Journal of Food Protection, Vol. 69, No. 4, 2006, Pages 729–738

Identifying the Components in Eggshell Membrane Responsible for Reducing the Heat Resistance of Bacterial Pathogens

GENE AHLBORN[1] AND BRIAN W. SHELDON[2]*

[1]*Department of Food Science and* [2]*Department of Poultry Science, North Carolina State University, Raleigh, North Carolina 27695, USA*

MS 05-490: Received 30 September 2005/Accepted 11 December 2005

ABSTRACT

The biological activity (*D*-value determination) of eggshell membrane (ESM) was examined to determine the membrane components and mechanisms responsible for antibacterial activity. Biological and enzymatic activities (i.e., β-*N*-acetylglucosaminidase [β-NAGase], lysozyme, and ovotransferrin) of ESM denatured with trypsin, lipases, or heat were compared with those of untreated ESM. Trypsin-treated ESM lost all biological activity (*D*-values at 54°C were 5.12 and 5.38 min for immobilized and solubilized trypsin, respectively) but showed no significant loss of enzymatic activities. Treatments with porcine lipase and a lipase cocktail did not impact biological or enzymatic activities. Heat denaturation of ESM (at 80 and 100°C for 15 min) resulted in significant decreases in biological activity (*D*-values of 3.99 and 4.43 min, respectively) and loss of β-NAGase activity. Lysozyme and ovotransferrin activities remained but were significantly reduced. Purified ESM and hen egg white components (i.e., β-NAGase, lysozyme, and ovotransferrin) were added to *Salmonella* Typhimurium suspensions (in 0.1% peptone water) at varying concentrations to evaluate their biological activity. *D*-values at 54°C were 4.50 and 3.68 min for treatment with lysozyme or β-NAGase alone, respectively, and 2.44 min for ovotransferrin but 1.47 min for a combination of all three components (similar to values for ESM). Exposure of *Salmonella* Typhimurium cells to a mixture of ovotransferrin, lysozyme, and β-NAGase or ESM resulted in significant increases in extracellular concentrations of Ca^{2+}, Mg^{2+}, and K^{+}. Transmission electron microscopic examination of *Salmonella* Typhimurium cells treated with a combination of ovotransferrin, lysozyme, and β-NAGase revealed membrane disruption and cell lysis. The findings of this study demonstrate that ovotransferrin, lysozyme, and β-NAGase are the primary components responsible for ESM antibacterial activity. The combination of these proteins and perhaps other ESM components interferes with interactions between bacterial lipopolysaccharides, sensitizing the outer bacterial membrane to the lethal affects of heat and possibly pressure and osmotic stressors.

In 1999, Mead and colleagues *(24)* in conjunction with the Centers for Disease Control and Prevention presented their data on the incidences of foodborne illnesses in the United States. They estimated that over 76 million cases of foodborne illness occur annually, resulting in more than 325,000 hospitalizations and more than 5,000 deaths. Although more than 250 foodborne diseases have been described, known bacteria (e.g., *Campylobacter*, *Salmonella*, and *Escherichia coli* O157:H7), viruses (e.g., Norwalk-like viruses and caliciviruses), and parasites (e.g., *Giardia* and *Cyclospora*) account for an estimated 14 million illnesses. It is difficult to calculate the exact cost of such illnesses; however, medical costs and lost wages due to foodborne salmonellosis, only one of many foodborne infections, have been estimated to be more than $1 billion per year *(5)*.

During the last decade, consumers have expressed an increasing demand for minimally processed foods and sustained functionality of naturally occurring bioactive ingredients. Several factors have fueled the public's interest in exploring options for minimally processed foods and bioactive compounds to enhance nutrition, including concerns about the safety and tolerance of synthetic preservatives, the suspected link between the overuse of subtherapeutic antibiotics as animal growth promotants and the development of multidrug resistance in microbes, and the increased media attention given to diet and health *(25)*.

Many products have been approved by regulatory agencies for use as direct food antimicrobials. However, the limited spectrum of antimicrobial activity of some of these substances has led to the continued search for more effective antimicrobials among naturally occurring compounds *(9)*. Although not often associated with food safety, eggshell waste has great potential for use in this area. In preliminary studies, Poland and Sheldon *(28)* demonstrated that components bound to the eggshell membrane (ESM) were capable of reducing the heat resistance and/or inhibiting the growth of selected gram-positive and gram-negative foodborne bacterial pathogens suspended in 0.1% peptone water. Reductions of 83 to 87% in thermal decimal reduction times (*D*-values) were observed for *Salmonella enterica* serovars Typhimurium and Enteritidis (at 54°C) and *E. coli* O157:H7 (at 52°C) and up to a 3-log reduction was observed for *Listeria monocytogenes* populations (at 37°C) following incubation with ESMs for 30 min.

The slightly pink ESM consists of an outer and inner membrane with thicknesses of approximately 50 and 15 μm, respectively. The outer membrane is located just inside the shell, and the inner membrane is located between the outer membrane and the albumen. ESM proteins are partially bound by a network of lysine-derived cross-links *(8)*.

* Author for correspondence. Tel: 919-515-5407; Fax: 919-515-7070; E-mail: brian_sheldon@ncsu.edu.

Their structure is similar to a meshwork of entangled threads, which aids in obstructing invading microorganisms. Lifshitz et al. *(20)* concluded that the inner shell membrane may be the most important single barrier to bacterial penetration into the egg and that shell membranes act as a bacterial filter and contain antibacterial substances.

Several compounds possessing known antimicrobial characteristics have been identified in the ESM. Elliott and Brant *(11)* were the first to report the presence of lysozyme in ESMs, and Winn and Ball *(41)* identified measurable amounts of β-*N*-acetylglucosaminidase activity within the membranes. Ovotransferrin was later identified by immunofluorescence as another component of the ESM *(14)*. These proteins and other unidentified compounds may contribute to the antimicrobial properties of the ESM.

Methods to extract these enzyme-rich ESMs are readily available *(22, 42)* and offer egg processors potential economic value as a natural processing adjuvant in food or pharmaceutical products to sensitize bacterial pathogens and spoilage organisms to heat or other treatments. However, a greater understanding of the ESM (i.e., the components and possible mechanisms of action responsible for the antimicrobial activity), which is the focus of our studies, is essential to better determine how the ESM may be used in practical applications.

MATERIALS AND METHODS

Chemicals. Chicken egg white lysozyme (EC 3.2.1.17), chicken egg white ovotransferrin (C-0755), porcine pancreatic lipase (EC 3.1.1.3, L-3126), wheat germ lipase (EC 3.1.1.3, L-3001), *Thermomyces lanuginosus* lipase (EC 3.1.1.3, L-0902), *Candida albicans* lipase (EC 3.1.1.3, L-4777), *Micrococcus lysodeikticus* ATCC 4689 (M-3770), and 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (N-9376) were all obtained from Sigma Chemical Company (St. Louis, Mo.). Tosyl-phenyl-chloro-ketone–treated bovine pancreas trypsin (T-1426; Sigma) was immobilized on controlled pore glass beads following the procedures described by Janolino and Swaisgood *(18)*. Brain heart infusion (BHI) broth and agar were obtained from Difco Laboratories (Becton Dickinson, Sparks, Md.). Hen egg white (HEW) ovotransferrin and β-*N*-acetylglucosaminidase (β-NAGase) were obtained using the extraction procedures described by Ahlborn and colleagues *(2)*. All other chemicals and buffers used were certified ACS (American Chemical Society) grade.

Bacteria and culture conditions. *Salmonella enterica* serovar Typhimurium (ST) ATCC 14028 was obtained from the American Type Culture Collection (Rockville, Md.), maintained in double-strength BHI broth plus 20% glycerol, and stored at −20°C. Working stock cultures were propagated by two successive transfers in BHI broth (37°C for 18 to 24 h) prior to use.

Individual 10-ml cultures were prepared by transferring a 0.1-ml subsample of ST to 10 ml of fresh BHI broth (37°C for 2 h). The cells were pelleted by centrifugation (5°C for 15 min at 8,000 × *g*), resuspended in sterile 0.1% peptone water (PW), and centrifuged again. Following resuspension in 10 ml of PW, the optical density at 600 nm was determined and adjusted to 0.5 (ca. 10^7 to 10^8 CFU/ml).

ESM extraction. ESMs were extracted with a modified commercial procedure described by Poland and Sheldon *(28)*. One hundred fresh, nonfertile White Leghorn (Hyline) eggs (obtained within 2 days of laying) were washed with a nylon brush in cool (15 to 18°C) water containing 200 ppm sodium hypochlorite and rinsed in sterilized deionized water. Eggs were broken, their contents were emptied, and residual albumen was removed by rinsing with distilled deionized water. Approximately one-third of the emptied eggshells were ground for 10 min in a food processor (model KFP600WH, KitchenAid, St. Joseph, Mich.) containing 400 ml of sterile water and then poured into a sterile container and left undisturbed for 5 min to allow shell fragments to settle. The top layer of the aqueous suspension containing the membrane fragments was decanted into a Buchner funnel containing Whatman no. 1 filter paper and vacuum dried for 10 min. An additional 400 ml of sterile water was added to the shell fragments to remove any residual membrane fragments remaining from the first process. This process was repeated with the remaining shells. Filter cakes of the compacted membrane fragments were removed from the filter paper, pooled, and stored in a sterile petri dish wrapped in aluminum foil and refrigerated until use (less than 1 week).

β-NAGase assay. The release of *p*-nitrophenol from 4-nitrophenyl *N*-acetyl-β-D-glucosaminide was achieved with a modification of the procedures of Lush and Conchie *(21)*, Donovan and Hansen *(10)*, and Winn and Ball *(41)*. The incubation mixture containing 0.01 g (±0.001 g) of membrane fragments, 0.9 ml of substrate (0.76 μmole 4-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0.1 M citrate buffer, pH 3.0), and 0.6 ml of distilled deionized water was incubated at 37°C for 25 min, and the reaction was stopped by adding 2.0 ml of 0.2 M Na_2CO_3. The mixture was briefly vortexed, the ESM was removed, and the absorbency at 400 nm was read in a narrow-path absorption cell (1-cm light path) with a UV 160U UV-Visible Recording Spectrophotometer (Shimadzu Corp., Kyoto, Japan) that contained a blank of the reaction solution minus the ESM. Enzyme activity was calculated according to equation 1:

$$\frac{\text{units}}{\text{mg}} = \frac{\Delta A_{400}/\text{total volume}}{\text{minutes} \times 10.8 \times \text{mg protein}} \quad (1)$$

where 10.8 is the extinction coefficient of the substrate at the given absorbance. ESM samples (treated and nontreated) were evaluated in triplicate, and the values are presented as the mean.

Lysozyme assay. Lysozyme activity was determined with an adaptation of the assay described by Shugar *(35)* as measured by the change in optical density (at 450 nm) following exposure of *Micrococcus lysodeikticus* to the ESM. A cell suspension was produced by adding 0.015% (wt/vol) lyophilized *M. lysodeikticus* cells (M-3770, Sigma) to 66 mM potassium phosphate buffer (pH 6.24) at 25°C. The resulting suspension absorbance (at 450 nm) was between 0.6 and 0.7. Random samples (0.01 ± 0.001 g) from various ESM treatments were added to 5.0 ml of the *M. lysodeikticus* suspension and mixed by inversion. After 2 min (designated as the optimal time to observe enzyme activity as indicated by a linear decrease in optical density), ESM fragments were removed, the decrease in absorbance was recorded, and enzyme activity was calculated according to equation 2:

$$\frac{\text{units}}{\text{mg solid}} = \frac{\Delta A_{450}/\text{time (in minutes)}}{0.001 \times \text{mg/solid/reaction mix}} \quad (2)$$

where 0.001 is the change in absorbance at 450 nm as per the unit definition. ESM samples (treated and nontreated) were evaluated in triplicate, and the values are presented as the mean.

Ovotransferrin activity. The iron-binding properties of the ESM were determined with a modified protocol described by Tranter et al. *(39)* and Crogennec et al. *(7)*. A 5-ml portion of a

solution (6 mg/ml) of filtered (0.45-μm pore size; Millipore, Bedford, Mass.) ferric ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) was added to 0.030 (±0.002) g of ESM. The solution was incubated with agitation (37°C for 30 min), and ESMs containing bound iron were washed three times in sterile deionized water and ashed in a muffle furnace (600°C for 24 h). The ash was acid digested (6 N HCl), and the iron content was determined by inductively coupled plasma emission spectroscopy. Ovotransferrin iron-binding activity was determined in triplicate ($n = 3$), and 6.5 μg/g was subtracted from the derived iron values to account for iron occurring naturally in the ESM.

***D*-value determinations.** Decimal reduction times (*D*-values) were determined with the combined methods of Poland and Sheldon *(28)* and the immersed sealed capillary tube (ISCT) procedure described by Schuman and colleagues *(34)* and Foegeding and Leasor *(12).* ST ATCC 14028 was selected because in preliminary trials these cells had the greatest sensitivity to ESM *(3, 9).* Mid-log-phase ST cell populations were produced by culturing cells for 24 h in 10 ml of BHI broth at 37°C in a rotating water bath shaker (model G76D, New Brunswick Scientific Co., Inc., Edison, N.J.), transferring 100 μl of the cell suspension into 10 ml of BHI broth, and incubating at 37°C for 2 h. Cells were harvested by centrifugation (4°C for 15 min at 8,000 × *g*), washed in sterile 0.1% PW, centrifuged again as described, and suspended in 10 ml of 0.1% PW (ca. 10^7 to 10^8 CFU/ml). Up to 1 g of the pooled and treated ESM fragment extracts was added to 20 ml of the PW bacterial suspension and incubated with agitation at 37°C for 30 min.

Following incubation, ESM fragments were removed from the PW bacterial suspension with a stomacher bag containing a nylon filter (Stomafilter P-type, Gunze Sangyo, Inc., Tokyo, Japan), and the inoculated PW (0.05 ml) was dispensed into individual glass capillary tubes (0.8 to 1.1 mm inside diameter, 90 mm long; 9530-4, Fisher Scientific, Pittsburgh, Pa.) using a syringe fitted with a 100-mm blunt needle (head space of approximately 4 mm). Filled capillary tubes were then heat sealed with a propane torch and kept on ice until heated (less than 30 min). Tubes were placed upright in a screen-covered test tube rack and rapidly submerged into a preheated (54°C) circulating water bath (model W19, Haake, Inc., Karlsruhe, Germany) with a temperature control module accurate to ±0.05°C (model DC1, Haake). At six to eight evenly spaced intervals, duplicate tubes were removed from the water bath and immersed in an ice-water slurry for 5 to 10 min. Capillary tubes were cleansed of any residual contamination by submerging the tubes in sodium hypochlorite (500 ppm, pH 6.5) for 5 s and then rinsing them three times in sterile deionized water. The clean capillary tubes were then aseptically transferred to individual test tubes containing 5 ml of sterile PW and finely crushed with a sterile glass rod. This initial 10^{-2} dilution was then agitated on a vortex mixer, serially diluted in PW, and spiral plated on BHI agar with an Autoplater 4000 (Spiral Biotech, Inc., Norwood, Mass.). Plates were incubated at 37°C for 18 to 24 h, and colonies were counted with a Microbiology International ProtoCOL automatic colony counting system (model 60000, Synoptics Ltd., Cambridge, UK).

Triplicate thermal inactivation trials were conducted, and survivor curves (ST log CFU per milliliter versus heating time) were plotted for each trial from the various treatments. Best-fit linear regression lines were drawn through the linear portion of each plot, and *D*-values were calculated as the negative reciprocal of the survivor slope obtained by regression analysis and presented as the mean of the three thermal inactivation trials.

Statistical analysis. Mean *D*-values obtained via the ISCT procedure for the control (no membrane) treatment and the experimental (membrane variables) treatment were compared in an analysis of variance (differences considered significant at $P \leq 0.05$), and each mean pair was compared using Student's *t* test (least significant difference defined at $P \leq 0.05$). The residual replicate-by-treatment mean square was used for testing the main effects (treatment and replicates). Enzyme activity results were analyzed with the general linear model and least squares means, with significance set at $P \leq 0.05$ *(33).*

MIC treatment. Seven sterile Erlenmeyer flasks were each filled with 10 ml of the PW bacterial suspension (ca. 7 to 8 log CFU/ml). Each flask was then inoculated with 1 (representing a 1:10 ratio), 0.5 (1:20 ratio), 0.33 (1:30 ratio), 0.25 (1:40 ratio), 0.2 (1:50 ratio), or 0.17 (1:60 ratio) gram of ESM, respectively. The seventh flask (without membrane) served as the control. Bacterial suspensions were incubated with agitation (150 rpm) at 37°C for 30 min. Following incubation, all suspensions were poured into a sterile filtering stomacher bag, and bacterial suspensions were removed with a sterile pipette and aseptically transferred to a sterile test tube (effectively removing all ESM fragments from the suspension). Samples were placed on ice (for <10 min) and then transferred to capillary tubes for *D*-value determinations.

ESM treated with trypsin and immobilized trypsin. Nine samples (1.5 g each) of ESM were placed in sterile 50-ml polypropylene graduated tubes (30 by 115 mm). Three tubes each received one of the following treatments in 46 mM Tris buffer (pH 8.1) with 12 mM $CaCl_2$: (i) 20 ml of trypsin (200 U/ml) in the Tris buffer; (ii) 20 ml of Tris buffer with 3 ml of immobilized trypsin (97.8 U/ml); (iii) 20 ml of Tris buffer as a control. Tubes were laid horizontally in a controlled environment incubator-shaker (series 25, New Brunswick Scientific, Edison, N.J.) and incubated (37°C at 100 rpm) for 3 h. Samples were removed, and ESM fragments were rinsed five times in sterile double-distilled water to remove any residual trypsin. Excess water was removed by pat drying the ESM fragments with sterile Kimwipes (Kimberly-Clark Corp., Roswell, Ga.). One gram of ESM was removed from each sample and added to 20 ml of the previously described bacterial suspension. Bacterial suspensions were incubated with agitation (150 rpm) at 37°C for 30 min. Following incubation, all suspensions were poured into a sterile filtering stomacher bag, and bacterial suspensions were removed with a sterile pipette and aseptically transferred to a sterile test tube (removing all ESM fragments from the suspension). Samples were placed on ice (for <10 min) and then transferred to capillary tubes. Remaining ESM fragments were evaluated for enzymatic activity.

Heat inactivation of ESM proteins. ESMs were placed in sterile deionized water, heated to either 80 or 100°C for 15 min, and rinsed with sterile water, and excess water removed. One gram from each sample was added to 20 ml of the bacterial suspension, incubated, and treated as previously described for determination of *D*-values and enzymatic activity.

Treatment of ESM with lipase. Three lipase treatments consisted of (i) a buffer control, (ii) porcine lipase (4,000 U), or (iii) a combination of porcine lipase (2,000 U), wheat germ lipase (300 U), *Thermomyces lanuginosus* lipase (5,000 U), and *Candida albicans* lipase (300 U) added to 100 ml of 50 mM sodium phosphate buffer (pH 7.0). Each treatment mixture was placed in a sterile 120-ml beaker containing 5 g of ESM from a common pool and a Teflon stir bar. Treatments were incubated (at 37°C for 4 h) with mild stirring (100 rpm) and stored (4°C) overnight. The following morning, treatments were removed from the refrigerator

and incubated for an additional 2 h (at 37°C and 100 rpm). Following incubation, ESM fragments were removed from the treatments and rinsed with five successive rinses of 300 ml of sterile double-distilled water (at which point lipase activity was no longer present in the ESM or rinsate samples). One gram of the ESM sample was removed and added to 20 ml of the bacterial suspension, and *D*-values were determined. Lipase activity was evaluated according to the procedures of Rajeshwara and Prakash *(30).*

***D*-value determination with purified components of ESM and HEW.** Because it is difficult to obtaining sufficient amounts of purified ESM proteins (i.e., β-NAGase and ovotransferrin), the ISCT procedure previously described was modified using 1 to 2 ml of bacterial suspensions (ca. 10^7 to 10^8 CFU/ml). *D*-values were determined by incubating (37°C for 30 min) various concentrations of the purified fractions of ovotransferrin (0.2 to 6.0 μM), lysozyme (0.02 to 0.2 μM), and β-NAGase (0.01 to 0.02 μM) with the bacterial suspension. *D*-values were calculated as previously described.

Transmission electron microscopy. Individual culture samples were prepared by transferring a 0.2-ml subsample of ST to 20 ml of fresh BHI broth and incubating the culture at 37°C for 2.5 h. The cells were pelleted by centrifugation (5°C for 15 min at 3,000 × *g*), resuspended in sterile 0.1% PW, and centrifuged again. Following resuspension in 10 ml of PW, the optical density at 600 nm was recorded (approximately 0.5 or 10^7 to 10^8 CFU/ml). Cells then received one of seven treatments: (i) control (no additional treatment), (ii) 1 g of fresh ESM, (iii) 0.2 μM ovotransferrin, (iv) 0.04 μM lysozyme, (v) 0.2 μM ovotransferrin and 0.04 μM lysozyme, (vi) 0.2 μM ovotransferrin and 0.01 μM β-NAGase, or (vii) 0.2 μM ovotransferrin, 0.04 μM lysozyme, and 0.01 μM β-NAGase. All samples were incubated with agitation at 37°C for 30 min, and the ESM fragments were removed. Samples were centrifuged as described, the supernatant was discarded, and the pellets were resuspended in 4 ml of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and fixed at 4°C for 1 week. Tubes were then centrifuged (7 min at 3,000 × *g*), and the supernatant was removed. Pellets were transferred to microfuge tubes with 1 ml of 0.1 M sodium cacodylate buffer, held for 10 min at 4°C, and centrifuged for 2 min. All samples were rinsed with the same buffer for two additional 15-min periods. Following removal of the last supernatant, 1.0 ml of 2% osmium tetroxide in 0.1 M sodium cacodylate buffer was added to resuspend the cells, and the cells were fixed for 2 h on ice in the dark. Samples were centrifuged as described, the osmium tetroxide was removed, and the samples were washed an additional two times in clean buffer before being held overnight at 4°C. The following day, samples were centrifuged (2 min at 3,000 × *g*), rinsed, and centrifuged again. The pellet was resuspended in 2% agarose (in 0.1 M sodium cacodylate buffer) and centrifuged (5 min at 3,000 × *g*), the samples were chilled on ice for 30 min, and the gelled agarose was removed from the microfuge tubes. The tips containing cell samples were cut off with a clean razor blade, cut into blocks, and placed in cold buffer. The blocks were rinsed with a brief distilled water wash and stained en bloc with 1% aqueous uranyl acetate in the dark (4°C for 12 h).

Following staining, samples were rinsed with a change of cold distilled water and then dehydrated with 1-h changes of cold 30, 50, 70, 95, and 100% EtOH warming to room temperature (23°C) and another two changes of 100% EtOH at room temperature. Infiltration proceeded 24 h later by subsequent changes of 1:2 Spurr's reagent:EtOH, 1:1 Spurr's reagent:EtOH, and 3:1 Spurr's reagent:EtOH and concluded with an 8-h change of 100% Spurr's reagent (23°C) and a 5-h change of 100% Spurr's reagent under a vacuum. Samples were embedded overnight with Epon 812–Araldite M epoxy resin mixture in BEEM capsules. Ultrathin sections were cut with diamond knives on an ultramicrotome (Um-03, Reichert, Austria), placed on 200-mesh nickel supporting grids for transmission electron microscopy, and stained with 4% uranyl acetate for 1 h and then with a 0.2% aqueous solution of Reynolds lead citrate *(31)* at room temperature. Each stain was followed by washes in triple-distilled water (1 min at 23°C). Samples were examined with an EM208 S transmission electron microscope (FEI/Philips, Eindhoven, The Netherlands) operated at 80 kV.

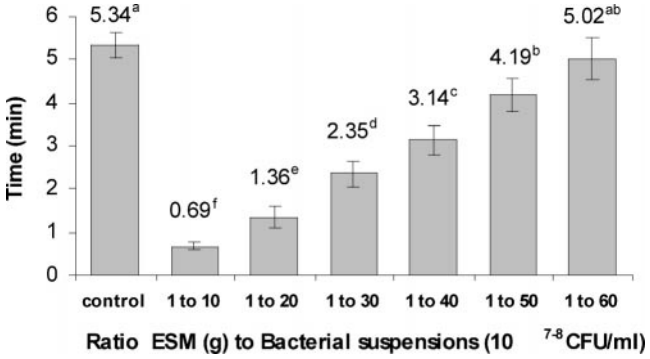

FIGURE 1. *Mean* D_{54}*-values (minutes) (*n = *3) for* Salmonella *Typhimurium following treatment (incubation at 37°C for 30 min) with various concentrations of eggshell membranes (grams) in bacterial suspensions. Mean* D*-values with different superscripts are significantly different (*$\alpha \leq 0.05$*).*

Atomic emission spectrometry. Loss of intracellular cations was determined with an adapted protocol from Aguilera and colleagues *(1).* A sample (3 ml) of ST cells was transferred from a 24-h stock culture to 200 ml of BHI broth and incubated in a circulating water bath (2 h at 37°C). Cells were centrifuged (15 min at 8,000 × *g*), washed three times in either Milli-Q or PW, and resuspended (A_{600} = 2.0) in either deionized Milli-Q water or 0.1% PW. Suspended cells (10 ml) were incubated at 37°C for 30 min in the presence or absence of ESM (2.0 g), ovotransferrin (0.5 μM), and a combination of ovotransferrin (0.5 μM), lysozyme (0.08 μM), and β-NAGase (0.01 μM). Aliquots (10 ml) were filtered through a stomacher bag containing a nylon filter and centrifuged for 10 min at 8,000 × *g*, and the supernatants were collected and filtered through a 0.45-μm-pore-size syringe. Ca^{2+}, Mg^{2+}, K^+, and Na^+ were quantified with an atomic emission spectrometry detector (UNICAM 929, Unicam Ltd., Cambridge, UK). Controls (bacteria free) containing only Milli-Q water or PW alone and with ESM, ovotransferrin, or a combination of ovotransferrin, lysozyme, and β-NAGase were evaluated independently. The trial was duplicated, and the samples were processed in duplicate.

RESULTS AND DISCUSSION

MICs. Figure 1 shows the effects of various concentrations of ESM on the *D*-values at 54°C (D_{54}) for ST 14028. At 54°C, the control treatment (no added ESM) required more than 5 min of heating time (5.34 min) to yield a 1-log reduction in the bacterial population. When ESM was added at a ratio of 1 g of ESM to 10 ml of the ST suspension, the D_{54}-value was significantly reduced more than sevenfold (to 0.69 min). Significant increases in the D_{54}-values were detected as the ratio of ESM concentration

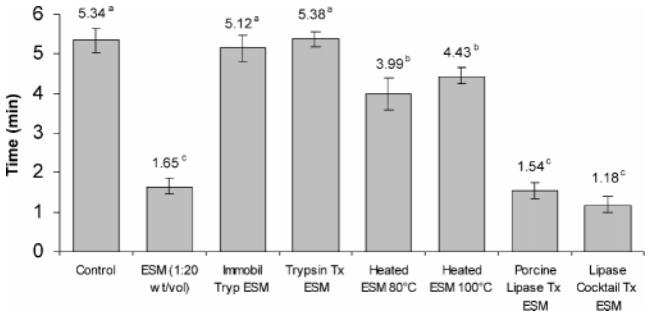

FIGURE 2. *Mean* D_{54}*-values (minutes) (*n = *3) for* Salmonella *Typhimurium following treatment (incubation at 37°C for 30 min) with treated eggshell membranes (1:20 ratio of ESM to bacterial suspension). Treatments: ESM (no alteration to shell membranes), Immobil Tryp ESM and Trypsin Tx ESM (shell membranes exposed to immobilized trypsin and suspended trypsin), ESM heated at 80°C for 15 min, ESM heated at 100°C for 15 min, ESM treated with porcine lipase, and ESM treated with a lipase cocktail. Mean* D*-values with different superscripts are significantly different (*$\alpha \leq 0.05$*).*

to cell population decreased. At a ratio of 1 g to 60 ml, there was no significant difference in the heat resistance of ST compared with that of the ESM-free control treatment (5.02 versus 5.34 min).

Trypsin-treated ESM. Because extraction and purification of the components of the ESM is difficult, an indirect approach was first taken to access the role of individual components in the observed antimicrobial activity of ESM. The biological activity (reduction in D_{54}-values) of ESM following exposure to various treatments is shown in Figure 2. Subjecting the ESM to suspended or immobilized trypsin resulted in a complete loss of antimicrobial activity, as indicated by significant increases in D_{54}-values (5.38 and 5.12 min, respectively) compared with that of the 1:20 ESM treatment (1.65 min). Trypsin is a protease that cleaves proteins between the lysine and arginine bond. Although the loss of biological activity was not surprising, when ESM was exposed to either solubilized or immobilized trypsin there were no significant reductions in β-NAGase activity and a slight decrease in lysozyme activity, as measured by the release of *p*-nitrophenol from 4-nitrophenyl *N*-acetyl-β-D-glucosaminide or a change in optical density (at 450 nm) following exposure to *M. lysodeikticus*, respectively (Table 1). These results may be explained in one of several ways: (i) the active sites responsible for enzymatic activity lack lysine-arginine bonds and are therefore unaffected by trypsin; (ii) the active sites are partially protected by membrane components; or (iii) trypsin is able to cleave specific sites in the enzyme, yielding peptide fragments adhered to the ESM that work cooperatively to produce the specific enzymatic activity. Unlike the enzyme activities, the iron-binding capabilities of ESM-bound ovotransferrin were significantly reduced by exposure to trypsin (Table 1).

Heat inactivation of ESM-bound components. Membranes subjected to heat treatments (80 and 100°C for 15 min) prior to exposure to ST cells lost significant biological activity (i.e., from 1.65 min to 3.99 and 4.43 min, respectively; Fig. 2). Although the heat-inactivated membranes retained some biological activity, it was greatly diminished. If protein components of ESM were primarily responsible for the reduction in *D*-values, heat denaturation of these proteins would occur to varying degrees with exposure to heat. ESM-bound β-NAGase was more susceptible to heat degradation, and no activity was detected after the 15-min heat treatments. ESM-bound ovotransferrin retained some activity, and lysozyme retained up to one third of its activity (14.0 ± 4.8 U/mg at 80°C and 4.3 ± 2.3 U/mg at 100°C) (Table 1). The retention of lysozyme activity is supported by the work of Masschalck and colleagues *(23)* in which partially heat-denatured lysozyme (80°C for 20 min) retained 11% of its activity.

Treatment of ESM with lipase. Lipid components, particularly fatty acids, have antimicrobial characteristics in some instances *(6, 17, 38)*. Although the lipid content (dry weight) of ESM is only 1.35% *(37)*, we treated ESM with porcine lipase and a lipase cocktail to determine whether lipid components may contribute to the observed antimicrobial activity. After treatment with the lipases, ESM was thoroughly rinsed to remove the lipase, and D_{54}-values were determined. ESM biological activity was not affected by treatment with the lipases (D_{54}-values of 1.54 and 1.18 min, respectively), indicating that lipid components are not probable contributors to ESM biological activity. These two lipase treatments also did not affect ovotransferrin, lysozyme, and β-NAGase activities (Table 1).

***D*-value determination for purified ESM and HEW components.** HEW was used as a source of identified ESM components (i.e., ovotransferrin, lysozyme, and

TABLE 1. *Enzymatic and biological activities of ESM components*[a]

ESM treatment	Biological activity (D_{54}, min)	Lysozyme activity (U/mg)	β-NAGase activity (U/mg)	Ovotransferrin activity (μg iron/g ESM)
Control	1.65 C	41.4 ± 3.9 A	12.2 ± 1.6 A	110.2 ± 10.6 A
Immobilized trypsin	5.12 A	36.7 ± 4.2 A	11.3 ± 1.1 A	45.7 ± 8.4 B
Trypsin (suspended)	5.38 A	37.8 ± 2.2 A	9.7 ± 1.8 A	39.7 ± 11.7 B
80°C for 15 min	3.99 B	14.0 ± 4.8 B	0 B	31.7 ± 12.0 BC
100°C for 15 min	4.43 B	4.3 ± 2.3 C	0 B	17.1 ± 4.3 C
Porcine lipase	1.54 C	42.8 ± 4.5 A	11.8 ± 1.4 A	100.1 ± 9.5 A
Lipase cocktail	1.18 C	40.6 ± 5.2 A	10.9 ± 1.7 A	104.7 ± 16.5 A

[a] Within columns, mean values ($n = 3$) with different letters are significantly different ($\alpha \leq 0.05$).

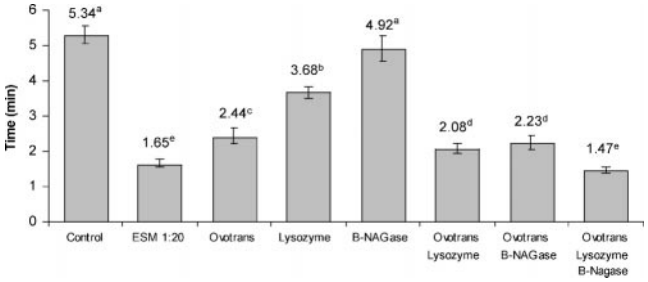

FIGURE 3. *Mean* D_{54}*-values (minutes) (*n *= 3) for purified egg white ovotransferrin (Ovotrans), lysozyme, ovotransferrin and lysozyme, and a ovotransferrin, lysozyme, and β-*N*-acetylglucosaminidase (β-NAGase) treatment compared with no treatment (control) and eggshell membrane (ESM)-treated* Salmonella *Typhimurium. Mean* D*-values with different superscripts are significantly different (*$\alpha \leq 0.05$*).*

β-NAGase). However, purified components from HEW and their ESM-bound counterparts may differ in both structure and activity (biological and enzymatic). Inhibitory concentrations equivalent to that exhibited by ESM (i.e., *D*-value reductions) were first determined for the purified HEW components using enzymatic values for lysozyme and β-NAGase activities as the reference *(3).*

Because it was difficult to obtaining sufficient amounts of β-NAGase, the 1.65 min D_{54}-value (1:20 ESM ratio) was chosen as the standard for antimicrobial evaluation. Preliminary studies (unpublished data) indicated that 0.04 μM lysozyme and 0.02 μM purified β-NAGase had enzyme activities equivalent to those of ESM-bound enzymes. Because ovotransferrin concentrations (as determined by iron-binding capabilities) varied greatly among ESM samples, D_{54}-values for a range of ovotransferrin concentrations (0.1 to 4.0 μM) were determined separately. Based on the *D*-value determination protocol described previously, 0.2 μM purified ovotransferrin resulted in significant reductions in D_{54}-values compared with the control. Increasing ovotransferrin concentrations above 0.2 μM did not significantly decrease D_{54}-values, but decreasing concentrations of ovotransferrin resulted in reduced biological activity (increased D_{54}-values) (data not shown).

Ovotransferrin alone at 0.2 μM significantly reduced D_{54}-values (2.44 versus 5.34 min) compared with that of the ST control (Fig. 3). Lysozyme at 0.04 μM also resulted in a significant reduction in D_{54}-values (3.68 min), although the values were not as low as those produced by ovotransferrin. Incubation of ST cells with 0.02 μM β-NAGase resulted in no significant reduction in D_{54}-values (4.78 min), and no reductions in bacterial populations were observed for any individual treatments (data not shown). D_{54}-values for treatment with lysozyme and ovotransferrin in combination were 2.08 min, and those for β-NAGase and ovotransferrin together resulted in a mean D_{54}-value of 2.40 min; neither of these values were different from that for ovotransferrin alone. A combination of all three components was required to yield D_{54}-values similar to those detected following ESM treatment (1.47 versus 1.65 min), indicating that these three components are primarily responsible for the antibacterial activity associated with ESM.

Atomic emission spectrometry. Extracellular concentrations of Ca^{2+}, Mg^{2+}, K^+, and Na^+ were determined following treatment of ST cells with ESM, purified ovotransferrin, or a combination of ovotransferrin, lysozyme, and β-NAGase in Milli-Q water. The release of these ions following treatment of ST cells with ESM in PW also was examined (Table 2). Aguilera and colleagues *(1)* demonstrated that ovotransferrin and other transferrins can cause selective ion efflux through bacterial membranes. They reported that extracellular concentrations of K^+ increased when *E. coli* cells were treated with transferrins, whereas Na^+ concentrations were not affected. Although the electrical potential of the cell ($\Delta\Psi$) was significantly reduced and even lost in some instances, they observed no loss of *E. coli* viability. Our findings were similar to those of Aguiler and colleagues; treatment of ST cells with ovotransferrin alone resulted in significant extracellular concentrations of K^+, whereas sodium concentrations were un-

TABLE 2. *Supernatant extracellular ion concentrations of* Salmonella *Typhimurium treated (37°C, 30 min) with eggshell membranes (ESM), ovotransferrin, or a combination of ovotransferrin, lysozyme, and β-*N*-acetylglucosaminidase (β-NAGase)*[a]

Sample ID	Ca (mg/liter)	K (mg/liter)	Mg (mg/liter)	Na (mg/liter)
Milli-Q water	<0.05 F	0.09 H	<0.05 F	<0.05 G
Milli-Q water, *Salmonella* Typhimurium	<0.05 F	1.30 E	<0.05 F	0.26 G
Milli-Q water, ESM	2.20 D	0.78 G	1.85 D	2.26 F
Milli-Q water, ESM, *Salmonella* Typhimurium	2.95 C	1.20 E	2.68 C	3.41 E
Milli-Q, ovotransferrin	<0.05 F	0.06 H	<0.05 F	17.14 D
Milli-Q, ovotransferrin, *Salmonella* Typhimurium	<0.05 F	0.97 F	<0.05 F	16.34 D
Milli-Q, ovotransferrin, lysozyme, β-NAGase	<0.05 F	<0.05 H	<0.05 F	19.16 C
Milli-Q, ovotransferrin, lysozyme, β-NAGase, *Salmonella* Typhimurium	0.45 E	2.15 D	0.46 E	43.01 A
Peptone	<0.05 F	2.63 C	<0.05 F	20.61 B
Peptone, *Salmonella* Typhimurium	0.16 F	2.69 BC	0.11 F	18.90 C
Peptone, ESM	5.27 B	2.80 B	4.00 B	20.28 B
Peptone, ESM, *Salmonella* Typhimurium	7.79 A	3.29 A	6.41 A	20.69 B

[a] Within columns, mean values ($n = 2$) with different letters are significantly different ($\alpha \leq 0.05$).

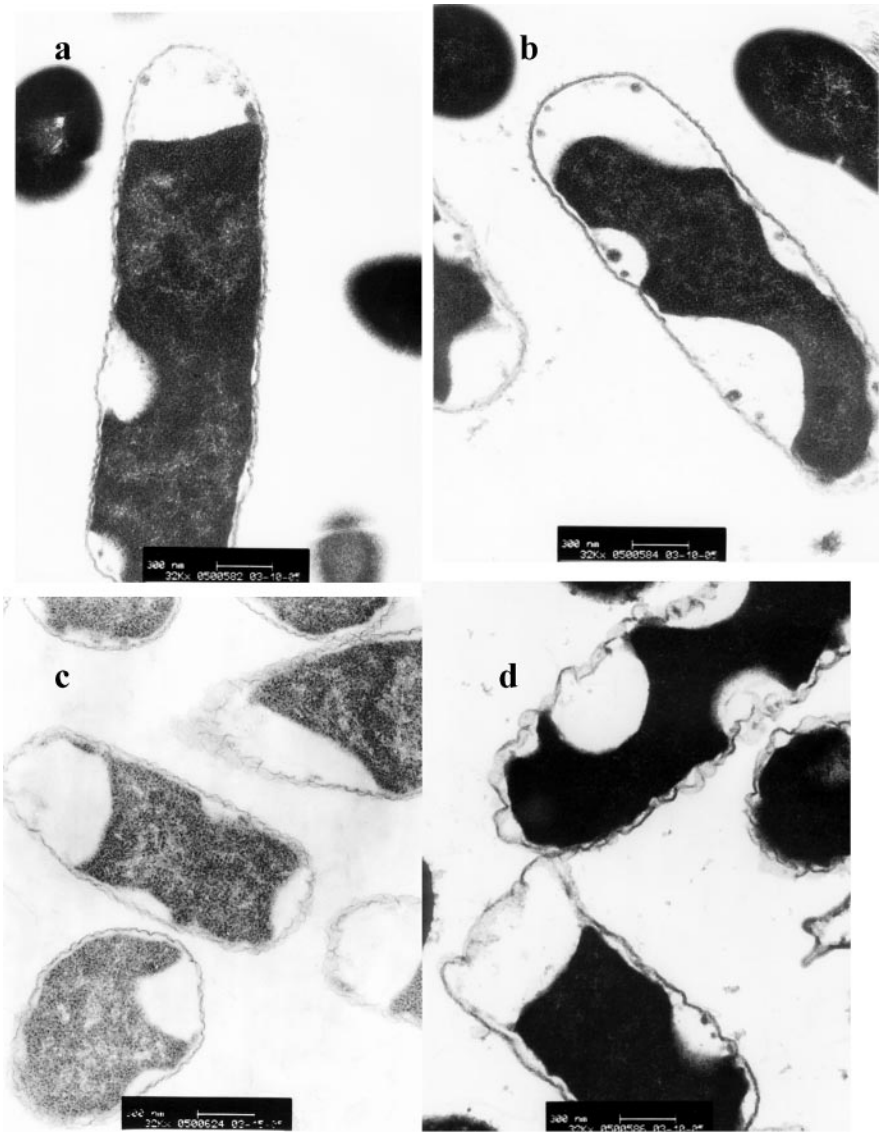

FIGURE 4. *Transmission electron micrographs of treated (37°C, 30 min)* Salmonella *Typhimurium, ×32,000 (magnification bar represents 300 nm). (a) Control cell receiving no treatment; (b) cell plus ovotransferrin; (c) cell plus eggshell membranes; and (d) cell plus ovotransferrin, lysozyme, and β-*N*-acetylglucosaminidase.*

affected. When lysozyme and β-NAGase were used in combination with ovotransferrin, significant concentrations of extracellular K^+, Na^+, Mg^{2+}, and Ca^{2+} were observed. Compared with the controls (ESM in Milli-Q water and ST suspended in Milli-Q water), an increase in extracellular concentrations of Mg^{2+}, Ca^{2+}, and Na^+ also were observed when ST cells were treated with ESM. ESM treatments in PW containing ST resulted in significant cellular losses of K^+, Na^+, Mg^{2+}, and Ca^{2+} concentrations compared with ESM treatments in Milli-Q water. However, for Na^+ there were no significant differences between the treatment composed of PW, ESM, and ST and two (PW and PW-ESM) of the three experimental controls. The difference in ion loss between Milli-Q water and PW treatments may be an artifact of the increased ionic strength of the PW (specifically the higher sodium content), creating a hypertonic environment and destabilizing the overall integrity of the cell.

Transmission electron microscopy. The outer membrane normally functions as a protective barrier for gram-negative bacteria and lies outside the thin peptidoglycan layer. It prevents or slows the entry of toxic substances (i.e., bile salts or antibiotics) that might kill or injure the bacterium. The lipopolysaccharide (LPS) layer constitutes the majority of the outer membrane and is located adjacent to the exterior peptidoglycan layer. Although complex, the outer membrane is not impermeable and can permit the passage of small molecules (ca. 600 to 700 Da) through porin proteins, which cluster together and span the outer membrane *(29).*

LPS associations in gram-negative bacteria also are stabilized by the presence of divalent cations (i.e., Mg^{2+} and Ca^{2+}) in the outer membrane that serve to decrease electrostatic repulsions and increase LPS-LPS associations. These strong associations seem to be the primary reason why large hydrophilic molecules and most hydrophobic and amphiphilic molecules are prevented from gaining access to the cell *(26, 27).* Alterations to the LPS, including removal of the divalent cations that stabilize the outer membrane, result in compromised functioning of this lipid bilayer barrier *(40).*

Transferrins, including ovotransferrin, bind aluminum, copper, and zinc in the identical manner of iron uptake *(13,*

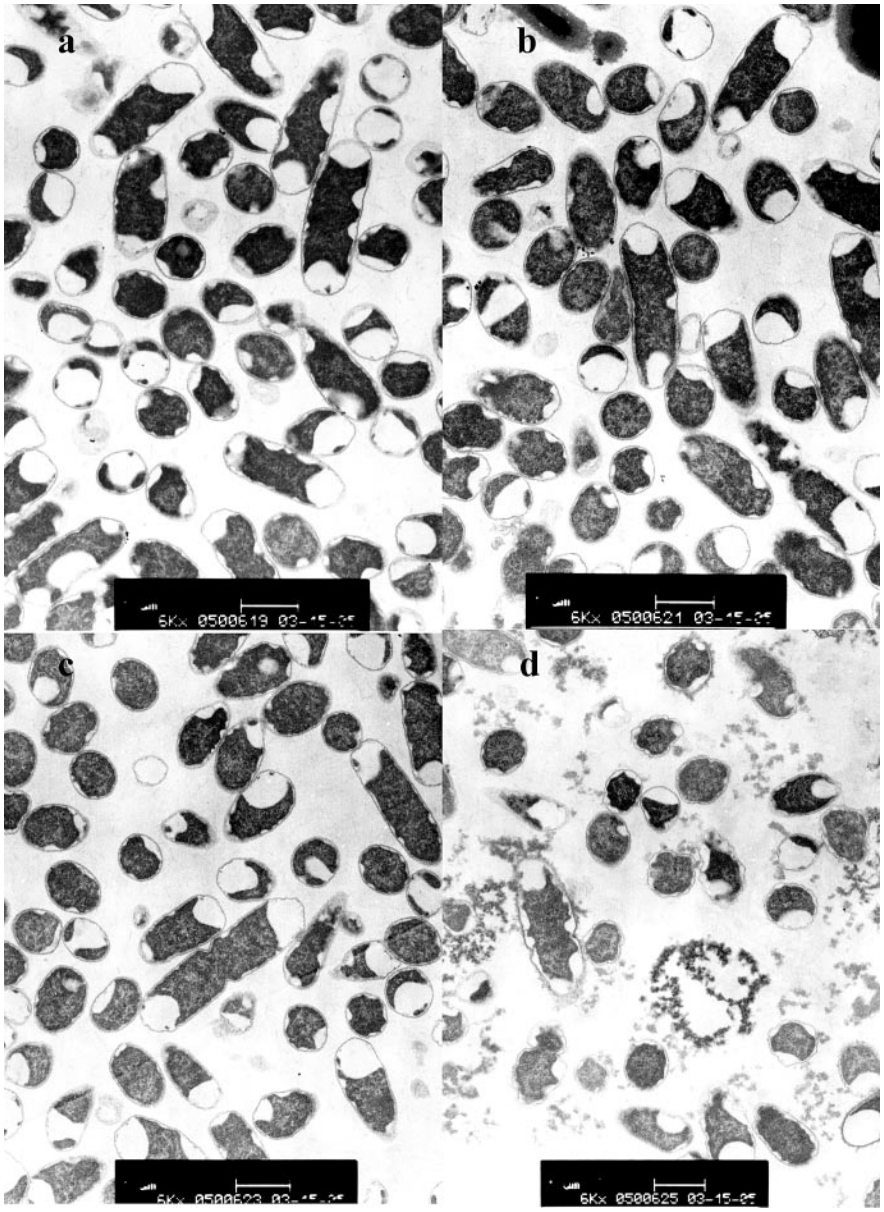

FIGURE 5. *Transmission electron micrographs of treated (37°C, 30 min)* Salmonella *Typhimurium, ×6,000 (magnification bar represents 1 μm). (a) Control cell receiving no treatment; (b) cell plus ovotransferrin; (c) cell plus eggshell membranes; and (d) cell plus ovotransferrin, lysozyme, and β-*N*-acetylglucosaminidase.*

19). Bovine lactoferrin binds calcium *(32).* Ovotransferrin alone appears to be unable to bind or affect the Mg^{2+} and Ca^{2+} cations through similar actions; however, ovotransferrin with the addition of lysozyme and β-NAGase (or ESM-bound ovotransferrin, lysozyme, and β-NAGase) are able to affect cellular losses of these and possibly other cations. Whether the Mg^{2+} and Ca^{2+} in the resulting extracellular fluid is from the LPS or from inside the cell itself requires more analysis.

Through transmission electron micrographs (Figs. 4 and 5), ST cells treated with ESM and purified enzyme exhibit some indication of cellular disruption in the outer membrane–LPS integrity. Little difference can be observed between the control samples (Fig. 4a) and ovotransferrin-treated cells (Fig. 4b) and ESM treated cells (Fig. 4c). However, ST cells treated with a combination of ovotransferrin, lysozyme, and β-NAGase exhibit a change in cellular integrity (Fig. 4d); outer membranes are jagged (or misshapen), and cellular material is scattered throughout the micrograph (Fig. 5d), indicating cell lysis. There was indication of intercellular protein loss from treatment with both ESM and purified membrane components (0.03 and 0.08 μg/ml, respectively; data not shown).

The outer membrane and LPS-LPS associations help prevent larger molecules from gaining access to the cell. Loss of LPS Mg^{2+} and Ca^{2+} could explain the observed disruption to the outer membrane and LPS structure. In this case, hydrolases such as lysozyme and β-NAGase may gain access to the peptidoglycan and further disrupt cellular integrity. This hypothesis is supported by the work of Hancock and Wong *(15),* who assessed the impact of nitrocefin on the permeability of *Pseudomonas aeruginosa* cells in the presence of the cation chelator EDTA. They theorized that EDTA sequestered Mg^{2+} ions essential for LPS-LPS cross-bridges. They also observed that the addition of Mg^{2+} ions countered the permabilizing effect by perhaps saturating EDTA with the supplemented Mg^{2+} ions, thereby preventing any further action on cellular Mg^{2+}. Stan-Lotter and colleagues *(36)* also demonstrated that the addition of cations (Mg^{2+} and Na^{+}) to the growth medium substantially

reduced the inhibitory effect of several antibiotics and the dye gentian violet on ST mutants. More recently, Branen and Davidson *(4)* demonstrated that the addition of either EDTA or lactoferrin enhanced the activity of nisin, monolaurin, and lysozyme against *L. monocytogenes, E. coli,* and *Salmonella* Enteritidis. These findings suggest that ovotransferrin combined with lysozyme and β-NAGase may remove cations essential for proper LPS stability.

Differences in *D*-values resulting from treatment of bacteria cells with ESM versus the purified components might be attributed to inaccessibility. The purified ovotransferrin and enzymes are able to access the cell structures, whereas ESM lysozyme and β-NAGase are bound in the matrix of the ESM and are probably not able to associate as readily with the cells. Although damage to the outer membrane by ESM (i.e., loss of divalent cations) may not be bactericidal, it may result in a weakened cell that exhibits greater susceptibility to damage from heat, pressure, and osmotic stress. Hitchener and Egan *(16)* demonstrated this effect by exposing *E. coli* K-12 cells to EDTA and sublethal heat (48°C). Although sublethal heat treatments alone resulted in the release of less than 20% of LPS components and EDTA produced some structural damage to the LPS, these individual treatments did not impact death rates of the bacteria. However, the combination of sublethal heat and EDTA resulted in a 50% loss of the LPS components from the cells and a significant decrease in *D*-values at 48°C. The addition of 0.5 mM Mg^{2+} to the heating medium also protected the cells from death and structural injury. Another possible reason for the decreased activity of ESM-bound components may be the high concentration of cations in the ESM itself, including high concentrations of Na^{+}, Ca^{2+}, Cu^{2+}, and to a lesser extent Mg^{2+}, which may interfere or limit sequestering or binding of extraneous cations.

The concentration of ST cells treated with ESM was reduced by 0.4 (±0.1) log unit after incubation for 30 min at 37°C versus a 0.8 (±0.1)-log reduction for cells treated with a combination of the purified proteins. This difference may be attributed to the decreased accessibility of ESM-bound enzymes and proteins.

Ovotransferrin, lysozyme, and β-NAGase from the ESM are the primary components responsible for the antibacterial activity exhibited by the ESM. The combination of these proteins and perhaps other ESM components is able to interfere with LPS-LPS interactions, sensitizing the outer membrane to the lethal effects of heat and possibly pressure and osmotic stressors. Purified proteins from HEW produced a cellular effect similar to but greater than that of the ESM-bound components.

Additional studies are needed to determine the true potential of ESM (and its components) as a natural antimicrobial or processing aid in heat-sensitive foods and pharmaceutical products. The impact of cations (e.g., Ca^{2+} and Mg^{2+}) and other food components (e.g., proteins, carbohydrates, and lipids) present in the matrix of foods and pharmaceutical products on ESM-mediated antimicrobial activity has not been fully explored. The economic feasibility and an understanding of how physical barriers and exposure conditions (temperature and time parameters) affect the efficacy of ESM must also be evaluated. However, the application of ESM or its components may lead to the reduction of thermal process requirements for foods (lower process temperatures and times) and yield products with extended shelf lives and reduced populations of microorganisms. Reduced thermal processing requirements may also result in food products that have higher concentrations of nutrients, improved functionality, and lower processing costs.

REFERENCES

1. Aguilera, O., L. M. Quiros, and J. F. Fierro. 2003. Transferrins selectively cause ion efflux through bacterial and artificial membranes. *FEBS Lett.* 548:5–10.
2. Ahlborn, G. J., D. Clare, B. W. Sheldon, and R. Kelly. Identification of eggshell membrane proteins and purification of ovotransferrin and β-*N*-acetylglucosaminidase from hen egg white. Submitted for publication.
3. Ahlborn, G. J., and B. W. Sheldon. Enzymatic and biological activity in egg shell membranes as influenced by layer and storage variables. Submitted for publication.
4. Branen, J. K., and P. M. Davidson. 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. *Int. J. Food Microbiol.* 90:63–74.
5. Centers for Disease Control and Prevention. 2003. Public Health Laboratory Information System surveillance data, *Salmonella* annual summaries. Available at: http://www.cdc.gov/ncidod/dbmd/phlisdata/salmonella.htm. Accessed 2 August 2005.
6. Coonrod, J. D. 1987. Role of surfactant free fatty acids in antimicrobial defenses. *Eur. J. Respir. Dis. Suppl.* 153:209–214.
7. Crogennec, T., F. Nau, S. Pezennec, M. Piot, and B. Gerard. 2001. Two-step chromatographic procedure for the preparation of hen egg white ovotransferrin. *Eur. Food Res. Technol.* 212:296–301.
8. Crombie, G., R. Snider, B. Faris, and C. Franzbrau. 1981. Lysine-derived cross-links in the egg shell membrane. *Biochim. Biophys. Acta* 640:365–367.
9. Davidson, P. M., and S. Zivanovic. 2003. The use of natural antimicrobials, p. 22–45. *In* P. Zeuthen and L. Bogh-Sorensen (ed.), Food preservation techniques. CRC Press, Boca Raton, Fla.
10. Donovan, J. W., and L. U. Hansen. 1971. The β-*N*-acetylglucosaminidase activity of egg white. 1. Kinetics of the reaction and determination of the factors affecting the stability of the enzyme in egg white. *J. Food Sci.* 36:174–177.
11. Elliott, L. E., and A. W. Brant. 1957. Effect of saline and eggshell membrane on bacterial growth. *Food Res.* 22:241–250.
12. Foegeding, P. M., and S. B. Leasor. 1990. Heat resistance and growth of *Listeria monocytogenes* in liquid whole egg. *J. Food Prot.* 53:9–14.
13. Garratt, R. C., R. W. Evans, S. S. Hasnain, P. F. Lindley, and R. Sarra. 1991. X.a.f.s. studies of chicken dicupric ovotransferrin. *Biochem. J.* 280:151–155.
14. Gautron, J., M. T. Hincke, M. Panheleux, J. M. Garcia-Riuz, T. Boldicke, and Y. Nys. 2001. Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. *Connect. Tissue Res.* 42:255–267.
15. Hancock, R. E., and P. G. Wong. 1984. Compounds which increase the permeability of the *Pseudomonas aeruginosa* outer membrane. *Antimicrob. Agents Chemother.* 26:48–52.
16. Hitchener, B. J., and A. F. Egan. 1977. Outer-membrane damage in sublethally heated *Escherichia coli* K-12. *Can. J. Microbiol.* 23:311–318.
17. Isaacs, C. F., and H. Thormar. 1991. The role of milk-derived antimicrobial lipids as antiviral and antibacterial agents. *Adv. Exp. Med. Biol.* 310:159–165.
18. Janolino, V. G., and H. E. Swaisgood. 1982. Analysis and optimization of methods using water-soluble carbodiimide for immobili-

zation of biochemicals to porous glass. *Biotechnol. Bioeng.* 24:1069–1080.
19. Li, Y. Q., and B. S. Yang. 2004. Aluminum ion removal from monoaluminum ovotransferrin by pyrophosphate. *Chin. J. Chem.* 22: 1153–1157.
20. Lifshitz, A., R. C. Baker, and H. B. Naylor. 1963. The relative importance of chicken egg exterior structures in resisting bacterial penetration. *J. Food Sci.* 28:94–99.
21. Lush, I. E., and J. Conchie. 1966. Glycosidases in the egg albumen of the hen, the turkey and the Japanese quail. *Biochim. Biophys. Acta* 130:81–86.
22. MacNeil, J. H. January 2001. Method and apparatus for separating a protein membrane and shell material in waste egg shell. U.S. patent 6,176,376.
23. Masschalck, B., R. Van Houdt, E. G. Van Haver, and C. W. Michaels. 2001. Inactivation of gram-negative bacteria by lysozyme, denatured lysozyme, and lysozyme-derived peptides under high hydrostatic pressure. *Appl. Environ. Microbiol.* 67:339–344.
24. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
25. Naidu, A. S. (ed.). 2000. Natural food antimicrobial systems, p. 1–16. CRC Press, Boca Raton, Fla.
26. Nikaido, H., and T. Nakae. 1979. The outer membrane of gram-negative bacteria. *Adv. Microb. Physiol.* 20:163–250.
27. Nikaido, H., and M. Vaara. 1987. Outer membrane, p. 7–22. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Typhimurium, vol. 1. American Society for Microbiology, Washington, D.C.
28. Poland, A. L., and B. W. Sheldon. 2001. Altering the thermal resistance of foodborne bacterial pathogens with an eggshell membrane waste by-product. *J. Food Prot.* 64:486–492.
29. Prescott, L. M., J. P. Harley, and D. A. Klein. 1999. Prokaryotic cell structure and function, p. 37–71. *In* Microbiology, 4th ed. WCB/McGraw-Hill, Boston.
30. Rajeshwara, A. N., and V. Prakash. 1994. Structural stability of lipase from wheat germ. *Int. J. Pept. Protein Res.* 44:435–440.
31. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electronopaque stain in electron microscopy. *J. Cell Biol.* 17:208–212.
32. Rossi, P., G. Francesco, A. Boffi, M. Ajello, P. Valenti, E. Chiancone, and G. Antonini. 2002. Ca2+ binding to bovine lactoferrin enhances protein stability and influences the release of bacterial lipopolysaccharide. *Biochem. Cell Biol.* 80:41–48.
33. SAS Institute, Inc. 1990. SAS/STAT user's guide, version 6, 4th ed. SAS Institute, Inc., Cary, N.C.
34. Schuman, J. D., B. W. Sheldon, and P. M. Foegeding. 1998. Thermal resistance of *Aeromonas hydrophila* in liquid whole egg. *J. Food Prot.* 60:231–236.
35. Shugar, D. 1952. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochim. Biophys. Acta* 8:302–309.
36. Stan-Lotter, H., M. Gupta, and K. E. Sanderson. 1979. The influence of cations on the permeability of the outer membrane of *Salmonella* Typhimurium and other gram-negative bacteria. *Can. J. Microbiol.* 25:475–485.
37. Suyama, T., H. Nakamura, M. Ishida, and S. Adachi. 1977. Lipids in the exterior structures of the hen egg. *J. Agric. Food Chem.* 25: 799–803.
38. Thormar, H., C. E. Isaacs, H. R. Brown, M. R. Barshatzky, and T. Pessolano. 1987. Inactivation of enveloped viruses and killing of cells by fatty acids and monoglycerides. *Antimicrob. Agents Chemother.* 31:27–31.
39. Tranter, H. S., N. H. C. Sparks, and R. G. Board. 1983. A note on the structure and iron-binding properties of egg-shell membranes. *Br. Poult. Sci.* 24:123–130.
40. Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* 56:395–411.
41. Winn, S. E., and H. R. Ball, Jr. 1975. β-*N*-acetylglucosaminidase activity of the albumen layers and membranes of the chicken's egg. *Poult. Sci.* 54:799–805.
42. Winn, S. E., and H. R. Ball, Jr. (North Carolina State University). 1996. Personal communication.