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Note

Effect of Repeated Pressure Treatment on Breakdown of Clumps of Bacterial Spores

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The effects of repeated pressure treatment (RP treatment) and continuous pressure treatment (CP treatment) at 25°C, 400 MPa for 30 min on the breakdown of spore clumps were investigated and RP treatment was found to be effective in breaking the clumps as the ratio of clumps decreased with increase in decompression time. It was deduced that this breakdown effect was one reason for the higher sterilization effect of RP treatment than CP treatment.

Keywords: hydrostatic pressure, repeated pressurization, decompression, bacterial spore, clump

The effects of hydrostatic pressure treatments on the inactivation of microorganisms were reported 100 years ago (Hite, 1899), and the application of such technology to food processing has increased in Japan (Hayashi, 1992).

In food sterilization, inactivation of dormant bacterial spores is an important objective. Dormant bacterial spores are highly resistant to many physical and chemical agents including heat, drying, radiation and chemicals such as hydrogen peroxide (Gould, 1983).

In hydrostatic pressure treatments, bacterial spores were also found to be resistant, surviving up to 1200 MPa (Larson *et al*., 1918; Johnson & ZoBell, 1949; Timson & Short, 1965; Sale *et al*., 1970). Therefore, the sterilizing effects of hydrostatic pressure on bacterial spores in combination with heat (Gould, 1973; Hayakawa *et al*., 1994a, Hayakawa *et al*., 1994b; Mallidis & Drizou, 1991; Okazaki *et al*., 1994; Roberts & Hoover, 1996), irradiation (Crawford *et al*., 1996), low pH (Roberts & Hoover, 1996) and bacteriocins such as nisin (Roberts & Hoover, 1996) have been studied.

In general, hydrostatic pressurization can initiate germination of dormant bacterial spores in a germinator free solution (Clouston & Wills, 1969; Wuytack *et al*., 1998). Thus, in the sterilization of bacterial spores germination of dormant spores by hydrostatic pressure treatment and then inactivating them by physical or chemical treatments including hydrostatic pressure could be effective.

We have indicated that repeated pressure treatment (repeated compression, pressurization and decompression) was effective to inactivate bacterial spores (Furukawa *et al*., 2000a; Furukawa *et al*., 2000b; Furukawa *et al*., 2003). Spores were more inactivated by RP treatment than by continuous pressure treatment (consisting of 1 cycle of 30 min pressurization; CP treatment). Germinated spores were increasingly injured by the repeated decompression. In RP treatment, spores were first germinated and then were injured by repeated decompression (Furukawa *et al*., 2000a; Furukawa *et al*., 2000b; Furukawa *et al*., 2003).

In the sterilization of spores by hydrostatic pressure treatment, the spore clumps appeared to decrease the inactivation ratio (Furukawa *et al*., 2001a; Furukawa *et al*., 2002). We hypothesized that RP treatment broke the spore clumps by the impulsive force generated by repeated decompression. Therefore, the breakdown of the spore clumps was believed to contribute to enhancement of the inactivation of spores by this RP treatment. In this study, the effect of RP treatment on breaking the clumps of bacterial spores was investigated.

The bacterium used was *Bacillus subtilis* IFO 13722, obtained from the Institute for Fermentation (Osaka).

Log phase cultures of *B. subtilis* IFO 13722 grown in nutrient broth (Eiken Chemical Co., Ltd., Tokyo) were transferred to soil infusion agar plates (Berry & Brandshaw, 1980). The medium consisted of nutrient agar (Eiken Chemical Co., Ltd.) plus soil extract. The plates were incubated at 37°C for 10 days.

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 boltexed in sterile distilled water and centrifuged at $4000\times g$ for 30 min, and then the supernatant was decanted. This process was repeated three times, and the solution was 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}.

Spore suspensions were sealed in sterile screw-capped plastic tubes (1.5 ml capacity; Greiner Labortechnik Co., Ltd., Germany), which were transferred to the container of the pressurization apparatus. The equipment used was a prototype pressurization apparatus (Hayakawa *et al*., 1994a). The period needed to achieve the treatment pressure was approximately 30 s, and the decompression period was less than 1 s. Temperature of the pressure cell was regulated by a thermocontrolled water bath (Haake GH, Germany). Spore suspensions were treated at 400 MPa, 25°C for 30 min by repeated pressure treatment (2×15 min, 3×10 min, 4×7.5 min, 5×6 min and 6×5 min pressurization; RP treatment) and continuous pressure treatment (1 cycle of 30 min pressurization; CP treatment). In this manuscript, hydrostatic pressure treatment includes the CP and RP treatment.

The numbers of single spores and spore clumps before and

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Table 1. Size distribution of spore clump in spore solutions of *Bacillus subtilis*.

Size of spore clump (μm)	Single spore	2.5–5.0	5.0–7.5
Distribution of spore and spore clump (%)	87.1±3.8	12.9±3.8	0.00±0.0

after RP treatment were measured with a Thoma counter by a phase microscope (Toda & Aiba, 1966), and the fraction of single spores and spore clumps was calculated. Spore clump ratio was calculated by the following equation, spore clump ratio= number of spore clumps/(number of single spores+number of spore clumps).

All experiments were done in triplicate. The data presented are the means of three replicate experiments (data not shown). In this study, only the effect of RP treatment on the breakdown of the spore clumps was investigated.

Inactivation ratios of spores were approximately 1 order in CP and 1.7 orders in RP at 400 MPa, 25°C for 30 min.

By size distribution in spore suspensions (Table 1), there were 12.9% clumps (2.5–5.0 μm), and no clumps larger than 5.0 μm in the spore suspensions. In a previous study, it was indicated that the 5 μm spore clumps are comprised of approximately 90 spores, and such clumps were approximately 5 times more heat resistant than a single spore, i.e., spore clumps decreased the heat resistance (Toda & Aiba, 1966). A previous study on the sterilization of spores by hydrostatic pressure treatment suggested that the clumps of spores decreased the inactivation ratio (Furukawa *et al*., 2001a; Furukawa *et al*., 2002). Therefore, the high inactivation effect of RP treatment was attributable to the spore clump-breakdown effect of the treatment.

The effect of RP treatment on the breakdown of the spore clumps was investigated (Fig. 1). Ratios of the spore clumps (to the single spores and clumps) decreased as the number of decompressions in the process increased. More than 50% of spore clumps were broken by one decompression, and a clump with 74% spores was broken by six decompressions. Single spores composing the clumps were probably released by the first decompression, and were injured and inactivated by the second decompression. This result confirmed the previous study on the effect of the number of repeated decompressions to inactivate *B. subtilis* spores (Furukawa *et al*., 2001b).

In RP treatment, spores were probably released from clumps when the clumps were broken. Such breakdown was caused by the impulsive force generated by repeated decompression. RP treatment was shown to be effective in breaking the clumps, and it was deduced that this breakdown effect was one of the reasons for the high sterilization effect of this treatment.

In RP treatment, spores were first germinated and then were injured by repeated decompression (Furukawa *et al*., 2000a; Furukawa *et al*., 2000b; Furukawa *et al*., 2003). Here, only pressurization was needed to germinate the spores (Furukawa *et al*., 2000a). This time, a new mechanism was proposed to inactivate the spores by RP treatment. Spores, initially composing the clumps and released from them by the first decompression were germinated by pressurization, these released single spores were then injured and inactivated by a second decompression.

These results showed that RP treatment could decrease the processing temperature and pressure to inactivate the bacterial spores. Lowering the processing temperature decreases the heat damage of processed foods. From the viewpoint of applying high pressure treatment to food industry, decreasing the processing pressure, i.e., decreasing the cost of high pressure equipment, is most important.

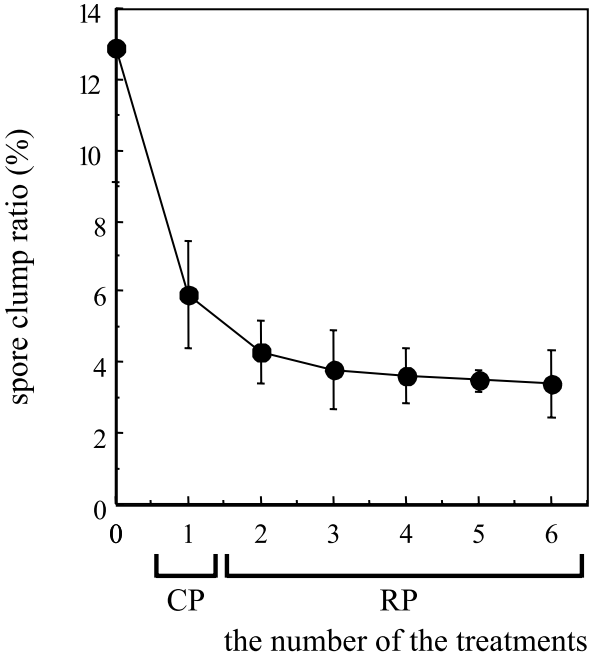

Fig. 1. Effect of decompression time on the spore clump ratio at 25°C, 400 MPa for 30 min.

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