Sporicidal activities and mechanism of surfactant components against *Clostridium sporogenes* spores

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Abstract The sporicidal activities of seven kinds of antimicrobial agent were investigated in order to screen for novel inactivation agents to apply to *Clostridium sporogenes* spores. Antimicrobial agents based on surfactant components, as poly-L-lysine, thiamine dilaurylsulfate, and torilin, were more effective than other agents. The degree of spore reduction with 1–2% surfactant components was 1.5–2.5 log CFU/mL. The HLB value (hydrophile-lipophile balance) related to denature protein of spores coat on surfactants with sporicidal activity was ranged from 6 to 16. Average HLB value and spore killing effect was inversely correlated. The proteins on spore structures seemed to be disorganized due to binding between polar groups of coats and hydrophilic and hydrophobic groups of surfactant components, resulting in killing of spores. The components that were effective to inactivate *C. sporogenes* spores had a chemical structure containing CH₃, OH, COOH, sulfate groups, and a double bond. Furthermore, hydrophobic surfactants were more effective than hydrophilic surfactants in inactivating spores. This was likely due to the type of hydrophobic surfactant and to the involvement of hydrophobic interactions on coat of spores.

Keywords *Clostridium sporogenes* spores · Surfactant components · Hydrophobic · Hydrophilic · HLB value

Introduction

Among the 90 known *Clostridium* spp., at least 30 species are associated with human diseases, and hence the major sterilizing targets in various foods are *Clostridium* endospores. *Clostridium botulinum* exists commonly in soil; it is an anaerobic, gram-positive, rod-shaped bacterium that produces oval, subterminal endospores, and only produces the botulinum neurotoxin. *Clostridium perfringens* is a spore-forming bacterium that produces bowel-irritating toxins and is a part of the normal flora in the intestinal tracts of animals (Peleg and Cole 2000; Huang et al. 2004). Since it is also one of the most common sources of food poisoning in humans and can cause serious illness, however, special attention must be paid to processing and preserving foods so that any *C. perfringens* spores are either inactivated or prevented from germinating or outgrowth (Huang et al. 2004).

The spore-forming microorganisms associated with *Clostridium* strains (e.g., *C. botulinum*, *C. perfringens*, and *C. sporogenes*) are difficult to control in industrial food production because they are highly resistant to both heat and chemical agents. From the perspective of food nutrition and safety, it is thus important to identify effective control methods such as physical treatments and natural sporicidal agents.

Physical treatments, such as dehydration, low-temperature storage, pasteurization, thermal sterilization, and nonthermal methods such as irradiation and ultra-high-pressure processing can be used to control *Clostridium* strains in foods.

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Chemical agents exhibiting sporicidal activity include glutaraldehyde, formaldehyde, chlorine-releasing agents, peroxygens, ethylene oxide, and ozone. Glutaraldehyde inhibits spore germination by sealing the outer layers of spores, while formaldehyde is able to penetrate into the interior of the bacterial spore (Russell 1990; Kim et al. 1993). Ethylene oxide inactivates spores by combining with various chemical groups in proteins and nucleic acids, and hydrogen peroxide removes the protein coat from spores; other antibacterial agents also interact with the outer spore layers (Russell 1990; Nishina et al. 1995). Nisin acted bactericidally against vegetative cells, and it inhibited the germination and outgrowth of the spores when added after the heat-activation stage. Therefore, nisin is very important for the effective control of spores during the heating process of foods, and it may be possible to apply nisin at the stage of processing that coincides with the most sensitive stage of spore transformation (Chung and Yousef 2007a, b). The more powerful bactericides may be inhibitory to spore germination or outgrowth, or both (i.e., they have sporostatic rather than sporicidal properties). Some of these chemical food preservatives have a long history of safe use (Russell 1990; Nishina et al. 1995; Kim et al. 1993; Atrih and Foster 2002).

Chemical treatments with salt, acids, nitrite, ascorbate or isoascorbate, polyphosphates, sugar or syrups, antioxidants, smoke and its components, seasonings, spices, extenders, and binders can also be used to control *C. botulinum* in various processed foods (Chaibi et al. 1997; Chung and Yousef 2007a; Yang et al. 2009). In the case of *C. botulinum* 62A, eucalyptus, chamomile, savage carrots, vervain, grapefruit, and orange oils were found to be sporostatic. Specifically, these essential oils (EOs) inhibited one or more stages of the spore cycle, depending on the EO and its concentration. Eucalyptus, orange, artemisia, and cedar oils (at 300 ppm), and grapefruit, savage carrots, and vervain (at 400 ppm) inhibited the growth of *C. botulinum* spores by inhibiting the commitment of spores to germinate by blocking their germination in L-alanine (Chaibi et al. 1997).

Chronic diarrhea caused by *C. perfringens* can be easily treated with antibiotics, the most commonly used being ampicillin, amoxicillin, metronidazole, and erythromycin. However, excessive use of antibiotics can cause the bacterium to develop resistance. Thus, alternative methods of preventing infection have been sought (Peleg and Cole 2000; Huang et al. 2004).

In the present study, the sporicidal activities of seven kinds of antimicrobial agents, known as antibacterial components were investigated in order to screen for novel sterilization materials to apply to *Clostridium* spores. The objectives of this research were to measure the sporicidal activities of various chemical ingredients with surfactant

components, which exhibit a wide spectrum of activity against *Clostridium* spores, and to study the mechanism underlying the sporicidal effect on intact spores. In this study, *C. sporogenes* that does not produce botulinum neurotoxins was used for safety on experiment.

Materials and methods

Preparation of spore suspensions

The *Clostridium sporogenes* (PA3679) used as a test microorganism in this study was obtained from the culture collection of the Food and Bioprocess Engineering Laboratory of Yonsei University. The spore suspension was prepared by inoculating 20 mL of cooked meat media (CMM, Difco, Detroit, MI, USA) with 0.1 mL of *C. sporogenes*; the mixture was then heated to 90 °C (center temperature of tube containing spore suspension) for 10 min to induce heat shock and to activate spore germination followed by incubation at 35 °C overnight under anaerobic conditions using an anaerobic incubator (CI-173 CO₂ Incubator, UITECH Corp., Suwon, Korea) (Byun et al. 2011). The CMM culture was then inoculated into 200 mL of sporulation medium (SM, Difco, Detroit, MI, USA) used for promoting sporulation and incubated at 30 °C for 8 h in an anaerobic incubator. The SM (200 mL) was then further incubated at 30 °C for 4 days in anaerobic conditions to obtain 90% level of spore condition by inoculation into 1.8 L of SM. The cell-to-spore transition was examined with the aid of a light microscope (Ni-U biological microscope, Nikon Corp., Tokyo, Japan). When the spore condition was observed about 90% level, the spore suspension was stirred. Spores were collected by centrifugation with a centrifuge (Centrifuge 5810 R, Eppendorf Corp., Hamburg, Germany) and subsequently washed by repeated centrifugation/resuspension. The first centrifugation was conducted at 1700 × g for 10 min, with subsequent washing three times in sterile distilled water at 4 °C by centrifugation (1200 × g for 10 min). After washing, the spore pellet was resuspended in 5 mL of sterile distilled water. Microcentrifuge tubes (Eppendorf Corp., Hamburg, Germany) containing 0.1 mL of *C. sporogenes* spore suspension were stored at −80 °C for maintaining of intact spore condition until used. The concentration of the spore suspension was examined by spreading on MTEYE medium (Difco, Detroit, MI, USA) containing 5% egg yolk and 0.5% yeast extract) (Yang et al. 2009).

Antimicrobial activity

This study investigated the sporicidal effects of the seven following antimicrobial components (0.5–2.0%, w/w) on *C. sporogenes* spores: immunoglobulin in yolk (IgY, Sigma-Aldrich Inc., Saint Louis, MO, USA), antimicrobial peptide (Sigma-Aldrich Inc., Saint Louis, MO, USA), sodium lactate (Sigma-Aldrich Inc., Saint Louis, MO, USA), nitrite (Sigma-Aldrich Inc., Saint Louis, MO, USA), surfactants including poly-L-lysine (ϵ -polylysine, Sigma-Aldrich Inc., Saint Louis, MO, USA), thiamine dilaurylsulfate (TLS, Sigma-Aldrich Inc., Saint Louis, MO, USA), and the ethanol extract of *Torilis japonica* with torilin. The specific IgY could be obtained from egg yolks from laying hens immunized with bacterial cells included *C. perfringens* and *B. cereus*, and the antimicrobial peptide was produced by probiotic bacteria as *Lactobacillus* strains. Commercial production of poly-L-lysine was achieved by natural fermentation of *Streptomyces albulus*. The chemical constituents of *T. japonica* fruit include many sesquiterpenoids such as torilin, essential oils, and hemiterpenoids. Six kinds of antimicrobial agents with reagent grade (Sigma-Aldrich Inc., Saint Louis, MO, USA) of 95–99% purity except the ethanol extract of *Torilis japonica* (Cho et al. 2007) were used for this study.

The antimicrobial activity of antimicrobial agents against *C. sporogenes* spores was measured by colony counting on McClung-Tobae differential plating medium according to Murray et al. (2007). In brief, 500 μ L of each of the seven kinds of antimicrobial agent and 500 μ L (vol/v) of pure spore suspension were mixed in test tubes. The contact time between antimicrobial agents and *C. sporogenes* spores was determined as 2 h at 30 °C. Our preliminary results showed that the antimicrobial effect of antimicrobial agents increased by 2 h and the contamination level of *C. sporogenes* spores was remained constant after 2 h. The suspensions were then washed three times by repeated centrifugation and resuspension with 1 mL of sterile 0.85% NaCl solution to negate any effects of residual antimicrobial components.

The final spore pellets were resuspended in 1 mL of sterile 0.85% NaCl solution. Each 0.1 mL of spore suspension was inoculated into MTEYE medium by spreading, and then incubated at 37 °C for 24 h in anaerobic conditions. The number of viable spores was determined using the standard colony count method in MTEYE medium.

Calculation of HLB value

The ability of a surfactant to denature protein was expressed by the HLB (hydrophile-lipophile balance) value related to degree of hydrophilic or lipophilic determined by

calculating values for the different regions of the molecule defined as

$$\text{HLB value} = \sum J_w - \sum J_o + 7$$

where J_w is the hydrophilic group number and J_o is the hydrophobic group number (Fujita and Suzuki 1992). The hydrophilic groups were $-\text{SO}_4\text{Na}^+$, $-\text{COO}^-\text{K}^+$, $-\text{O}-$, $-\text{COOH}$, and $-\text{OH}$, while the hydrophobic groups were $-\text{CH}_3$, $-\text{CH}_2-$, and $=\text{CH}-$. A surfactant with higher HLB value has higher hydrophilicity.

Statistical analysis

The experiments were repeated at least five times for each sample. All the data are expressed as averages of five replicate measurements and the standard deviation. The statistical analysis was conducted using Minitab (MTB13, Minitab Inc., State College, PA, USA). In order to verify to significant differences between the samples, analysis of variance and Duncan's multiple range test were carried out ($p < 0.05$).

Results and discussion

Deactivation activities of the antimicrobial agents

C. sporogenes PA 3679 is a nonpathogenic, putrefactive, spore-forming anaerobe, and its spores have been successfully used in studies of the inactivation processes of *C. botulinum* spores. The inactivation effects against *C. sporogenes* spores with 0.5–2.0% (w/w) solutions of the four tested commercial antimicrobial agents with bacteriostatic and bactericidal known to possess possibility of sporostatic and sporicidal abilities are compared in Fig. 1. Sodium lactate and antimicrobial peptide can inhibit spore growth in the early germination stage of spores (Russell 1990; Kim et al. 1993; Hamouda and Baker 2002; Chung and Yousef 2007a, b). The deactivation activities of IgY, antimicrobial peptide bacteriocin, sodium lactate, and nitrite were weak, with sporicidal values of about 0.5 log CFU/mL reduction. In general, sporicidal activities of spores are only considered effective at a 1–2 log CFU/mL reduction (corresponding to 90–99% inactivation). Therefore, the commercial antimicrobial agents tested in this study were unable to generate a detectable reduction of intact spores.

Sporicidal mechanism of surfactant components

The surfactants components of 0.5–2.0% (w/w) poly-L-lysine and thiamine dilaurylsulfate in various commercial

Fig. 1 Comparison of the inactivation effects of various antimicrobial components against *Clostridium sporogenes* spores: **a** immunoglobulin in yolk (IgY), **b** antimicrobial peptide, **c** sodium lactate, and **d** sodium lactate with 5 ppm nitrite. Each vertical bar indicates the standard deviation. Values marked above the bar with different letters are significantly different by ANOVA with Duncan's multiple range test at $p < 0.05$

natural antimicrobial agents exhibited significantly ($p < 0.05$) remarkable deactivation effects, with 1–2 log CFU/mL reductions of spores (Fig. 2a, b). Poly-L-lysine and thiamine dilaurylsulfate inhibit spore germination by acting as a surfactant with hydrophilic and hydrophobic properties, thereby destroying the cell structure such as coat and cortex of spore (Matsushashi et al. 1995; Ko and Kim 2004; Chung and Yousef 2007a; Wei et al. 2014).

We have previously revealed that a 1% crude ethanol extract of *T. japonica* exhibited particularly high sporicidal activities against *Bacillus subtilis*, reducing the spore count by about 3.5 log CFU/mL (Cho et al. 2007). The active antimicrobial compound was isolated and purified from the hexane layer, and identified as torilin ($C_{22}H_{32}O_5$). Torilin reportedly exhibits anti-inflammatory, anticancer, and anti-invasive activities in human fibrosarcoma cells. Torilin immediately reduced vegetative cell counts by five- to sixfold, and reduced spores counts by one order of magnitude. Torilin functions as a surfactant with both hydrophilic and hydrophobic properties via a process related to denaturation of various proteins (Cho et al. 2007).

The present study investigated the sporicidal activity of *T. japonica* with a torilin component against *C. sporogenes* spores, and found that a 1–2% crude ethanol extract of *T. japonica* exerted effective sporicidal effects, reducing the

spore count by 1.5–2.5 log CFU/mL (Fig. 2c). The sporicidal activity of 1% ethanol extract of *T. japonica* for *B. subtilis* spores was higher than for *C. sporogenes* spores, causing a 3.5 log reduction (Cho et al. 2007). This differential sporicidal effect was assumed to be due to significant differences between the coatings of these two species (Chaibi et al. 1997; Atrih and Foster 2002; Leggett et al. 2012). Such species-specific differences in coat composition could impact upon the biocidal formulation and environmental stresses, including the disinfectants and sterilants required to rapidly overcome the defense provided by the spore coat (Leggett et al. 2012).

The integrity of structures such as the coating of the three layers (inner, middle, and outer) on spore coat, and the cortex, protoplast membrane, and core have been known to be vital to spores (Russell 1990; Atrih and Foster 2002). In this study, the ability of each surfactant to denature protein of spores coat was expressed by its HLB value based on degree of hydrophilic or lipophilic (Fujita and Suzuki 1992; Huang et al. 2004). The HLB value of most of the chemical surfactants ranged from 10 to 17, and the average HLB value of the surfactants with sporicidal activities exhibiting a reduction of 1.5–2.5 log was 11 and ranged from 6 to 16, which is similar to the values of various chemical surfactants. Torilin and thiamine dilaurylsulfate showed the lowest and highest value of HLB,

Fig. 2 Comparison of the inactivation effects of three surfactant-based antimicrobial components against *C. sporogenes* spores: **a** poly-L-lysine and **b** thiamine dilaurylsulfate and **c** crude ethanol extract of *Torilis japonica* fruit with torilin. Vertical bar represents standard deviation. Values marked above the bar with different letters are significantly different by ANOVA with Duncan's multiple range test at $p < 0.05$

respectively. We carried out to analyze for correlation between HLB value and spore killing activity for interpretation of inactivation mechanism. Correlation between average HLB value and spore killing effect was an inverse increased linear function (Fig. 3).

Fig. 3 Correlation between HLB value and spore killing activity on various surfactants such as torilin in crude ethanol extract of *Torilis japonica* fruit, poly-L-lysine and thiamine dilaurylsulfate against *C. sporogenes* spores (filled circle: spore count (CFU/mL), filled square: inactivation ratio (%)). Vertical bar represents standard deviation. Each value were compared with the control at $p < 0.05$ Duncan's test and vertical bar represents standard deviation

The structure of protein on spore coats appears to be disorganized and distorted due to binding between the polar group of spore coats and the hydrophilic ($-\text{SO}_4\text{Na}^+$, $-\text{COOH}$, and $-\text{OH}$) and hydrophobic ($-\text{CH}_3$ and $=\text{CH}-$) groups of surfactant components by inference with the result of Fig. 3 (Hamouda and Baker 2002; Huang et al. 2004). The components that are effective at deactivating *C. sporogenes* spores, causing reductions of 1.5–2.5 log CFU/mL form chemical structures with CH_3 , OH , COOH , and sulfate groups, and a double bond. The hydrophobic surfactants were more effective than the hydrophilic surfactants for inactivating *Clostridium* spores due to the hydrophobic nature of the spores and their coating.

Conclusion

This study demonstrated that the surfactants can inactivate the *Clostridia* spores. *C. sporogenes* spores were effectively reduced by 1–2% surfactant components as poly-L-lysine, thiamine dilaurylsulfate, and torilin with chemical structures containing CH_3 , OH , COOH , sulfate groups, and a double bond. From the results of this study, the sporicidal mechanism was seemed to involve a two-step: (1) the hydrophobic binding of the surfactant on the surface of the outer coat first destroys the protein structure of coat, and

then (2) the components penetrate into the interior, continuously decomposing the cortex area with very thick layer of specialized peptidoglycan. Further research is currently being carried out to identify the detailed antimicrobial mechanisms and screen for more effective surfactant components and to assess the potential of screening components extracted from various herbs and medicinal plants, with a view to developing novel natural antimicrobial agents for deactivation of *Clostridia* spores.

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