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

Taisuke Watanabe, Soichi Furukawa,* Junichi Hirata, Tetsuya Koyama, Hirokazu Ogihara, and Makari Yamasaki

Department of Food Science and Technology, College of Bioresource Sciences, Nihon University, Fujisawa-shi, Kanagawa 252-8510, Japan

Received 12 May 2003/Accepted 22 September 2003

High-pressure CO₂ 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.

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 CO₂ 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, CO₂ 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 CO₂ treatment (1, 4, 5, 16, 18); for example, CO₂ treatment can substantially inactivate bacterial spores at temperatures above 50°C. Another study showed that *Geobacillus stearothermophilus* spores were poorly inactivated by CO₂ 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 CO₂ treatment on inactivation of the spores of five bacteria, including *G. stearothermophilus*. We also investigated the effect of CO₂ 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.).

*Corresponding author. Mailing address: Laboratory of Food Microbiology, Department of Food Science and Technology, College of Bioresource Sciences, Nihon University, 1866, Kameino, Fujisawa-shi, Kanagawa 252-8510, Japan. Phone: 81-0466-84-3973. Fax: 81-0466-84-3973. E-mail: furukawa@brs.nihon-u.ac.jp.*
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 Mn2+/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 with 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 10⁶ 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.

CO2 treatment. Spore suspensions (10 ml) were poured into sterile stainless steel 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 CO2. 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 (CO2 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 (CO2 treatment z value) was defined as the temperature required to decrease the CO2 treatment D value 10-fold in the CO2 treatment experiments. CO2 treatment z values were calculated by determining the negative reciprocals of the slopes of the log CO2 treatment D value curves (log CO2 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 CO2 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/N₀) = −kt, where N₀ and N are the average viable spore counts measured prior to the CO2 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/N₀) 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 − (Eₐ/R)(1/T), where A is the frequency factor, R is the gas constant (8.314 J/mol • K), and Eₐ is the activation energy of the CO2 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...
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 CO₂ treatment.** Figure 4 shows the data for survival of the bacterial spores after treatment with CO₂ at 35°C and 30 MPa. The survival curves exhibited tailing, and CO₂ treatment $D$ values were calculated from the linear portions of the survival curves. The calculated CO₂ treatment $D$ values are shown in Table 1. Based on the CO₂ 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 CO₂ treatment. The CO₂ 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 CO₂ treatment was not correlated with resistance to heat and pressure.

**Effect of CO₂ 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 CO₂ treatment at 35°C (18). However, we speculated that CO₂ treatment would inactivate G. stearothermophilus spores at higher temperatures. Therefore, we examined the effect of temperature on inactivation of G. stearothermophilus spores by CO₂ treatment at 30 MPa. We found that the inactivation rate increased proportionally with the treatment temperature (Fig. 5). The calculated CO₂ treatment $D$ values are shown in Table 1.
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 CO₂ treatment for 120 min at 95°C and 30 MPa.

**CO₂ treatment z values for inactivation of *G. stearothermophilus* spores.** Next, we examined the kinetics of CO₂ inactivation of *G. stearothermophilus* spores at various temperatures. Figure 6 shows the effect of temperature on the log CO₂ treatment D values. There were two straight portions in the regression lines in the log CO₂ treatment D value graphs, and CO₂ treatment z values were calculated from the slopes of the straight portions of the regression lines. CO₂ treatment z values are shown in Table 3. The CO₂ 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 CO₂ treatment.** Figure 6 also shows the Arrhenius plots of the logarithm of the rate constant for inactivation of *G. stearothermophilus* spores by CO₂ 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).

**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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂, heat, and pressure.

*G. stearothermophilus* spores were inactivated approximately 5 orders of magnitude by CO₂ 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, CO₂ treatment was substantially more effective than pressure treatment alone for inactivating *G. stearothermophilus* spores.

The CO₂ 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 CO₂ treatment decreased the CO₂ 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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temp (°C)</th>
<th>D value (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ (30 MPa)</td>
<td>35</td>
<td>345</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>29.9</td>
</tr>
<tr>
<td>Heat</td>
<td>95</td>
<td>2,450</td>
</tr>
</tbody>
</table>

**TABLE 3. Effect of temperature on the CO₂ treatment z value and activation energy of *G. stearothermophilus* spores subjected to CO₂ treatment at 30 MPa**

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>CO₂ treatment-z value (°C)</th>
<th>Activation energy (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35–85</td>
<td>135</td>
<td>18.0</td>
</tr>
<tr>
<td>75–95</td>
<td>25.7</td>
<td>94.5</td>
</tr>
</tbody>
</table>

FIG. 6. Effect of temperature on log CO₂ treatment D values and Arrhenius plots of the logarithm of the rate constant for inactivation of *G. stearothermophilus* spores at 30 MPa.
at 95°C. Thus, we predicted that the four Bacillus strains would be inactivated easily by CO₂ 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 CO₂ treatment (9). Clearly, the spores that germinated during CO₂ treatment at a higher temperature were heat inactivated at 95°C. In addition, reduction of the pH by CO₂ might contribute to the inactivation of the bacterial spores.

The CO₂ 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 CO₂ treatments, there were two distinct linear portions of the log CO₂ 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 CO₂ 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 CO₂ treatment appears to be different from the mechanism of bacterial spore inactivation by heat treatment.

Similar to the results of the CO₂ treatments, there were two straight portions in the regression lines of the Arrhenius plots for pressure treatment at 100 MPa (25). Similarly, both CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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, CO₂ treatment is more effective than both heat treatment and pressure treatment for inactivating bacterial 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 CO₂ 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×D concept (21). Based on this calculation, treatment for 120 min at 95°C and 30 MPa is sufficient for sterilization by CO₂ treatment. CO₂ 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 CO₂ 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.

### REFERENCES


### TABLE 4. Activation energies for inactivating bacterial spores by heat, pressure, and CO₂ treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pressure (MPa)</th>
<th>Activation energy (kJ/mol)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>0.1</td>
<td>221–351</td>
<td>21, 33</td>
</tr>
<tr>
<td>Pressure</td>
<td>100</td>
<td>143–247</td>
<td>24</td>
</tr>
<tr>
<td>CO₂</td>
<td>200</td>
<td>198–270</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>94.5†</td>
<td>This study</td>
</tr>
</tbody>
</table>

† The data include data for G. stearothermophilus spores.