Food Sci. Biotechnol. Vol. 17, No. 3, pp. 603 ~ 607 (2008)

Food Science and Biotechnology

Effect of Commercial Antimicrobials in Combination with Heat Treatment on Inactivation of *Bacillus cereus* Spore

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Abstract Thirteen commercial antimicrobial products were examined to assess the sporicidal activity against *Bacillus cereus* spores at room temperature, 60 and 85°C. Neither the antimicrobials showed detectable antimicrobial activity against the *B. cereus* spores nor induced spore germination after the treatment at 0.5 or 1.0%(w/v, v/v) commercial antimicrobial agents at room temperature for 0.5 to 4 hr. However, when the antimicrobials such as chitosan, lactic acid, fermented pollen, grapefruit extract were applied with heat at 85°C for 30 min, more than 1 log CFU/mL spores were additionally inactivated compared to only heat treatment without antimicrobials. Imposition of 60°C to *B. cereus* spores with the higher concentration of 5.0%(v/v) lactic acid or 2.5%(w/v) thiamine dilaurylsulfate for the longer time incubation of 24 hr resulted in 3 log CFU/mL spore inactivation. This work showed that low concentrations of commercial antimicrobials by themselves did not inactivate *B. cereus* spores. However, when physical processes such as heat were combined together, antimicrobials showed a synergistic effect against *B. cereus* spores.

Keywords: *Bacillus cereus*, spore, commercial antimicrobial, inactivation

Introduction

Bacterial spores are one of the main targets for inactivation in food industry to manufacture safe food products with extended shelf life because they are very resistant to physical treatment such as heat, freezing (1-3), and even harsh chemical treatments such as hydrogen peroxide (4-6). It is well known that the resistant properties and the dormancy of spores enable them to survive for long periods if there is no spore activation process (7). Synthesis α/β-type small acid-soluble proteins (SASP) and the saturation to DNA during spore forming (8,9) contribute to the physical and chemical resistance of spores with the spores characteristics of the decreased spore water content, spore mineralization, spore multi-layer structure, and DNA repair during spore germination (10-13).

The main food poisoning spore-formers are *Bacillus cereus*, *Clostridium botulinum*, and *Clostridium perfringens*. Sometimes *Bacillus subtilis* and *Bacillus licheniformis* have been reported as the cause of food poisoning outbreaks (14). The easily detectable spore-forming bacterium in foods, *B. cereus*, is present in soil, dust, and water and is an aerobic rod shaped spore-former. *B. cereus* produces diverse extracelluar toxins and enzymes, such as lecithinase, proteases, β-lactamase, sphinogomyelinase, cerolysin, and hemolysin BL. There are two (emetic and diarrhoeal) types of *B. cereus* food poisoning (15-17). The food industry make efforts to produce foods which are not heat processed and are less acidic and less salty in accordance with consumer preferences (18,19). Therefore, it is a big concern in food industry that controlling spore-forming bacteria, which can form spore and then germinate and grow wherever good growth conditions are found. If the spores remain during processing and germinate later in the food product without achievement of microbial criteria during sterilization processing, it fails to meet the standard requirement and gives rise to face processing difficulty.

Meanwhile, not only the demand for minimally processed foods to consume the natural food flavor and taste without triggering any harmful effect on the health by chemical preservatives but also the requirement such as natural antimicrobials containing naturally occurring bioactive ingredients from food (considered as generally recognized as safe, GRAS) is increasing (20). Antimicrobial agents from nature are diverse and abundant in the environment. Natural antimicrobials have been widely classified to lacto-antimicrobials (lactoferrin, lactoperoxidase), ovo-(lysozyme, ovoglobulin, avidin), phyto-(saponins, flavonoids, catechins), bacto-(probiotics, nisin, pediocins), and acid-(lactic acid, acetic acid, citric acid) depending on the source (20). It has been reported that nisin has been shown to be useful to inhibit the growth of *B. cereus* cells during soybean sprout storage (21). To inhibit spore germination and outgrowth during food processing and distribution, natural antimicrobials would offer great advantages in the food industry and some products are currently in use.

As the spore model of food-poisoning spore former, *B. cereus* spores were applied to various commercial antimicrobials relatively stable under heat to assess which and how natural antimicrobials are effective on inactivating *B. cereus* spores under heat treatment. This study also aims to investigate the possibility of natural antimicrobials use as a huddle during food processing.

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Received September 20, 2007; Revised October 31, 2007;
Accepted November 1, 2007

Materials and Methods

Strains and *B. cereus* spore manufacture *B. cereus* ATCC 11778 and ATCC 14579 strains were grown on nutrient broth (Difco Laboratories, Sparks, MD, USA) and the subculture were grown at nutrient agar (Difco) containing 0.001%(w/v) $MnSO_4 \cdot H_2O$ at 35°C for 5 days. Formation of the spores was checked by phase contrast microscopic method. When more than 95% of the cells formed spores, the spores were harvested by adding 5 mL physiological saline to each plate and collected by using sterile spreader. The harvested spores were washed with distilled water and centrifuged at 4,500×g for 10 min. The pure spores were stored at 4°C up to 1 month.

Heat treatment and D-values Before the viable spores were counted, all the preparations were heated at 80°C for 10 min to kill any remaining vegetative cells in this study. For inactivation studies, 1 mL suspensions (10^8 spores/mL) were heated at 85 or 90°C. Treated and untreated control spore suspensions were serially diluted and surface plated on nutrient agar (Difco). The plates were incubated for 48 hr at 35°C before the colonies were counted. The D-values of the spores each strain at 85 and 90°C were obtained and calculated by plotting the thermal death curve (22).

Spore inactivation by antimicrobials For inactivation studies, 1.8 mL mixture of the 2 strains (1:1) spore suspensions (10^{6-7} spores/mL) were mixed to 0.2 mL of 10%(w/v) antimicrobials (commercial products containing nisin, lactoferrin, polylysin, mustard, Japanese apricot, garlic, grapefruit seed extract, or green tea extract) or 10%(v/v) commercial products (containing chitosan, ethanol, lactic acid, or fermented pollen) at 25°C for 30 min to adjust to 1% final antimicrobials as the suggestion by the manufacturers (Table 1). An antimicrobial product containing thiamine dilaurylsulfate was prepared and the final application antimicrobial concentration to *B. cereus* spores was 0.5%(w/v). Green tea extract and thiamine dilaurylsulfate was dissolved in 70% ethanol and the others were dissolved in water and prepared. The cells suspended in antimicrobials were washed by centrifugation at the maximum speed in a centrifuge to remove the antimicrobials to stop the effect of antimicrobial agents on the spores after treatment (23). Treated and untreated spore suspensions were serially diluted and surface plated on nutrient agar. The plates were incubated for 48 hr at 35°C and the colony numbers were enumerated.

Spores germination Half volumes (2.5 mL) of the washed spores after antimicrobials treatment at room temperature were heated at 80°C for 10 min to kill any germinated spores and then cooled down on ice (24). The spore suspensions were serially diluted and surface plated on nutrient agar. The plates were incubated for 48 hr at 35°C before the colonies were counted. The spore numbers ungerminated after heat treatment at 80°C for 10 min were calculated as the number of spores that did not germinate in the presence of the antimicrobials.

Application of various concentrations of antimicrobials and heat treatment combination The spore suspensions were imposed to the 0.5 to 5%(w/v or v/v) antimicrobials at 60 or 85°C for 30 min up to 48 hr. The survival or ungerminated spore numbers were measured as described above.

Results and Discussion

Effect of commercial antimicrobials on inactivation of *B. cereus* spores at room temperature In advance, D_{85}-values of *B. cereus* ATCC 11778 (29.2 min) and ATCC 14579 (38.3 min) were compared to investigate the effect of heat on inactivation of *B. cereus* spores without antimicrobial treatment. D_{90}-value of ATCC 11778 (7.3 min) and ATCC 14579 (13.3 min) was approximately 3 times shorter than D_{85}-values of the 2 strains, respectively (Table 2). To investigate the sporicidal activity of commercial antimicrobials on *B. cereus* spores, 1%(w/v or v/v) the standard concentration of antimicrobials was applied on the spore cocktail of *B. cereus* ATCC 11778 and ATCC 14579. There is food itself showing antimicrobial activity such as garlic, Japanese apricot, and mustard. Grapefruit seed extract is in controversial as food additives. However this

Table 1. Commercial antimicrobial components, the recommended concentrations according to the manufacturers, and the pH value in the spore suspension containing the recommended concentration of antimicrobial

Commercial antimicrobials	Active ingredients (product type)	Recommended concentration (v/v, w/v)	pH
Nisin	Nisin (powder)	-	4.5
Lactoferrin	Lactoferrin (powder)	1.0%	4.0
Polylysin	Polylysin (powder)	0.5-1.0%	5.0
Chitosan	Chitosan (liquid)	1,000 ppm	4.0
Mustard	70% Mustard (powder)	-	5.0
Japanese apricot	50% Japanese apricot (liquid)	-	4.5
Garlic	Garlic (powder)	-	5.5
Ethanol	70% Ethanol (liquid)	1.0-2.0%	6.0
Lactic acid	Lactic acid (liquid)	0.5-2.5%	2.0
Fermented pollen	Fermented pollen (liquid)	0.005-0.5%	4.5
Grapefruit seed extract	Grapefruit seed extract (liquid)	-	4.5
Green tea extract	Green tea extract (powder)	0.1-1.0%	5.0
Thiamin dilaurylsulfate	Thiamin dilaurylsulfate (powder)	0.005-0.5%	4.5

Table 2. The D-values of *B. cereus* ATCC 11778 and 14579 spores at 85 and 90°C

Strain	Temperature (°C)	D-value (min)
B. cereus ATCC 11778	85	29.2
	90	7.3
B. cereus ATCC 14579	85	38.3
	90	13.7

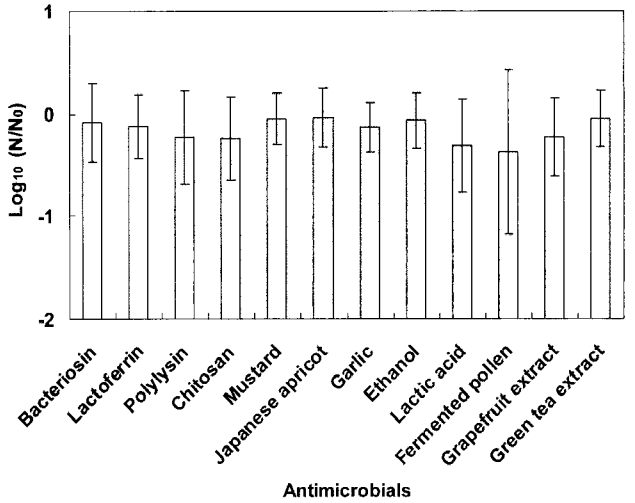

Fig. 1. Effect of commercial animicrobial treatments for 30 min at 25°C on the survival numbers of *B. cereus* spores of ATCC 11778 and 14579.

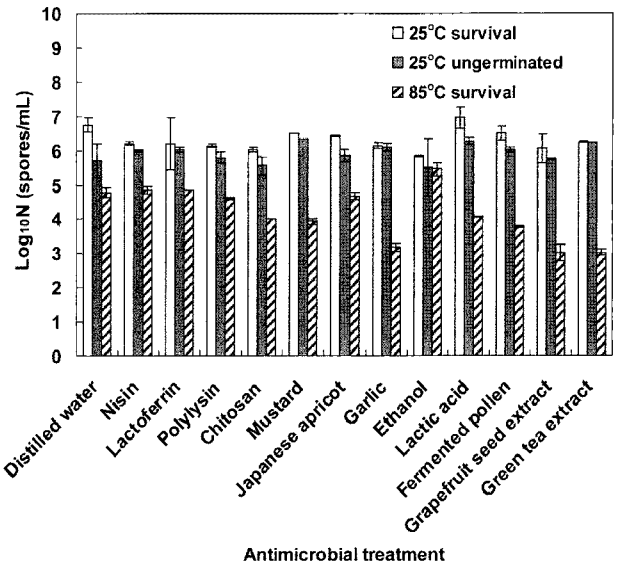

Fig. 2. The resistance of *B. cereus* spores to antimicrobial treatment at 25 and at 85°C for 30 min, respectively.

study focused on evaluating the different antimicrobial sources against *B. cereus* spores. All the 12 commercial antimicrobial treatment (nisin, lactoferrin, polylysin, chitosan, mustard, Japanese apricot, garlic powder, ethanol, lactic acid, grapefruit seed extract, green tea extract, or fermented pollen) for 30 min at room temperature (25°C) reduced the spore numbers by less than 0.5 log CFU/mL (Fig. 1). The sporicidal activity of commercial antimicrobials at room temperature was negligible compared to heat treatment such as 85 or 90°C. Commercial antimicrobials are the products by using the main materials showing great antimicrobial activity with wide spectrum against food-borne pathogens, spoilage bacteria, yeast, and molds to improve food safety and to extend the shelf-life of food products (19). To widen the antimicrobial spectrum of commercial antimicrobials, additional sub-materials such as acid or ethanol are normally mixed accordingly some of commercial antimicrobials were shown to be acidic (Table 1). Therefore, it has been pointed out that it is difficult to distinguish whether microorganisms are inactivated by the action of the main antimicrobial material itself or by the effect of acid which is also antimicrobial material such as acetic acid or lactic acid mixed in the product. Nevertheless the commercial product examined in this study did not show the sporicidal effect on *B. cereus* spores after short time application at 25°C. The low sporicidal activity of the commercial antimicrobials might be attributed to the spore's multi-layered structure much more complex than the vegetative cell's because it is widely known that the spore cortex encases the protoplast and the spore coat plays also an important role in biocide resistance by limiting penetration to the protoplast (13).

Effect of commercial antimicrobials on germination of *B. cereus* spores It is necessary to know if antimicrobials have an effect on spore germination because germinated spores are much more sensitive to physical and chemical treatment than the spores (3). If antimicrobials can induce spore germination and the antimicrobials have the antimicrobial activity against the germinated spores, which may be powerful tool to control bacterial spores. On the contrary, some portions of the germinated spores remain and the germinated spores outgrow later on, it can cause the problem in food safety and quality (25). Therefore investigating the action of antimicrobials on spore is required to use appropriate processing tool in food industry.

The spores 12 antimicrobial treated for 30 min at 25°C hardly induced spore germination. The ungerminated spore numbers of *B. cereus* were nearly similar to the survived spore numbers after antimicrobial treatment at 25°C (Fig. 2). However, when the spores were tested on the same concentration (1%, w/v or v/v) of the antimicrobials, the number of spores survived and ungerminated at 85°C were 1-2 log CFU/mL less than those at 25°C depending on the antimicrobials. The antimicrobials reduced more than 2 log CFU/mL spores were the product containing garlic, lactic acid, fermented pollen, grapefruit seed extract, or green tea extract (Fig. 2). Because it has known that heat at 85°C can inactivate almost the germinated spores, the number of survival spores was the ungerminated spores. This study emphasized that some antimicrobials can inactivate more spores when heat treatment was combined, in this case antimicrobials can play a role as a huddle to inactivate *B. cereus* spores. Because the D_{85}-values were approximately 30 min (Table 1), it was concluded that the treatments of antimicrobial at 25°C for 30 min did not induce any significant spore reduction (Fig. 1 and 2), accordingly the additional 1 log CFU/mL reduction could be resulted from

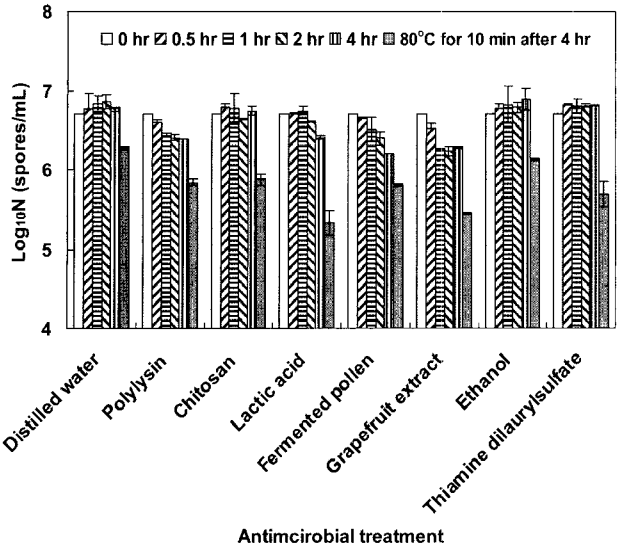

Fig. 3. Time to kill assay of *B. cereus* spores by antimicrobial treatments up to 4 hr.

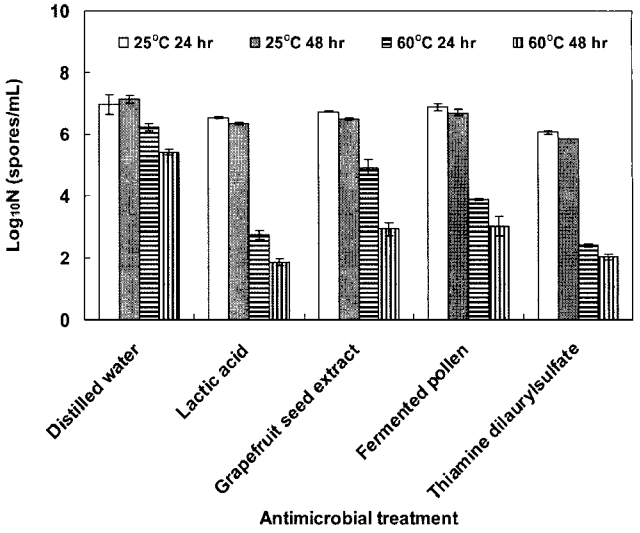

Fig. 4. The effect of 5 times more concentrated antimicrobials application on *B. cereus* spores at 25 and 60°C.

synergistic effect of some antimicrobial treatment and heat combination. Recently, some researchers had interest in finding GRAS materials blocking spore germination to prevent spore germination during food distribution or storage (26). Meanwhile some scientists have worked on inducing spore germination to kill the spores effectively by using high hydrostatic pressure (27,28). Cho (29) showed that the extracted antimicrobial material from Chinese medicine materials not only has sporicidal activity but also induces spore germination. This work showed that the commercial antimicrobials tested in this study do not play the role in inducing *B. cereus* spore germination at room temperature for 30 min. Therefore it is necessary to check the antimicrobial activity for spore killing and germination for the longer application time.

Time to kill assay To monitor the antimicrobial activity against *B. cereus* spores for the longer application time, *B. cereus* spores were applied on the selected 7 antimicrobials for 0.5-4 hr. The control spores in distilled water were also compared to see if there is any change in numbers of spores depending on incubation time (Fig. 3). Some of the antimicrobials such as grapefruit seed extract showed about 0.5 log reduction by 4 hr incubation at room temperature. Most of the selected antimicrobials did not show significant reduction in spore numbers even after 4 hr incubation. However, when 80°C for 10 min heat was additionally applied on the spores, 1%(v/v) lactic acid, 1%(v/v) grapefruit seed extract, and 0.5%(w/v) thiamine dilaurylsulfate dissolved in ethanol resulted in more spore inactivation level than the other antimicrobials. The reduced number of the *B. cereus* spores could be sensitized spores by the longer time incubation such as 4 hr with the antimicrobials and the subsequent heat treatment at 80°C for 10 min.

Combination effect of the increased concentration of antimicrobials and pasteurization This study showed that low concentration of antimicrobials (0.5-1.0%) did not show sporicidal effect on *B. cereus* spores. Accordingly, it is required to check if the application concentration is appropriate to kill *B. cereus* spore. To investigate the effect of the higher concentration of antimicrobials on *B. cereus* spores, 5 times more concentrated antimicrobials [5%(v/v) lactic acid, 5%(v/v) grapefruit seed extract, 5%(v/v) fermented pollen, and 2.5%(w/v) thiamine dilaurylsulfate dissolved in ethanol] were applied on *B. cereus* spores. To compare the sprocidal effects of the selected antimicrobials on *B. cereus* between at room temperature and at 60°C, the survival spore numbers after the longer time incubation up to 48 hr at the 2 temperatures were compared (Fig. 4). When the spores were heated up to 60°C with the commercial antimicrobials, *B. cereus* spore number was decreased by 2.5-3.5 log CFU/mL (Fig. 4). Because the spores suspended only in distilled water at 60°C for 48 hr were inactivated by more than 1.5 log CFU/mL, 1-2 log CFU/mL spores could be inactivated by the action of the antimicrobials at 60°C. Although the concentration of the commercial product containing thiamine dilaurylsulfate (2.5%) was lower than the other products (5.0%), the reduction level *B. cereus* spore was the greatest in thiamine dilaurylsulfate (2.5%) both at room temperature and at 60°C. The concentration of 5% lactic acid treatment for 24 hr with 60°C also inactivated *B. cereus* spores by up to 3 log CFU/mL while that treatment at room temperature showed only less than 1 log CFU/mL reduction. This work showed that all the commercial antimicrobials known for playing antimicrobial role in vegetative cells do not play dramatic sporicidal role in inactivating *B. cereus* spores at room temperature except 2.5% thiamine diaurylfulfate which showed the inactivation level of slightly over 1 log CFU/mL *B. cereus* spores after 24 hr application. However, the potential impact of antimicrobials will encourage food industry to apply to control bacterial spores as a huddle to sensitize the spores rather than the single step in food processing units. Kim *et al.* (30) and Lee *et al.* (31) also reported the use of natural antimicrobial materials such as the methanol extract from various food and herbs or linolenic acid with preservatives to result in the synergistic antimicrobial effect. The antimicrobials addition would play a role in reducing the level of chemical preservatives

or harsh physical food processing therefore food quality would be improved in the food industry. This research is the preliminary work before applying onto real food model, however, could be useful as the basic data of commercial antimicrobials application on *B. cereus* spores. If antimicrobials can inhibit spore germination or even reduce spore numbers in the real food models, the use of natural antimicrobials will contribute in terms of the longer shelf life of the food products as well as cost savings in the processing.

Achnowledgments

This work was supported by the CJ grant and the Research Specialization Program of Korea Food Research Institute.

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