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Original Research Article

Evaluation Injury Characteristics of *Bacillus* Spores by Combination of Hydrostatic Pressure with Alkaline Electrolyzed Water

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ABSTRACT

The effect of combining procedures of high hydrostatic pressure (HHP) with alkaline electrolyzed water (AlEW) on the injury characteristics of bacterial spores was investigated. Effective reductions of survival *Bacillus subtilis* spores by a combination treatment of AlEW with HHP were obtained after the treated spores were heat shocked at 80°C for 15 min. No survival was observed by applying 100 MPa of HHP treatment. Approximately 90 to 99% of *Bacillus* spores treated by HHP with AlEW were injured or lost heat resistance and there were 1 to 2 logs differences in the survival comparing with the unheated spores. Double culture method (DCM) for assessing the injury characteristics of bacterial spores was performed by using trypticase soy broth supplemented with chloramphenicol (CP) or rifampicin (RFP), which were related to the synthesis inhibition of protein and RNA, respectively. Severe injury of the treated bacterial spores assessed by RFP supplemented broth was obtained at 50 MPa with and without AlEW. Especially at 50 MPa, considerable synergistic effect of HHP and AlEW on spore injury was observed. These results suggested that injury associated with the biosynthesis of some enzymes was observed at relatively low pressure level.

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INTRODUCTION

Thermal processing is a major method for reducing the number of microorganisms in various food commodities. However, huge amount of thermal energy to inactivate bacterial spores, which are often existing in various agricultural commodities and have high resistance against physical stresses, results in the degradation of internal/external quality of fresh/processed foods, such as flavor, texture, surface color, functional components (Miyamoto, 2009). In order to reduce the negative effect on the quality of foods by thermal energy, various non-thermal antimicrobial technologies have been investigated for the actual application as an alternative. Although there are disadvantages about the inactivation process of bacterial spores (Ogino and Nishiumi, 2015), high hydrostatic pressure (HHP) treatment has been appraised as the useful technology with small degradation of food quality.

Bacterial spores have high resistance against various chemical and physical stresses as previously described; it was also well known that the injured or germinated bacterial spores induced by chemical/physical treatment lost their resistance against the antimicrobial processes without severe condition (Sonoike et al., 1997). Some researchers reported that HHP had a potential for reducing heat resistance of bacterial spores (Tsuchido et al., 1992). Comparing with a single heat treatment, additional 3-4 logs reduction of bacterial spores, previously treated by a 200 MPa of HHP at 50°C for 10 min, was obtained at 80°C (Bartlett, 2002). A further reduction of pressure level is really important to be considered for the application of HHP antimicrobial processing in the food industry by appropriately combining the procedure with additional technologies.

In our previous study, the combination of HHP with alkaline electrolyzed water (AlEW) generated by electrolysis of diluted saline solution in a cathode side of diaphragm tank. The system had a potential to reduce the heat resistance of bacterial spores. Although the reduction of heat resistance was considered as a result from spore injury by the combining treatment, the detailed mechanisms of the injury process had not been elucidated.

Double culture method (DCM) using standard culture media, such as trypticase soy agar (TSA) with/without typical growth-inhibiting agents has been used to determinate of the number of injured cells treated by an inactivation process. DCM is useful for evaluation of the number of injured cell (Tsuchido, 1999), however, there are some disadvantages for the clarification of detail mechanisms of injury process. For a further understanding of the injury characteristics of bacterial spores treated by AlEW with HHP, the modified DCM was required to be applied.

In this study, it was investigated the evaluation of injury characteristic of bacterial spores treated by AlEW, HHP and their combination using DCM applied with antibiotics supplemented growth media.

MATERIALS AND METHODS

Tested bacterium and preparation of spore suspension

Bacterial spores used in this study were *Bacillus subtilis* NBRC3134 obtained from National Institute of Technology and Evaluation (NITE, Japan). Stock culture of B. *subtilis* was transferred to trypticase soy broth (TSB, Becton Dickinson and Company, USA), and cultured at 30°C for 24 h. A 0.1 mL of the cultured broth was applied and spread onto standard method agar (SMA, Nissui Pharmaceutical Co. Ltd., Tokyo) plates supplemented with 1 g/L of $MgSO_4 \cdot 7H_2O$ (Wako Pure Chemicals Ltd., Tokyo) and incubated at 30°C for 7 days for spore formation. Spores were collected by flooding the cultured agar surface with sterile physiological saline (PS) solution, and then scraping the surface with a sterile glass rod. Harvested spores were washed three times in sterile PS solution by centrifugation at 3500×g for 10 min, followed by heat treatment at 70°C for 1 h in order to kill the vegetative cells. The spore concentration of prepared suspension was approximately 10^8-10^9 colony forming unit per milliliter (CFU/mL).

AlEW contracting treatment

AlEW was generated using an electrolysis apparatus (ROX-20TA, HOSHIZAKI Electric Co. Ltd., Aichi, Japan). The current passing through the electrolysis apparatus was set at 10 A, and the voltage between the electrodes was set at 10 V. Oxidation reduction potential and pH were -835 mV and 11, respectively. PS solution was used as a control water.

Contacting treatment of bacterial spores with AlEW was performed by suspending in test solution in plastic tube for 1 min, and centrifuged at 3500×g for 5 min. The pellets of bacterial spores on the bottom of plastic tube was re-suspended by vigorous pipetting and vortex-shaking for 3 min. This process was performed two times, and treated spores were finally suspended with PS solution.

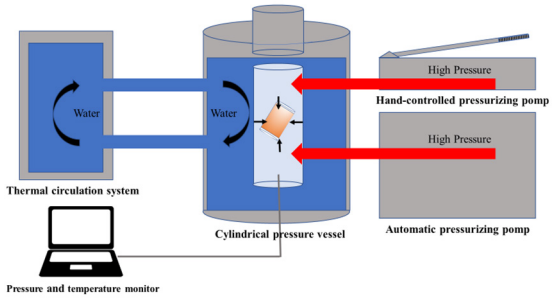

Fig.1. Schematic diagram of pressurization apparatus

HHP treatment

A 5 mL of spore suspension was poured into plastic bag without air, and aseptically heat sealed to prepare 2x5 cm^2 pouch for the following treatment. HHP treatment was immediately performed by the pressurization apparatus (Fig. 1, Echigo Seika Inc., Niigata, Japan) with a cylindrical pressure vessel (inside volume = 300 mL). Internal temperature of vessel was controlled by thermal circulation system (LBX-350, AS ONE, Co., Osaka, Japan) at 50°C. HHP conditions were prepared by the combined pressurization system equipped both automatic and hand-controlled pressurizing pump connected to the vessel. HHP treatments were performed at 30, 50 and 100 MPa for 1 hour. Internal pressure and temperature during treatment were monitored by sensors equipped in the vessel. Rates of pressurization and depressurization were approximately 20 MPa/sec and 100 MPa/sec, respectively.

Evaluation of spore survival

The treated pouch was immediately cooled down to ambient temperature, and aseptically opened by sterile scissors. Treated suspension was transferred to glass test tube, and subjected to heat shock at 80°C for 15 min in the electric heating block in order to kill the injured cells. A 1 mL of spore suspension was serially diluted by PS, and a 0.1 mL was applied and evenly spread onto TSA plate (Becton Dickinson and Company, USA). TSA plates were incubated at 30°C for 24 h, and the survival counts were calculated by the number of colonies appeared after incubation.

Evaluation of injury characteristics of bacterial spores

The treated pouch was immediately cooled down to ambient temperature, and aseptically opened by sterile scissors. A 0.1 mL of spore suspension was inoculated in TSB supplemented with chloramphenicol (CP, Wako Pure Chemical Co.) or rifampicin (RFP, Wako Pure Chemical Co.), for evaluation of the injury characteristics related to synthesizes of protein (enzyme) or RNA, respectively (Sawai et al., 1995). The concentration of CP and RFP in TSB was 100ppm, which was the maximum level without influence on the growth of B. subtilis spores used in this study. After incubation for 24 h, a 200 mL of cultured media was transferred into microtiter plate, and the optical density (OD) at 600 nm was measured by microtiter plate reader (Elx800UV, Biotek Instruments, Inc., Vermont, USA).

Injury characteristics of bacterial spores were determined by injury ratio (I_R) calculated by following equation;

$$I_R = \frac{N_I - N_{AI}}{N - N_A}$$

Where I_R is the injury ratio to the intact spores. N, N_A, N_I and N_{AI} are **ODs of cultured pure TSB inoculated with untreated bacterial spores**, cultured TSB supplemented with antibiotics inoculated with the untreated bacterial spores, **cultured pure TSB inoculated with HHP or a combination of AlEW and HHP treated bacterial spores, and cultured TSB supplemented with antibiotics inoculated with HHP or a combination of AlEW and HHP treated bacterial spores**, respectively. In case the calculated value of I_R is more than 1, it was considered that some specific properties of bacterial spores were influenced by single or synergistic effect of AlEW, HHP and its combination.

All experiments were performed triplicate and the means of spore survival and injury ratio were calculated. Statistically significant difference in survival of bacterial spores between before and after treatment were determined at $p < 0.05$ by Student's t-test.

RESULTS AND DISCUSSION

It is well known that microbicidal efficiency of HHP treatment accelerate with an increase in the pressure level. In this study, HHP treatment with 100 MPa had a potential to inactivate bacterial spores compared with that at 30 MPa as shown in Figure 2. In addition, 0.5-1.5 logs reductions of spore survival was obtained by the combination of AlEW and HHP regardless of differences in the pressure levels. Approximately 90-95% of bacterial spores were injured and lost heat resistance by single HHP or its combination with AlEW less than 50 MPa, because the heat shock treatment at 80°C for 15min after HHP with/without AlEW could inactivate 1-1.5 logs of bacterial spores at 30 and 50 MPa. On the other hand, all bacterial spores pressurized at 100 MPa both with and without AlEW could be injured and lost heat resistance since no survivals were obtained after a heat shock treatment at 80°C.

In DCM procedure, as the injury assessment of bacteria treated by some bactericidal technology, typical growth agar media salt-supplemented or water activity-controlled were generally applied for the recognition of the difference in the counted number of the appeared colonies (Tsuchido and Sakamoto, 2018). However, typical colony counting procedure was difficult to understand the injury characteristics of the stressed bacteria. Therefore in this study, the optical densities of growth culture broth supplemented with two antibiotics of CP and RFP before/after incubation were applied to the evaluation of injury characteristics of bacterial spores. CP and RFP have specific inhibiting influences on the protein (enzyme) and RNA synthesizes (Cundliffe and McQuillen, 1967; Sensi, 1983), and the injury characteristics could be assessed by the differences in the sensitivities of injured and intact bacterial cells.

Figure 3 shows the calculated injury ratio obtained from the measured OD values of TSB supplemented with CP and RFP. The higher sensitivities of treated bacterial spores against CP supplemented broth were obtained in comparison with the control sample. AlEW treatments did not have synergistic effect on the injury ratio at 50 MPa. On the other hand, high injury ratios of the treated bacterial spores assessed by RFP supplemented broth were also obtained at 50 MPa with and without AlEW comparing with control sample. A considerable synergistic effect of AlEW on spore injury was observed. These results suggested that injury related to the biosynthesis of proteins such as enzymes (Setlow et al., 2003) was observed at relatively low pressure level, whereas the injury related to RNA synthesis was influenced by slightly higher pressure level. Further studies are needed to clarify the mechanisms of the synergistic efficiencies observed at 30/50 MPa with CP broth and at 50 MPa with RFP broth. It was considered that AlEW synergistic effect could appear under slightly stressed conditions with HHP for bacterial spores.

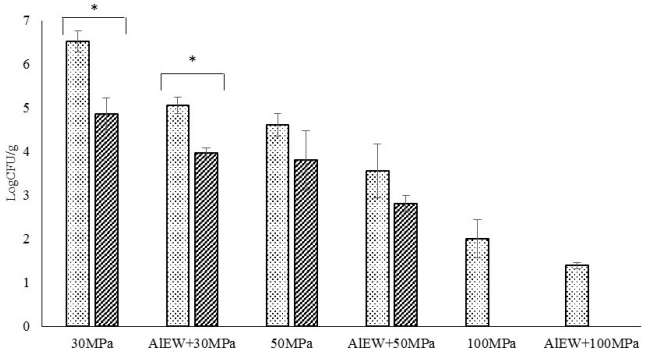

Fig. 2 Survival of bacterial spores treated with/without alkaline electrolyzed water and high hydrostatic pressure followed by heat treatment. ▨ : before heating, ▨ : after heating

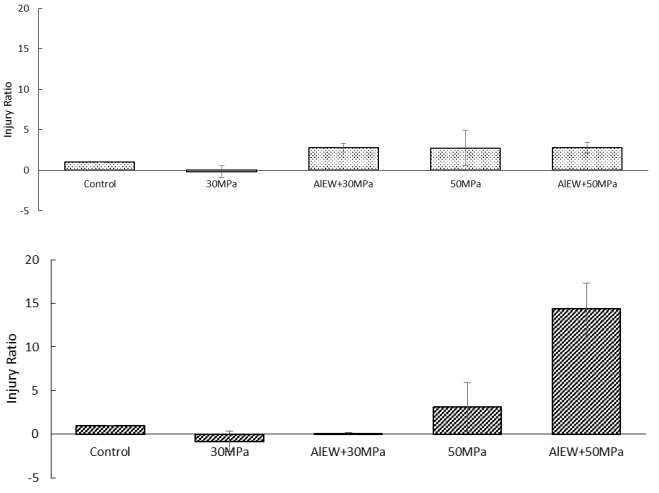

Fig. 3. Injury ratio of Bacillus subtilis spores treated by HHP with or without AlEW cultured with chloramphenicol (▨) or rifampicin (▨) supplemented trypticase soy broth.

Although most of microorganisms are injured when exposed to various stresses, it is well known that microbes have a potential to develop a specific function corresponding to the type of stress and recover as they grow (Tsuchido, 1999). Considering about HHP treatment, typical injury of bacterial cells could be the enzyme denaturation and the destruction of cell membrane during physical process. The combination of AlEW and HHP might accelerate the injury of bacterial spores related to HHP treatment.

CONCLUSIONS

In this study, it was obviously clarified that the HHP treatment is could injure bacterial spores through synthesis of protein and RNA, and the combination of AlEW and HHP increased the efficient of injury to bacterial spores. However, further investigations are strongly required for the clarification of the relationship between HHP and AlEW injury characteristics and reduction of heat resistance. The analysis of molecular level such as gene expression would be important to evaluate the detail mechanism of HHP injury. And the possibility of the application of combining technology of HHP with ALEW should be considered in the further studies.

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