Note

Enhanced Inactivation of *Bacillus subtilis* Spores by Carbonation with Heating in the Presence of Monoglycerol-caprate

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We have reported that the inactivation effect of carbonation with heating (CH; 80°C, 5 MPa, 30 min) on *Bacillus subtilis* spores was enhanced by the addition of monoglycerol-caprate (MC10). The aim of this study was to obtain aspects of the mechanism of this enhanced inactivation. The addition of MC10 (0.05%) to spores suspended in nutrient broth (NB) at pH 3.2 increased the inactivation of spores by heating (HT, 80°C) alone and with pressurization (5 MPa nitrogen gas). However, no inactivation effect was observed in NB at pH 6.8. The results showed that MC10 increased the number of spores with decreased resistance to heat and pressure under acidic conditions. The addition of MC10 also enhanced the ratio of spores germinated by CH. The bacteriostatic effect of MC10 was enhanced when combined with CH. This enhanced bacteriostatic effect might be responsible for inducing the high inactivation effect of CH with MC10.

Keywords: carbonation, monoglycerol-caprate, germination, *Bacillus subtilis*, spore

Introduction

Carbonation treatment is carried out by solubilizing carbon dioxide (CO2) into liquid foods under pressure. Dissolved CO2 generates protons, illustrated by the following equation, leading to the acidification of liquid food.

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+ \\
\text{HCO}_3^- \leftrightarrow \text{CO}_3^{2-} + \text{H}^+
\]

Attempts have been made to apply carbonation treatment for the pasteurization of acidic liquid foods. However, removal of CO2 gas from liquid food after carbonation treatment can recover the pH of the food to near the original level. Therefore, carbonation can be applied to liquid food with neutral pH, and is likely to replace or partially substitute currently and widely applied thermal processes (Spilimbergo and Bertucco, 2003). Previous studies have shown that carbonation with mild heating (<10 MPa, 20 – 40°C) effectively inactivates vegetative bacterial cells and fungal cells (Watanabe et al., 2003). However, spores of various *Bacillus* species are metabolically dormant and are thus highly resistant to environmental stress factors (Ghosh and Setlow, 2009) including carbonation (Watanabe et al., 2003).

Fatty acid esters are used as a food preservative because of their bacteriostatic effect on bacterial spores and inactivation effect on Gram-positive vegetative bacterial cells (Shibasaki, 1979). We previously demonstrated that the addition of monoglycerol-caprate (MC10) and monoglycerol-laurate (MC12) increased the inactivation effect of carbonation with heating (CH) (80°C, 5 MPa, 30 min) on *B. subtilis* spores, and the combination treatment prevented growth during 30-day storage (Klangpetch et al., 2013). We also revealed the significant inactivation effect of combined treatment of CH with MC10 or MC12 on *Bacillus cereus*, *B. coagulans* and

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Geo Bacillus stearothermophilus spores (Klangpetch et al., 2013; Nakai et al., 2014). However, the mechanism of the enhanced inactivation effect is still uncertain.

The aim of this study is to obtain aspects of the mechanism of the enhanced inactivation of B. subtilis spores by CH in the presence of MC.

Materials and Methods

Preparation of spore suspension B. subtilis 168 was kindly provided by Dr. J. Sekiguchi, Shinshu University. Spores were formed and prepared as previously reported (Klangpetch et al., 2013). The spore suspension was diluted with nutrient broth (NB; Difco Inc., Detroit, MI, USA) to give a spore concentration of about 10^8 (CFU/mL) as a liquid food model. The pH of NB was 6.8 (original) or 3.2 (acidified with HCl). Monoglycerol-caprate (MC) was kindly provided by Taiyo Kagaku Co. (Mie, Japan). Its molecular weight and hydrophile/lipophile balance were 246 and 6.5, respectively. MC was added to the spore/NB suspension at a final concentration of 0.005% or 0.05%, and cooled on ice until use.

Carbonation with heating (CH) and heat treatment (HT) A schematic diagram of the apparatus for CH is shown in a previous paper (Noma et al., 2011). A test tube containing 1 mL spore suspension was immersed in salad oil at 80°C in a CO2-dissolving vessel. CO2 gas was introduced into the vessel at 5 MPa and was dissolved into the spore suspension with stirring. After standing for 10 or 30 min, CO2 gas in the vessel was released via a pressure control valve. The time to reach the desired pressure was about 1 min; this was not included in the treatment time. To investigate the contribution of pressurization on the inactivation effect of CH, nitrogen gas was used for pressurization treatment instead of CO2 gas. For HT, the spore suspension in the test tube was heated at 80 or 85°C for 30 min in a water bath. The time to reach the desired temperature was about 1 min; this was not included in the treatment time. The spore suspension subjected to CH and heat was immediately cooled by immersion in ice-cooled water.

Determination of viable spores Viable spore counts were determined by the plate count method. Appropriate serial dilutions of the spore suspension were prepared with water, and 100 μL of the diluted suspension was plated onto nutrient agar (Difco, Inc.). The number of viable spores was enumerated as CFU/mL after incubation at 30°C for 24 h.

Estimation of germination The degree of spore germination was estimated by heat resistance and refractility of spores. Heat resistance of B. subtilis spores in the presence or absence of MC was determined by investigating the viable spore count after additional heat treatment at 80°C for 15 min. Refractility of spores before and after CH in the presence of 0.05% MC was determined using a phase contrast microscope (BX50; Olympus Co., Tokyo, Japan).

Estimation of bacteriostatic activity Spores with or without CH in the presence or absence of 0.005% MC were released at 30°C for 16 h with shaking in a microtiter plate. Proliferation of spores was determined before and after incubation using the phase contrast microscope.

Determination of dipicolinic acid (DPA) The concentration of DPA released from spores was measured as previously reported (Shibata et al., 1993). Briefly, 100 μL of spore suspension was mixed with both 100 μL of 1 mM terbium chloride (Nactalai Tesque, Kyoto) solution and 800 μL of 25 mM Tris/HCl buffer (pH 7.5). Then, fluorescence intensity was measured using a fluorescence spectrophotometer (RF-5300PC; Shimadzu, Kyoto, Japan) at an excitation wavelength of 282 nm and an emission wavelength of 544 nm.

Statistical analysis The statistical significance of DPA release was assessed using a Student’s t test (P < 0.05 or 0.01) following the F test (P < 0.05).

Results and Discussion

We have found that the addition of MC enhanced the inactivation effect of CH on B. subtilis spores by 3-4 orders of magnitude (Klangpetch et al., 2013). In the present study, we obtained aspects of the mechanism of this enhanced inactivation in B. subtilis spores.

Factors influencing the inactivation of B. subtilis spores by CH in the presence of MC The spore suspension is expected to be acidified to pH 3.2 during CH (Spilimbergo et al., 2005), and we have previously obtained enhanced inactivation in the presence of MC under CH conditions of 80°C and 5 MPa. Then, in order to assess the contribution of CH-related factors, i.e. heating, pressurization, and acidification, to the high inactivation effect of CH in the presence of MC, we designed the following experiments. The B. subtilis spores suspended in NB at pH 6.8 or 3.2 were subjected to HT, pressurization with N2 gas (PT), and HT under pressurization with N2 gas (HPT).

Fig. 1(A) shows the viable spore counts after each treatment in the absence of MC. The pH downshift of the spore suspension from pH 6.8 to 3.2 did not decrease the viable count of untreated spores. PT did not induce pronounced inactivation at either pH condition. For HT, spores in NB at pH 6.8 were not inactivated, while those at pH 3.2 were inactivated by approximately 2 log-order. This result corresponds to a previous paper (Wuytack and Michiels, 2001). For HPT, no viable loss was observed at pH 6.8; however, an approximately 2.3 order of magnitude decrease in spore count was demonstrated at pH 3.2. These results suggest that the combination of pH downshift and heating is important for inactivating B. subtilis spores by CH; while pH downshift alone, pressurization alone, and combination of pressurization and pH downshift are not involved in spore inactivation by CH. This corresponds with our previous paper describing that inactivation by carbonation was observed with heating above a certain temperature, while an increase in treatment pressure from 1 to 5
MPa did not enhance the decrease in viable spore counts in NB (Klangpetch et al., 2013).

Fig. 1(B) shows viable spore counts after treatments in the presence of MC$_{10}$. No apparent inactivation was induced in untreated spores by the pH downshift from 6.8 to 3.2. Similarly, viable counts did not differ between pH 6.8 and 3.2 after PT. These results corresponded with those in the absence of MC$_{10}$. For HT, spores were not inactivated at pH 6.8, while they were inactivated by about 5 log-order at pH 3.2. The difference in inactivation effect between pH 6.8 and 3.2 in the presence of MC$_{10}$ was greater than that in its absence, suggesting that MC$_{10}$ increase the number of the spores with lowered heat resistance of spores under acidic pH. For HPT, no spore inactivation was observed in NB at pH 6.8. However, an approximately 6.2 log-order inactivation was obtained at pH 3.2, and the degree of this inactivation was greater than with HT. This result corresponds with our previous study demonstrating that counts of inactivated spores with CH treatment in the presence of MC$_{10}$ increased in a pressure-dependent manner (Klangpetch et al., 2013). It is suggested that MC$_{10}$ raised the number of spores with decreased resistance to pressure under heating at acidic pH. In addition, these results clearly indicate that three factors in CH, i.e., acidification of the spore suspension, heating, and pressurization, were essential for obtaining the high inactivation effect of CH in the presence of MC$_{10}$.

Effect of CH in the presence of MC$_{10}$ on germination of B. subtilis spores It is thought that dormant spores are easily inactivated by several pasteurization methods after germination (Watanabe et al., 2003). Pressure-mediated inactivation of spores was achieved by first initiating germination and then inactivating the germinating spores via hydrostatic pressure treatment (Sale et al., 1970). Some decrease in resistance is observed at the initial stage of germination, and then loss of refractility, decrease in optical density, and increase in permeability of fluorescent dyes are induced in the process of spore germination (Hachisuka, 1988). We previously reported that CH induced these phenomena in B. subtilis spores suspended in water (Noma et al., 2011). This raises the possibility that MC$_{10}$ facilitates the germination of B. subtilis spores by CH. Therefore, the effect of CH in the presence of MC$_{10}$ on germination was assessed using heat resistance and refractility of spores. B. subtilis spores suspended in NB with or without 0.05% MC$_{10}$ were subjected to CH (80°C, 5 MPa for 10 or 30 min) and then heated (85°C for 30 min); the germination ratio through the loss of heat resistance was measured. Untreated spore counts were not significantly decreased by heating in both the presence and absence of MC$_{10}$ (Fig. 2A), as seen in Fig. 1. After CH for 10 min in the absence of MC$_{10}$, no apparent additional reduction in spore counts was induced by the subsequent heat treatment. However, an additional approximately 1.2 log order inactivation was observed by the subsequent heat treatment in the presence of MC$_{10}$.
The effect of CH in the presence of MC_{10} on spore refractility was visually assessed by microscopic observation (Fig. 2B). The untreated spores possessed a high degree of refractility in the presence of MC_{10}, while the spores after CH in the presence of MC_{10} showed decreased refractility. These results indicated that MC_{10} promoted the initial stage of CH-mediated germination.

**Bacteriostatic effect of CH and MC_{10} combination treatment**

It is expected that germination might facilitate the penetration of MC_{10} into spores and enhance the bacteriostatic effect of MC_{10}, leading to a high inactivation effect. Comparison of the bacteriostatic effect between CH with and without MC_{10} by microscopic observation is needed for normalization of the viable spore count. Therefore, the maximum concentration of MC_{10} with no additional viable loss when compared with CH alone was determined as 0.005% (data not shown). *B. subtilis* spores usually do not physiologically germinate and proliferate at pH < 4. The pH value of the spore suspension immediately after CH was about 5.6 (Nakai et al., 2013), indicating that the CH-induced pH downshift of the spore suspension does not seriously affect spore outgrowth and proliferation during subsequent incubation to estimate the bacteriostatic effect. Then, microscopic observation was performed to investigate the proliferation of spores subjected to CH in the presence of 0.005% MC_{10} (Fig. 3). Untreated spores proliferated after 16 h incubation in both the presence and absence of MC_{10}. Spores subjected to CH in the absence of MC_{10} also proliferated after the 16 h incubation. However, in the presence of MC_{10}, the germination process before outgrowth seemed to be inhibited and spores did not proliferate with incubation. These indicate that CH apparently enhances the bacteriostatic effect of MC_{10}.

To obtain evidence of the target of CH in the presence of MC_{10}, release of DPA from spores, which is initiated at the initial stage of germination, was also investigated after incubation at 30°C for 3 h (Fig. 4). DPA release reached a maximum level after 3 h incubation (data not shown). In untreated spores, the DPA level increased from 0.08 μg/mL to 1.2 μg/mL during incubation. In the presence of MC_{10}, the DPA concentration after incubation was 0.42 μg/mL, suggesting that MC_{10} inhibits any germination process(es) before DPA release. Besides, it is thought that spores resistant to MC_{10} proliferated after incubation for 16 h, as observed microscopically (Fig. 3). A high degree of DPA release was observed just after CH with and without MC_{10}, suggesting that CH physically induces DPA release. Subsequent incubation did not significantly increase DPA release in the absence of MC_{10}. This suggests that some of spores less damaged by CH germinated and proliferated during the incubation. Also, in the presence of MC_{10}, the DPA concentration was not significantly increased during the incubation. CH and MC_{10} combination treatment might inhibit DPA release in spores, inducing the enhanced inactivation effect of CH in the presence of MC_{10}.

In conclusion, we propose that the enhanced inactivation effect of CH in the presence of MC_{10} on *Bacillus subtilis* spores was obtained as follows. The three factors of CH, i.e., acidification of the spore suspension, heating, and pressurization, collaborated to increase the inactivation effect of CH in the presence of MC_{10}. MC_{10} induced germination to more spores than CH alone, allowing MC_{10} to act on the spores. As a result, the bactericidal effect of MC_{10} targeted a greater number of spores, inducing an enhanced, high inactivation effect. This study contributes to the development of a useful method to control bacterial spores in processed food by combining CH with low concentrations of MC_{10}.
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References