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# Gallic Acid-*Grafted*-Chitosan Inhibits Foodborne Pathogens by a Membrane Damage Mechanism

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**ABSTRACT:** In this study, antimicrobial activity of gallic acid-*grafted*-chitosans (gallic acid-*g*-chitosans) against five Gram-positive and five Gram-negative foodborne pathogens was evaluated. The minimum inhibitory concentrations (MICs) of gallic acid-*g*-chitosans ranged from 16 to 64 μg/mL against Gram-positive bacteria and ranged from 128 to 512 μg/mL against Gram-negative bacteria. These activities were higher than those of unmodified chitosan. The bactericidal activity of gallic acid-*g*-chitosan (I), which showed the highest antimicrobial activity, was evaluated by time-killing assay with multiples of MIC, and it was recognized to depend on its dose. The integrity of cell membrane, outer membrane (OM), inner membrane (IM) permeabilization experiments, and transmission electron microscopy (TEM) observation were conducted for elucidation of the detailed antimicrobial mode of action of gallic acid-*g*-chitosan. Results showed that treatment of gallic acid-*g*-chitosan (I) quickly increased the release of intracellular components for both *Escherichia coli* and *Staphylococcus aureus*. In addition, gallic acid-*g*-chitosan (I) also rapidly increased the 1-*N*-phenylanphthylamine (NPN) uptake and the release of β-galactosidase via increasing the permeability of OM and IM in *E. coli*. TEM observation demonstrated that gallic acid-*g*-chitosan (I) killed the bacteria via disrupting the cell membrane.

**KEYWORDS:** *Antimicrobial activity, Chitosan, Membrane permeation, Foodborne pathogens*

## 1. INTRODUCTION

Consumption of food contaminated with pathogenic bacteria or their toxins resulting in foodborne illness has been of vital concern to public health.[1] The microbial contamination problem in food industry is of great concern, and controlling pathogens are needed to reduce foodborne outbreaks and to ensure consumers a safe, wholesome, and nutritious food supply. It is well-known that the survival of microorganisms in food can lead to spoilage and can deteriorate the quality of food products or cause infection and illness.[2,3] Thus, food preservation is important in food industry since food products require a longer shelf life and greater assurance of freedom from foodborne pathogenic organisms.[4] Consequently, there is considerable research interest in the development of antimicrobial agents in order to prevent the growth of foodborne pathogens or to delay the onset of food spoilage.[5−7]

Chitosan is a carbohydrate biopolymer derived from deacetylation of chitin, the main component of crustacean exoskeletons. Because of the unique biological properties including biocompatible, biodegradable, and nontoxic nature, chitosan has been developed for biomaterials since it displays a broad spectrum of biological activities such as antioxidant, antitumor, antihypertensive, immuno-modulating, and anti-inflammatory activity.[8−12] In particular, antimicrobial activity of chitosan and its derivatives has been well documented. Chemical, physical, and biological factors including molecular weight, degree of deacetylation, pH, temperature, and species of bacteria affect chitosan's antimicrobial activity.[13] However, the exact mode of action of chitosan is still not fully understood, although several mechanisms have been elucidated for its antimicrobial activity.[14]

Recently, various functionalized chitosans with specific functional groups such as arginine, oleoyl, and aminoethyl were developed and were evaluated on their antimicrobial activity.[7,13,15] Antimicrobial activity of chitosan was improved by conjugating a functional group onto chitosan indicating that the conjugation strategy was thought to be a good method for development of novel chitosan derivatives. In our previous report, gallic acid-*g*-chitosans were developed, and their antioxidant, hepatoprotective, and enzyme inhibition activity were evaluated.[16−18] Biological activities of chitosan were agumented by grafting of gallic acid. Thus, as part of our ongoing investigation of biological activity of chitosan derivatives, antimicrobial activity of gallic acid-*g*-chitosans was investigated against foodborne pathogens as five Gram-positive and five Gram-negative bacteria. In addition, the mode of action of gallic acid-*grafted*-chitosan on model Gram-positive bacteria, *Staphylococcus aureus*, and Gram-negative bacteria, *Escherichia coli*, was evaluated using outer membrane (OM) and inner membrane (IM) permeability assays, cell membrane integrity, bactericidal activity, and transmission electron microscopy.

## 2. MATERIALS AND METHODS

**Materials.** The microorganisms tested for antibacterial activity were obtained from Korean Collection of Type Cultures (KCTC, Daejeon, Korea). *O*-Nitrophenyl-β-D-galactoside (ONPG) and 1-*N*-phenyl- naphthylamine (NPN) were

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Table 1. Minimum Inhibitory Concentrations (MICs) of Gallic Acid-*g*-Chitosans and Unmodified Chitosan

| strain | MIC (μg/mL) | | | | |
|---|---|---|---|---|---|
| | gallic acid-*g*-chitosan (I) | gallic acid-*g*-chitosan (II) | gallic acid-*g*-chitosan (III) | gallic acid-*g*-chitosan (IV) | unmodified chitosan |
| *Staphylococcus aureus* (KCTC 1927) | 32 | 32 | 32 | 32 | 128 |
| *Bacillus subtilis* (KCTC 1028) | 16 | 32 | 32 | 32 | 64 |
| *Bacillus cereus* (KCTC 3624) | 32 | 32 | 64 | 64 | 128 |
| *Enterococcus faecalis* (KCTC 2011) | 16 | 16 | 32 | 32 | 64 |
| *Listeria monocytogenes* (KCTC 3569) | 16 | 32 | 64 | 64 | 128 |
| *Escherichia coli* (KCTC 1682) | 256 | 512 | 512 | 512 | 1024 |
| *Klebsiella pneumoniae* (KCTC 2242) | 128 | 256 | 256 | 256 | 512 |
| *Pseudomonas aeruginosa* (KCTC 1637) | 256 | 256 | 512 | 512 | 512 |
| *Salmonella typhimurium* (KCTC 1925) | 128 | 256 | 256 | 512 | 512 |
| *Shigella flexneri* (KCTC 2998) | 256 | 256 | 512 | 512 | 1024 |

obtained from Sigma Chemical Co. (St. Loius, MO). All other reagents were of the highest grade available commercially.

**Antimicrobial Agents.** Four kinds of gallic acid-*g*-chitosans using different molar ratios of chitosan residue and gallic acid for the antimicrobial assay were prepared from our previous method.[19] To confirm successful synthesis, $^1$H NMR analysis was conducted, and the results were compared to those of Cho et al.[19] Unmodified chitosan: $^1$H NMR (400 MHz, D2O) $\delta$: 5.30 (1H, H-1), 3.63−4.35 (1H, H-2/6), 2.51 (H−Ac), 4.8 ($D_2O$). Gallic acid-*g*-chitosan: $^1$H NMR (400 MHz, $D_2O$) $\delta$: 7.63 (phenyl protons of gallic acid), 5.33 (1H, H-1), 3.65−4.36 (1H, H-2/6), 2.51−2.54 (H−Ac), 4.8 ($D_2O$). The gallic acid contents in the gallic acid-*g*-chitosans were determined by the Folin−Ciocalteau method, and the contents were 118.92 mg gallic acid/g gallic acid-*g*-chitosan for gallic acid-*g*-chitosan (I), 82.91 for gallic acid-*g*-chitosan (II), 67.62 for gallic acid-*g*-chitosan (III), and 53.87 for gallic acid-*g*-chitosan (IV).

**Determination of MIC Values.** Antimicrobial activity of gallic acid-*g*-chitosans against five Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecalis*, and *Listeria monocytogenes*) and five Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Shigella flexneri*) was evaluated using twofold serial broth dilution as follows. Bacteria culture ($10^6$−$10^7$ CFU/mL) grown in 5 mL Mueller Hinton broth (MHB, Difco, MI) which contained 1 mL of antimicrobial agent with various concentrations in 50 mM acetate buffer (pH 5.5) was incubated at 37 °C for 18 h. MIC was defined as the lowest concentration of antimicrobial agent at which the cell growth was not visible with the naked eye.

**Bactericidal Assay.** The bactericidal activity of gallic acid-*g*-chitosan was evaluated with a time-kill experiment. Briefly, gallic acid-*g*-chitosan was added to MHB inoculated with *E. coli* and *S. aureus* strain, which was adjusted to an estimated cell density of approximately $10^5$ CFU/mL, followed by culture at 37 °C for 24 h. The final gallic acid-*g*-chitosan concentrations consisted of the MIC, 2 × MIC, 4 × MIC, and 8 × MIC. Viable cell counts were estimated at various incubation times by the spread plate method. The plates were incubated at 37 °C for 24 h, and the colonies were counted.

**Cell Membrane Integrity.** Bacterial cell membrane integrity was examined by determination of the release of material absorbing at 260 nm.[20] Bacterial cultures grown were harvested by centrifugation at 11 000*g* for 10 min, were washed, and were resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance at 420 nm of 0.7. A 1.5 mL portion of antimicrobial agent solution was mixed with 1.5 mL of bacterial cell suspension, and the release over time of materials absorbing at 260 nm was monitored with UV spectrometer (SpectraMax M2$^e$, CA, United States).

**OM Permeabilization Assay.** OM permeabilization activity of water-soluble chitosans was determined by the NPN assay described by Ibrahim et al.[21] Bacterial cultures grown were harvested by centrifugation at 11 000*g* for 10 min, were washed, and were resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance at 420 nm of 1.0. Twenty microliters of 1 mM NPN was added into 1 mL of bacteria in a quartz cuvette, and background fluorescence was recorded using a SpectraMax M2$^e$, with 1 cm path length cuvettes, at excitation wavelength of 350 nm and emission wavelength of 429 nm. Then, various concentrations of antimicrobial agent were added. An increase in fluorescence because of partitioning of NPN into the OM was recorded as a function of time until there was no further increase in intensity. Control tests were performed to verify that the enhanced fluorescence was due to NPN uptake by bacteria.

**IM Permeabilization Assay.** IM permeabilization was determined by measuring the release of cytoplasmic $\beta$-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate.[21] Logarithmic-phase bacteria grown in nutrient broth containing 2% lactose were harvested, were washed, and were resuspended in 0.5% NaCl solution in order to adjust an absorbance at 420 nm of 1.2. A 200 μL bacterial suspension was pipetted into the wells of a standard microtiter plate, and then 10 μL of ONPG (30 mM) was added to each well. The production of *o*-nitrophenol over time was determined by monitoring the increase in absorbance at 420 nm using a spectrophotometer.

**Transmission Electron Microscopy (TEM).** *E. coli* and *S. aureus* were inoculated into MHB in the absence or presence of the gallic acid-*g*-chitosans and then were further incubated at 37 °C for 18 h. After the incubation, 1 mL aliquots were centrifuged at 11 000 rpm, were washed twice with phosphate-buffered saline (PBS) (pH 7.2), and were fixed with 2.5% glutaraldehyde in 0.1 M PBS. Samples were postfixed with 1% (w/v) $OsO_4$ in 0.1 M PBS for 2 h at room temperature, were washed once with the same buffer, were dehydrated in a graded series of ethanol solutions, and were embedded in Spurr low-viscosity embedding medium. Thin sections of the specimens were cut with a diamond knife on an Ultracut ultramicrotome and were double-stained with uranyl acetate and lead citrate.

## 3. RESULTS AND DISCUSSION

**Antimicrobial Activities of Gallic Acid-*g*-Chitosans.** The antimicrobial activity of chitosan has been well documented, and its activity was influenced by several factors including the type of chitosan, molecular weight, and some of its other physicochemical properties.[22] Recently, chitosan derivatives with specific moiety were developed to improve its antimicrobial activity,[13,23,24] and the results showed that chitosan derivatives exhibited better antimicrobial activity than that of unmodified chitosan. These results indicate that chemical modification is a good strategy for improving antimicrobial activity of chitosan.

Gallic acid is a naturally occurring phenolic compound, which is noteworthy for its antioxidant activity.[25] Currently, antimicrobial activity of gallic acid is reported.[26−28] In this study, therefore, we conjugated gallic acid onto chitosan backbone in order to improve antimicrobial activity of chitosan. For that, different molar ratios were used to conjugate gallic acid onto chitosan; thereby, we produced four kinds of gallic acid-*g*-chitosans bearing different amounts of gallic acid, and it could be expected to show enhanced antimicrobial activities. Ten bacteria strains, including five Gram-positive bacteria and five Gram-negative bacteria, were used to determine the antimicrobial spectrum of gallic acid-*g*-chitosans by twofold serial dilution method as described above. The activity appeared to vary among the tested bacteria. The unmodified chitosan showed the MICs of 64−128 μg/mL against five Gram-positive bacteria and the MICs of 512−1024 μg/mL against five Gram-negative bacteria (Table 1). However, gallic acid-*g*-chitosans showed higher antimicrobial activities than that of unmodified chitosan indicating that the conjugation of gallic acid improved the antimicrobial activity of chitosan. The MICs of four gallic acid-*g*-chitosans ranged from 16 to 64 μg/mL against Gram-positive bacteria and ranged from 128 to 512 μg/mL against Gram-negative bacteria. In addition, gallic acid-*g*-chitosan (I) possessed the highest antimicrobial activities of all the gallic acid-*g*-chitosans. As described above, gallic acid content in gallic acid-*g*-chitosans was in the order of gallic acid-*g*-chitosan (I) > gallic acid-*g*-chitosan (II) > gallic acid-*g*-chitosan (III) > gallic acid-*g*-chitosan (IV). These results indicated that gallic acid content in gallic acid-*g*-chitosans by the conjugation of gallic acid was a crucial role for improvement of antimicrobial activity of chitosan. The antimicrobial activity of gallic acid-*g*-chitosan (I) was 4 times stronger than that of unmodified chitosan. We also tested the antimicrobial activity of gallic acid alone as the same concentration of gallic acid calculated from gallic acid-*g*-chitosan (I), which contains 118.92 mg gallic acid/g gallic acid-*g*-chitosan (I), compared to the MICs of gallic acid-*g*-chitosan (I). However, gallic acid does not show antimicrobial activity against the tested bacteria (data not shown). Taguri et al.[28] reported the MIC values of gallic acid against *Bacillus cereus* (1067 μg/mL), *Bacillus subtilis* (1600 μg/mL), *Listeria monocytogenes* (1600 μg/mL), *Staphylococcus aureus* (533 μg/mL), *Escherichia coli* (600 μg/mL), *Klebsiella pneumoniae* (400 μg/mL), *Shigella flexneri* (267 μg/mL), and *Pseudomonas aeruginosa* (533 μg/mL). In this study, the MIC values of gallic acid-*g*-chitosan (I) ranged from 16 to 32 μg/mL against Gram-positive bacteria and ranged from 128 to 256 μg/mL against Gram-negative bacteria. Considering gallic acid content in these concentrations, gallic acid content in gallic acid-*g*-chitosan (I) was very lower than that of the literature MIC values. Thus, the gallic acid in gallic acid-*g*-chitosans was not solely responsible for the growth inhibition of the tested bacteria, and the synergy effect may exist by the conjugation of gallic acid onto chitosan.

Several researches revealed that polyphenols with gallate such as catechin gallate, epicatechin gallate, and epigallocatechin gallate possess higher affinity to cell membranes than those without gallate.[29,30] In addition, epigallocatechin gallate showed higher antimicrobial activity via damaging the lipid bilayer of liposomes than that of epicatechin, but epicatechin did not damage the lipid bilayer.[31] These results indicate that gallic acid would also have high affinity to the cell membrane. This might be the reason for why the antimicrobial activity of unmodified chitosan was improved by the conjugation of gallic acid. In other words, the affinity of chitosan to the bacterial cell membranes was improved by the conjugation of gallic acid.

In comparison with the MIC values of food antiseptics, gallic acid-*g*-chitosans showed higher antimicrobial activities that those of sorbic acid (6055 μg/mL for *E. coli* and 9262 μg/mL for *S. aureus*), benzoic acid (10 171 μg/mL for *E. coli* and 16 788 μg/mL for *S. aureus*), and propionic acid (15 000 μg/mL for *E. coli* and *S. aureus*).[32,33] This comparison indicates that gallic acid-*g*-chitosans may be useful for a food preservative.

**Bactericidal Assay.** Considering the MIC values of gallic acid-*g*-chitosans, the bactericidal effects of gallic acid-*g*-chitosan (I) against *E. coli* and *S. aureus* were confirmed by a time-kill curve experiment. As shown in Figure 1, *E. coli* ($5.8 \times 10^5$ CFU/mL) suspension without gallic acid-*g*-chitosan (I) was

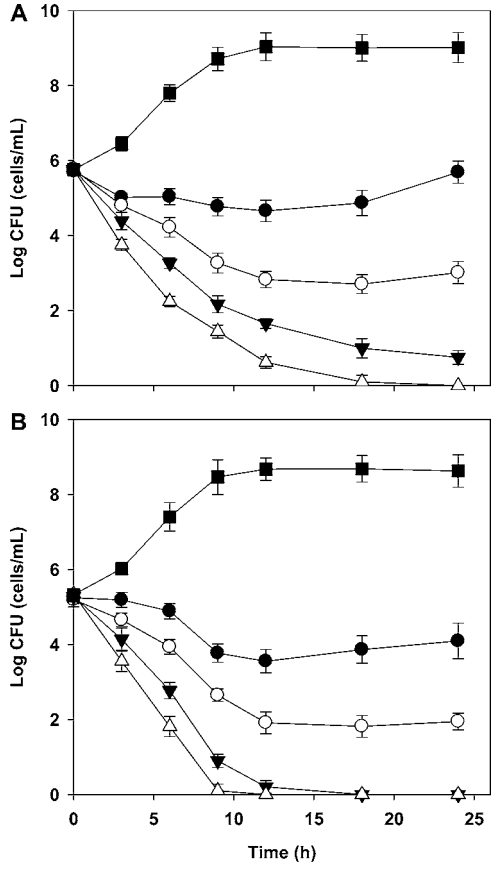


**Figure 1.** Bactericidal activity of gallic acid-*g*-chitosan (I) against (A) *E. coli*. and (B) *S. aureus*. MIC, ●; MIC × 2, ○; MIC × 4, ▼; MIC × 8, Δ; control, ■. The values represent means ± standard deviation (SD) ($n$ = 3).


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significantly increased to $1.4 \times 10^9$ CFU/mL after 24 h. However, the incubation with gallic acid-*g*-chitosan (I) at MIC suppressed bacterial growth for 24 h indicating a bacteriostatic effect. Over the MIC, gallic acid-*g*-chitosan (I) killed the bacteria, and 2.8 log reduction (2 × MIC), 5.0 log reduction (4 × MIC), and no viable cells were observed after being exposed to 8 × MIC for 24 h (Figure 1A). In the case of *S. aureus*, viable cells without gallic acid-*g*-chitosan (I) increased from $2.2 \times 10^5$ CFU/mL to $5.6 \times 10^8$ CFU/mL after 24 h. However, gallic acid-*g*-chitosan (I) treatment decreased viable cells, and 1.2 log reduction (MIC) and 3.4 log reduction (2 × MIC) were observed after 24 h. No viable cells were observed after being exposed to 4 × MIC for 18 h and to 8 × MIC for 12 h (Figure 1B).

**Integrity of Bacterial Cell Membranes.** The cytoplasmic cell membrane is a structural component, which may become damaged and functionally invalid during exposure to antimicrobial agents. Thus, release of intracellular components is a good indicator of membrane integrity. If the bacterial membrane becomes compromised by interaction with antimicrobial agents, first, small ions such as potassium and phosphate tend to leach out followed by large molecules such as DNA, RNA, and other materials. These intracellular components are easily detected by UV at 260 nm as an indication of membrane damage.[20]

As shown in Figure 2, total nucleotide leakage from *E. coli* and *S. aureus* as a function of incubation time with gallic acid-*g*-chitosan (I) was plotted. Upon addition of gallic acid-*g*-chitosan (I) to *E. coli*, there was a dramatic increase in $OD_{260}$ within 30 min, and thereafter, $OD_{260}$ slightly increased up to 90 min. The extent of nucleotide leakage by treatment of gallic acid-*g*-chitosan (I) was in a dose-dependent manner, which is agreeable with the findings for bactericidal activity. In the case of *S. aureus*, the extent of nucleotide leakage by treatment of gallic acid-*g*-chitosan (I) was higher than that of *E. coli*. This was probably due to the differences in cell wall structure and composition, and *S. aureus* does not have the OM to prevent the influx of foreign molecules.[20] These results indicate that gallic acid-*g*-chitosan (I) can bind to membrane components and can cause membrane permeabilization to varying extents.

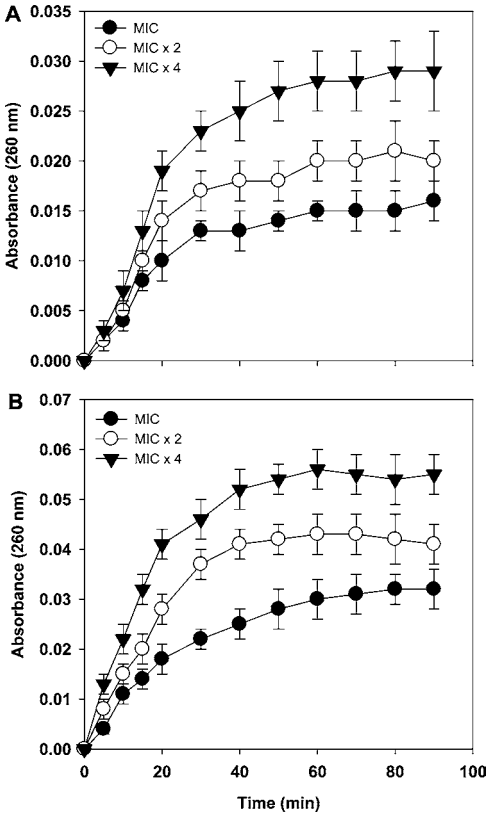


**Figure 2.** The release of intracellular components of (A) *E. coli* and (B) *S. aureus*. The values represent means ± SD ($n$ = 3).

**OM Permeabilization Assay.** Gram-negative bacteria such as *E. coli* have two cell envelope membranes. Thus, we examined the ability of gallic acid-*g*-chitosan (I) to interact with both OM and IM. To determine OM permeabilization, hydrophobic NPN probe was used. NPN is normally excluded from OM. However, when OM was damaged and functionally invalid by interaction with antimicrobial agents, NPN could partition into perturbed OM exhibiting increased fluorescence. Therefore, the increase of NPN fluorescence intensity can be used as an indicator for increased cell membrane permeability.

On the basis of this principle, the change of NPN fluorescence upon the addition of gallic acid-*g*-chitosan (I) is shown in Figure 3. The fluorescence increase by the addition of gallic acid-*g*-chitosan (I) to *E. coli* suspensions was dose- and time-dependent manner; it is indicated that *E. coli* cell membranes were damaged or functionally invalid by gallic acid-*g*-chitosan (I).

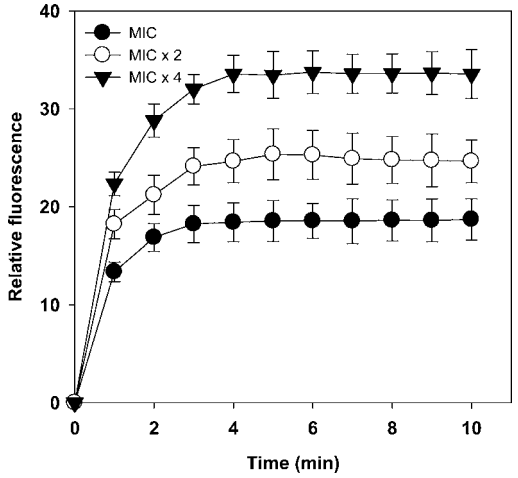


**Figure 3.** The uptake of NPN by *E. coli* suspensions treated with gallic acid-*g*-chitosan (I). The values represent means ± SD ($n$ = 3).

OM contains polyanionic lipopolysaccharide (LPS) stabilized by divalent cations; thus, polycationic antimicrobial agents could bind to the negatively charged O-specific oligosaccharide units of *E. coli* LPS thus disrupting the integrity of OM resulting in loss of the barrier function or blocking the nutrient flow with concomitant bacterial death because of depletion of the nutrients. Chitosan is a polycationic biopolymer because of the primary amines at the C-2 position; this is a major factor affecting antimicrobial activity of chitosan. Moreover, we conjugated gallic acid, which has high affinity to lipid bilayer in the cell membrane, onto chitosan. Thus, gallic acid-*g*-chitosan could interact with LPS and lipid bilayer in the cell membrane; thereby, the antimicrobial activity of gallic acid-*g*-chitosan appeared by disruption of OM.

**IM Permeabilization Assay.** Destabilization of OM is necessary to gain access to IM. Gallic acid-*g*-chitosan (I) could

interact with IM because OM permeation was demonstrated. Thus, we further examined IM permeabilization of *E. coli* as a function of cytoplasmic β-galactosidase release with bacteria grown in lactose-containing medium in order to operate lac operon in *E. coli*. As shown in Figure 4 the control suspensions,

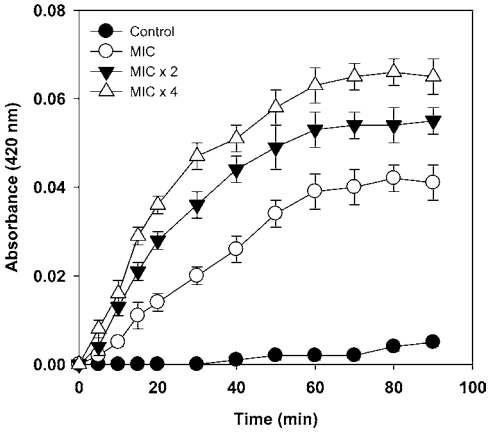


**Figure 4.** The release of cytoplasmic β-galactosidase activity of *E. coli* cells treated with gallic acid-*g*-chitosan (I). The values represent means ± SD ($n$ = 3).

there was a lag of about 40 min before cytoplasmic β-galactosidase was released very slowly, whereas the suspensions with gallic acid-*g*-chitosan (I) showed immediate release of cytoplasmic β-galactosidase followed by a progressive release up to 70 min to reach a steady state. The release of cytoplasmic β-galactosidase in *E. coli* was a dose-dependent manner, which was agreeable with the assay of integrity of cell membranes and OM pemeabilization.

**Transmission Electron Microscopy.** To further elucidate the nature of the killing mechanisms of gallic acid-*g*-chitosan (I), *E. coli* and *S. aureus* were treated with gallic acid-*g*-chitosan (I) for 18 h, and the bacteria were analyzed by TEM. Compared to the control, gallic acid-*g*-chitosan (I) treatment resulted in clear morphological changes (Figure 5 and 6). Untreated cells displayed a smooth and compact surface without release of intracellular components and notable ruptures or pores on the cell surface (Figures 5A and 6A). However, the cells' shape changed compared to those of

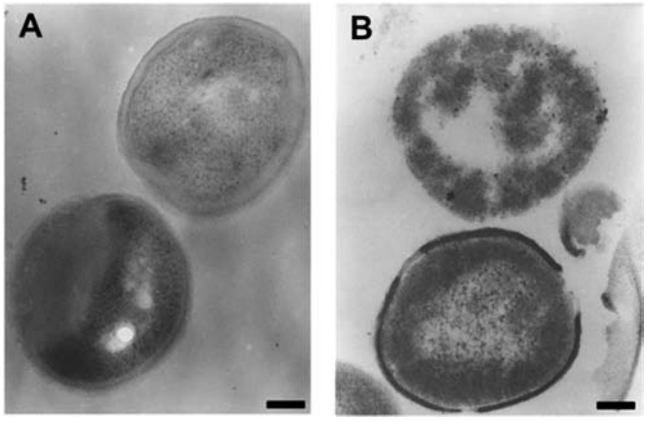


**Figure 5.** TEM of *S. aureus* treated with 128 μg/mL of gallic acid-*g*-chitosan (I) for 18 h. (A) Untreated *S. aureus*; (B) gallic-*g*-chitosan treated *S. aureus*. Scale bar: 100 nm.

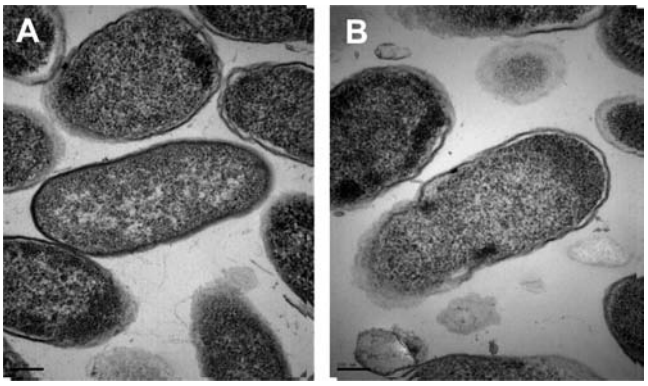


**Figure 6.** TEM of *E. coli* treated with 1024 μg/mL of gallic acid-*g*-chitosan (I) for 18 h. (A) Untreated *E. coli*; (B) gallic-*g*-chitosan treated *E. coli*. Scale bar: 100 nm.

untreated cells. *S. aureus* cells treated with gallic acid-*g*-chitosan (I) appeared to undergo cell membrane damage resulting in the release of their cellular components into the surrounding environment and finally becoming empty (Figure 5B). In the case of *E. coli*, the cell membrane was shrunk and was irregular by treatment of gallic acid-*g*-chitosan (I), and the cellular components were released. These results correlate with the integrity of cell membrane and OM and IM permeabilization.

The present study suggests that gallic acid-*g*-chitosans had good antimicrobial activity against foodborne pathogens. The integrity cell membrane and the OM and IM permeabilization experiments indicated that gallic acid-*g*-chitosans influenced the structure of the membrane and was speculated to interact with lipids on the cell membrane. TEM observations demonstrated the disruption of the cell membrane by gallic acid-*g*-chitosans. Chitosan and gallic acid are regarded as relatively safe; thus, these conjugation products, gallic acid-*g*-chitosans, could be used for food preservatives.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

(1) Kim, J. M.; Marshall, M. R.; Wei, C. Antibacterial Activity of Some Essential Oil Components against 5 Foodborne Pathogens. *J. Agric. Food Chem.* **1995**, *43*, 2839−2845.

(2) Yesil-Celiktas, O.; Kocabas, E. E. H.; Bedir, E.; Sukan, F. V.; Ozek, T.; Baser, K. H. C. Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations. *Food Chem.* **2007**, *100*, 553−559.

(3) Jacob, C.; Mathiasen, L.; Powell, D. Designing effective messages for microbial food safety hazards. *Food Control* **2010**, *21*, 1−6.

(4) Chorianopoulos, N.; Kalpoutzakis, E.; Aligiannis, N.; Mitaku, S.; Nychas, G. J.; Haroutounian, S. A. Essential oils of Satureja, Origanum, and Thymus species: Chemical composition and antibacterial activities against foodborne pathogens. *J. Agric. Food Chem.* **2004**, *52*, 8261−8267.

(5) Rurian-Henares, J. A.; Morales, F. J. Antimicrobial activity of melanoidins against *Escherichia coli* is mediated by a membrane-damage mechanism. *J. Agric. Food Chem.* **2008**, *56*, 2357−62.
(6) Tang, Y. L.; Shi, Y. H.; Zhao, W.; Hao, G.; Le, G. W. Discovery of a novel antimicrobial peptide using membrane binding-based approach. *Food Control* **2009**, *20*, 149−156.
(7) Xing, K.; Chen, X. G.; Liu, C. S.; Cha, D. S.; Park, H. J. Oleoyl-chitosan nanoparticles inhibits *Escherichia coli* and *Staphylococcus aureus* by damaging the cell membrane and putative binding to extracellular or intracellular targets. *Int. J. Food Microbiol.* **2009**, *132*, 127−133.
(8) Park, P. J.; Je, J. Y.; Kim, S. K. Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. *Carbohydr. Polym.* **2004**, *55*, 17−22.
(9) Kim, E. K.; Je, J. Y.; Lee, S. J.; Kim, Y. S.; Hwang, J. W.; Sung, S. H.; Moon, S. H.; Jeon, B. T.; Kim, S. K.; Jeon, Y. J.; Park, P. J. Chitooligosaccharides induce apoptosis in human myeloid leukemia HL-60 cells. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 6136−6138.
(10) Je, J. Y.; Park, P. J.; Kim, B.; Kim, S. K. Antihypertensive activity of chitin derivatives. *Biopolymers* **2006**, *83*, 250−254.
(11) Suzuki, K.; Mikami, T.; Okawa, Y.; Tokoro, A.; Suzuki, S.; Suzuki, M. Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydr. Res.* **1986**, *151*, 403−408.
(12) Cho, Y. S.; Lee, S. H.; Kim, S. K.; Ahn, C. B.; Je, J. Y. Aminoethyl-chitosan inhibits LPS-induced inflammatory mediators, iNOS and COX-2 expression in RAW264.7 mouse macrophages. *Process Biochem.* **2011**, *46*, 465−470.
(13) Tang, H.; Zhang, P.; Kieft, T. L.; Ryan, S. J.; Baker, S. M.; Wiesmann, W. P.; Rogelj, S. Antibacterial action of a novel functionalized chitosan-arginine against Gram-negative bacteria. *Acta Biomater.* **2010**, *6*, 2562−2571.
(14) Rabea, E. I.; Badawy, M. E. T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W. Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules* **2003**, *4*, 1457−1465.
(15) Je, J. Y.; Kim, S. K. Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. *J. Agric. Food Chem.* **2006**, *54*, 6629−6633.
(16) Cho, Y. S.; Kim, S. K.; Je, J. Y. Chitosan gallate as potential antioxidant biomaterial. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3070−3073.
(17) Senevirathne, M.; Jeon, Y. J.; Kim, Y. T.; Park, P. J.; Jung, W. K.; Ahn, C. B.; Je, J. Y. Prevention of oxidative stress in Chang liver cells by gallic acid-*grafted*-chitosans. *Carbohydr. Polym.* **2012**, *87*, 876−880.
(18) Cho, Y. S.; Kim, S. K.; Ahn, C. B.; Je, J. Y. Inhibition of acetylcholinesterase by gallic acid-*grafted*-chitosans. *Carbohydr. Polym.* **2011**, *84*, 690−693.
(19) Cho, Y. S.; Kim, S. K.; Ahn, C. B.; Je, J. Y. Preparation, characterization, and antioxidant properties of gallic acid-*grafted*-chitosans. *Carbohydr. Polym.* **2011**, *83*, 1617−1622.
(20) Chen, C. Z.; Cooper, S. L. Interactions between dendrimer biocides and bacterial membranes. *Biomaterials* **2002**, *23*, 3359−3368.
(21) Ibrahim, H. R.; Sugimoto, Y.; Aoki, T. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochim. Biophys. Acta, Gen. Subj.* **2000**, *1523*, 196−205.
(22) Park, P. J.; Je, J. Y.; Byun, H. G.; Moon, S. H.; Kim, S. K. Antimicrobial activity of hetero-chitosans and their oligosaccharides with different molecular weights. *J. Microbiol. Biotechnol.* **2004**, *14*, 317−323.
(23) Jia, Z. S.; Shen, D. F.; Xu, W. L. Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydr. Res.* **2001**, *333*, 1−6.
(24) Xiao, B.; Wan, Y.; Zhao, M. Q.; Liu, Y. Q.; Zhang, S. M. Preparation and characterization of antimicrobial chitosan-*N*-arginine with different degrees of substitution. *Carbohydr. Polym.* **2011**, *83*, 144−150.
(25) Yilmaz, Y.; Toledo, R. T. Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *J. Agric. Food Chem.* **2004**, *52*, 255−260.
(26) Chanwitheesuk, A.; Teerawutgulrag, A.; Kilburn, J. D.; Rakariyatham, N. Antimicrobial gallic acid from Caesalpinia mimosoides Lamk. *Food Chem.* **2007**, *100*, 1044−1048.
(27) Akiyama, H.; Fujii, K.; Yamasaki, O.; Oono, T.; Iwatsuki, K. Antibacterial action of several tannins against *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **2001**, *48*, 487−491.
(28) Taguri, T.; Tanaka, T.; Kouno, I. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. *Biol. Pharm. Bull.* **2006**, *29*, 2226−2235.
(29) Kajiya, K.; Kumazawa, S.; Nakayama, T. Effects of external factors on the interaction of tea catechins with lipid bilayers. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2330−2335.
(30) Kajiya, K.; Kumazawa, S.; Nakayama, T. Steric effects on interaction of tea catechins with lipid bilayers. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2638−2643.
(31) Ikigai, H.; Nakae, T.; Hara, Y.; Shimamura, T. Bactericidal catechins damage the lipid bilayer. *Biochim. Biophys. Acta* **1993**, *1147*, 132−136.
(32) Eklund, T. The antimicrobial effect of dissociated and undisociated sorbic acid at different pH levels. *J. Appl. Microbiol.* **1983**, *54*, 383−389.
(33) Eklund, T. Inhibition of microbial growth at different pH levels by benzoic and propionic acids and esters of *p*-hydroxybenzoic acid. *Int. J. Food Microbiol.* **1985**, *2*, 159−167.