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Inactivation of Bacterial Spores by High Pressure and Food Additive Combination

Yoon-Kyung Chung*

Department of Nutrition and Culinary Science Hankyong National University Ansung-si, Kyonggi-do 456-749, Korea

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Antimicrobial efficacy of high pressure (HP) can be enhanced by the application of additional hurdles. The objective of this study was to assess the enhancement in pressure lethality by *tert*-butylhydroquinone (TBHQ) treatment, against bacterial spores that are considered significant in the food industry. Spores of *Clostridium sporogenes*, *Bacillus cereus* and *B. subtilis* were prepared. Spore suspensions containing TBHQ (200 ppm, dissolved in dimethyl sulfoxide, DMSO) were pressurized at 650 or 700 MPa at 54-72°C for 5 min. Inactivation of bacterial spores resulted only with HP treatment. The population of *B. subtilis* spores was more inactivated by HP than those of *B. cereus* and *C. sporogenes* spores. Inactivation of *C. sporogenes* spores using pressure was more affected by the germinated population, compared to *Bacillus* spores. The inactivation of *Bacillus* spores increased when pressurized at 70°C, compared to 54°C. On the other hand, the degree of germination-induced lethality for *Bacillus* spores decreased at 70°C. When spores were treated with a combination of DMSO-HP and TBHQ-HP, these treatments seemed to protect the spores against HP, especially at 54°C. Further mechanistic studies involved in inducing germination by HP and using a subsequent sporicidal agent will be needed for a better understanding of bacterial spore inactivation.

Key words : Bacterial spores, high pressure, tert-butylhydroquinone, dimethylsulfoxide, germination.

Introduction

High-pressure (HP) processing has been considered as a non-thermal sterilization method, since microbial inactivation can be achieved on a reduced temperature level. Although the exact mechanism of the inactivation of bacterial spores is still unknown [10,28], several studies indicated that inactivation of bacterial spores takes place subsequent to the pressure-induced germination [7,25]. It has been proposed, that high pressure triggers the germinant receptor system to initiate an enzymatic chain reaction, which leads to the degradation of the cortex [9]. The rehydration process is accompanied by a loss of heat resistance [26]. These findings resulted in several process strategies such as applying heat treatment subsequent to pressure in order to inactivate the germinated, sensitized spores [4]. Studies indicate that the spore inactivation rate rises with increasing temperature. On the other hand, raising the pressure does not increase the spore inactivation rate when the system is kept at certain temperature levels. Mallidis and Drizou [17] reported that an increase in pressure (3-30 MPa) did not result in a higher degree of inactivation at temperatures between 117 and 125°C. Rovere *et al.* [23] obtained similar results at a temperature between 60 and 70°C and a pressure range between 700 and 900 MPa.

Food processors often combine lethal treatments to assure food safety. Process combinations that act synergistically against bacterial spores are most desirable, and improvements in spore inactivation by HP processing have been achieved when the pressure treatments were used in conjunction with bacteriocins and chemical preservatives [6,16,27]. Several studies indicated that essential oils prevented the germination and delayed the growth and toxin production of *Clostridium botulinum* spores [3,12,22]. It was reported that the variability in antimicrobial activities of essential oils had been attributed to the differences in their active fraction. Essential oils have the greatest inhibitory effect when they contain phenolic compounds [8,15]. In addition, rosemary oil, which contains phenolics as the main active components, was sporicidal against *B. cereus* spores at a lower concentration (≥170 ppm) than other essential oils with non-phenolic active components; the latter showed sporicidal effects at ≥300 ppm [3]. Al-Khayat and Blank [1] indicated that eugenol, isoeugenol, gingerol, and zingerone, were all sporostatic against *B. subtilis* spores.

Combining HP and phenolics is a promising strategy to improve the safety of minimally- or nonthermally-processed

***Corresponding author**
Tel : +82-31-670-5184, Fax : +82-31-670-5187
E-mail : ykchung@hknu.ac.kr

foods. The enhanced antimicrobial activity of HP by *tert*-butylhydroquinone (TBHQ) was reported against food borne pathogens such as *Listeria monoytogenes* and *Escherichia coli* O157:H7 [5,18,29]. The cell membrane is suggested to be one of the targets for HP [4]. The lipophilic phenolic compounds may also target the cytoplasmic membrane. The hydroxyl group on some phenolic compounds such as TBHQ may also contribute to their antimicrobial action by destabilizing the cytoplasmic membrane. Therefore, physical damage to the cell membrane may be enhanced by phenolic ingredients during the pressure treatment. In addition, considering the antioxidant properties of TBHQ, presence of this compound during pressure treatment may have altered the cell metabolic pathways governing redox homeostasis [5,18,29]. However, studies depicting this synergy against bacterial spores are lacking. Therefore, the objective of this study was to assess the antimicrobial effect of TBHQ and HP combination against some spores which have importance in the food industry.

Materials and Methods

Strains and media

C. sporogenes OSU392, *B. cereus* ATCC 14579, *B. subtilis* OSU494 were used. *Bacillus* species were grown in tryptose broth (TB; Difco, Becton Dickinson and Co., Sparks, MD, USA). *C. sporogenes* was grown in tryptone-peptone-glucose-yeast extract (TPGY) broth anaerobically. *C. sporogenes* were incubated in an anaerobic chamber containing an atmosphere of 5% H_2, 10% CO_2, and 85% N_2 (Forma Scientific Inc., Marietta, OH, USA). Stock cultures were stored at -80°C in TB or TPGY broth containing 40% (v/v) glycerol. Cultures were transferred in the corresponding broths two times prior to use.

Spore preparation

Spore suspensions of *C. sporogenes*, *B. cereus*, and *B. subtilis* were prepared as described by Sala *et al.* [24] and Khadre and Yousef [13] and cleaned using the method described by Billon *et al.* [2]. Aliquots of 10 µl overnight cultures of each strains were streaked onto nutrient agar (NA; Difco, Becton Dickinson and Co., Sparks, MD, USA) supplemented with 500-ppm dextrose (Difco, Becton Dickinson and Co., Sparks, MD, USA) and 10-ppm manganese chloride and TPGY agar supplemented with 10-ppm manganese chloride, for *Bacillus* strains and *C. sporogenes*, respectively. Plates were incubated aerobically for *Bacillus* strains and anaerobically for *C. sporogenes* at 37°C until >90% of the cells are sporulated. Sporulation was verified by observing the refractile spores using phase-contrast microscope. Spores were harvested by adding 10-ml cold sterile deionized water on each plate, and releasing the colonies containing spores from the surface of the agar with the use of a sterile disposable inoculation loop. Collected spores were washed four times by centrifugation (8,000× *g* for 20 min at 4°C) and resuspension in sterile deionized water. After an additional centrifugation, the resulting pellet was cleaned to separate the free spores from vegetative cells and debris. The cleaning process consisted of aseptically treating each pellet with 100-ml of lysozyme solution (200-µg/ml of lysozyme in 0.05 M potassium phosphate buffer [KH_2PO_4 and K_2HPO_4], pH 8.1 and filter sterilized) for 30 min at 45°C. Then 100-ml trypsin solution (100 µg/ml trypsin in 0.05 M potassium phosphate buffer [KH_2PO_4 and K_2HPO_4], pH 8.1 and filter sterilized) was added and the mixture was incubated for 2 hr. After centrifugation of enzyme-treated spores, the spore crops were washed 3 times in sterile deionized water by centrifugation at 8,000× *g* for 20 min at 4°C. After the last centrifugation, the spore pellets were resuspended in sterile deionized water. The spore suspensions were checked microscopically to ensure that it is free from vegetative cells. The spore suspension was stored at 4°C until used.

Pressure and additive treatments

Spore suspension (1.6 ml) was transferred to a sterile stomacher bag, and 400 µl of additives (DMSO only or TBHQ in DMSO) was added into the sample bag at 200 ppm. Sample bags were sealed with vacuum, and treated with HP at 650 or 700 MPa at 54-72°C for 5 min. Pressure treatments were performed using a hydrostatic food processor (Quintus QFP6, Flow Pressure Systems, Kent, WA, USA) containing 1:1 (vol/vol) glycol/water pressure transmitting fluid (Houghto-Safe 620 TY, Houghton International Inc., Valley Forge, PA, USA). The press consists of a jacketed vessel with end closures, having a 2-liter capacity and is designed to operate at up to 900 MPa (130.5 kpsi). Initial temperatures for samples and pressure-transmitting fluid in the vessel were adjusted to compensate for temperature rise during adiabatic heating upon compression. Based on the measurements done in this study, adiabatic heating was 3 to 4°C per 100 MPa rise in pressure.

Treated spores were heat-shocked (75°C for 10 min) for

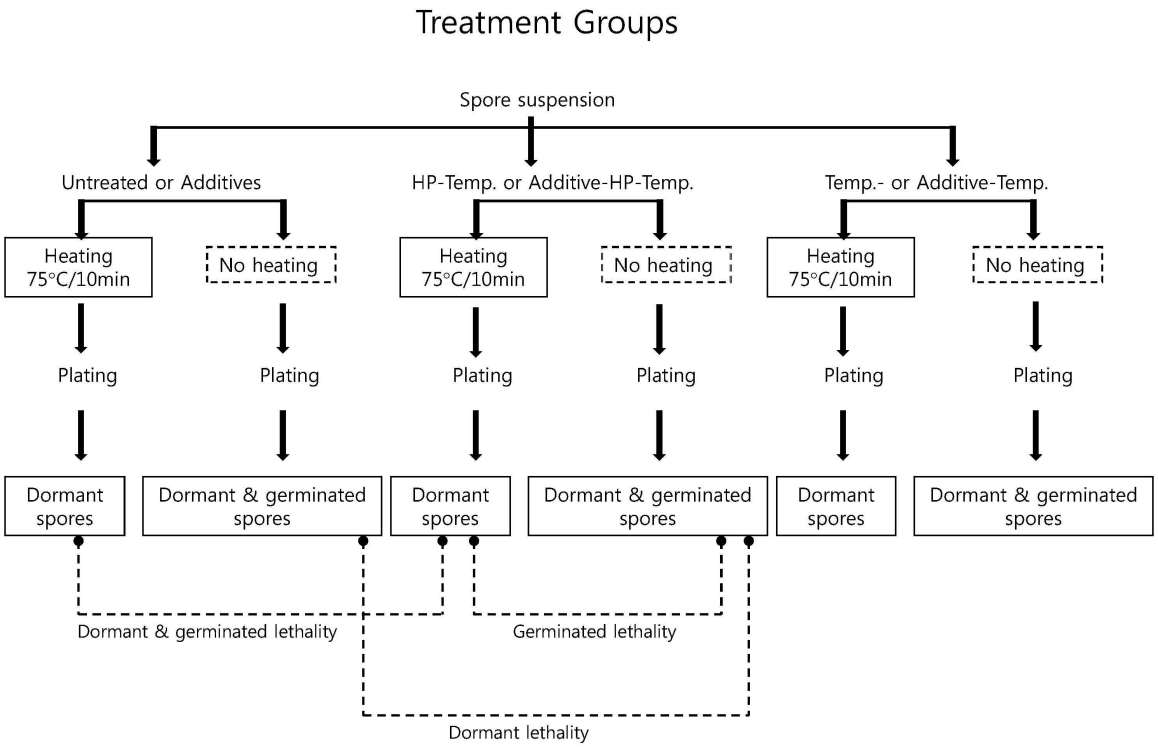

Fig. 1. Treatment groups used in this study.

dormant spore counts, or were plated without heating for counting dormant and germinated spores. Treatment groups are shown in Fig. 1 to clarify the terminology, such as germinated lethality and dormant lethality. Heat-TBHQ combination treatment was also used as a control, but these data are not shown in the figures.

Results and Discussion

When control treatments were tested, TBHQ alone (200 ppm), heat (72°C for 5 min) and TBHQ-heat combination resulted in 0-0.2 log reduction of the bacterial spores (data not shown). This suggests that HP is necessary to inactivate the spores. Though the mechanistic background of the inactivation of bacterial spores is still a matter of discussion, there exists considerable evidence that inactivation of bacterial spores takes place subsequent to the pressure-induced germination [19-21,30].

The population of *B. subtilis* spores was more inactivated by HP than those of *B. cereus* and *C. sporogenes* spores (Fig. 2). Inactivation of *C. sporogenes* spores by pressure was more affected by the germinated population, compared to *Bacillus* spores. In other words, total inactivated populations of *B. cereus* and *C. sporogenes* spores were similar, but the lethality due to the germination was higher in *C. sporogenes* than *B. cereus* when pressurized at 700 MPa and 72°C. Combining TBHQ with HP did not produce more inactivation of *Bacillus* spores. In addition, TBHQ-HP combination treatment decreased the inactivated and germinated population of *C. sporogenes* spores, compared to HP alone (Fig. 2). It may

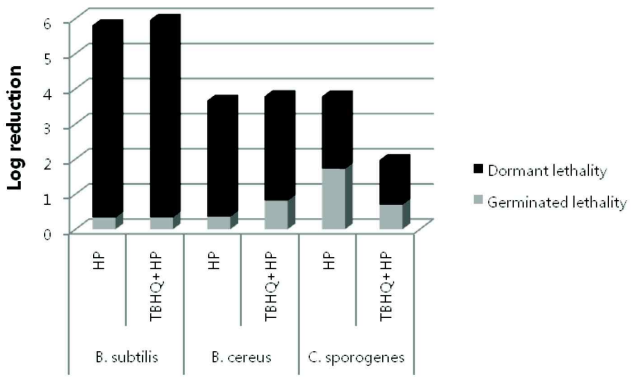

Fig. 2. Inactivation of bacterial spores by high pressure (700 MPa, 72°C, 5 min) and *tert*-butylhydroquinone (200 ppm, dissolved in DMSO) combination. Each bar represents the average values of two independent experiments.

indicate that TBHQ prevented the germination and delayed the outgrowth of *C. botulinum* spores. It was also reported in earlier studies that some essential oils, which contain phenolic compounds, prevented the germination of *C. botulinum* spores [3,12,22]. Otherwise, it may suggest that TBHQ exerts its antioxidant property in anaerobes such as *C. sporogenes*, due to the alteration of cell metabolic pathways governing redox homeostasis [18]. It needs further investigation for the exact mechanism.

Some studies reported that the levels of pressure and temperature where spore inactivation is maximized vary with the spore strain. While some strains such as *B. coagulans* and *B. subtilis* have an optimal pressure which maximizes the inactivation, *B. stearothermophilus* spores inactivated in proportional to the increased level of pressure and temperature [11,25]. Mallidis and Drizou [17] reported that the increase in pressure level (3-30 MPa) did not result in a higher degree of inactivation when the system is kept at certain temperature levels (between 117 and 125°C). Therefore, this study examined the effect of temperature on the pressure-induced germination and inactivation of *Bacillus* spores. When *B. cereus* spores were treated with 650 MPa for 5 min at two different temperatures, 54 and 70°C, the degree of inactivation resulted from HP was similar at both temperatures (Fig. 3). However, the lethality due to the germinated population was only observed at 54°C (Fig. 3A). The inactivation of *B. subtilis* spores increased when pressurized at higher temperature (70°C) (Fig. 4). On the other hand, as in the case of *B. cereus* spores, the degree of germination-induced lethality for *B. subtilis* spores also decreased at 70°C (Fig. 4B). Other studies also suggested that the germination system was impaired when spores were treated with high temperature (ex. 30°C vs. 70°C) [14] and high pressure (ex. 100 MPa vs. 600 MPa) [30].

When spores were treated with DMSO-HP and TBHQ-HP combination, these treatments seemed to protect the spores against HP, especially at 54°C (Figs. 3A and 4A). The degree of inactivation decreased when treated with combination, compared to HP alone. While DMSO was intended to serve only as a solvent for TBHQ, DMSO exhibited some pro-

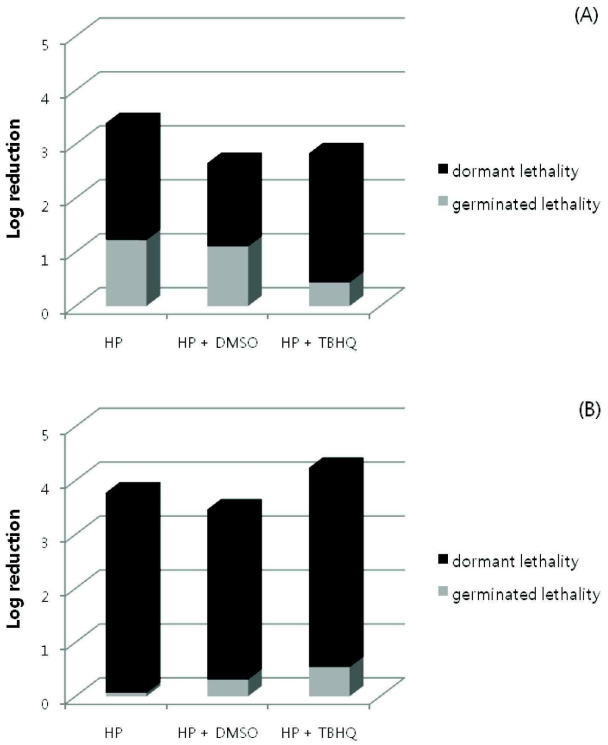

Fig. 3. Inactivation of *Bacillus cereus* ATCC 14579 spores by high pressure (650 MPa, 5 min), DMSO-HP combination, and *tert*-butylhydroquinone (200 ppm, dissolved in DMSO)-HP combination (A) at 54°C (B) at 70°C. Each bar represents the average values of two independent experiments.

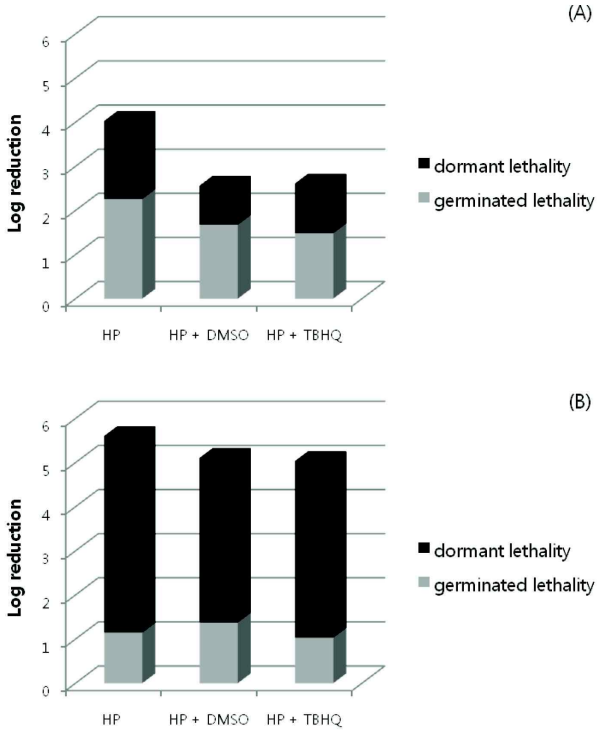

Fig. 4. Inactivation of *Bacillus subtilis* OSU494 spores by high pressure (650 MPa, 5 min), DMSO-HP combination, and *tert*-butylhydroquinone (200 ppm, dissolved in DMSO)-HP combination (A) at 54°C (B) at 70°C. Each bar represents the average values of two independent experiments.

tection effect against the spores. This study examined the inactivation of bacterial spores using HP and TBHQ combination. Mechanisms involved in inducing germination by HP and using a subsequent sporicidal agent such as TBHQ will be needed for the future investigation.

Acknowledgement

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초록 : 초고압과 식품첨가물 병용을 이용한 세균 포자의 살균

정윤경*
(국립한경대학교 영양조리과학과)

초고압의 미생물에 대한 살균 효과는 다른 제어 방법을 더 적용함으로써 증대 될 수 있다. 본 연구의 목적은 식품 산업에서 중요한 세균 포자들을 *tert-butylhydroquinone (TBHQ)*를 처리하여 초고압 살균효과를 증진시킴을 알아보고자 한다. *Clostridium sporogenes*, *Bacillus cereus*, *Bacillus subtilis*의 포자를 준비하였다. Dimetylsulfoxide (DMSO)에 용해시킨 TBHQ 200 ppm을 함유한 포자액을 650 또는 700 MPa로, 54-72°C에서 5분간 처리하였다. 세균 포자의 사멸은 초고압 처리 이후에만 일어났다. *Bacillus subtilis* 포자가 *B. cereus*나 *C. sporogenes* 포자보다 초고압에 의해 더 많이 사멸되었다. *C. sporogenes* 포자의 사멸은 *Bacillus* 포자에 비해서 초고압에 의한 germination이 더 일어나면서 이루어 졌다. *Bacillus* 포자는 54°C에서 초고압 처리 때 보다 72°C에서 처리했을 때 더 많이 사멸되었다. 하지만 germination에 의한 사멸은 70°C에서 감소되었다. 포자를 DMSO와 초고압, TBHQ와 초고압의 병용 처리를 했을 때, 특히 54°C에서는 포자 사멸이 덜 일어났다. 세균 포자의 사멸에 대한 좀 더 나은 이해를 위해서는 초고압에 의한 germination 과 다른 포자 억제 물질과의 상호 작용에 대한 메커니즘 연구들이 필요하다.