Effect of the Combination of High Hydrostatic Pressure and Alkaline Electrolyzed Water on the Reduction of Heat Resistance of Bacterial Spores

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The effect of combined use of alkaline electrolyzed water (AlEW) on the reduction of heat resistance of bacterial spores by high hydrostatic pressure processing (HPP) was investigated in this study. No reduction of heat resistance of bacterial spores, which was defined as the spore survival by heat treatment at 80°C for 15 min, was observed by the treatment of single HPP with 30MPa at 50°C even for 6 hours. However, a 3-log decrease in the viable bacterial spores was obtained by the combination of AlEW pretreatment with 1 hour of HPP treatment. An additional 2 hours duration of HPP treatment could inactivate more 2 logs of the viable bacterial spores. The obtained D value of bacterial spores treated by HPP was decreased to one-eighth by the pretreatment with AlEW when compared with the control sample. In case of the temperature during HPP treatment was 70°C, bacterial spores did not reduce its heat resistance with lower pressuring levels. In case of the temperature during HPP treatment is high with lower pressure levels, bacterial spores did not reduce its heat resistance even when AlEW was combined as the pretreatment. It was considered that the decrease in heat resistance by AlEW was resulted from the weakening of surface layer of spores by protein dissolution with alkaline substance. No clear effect of high negative redox potential, which is a unique property of AlEW, on the reduction of heat resistance was recognized.

Key words : High Hydrostatic Pressure / Alkaline Electrolyzed Water / Bacterial Spores / Heat Resistance.

INTRODUCTION

In the food industry, ensuring the microbiological safety of food is extremely important, and the introduction of a reliable microbicidal technique such as heat treatment is surely required. In addition, since the achievement of high quality as well as high safety of food is required, non-thermal technologies which does not rely on thermal energy is also widely applied (Zhang et al., 2019). Various chemical and non-thermal physical techniques have been applied in a practical situation of food processing. Among them, high hydrostatic pressure processing (HPP) is already installed in various food companies since it has advantageous as low energy, uniform treatment (Yamamoto, 2017). However, in the case that bacterial spores which formed by Bacillus and Clostridium and have extremely high resistance for stresses are the target organism in microbicidal process. Effective sterilizing performance by solo-HPP is not obtained because previous research indicated that more than several hundred MPa of pressure value was required for the expected level of spore inactivation (Sonoike, 1997, San Martin et al., 2002, Patterson, 2005, Nakayama et al., 1996, Syed et al., 2016). On the other hand, it has been reported that HPP with medium level induce spore germination and reduce heat resistance, and then the following or simultaneous heat treatment can kill bacterial spores without high temperature level (Gould and Sale, 1970, Wimalaratne and Farid, 2008, Okazaki et al., 2000, Reineke et al., 2011, Kobayashi et al., 2014). The reduction treatment
of heat resistance of bacterial spores mainly based on such medium HPP (~200MPa) is also recognized by the simultaneous treatment with organic acid, nisin, and carbon dioxide, etc. (Kalchayanand et al., 1998, 2003). Although there are various reports suggesting that the combining treatment with HPP, high level of pressure such as 200-400MPa is required for the inactivation of bacterial spores. The huge and expensive equipment resulted from high pressure level prevents the introduction of HPP to small and middle-sized enterprises, which are the majority of the food manufacturing industry. If it is possible that the further reduction of the pressure level of HPP for the reduction treatment of heat resistance, it is expected that the introduction of high-pressure technology will proceed even for the production of processed foods containing bacterial spores.

The heat resistance of spores is due to a high internal dehydration state (Nakashio and Gerhardt, 1985), and it is important to induce a physiological or non-physiological germination state in order to reduce the heat resistance (Moir and Smith, 1990). Therefore, it is considered necessary to weaken the outer shell and efficiently introduce water into the inside of bacterial spores. However, the high hydrophobicity of the spore surface prevents easy direct contact of water molecules (Wiencek et al., 1990). In addition, since a physically strong cortex layer is formed on the surface layer of the spore, it is difficult to introduce water into the inside by simple HPP treatment. From these backgrounds, we examined the reduction of heat resistance of bacterial spores by the combination of alkaline electrolyzed water (AlEW) and HPP in this study. AlEW is one of the functional waters generated on the cathode side in a diaphragm-type electrolyzer (Huang et al., 2008). Although it does not have a high microbicidal potential like acidic electrolyzed water generated on the anode side (Ovissipour et al., 2015), several researchers reported that it can be used as a supporting agent that assists the effects of other disinfectants including acidic electrolyzed water by removal of protein residue (Koseki et al., 2004, Rahman et al., 2011, Xie et al., 2012, Hao et al., 2015). In addition, since AlEW is known to have an emulsifying effect (Takehara et al., 2001), it is supposed to have the potential of lowering the high hydrophobicity of spore surface and enhancing the contact of water with the outer layer of bacterial spores. Furthermore, since AlEW shows a negative ORP (Oxidation Reduction Potential) value, the strong disulfide bond of the protein consisting the outer layer of spores, such as spore coat and cortex, could be reduced to the SH group, and make them weakened physically, chemically and electrically. However, there is little information that the investigation of combining treatment of HPP with AlEW for the reduction process of heat resistance of bacterial spores. In this study, we examined the effect of combining treatment of low level HPP with AlEW on the heat resistance of Bacillus subtilis spores.

**MATERIALS AND METHODS**

**Test bacterium**

Bacillus subtilis subsp. subtilis NBRC3134 (B. subtilis) obtained from the collection of National Institute of Technology and Evaluation Biological Resource Center – Japan (NBRC), were used in this study. The obtained bacterium was hydrated by 0.1% peptone, water, and cultured by nutrient broth containing Hipolypeptone (Nihon Pharmaceutical Co., Ltd., Tokyo): 10g, Yeast extract (Becton Dickinson and Company, Franklin Lakes, NJ, USA): 2g, MgSO4•7H2O (FUJIFILM Wako Pure Chemical Co., Osaka): 1g, for 1 L of deionized water. Cultured B. subtilis cells were suspended in glycerol medium and stored at -80°C.

**Preparation of spore suspension**

The frozen sample was thawed, and cultured with trypticase soy broth (TSB: Becton Dickinson and Company) at 30°C for 24 hours. The cultured TSB was applied onto the surface of a magnesium sulfate-containing nutrient agar medium (HiPolyptone (Nihon Pharmaceutical): 10g, Yeast extract (Becton Dickinson and Company): 2g, MgSO4•7H2O (FUJIFILM Wako Pure Chemical): 1g, Agar (FUJIFILM Wako Pure Chemical Co.): 15g for 1 L of deionized water), and cultured at 30°C for 1 week in order to form spores. The formed bacterial spores were harvested by stainless steel spreader and suspended in sterile distilled water. A cell pellet was prepared by centrifugation (3000×g, 10 min, himac CT15RE, Koki Holdings Co., Ltd., Tokyo), and re-suspended by pipetting with another sterile distilled water. This process was repeated three times for washing bacterial spores. The pretreated bacterial spores were diluted with sterile distilled water to an optical density of 2 (ca. 10⁷ cells/mL) at 490 nm by spectrophotometer (U-2900, Hitachi High-Tech Science Co, Tokyo). The spore suspension was transferred into glass test tube, and heated at 70°C for 60 min with heat-block (DTU-1B, TAITEC Co., Saitama, Japan) in order to kill vegetative type cell. The prepared spore suspension was stored at 4°C in refrigerator until use.

**Test solutions and its contacting process with bacterial spores**

AlEW used in this study was obtained by an electrolyzed water generator (ROX10WB, Hoshizaki Co., Aichi, Japan) set at 10A of electric current and 10V of voltage. The ORP, EC (Electric Conductivity) and pH were -835mV, 2.22mS/cm and 10.5, respectively, produced from 0.1% concentration of saline solution. Saline solu-
tion was prepared by sodium chloride (NaCl, FUJIFILM Wako Pure Chemical) and reverse osmosis (RO) water with 0.3 to 1.0 MΩcm, and provided to electrolyzed generator tank. No active chlorine was detected in the generated AIEW by using chlorine meter (AQ-202P, SIBATA SCIENTIFIC TECHNOLOGY Ltd., Saitama, Japan).

For the evaluation of the reduction potential of AIEW comparing with other solution at same pH level, sodium hydroxide (NaOH, FUJIFILM Wako Pure Chemical), potassium hydroxide (KOH, FUJIFILM Wako Pure Chemical) and sodium carbonate (Na2CO3, FUJIFILM Wako Pure Chemical) were used. Deionized water with 15MΩcm was also used as the control liquid.

Besides, for the evaluation of SH group by the reduction of disulfide bonds, 100M concentration of dithiothreitol (DTT, nacalai tesque Inc., Kyoto), tris (2-carboxyethyl) phosphine (TCEP, nacalai tesque) and ascorbic acid (AsA, FUJIFILM Wako Pure Chemical) were used.

A 2 mL of the prepared spore suspension was centrifuged at 3500×g for 5 min in a 2 mL plastic tube to obtain spore pellet at the bottom, and supernatant was discarded. A 2 mL of test solution was poured into tube and thoroughly mixed with spore pellet by pipetting and vortexing for 1 min, and then centrifuged again. This process was repeated two times, and finally suspended in 2 mL of sterile distilled water was used as a spore sample for HPP treatment.

High hydrostatic pressure processing

Approximately 10 mL of spore suspension sample contacted with various test solution was heat-sealed in a 2×5cm² plastic bag pouch (RN-1018H, Meiwa Pax Co., Ltd., Osaka) without air. Sample pouch was set into the vessel of semi-automatic hydrostatic pressure device (S2-250/0.3, Echigo Seika Co., Ltd, Niigata, Japan) with tap water shown in Fig. 1, and pressure treatment was performed at target level. The vessel volume and pressuring speed are 300mL and 20 MPa/sec, respectively. The pressuring conditions in this study were set the combination of 30-50MPa at 30-70°C for the duration of time. About 2, 3 and 3.5 seconds were required to reach target pressure levels of 30, 40 and 50, respectively. The temperature was maintained by water circulator (LBX-300, AS ONE Co., Osaka), and the target pressure level was kept constant while monitoring the pressure sensor installed inside the vessel during treatment. The pressure was reduced to atmospheric level after treatment, the heat resistance of bacterial spores was evaluated following methodology. The time required for decompression was less than one second.

Evaluation of heat resistance

The HPP treated pouch of spore suspension was taken out and heated at 80°C for 15 min with hot water bath in order to inactivate the bacterial spores which lost heat resistance by HPP. The heat-treated pouch was cooled down to 20°C immediately, and aseptically opened by scissors. Then bacterial suspension was serially diluted by sterile distilled water. A 0.1 mL of diluted suspension was applied onto standard method agar (Nissui Pharmaceutical Co., Ltd., Tokyo) plate, and the appeared colonies were counted for incubation at 30°C for 24 hours. The heat resistance of bacterial spores before/after HPP treatment was evaluated by the logarithm of colony forming unit per milliliter.

Measurement of protein

The eluted protein by HPP treatment was quantitatively evaluated by Bradford method. The HPP treated sample was centrifuged (15000×g, 10 min). A 0.03 mL of the obtained supernatant was mixed with 1.5 mL of Bradford reagent (PierceTM Coomassie Plus (Bradford) Assay kit, ThermoFisher Scientific, Tokyo), then the optical density at 595 nm was measured with spectrophotometer (Hitachi High-Tech Science).

Measurement of the amount of thiol group

Quantitative evaluation of thiol group was conducted by DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) method using a thiol assay kit (Redox Assay, Metallogenics Co., Ltd., Chiba, Japan) accordingly to the manufacturer’s instruction. The HPP treated sample was centrifuged (15000×g, 10 min), and a 20 µL of the obtained supernatant was transferred to 96 well microtitor plate (Thermo Fisher Scientific K.K., Tokyo), and mixed with the provided reagents individually. The optical densities of the colored samples at 412 nm were measured by microplate reader (ELx800UV, BioTek Instruments, Inc.)
A 1 mM concentration of N-Acetyl-L-cysteine (Nacalai Tesque Inc.) and deionized water were used as standard and blank samples, respectively.

Statistical analysis

All data obtained in this study were the means of 5 replicates. Regarding statistically significant differences, Student’s t-test was used for comparison between the two groups, and one-way ANOVA was used for multiple groups using Tukey-Kramer’s multiple range test by SPSS 26 (IBM Japan, Tokyo).

RESULTS AND DISCUSSION

The effect of HPP treatment with/without AlEW contact on the heat resistance of bacterial spores, which was defined as the number of survived bacterial spores by heat treatment at 80°C for 15 min, was shown in Figure 2. The heat resistance of bacterial spores did not reduce by single HPP treatment at 50°C with 30MPa even for continuous 6 hours, but 2 to 3 logs reduction of viable number of bacterial spores were obtained by the combination of AlEW with HPP for 1 hour. An additional 2 logs of viable spores were inactivated by further 2 hours duration of HPP treatment. When bacterial spores were pretreated with AlEW, the D value (time required to kill 90% of viable spores), which was obtained from the linearly decreasing portion of inactivation curve, was approximately 0.67 (hr). This obtained D value was approximately one-eighth in comparison to that of control sample (5.3 (hr)). However, even if the sample pretreated with AlEW was subjected to HPP treatment for a long time of 3 hours or more, no further reduction of heat resistance of bacterial spores was observed. It seemed that there was a clamp of bacterial spores, or a various group with different heat resistance, therefore the linear decrease in the viable count was not observed (Tay et al., 2003).

Figure 3 shows the changes in the heat resistance of bacterial spores treated by HPP with various combinations of pressure and temperature with and without AlEW pretreatment. The heat resistances of bacterial spores were decreased as the pressuring level increased, and the more effective inactivation was recognized in the bacterial spores pretreated with AlEW. However, even when the temperature during HPP treatment was higher, the obtained results showed that the heat resistance of bacterial spores was not effectively reduced. Heat resistance of bacterial spores pretreated with AlEW decreased at 30, 40 and 50°C during HPP treatment. But at 70°C with 30 and 40 MPa of HPP treatment, there was no reducing effect on the heat resistance of bacterial spores pretreated with AlEW at all. Even when the treatment pressure was 50 MPa, there was only the effect of reducing the heat resistance to the same extent as when HPP treatment was performed at 40 or 50°C. This result was similar to the previous report that there was an optimum temperature in the reduction of heat resistance of bacterial spores by low level HPP treatment (Furukawa and Hayakawa, 2001).

In addition to the physical and chemical damage to the outer layer of bacterial spores affecting the changes in heat resistance observed in this study, the activation properties of macromolecules such as enzymes existing in cells also need to be considered for explaining this result. Among the enzyme proteins existing in the core, those involved in germination play an important role in reducing the heat resistance of bacterial spores, and it is thought that their activity is enhanced by external stimulation and water influx (Foster and Johnstone, 1990). It is thought that these germination-related enzymes also have an optimal temperature range for activation (Gould and Sale, 1970), and heat resistance supposed to be decreased due to germination caused by the combined use of AlEW and HPP treatment up to about 50°C. However, it is presumed that the temperature was too high to germinate at 70°C during HPP treatment, and as a result, high heat resistance was maintained during the process. The decrease in heat resistance obtained in the combination of 50 MPa of HPP at 70°C with AlEW was not supposed an enzymatic germination. This reduction of heat resistance could be resulted from a structural weakening of spore coat and cortex layer due to a dissolution by NaOH contained in AlEW.

The outer layer of the bacterial spores having heat resistance is formed by a cortex layer and spore coat...
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ened by alkaline substance contained in AIEW, and enhanced the decrease in heat resistance due to the following HPP treatment. In order to verify the difference from the simple alkaline reagent, the same pH aqueous solution of NaOH, KOH, and Na₂CO₃ as AIEW was used, and the difference in the influence of HPP treatment on the reduction of heat resistance was examined.

Figure 4 shows the difference in heat resistance when HPP treatment was performed at 30MPa for 1 hour at 30°C and 50°C. At 30°C of the holding temperatures, no decrease in heat resistance was observed as with AIEW, whereas the combination of HPP with any alkaline

FIG. 3. Effect of temperature during high pressure processing (HPP) for 1 hour on the survival of bacterial spores pretreated with/without alkaline electrolyzed water (AIEW). Survival number of bacterial spores after heat treatment at 80°C for 15 min were defined as the heat resistance. Data are presented as mean ± standard error (n=5). Asterisks indicate significant difference in spore survival between HPP and HPP with AIEW at p<0.05 (*) and p<0.01 (**). Different large and small letters indicate significant differences in spore survival among different treatment temperature during HPP and HPP with AIEW, respectively.

FIG. 4. Survival of the heat-treated bacterial spores pretreated by high hydrostatic pressure processing (HPP) at 30MPa for 1 hour combined with various alkaline solution at 30°C (□) and 50°C (■). Survival number of bacterial spores after heat treatment at 80°C for 15 min were defined as the heat resistance. Data are presented as mean ± standard error (n=5). Asterisk indicates significant difference in spore survival between different treatment temperature at p<0.01.

FIG. 5. Amount of protein in the supernatant of spore suspension treated with the combination of HPP at 30MPa, 50°C for 1 hour with different alkaline solution (PS; physiological saline). Data are presented as mean ± standard error (n=5). Different letters mean significant difference among the amount of protein treated with solutions at p<0.05.
like crystalline proteins with high content of cysteine and cystine. Whereas the amounts of thiol groups produced by the reduction treatment of disulfide bond with AsA and AlEW were small, that is almost the same as physiological saline, a large amount of thiol groups were produced by DTT and TCEP treatment (Fig. 7). From this result, it was inferred that the reducing effect of AlEW on the disulfide bond of the protein existing in bacterial spores had little effect on the reduction of heat resistance.

**CONCLUSION**

Several researchers have already reported that AlEW has little direct disinfecting potential of food and food processing environment so far. Therefore, AlEW is used as a pretreatment agent expecting a supporting role for the washing and decontaminating potential of other antimicrobial processing. In this study, it was clarified that heat resistance of bacterial spores was effectively reduced when used in combination with low level of HPP, and it was considered that the effect was resulted from the structural weakening due to alkaline dissolution of some proteins in the surface layer of spores. On the other hand, the effect of high reducibility potential, which is one of the characteristics of AlEW, on the heat resistance of bacterial spores was not clear. Since decreasing the pressure level of HPP treatment is extremely valuable industrially, we will continue to investigate more effective combinations for reducing the heat resistance of bacterial spores and accumulate data that will contribute to the introduction into the food industry.
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