Effect of Ethylenediaminetetraacetate and Lysozyme on the Antimicrobial Activity of Ovotransferrin Against Listeria monocytogenes

K. Y. Ko,* A. F. Mendonca,† and D. U. Ahn*1

*Department of Animal Science, and †Department of Food Science and Human Nutrition, Iowa State University, Ames 50011

ABSTRACT This study evaluated the effect of EDTA and lysozyme on the antibacterial activities of activated ovotransferrin against 5 strains of Listeria monocytogenes. First, a disc test was performed to screen the concentrations of EDTA or lysozyme that showed antibacterial activities in ovotransferrin (O) or ovotransferrin in 100 mM NaHCO3 (OS) solution. Turbidity and viability tests were conducted using O or OS solution combined with either lysozyme (OL and OSL) or EDTA (OE and OSE). Also, OS combined with 2 mg/mL of lysozyme (OSL) or 1 mg/mL of EDTA (OSLE), or both, was applied on commercial hams to determine if the solutions show antibacterial activities on meat products. The effect of initial cell population on the antibacterial activities of ovotransferrin combined with either EDTA or lysozyme was also determined. The L. monocytogenes started to grow after 1 d of incubation in the presence of >2.0 mg/mL of lysozyme. The OL groups showed weak antibacterial activities against L. monocytogenes in brain heart infusion broth culture, and their activities were bacteriostatic. The OSL groups were bactericidal against L. monocytogenes, resulting in 1 log reduction from initial cell population. Even though OSL showed stronger antibacterial activity than OS, lysozyme had no significant effect on antibacterial activity of OS against L. monocytogenes. Also, EDTA itself at 1.0 and 2.0 mg/mL was bacteriostatic against 5 strains of L. monocytogenes. They were more susceptible to EDTA than lysozyme, and OSE1 and OSE2 had bactericidal activity against L. monocytogenes. There was a significant difference in the survivor cell populations between OS and OSE groups (P < 0.05). Therefore, EDTA enhanced the antibacterial activity of OS against L. monocytogenes. However, ovotransferrin plus either lysozyme or EDTA, or both, did not show any antibacterial effect in commercial hams during storage at 10°C. In addition, the initial population of L. monocytogenes cells influenced the antibacterial activity of OSL or OSE.

Key words: ovotransferrin, antibacterial activity, ethylenediaminetetraacetate, lysozyme, Listeria monocytogenes

INTRODUCTION

Natural antimicrobial agents that originate from plant, animal, and bacterial sources are expected to provide great satisfaction to consumers who are sensitive to health and safety issues. Ovotransferrin, the second major egg white protein, is an iron-binding glycoprotein that transports and scavenges Fe (III) in eggs of poultry (Kurokawa et al., 1995). Antibacterial properties of ovotransferrin against a variety of microorganisms including Escherichia coli (Schade and Caroline, 1944; Valenti et al., 1983), Pseudomonas spp., Streptococcus mutans (Valenti et al., 1983), Staphylococcus aureus, Bacillus cereus (Ibrahim, 1997), Salmonella Enteritidis (Baron et al., 1997, 2000), and Candida (Valenti et al., 1985) have been reported. However, the antimicrobial mechanisms of ovotransferrin are not fully defined yet. The iron-binding capability of ovotransferrin was initially believed to be the major antimicrobial action of ovotransferrin. However, recent studies demonstrated that direct interactions of ovotransferrin with bacterial surface were the major cause of antimicrobial action of ovotransferrin (Arnold et al., 1981; Valenti et al., 1983, 1985; Ibrahim, 1997; Ibrahim et al., 2000).

Listeria monocytogenes can proliferate in the presence of curing salt at refrigerated temperature (Lou and Yousef, 1999) and can colonize, multiply, and persist on processing equipment or plant substances (Rocourt and Seeliger, 1985). The strains are sensitive to heat treatment and can be easily inactivated by cooking. However, ready-to-eat (RTE) meat products such as frankfurters, and deli meats have high incidence rates of listeriosis due to contamination during multiple handling and processing steps performed after cooking (Giovannacci et al., 1999; Uyttendaele et al., 1999; Lunden et al., 2003). The Centers for Disease Control and Prevention (CDC, 2002) reported that 2,500 people suffer from listeriosis annually in the
United States and 1 in 5 die from the disease (http://www.fsis.usda.gov/oppde/larc). Due to this risk, the USDA currently established a zero-tolerance policy for *L. monocytogenes* in RTE meat products. Therefore, developing potent antimicrobials against *L. monocytogenes* for RTE meat is necessary. According to a previous study, apo-ovotransferrin alone was not effective in controlling *L. monocytogenes* in brain heart infusion (BHI) broth (our unpublished data). To improve the antimicrobial activities of ovotransferrin, therefore, combinations of several antimicrobial agents are necessary (Sofos et al., 1998). The combinations of materials with different antimicrobial mechanisms are expected to increase antibacterial effectiveness, because simultaneous attacks on different targets are likely to increase the sensitivity of the microorganism to the environment. The use of ovotransferrin combined with EDTA or lysozyme, or both, as antimicrobials on the surface of meat or meat products is appealing to the meat industry, because all the components are generally regarded as safe.

Lysozyme, a single peptide protein with a molecular weight of 14.6 kDa, catalyzes the hydrolysis of 1,4-glycosidic linkages between N-acetylmuramic acid and N-acetylglycosamine of cell wall peptidoglycan (Proctor and Cunningham, 1988). Therefore, the antimicrobial activity of lysozyme is ascribed primarily to the enzymatic lyses of peptidoglycan in the cell wall of microorganisms (Branen and Davidson, 2004). The application of lysozyme in foods has been limited, because gram-negative bacteria are protected by an outer membrane, which is relatively resistant to antimicrobial activities of lysozyme (Gill and Holley, 2003). However, lysozyme gained considerable interest for use in food systems, because it is a natural enzyme produced by animals and it activity targets a specific cellular structure of microorganisms (Proctor and Cunningham, 1988).

Ethylene-diaminetetraacetate has been used in several food products as a chelating agent to prevent oxidation (Hansen et al., 2001). Although the mechanism of antibacterial effectiveness by EDTA is not fully understood, its chelating property to divalent cations like Ca$^{2+}$ or Mg$^{2+}$ should be involved (Shelef and Seiter, 1993). Ethylene-diaminetetraacetate inhibits the growth of microorganisms by depriving Mg$^{2+}$, Ca$^{2+}$, and Fe$^{2+}$, which are essential factors for microbial growth, from microorganisms (Banin et al., 2006). Ethylene-diaminetetraacetate is reported to enhance the antimicrobial activity of lactoferrin by destabilizing the outer membrane of bacteria and increasing the permeability of divalent cations (Coughlin et al., 1983; Vaara, 1992; Boland et al., 2004). Also, chelating agents facilitate the detachment of cells from biofilm and enhance the killing of biofilm-producing microorganisms by depriving Mg$^{2+}$ associated with lipopolysaccharides (Banin et al., 2006).

The objective of this study was to enhance the antimicrobial activity of ovotransferrin solution supplemented with 100 mM sodium bicarbonate against a cocktail of 5 strains of *L. monocytogenes* by combining with lysozyme or EDTA. Many studies related to antibacterial activity of ovotransferrin in vitro have been reported, but little work has been done to apply ovotransferrin as an antimicrobial agent in meat or meat products. Therefore, the present study applied ovotransferrin in 100 mM NaHCO$_3$ plus lysozyme or EDTA, or both, on commercial hams to determine their effect on the growth of *L. monocytogenes* in meat products.

**MATERIALS AND METHODS**

**Ovotransferrin**

Apo-ovotransferrin (iron-free) used in this study was prepared by the method of Ko et al. (our unpublished data). After iron saturation of 2x diluted egg white solution, holo-ovotransferrin (iron-saturated) was separated using ethanol. Iron was removed from the holo-ovotransferrin using AG 1-X2 resin (chloride form, Bio-Rad Laboratories Inc, Hercules, CA) and then freeze-dried. The dried apo-ovotransferrin was dissolved in 0.15 M NaCl solution, and the pH was adjusted to 7.4. The purity of ovotransferrin used in this study was around 80%, and the residual iron in prepared apo-ovotransferrin solution was less than 0.5 mg/kg. Prior to microbial study, the ovotransferrin solution was sterilized by passing through a 0.22-μm syringe filter (Whatman Inc., Floribam Park, NJ).

**Bacterial Strains**

Five different strains of *L. monocytogenes* (NADC 2045, H7962, H7969, H7762, and H7596) were used. Before inoculation, each strain was individually cultured on BHI broth (Remel Inc., Lenexa, KS) for 24 h at 35°C and harvested twice to activate the strain appropriately. Each strain culture solution (5 mL) was transferred to a sterile 100 mL flask and centrifuged at 100,000 × g for 10 min at 4°C. The pellet was collected, washed with saline, and then resuspended in 25 mL of saline. An inoculation cocktail was prepared by mixing equal volumes of each cell suspension to have approximately the same population of 5 strains of *L. monocytogenes*. The number of inoculated *L. monocytogenes* strain was analyzed on BHI agar plates after serial dilution with 0.1% peptone water (Difco Laboratories, Detroit, MI) and incubation at 35°C.

**Formation of Clear Zone**

Before analyzing the antibacterial activity through viability and turbidity tests, the disc diffusion method (Bauer et al., 1966) was used to determine the concentration of EDTA (disodium salt, Fisher Scientific, Fairlawn, NJ) or lysozyme (from egg white, Sigma-Aldrich Inc., St. Louis, MO) capable of promoting antibacterial activity of ovotransferrin. A *L. monocytogenes* cocktail containing an equal amount of each strain was diluted in series, and then 0.1 mL of cell suspension corresponding to 10$^5$ or 10$^6$ cfu/mL was spread homogeneously on the BHI agar.
media plates. The BHI agar plates inoculated with *L. monocytogenes* were dried for 30 to 60 min in an incubator at 35°C. Ethylenediaminetetraacetic acid (1, 0, 1.5, 2.0, or 2.5 mg/mL) or lysozyme (1.0, 1.5, 2.0, or 2.5 mg/mL) was added to ovotransferrin solution or ovotransferrin solutions in 100 mM NaHCO₃ (OS). All solutions prepared were sterilized using 0.22-μm syringe filters (Whatman Inc.) before performing this experiment. Ovotransferrin solutions (120 μL) containing different concentrations of EDTA or lysozyme were dispensed to sterilized glass microfiber filter (2.1-cm diameter, Whatman Inc.) on the dried BHI agar media. Three replications were prepared. The BHI agar plates were incubated at 35°C for 24 to 48 h, and then antibacterial activity of each ovotransferrin solution against *L. monocytogenes* was estimated through the existence of clear zones.

### Turbidity Test

The antimicrobial capacity of ovotransferrin solutions combined with EDTA or lysozyme against the growth of *L. monocytogenes* was analyzed by measuring the turbidity of solution after incubation in a BHI broth culture at 35°C. Disc diffusion test indicated that when ≥2.0 mg/mL of EDTA and ≥1.5 mg/mL of lysozyme were combined with ovotransferrin, the solutions had antibacterial activity against *L. monocytogenes* (Table 1). After 2 mL of 40 mg/mL ovotransferrin solution was added to 2 mL of 2×-strength BHI broth; stock solutions of EDTA or lysozyme were added to the media including ovotransferrin (O) or OS. The final concentration of lysozyme was adjusted to be 2.0 mg/mL (OL2 and OSL2), 2.5 mg/mL (OL2.5 and OSL2.5), or 3.0 (OL3 and OSL3) mg/mL, and that of EDTA was adjusted to 1.0 mg/mL (OE1 and OSE1), 1.5 mg/mL (OE1.5 and OSE1.5), or 2.0 mg/mL (OE2 and OSE2.5). Also, 40 μL of cell suspension containing 10⁶ to 10⁷ level of activated *L. monocytogenes* was inoculated to the prepared solution to make the initial population of *L. monocytogenes* 10⁴ to 10⁵ cfu/mL. Ethylenediaminetetraacetic acid and lysozyme solution without ovotransferrin were prepared to determine the antibacterial activity of EDTA or lysozyme alone.

After inoculation, the samples were incubated at 35°C for 36 h. Proliferation of *L. monocytogenes* in each treatment was evaluated by measuring its turbidity with a spectrophotometer at 620 nm every 3 to 6 h.

### Viability Analysis

The antibacterial activities of ovotransferrin plus lysozyme (OSL) and ovotransferrin plus EDTA (OSE) were investigated using a viability test. First, 2 mL of ovotransferrin solution (40 mg/mL) was added to 2 mL of 2×-concentrated BHI broth media and then 100 mM sodium bicarbonate was added. Stock solution of either EDTA or lysozyme was added to the BHI broth culture contained with ovotransferrin alone or OS. The final concentration of lysozyme and EDTA in solution was identical to that of the turbidity test. Also, 40 μL of cell suspension containing 10⁶ to 10⁷ cells of activated *L. monocytogenes* was inoculated to make initially 10⁴ to 10⁵ cfu/mL in BHI broth culture. Ethylenediaminetetraacetate or lysozyme alone was prepared as a control group. After inoculation, the prepared culture solutions were incubated at 35°C for 36 h. The number of viable cells was analyzed by spreading each culture solution after diluting (1:10) with 0.1% sterile peptone water (Difco Laboratories), spread-

---

**Table 1.** Effect of ovotransferrin combined with NaHCO₃ and EDTA or NaHCO₃ and lysozyme on clear zone formation on brain heart infusion (BHI) agar media plates inoculated with *Listeria monocytogenes* during 35°C incubation for 24 to 48 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/mL)</th>
<th>Control</th>
<th>OTF¹</th>
<th>OTF + NaHCO₃²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–/+</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>–</td>
<td>+/-</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>+/+</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>++</td>
<td>+++</td>
<td>+/−</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0</td>
<td>−−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>++</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

¹− = no clear zone formed on BHI agar media plate. The strength of the clear zone was described as + (weak), ++ (middle), and +++ (strong) by its size or clarity on BHI agar. (n = 3).
²20 mg/mL of ovotransferrin.
³100 mM NaHCO₃ was used at this study.
ing 0.1 mL of diluted samples homogeneously on a BHI agar plate, and incubating at 35°C for 24 to 36 h. The number of survivors on BHI agar plates was counted as colony-forming units per milliliter of sample.

**Effect of Initial Cell Number**

This study was performed to determine if the populations of initial cells affect the antibacterial activity of ovotransferrin combined with various additives. The BHI broth media containing ovotransferrin were prepared with the same method described above. Stock solution of either EDTA or lysozyme was added to OS so that the final concentration of lysozyme and EDTA in the ovotransferrin solution became 2 mg/mL of lysozyme (OSL2) or 1 mg/mL of EDTA (OSE1), respectively. Ethylenediaminetetraacetate (2 mg/mL) or lysozyme (1 mg/mL) alone was prepared to identify antibacterial activity of the solution itself against *L. monocytogenes*. Also, 40 μL of cell suspension containing 10^5, 10^6, or 10^7 cells of activated E. coli O157:H7 was inoculated to 4 mL of BHI broth media to make the number of *L. monocytogenes* 10^4, 10^5, or 10^6 cfu/mL. After the prepared culture solutions were incubated at 35°C for 24 to 36 h, the viability of each treatment was measured using the same method as above.

**Application of Ovotransferrin to Hams**

Commercial hams were purchased from local retail stores. Hams were sliced to 0.2-cm-thick pieces and vacuum-packaged (−1 bar of vacuum with 10-s dwell time) in low oxygen-permeable bags (nylon/polyethylene, 9.3 mL of O _2/_m² per 24 h at 0°C, Koch, Kansas City, MO). Before inoculation, each package was aseptically opened using alcohol-sterilized scissors. Activated *L. monocytogenes* cocktail stock suspension (0.1 mL) was inoculated aseptically on the surface of sliced ham to make the initial cell number around 10^3 cfu/mL. After manually mixing for 30 s to distribute the inocula evenly, the packaged samples were randomly divided into 7 groups. Lysozyme or EDTA, or both, was added to ovotransferrin solution, including 100 mM NaHCO _3_ and 0.15 M NaCl. Therefore, the final treatments comprised of 2 mg/mL of lysozyme (L), 2 mg/mL of lysozyme plus 1 mg/mL of EDTA (LE), 20 mg/mL of ovotransferrin plus 2 mg/mL of lysozyme (1OSL), 20 mg/mL of ovotransferrin plus 2 mg/mL of lysozyme and 1 mg/mL of EDTA (1OSLE), 30 mg/mL of ovotransferrin plus 2 mg/mL of lysozyme (2OSL), and 30 mg/mL of ovotransferrin plus 2 mg/mL of lysozyme and 1 mg/mL of EDTA (2OSLE). Each prepared ovotransferrin solution (1 mL) was distributed evenly on the surface of a ham. Control group was prepared by inoculating *L. monocytogenes* to hams without adding any solution, and all hams were stored in a 10°C incubator.

Viable *L. monocytogenes* cells on the ham slices were analyzed after 0, 2, 5, 10, 15, 22, and 29 d of storage: 30 to 50 mL of sterile 0.1% peptone water solution was added to each vacuum-package bag followed by pummeling for 1 min in a stomacher at normal speed. After serial dilution of the sample with 0.1% peptone water, 0.1 mL of diluted sample was spread homogeneously on MOX media plates (Difco Laboratories) in duplicate. Survivors were enumerated as colony-forming units per milliliter by the same method as above.

**Statistical Analysis**

All experiments were replicated 3 times, and the data were analyzed using the JMP software (version 5.1.1, SAS Institute, Cary, NC). The differences in the mean values were compared using Tukey honestly significantly different test, and means values and standard error (SEM) were reported (Kuehl, 2000). Statistical significance for all comparisons was obtained at P < 0.05.

**RESULTS AND DISCUSSION**

**Formation of Clear Zone**

Lysozyme at 1.0 and 1.5 mg/mL did not form clear zones, but 2.0, 2.5, and 3.0 mg/mL of lysozyme did. The strength of clear zone formed by lysozyme combined with ovotransferrin or OS was similar when compared with lysozyme (Table 1). *Listeria monocytogenes* was susceptible to ≥2.0 mg/mL of lysozyme. The antimicrobial activity of lysozyme has long been believed to be due to its bacteriolytic actions, which hydrolyze β-1,4-glycosidic bond in peptidoglycan of gram-positive bacteria that do not possess any outer membrane, but gram-negative bacteria with outer membrane is reported to be relatively resistant to lysozyme (Davies et al., 1980; Spitznagel, 1984; Davis and Reeves, 2002). The antibacterial action of lysozyme against *L. monocytogenes* in vegetables was strong but was weak in animal-derived foods such as pork sausage and cheese (Hughey et al., 1989). Carminati and Carini (1989) reported that no antimicrobial activities were exhibited by lysozyme against *L. monocytogenes* in whole milk.

Clear zones were observed at 1.5 and 2.0 mg/mL of EDTA but not at 1.0 mg/mL. When EDTA was combined with ovotransferrin or OS, the strength of clear zone decreased (Table 1). Zhang and Mustapha (1999) demonstrated that EDTA itself was not lethal to *L. monocytogenes* under the concentrations permitted in foods. Ethylenediaminetetraacetate is usually used in combination with other preservatives and promotes the suppression of *L. monocytogenes* in meat (Monk et al., 1996; Parente et al., 1998), but sometimes EDTA seems to modulate the antibacterial activity of antimicrobials such as nisin (Zhang and Mustapha, 1999). This study indicated that more than 1.5 mg/mL of EDTA inhibited the growth of *L. monocytogenes*. However, when EDTA was combined with ovotransferrin or OS, the strength of clear zone decreased. Based on the disc test, more than 2 mg/mL of lysozyme or 1 mg/mL of EDTA was chosen in turbidity and viability tests, and tests were performed to identify EDTA and lysozyme effects on antibacterial activities of ovotransferrin.

**Lysozyme on Antibacterial Activity of Ovotransferrin**

As shown in Figure 1, *L. monocytogenes* entered log phase after 7 to 8 h incubation at 35°C in BHI broth.
culture. They seemed to face stationary phase after 24 h of incubation and then death. Lysozyme at 2 mg/mL delayed the growth of *L. monocytogenes* for 24 h, whereas lysozyme at 2.5 mg/mL and 3 mg/mL delayed it for 30 and 36 h, relatively. Therefore, *L. monocytogenes* seemed to have a lag time to adapt to a new environment when lysozyme was added (Figure 1). Even though lysozyme alone delayed the growth of *L. monocytogenes* during the initial period, *L. monocytogenes* started to grow after 24 to 36 h of incubation at 35°C. The turbidity values obtained from all OL or OSL groups were lower than 0.1 as in OS treatment (Figure 1). According to turbidity test, *L. monocytogenes* was found to be controlled by OL or OSL groups even though they were resistant to lysozyme alone under the concentrations used in this study.

In the viability test, which measures the number of viable *L. monocytogenes* cells after 48 h of incubation at 35°C, *L. monocytogenes* growth for 2.0, 2.5, or 3.0 mg/mL of lysozyme was similar (*P < 0.05) to the control (Figure 2). The activities of lysozyme against *L. monocytogenes* are reported to vary depending upon food products or media: *L. monocytogenes* are susceptible to lysozyme in media and phosphate buffer, whereas they are highly resistant to lysozyme in whole milk containing divalent cations such as Ca<sup>2+</sup> and Mg<sup>2+</sup> (Kihm et al., 1994). Because BHI media used in this study is relatively nutrient-rich media, injured cells of *L. monocytogenes* by lysozyme treatment should have been repaired easily in the media. Thus, they showed resistance to 2.0 to 3.0 mg/mL of lysozyme. Another possible explanation for resistance of *L. monocytogenes* to lysozyme may be attributed to using a cocktail of 5 different strains of *L. monocytogenes* instead of using a specific strain.

The number of viable cells obtained when using OS slightly increased from 4.6 log cfu/mL, the number of the initial inoculation, to 4.9 log cfu/mL during 48 h of incubation at 35°C. Therefore, OS seemed to be bacteriostatic against *L. monocytogenes*. Also, the number of viable *L. monocytogenes* cells in OL2, OL2.5, and OL3 increased from 4.6 log cfu/mL, the number of initial inoculated cell, to 5.1, 7.2, and 6.0 log cfu/mL, respectively (Figure 2). The OL groups showed a weak antibacterial activity against *L. monocytogenes* compared with control. However, OSL groups showed a stronger antilisterial activity against *L. monocytogenes* than that of OL groups. In addition, OSL2.5 and OSL3.0 were listericidal considering their changes from 4.6 log cfu/mL to 3.3, 3.0, or 2.9 log cfu/mL, respectively. Also, there was significant difference in antibacterial activity against *L. monocytogenes* between OL and OSL under lysozyme at 2.5 and 3.0 mg/mL (Figure 2). Therefore, this study demonstrates that 100 mM sodium bicarbonate enhanced the antimicrobial activity of OL against *L. monocytogenes*, whereas lysozyme at 2.0, 2.5, or 3.0 mg/mL did not clearly show effect on antibacterial activity of OS toward *L. monocytogenes*.

Payne et al. (1994) demonstrated that a combination of 15 mg/mL of lactoferrin and 0.15 mg/mL of lysozyme delayed the growth of *L. monocytogenes* in ultra high temperature milk, whereas Boesman-Finkelstein and Finkelstein (1985) reported that synergistic activity by lactoferrin plus lysozyme was not clearly found. Cultures inoculated with the strains at early exponential phase are relatively susceptible to lactoferrin, whereas those at the early stationary phase are comparatively resistant to the antimicrobial (Arnold et al., 1981). However, using multiple *L. monocytogenes* strains at the stationary phase, and nutrient rich media, resulted in a weak synergistic effect of lysozyme on antibacterial activity of ovotransferrin.

### EDTA on Antibacterial Activity of Ovotransferrin

Ethylenediaminetetraacetic acid at 1.0, 1.5, or 2.0 mg/mL; OS; OE group; and OSE group showed <0.1
Ethylenediaminetetraacetate chelates divalent cations of outer membrane (Coughlin et al., 1983; Vaara, 1992; Boland et al., 2004), which enhanced the activity of lactoferrin through the destabilization of outer membrane of gram-negative bacteria and increased membrane permeability. Negatively charged head groups of phosphatidylglycerol and cardiolipin, which are predominant phospholipids existing in cytoplasmic membrane of *L. monocytogenes* (O’Leary and Wilkinson, 1988), can be neutralized by divalent cations such as Mg$^{2+}$ and Ca$^{2+}$. As a result, a more rigid membrane is formed by condensation of membrane lipids (Harwood and Russel, 1984) and becomes less efficient for nisin insertion and pore formation (Abee et al., 1994). As EDTA is combined with nisin and leucosin, their bactericidal effect against *L. monocytogenes* in tryptone soy broth supplemented with 0.6% yeast extract is enhanced (Parente et al., 1998) probably due to the chelating property of EDTA to divalent cations that play a role to protect *L. monocytogenes* (Abee et al., 1994). Aguilera et al. (2003) reported that transferrin family proteins including ovotransferrin interacted directly with cell membrane and resulted in dissipation of electrical potential inside cells.

In conclusion, EDTA increased the antibacterial activity of OS against *L. monocytogenes*. However, antimicrobial effects by EDTA itself seemed to be the major contributor to the listericidal activity of OSE. Antibacterial activity of the combinations consisting of EDTA and OS, which were shown to be bacteriostatic, was likely to be ascribed to the additive interactions rather than synergistic effect.

**Cell Number Effect**

The OS treatment to BHI broth media inoculated with $10^4$ and $10^5$ cfu/mL produced a 1-log increase from the initial cell number during 35°C incubation for 48 h. However, the viable cells in BHI broth culture inoculated with $10^6$ cells and treated with OS did not indicate any increase (Figure 5). The BHI broth culture added with OSL2 and inoculated with $10^4$, $10^5$, or $10^6$ cfu/mL of *L. monocytogenes* resulted in 3.3, 3.7, or 4.5 log cfu/mL of viable cells, respectively, after 48 h of incubation at 35°C. The BHI broth inoculated with $10^4$, $10^5$, or $10^6$ cfu/mL of *L. monocytogenes* cells and OSE1 showed 3.1, 4.0, or 4.4 log cfu/mL of viable cells, respectively (Figure 5). These results demonstrate that OSL2 or OSE1 had listericidal activity against *L. monocytogenes*, and the number of initial cells existing in the media affected the antibacterial activity of OSL2 and OSE1 ($P < 0.05$). However, the influence by initial cell population on antimicrobial actions of ovotransferrin against *L. monocytogenes* was not overwhelming.

**Ovotransferrin Application on Commercial Hams**

*Listeria monocytogenes* with $10^4$ initial cells grew slowly in commercial hams to $10^2$ cfu/mL during storage at 10°C for 29 d. In contrast to in vitro test, 1OSL, 1OSLE, 2OSL, and 2OSLE did not show any antimicrobial activity.
against *L. monocytogenes*. No significant difference between control and all treatments were detected (Table 2). This suggested that although OSL2 and OSE1 showed clear bactericidal effect against *L. monocytogenes* in model systems, they did not have antibacterial activities against *L. monocytogenes* in commercial hams during storage.

Antibacterial activities of some antimicrobials shown in buffer or laboratory media sometimes can be modulated in nutrient media or in the presence of cations and salts, which are capable of supporting the growth of microorganisms (Bellamy et al., 1992; Jones et al., 1994; Branten and Davidson, 2000). Murdock et al. (2007) reported that the inhibitory activities of lactoferrin against *L. monocytogenes* could be eliminated or reduced in some media like trypticase soy broth, because they provided many divalent cations that might affect the antibacterial activity of lactoferrin. Exogenous Ca$^{2+}$ and Mg$^{2+}$ may block the biological activity of lactoferrin by forming lactoferrin

---

**Figure 4.** Antibacterial activity of ovotransferrin (20 mg/mL) combined with 100 mM NaHCO$_3$ or EDTA (1 mg/mL), or both, against the growth of *Listeria monocytogenes* in brain heart infusion broth culture during 35°C incubation for 48 h. **Different letters show that the means are significantly different (**P** < 0.05, n = 4). C = control, only 10$^4$ cells of *L. monocytogenes*; E1 = 1 mg/mL of EDTA; E2 = 2.0 mg/mL of EDTA; OS = 20 mg/mL of ovotransferrin + 100 mM NaHCO$_3$; OSE1 = OS + E1; OSE2 = OS + E2.

**Figure 5.** Effect of initial cell population on the antibacterial activity of ovotransferrin (20 mg/mL) solution combined with 100 mM NaHCO$_3$ and EDTA (2 mg/mL) or lysozyme (1 mg/mL) against *Listeria monocytogenes* in brain heart infusion broth culture during 35°C incubation for 48 h. **Different letters show that the means are significantly different (**P** < 0.05, n = 4). A = 10$^4$ cfu/mL of BHI broth; B = 10$^5$ cfu/mL of BHI broth; C = 1$^6$ cfu/mL of BHI broth; OS = 20 mg/mL of ovotransferrin + 100 mM NaHCO$_3$; OSL2 = OS + 2 mg/mL of lysozyme; OSE1 = OS + 2 mg/mL of lysozyme + 1 mg/mL of EDTA.
concentrations of Ca²⁺ and Mg²⁺ or inhibiting binding to tetramers, increasing iron strength in the presence of high antibacterial effect against be diluted in a certain part of the product so that low the surface of hams, and the concentration was likely to upon dose of ovotransferrin. In the present study, 1 mL of 20 to 30 mg/mL of ovotransferrin was distributed on antimicrobial activity of ovotransferrin is dependent ovotransferrin on the surface of meat products. Generally, to the products, state of strains in inocula, or stability of distribution problems or dilution effects of ovotransferrin bials may be recovered more easily in food systems than rich environment, injured microorganisms by antimicrobial action of ovotransferrin was not significant. Contrary to lysozyme at 2.0, 2.5, or 3.0 mg/mL did not show significant effect on antibacterial activity of OS toward L. monocytogenes. It was found that EDTA alone at 1 mg/mL was bacteriostatic against L. monocytogenes, and OSE1 was bactericidal. However, it was difficult to determine whether EDTA had a synergistic effect on ovotransferrin, because EDTA itself had a significant antibacterial activity against L. monocytogenes in BHI broth culture. The initial number of cells in media affected the antibacterial activity of OSE or OSL toward L. monocytogenes, but the effect of cell population on antimicrobial action of ovotransferrin was not significant. Contrary to in vitro test, OSL, OSE1, L, and LE did not show any antimicrobial activities in commercial hams during incubation at 10°C for 29 d. There was apparent difference in antibacterial activities of ovotransferrin between model systems and meat products. This study suggested that OSL2.5 to 3.0 had a great potential as a natural antimicrobial agent to control L. monocytogenes in vitro, but further studies are needed to improve the antimicrobial activity of ovotransferrin in food products.

Table 2. Number of viable cells of Listeria monocytogenes (log10 cfu/mL) on commercial hams treated with or without ovotransferrin in 100 mM NaHCO₃ (OS) or lysozyme (2 mg/mL), or both, or NaHCO₃ or EDTA (1 mg/mL), or both, during 10°C storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>22</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.5ᵃ ± 0.02</td>
<td>4.6ᵃ ± 0.2</td>
<td>5.0ᵃ± 0.0</td>
<td>5.3ᵃ± 0.4</td>
<td>5.8ᵃ± 0.0</td>
<td>5.4ᵃ± 0.2</td>
<td>5.3ᵃ± 0.4</td>
</tr>
<tr>
<td>1OSL</td>
<td>4.3ᵃ ± 0.1</td>
<td>4.7ᵃ± 0.3</td>
<td>5.1ᵃ ± 0.1</td>
<td>5.5ᵃ± 0.1</td>
<td>5.6ᵃ± 0.4</td>
<td>5.5ᵃ± 0.9</td>
<td>5.2ᵃ± 0.3</td>
</tr>
<tr>
<td>1OSLE</td>
<td>4.4ᵃ ± 0.1</td>
<td>4.6ᵃ± 0.0</td>
<td>5.0ᵃ± 0.0</td>
<td>5.1ᵃ± 0.2</td>
<td>5.9ᵃ± 0.3</td>
<td>5.6ᵃ± 0.2</td>
<td>6.0ᵃ± 0.4</td>
</tr>
<tr>
<td>2OSL</td>
<td>4.4ᵃ± 0.0</td>
<td>4.6ᵃ± 0.0</td>
<td>5.0ᵃ± 0.0</td>
<td>5.2ᵃ± 0.2</td>
<td>5.2ᵃ± 0.1</td>
<td>5.5ᵃ± 0.0</td>
<td>5.4ᵃ± 0.6</td>
</tr>
<tr>
<td>2OSLE</td>
<td>4.3ᵃ± 0.1</td>
<td>4.6ᵃ± 0.1</td>
<td>4.9ᵃ± 0.2</td>
<td>5.1ᵃ± 0.1</td>
<td>5.6ᵃ± 0.2</td>
<td>5.7ᵃ± 0.3</td>
<td>5.4ᵃ± 0.2</td>
</tr>
<tr>
<td>L</td>
<td>4.3ᵃ± 0.0</td>
<td>4.5ᵃ± 0.1</td>
<td>4.9ᵃ± 0.2</td>
<td>5.1ᵃ± 0.3</td>
<td>5.4ᵃ± 0.3</td>
<td>5.0ᵃ± 1.0</td>
<td>5.4ᵃ± 0.5</td>
</tr>
<tr>
<td>LE</td>
<td>4.4ᵃ± 0.2</td>
<td>4.5ᵃ± 0.1</td>
<td>5.0ᵃ± 0.1</td>
<td>5.1ᵃ± 0.1</td>
<td>5.5ᵃ± 0.5</td>
<td>5.6ᵃ± 0.3</td>
<td>6.2ᵃ± 0.6</td>
</tr>
</tbody>
</table>

ᵃMeans within a column with no common superscript differ (P < 0.05, n = 3).
ᵇMeans within a column with no common superscript differ (P < 0.05, n = 3).
ᶜControl = only L. monocytogenes inoculated; 1OSL = 20 mg/mL of ovotransferrin + 100 mM NaHCO₃ + 2 mg/mL of lysozyme; 1OSLE = 1OSL + 1 mg/mL of EDTA; 2OSL = 30 mg/mL of ovotransferrin + 100 mM NaHCO₃ + 2 mg/mL of lysozyme; 2OSLE = 2OSL + 1 mg/mL of EDTA; L = 2 mg/mL of lysozyme; LE = 2 mg/mL of lysozyme + 1 mg/mL of EDTA.
ᵈMean ± standard deviation.
REFERENCES


Payne, K. D., S. P. Oliver, and P. M. Davidson. 1994. Comparison of EDTA and apo-lactoferrin with lysozyme on the growth