Enzymatic and Microbiological Inhibitory Activity in Eggshell Membranes as Influenced by Layer Strains and Age and Storage Variables

G. Ahlborn* and B. W. Sheldon†,1

*Department of Food Science and †Department of Poultry Science, North Carolina State University, Raleigh, North Carolina 27695

ABSTRACT Eggshell membranes (ESM) have been shown to exhibit antibacterial activity. The purpose of this study was to evaluate the enzymatic and biological [decimal reduction times (D-values)] activities of ESM as a function of bird breed, age, and ESM stabilization treatments. Younger White Leghorn (WL) hens produced ESM with 28% higher lysozyme activity than Rhode Island Red (RIR) layers. In contrast, older WL layers produced ESM with 17% less lysozyme activity than ESM from RIR layers. Similarly, β-N-acetylglucosaminidase (β-NAGase) ESM activities differed by hen age within breeds with younger hens yielding 14 to 16% more enzyme activity. D54ºC-values of Salmonella Typhimurium cells preexposed to WL ESM did not differ as a function of bird age (33, 50, and 81 wk). The ESM Lysozyme and β-NAGase activities varied somewhat over a 6-mo storage study after treatment with 1 of 5 stabilization methods [i.e., storage at 4°C, −20°C, or ambient air storage after freeze drying, air drying (23°C), or forced-air drying (50°C)]. Both air and forced-air drying yielded significant reductions in β-NAGase and lysozyme ESM activity (ca 12 to 30%) after the initial 24 h and then remained fairly stable during the extended storage. Freeze-dried samples retained the most enzymatic activity (95%) throughout the 6-mo trial, whereas refrigerated ESM lost 20 and 18% of the β-NAGase and lysozyme activities, respectively. Frozen ESM lost 22% of the β-NAGase activity, whereas lysozyme was nearly unaffected after 6 mo. The ESM biological activities against S. Typhimurium were not adversely impacted by layer breed or age. No significant loss in biological activity of ESM was detected 24 h after processing or after 6 mo of storage for refrigerated, frozen, and freeze-dried membranes, whereas significant reductions were observed for air- and heat-dried ESM. These findings demonstrate that ESM enzyme and biological activities are relatively constant across layer breeds and over extended storage. Based on these and other findings, ESM may have potential commercial value as a processing adjuvant in food and pharmaceutical product applications.

(Key words: eggshell membrane, β-N-acetylglucosaminidase, lysozyme, stability, inhibitory activity)

INTRODUCTION

Numerous bacterial pathogens are transmitted via the food chain. In 1999 the Centers for Disease Control and Prevention reported that an estimated 76 million persons contract foodborne illnesses each year in the United States (Mead et al., 1999). Although more than 250 foodborne diseases have been described, identified bacteria (e.g., Campylobacter, Salmonella, Escherichia coli O157:H7), viruses (e.g., Norwalk-like, caliciviruses), and parasites (e.g., Giardia, Cyclospora) account for an estimated 14 million illnesses. It is difficult to calculate the exact cost as a result of such illnesses; however, medical costs and lost wages due to foodborne salmonellosis have been estimated to be more than $1 billion per year (Centers for Disease Control and Prevention, 2003). This information along with increasing consumer demands for naturally or minimally processed foods (Ray, 1992; Zeuthen and Bogh-Sorensen, 2003) has opened the doors for food processors to explore new options in food safety.

Although not generally associated with food safety, eggshell membranes (ESM) may provide an option for processors to decrease processing times and temperatures in some of these minimally processed foods. In preliminary studies, Poland and Sheldon (2001) demonstrated that ESM-bound components are capable of reducing the heat resistance or inhibiting the growth of selected gram-positive and gram-negative foodborne bacterial pathogens suspended in 0.1% peptone water [i.e., 83 to 87%
reduction in thermal decimal reduction times (D-values) for *Salmonella enterica* serovars Typhimurium (ST; D$_{54^°C}$) and Enteritidis (D$_{54^°C}$), *E. coli* O157:H7 (D$_{52^°C}$) and up to a 3 log reduction in *L. monocytogenes* populations following incubation for 45 min at 37°C. Ahlborn and Sheldon (unpublished data) have identified that lysozyme, β-N-acetylglucosaminidase (β-NAGase), and ovotransferrin are the primary components in ESM responsible for decreasing the heat resistance of these selected foodborne bacterial pathogens. Methods to extract these enzyme-rich shell membranes are readily available (MacNeil, 2001; Poland and Sheldon, 2001) 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.

If ESM are to be used as a food safety hurdle, it would be beneficial to correlate measurable enzymatic activity with the antimicrobial (or biological) activity as well as to determine if there are differences in enzymatic and biological activity as a function of layer breeds and age and methods of stabilizing (or preparing) ESM for subsequent use. The purpose of this study was to examine the effect of layer breed, bird age, membrane stabilization treatment, and storage time on the enzymatic and biological activity of lysozyme and β-NAGase in ESM.

**MATERIALS AND METHODS**

**Materials**

The ST ATCC 14028 was obtained from the American Type Culture Collection (Rockville, MD) and stock cultures were maintained in sterile double-strength brain heart infusion (BHI) broth plus 20% (vol/vol) glycerol and stored at −20°C. Lyophilized *Micrococcus lysodeikticus* ATCC 4689 cells (M-3770) and 4-nitrophenyl N-acetyl-β-D-glucosaminide (N-9376) were obtained from Sigma Chemical Co. (St. Louis, MO). The BHI broth and agar were obtained from Difco Laboratories (Detroit, MI). All other chemicals and buffers used were reagent grade.

**Experimental Design**

**Layer Breed and Age Trials.** Fifty eggs per group were collected from White Leghorn (WL) and Rhode Island Red (RIR) layers at 25 to 27 and 78 to 80 wk of age. Birds were housed at Carolina Eggs (Nashville, NC) and the North Carolina Department of Agriculture and Consumer Services Piedmont Research Station (Salisbury, NC) and were fed standard corn and soybean layer rations under a 16L:8D cycle. Membranes were extracted and prepared as described below with subsequent analysis of the enzymatic activities of lysozyme and β-NAGase.

**Enzymatic Vs. Biological Activity.** Fifty fresh eggs from WL layers at 33, 50, and 81 wk of age were collected, and their membranes were extracted using the more commercial-like methods described below. Lysozyme and β-NAGase activity were measured and heat inactivation D$_{54^°C}$-values determined for each group of membranes.

**Membrane Stabilization Trials.** Membranes from 100 eggs produced by WL layers (25 to 27 wk) were evaluated. After extraction (described later), the pooled ESM were randomly divided into 5 subgroups, and lysozyme and β-NAGase activities were determined. Each subgroup was randomly assigned to 1 of 5 membrane stabilization treatments: (1) refrigerated and stored at 4°C, (2) frozen and stored at −20°C, (3) lyophilized (freeze-dried under identical conditions as described below for determining solids content), (4) dried at ambient temp (ca 23°C) for 72 h, and (5) dried at 50°C for 36 h in a forced-air convection oven. The later 3 subgroups were maintained at room temperature after the stabilization treatment. The ESM subgroups were then assayed for lysozyme and β-NAGase activity 24 h after the stabilization treatments and then at 1, 2, 4 and 6 mo under the described storage conditions. The D$_{54^°C}$-values against ST were determined for treated ESM 24 h post treatment and after 6 mo of storage.

**Methods**

**Membrane Extraction.** Eggs that were 1 to 4 d old were washed with a nylon brush in cool (15 to 18°C) water containing 100 ppm sodium hypochlorite and then rinsed in sterilized deionized water. Eggs were broken, their contents emptied, and residual albumin removed by rinsing with distilled, deionized water. Membranes were carefully extracted by hand (ensuring that both the inner and outer membrane remained intact), and up to 30 samples (4.7 mm diameter) were excised from each membrane using a die cutter. Excised disks from identical layer experimental variables were pooled together, randomly divided in subgroups of 10 disks each (totaling 0.01 ± 0.002 g), and treated as described in the experimental design.

Because removal of shell membranes by hand would prove impractical for commercial application, a commercial-like process was used to extract ESM using a modified procedure described by Winn and Ball (1975) and Poland and Sheldon (2001) for the comparison of enzymatic vs. biological activity. Briefly, eggs were washed and cleaned of surface contamination and rinsed as described above. The emptied eggshells were ground for 10 min in a food processor (model KFP600WH, KitchenAid, St. Joseph, MI) that contained 400 mL of sterile water before being poured into a sterile container and were left undisturbed for 5 min to allow shell fragments to settle. The top layer of the aqueous suspension containing the membrane fragments were decanted into a Buchner funnel containing Whatman no. 1 filter paper and then 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. Filter cakes (from the same egg experimental variables) of the compacted membrane fragments were removed from the filter paper,
pooled together, stored in a sterile Petri dish wrapped in aluminum foil, and refrigerated until used (less than 24 h).

The Kjeldahl method was used to determine total protein content required in the enzyme assay equations. Percentage of protein was obtained using the conversion factor 6.25 from the average of 3 determinations from each sample. Solids content was determined by weighing extracted membranes before and after freeze-drying. Briefly, ESM were submersed in liquid nitrogen for 10 min. To prevent the ESM from thawing, membranes were quickly removed from the liquid nitrogen and placed on the precooled plate of a freeze dryer (LabConco Series 5 Freeze Dryer, LabConco Corp., Kansas City, MO) and dried for 18 h at −20°C under 10⁻² Torr of pressure.

**Lysozyme Assay.** Lysozyme activity was determined using an adaptation of the assay described by Shugar (1952) as measured by the change in optical density (450 nm) following exposure of *Micrococcus lysodeikticus* to the ESM. A cell suspension was produced by adding 0.015% (wt/vol) lyophilized *Micrococcus lysodeikticus* cells to 66 mM potassium phosphate buffer (pH 6.24) at 25°C. The resulting suspension absorbance (450 nm) was between 0.6 and 0.7. ESM samples [each sample comprised of 10 disks (4.7 mm diameter totaling 0.01 ± 0.002 g) or 0.01 ± 0.001 g of membrane fragments] from the treatment groups 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 (unpublished data)], the disks were removed, the decrease in absorbance recorded, and enzyme activity calculated according to equation 1. Twenty samples (n = 20) were evaluated for each of the 5 stabilization treatment groups and 3 age groups for the biological vs. enzymatic trial.

\[
\text{units per milligram of solid} = \frac{\Delta A_{450}/\text{time (in min)}}{0.001 \times \text{mg/solid per reaction mix}} \tag{1}
\]

where 0.001 = change in absorbance at A₄₅₀nm as per the unit definition.

**β-NAGase Assay.** The release of p-nitrophenol from 4-nitrophenyl N-acetyl-β-D-glucosaminide was followed using a modified procedure of Lush and Conchie (1966), Donovan and Hansen (1971), and Winn and Ball (1975). The incubation mixture containing 10 ESM disks (4.7 mm diameter totaling 0.01 ± 0.002 g) or 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 pH 3.0 citrate buffer), and 0.6 mL of distilled, deionized water was incubated at 37°C for 25 min. The reaction was stopped by adding 2.0 mL of 0.2 M Na₂CO₃ to the incubation mixture. The mixture was then briefly vortexed, the ESM disks were removed, and the absorbency (at 420 nm) of the solution was read in a narrow path absorption cell (1-cm light path) using a Shimadzu UV 160U UV-Visible Recording Spectrophotometer (Shimadzu Corp., Kyoto, Japan) blanked against the reaction solution minus the ESM disks. Enzyme activity was calculated according to equation 2. Sample sizes were identical to the lysozyme assay.

\[
\text{units per milligram} = \frac{\Delta A_{450}/\text{total volume}}{\text{minutes} \times 10.8 \times \text{milligrams of protein}} \tag{2}
\]

where 10.8 = extinction coefficient of the substrate at the given absorbance.

**Bioassay (D-value Determination).** The D-values were determined using the combined methods of Poland and Sheldon (2001) and the immersed sealed capillary tube procedure described by Schuman and colleagues (1998). The ST ATCC 14028 (ST) was selected on the grounds that in preliminary trials cells from ST showed the greatest sensitivity to ESM (Poland and Sheldon, 2001; Ahlborn and Sheldon, unpublished data). Midlog phase cell populations of ST 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, NJ) followed by transfer of 100 µL of the cell suspension into 10 mL of BHI broth and incubation at 37°C for 2 h. Cells were harvested by centrifugation (15 min at 4°C at 8,000 × g), washed in sterile 0.1% peptone water (PW) and suspended in 10 mL of 0.1% PW (ca 10⁸ cfu/mL). Zero or 1 g of the pooled ESM fragment extract was added to 20 mL of the peptone bacterial suspension and incubated with agitation at 37°C for 30 min.

After incubation, ESM were removed from the peptone bacterial suspension in 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 i.d. × 90 mm long; model 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 using a propane torch and kept on ice until heated (less than 30 min). Tubes were placed upright in a mesh-screen-covered test tube rack and rapidly submersed into a preheated (54°C) circulating water bath (model W19, Haake, Inc., Karlsruhe, Germany) with a temperature control module (model DC1, Haake, Inc.) accurate to ±0.05°C. At 6 to 8 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 immersed in sodium hypochlorite (500 ppm, pH 6.5) for 5 s and then rinsed 3 times in sterile, distilled deionized water. Tubes were aseptically transferred to individual test tubes containing 5 mL of sterile PW and finely crushed using 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 using the Spiral Biotech Autoplater 4000 (Spiral Biotech Inc, Norwood, MA). Plates were incubated at 37°C for 18 to 24 h and enumerated using a Microbiology International ProtoCOL automatic colony counting system (model 60000, Synoptics Ltd., Cambridge, UK).

Triplicate thermal inactivation trials were conducted and survivor curves (log viable ST/mL vs. heating time)
RESULTS AND DISCUSSION

Breed and Age Trial

Figure 1 shows the enzymatic activity of lysozyme and β-NAGase as influenced by layer breed and age. Shell membrane lysozyme activity (43.4 U/mg) was greatest in the WL layers at 25 to 27 wk of age. The ESM from 78- to 80-wk-old WL layers had significantly lower lysozyme activities (17.1%) than ESM from the younger birds. Contrary to the differences observed in WL layers, no difference in ESM lysozyme activity was detected between young and old RIR layers. The ESM lysozyme activity from 25- to 27-wk-old WL layers was 28% greater than the RIR counterparts; however, no significant breed difference was observed for membranes extracted from the 78- to 80-wk-old layers. The β-NAGase activity was highest in the ESM extracted from 25- to 27-wk-old WL and RIR layers (13.2 and 12.6 U/mg respectively). A significant reduction in β-NAGase activity within the WL breed was observed in ESM extracted from the older birds (14.4%).

Comparison of Enzymatic and Biological Activity

The time and resources required to determine biological activity (through heat inactivation trials) can be costly and time consuming. Thus, the relationship between biological and enzymatic activities was determined for lysozyme and β-NAGase. Figure 2 depicts the enzymatic and biological (D5_4°C-values) activity of lysozyme and β-NAGase detected in ESM from WL layers at 3 different ages (33, 50, and 81 wk).

Lysozyme and β-NAGase activities were greatest in the ESM from 33-wk-old layers. A significant reduction in ESM enzyme activities was found in the 50-wk-old layers. At 81 wk, a slight numerical increase in lysozyme and β-NAGase activities was observed in the ESM, although not significantly different than membranes from 50-wk-old layers. This reduction in enzyme activity may correspond to the hen’s laying cycle. Egg production begins at approximately 19 wk of age, reaches a maximum between 24 to 28 wk of age, and then gradually declines as the layers reach the forced molting period (through feed restriction or reduced caloric intake) in which egg production declines significantly. After the forced molting period (around 60 to 64 wk of age), egg production increases again, reaching a peak output at approximately 72 to 78 wk of age, and thereafter is followed by a decline in egg production. It is well established that hormone levels such as estrogen (Beck and Hansen, 2004), corticosterone, and thyroid hormones (Davis et al., 2000) change throughout the egg-laying cycle. We hypothesize that perhaps as egg production decreases, other hormonal and physiological changes occur in the hen such as a reduction in lysozyme and β-NAGase levels in the oviduct, albumen, and shell membrane. After molting, an increase in the expression of these enzymes should coincide with the increase in egg production after restoration of normal hormonal levels.

In comparing the enzymatic and biological activities, a significant inverse correlation was observed for lysozyme (r = 0.998) and β-NAGase (r = 0.992). Shell membranes from 33-wk-old layers (having the highest lysozyme and β-NAGase activity) produced the lowest D-value (1.9 min). However, no significant differences in D-values were observed across hen age groups. After molting, the 81-wk-old layers produced similar lysozyme and β-NAGase ESM activities as the 33- (lysozyme) and 50-wk-old layers (lysozyme and β-NAGase). Despite significant decreases in lysozyme and β-NAGase between layers at 33 and 50 wk, the lack of significant differences in D-values may indicate that ovotransferrin, the third component responsible for the observed antibacterial properties, may have a greater role than the 2 enzymes and perhaps be key to the antimicrobial activity of the ESM. Layer breeds within age groups did not influence D-values (unpublished data). Thus, age and breed apparently do not adversely affect the biological activity of ESM.
Membrane Stability Trial

After extraction from the shell, representative membrane samples were taken and enzymatic activities were determined from pooled samples prior to subjecting sub-samples to 1 of 5 stabilization treatments. No significant difference was observed in the samples prior to the processing treatments with lysozyme and β-NAGase activity averaging 42.4 (± 3.3) and 13.5 (± 0.75) units/mg, respectively. Figure 3 depicts the activity of β-NAGase subjected to the 5 treatments over a 6-mo period. Twenty-hours after treatment, no significant change in enzyme activity was observed in the refrigerated, frozen, and freeze-dried samples (statistical data not shown). In contrast, air- and heat-dried samples lost 18 and 31% of their respective activities. The 3 drying methods (freeze, air, and heat drying) produced fairly consistent and stable enzyme activity over the next 6 mo. However, the β-NAGase activity of refrigerated and frozen ESM gradually decreased over time, and samples lost approximately 20 and 22% of their enzyme activities, respectively, at the end of 6 mo.

Similar trends were observed with lysozyme activity as illustrated in Figure 4. After treatment, the greatest loss of activity after 24 h was detected in the air- and heat-dried samples (16 and 31%, respectively), after which no significant further loss was observed in the succeeding 6 mo. After 6 mo, lysozyme activity had declined by 18% in refrigerated samples, although there were no significant decreases in frozen (4%) and freeze-dried membranes (< 1%) throughout the extended storage.

With the exception of the initial 24-h period for air- and heat-dried ESM, enzyme activity remained reasonably stable over the 6 mo of storage. Lysozyme activities were also generally more variable than β-NAGase (larger standard deviations). These larger variances may be explained by data presented by Hincke et al. (2000) who observed...
Figure 4. Lysozyme activity as influenced by membrane stabilization method and storage time. Refrigerated and stored at 4°C (◇), frozen and stored at −20°C (◆), lyophilized (freeze-dried; ▲), dried at ambient temp (ca 23°C) for 72 h (▲), and dried at 50°C for 36 h in a forced-air convection oven (☆). Means with different letter superscripts across treatments differ significantly (P ≤ 0.05) (n = 20).

using a colloidal-gold immunocytochemical localization detection method that lysozyme was distributed heterogeneously throughout the ESM membrane. The apparent random distribution of lysozyme could explain the larger deviations observed within treatment subgroups and samples despite a representative sampling and pooling protocol. This hypothesis was supported by preliminary trials in which we examined the lysozyme and β-NAGase activity of 20 (4.7 mm diameter, 0.4 to 0.5 g each) excised disks taken from different sections of an egg. Lysozyme activity varied by as much as 270% (20.4 to 55.1 units/mg), whereas β-NAGase activity only varied by as much as 64% (9.1 to 14.2 units/mg; unpublished data).

Figure 5 presents the D54°C-values (min) for ST preexposed (37°C, 30 min) to ESM following the membrane stabilization treatments. No difference was observed in D-values from bacteria exposed to fresh, frozen, and freeze-dried samples taken 24 h after their respective treatments. There was a significant loss of biological activity in membranes that were air dried, and D-values were
only slightly lower than the control treatment (4.2 compared with 5.3 min, respectively). Heat stabilized membranes also lost significant biological activity (D-value = 4.8 min) and were not different than air-dried membranes or the control (no ESM treatment). No significant differences in D-values within processing treatments were detected between the 24-h and 6-mo samples, indicating that the components responsible for increasing the heat sensitivity of ST were stable.

In conclusion, only lysozyme activity was significantly greater in the membrane of WL layers, with no differences observed with β-NAGase. These significant differences in lysozyme activity did not correlate to significant differences in the biological activity of ESM between breeds. Although the focus of this study was directed only at lysozyme and β-NAGase, the properties and activity of ESM ovotransferrin, the other primary component presumed to contribute to the antibacterial activity of the ESM, have not yet been fully evaluated. Preliminary data indicates that the chelating (iron-binding) properties of membrane bound ovotransferrin follow similar stability and activity trends as observed with lysozyme and β-NAGase (unpublished data). However, further evaluation is required to make a complete assessment.

The application of the ESM (and its components) as a natural antimicrobial may lead to their use as processing aids in heat-sensitive food and pharmaceutical products resulting in a reduction of thermal process requirements (lower process temperatures and times) yet still attaining a product with extended shelf life and reduced levels of microorganisms. Reduced thermal processing requirements may also result in food products that have higher nutrient levels, improved functionality, and potentially lower processing costs. Consumers will perhaps also perceive these ingredients as natural and more acceptable, whereas egg processors will gain a new value-added product having potentially significant market value.

If food and pharmaceutical processors are able to use ESM as a processing adjuvant, the economic feasibility of the ESM extraction and stabilization methods will need to be thoroughly evaluated. Although freeze-drying maintained the greatest activity over time, any benefit captured by using freeze-dried ESM having higher enzymatic and biological activity may be offset by the cost of the freeze-drying process. Thus, other stabilization methods may be more practical. More investigations are needed to evaluate the effectiveness of ESM in actual food or pharmaceutical applications to realize its real market potential. The effects of food components (i.e., proteins, fats, and carbohydrates), physical barriers, and exposure temperatures and time on ESM biological activity are a few challenges that must be explored.

**ACKNOWLEDGMENTS**

The authors thank the American Egg Board for partial financial support of this research.

**REFERENCES**


