein of origin, peptide length and mass, slice distribution, and scores; and annotated spectra of the histone H4 peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Wellman-Labadie, O.; Picman, J.; Hincke, M. T. Antimicrobial activity of the anserifom outer eggshell and cuticle. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2008**, *149*, 640–649.
(2) Fernandez, M. S.; Moya, A.; Lopez, L.; Arias, J. L. Secretion pattern, ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation. *Matrix Biol.* **2001**, *19*, 793–803.
(3) Baker, J. R.; Balch, D. A. A study of the organic material of hen's eggshell. *Biochem. J.* **1962**, *82*, 352–361.
(4) Chavez, C.; Knape, K. D.; Coufal, C. D.; Carey, J. B. Reduction of eggshell aerobic plate counts by ultraviolet irradiation. *Poult. Sci.* **2002**, *81*, 1132–1135.
(5) De Reu, K.; Grijspeerdt, K.; Heyndrickx, M.; Uyttendaele, M.; Herman, L. The use of total aerobic and Gram-negative flora for quality assurance in the production chain of consumption eggs. *Food Control* **2005**, *16*, 147–155.
(6) De Reu, K.; Grijspeerdt, K.; Herman, L.; Heyndrickx, M.; Uyttendaele, M.; Debevere, J.; Putirulan, F. F.; Bolder, N. M. The effect of a commercial UV disinfection system on the bacterial load of shell eggs. *Lett. Appl. Microbiol.* **2006**, *42*, 144–148.
(7) Board, R. G.; Tranter, H. S. The Microbiology of Eggs. In *Egg Science and Technology*, 3rd ed..; Stadelman, W. J., Cotterill, O. J., Eds.; AVI Publishing Company: Westport, CT, 1986; pp 75−96.
(8) Sparks, N. H. C.; Board, R. G. Cuticle, shell porosity and water intake through hen's eggshells. *Br. Poult. Sci.* **1984**, *25*, 267–276.
(9) Wellman-Labadie, O.; Picman, J.; Hincke, M. T. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. *Br. Poult. Sci.* **2008**, *49*, 133–143.
(10) Wellman-Labadie, O.; Picman, J.; Hincke, M. T. Comparative antimicrobial activity of avian egg white protein extracts. *Br. Poult. Sci.* **2008**, *49*, 125–132.
(11) Wellman-Labadie, O.; Picman, J.; Hincke, M. T. Enhanced c-type lysozyme content of wood duck (*Aix sponsa*) egg white: an adaptation to cavity nesting? *Physiol. Biochem. Zool.* **2008**, *81*, 235–245.

(12) Hincke, M. T.; Gautron, J.; Panheleux, M.; Garcia-Ruiz, J.; McKee, M. D.; Nys, Y. Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. *Matrix Biol.* **2000**, *19*, 443–453.
(13) Panheleux, M.; Bain, M.; Fernandez, M. S.; Morales, I.; Gautron, J.; Arias, J. L.; Solomon, S. E.; Hincke, M.; Nys, Y. Organic matrix composition and ultrastructure of eggshell: a comparative study. *Br. Poult. Sci.* **1999**, *40*, 240–252.
(14) Panheleux, M.; Nys, Y.; Williams, J.; Gautron, J.; Boldicke, T.; Hincke, M. T. Extraction and quantification by ELISA of eggshell organic matrix proteins (ovocleidin-17, ovalbumin, ovotransferrin) in shell from young and old hens. *Poult. Sci.* **2000**, *79*, 580–588.
(15) Gautron, J.; Hincke, M. T.; Panheleux, M.; Garcia-Ruiz, J. M.; Boldicke, T.; Nys, Y. Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. *Connect. Tissue Res.* **2001**, *42*, 255–267.
(16) Wellman-Labadie, O.; Lakshminarayanan, R.; Hincke, M. T. Antimicrobial properties of avian eggshell-specific c-type lectin-like proteins. *Febs Lett.* **2008**, *582*, 699–704.
(17) Xing, J.; Wellman-Labadie, O.; Gautron, J.; Hincke, M. T. Recombinant eggshell ovocalyxin-32: expression, purification and biological activity of the glutathione s-transferase fusion protein. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2007**, *147*, 172–177.
(18) Mine, Y.; Oberle, C.; Kassaify, Z. Eggshell matrix proteins as defense mechanism of avian eggs. *J. Agric. Food Chem.* **2003**, *51*, 249–253.
(19) Miksik, I.; Charvatova, J.; Eckhardt, A.; Deyl, Z. Insoluble eggshell matric proteins: their peptide mapping and partial characterization by capillary electrophoresis and high-performance liquid chromatography. *Electrophoresis* **2003**, *24*, 843–852.
(20) Miksik, I.; Charvatova, J.; Eckhardt, A.; Deyl, Z. Peptide mapping by capillary electrophoresis with pluronic F127. *J. Chromatogr., B* **2004**, *800*, 155–160.
(21) Miksik, I.; Eckhardt, A.; Sedlakova, P.; Mikulikova, K. Proteins of insoluble matrix of avian (*Gallus gallus*) eggshell. *Connect. Tissue Res.* **2007**, *48*, 1–8.
(22) Miksik, I.; Sedlakova, P.; Lacinova, K.; Pataridis, S.; Eckhardt, A. Determination of insoluble avian eggshell matrix proteins. *Anal. Bioanal. Chem.* **2010**, *397*, 205–214.
(23) Tokuyama, R.; Takahashi, Y.; Tomita, Y.; Tsubouchi, M.; Yoshida, T.; Iwasaki, N.; Kado, N.; Okezaki, E.; Nagata, O. Structure-activity relationships (SAR) studies on oxazolidinone antibacterial agents. Relationships between lipophilicity and antibacterial activity in 5-thiocarbonyl oxazolidinones. *Chem. Pharm. Bull.* **2001**, *49*, 353–360.
(24) Steinberg, D. A.; Lehrer, R. I. Designer assays for antimicrobial peptides. Disputing the one-size-fits-all theory. *Methods Mol. Biol.* **1997**, *78*, 169–186.
(25) Reneerkens, J.; Piersma, T.; Damste, J. S. S. Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues. *J. Exp. Biol.* **2005**, *208*, 4199–4202.
(26) Shevchenko, A.; Tomas, H.; Havliè, J.; Olsen, J. V.; Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **2006**, *1*, 2856–2860.
(27) Rappsilber, J.; Ishihama, J.; Mann, M. Stop and Go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **2003**, *75*, 663–670.
(28) Mann, K.; Macek, B.; Olsen, J. V. Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. *Proteomics* **2006**, *13*, 3801–3810.
(29) Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized ppb-range mass accuracies and proteome-wide quantification. *Nat. Biotechnol.* **2009**, *26*, 1367–1372.
(30) Cox, J.; Matic, I.; Hilger, M.; Nagaraj, N.; Selbach, M.; Olsen, J. V.; Mann, M. A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat. Protoc.* **2009**, *4*, 698–705.
(31) Seviour, E. M.; Board, R. G. Bacterial growth in albumen taken from the eggs of domestic hens and waterfowl. *Br. Poult. Sci.* **1972**, *13*, 557–575.
(32) Jennes, W.; Dicks, L. M.; Verwoerd, D. J. Enterocin 012, a bacteriocin produced by *Enterococcus gallinarum* isolated from the intestinal tract of ostrich. *J. Appl. Microbiol.* **2000**, *88*, 349–357.
(33) Shawkey, M. D.; Pillai, S. R.; Hill, G. E. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. *J. Avian Biol.* **2003**, *34*, 345–349.
(34) Bandyopadhyay, A.; Bhattacharyya, S. P. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface bacteria of fowl. *Indian J. Exp. Biol.* **1996**, *34*, 48–52.
(35) Bandyopadhyay, A.; Bhattacharyya, S. P. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface fungi of fowl. *Indian J. Exp. Biol.* **1999**, *37*, 1218–1222.
(36) Patat, S. A.; Carnegie, R. B.; Kingsbury, C.; Gross, P. S.; Chapman, R.; Schey, K. L. Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. *Eur. J. Biochem.* **2004**, *271*, 4825–4833.
(37) Kawasaki, H.; Isaacson, T.; Iwamuro, S.; Conlon, J. M. A protein with antimicrobial activity in the skin of Schlegel's green tree frog *Rhacophorus schlegelii* (Rhacophoridae) identified as histone H2B. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 1082–1086.
(38) Bergsson, G.; Agerberth, B.; Jornvall, H.; Gudmundsson, G. H. Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). *FEBS J.* **2005**, *272*, 4960–4969.
(39) Silphaduang, U.; Hincke, M. T.; Nys, Y.; Mine, Y. Antimicrobial proteins in chicken reproductive system. *Biochem. Biophys. Res. Commun.* **2006**, *340*, 648–655.
(40) Rahman, M. A.; Moriyama, A.; Iwasawa, A.; Yoshizaki, N. Cuticle formation in quail eggs. *Zool. Sci.* **2009**, *26*, 496–499.

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