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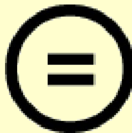

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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Antibacterial characterization of erythorbyl laurate against heat-treated spore of *Geobacillus stearothermophilus*

Geobacillus stearothermophilus 포자에 대한 erythorbyl laurate의 항균 특성 규명

February, 2019

The Graduate School

Seoul National University

Department of Agricultural Biotechnology

Shin, Hyukjin

석사학위논문

Antibacterial characterization of erythorbyl laurate against heat-treated spore of *Geobacillus stearothermophilus*

지도교수 장 판 식

이 논문을 석사학위 논문으로 제출함

2019년 2월

서울대학교 대학원

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신혁진의 석사 학위논문을 인준함

2019년 2월

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Abstract

Erythorbyl laurate (EL), an amphiphilic substance produced by lipase-catalyzed esterification with erythorbic acid and lauric acid, is a multifunctional emulsifier having antioxidant, antibacterial, and emulsifying properties. The bacteriostatic and bactericidal effects of EL against Gram-positive foodborne bacteria have been demonstrated, but its antibacterial activity against thermophilic bacteria and spores, including in an emulsion system, has not. EL is structurally similar to sucrose fatty acid esters and the antibacterial property of EL is also similar to that of sucrose fatty acid esters. Since the antibacterial activity of sucrose fatty ester was confirmed against thermophilic spores, EL was expected to have antibacterial activity.

The antibacterial activity of EL against *Geobacillus stearothermophilus* in aqueous medium and an oil-in-water (O/W) emulsion was evaluated. Antibacterial activity was verified based on dipicolinic acid (DPA) release. The relevance of EL for the food industry was determined in an antibacterial assay in canned milk coffee.

The bactericidal concentrations of EL against vegetative cells were 200 ppm in the aqueous system and 1,500 ppm in the O/W emulsion. EL inhibited heat shocked spore germination at concentrations of 200 ppm in the

aqueous system and 2,000 ppm in the O/W emulsion. Sporicidal effects in the O/W emulsion were achieved with 7,500 ppm. In DPA assay, increasing concentrations of EL decreased DPA release, indicating that germination of heat shocked spore was inhibited. The antibacterial activity of EL in canned milk coffee showed that the number of viable heat shocked spores decreased with increasing EL concentration. Furthermore, the antibacterial effect was maintained for 14 days.

Keywords: erythorbyl laurate, antibacterial properties, O/W emulsion, thermophilic spore, canned milk coffee

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1. Introduction

Spore-forming bacteria survive nutrient starvation, such as the lack of carbon and nitrogen sources for growth, by forming spores that are resistant to heat, chemicals, and radiation. Heat resistance is conferred by calcium–dipicolinic acid (Ca–DPA) complexes, which bind to water inside the spore core, resulting in dehydration and therefore the reduction of heat transfer (Paidhungat, Setlow, Driks, & Setlow, 2000). Most bacteria and spores are killed by 12D (D, decimal reduction time) heat treatment of *Clostridium botulinum*, which is the standard “botulinum cook” for low-acid canned foods in the food industry (Stumbo, Purohit, & Ramakrishnan, 1975). However, thermophilic spores produced by thermophilic bacteria, such as *G. stearothermophilus* and *Moorella thermoacetica*, can survive heat treatment because of their much higher heat resistance than other spores (Brown, 1994).

Germination of the surviving spores occurs during their exposure to temperatures over 43°C and results in the formation of vegetative cells (Ghani, Farid, & Chen, 2002; Ng & Schaffner, 1997). As the average temperature of a heat cabinet is 50–60°C, the optimum temperature range for the growth of *G. stearothermophilus*, canned foods stored under these

conditions will likely become contaminated (Feeherry, Munsey, & Rowley, 1987). The flat-sour spoilage that results from vegetative cell growth causes an off flavor and decreases the pH of the food (Nakayama & Shinya, 1981). Moreover, spoilage is not noticed easily, given the lack of abnormality in the appearance of the cans or their contents, due to the absence of gas production during the growth of vegetative cell of *G. stearothermophilus* (Fields, 1970). Increased treatment temperature or time can kill *G. stearothermophilus* spores, but may also reduce the nutrient content of the food or alter its color, flavor, and texture (Head, Cenkowski, Holley, & Blank, 2008). Therefore, the food industry has used additives such as monolaurin and sucrose fatty acid esters, including sucrose laurate, sucrose palmitate, and sucrose stearate, to control contamination by spores (Bevilacqua, Sinigaglia, & Corbo, 2008; Shearer, Dunne, Sikes, & Hoover, 2000; Yokota, Fujii, & Goto, 2008).

Several emulsion-based foods (*e.g.*, milk coffee, cocoa and soy milk) are stored in heat cabinets or hot vending machines at 50–60°C, which is the optimum temperature for the growth of *G. stearothermophilus* (Sakanaka, Juneja, & Taniguchi, 2000). Therefore, sucrose palmitate, sucrose stearate, and other sucrose fatty acid esters are used widely in these products to inhibit spore germination and flat-sour spoilage (Nakayama, Sonobe, & Shinya, 1982; Tomida, Suwa, Machida, Nishimura, & Makino, 1991). The

antibacterial activity of sucrose fatty acid esters involves pore formation on the cell membrane and a subsequent change in membrane permeability (Zhao, Zhang, Hao, & Li, 2015). As the composition of the spore inner membrane is similar to that of the vegetative cell membrane, sucrose fatty acid esters presumably interact in an essentially identical manner with both bacterial life stages (Popham, 2002; Setlow, 2006).

Erythorbyl laurate (6-*O*-lauroyl-erythorbic acid, EL), an amphiphilic substance produced by the lipase-catalyzed esterification of erythorbic acid and lauric acid, is a multifunctional emulsifier with antioxidant and antibacterial properties (Park, Lee, Jo, Choi, Lee, & Chang, 2017). The bacteriostatic and bactericidal effects of EL against Gram-positive foodborne bacteria, such as *Staphylococcus aureus*, *Listeria monocytogenes,* and *Bacillus cereus*, are confirmed. In these species, EL targets the cell membrane and changes its permeability (Park, Jo, Yu, Park, Choi, Lee, et al., 2018). It is well known that sucrose fatty acid ester could control *G. stearothermophilus* spores in emulsion-based foods (Lamo-Castellví, Ratphtagsanti, & Balasubramaniam, 2013; Shintani, 2006). EL is structurally similar to sucrose fatty acid esters and the antibacterial property of EL is also similar to that of sucrose fatty acid esters. Accordingly, EL is also expected to effectively prevent the germination of *G. stearothermophilus* spores in

emulsion-based foods

Therefore, the objective of this study was to evaluate the antibacterial activity of EL against *G. stearothermophilus* spores and vegetative cells in an aqueous system and an oil-in-water (O/W) emulsion system. A quantitative analysis of DPA released from spores and transmission electron microscopic (TEM) analysis of EL-treated spores were used to demonstrate and confirm the antibacterial activity of EL. The relevance of EL for the food industry was then assessed in an assay involving a commercially available canned milk coffee.

2. Materials and methods

2.1. Materials

Erythorbic acid (≥98.0%) was purchased from Fisher Scientific (Seoul, Korea), and lauric acid (≥99.0 was purchased from Deajung Chemicals & Metals Co., Ltd. (Siheung, Gyonggi-do, Korea). Immobilized lipase from *Candida antarctica* (EC 3.1.1.3, triacylglycerol hydrolase, Novozym® 435) was obtained from Novozymes (Bagsvaerd, Denmark). The catalytic activity of the enzyme was 7,000 PLU/g (PLU, mmol propyl laurate synthesized per min at 60°C). *G. stearothermophilus* ATCC 7953 was purchased from the Korean Culture Center of Microorganisms (Seoul, Korea), and 2,6-Pyridinedicarboxylic acid (DPA, ≥99.0%) and terbium(III) chloride hexahydrate ($TbCl_3$, ≥99.9% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Soybean oil, used without further purification, was purchased from Ottogi (Seoul, Korea). Tween 20 and Tween 80 were provided by Ilshinwells Co. (Cheongju, Korea). Milk coffee was kindly gifted from Dongsuh Foods Co. (Seoul, Korea). All other chemicals used in this study were of analytical grade.

2.2. Preparation of spore suspension

G. stearothermophilus ATCC 7953 was inoculated in 5.0 mL tryptic soy broth (TSB) and incubated at 55°C for 18 h. Two hundred microliters of suspension were cultured in tryptic soy agar at 55°C for 10 days. The bacterial colonies that developed on the agar medium were flooded with 5.0 mL sterilized water, and the surface growth was then gently collected with a loop. The resulting suspension was washed four times with cold distilled water by centrifugation for 15 min at 4°C and 5,000×*g*. The pellets were resuspended in sterilized water and heat shocked at 100°C for 10 min to kill all of the vegetative cells. Spore suspensions with spore concentrations of 1.0×10^7 cells/mL (counted on an agar plate) were stored at 4°C and used in the experiments.

2.3. Preparation of O/W emulsion

The O/W emulsion (5.0%, w/w) was composed of soybean oil, which had no antibacterial activity, emulsifiers (Tween 20 and Tween 80, 1.0–2.0%) and TSB (94.0–95.0%). The three components were mixed thoroughly in a screw-cap vial and then vortexed (WiseMix VM-10, Daihan, Wonju, Korea)

for 2 min. The resulting coarse O/W emulsion was sonicated for 20 min at 20 kHz using an ultrasonicator (ULH-700S, Jeiotech, Daejeon, Korea) and the following cycle settings: 210 W sonication power, 1 s pulse on and 4 s pulse off at 4°C. The suspension of *G. stearothermophilus* heat shocked spores was diluted to 5 mL with the O/W emulsion to yield a final concentration of 10^4 colony-forming units (CFU)/mL. The samples were then shake-incubated for 48 h at 55°C and 220 rpm. During incubation, 200 μL aliquots were removed after 0, 4, and 8 h, diluted serially with distilled water and spread on an agar plate. The number of colonies was counted after incubation of the plates for 24 h at 55°C.

2.4. Antibacterial assay in the aqueous system

G. stearothermophilus in TSB medium was shake-incubated at 55°C and 220 rpm for 18 h. The concentration of the suspension was measured based on the absorbance at 600 nm of the 0.5 McFarland standard, using an ultraviolet–visible spectrophotometer (UV-2075, Jasco, Tokyo, Japan).

To identify antibacterial activity against vegetative cells, EL was diluted in TSB medium to obtain concentrations ranging from 100 to 1,600 ppm. EL

was dispersed in TSB using a 20-kHz ultrasonicator (ULH-700S, Jeiotech) for a total of 2 min at 4°C: each cycle consisted of 1 s pulse on and 4 s pulse off at 210 W sonication power. The samples were then transferred to glass tubes containing 5.0 mL bacterial suspension (10^4 CFU/mL) and shake-incubated at 55°C and 220 rpm for 48 h. During incubation, 200 μL aliquots were removed after 0, 3, 6, 9, 24, and 48 h, diluted serially with distilled water and spread on agar plates. The colonies that developed after incubation at 55°C for 24 h were then counted. To evaluate the antibacterial activity of EL against with heat shocked and without heat shocked spores, the same procedure was repeated, except the bacterial suspension was replaced with a spore suspension.

2.5. Antibacterial assay in the O/W emulsion system

O/W emulsion was prepared as described above. The antibacterial activity of EL against vegetative cells and spores in the emulsion was determined as described above for the aqueous system.

2.6. Quantitative analysis of DPA released from spores

The release of DPA from spores was determined by measuring DPA concentrations in the supernatants of EL-treated and untreated spore suspensions. Tb–DPA fluorescence was monitored using a fluorescence spectrometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 270 nm and an emission wavelength of 543 nm. Specifically, stock solutions of $TbCl_3$ and DPA were prepared in 50 mM Tris-HCl buffer (pH 8.0) to obtain a final concentration of 1 mM each. In the experiments, 600 μL $TbCl_3$ diluted to a final concentration of 100 μM was mixed with 600 μL DPA at concentrations ranging from 2,000 to 20,000 nM. The fluorescence of the samples was measured using a fluorescence spectrometer.

A spore suspension of *G. stearothermophilus* was autoclaved at 121°C for 30 min to release all DPA from the spores. The autoclaved spore suspension was diluted in distilled water to final concentrations of 20–200,000 CFU/mL. Samples containing 600 μL of 100 μM $TbCl_3$ and 600 μL of diluted spore suspension were prepared, and their fluorescence after filtration (pore size, 0.2 μm) was determined using a fluorescence spectrometer.

Heat shocked spore suspension of *G. stearothermophilus* in 60 μL

distilled water was diluted to 600 μL in the O/W emulsion containing various concentrations of EL (final concentrations, 2,000–10,000 ppm) to obtain a final spore concentration of 200,000 CFU/mL. After incubation at 55°C for 48 h, the suspensions were mixed with 100 μM $TbCl_3$, and their fluorescence after filtration (pore size, 0.2 μm) was determined using a fluorescence spectrometer.

2.7. TEM analysis

EL-untreated (negative control) and -treated (4,000 ppm for 24 h at 55°C) heat shocked spore suspensions were harvested by centrifugation at 10,000×*g* for 10 min. The pellets were fixed for 4 h at 4°C in modified Karnovsky's fixative containing 2.0% paraformaldehyde and 2.0% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2), and then washed with 50 mM sodium cacodylate buffer. Post-fixation was performed with 2.0% osmium tetroxide in 100 mM sodium cacodylate buffer for 2 h at 4°C, followed by washing with distilled water at room temperature. After overnight treatment with 0.5% uranyl acetate, the samples were dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, and 100%), treated with 100% propylene oxide and infiltrated with Spurr's resin. The samples were

then dried in a 70°C oven for 24 h and visualized on a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) under standard conditions (Y.-H. Kim & Chung, 2011).

2.8. Antibacterial assay in commercialized canned milk coffee

Coffee medium containing various concentrations of EL (100, 200, 300, 400, and 500 ppm) was prepared by ultrasonic emulsification and then used to prepare a heat shocked spore suspension containing 10^4 CFU/mL. A conical tube containing 5.0 mL heat shocked spore-inoculated coffee medium, with or without EL, was then heated for 0–16 min in an oil bath at 120°C (F_0=8.0), followed by rapid cooling on ice and shake-incubation at 55°C and 220 rpm. The numbers of surviving spores on 7 and 14 days were counted on agar plates.

2.9. Statistical analysis

Statistical analysis was performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Experiments were conducted in triplicate. Differences between mean values were assessed using Duncan's multiple range test. *P*

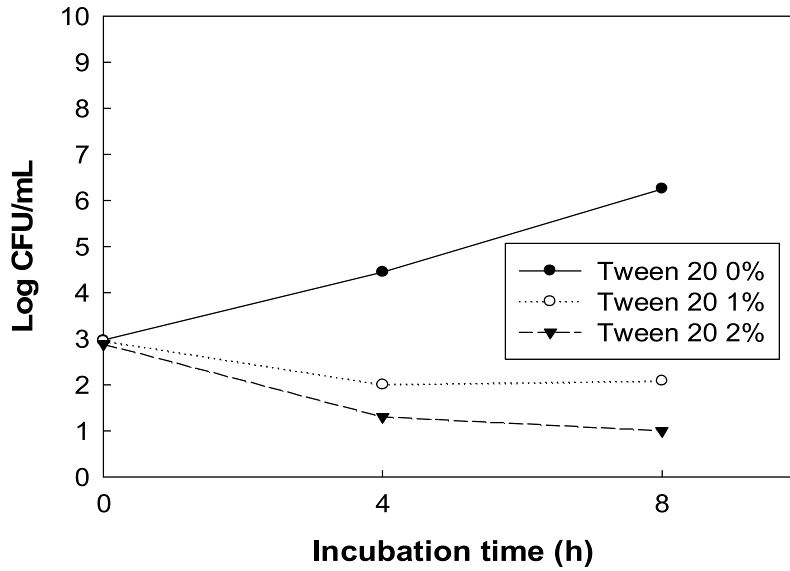
values <0.05 were considered to indicate statistical significance.

3. Results and discussion

3.1. Effects of emulsifiers on heat shocked spores

To evaluate the antibacterial activity of EL, the effect of the supplemented emulsifier on the spores should be minimal. Among the polysorbates used widely as emulsifiers in the food industry, polysorbate 20 (Tween 20) and polysorbate 80 (Tween 80), are suitable for the stabilization of O/W emulsions because the hydrophilic/lipophilic balance value of Tween 20 is 16.7 and that of Tween 80 is 15.0 (Bąk & Podgórska, 2016; Fuller, Considine, MacGibbon, Golding, & Matia-Merino, 2018). In the Tween-20–treated group, 8 h exposure to concentrations of 1.0% and 2.0% decreased the heat shocked spore concentrations. In contrast, treatment with Tween 80 under the same conditions had no sporicidal effect (Fig. 1). Accordingly, 1.0% of Tween 80 was selected as the emulsifier concentration used to stabilize the O/W emulsion. The stability of the O/W emulsion with 1.0% Tween 80 during the experiment was confirmed by measurement of the creaming index (data not shown).

(a)



(b)

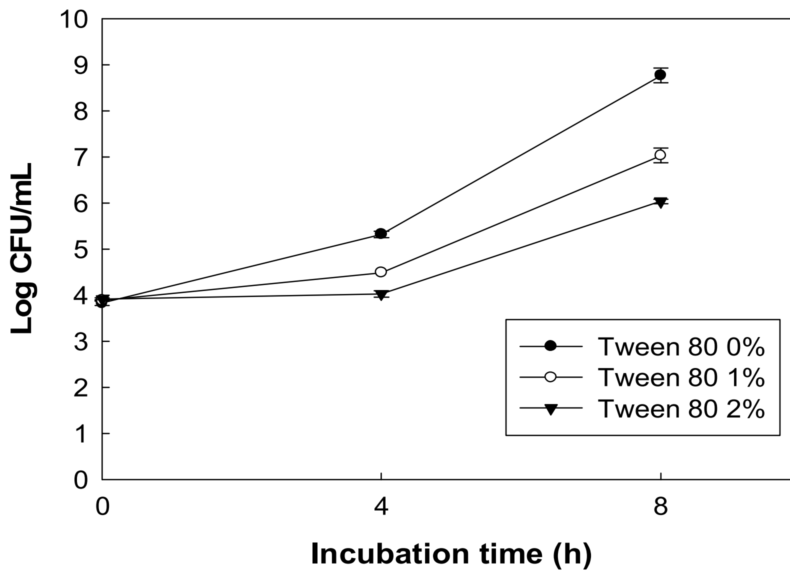


Fig. 1. Antibacterial effect of Tween 20 (a) and Tween 80 (b) on the growth of germinated *Geobacillus stearothermophilus* heat shocked spores.

3.2. Antibacterial activity of EL in the aqueous system

TSB medium contains sufficient levels of nutrients to ensure the growth of thermophilic spores (Ma, Davidson, & Zhong, 2013). Thus, it was suitable for measurement of the antibacterial activity of EL against vegetative cells and heat shocked spores of *G. stearothermophilus* in an aqueous system (Devlieghere, Vermeulen, & Debevere, 2004). The sporulation cycle of *G. stearothermophilus* is shown in Fig. 2.

In the aqueous system, a 3.0-log reduction of vegetative cells was attained after 6 h incubation with 200 ppm EL (Fig. 3), whereas the same concentration did not reduce the number of heat shocked spores (Fig. 4). Spores that do not germinate remain dormant before spreading on agar medium, where they form a single colony and the concentration remains unchanged (Paredes-Sabja, Setlow, & Sarker, 2011). However, in our experiment, if the spores had germinated in the aqueous system, 200 ppm EL would have killed the vegetative cells, thereby reducing the spore concentration. Our results demonstrate that 200 ppm EL had a bactericidal effect on vegetative cells and, as the initial concentration of heat shocked spores was maintained, inhibited heat shocked spore germination in the aqueous system.

The antibacterial activity of EL derives from its targeting of the bacterial cell membrane and alteration of membrane permeability (Park, et al., 2018). As the composition of the spore inner membrane is similar to that of the vegetative cell membrane, the mechanism underlying the effects of EL on vegetative cells and spores was presumably the same (Popham, 2002; Setlow, 2006).

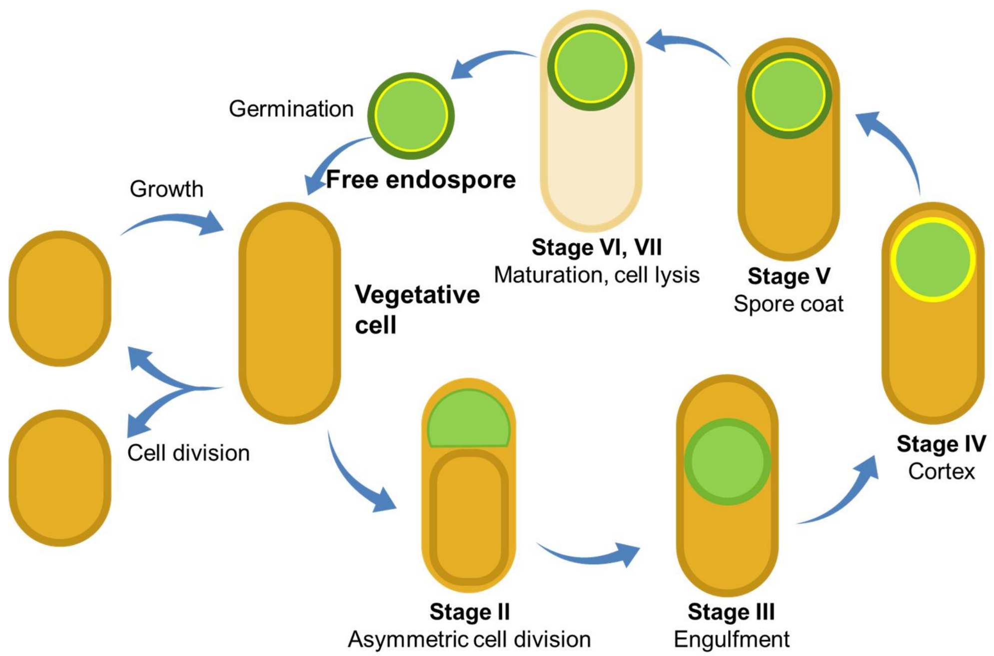


Fig. 2. The sporulation cycle of *Geobacillus stearothermophilus*.

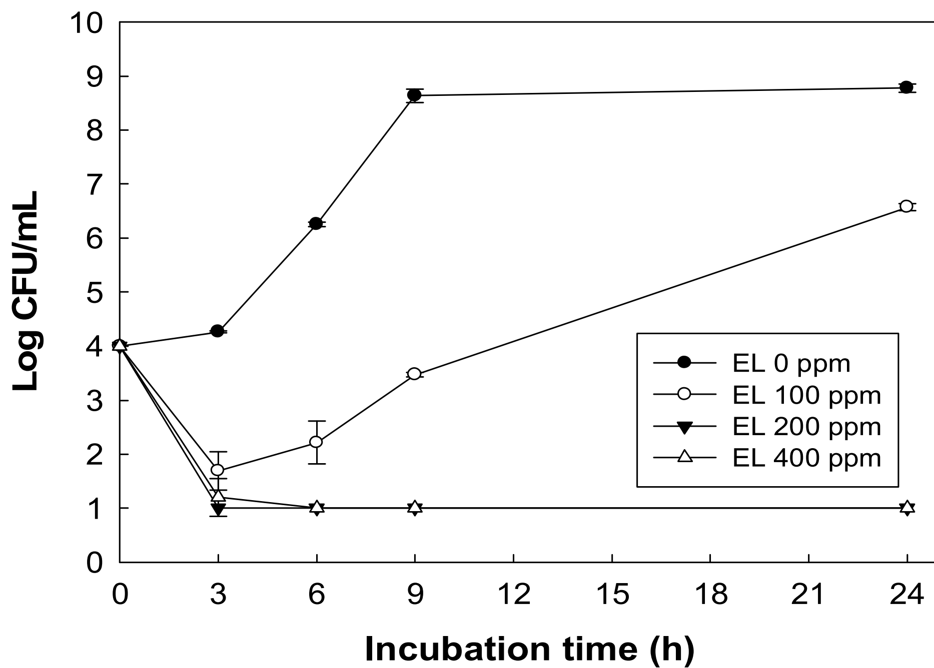


Fig. 3. Growth curves of *Geobacillus stearothermophilus* vegetative cells treated with 100, 200, and 400 ppm erythorbyl laurate (EL) in an aqueous system. The samples were shake-incubated at 55°C and 220 rpm for 24 h, after which the numbers of colonies were counted.

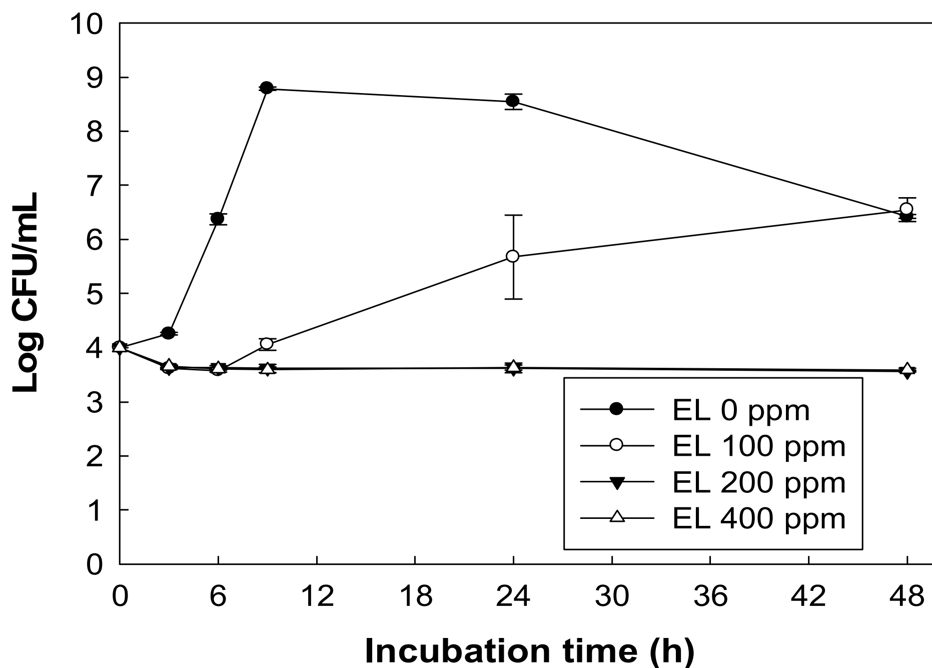


Fig. 4. Growth curves of germinated *Geobacillus stearothermophilus* heat shocked spores treated with 100, 200, and 400 ppm erythorbyl laurate (EL) in an aqueous system. The samples were shake-incubated at 55°C and 220 rpm for 24 h, after which the numbers of colonies were counted.

3.3. Antibacterial activity of EL in the O/W emulsion system

The antibacterial activity of EL in an O/W emulsion may differ from that in an aqueous system, due to differences in the solubility of EL under the respective conditions (Munoz-Bonilla & Fernández-García, 2012). Thus, for potential applications in O/W emulsion–based foods, the antibacterial activity of EL was also tested in an O/W emulsion system (Char, Cisternas, Pérez, & Guerrero, 2016).

In the O/W emulsion, 6 h incubation with 1,500 ppm EL resulted in a 3.0-log reduction in the vegetative cell number (Fig. 5) and 2,000 ppm EL resulted in no reduction in the number of heat shocked spores. However, a 2.7-log reduction in the number of heat shocked spores was achieved with 7,500 ppm EL, and a 3.0-log reduction was achieved with 10,000 ppm EL (Fig. 6). Thus, 1,500 ppm EL in an O/W emulsion system had a bactericidal effect on vegetative cells and inhibited heat shocked spore germination, whereas the effect of EL was sporicidal at concentrations more than 7,500 ppm.

The presence of a sporicidal effect in the O/W emulsion, but not in the aqueous system may have been due to the limitation on the amount of EL that could be added to the latter, where EL concentrations over 1,600 ppm resulted in aggregation of the compound (Rosas-Piñón, Mejía, Díaz-Ruiz,

Aguilar, Sánchez-Nieto, & Rivero-Cruz, 2012). By contrast, in the O/W emulsion system, no such limit existed and a sporicidal effect was observed at high EL concentrations.

The minimum bactericidal concentration of EL was 7.5-fold higher in the O/W emulsion than in the aqueous system. The same phenomenon was observed for the minimum concentration inhibiting heat shocked spore germination, which was 200 ppm in the aqueous system and 2,000 ppm in the O/W emulsion. Thus, for vegetative cells and heat shocked spores, the antibacterial activity of EL was 7.5 to 10.0-fold lower in the O/W emulsion than in the aqueous system.

In the O/W emulsion, EL may have been located at the interfaces of droplets stabilized with the Tween 80 emulsifier (Park, Lee, Jo, Choi, Lee, & Chang, 2017). Unlike the freely dispersed form of EL in an aqueous system, the amount of EL in the emulsion able to contact the vegetative cell membrane is reduced in an O/W emulsion, where EL participates in micelle formation (Laatiris, El Achouri, Infante, & Bensouda, 2008). In addition, when the target of the antibacterial action is the cell membrane, hydrophilic/hydrophobic balance is important for the interaction of the amphipathic substance with the bacterial cell membrane (Mingeot-Leclercq & Décout, 2016). However, the outer portion of a micelle is hydrophilic and

the inner portion is hydrophobic, such that the hydrophilic/hydrophobic balance in the O/W emulsion containing EL would have been biased toward hydrophilicity. Consequently, the interaction of EL with the cell membrane, and thus the antibacterial activity of EL, would have been reduced (Engler, Tan, Ong, Coady, Ng, Yang, et al., 2013).

Sucrose palmitate, a sucrose fatty acid ester, is a widely used additive for the control of spores in canned foods (Nakayama, Sonobe, & Shinya, 1982). The amphipathic structure of sucrose palmitate allows it to interact with the cell membrane and accounts for its antibacterial activity. Specifically, sucrose palmitate interacts with the cell membrane to form a pore, which increases membrane permeability. The antibacterial activity of sucrose palmitate on the inner membranes of spores and the cell membranes of vegetative cells presumably occurs via the same mechanism. As the structure of EL resembles that of sucrose palmitate, *i.e.*, a hydrophilic head group and hydrophobic fatty acid tails (Fig. 7), the activity of EL on the spore inner membrane should also be similar.

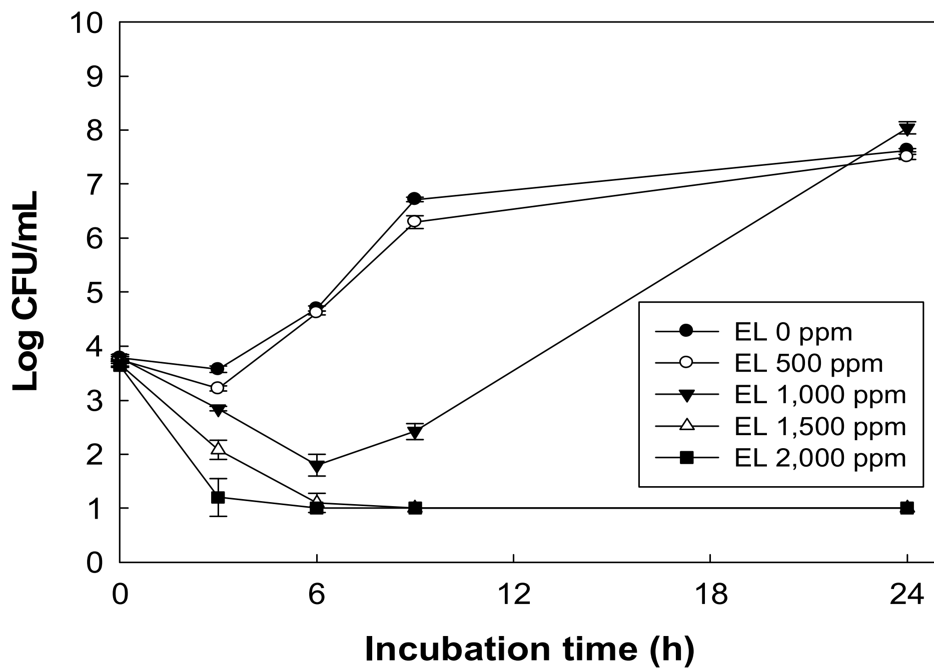


Fig. 5. Growth curves of *Geobacillus stearothermophilus* vegetative cells treated with 500, 1,000, 1,500, and 2,000 ppm erythorbyl laurate (EL) in an oil-in-water (O/W) emulsion stabilized with 1.0% Tween 80. The samples were shake-incubated at 55°C and 220 rpm for 24 h, after which the numbers of colonies were counted.

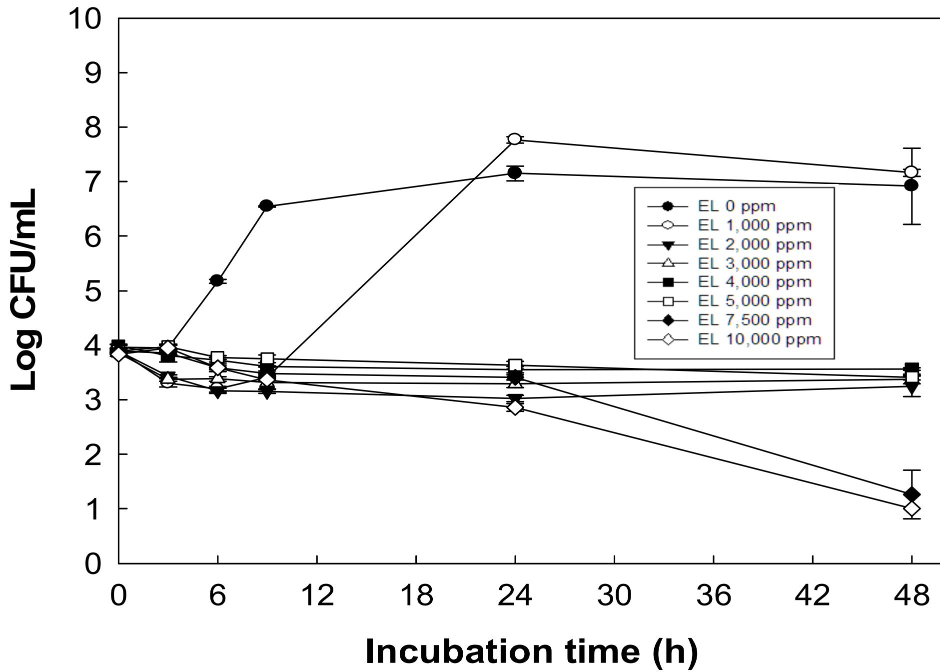
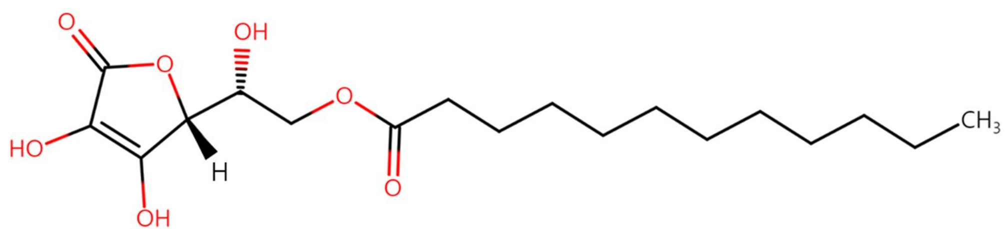


Fig. 6. Growth curves of germinated *Geobacillus stearothermophilus* heat shocked spores treated with 1,000, 2,000, 3,000, 4,000, 5,000, 7,500, and 10,000 ppm erythorbyl laurate (EL) in an oil-in-water emulsion system. The samples were shake-incubated at 55°C and 220 rpm for 24 h, after which the numbers of colonies were counted.

(a)



(b)

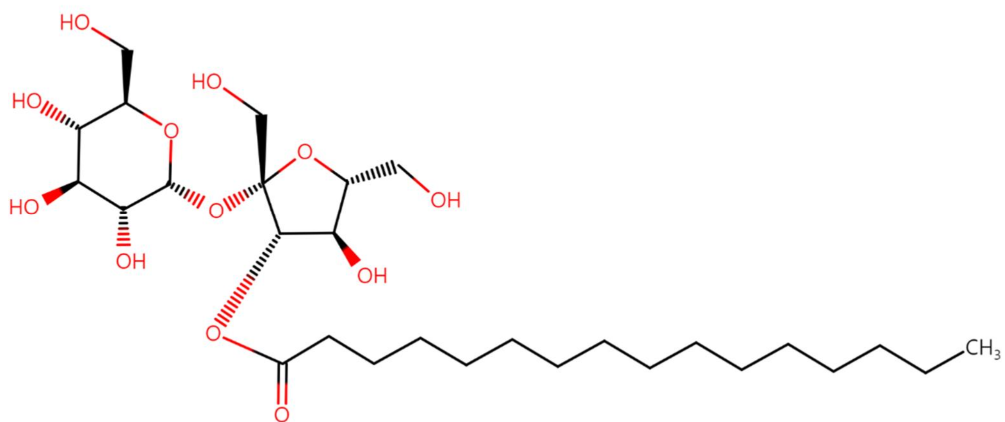


Fig. 7. Structures of erythorbyl laurate (a) and sucrose palmitate (b).

3.4. Confirmation of the germination inhibitory activity of EL

A DPA assay was conducted to obtain further evidence that EL inhibits heat shocked spore germination. DPA binds to calcium ions in the spore core, resulting in Ca–DPA formation (Magge, Granger, Wahome, Setlow, Vepachedu, Loshon, et al., 2008). Spore germination results in the release of Ca–DPA, whereas DPA remains inside the core when germination is inhibited (Francis, Allen, & Sorg, 2015). In a solution containing DPA and $TbCl_3$, DPA binds to Tb to form Tb–DPA, which has an excitation wavelength of 270 nm and an emission wavelength of 543 nm, allowing its detection using a fluorescence spectrometer (Fichtel, Köster, Rullkötter, & Sass, 2007).

Indeed, in the DPA assay, the DPA concentration (0–2,100 nM) was proportional to the number of relative fluorescence units (Fig. 8). The amounts of DPA released from autoclaved spores at concentrations of 4.0 and 5.0-log CFU/mL differed significantly (Fig. 9). As the amount of DPA that can be released from heat shocked spores is limited, but can be analyzed statistically, DPA analysis was used in this study to verify the inhibition of heat shocked spore germination.

The results showed the concentration-dependent inhibition of heat shocked spore germination by EL (Fig. 10). In addition, they indicated that EL did not

interact with the vegetative cells germinated from spores but only with the spores.

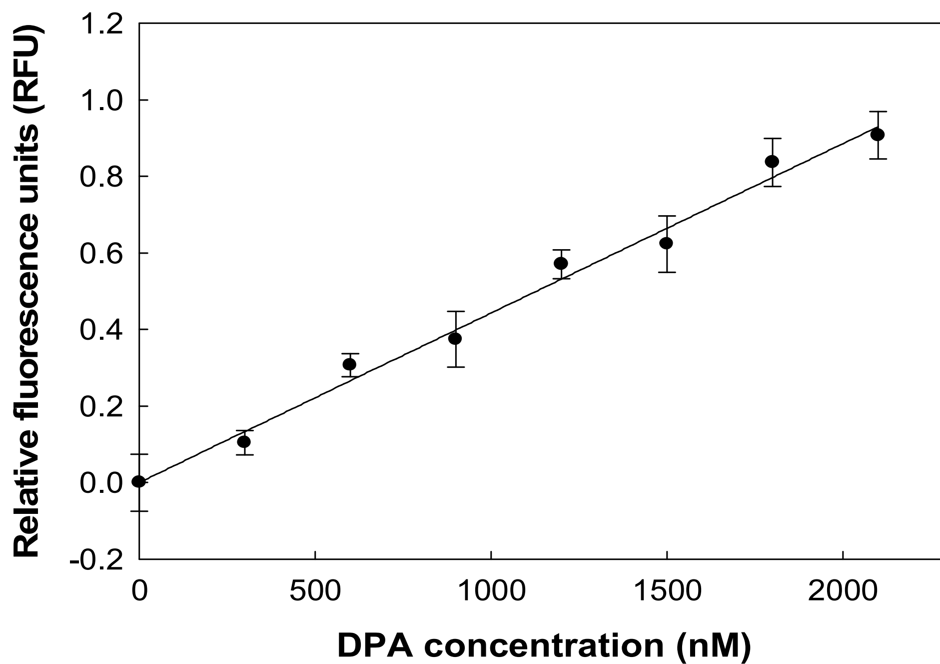


Fig. 8. Dipicolinic acid (DPA) standard curve. DPA concentrations were measured using a fluorescence spectrometer.

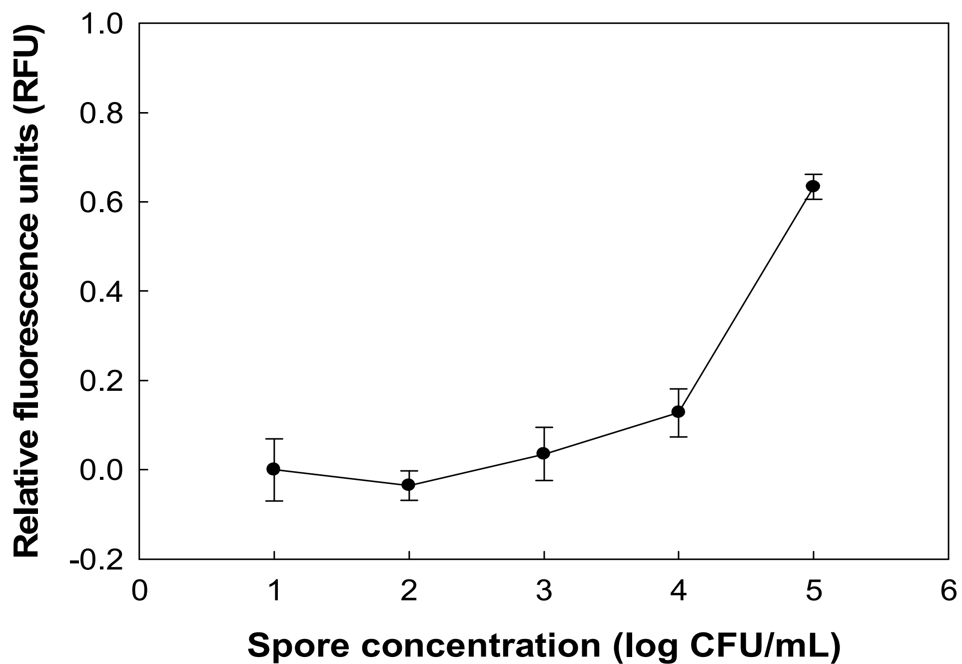


Fig. 9. Quantitative analysis of dipicolinic acid (DPA) released from a *Geobacillus stearothermophilus* heat shocked spore suspension autoclaved at 121°C for 30 min.

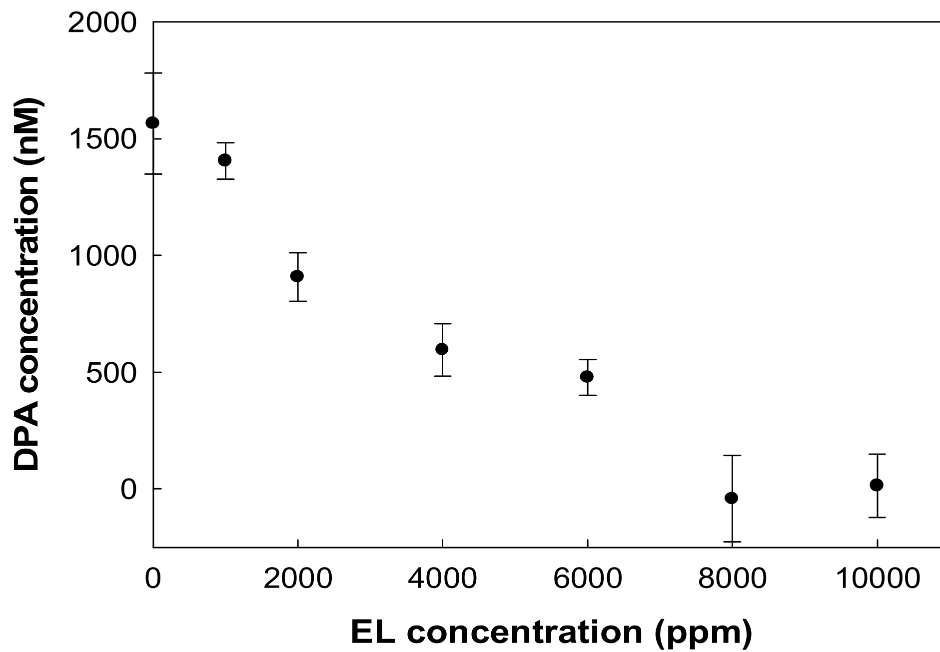


Fig. 10. Quantitative analysis of dipicolinic acid (DPA) released from erythorbyl laurate (EL)-treated *Geobacillus stearothermophilus* heat shocked spores.

3.5. TEM visualization of spores

The structural changes induced by EL in heat shocked spores were examined by TEM analysis. In spores not treated with EL, the cores remained circular shape and the inner membranes were clear; in spores treated with 4,000 ppm EL, the core shapes were altered and the inner membranes were no longer clear (Fig. 11), presumably due to the EL-induced changes in membrane permeability and the subsequent migration of materials from the core and cortex (Rao, Wang, Chen, & Liao, 2016).

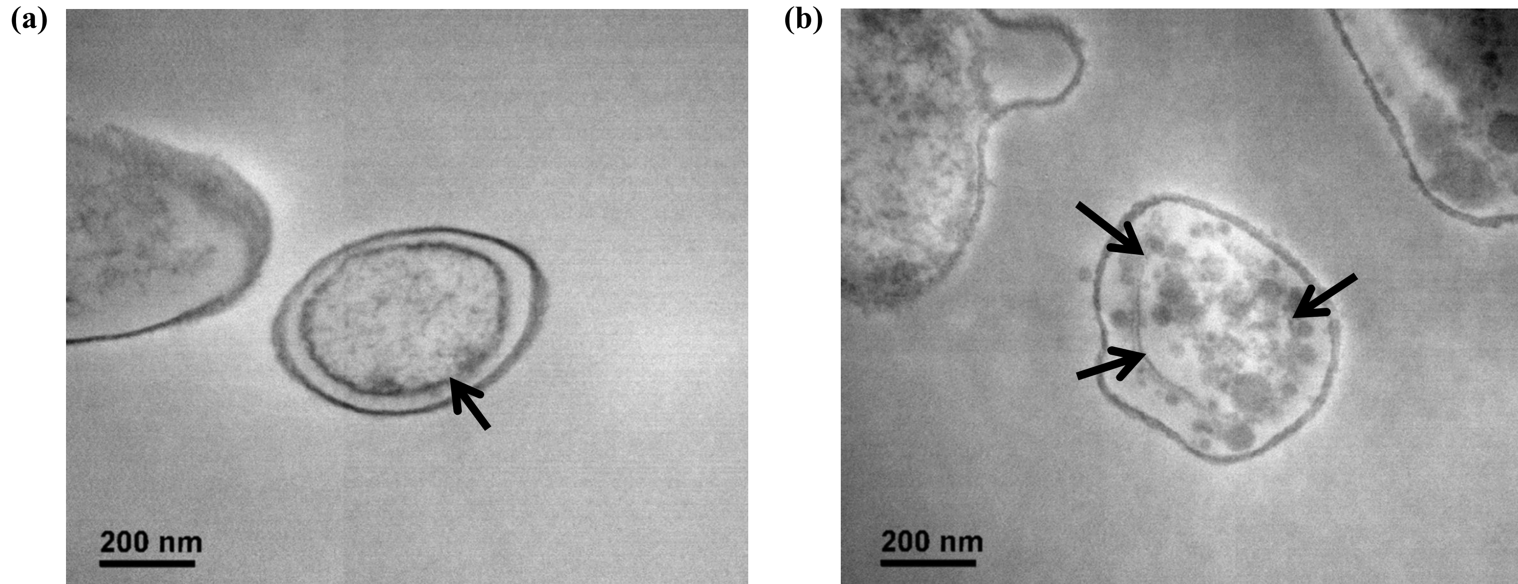


Fig. 11. Transmission electron micrographs of *Geobacillus stearothermophilus* heat shocked spores. Spores in an oil-in-water (O/W) emulsion were incubated without erythorbyl laurate (a) and with 4,000 ppm erythorbyl laurate (b). The arrow indicates the spore inner membrane.

3.6. Antibacterial assay in commercialized canned milk coffee

To investigate the applicability of EL for commercially available canned foods, the effect of EL on canned milk coffee stored in a heat cabinet (temperature, 55–60°C) for 14 days was determined. Heat treatment at 120°C for 4 min is the standard in the food industry to kill pathogenic spores, and F_0=8.0 is sufficient time required for sterilization.

At an initial heat shocked spore concentration of 4.0-log CFU/mL, all spores were killed (3-log reduction) after 16 min of heat treatment at 120°C (Fig. 12). After heat treatment at F_0=8.0 and subsequent incubation for 7 days at 55°C, a 0.7-log reduction in heat treated spores was observed in canned milk coffee not treated with EL. In canned milk coffee treated with 500 ppm EL, a concentration consistent with food industry standards, a 3.0-log reduction in heat treated spores occurred (Table 1). The heat treated spore concentration decreased proportionally as the EL concentration increased, and the effect was maintained for 14 days, the recommended duration for products in a heat cabinet.

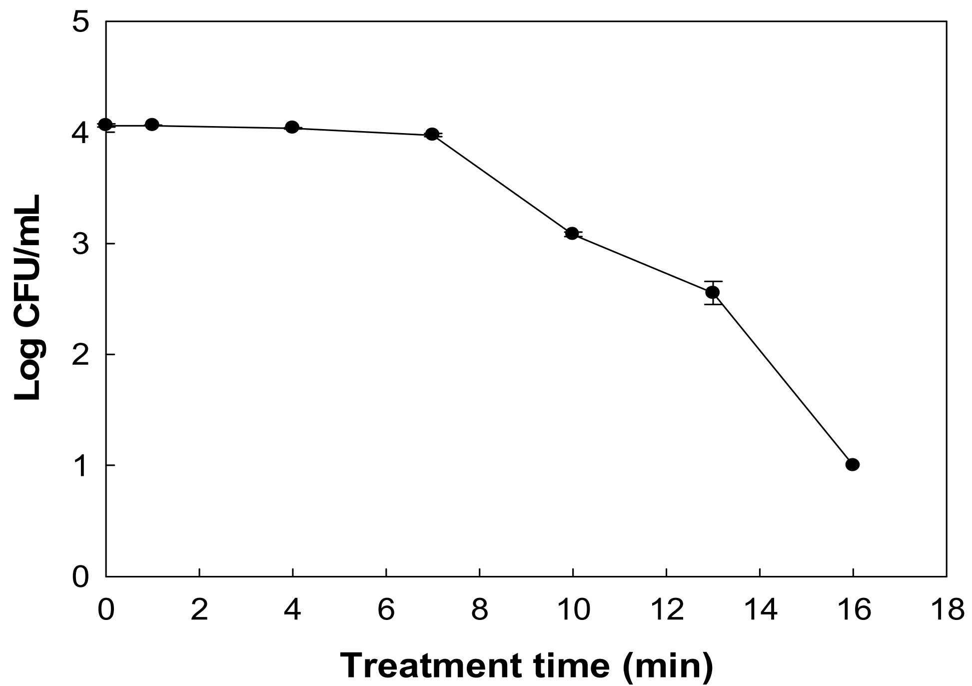


Fig. 12. Reductions in the numbers of viable heat shocked spores depending on the treatment (120°C in an oil bath) time.

Table 1. Effects of erythorbyl laurate (EL) on heat-treated (F_0=8.0) *Geobacillus stearothermophilus* heat shocked spores in canned milk coffee after 7 and 14 days

EL concentration (ppm)	Viable spore count (log CFU/mL)		
	Before treatment	After treatment	
		7 days	14 days
0	4.11 ± 0.01*, [a]	3.43 ± 0.05[a]	3.38 ± 0.05[a]
100	4.11 ± 0.01[a]	3.33 ± 0.10[a]	3.37 ± 0.03[a]
200	4.12 ± 0.02[a]	2.96 ± 0.07[b]	2.94 ± 0.12[b]
300	4.11 ± 0.01[a]	2.25 ± 0.21[c]	2.38 ± 0.16[c]
400	4.10 ± 0.02[a]	1.78 ± 0.20[d]	1.78 ± 0.39[d]
500	4.09 ± 0.01[a]	ND**, [e]	ND[e]

Values with different superscripts in each column differ significantly from each other, as determined by Duncan's multiple range test (p<0.05).

*Mean ± standard deviation.

**ND, not detected.

4. Conclusions

In this study, the antibacterial properties of EL against thermophilic heat shocked spores and the application of EL as a food additive to prevent spoilage were investigated. For vegetative cells, the bactericidal concentration of EL was 7.5-fold higher in the O/W emulsion than in the aqueous system. The same phenomenon was observed for sporostatic concentration of EL to heat shocked spores which was 10.0-fold higher in the O/W emulsion than in aqueous system. The difference in the antibacterial activity of EL in the two systems is presumably due to the reduced contact opportunities and decreased interactions of EL with cell membranes in the O/W emulsion.

The antibacterial action of EL could be attributed to the inhibition of heat shocked spore germination, as demonstrated quantitatively by the measurement of DPA release from spores. In the experiments using commercial canned milk coffee, heat shocked spore suppression increased with the EL concentration, as shown in the DPA and antibacterial assays.

In summary, this study characterized the antibacterial activities of EL against vegetative cells and heat shocked spores of *G. stearothermophilus*. Despite exhibiting less antibacterial activity, EL remained effective in the O/W emulsion and can thus be applied to prevent the contamination of

emulsion-based foods.

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국문초록

식품 산업에서 미생물 오염은 식품의 품질을 저하시키기에 매우 중대한 문제이다. 특히 호열성 포자는 높은 내열성을 지녀 열을 통한 살균처리에서 살아남아 식품의 오염을 유발할 수 있다. 식품 산업에서는 호열성 포자를 제어하기 위해 다양한 방법을 사용하고 있으며 그 중 첨가물의 사용은 그 중 가장 널리 이용되는 방법이다.

Erythorbyl laurate (EL)은 친수성의 erythorbic acid와 소수성의 lauric acid가 lipase 효소에 의해 esterification을 통해 결합된 양친매성 물질로 항산화, 항균, 유화 특성을 갖는 다기능성 유화제이다. 선행 연구를 통해 EL이 다양한 그람 양성 식품유해균에 대한 항균 활성을 나타냈으며 EL이 세포막에 작용하여 항균력을 나타냄을 확인하였다. 현재 산업에서 포자를 제어하기 위해 첨가 중인 자당지방산에스테르와 양친매성 구조와 항균 기작이 유사하기에 EL 또한 포자를 제어할 수 있을 것으로 예상하였다.

수중유적형 유화계에서는 EL의 용해도 차이로 인해 수상계에서와 항균 활성이 다르게 나타날 수 있으며 호열성 포자가 오염되기 쉬운 온장 보관하는 제품들 중 유화-제형의 제품이 다수 존재하기에 EL의 식품 산업 적용을 위해 수상계와 수중유적형 유화계에서 각각 항균 활성을 측정하였다.

수상계에서 호열성 포자형성균인 *Geobacillus stearothermophilus*에 대한 EL의 항균 활성을 측정한 결과 균에 대해서는 200 ppm의 EL 처리 시 3.0-log reduction 효과를 나타냈으며 열 충격을 받은 포자에 대해서는 200 ppm의 EL 처리 시 초기 농도가 유지되었다. 포자가 발아하지 못한다면 한천 배지에 도말 되기 전까지 포자 상태를 유지하기에 하나의 포자가 하나의 콜로니를 형성하게 된다. 만약 200 ppm의 EL 처리에서 포자가 발아했다면 발아된 균이 사멸하기 때문에 포자 농도가 감소했을 것이다. 따라서 200 ppm의 EL 처리는 균에 대해서는 최소 사멸 농도이며 열 충격을 받은 포자에 대해서는 포자의 발아를 억제하는 농도임을 나타낸다.

반면 수중유적형 유화계에서는 1,500 ppm의 EL 처리 시

균에 대해서는 3.0-log reduction을 나타냈으며 열 충격을 받은 포자에 대해서는 2,000 ppm의 EL 처리 시 초기 농도를 유지하였으며 7,500 ppm의 EL 처리 시 포자 농도에 2.7-log reduction을 나타냈다. 이는 1,500 ppm의 처리가 균에 대해서는 최소 사멸 농도이며 열 충격을 받은 포자에 대해서는 2,000 ppm의 처리가 발아를 억제하는 농도임을 나타낸다. 반면 7,500 ppm의 EL은 발아되지 않은 포자에 미친 영향이기에 이는 포자를 사멸시키는 농도이다.

수상계와 수중유적형 유화계에서 항균 활성을 비교하면 균 사멸 농도와 열 충격을 받은 포자 발아 억제 농도에서 수중유적형 유화계에서 항균 활성 농도가 7.5-10.0배 높은 것을 확인할 수 있다. 이는 수중유적형 유화계의 항균 활성이 수상계보다 낮은 것을 의미하며 이 차이는 수중유적형 유화계에서 EL의 세포막에 대한 접촉 기회 감소와 세포막과의 hydrophobic interaction이 감소되어 나타난 것으로 추론된다.

EL의 항균 활성이 포자에 대한 활성이 맞는지 확인하기 위해 포자가 발아할 때 방출되는 DPA를 검출하는 실험을 진행하였

다. 그 결과 EL 처리 농도가 증가함에 따라 DPA 방출량이 줄어드는 것을 확인했으며 이는 EL 처리 농도에 따라 열 충격을 받은 포자의 발아를 억제하는 것을 의미하기에 EL이 포자에 작용함을 나타낸다.

식품산업에 적용 가능성을 평가하기 위해 실제 판매되는 캔 밀크커피에 EL을 첨가 후 항균 활성을 확인한 결과 EL 처리 농도에 따라 농도 비례적인 포자 저감화 효과를 확인했으며 그 효과가 온장고 보관 권장 기간인 14일 동안 유지됨을 확인할 수 있었다.

본 논문에서는 erythorbyl laurate의 호열성 포자에 대한 항균력이 수상계보다 수중유적형 유화계에서 떨어졌지만 수중유적형 유화계에서도 그 활성이 여전히 유지됨을 확인하였으며 EL이 포자 발아 억제에 작용함을 밝혔다. 또한 제품 실험을 통해 erythorbyl laurate를 식품 산업에 적용 가능함을 확인하였으며, 이는 상용화를 위한 기초자료로 활용될 수 있을 것으로 예상된다.

주요어: Erythorbyl laurate, 항균 특성, 수중유적형 유화계, 호열성

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