

EDTA and Lysozyme Improves Antimicrobial Activities of Ovotransferrin against *Escherichia coli* O157:H7

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Summary and Implications

The aim of this study was to evaluate the effect of ethylenediaminetetraacetic acid (EDTA) or/and lysozyme on the antibacterial activity of ovotransferrin against *E. coli* O157:H7. Ovotransferrin solution (20 mg/ml) containing 100 mM-NaHCO₃ (OS) was added with EDTA (2.0 or 2.5 mg/ml), lysozyme (1.0, 1.5, or 2.0 mg/ml) or both were prepared. Antibacterial activities of OS (20 mg/ml ovotransferrin + 100 mM-NaHCO₃), OSE (OS+ EDTA), or OSL (OS + lysozyme) against *E. coli* O157:H7 in model systems were investigated by turbidity and viability tests. Also, OSE, OSL or OSEL (OS + EDTA + lysozyme) was applied on irradiated pork chops and commercial hams to determine if the solutions have antibacterial activity on meat products. The effect of initial cell population on the antibacterial activity of ovotransferrin and EDTA or lysozyme combinations was also determined. EDTA at 2 mg/ml plus OS (OSE2) induced 3 ~ 4 log reduction in viable *E. coli* O157:H7 cells in brain heart infusion (BHI) broth media, and 1 mg/ml lysozyme plus OS (OSL1) resulted in 0.5 ~ 1.0 log reduction during 35 °C incubation for 36 hr. However, OSE or OSEL did not show significant antibacterial effect on pork chops and hams during storage at 10 °C. The initial cell number in media did not affect the antibacterial activity of OSE or OSEL against *E. coli* O157:H7. This study demonstrates that combinations of ovotransferrin, NaHCO₃, and EDTA (OSE) have potential to control *E. coli* O157:H7.

Introduction

Natural antimicrobials that are environmentally friendly, medically acceptable, and highly effective and economical to manufacture have gained attention due to consumer desires for natural food products. Ovotransferrin is a major contributor to the egg's defense against microbial infection and rotting. Initially, the iron binding capability of ovotransferrin, which limits the availability of iron required for microbial growth, was considered as the major antibacterial mechanism of ovotransferrin. However, subsequent studies have suggested that the antimicrobial action of ovotransferrin could be intimately related to direct interactions of ovotransferrin with bacterial surface membrane, which

result in damage of outer membranes of microorganisms or destroy microbial function such as proton motive force.

E. coli O157:H7 possesses specific cell-surface appendages that facilitate their adhesion to host-tissue-matrix components so that it can tightly attach to beef tissues. In addition, illness caused by *E. coli* O157:H7 are frequently linked to cattle and their products such as undercooked ground beef and raw milk.

The combinations of antimicrobials with different mechanisms can increase the antibacterial effectiveness. Ethylenediaminetetraacetic acid (EDTA) has been used in a number of food products as a chelating agent to prevent oxidation and deteriorative reactions, which are catalyzed by metal ions. Also, EDTA is known to enhance the effectiveness of antimicrobials and antibiotics, especially against gram-negative bacteria. EDTA destabilizes outer membrane of gram-negative bacteria by chelating Ca²⁺ and Mg²⁺ salts that function as bridges between lipopolysaccharides (LPS) of microbial outer membrane, and thus results in the release of LPS from gram-negative bacteria.

Lysozyme catalyzes the hydrolysis of 1,4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in cell wall peptidoglycan. As the cell walls of gram-negative bacteria are protected by an outer membrane, gram-negative microorganisms are relatively resistant to antimicrobial activity of lysozyme, thus the application of lysozyme in foods has been limited. However, lysozyme gained considerable interest for use in food systems because it is a natural enzyme produced by many animals and its activity targets a specific cellular structure of microorganism.

The aim of the present study was to promote the antimicrobial activity of OS against *E. coli* O157:H7 through combining EDTA and/or lysozyme and to identify the possibility about application of the combinations, which are comprised of EDTA or/and lysozyme, ovotransferrin, and 100 mM-NaHCO₃ on commercial hams and irradiated pork chops to control the growth of *E. coli* O157:H7.

Material and Methods

Apo-ovotransferrin (iron-free) used in this study was prepared by the method of Ko and Ahn (2008). Five different strains of *E. coli* O157:H7 (ATCC 43890, C467, FRIK 125, ATCC 43895, and 93-062) were used. The antimicrobial capacities of ovotransferrin solutions combined with EDTA or lysozyme against *E. coli* O157:H7 were determined by measuring the turbidity. The antibacterial activity of ovotransferrin plus either EDTA or lysozyme was investigated using a viability test.

Fresh boneless pork loin chops were purchased, sliced, vacuum-packaged in low oxygen-permeable bags and irradiated at 5 kGy to kill bacteria, which may exist in the pork chop. Each package was aseptically opened and inoculated 10^6 CFU/ml of *E. coli* O157:H7 cocktail stock suspension (0.2 ml) on the surface of each pork chop.

Commercial hams were purchased, sliced and inoculated aseptically on the surface of sliced ham to make 10^3 CFU/cm². The number of surviving bacteria was enumerated using the same method as in the pork chop study after incubating for 0, 3, 8, and 13 days.

Results and Discussion

E. coli O157:H7 in BHI broth multiplied rapidly after 6 hr incubation and entered the stationary phase after 10 hr incubation at 35 °C. *E. coli* O157:H7 in the media containing 2 mg/ml or 2.5 mg/ml EDTA alone showed a lag time for 10 hr and entered stationary phase after 18 hr of incubation (Figure. 1), indicating that *E. coli* O157:H7 strains to have resistance to 2 mg/ml or 2.5 mg/ml EDTA. OS, OSE2 (OS + 2 mg/ml EDTA) and OSE2.5 (OS + 2.5 mg/ml EDTA) treatments had ≤ 0.1 of optical density at 620 nm (Figure. 1) indicating that these treatments inhibited the growth of *E. coli* O157:H7 during 35 °C incubation. The number of survivors in 2 mg/ml EDTA alone was similar to that of control. However, when 2 mg/ml EDTA was added to OS, the antibacterial activity of OS was found to increase significantly, indicating to be bactericidal against *E. coli* O157:H7 (Figure. 3). In conclusion, even though OS was bacteriostatic against *E. coli* O157:H7, the combination comprised of ovotransferrin, NaHCO₃, and EDTA (OSE2) resulted in 3 ~ 4 log reduction of *E. coli* O157:H7. Therefore, OSE2 has a potential as an antibacterial agent for controlling *E. coli* O157:H7.

In turbidity test, lysozyme itself at 1.0, 1.5, and 2.0 mg/ml could not inhibit the growth of *E. coli* O157:H7. When both 1 mg/ml or 2 mg/ml lysozyme were combined with OS, however, the combinations (OSL group) had antibacterial activity against *E. coli* O157:H7 (Figure 2). Even though the combination (OSL1= OS + 1mg/ml of lysozyme) was bacteriostatic against *E. coli* O157:H7, indicating that the antimicrobial activity of OSL1 was due to OS. Therefore, 1 mg/ml of lysozyme did not affect the antimicrobial activity of OS against *E. coli* O157:H7 significantly. It can be assumed that use of several strains, as in this study, might reduce the lysozyme effect on antibacterial activity of ovotransferrin by inducing rapid repairing of some strains.

After 24 h incubation at 35 °C, OS did not restrain completely the growth of *E. coli* O157:H7. Also, the antibacterial action of OS against *E. coli* O157:H7 indicated to be more effective in 10^4 CFU/ml of initial cell number than 10^6 CFU/ml. However, antibacterial activities of OSE and OSEL, which were bactericidal against *E. coli* O157:H7, were not affected by the number

of initial bacteria (Figure. 4). In addition, combinations (EL) consisting of EDTA (2 mg/ml) and lysozyme (1 mg/ml) could not inhibit the growth of *E. coli* O157:H7 in BHI broth media when inoculated with 10^5 CFU/ml and 10^6 CFU/ml cells initially. When EL was combined with OS (OSEL), the solution exhibited a strong anti-bactericidal activity against *E. coli* O157:H7 in BHI broth by a reduction around 1 log CFU/ml.

E. coli O157:H7 inoculated at 10^4 CFU/cm² grew on the pork chop and increased to 10^7 CFU/ml after 10 °C storage for 12 days. Also, 20 mg/ml OS plus 2 mg/ml EDTA (IOSE), 20 mg/ml OS combined with 2 mg/ml EDTA and 1 mg/ml lysozyme (IOSEL), 30 mg/ml OS plus 2 mg/ml EDTA (2OSE) or 30 mg/ml OS combined with 2 mg/ml EDTA and 1 mg/ml lysozyme (2OSEL) did not show any antimicrobial activities (Table 1). *E. coli* O157:H7 grew better in pork chops, which contain more drip by defrosting than commercial hams, whereas viable *E. coli* O157:H7 cells inoculated on hams seemed to decrease slowly unlike the pork chops. Also, IOSE, IOSEL, 2OSE or 2OSEL treated on hams did not show any antibacterial activity against *E. coli* O157:H7 and there was no significant difference in the number of survivors between control and other treatments in both pork chops and commercial hams ($p < 0.05$) (Table 2).

In contrast to the *in vitro* tests, lack of antibacterial activity of ovotransferrin in pork chops and commercial hams may be due mainly to divalent ions such as Ca²⁺ and Mg²⁺, which are assumed to exist on hams and pork chops. The results obtained from application of antimicrobials to foods or products are found to be significantly different from those of the model system. Generally, food products provide a nutrient-rich environment so that microorganisms injured by certain antimicrobials are considered to be recovered more easily in the systems than under cell starvation conditions or laboratory media. Also, distribution or dilution effect of ovotransferrin on the products could be another explanation for the low antibacterial activity of ovotransferrin in pork chops and hams. Even though in the present study 20 ~ 30 mg/ml of ovotransferrin was distributed on the surface of pork chops and hams, the concentration was likely to be diluted on the surface of the products. In addition, significant amount of drip formed during the defrosting process in pork chops might prevent the complete incorporation of ovotransferrin on the surface of the pork chops. Therefore, this study demonstrates that antimicrobial action by ovotransferrin in pork chop and hams seemed to be modulated due to assumed reasons described above. And, it is necessary to overcome some limits and approaches to various study required for application of ovotransferrin on meat or meat products.

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Table 1. Changes of the number of *E. coli* O157:H7 survivors on e-beam irradiated pork chops treated with or without ovotransferrin solutions contained 100 mM NaHCO₃ plus either EDTA (2 mg/ml) or/and lysozyme (1 mg/ml) during 10 °C storage.

| Treatments | Number of viable cells (log ₁₀ CFU/ml) | | | | |
|------------|---|------------------------|------------------------|------------------------|------------------------|
| | Storage period (days) | | | | |
| | 0 | 2 | 4 | 8 | 12 |
| Control | 4.1 ^a ± 0.1 | 4.2 ^a ± 0.1 | 5.0 ^a ± 0.1 | 5.2 ^a ± 0.1 | 7.3 ^a ± 0.1 |
| 1OSE | 4.1 ^a ± 0.1 | 4.5 ^a ± 0.2 | 4.7 ^a ± 0.6 | 6.0 ^a ± 1.5 | 7.2 ^a ± 0.7 |
| 1OSEL | 4.2 ^a ± 0.1 | 4.0 ^a ± 0.2 | 5.0 ^a ± 0.8 | 5.9 ^a ± 0.7 | 7.2 ^a ± 1.3 |
| 2OSE | 4.2 ^a ± 0.0 | 4.3 ^a ± 0.6 | 5.4 ^a ± 0.6 | 6.2 ^a ± 0.3 | 6.9 ^a ± 1.0 |
| 2OSEL | 4.0 ^a ± 0.2 | 4.2 ^a ± 0.1 | 5.9 ^a ± 0.4 | 5.9 ^a ± 1.2 | 7.0 ^a ± 1.3 |

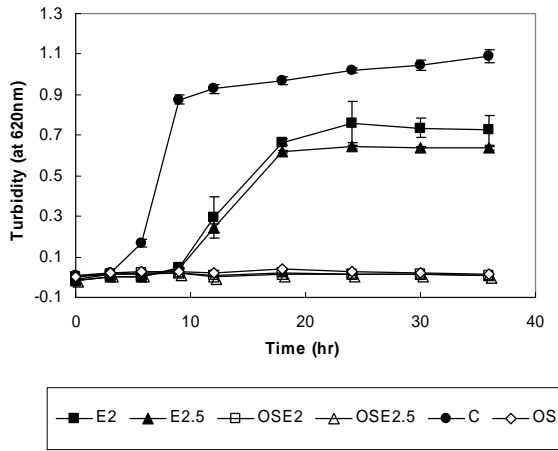
^aValues with different superscripts within a column are significantly different (p < 0.05). n = ; Control: Only *E. coli* O157:H7; 1OS or 2OS: Ovotransferrin (20 mg/ml = 1 or 30 mg/ml = 2) + 100 mM-NaHCO₃; 1OSE: 1OS + 2 mg/ml EDT; 1OSEL: 1OS + 2 mg/ml EDTA + 1 mg/ml lysozyme; 2OSE: 2OS + 2 mg/ml EDT; 2OSEL: 2OS + 2 mg/ml EDTA + 1 mg/ml lysozyme

Table 2. Survivors of *E. coli* O157:H7 in commercial hams treated with or without ovotransferrin solutions contained 100 mM-NaHCO₃ plus EDTA or/and lysozyme during storage at 10 °C.

| Treatment | Number of viable cells (log ₁₀ CFU/g) | | | |
|-----------|--|------------------------|------------------------|------------------------|
| | Storage period (days) | | | |
| | 0 | 3 | 8 | 13 |
| Control | 3.7 ^a ± 0.0 | 3.8 ^a ± 0.2 | 3.3 ^a ± 0.4 | 2.8 ^a ± 0.1 |
| 1OSE | 3.5 ^a ± 0.0 | 3.5 ^a ± 0.1 | 3.6 ^a ± 0.1 | 2.9 ^a ± 0.2 |
| 1OSEL | 3.5 ^a ± 0.1 | 3.5 ^a ± 0.3 | 3.2 ^a ± 0.3 | 3.2 ^a ± 0.3 |
| 2OSE | 3.4 ^a ± 0.1 | 3.5 ^a ± 0.1 | 3.7 ^a ± 0.3 | 2.7 ^a ± 0.2 |
| 2OSEL | 3.3 ^a ± 0.2 | 3.5 ^a ± 0.3 | 3.1 ^a ± 0.5 | 2.7 ^a ± 0.2 |

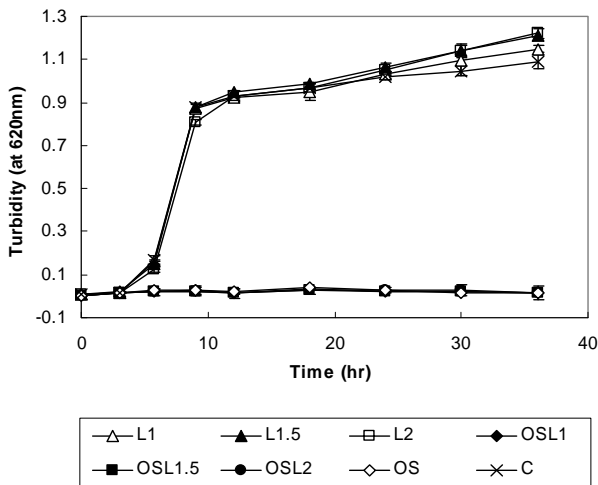
^aValues with different superscripts within a column are significantly different (p < 0.05). n = 3
Control: Only *E. coli* O157:H7; 1OS or 2OS: Ovotransferrin (20 mg/ml = 1 or 30 mg/ml = 2) + 100 mM-NaHCO₃; 1OSE: 1OS + 2 mg/ml EDT; 1OSEL: 1OS + 2 mg/ml EDTA + 1 mg/ml lysozyme; 2OSE: 2OS + 2 mg/ml EDTA; 2OSEL: 2OS + 2 mg/ml EDTA + 1 mg/ml lysozyme

Figure 1. Turbidity of BHI broth cultures inoculated *E. coli* O157:H7 and ovotransferrin (20 mg/ml) solution combined NaHCO₃ or/and EDTA during 35 °C incubation.



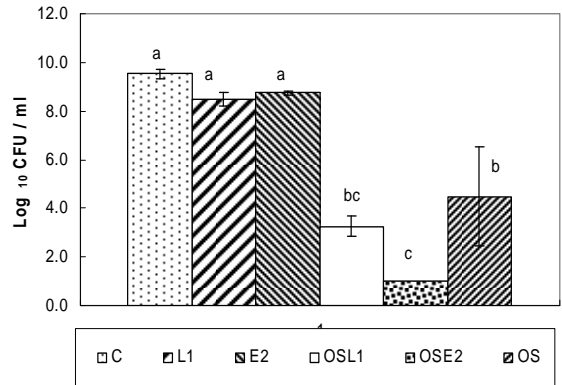
C: control, only 10⁴ CFU/ml of *E. coli* O157:H7; E2: 2 mg/ml EDT; E2.5: 2.5 mg/ml EDT; OS: 20 mg/ml ovotransferrin + 100 mM-NaHCO₃; OSE2: OS + 2 mg/ml EDT; OSE2.5: OS + 2.5 mg/ml EDTA.

Figure 2. Turbidity of BHI broth cultures inoculated *E. coli* O157:H7 and ovotransferrin (20 mg/ml) combining NaHCO₃ or/and lysozyme during 35 °C incubation.



C: control, only 10⁴ CFU/ml of *E. coli* O157:H7; L1: 1 mg/ml Lysozyme; L1.5: 1.5 mg/ml Lysozyme; L2: 2 mg/ml lysozyme; OS: 20 mg/ml ovotransferrin + 100 mM NaHCO₃; OSL1: OS + 1 mg/ml lysozyme; OSL1.5: OS + 1.5 mg/ml lysozyme; OSL2: OS + 2 mg/ml lysozyme

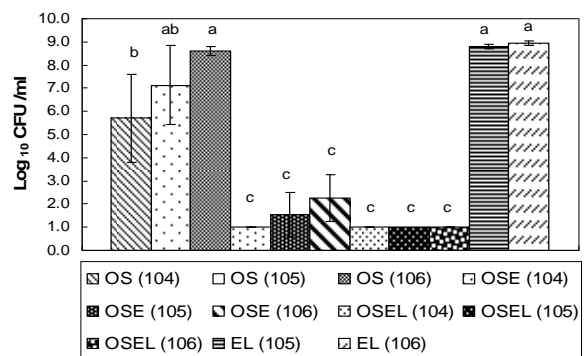
Figure 3. Antibacterial activity of ovotransferrin (20 mg/ml) containing 100 mM NaHCO₃ and lysozyme (1 mg/ml) or EDTA (2 mg/ml) against the growth of *E. coli* O157:H7 in BHI broth culture during 35 °C incubation for 36 h.



^{a-c}Different letters shows that the means are significantly different (P < 0.05). n=3.

C: control, only 10⁴ CFU/ml of *E. coli* O157:H7; L1: 1 mg/ml Lysozyme; E2: 2 mg/ml EDTA
OS: 20 mg/ml ovotransferrin + 100 mM-NaHCO₃; OSL1: OS + 1 mg/ml lysozyme
OSE2: OS + 2 mg/ml EDTA

Figure 4. Effect of initial cell population on antibacterial activity of ovotransferrin (20 mg/ml) combining 100 mM NaHCO₃ and EDTA (2 mg/ml) or Lysozyme (1 mg/ml) against *E. coli* O157:H7 in BHI broth culture during 35 °C incubation for 24 h.



^{a-c}Different letters shows that the means are significantly different (P < 0.05). n=3.

10⁴ : 10⁴ CFU/ ml BHI broth; 10⁵ : 10⁵ CFU/ ml BHI broth; 10⁶ : 10⁶ CFU/ ml BHI broth ; OS: 20 mg/ml ovotransferrin + 100 mM-NaHCO₃ ; OSE: OS + 2 mg/ml EDTA; OSEL: OS + 2 mg/ml of EDTA + 1 mg/ml lysozyme; EL: 2 mg/ml EDTA + 1 mg/ml lysozyme