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# Inactivation of *Geobacillus stearothermophilus* Spores by High-Pressure Carbon Dioxide Treatment

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**High-pressure $CO_2$ treatment has been studied as a promising method for inactivating bacterial spores. In the present study, we compared this method with other sterilization techniques, including heat and pressure treatment. Spores of *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, and *Geobacillus stearothermophilus* were subjected to $CO_2$ treatment at 30 MPa and 35°C, to high-hydrostatic-pressure treatment at 200 MPa and 65°C, or to heat treatment at 0.1 MPa and 85°C. All of the bacterial spores except the *G. stearothermophilus* spores were easily inactivated by the heat treatment. The highly heat- and pressure-resistant spores of *G. stearothermophilus* were not the most resistant to $CO_2$ treatment. We also investigated the influence of temperature on $CO_2$ inactivation of *G. stearothermophilus*. Treatment with $CO_2$ and 30 MPa of pressure at 95°C for 120 min resulted in 5-log-order spore inactivation, whereas heat treatment at 95°C for 120 min and high-hydrostatic-pressure treatment at 30 MPa and 95°C for 120 min had little effect. The activation energy required for $CO_2$ treatment of *G. stearothermophilus* spores was lower than the activation energy for heat or pressure treatment. Although heat was not necessary for inactivation by $CO_2$ treatment of *G. stearothermophilus* spores, $CO_2$ treatment at 95°C was more effective than treatment at 95°C alone.**

Dormant bacterial spores are highly resistant to many physical treatments and chemical agents, including heat, drying, radiation, and chemicals such as hydrogen peroxide (11), and inactivation of such spores is the main objective of food sterilization. Currently, heat, radiation, and chemical preservatives are the major food sterilization methods (29). Of these methods, moist heat treatment is most commonly used (32). Moist heat at temperatures below 100°C is defined as a disinfection process (29), which is meant to destroy disease-causing or other harmful microorganisms but not to kill bacterial spores (2). On the other hand, moist heat at 121°C or higher temperatures is defined as a sterilization process (29), which destroys all forms of life, especially microorganisms, including bacterial spores (2). The high temperature required for heat sterilization can damage the nutritive value, color, and taste of foods (17).

Hydrostatic pressure treatment can also kill dormant bacterial spores (3, 12, 34). However, at moderate temperatures, extremely high pressures (more than 600 MPa) are required to inactivate bacterial spores (19, 30). Such a pressure treatment would be difficult to apply to the food industry (19, 30).

There have been many studies of the effects of high-pressure $CO_2$ treatment, including gaseous and supercritical states, on inactivation of bacteria at moderate temperatures (20 to 40°C) and pressures (5 to 35 MPa) (6, 15, 22). At moderate temperatures and pressures, $CO_2$ treatment significantly inactivates bacterial vegetative cells, whereas pressure treatment alone has little effect (19, 30). There have been some previous studies of inactivation of bacterial spores by $CO_2$ treatment (1, 4, 5, 16, 18); for example, $CO_2$ treatment can substantially inactivate bacterial spores at temperatures above 50°C. Another study showed that *Geobacillus stearothermophilus* spores were poorly inactivated by $CO_2$ treatment at 35°C and 20 MPa (18).

The *G. stearothermophilus* spore is one of the most heat-resistant spores of aerobic microorganisms. *G. stearothermophilus* usually causes flat sour spoilage of canned liquid foods, such as coffee, during storage in automatic vending machines. Because of the heat resistance of this microorganism's spores, they are often used as a biological indicator to evaluate the effectiveness of sterilization processes (7, 20, 26). In general, foods are cooked at temperatures around 100°C, and it is assumed that the foods are not damaged by heat treatment at temperatures below 100°C. For commercial applications, the sterilization period is calculated by using the $5 \times D$ (decimal reduction time) concept (21). Based on this concept, 5-log-order killing of bacterial spores (especially *G. stearothermophilus* spores) at temperatures below 100°C is desired to preserve food quality while providing sterilization. For this reason, in the present study we compared the effects of heat, pressure, and $CO_2$ treatment on inactivation of the spores of five bacteria, including *G. stearothermophilus*. We also investigated the effect of $CO_2$ treatment on inactivation of *G. stearothermophilus* spores at temperatures below 100°C.

## MATERIALS AND METHODS

**Bacteria.** *Bacillus coagulans* JCM2257 and *Bacillus licheniformis* JCM2505 were obtained from the Japan Collection of Microorganisms (Saitama, Japan). *Bacillus cereus* IAM1110 and *Bacillus subtilis* IAM1069 were obtained from the Institute of Molecular and Cellular Biosciences (Tokyo, Japan). *G. stearothermophilus* ATCC 12980 was obtained from the American Type Culture Collection (Manassas, Va.).

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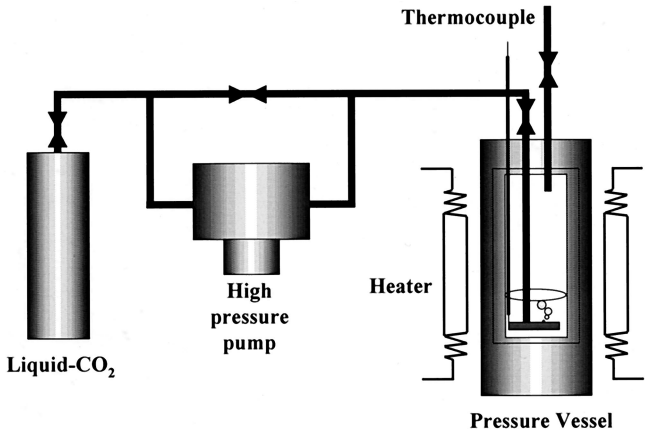


FIG. 1. Apparatus for high-pressure $CO_2$ treatment.

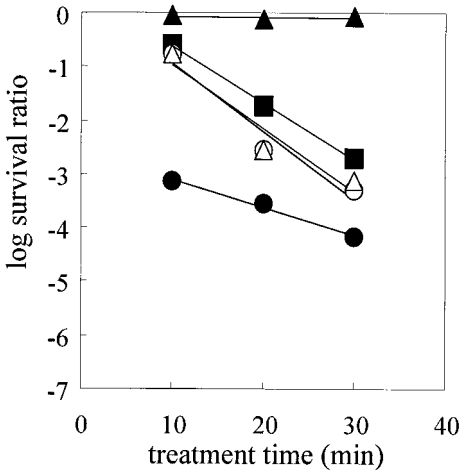


FIG. 2. Survival curves for *G. stearothermophilus* (▲), *B. coagulans* (■), *B. licheniformis* (○), *B. cereus* (△), and *B. subtilis* (●) spores after heat treatment at 85°C.

**Media and culture conditions.** Overnight cultures of *Bacillus* and *Geobacillus* strains grown in nutrient broth (Difco, Detroit, Mich.) were transferred to sporulation agar plates, which consisted of nutrient agar (Difco) containing 1 μg of $Mn^{2+}$/ml. The plates were incubated at 37°C (*Bacillus*) or 55°C (*Geobacillus*) for 10 days.

**Preparation of spore suspensions.** Spores were collected by flooding the surface of a culture with sterile distilled water and then scraping the surface with sterile microscope slides. The spores collected were washed three times by centrifugation at 8,000 × *g* for 10 min, resuspended in sterile distilled water, and stored at 4°C until they were used. Suspensions were diluted to obtain approximately $10^6$ CFU/ml.

**Heat treatment.** Spore suspensions (2 ml) were transferred into glass test tubes (10 by 100 mm), which were then immersed in a water bath equilibrated at 85°C for 20 or 40 s or 1, 3, 5, 60, 120, or 180 min.

**Pressure treatment.** Spore suspensions were sealed in sterile screw-cap plastic tubes (capacity, 5 ml; Greiner Labortechnik Co., Ltd., Frickenhausen, Germany) that were pressurized with a prototype pressurization apparatus (24). The time needed to achieve the treatment pressure was approximately 60 s for 200 MPa. The decompression time was approximately 10 s. The temperature of the pressure cell was regulated with a temperature-controlled water bath. Combinations of hydrostatic pressure (200 MPa) and temperature (35 and 95°C) and a total holding period of 120 min were used in this study.

**$CO_2$ treatment.** Spore suspensions (10 ml) were poured into sterile stainless test tubes (104 by 17 mm). Each test tube was placed in the vessel of a prototype pressurization apparatus (AKICO Co., Tokyo, Japan) (Fig. 1), and the tube was pressurized with $CO_2$. Approximately 3 min was needed to achieve a treatment pressure of 30 MPa, and the decompression time was approximately 2 min. The temperature in the pressure cell was regulated with a temperature-controlled heater. Combinations of pressure (30 MPa), temperature (35, 55, 65, 75, 85, and 95°C), and total holding period (0, 30, 60, and 120 min) were used in this study.

**Measurement of the number of surviving spores.** The number of surviving spores was determined by the viable plate count method by using nutrient agar (Difco). Prior to counting, the plates containing *G. stearothermophilus* were incubated at 55°C for 24 h, while the four *Bacillus* strains were incubated at 37°C for 24 h.

***D* and *z* values.** Decimal reduction times for heat treatment (heat treatment *D* values), decimal reduction times for hydrostatic pressure treatment (pressure treatment *D* values), and decimal reduction times for high-pressure carbon dioxide treatment ($CO_2$ treatment *D* values) were calculated from the negative reciprocals of the slopes of the regression lines from the straight portions of the survival curves. The high-pressure carbon dioxide *z* value ($CO_2$ treatment *z* value) was defined as the temperature required to decrease the $CO_2$ treatment *D* value 10-fold in the $CO_2$ treatment experiments. $CO_2$ treatment *z* values were calculated by determining the negative reciprocals of the slopes of the log $CO_2$ treatment *D* value curves (log $CO_2$ treatment *D* value versus temperature).

**Arrhenius plot.** Rahn suggested that microbial destruction was due to inactivation of a single critical molecule in the cell and, therefore, could be assumed to follow first-order kinetics (28). Therefore, to evaluate the effects of $CO_2$ treatment on the rates of inactivation of bacterial spores, a simplified mathematical model based on first-order kinetics was used (17, 27): $dN/dt = -kN$, or, in the integrated form, $\ln (N/N_0) = -kt$, where $N_0$ and $N$ are the average viable spore counts measured prior to the $CO_2$ treatment (i.e., control) and at time $t$ (including time zero), respectively, and $k$ is the specific death rate constant or death rate coefficient, which is a function of temperature. The slope of the linear regression analysis of $\ln (N/N_0)$ versus time $t$ is equal to $-k$. A survival curve for each spore type was plotted for each temperature. Regression lines were then generated within the linear portions of the survival curves.

The temperature dependence of the rate constant $k$ can generally be expressed with the Arrhenius equation (31). The natural logarithm of the absolute value of the specific death rate ($\ln k$) as a function of the reciprocal absolute temperature ($T$) is plotted as follows: $\ln k = \ln A - (E_a/R)(1/T)$, where $A$ is the frequency factor, $R$ is the gas constant (8.314 J/mol • K), and $E_a$ is the activation energy of the $CO_2$ treatment inactivation process (in joules per mole), which also provides a measure of the temperature sensitivity of the bacterial spores under various conditions.

**Statistical analysis.** All experiments were repeated three times. In each experiment, one sample was used. The data presented below are the means of three replicate experiments.

## RESULTS

**Evaluation of the heat resistance of the spores of five species of bacteria.** Figure 2 shows the data for inactivation of spores of five species of bacteria by heat treatment at 85°C. The survival curves exhibited tailing, and heat treatment *D* values for five species were calculated from the linear portions of the survival curves (Table 1). According to the heat treatment *D* values, the *G. stearothermophilus* spores were the most heat-resistant spores among the five types of spores (significant inactivation was not observed in 180 min), while the *B. licheniformis* spores were the most heat sensitive.

**Evaluation of the resistance of the spores of five species of bacteria to hydrostatic pressure treatment.** Figure 3 shows the data for survival of the spores of five species of bacteria after treatment at 65°C and 200 MPa. The survival curves exhibited tailing, and pressure treatment *D* values for the five species were calculated from the linear portions of the survival curves. The pressure treatment *D* values calculated are shown in Table 1. Based on the pressure treatment *D* values, the *G. stearothermophilus* spores were the most resistant spores among the spores of the five strains, while the *B. cereus* spores were the most sensitive. The pressure treatment *D* values for the *G. stearothermophilus* spores were approximately 11 times higher

TABLE 1. Effect of heat treatment at 85°C, pressure treatment at 65°C and 200 MPa, and $CO_2$ treatment at 35°C and 30 MPa on the resistance of bacterial spores

| Organism | Treatment | *D* value (min) |
|---|---|---|
| *G. stearothermophilus* | Heat | ∞ |
| | Pressure | 75.2 |
| | $CO_2$ | 385 |
| *B. subtilis* | Heat | 19.0 |
| | Pressure | 9.3 |
| | $CO_2$ | 1,667 |
| *B. coagulans* | Heat | 9.5 |
| | Pressure | 6.9 |
| | $CO_2$ | 164 |
| *B. cereus* | Heat | 8.5 |
| | Pressure | 6.9 |
| | $CO_2$ | 133 |
| *B. licheniformis* | Heat | 7.9 |
| | Pressure | 8.5 |
| | $CO_2$ | 182 |

than those for *B. cereus* spores. Based on the results obtained, except for *G. stearothermophilus*, there was no clear relationship between heat resistance and pressure resistance in the bacterial spores.

**Evaluation of the resistance of the spores of five species of bacteria to high-pressure $CO_2$ treatment.** Figure 4 shows the data for survival of the bacterial spores after treatment with $CO_2$ at 35°C and 30 MPa. The survival curves exhibited tailing, and $CO_2$ treatment *D* values were calculated from the linear portions of the survival curves. The calculated $CO_2$ treatment *D* values are shown in Table 1. Based on the $CO_2$ treatment *D* values, the *B. subtilis* spores were the most resistant spores among the spores of the five strains, while the *B. cereus* spores were the most sensitive. The *G. stearothermophilus* spores were more sensitive than other spores to $CO_2$ treatment. The $CO_2$ treatment *D* values for *B. subtilis* spores were approximately 13 times higher than those for *B. coagulans* spores, indicating that resistance of the spores to $CO_2$ treatment was not correlated with resistance to heat and pressure.

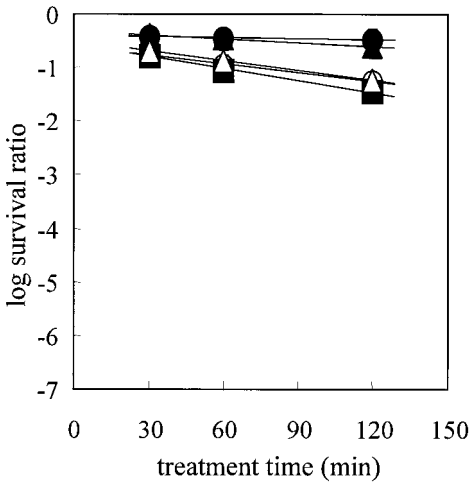


FIG. 4. Survival curves for *G. stearothermophilus* (▲), *B. coagulans* (■), *B. licheniformis* (○), *B. cereus* (△), and *B. subtilis* (●) spores after $CO_2$ treatment at 35°C and 30 MPa.

**Effect of $CO_2$ treatment on inactivation of *G. stearothermophilus* spores at temperatures below 100°C.** Previous studies showed that it is difficult to completely inactivate *G. stearothermophilus* spores by $CO_2$ treatment at 35°C (18). However, we speculated that $CO_2$ treatment would inactivate *G. stearothermophilus* spores at higher temperatures. Therefore, we examined the effect of temperature on inactivation of *G. stearothermophilus* spores by $CO_2$ treatment at 30 MPa. We found that the inactivation rate increased proportionally with the treatment temperature (Fig. 5). The calculated $CO_2$ treatment *D*

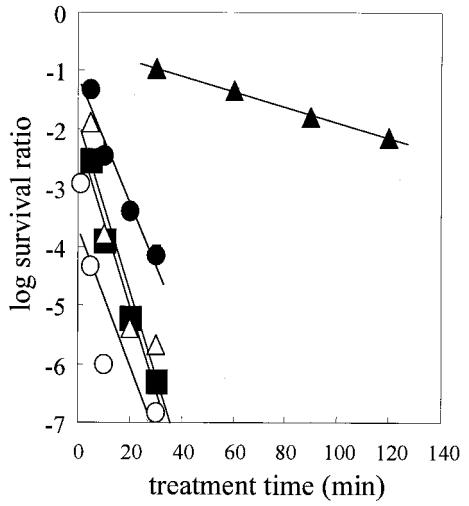


FIG. 3. Survival curves for *G. stearothermophilus* (▲), *B. coagulans* (■), *B. licheniformis* (○), *B. cereus* (△), and *B. subtilis* (●) spores after pressure treatment at 65°C and 200 MPa.

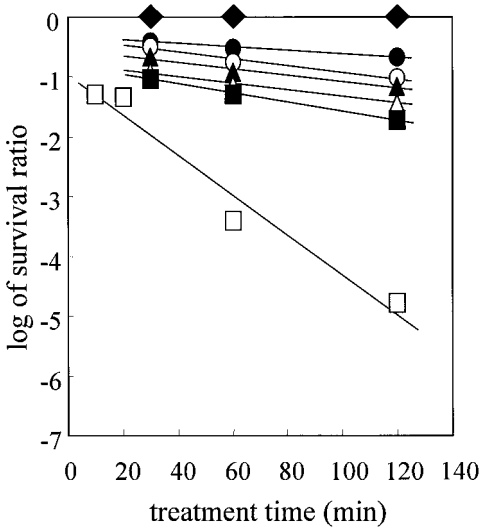


FIG. 5. Effect of temperature on survival of *G. stearothermophilus* spores at 30 MPa and 35°C (●), 55°C (○), 65°C (▲), 75°C (△), 85°C (■), and 95°C (■). ♦, survival curve for *G. stearothermophilus* spores after heat treatment at 95°C.

TABLE 2. *D* values for *G. stearothermophilus* spores

| Treatment | Temp (°C) | *D* value (min) |
|---|---|---|
| $CO_2$ (30 MPa) | 35 | 345 |
| | 55 | 182 |
| | 65 | 196 |
| | 75 | 179 |
| | 85 | 130 |
| | 95 | 29.9 |
| Heat | 95 | 2,450 |

values are shown in Table 2. Although temperatures from 35 to 85°C did not inactivate the *G. stearothermophilus* spores, the spores were inactivated by $CO_2$ treatment for 120 min at 95°C and 30 MPa.

**$CO_2$ treatment *z* values for inactivation of *G. stearothermophilus* spores.** Next, we examined the kinetics of $CO_2$ inactivation of *G. stearothermophilus* spores at various temperatures. Figure 6 shows the effect of temperature on the log $CO_2$ treatment *D* values. There were two straight portions in the regression lines in the log $CO_2$ treatment *D* value graphs, and $CO_2$ treatment *z* values were calculated from the slopes of the straight portions of the regression lines. $CO_2$ treatment *z* values are shown in the Table 3. The $CO_2$ treatment *z* values were 135°C (35 to 85°C) and 25.7°C (75 to 95°C), respectively. These results indicate that an increase in the inactivation rate depends on the increase in temperature more for the high temperature range than for a lower temperature range (Table 3).

**Determination of the activation energy for inactivation of *G. stearothermophilus* spores by $CO_2$ treatment.** Figure 6 also shows the Arrhenius plots of the logarithm of the rate constant for inactivation of *G. stearothermophilus* spores by $CO_2$ treatment. There were two linear portions in the regression lines, which were used to calculate the activation energies. The activation energies were 18.0 kJ/mol (35 to 65°C) and 94.5 kJ/mol (75 to 95°C).

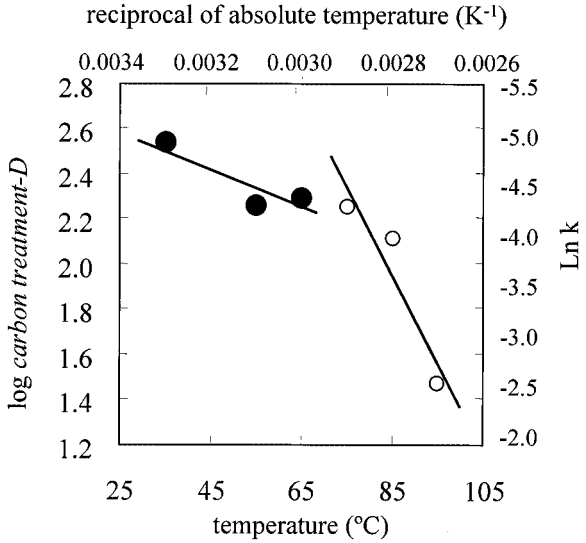


FIG. 6. Effect of temperature on log $CO_2$ treatment *D* values and Arrhenius plots of the logarithm of the rate constant for inactivation of *G. stearothermophilus* spores at 30 MPa.

TABLE 3. Effect of temperature on the $CO_2$ treatment *z* value and activation energy of *G. stearothermophilus* spores subjected to $CO_2$ treatment at 30 MPa

| Temp (°C) | $CO_2$ treatment-z value (°C) | Activation energy (kJ/mol) |
|---|---|---|
| 35–85 | 135 | 18.0 |
| 75–95 | 25.7 | 94.5 |

## DISCUSSION

Previous studies have shown that *G. stearothermophilus* spores are highly resistant to heat (7, 20, 26). Similarly, we found that at 85°C the heat treatment *D* value for *G. stearothermophilus* spores was remarkably higher than the values for four other species. Other studies have shown that bacterial spores cannot be effectively inactivated by pressure treatment at room temperature (8, 10, 23, 25, 30). Because we were interested in the combined effects of pressure and heat, we examined the ability of pressure to sterilize spores at 65°C. We found that the spores used in this study were not inactivated by heat treatment at 70°C for up to 540 min (data not shown). We also found that the pressure treatment *D* value for *G. stearothermophilus* spores was approximately 11 times higher than the value for *B. cereus* spores. Except for *G. stearothermophilus*, there was no clear relationship between the heat resistance and the pressure resistance of bacterial spores. This result partially agreed with the results of a previous study (23). However, in the previous study, spores were subjected to a pressure treatment at 5 to 10°C and 588 MPa, and the spores of only two of the six *Bacillus* species used were significantly inactivated (23). Therefore, it is difficult to compare the results of this study and the results of the previous study.

The effect of $CO_2$ treatment on inactivation of bacterial spores was investigated at 35°C and 30 MPa. Based on our results, *B. subtilis* spores were the most resistant spores among the spores of the five species tested, while *B. coagulans* spores were the most sensitive. The *G. stearothermophilus* spores were not the most resistant spores. Specifically, the $CO_2$ treatment *D* value for *B. subtilis* spores was approximately 13 times higher than that for *B. coagulans* spores and was approximately 4.3 times higher than that for *G. stearothermophilus* spores. The $CO_2$ treatment resistance did not correlate with heat resistance and pressure resistance. These results suggest that there are different mechanisms for inactivation of bacterial spores by $CO_2$, heat, and pressure.

*G. stearothermophilus* spores were inactivated approximately 5 orders of magnitude by $CO_2$ treatment for 120 min at 95°C and 30 MPa. In contrast, *G. stearothermophilus* spores were inactivated only approximately 50% by pressure treatment for 120 min at 95°C and 30 MPa (data not shown). Therefore, $CO_2$ treatment was substantially more effective than pressure treatment alone for inactivating *G. stearothermophilus* spores.

The $CO_2$ treatment *D* value for *G. stearothermophilus* spores at 95°C was 29.9 min, and the atmospheric treatment *D* value at 95°C was 2,450 min. Therefore, the increase in the temperature during $CO_2$ treatment decreased the $CO_2$ treatment *D* value for *G. stearothermophilus* approximately 80-fold at 95°C. In contrast to *G. stearothermophilus* spores, the spores of the four *Bacillus* strains were easily inactivated by heat treatment

at 95°C. Thus, we predicted that the four *Bacillus* strains would be inactivated easily by $CO_2$ treatment at 95°C and 30 MPa. This treatment is also effective in inactivating *G. stearothermophilus* spores.

*G. stearothermophilus* spores are effectively inactivated by pressure treatment at higher temperatures (8, 13, 14). During pressure treatment, dormant bacterial spores germinated under hydrostatic pressure, and the germinated spores were inactivated (3, 12, 34). Similar to the results obtained with the pressure treatment, the bacterial spores germinated during $CO_2$ treatment (9). Clearly, the spores that germinated during $CO_2$ treatment at a higher temperature were heat inactivated at 95°C. In addition, reduction of the pH by $CO_2$ might contribute to the inactivation of the bacterial spores.

The $CO_2$ treatment *z* values for *G. stearothermophilus* spores were 135°C (35 to 85°C) and 25.7°C (75 to 95°C). The inactivation rates at the higher temperature range were more dependent on the increase in temperature than were those at the lower temperature range. In general, during heat treatment, the regression plots of log heat treatment *D* values versus temperatures were linear (17). However, for $CO_2$ treatments, there were two distinct linear portions of the log $CO_2$ treatment *D* value plots, and there were two corresponding linear portions in the Arrhenius plots (Fig. 6). The activation energies were 18.0 kJ/mol (35 to 65°C) and 94.5 kJ/mol (75 to 95°C) (Table 3). The Arrhenius plots for heat treatments also were linear (27). Based on the $CO_2$ treatment *z* values and activation energies, the inactivation mechanisms appear to be different at 85 to 95°C. In addition, the mechanism of bacterial spore inactivation by $CO_2$ treatment appears to be different from the mechanism of bacterial spore inactivation by heat treatment.

Similar to the results of the $CO_2$ treatments, there were two straight portions in the regression lines of the Arrhenius plots for pressure treatment at 100 MPa (25). Similarly, both $CO_2$ treatment and pressure treatment initiate bacterial spore germination (9). Thus, inactivation of bacterial spores occurs in two steps. We therefore expected that the *G. stearothermophilus* spores that germinated during $CO_2$ treatment would be more effectively inactivated at 85 to 95°C than at a lower temperature. Microbial destruction by heat treatment has been shown to be due to inactivation of a single critical molecule in the cells and spores and therefore could be assumed to follow first-order kinetics (28). In other words, inactivation of microorganisms by heat treatment occurs in a single step, while inactivation by $CO_2$ treatment occurs in two steps.

In addition to this evidence indicating that there is a difference in the mechanisms of inactivation, we found differences in the activation energies for bacterial spore inactivation by heat and $CO_2$ treatments. The activation energy for inactivation by heat treatment ranged from 221 to 347 kJ/mol (21), and the activation energy for *G. stearothermophilus* spores was 351 kJ/mol (33) (Table 4). In contrast, the activation energy for inactivation by $CO_2$ treatment was 94.5 kJ/mol at 75 to 95°C. Therefore, from the perspective of energy consumption, the spores were more effectively inactivated by $CO_2$ treatment than by heat treatment.

For pressure treatments at 90 to 110°C, the activation energies for inactivation of bacterial spores ranged from 143 to 247 kJ/mol at 100 MPa and from 198 to 270 kJ/mol at 200 MPa (25). These data, however, did not include the data for *G. stearothermophilus*, but we predicted that the activation energy for inactivating *G. stearothermophilus* spores is higher than the activation energies for inactivating *Bacillus* spores. Therefore, $CO_2$ treatment is more effective than both heat treatment and pressure treatment for inactivating bacterial spores.

TABLE 4. Activation energies for inactivating bacterial spores by heat, pressure, and $CO_2$ treatments

| Treatment | Pressure (MPa) | Activation energy (kJ/mol) | Reference(s) |
|---|---|---|---|
| Heat | 0.1 | 221–351[a] | 21, 33 |
| Pressure | 100 | 143–247 | 24 |
| | 200 | 198–270 | 24 |
| $CO_2$ | 30 | 94.5[a] | This study |

[a] The data include data for *G. stearothermophilus* spores.

Many foods are cooked at approximately 100°C. For this reason, it is desirable to kill bacterial spores by heat treatment at temperatures less than 100°C. Our results show that $CO_2$ treatment at temperatures below 100°C can effectively inactivate bacterial spores, including those of *G. stearothermophilus*. For commercial food sterilization, the sterilization period is calculated based on the $5 \times D$ concept (21). Based on this calculation, treatment for 120 min at 95°C and 30 MPa is sufficient for sterilization by $CO_2$ treatment. $CO_2$ treatment can therefore decrease the processing temperature and pressure needed to inactivate bacterial spores, including spores of *G. stearothermophilus*. Decreasing the processing temperature should decrease the amount of heat damage and the required processing pressure and thereby reduce the cost of sterilization equipment.

We investigated the effect of a 120-min treatment with $CO_2$ at 95°C and 30 MPa on the quality of commercially available milk, orange juice, coffee, and soup. Except for milk, which was coagulated, we found no noticeable reduction in quality.

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