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Note

High Pressure Carbon Dioxide Decreases the Heat Tolerance of the Bacterial Spores

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Effect of high pressure carbon dioxide treatment (35˚C, 6.5 MPa) (HPCT) on the heat tolerance of bacterial spores was investigated. Spores of *Bacillus coagulans* and *B. licheniformis* were subjected to HPCT following heat treatment (80, 85 and 90˚C for 30 min) (HT). Spores of both types were sterilized completely in 30 min-90˚C HT after HPCT. These results indicated that HPCT greatly decreased the heat resistance of spores.

Keywords: carbon dioxide, heat, bacterial spore, sterilization, inactivation

In food sterilization, inactivation of dormant bacterial spores is the main objective. To inactivate bacterial spores, heat sterilization, the most commonly used method in food industry, usually results in detrimental changes in the nutritive value, color and flavor of foods (Joslyn, 1991; Walker & LaGrange, 1991). In contrast, hydrostatic pressure treatment (HPT) can inactivate microorganisms without altering the flavor or nutrient components in foods (Cheftel, 1992).

Dormant bacterial spores are highly resistant to many physical treatments including heat, drying, radiation and chemical agents such as hydrogen peroxide (Gould, 1983), but germinated spores are not resistant. Thus, it is believed to be effective for dormant spores to be germinated firstly and then to inactivate those that are germinated.

HPT can initiate germination of dormant bacterial spores in a germinator free solution (Clouston & Wills, 1969; Gould & Sale, 1970; Wuytack *et al*., 1998). In the inactivation of bacterial spores by HPT, bacterial spores first germinated by hydrostatic pressure, and then the germinated spores, now sensitive to this type of pressure, were inactivated if the pressure was sufficiently high (Gould & Sale, 1970). At moderate temperature, HPT had to be extremely high above 600 MPa to inactivate the germinated bacterial spores (Sonoike, 1997). The combination of HPT with heat treatment (HT) is thus a promising method to inactivate bacterial spores (Gould, 1973; Mallidis & Drizou, 1991; Okazaki *et al*., 1994; Roberts & Hoover, 1996), because germinated spores were easily inactivated by HT alone.

There have been many studies on the effects of high pressure carbon dioxide treatment (HPCT) on the inactivation of bacteria under moderate temperature (approximate 20 to 40˚C) and pressure (approximately 5 to 35 MPa) (Nakamura *et al*., 1994; Ishikawa *et al*., 1995; Erkmen, 2001; Shimoda *et al*., 2001, 2002). For the sterilization of bacterial spores, it has been thought to be effective to decrease the heat tolerance of bacterial spores, and then to inactivate them by subsequent HT. In this study, we investigated the effect of HPCT and subsequent heat treatment on the inactivation of bacterial spores.

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Materials and Methods

Bacteria The bacteria used were *Bacillus coagulans* JCM2257 and *Bacillus licheniformis* JCM2505, and were obtained from the Japan Collection of Microorganisms (Saitama, Japan).

Media and culture conditions Overnight cultures of *Bacillus* grown in nutrient broth (Difco, USA) were transferred to sporulation agar plates. The medium consisted of nutrient agar (Difco) containing 1 μg/*l* Mn^{2+}. Two hundreds microliters (approximately 10^9 CFU/ml) of overnight culture was poured on each sporulation agar plates and the plates were incubated at 37˚C for 10 days.

Preparation of spore suspensions Spores were collected by flooding the surface of the culture with sterile distilled water followed by scraping the surface with a sterile microscope slide glass. After collection, spores were washed three times by centrifugation at 8000×*g* for 10 min, resuspended in sterile distilled water and stored at 4˚C until use. Suspensions were diluted to give approximately 10^6 colony forming units (CFU) ml^{-1}.

High pressure carbon dioxide treatment (HPCT) and heat treatment (HT) Spore suspensions (10 ml) were poured into a sterile test tube (104×17 mm) and the test tube was placed in a preheated sterilization vessel, and then spores were treated. The equipment used was a prototype pressurization apparatus (AKICO Co., Japan) (Furukawa *et al*., 2003). Temperature of the sterilization vessel was regulated by a thermocontrolled heater at 35˚C. CO_2 was gradually forced into the vessel until the pressure reached 6.5 MPa; the time required to achieve this pressure was approximately 1 min. The sample was kept for 10, 30, 60 min at a constant temperature and pressure during each experiment. Following the treatment, the decompression time was approximately 2 min. Then, a portion of the samples (2 ml) was transferred into glass test tubes (10×100 mm) and incubated at 80, 85 and 90˚C for 30 min.

Measurement of surviving spores Viable spores were determined by plating 100 μl of appropriately diluted samples onto nutrient agar (Difco). The plates were incubated at 37˚C for

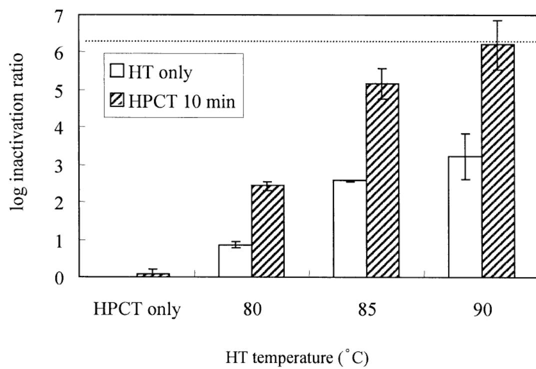

Fig. 1. Effect of HPCT at 35°C, 6.5 MPa and the following HT at 80, 85, 90°C for 30 min on the inactivated ratio of *B. coagulans* JCM2257 spores. Dotted line indicates the complete sterilization.

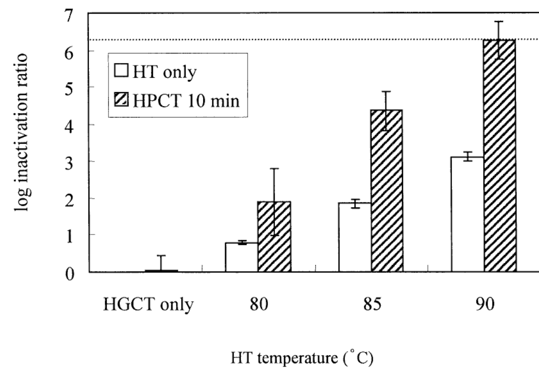

Fig. 2. Effect of HPCT at 35°C, 6.5 MPa and the following HT at 80, 85, 90°C for 30 min on the inactivated ratio of *B. licheniformis* JCM2505 spores. Dotted line indicates the complete sterilization.

24 h, and colonies were counted.

Statistical analysis All experiments were done in triplicate, and the data presented are the means of the triplicate experiments. Significant differences between surviving and ungerminated spores were assessed with 5% level of significance ($p < 0.05$) by Student's t test.

Results and Discussion

The effects of high pressure carbon dioxide treatment (HPCT) at 35°C at 6.5 MPa and subsequent heat treatment (HT) at 80, 85 and 90°C for 30 min on the bacterial spores were investigated. Figure 1 shows the effect of this action on the inactivation of spores of *B. coagulans* JCM2257. Approximately 1 order (80°C), 2.5 orders (85°C) and 3 orders (90°C) of the log inactivation ratio of *B. coagulans* spores were inactivated by HT for 30 min, respectively.

In HT, *B. coagulans* spores were not completely sterilized. The effect of HPCT subsequent HT on the inactivation of these spores was then investigated. Approximately, 2.5 orders (80°C), 5 orders (85°C) and 6 orders (90°C) of the log inactivation ratio of *B. coagulans* spores were inactivated by HPCT, respectively.

Figure 2 shows the effect of HPCT and the following HT on the inactivation of the spores of *B. licheniformis* JCM2505. Approximately 1 order (80°C), 2 orders (85°C) and 3 orders (90°C) of the log inactivation ratio of these spores were inactivated by HT for 30 min, respectively. In HT, *B. licheniformis* spores were not completely sterilized, and the effect of HPCT and subsequent HT at 80, 85 and 90°C for 30 min on their inactivation was investigated. Approximately 2 orders (80°C), 4 orders (85°C) and 6 orders (90°C) of the log inactivation ratio of these spores were found to have been inactivated. There was no significant difference among the surviving spores in HPCT at 10, 30 and 60 min and the following HT. Therefore, only the results of 10 min are indicated (Figs. 1 and 2).

Both spores were sterilized completely by HPCT and subsequent HT at 90°C. From these results, it was concluded that HPCT decreased the heat tolerance of bacterial spores.

It was previously indicated that the hydrostatic pressure treatment (HPT) increased the heat sensitivity of bacterial spores by initiating their germination (Clouston & Wills, 1969; Gould & Sale, 1970; Wuytack *et al*., 1998). In the inactivation of bacterial spores by HPT, bacterial spores first germinated by hydrostatic pressure, and then the germinated spores, now sensitive to such pressures, were inactivated if the pressure was sufficiently high (Gould & Sale, 1970). It was indicated that the germination effect of HPCT was also the main reason for the inactivation of the spores (Watanabe *et al.,* 2002).

This study showed that HPCT decreases the heat tolerance of bacterial spores. From the viewpoint of applying HPCT to industrial food sterilization, increase of the inactivation ratio of bacterial spores is thought to be very important. In general, many boiled foods are cooked at around 100°C. Food quality is not believed to be changed detrimentally by HT around 100°C. With the combination of HPCT, we succeeded in the effective sterilization by HT below 100°C.

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