Inhibitory Effect of Spice Powders on the Development of Heated and Irradiated *Bacillus subtilis* Spores as Evaluated by Calorimetry

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Inhibitory effects of the powders of paprika, red pepper, black pepper, sage, oregano and thyme in a solid medium after heat treatment and gamma-irradiation on the development from spore of *Bacillus subtilis* were examined using calorimetry. Based on the $f(t)$ curve (Antoce et al., 1996) from the thermogram obtained, two parameters, the growth rate constant and the growth retardation time, were used to evaluate the inhibitory effect. The inhibitory effects of paprika and red pepper powders were enhanced by the spore pretreatment with heat, but not significantly with irradiation. The inhibitory enhancement by preheating depended upon the kind of spices used. Sage, oregano and thyme powders per se inhibited the development from spores completely even at a low concentration of 0.04 g/ml. Inhibitory effects of paprika and red pepper powders were obviously observed with heat treatment but not with irradiation. With black pepper powder, by contrast, substantial enhancement was neither observed with heat treatment nor gamma-irradiation. The results suggested that the addition of those spice powders might be useful in the thermal inactivation process of solid foods contaminated with *Bacillus subtilis* spores.

Key words : *Bacillus subtilis* spore / Spice / Heat treatment / Gamma irradiation / Calorimetry.

INTRODUCTION

Powdered raw spices are often contaminated by a substantial level of bacterial spores (Banerjee et al., 2003; Sagoo et al., 2009). These spores are highly resistant and thus often survive various killing treatments such as heating and ionizing radiation. The Japanese Food Sanitation Act indicates that the number of bacterial spores present must be below 1,000 spores/g for the safe commercial use of spices (Japan Food Hygiene Association, 2015). To meet these standards, in Japan, superheated steam is the most frequently applied treatment to spices, but this treatment often causes the deterioration of flavor and native color (Sadecka et al., 2010).

Ionizing radiation provides an attractive alternative for spice decontamination, as it is less detrimental to the spice quality because the rise in temperature induced by radiation is small and the original flavors and colors are hardly lost. In fact, spice irradiation has been put into practical use in 57 countries in the world at present (Kume et al., 2009) although it has not yet been approved in Japan. On the other hand, there is little information regarding the bacterial developmental behavior including the recovery of spores from injury of and the development from spores that have survived the sterilization process.

However, it is well known that spices themselves
possess antimicrobial activities and many of those contain different characteristic essential oils as inhibitory compounds (Miradinovic, 2000; Roldan et al., 2010; Zarringhalam, 2013). Based on these features, it is hypothesized that heated or irradiated bacteria and their spores contaminated contaminating spices may be damaged and then become sensitive to some antimicrobial compound(s) that are found in spices. Furuta et al. (2010) reported that the growth of the survivor fraction of contaminated bacteria in superheated-steam treated paprika powder (110°C for 50 sec) showed a significant delay compared that in with untreated paprika powder when mixed with minced sausage. On the other hand, no growth delay observed when the meat was mixed with paprika powder treated with 60Co gamma-irradiation at 10 kGy. Therefore, it is suggested that some compound(s) in paprika may inhibit the development from spore injured by superheated steam treatment. In addition, Sakai et al. (2012) reported that paprika powder per se did inhibit the development from spore of B. subtilis in minced sausage. However, they have not yet investigated whether the spores are sensitized to paprika powder by heat treatment.

In order to monitor bacterial growth in a solid and heterogeneously mixed food and food materials, investigators have found, the calorimetric method to be very useful (Antoce et al., 1996; Koga et al., 2008). Calorimetry measures the evolution of metabolic heat during bacterial propagation, enabling the growth patterns of bacteria in the absence or presence of spices and their constituent compounds to be monitored in a nondestructive manner.

The purpose of this study is to clarify how different spice powders, such as paprika, red pepper and black pepper, inhibit the development of heated or irradiated spores in Trypticase soy agar (TSA, Becton Dickinson and Co., U. S. A.) as a solid model.

**MATERIALS AND METHODS**

**Bacterial strain and preparation of spore suspensions**

The bacterial strain Bacillus subtilis ATCC6633 (obtained from Mesa Labs, Inc., U. S. A.) was used throughout this study. Before spore preparation, a culture from the spore suspension was streaked on a TSA plate and the plate was incubated at 37°C for 1 d. After that, a single colony was picked up from the cultured plate and one loopful of the colony was inoculated into a test tube containing Trypticase soy broth (TSB, Becton Dickinson and Co., U. S. A.). The tube was then incubated overnight at 37°C with shaking at 120 rpm. For spore preparation, 100 µl of culture was inoculated on plates of TSA supplemented with 0.02 g/l of MnCl₂ · 4H₂O, 0.25 g/l of MgSO₄ · 7H₂O, 3 mg/l of FeSO₄ · 7H₂O and 0.15 g/l of CaCl₂ · 2H₂O (Koshikawa, 2004). After incubation at 37°C for 7 d, spores were collected by immersing the surface of the agar plate with physiological saline and then scraping the surface with a plastic spatula. After being harvested, spores were washed twice by centrifugation at 10,000 × g at 4°C for 15 min and then resuspended in fresh physiological saline solution. A sporulation rate of more than 80% was achieved, as determined by phase contrast microscopy (Olympus BX51, Japan). Following quantification with a flow cytometer (BACT analyzer, Sysmex, Japan), the spore number in the resultant suspension was adjusted to 10⁷ spores/ml before storage at 4°C until use.

**Heat treatment and gamma-irradiation of B. subtilis spores**

A plastic microtube containing 0.9 ml of 50 mM potassium phosphate buffer (KPB, pH 7.0) was preheated at 100°C in a water bath. Subsequently, 0.1 ml of spore suspension (10⁷ spores/ml) was quickly suspended in the preheated microtube and the microtube was put into the water bath to be heated for 5, 10, 15 and 20 min.

Gamma-irradiation was performed in the irradiation facilities of the Radiation Research Center, Osaka Prefecture University, Japan. One milliliter of spore suspension containing 10⁷ spores was added to a test tube, and the tube was irradiated with 60Co gamma-ray at a dose rate of 3.0 kGy/h. The spore suspension was irradiated at 0.5, 1.0, 1.5, and 2.0 kGy.

**Viability assay**

After heat treatment and gamma-irradiation, each spore suspension was diluted serially with 50 mM KPB (pH 7.0) and then spread onto TSA plates. The plates were incubated at 37°C for 1 d. After incubation, the number of colonies was counted in order to obtain a survival curve. Three replicate experiments per each treatment were performed, and the resultant data were averaged.

**Spice powders**

The powders of paprika, red pepper, black pepper, sage, oregano, and thyme were obtained from K. Kobayashi, Inc. (Kobe, Japan). Before use, bacteria that remained on the surface of the spice powder were killed by irradiation at 20 kGy using a 60Co source. The resultant dose rate was 10 kGy/h.

**Preparation of the model solid food sample and calorimetry**

Five milliliters of solidified TSA were prepared in 25 ml sterilized glass vials. After that, the TSA solid was minced
with a spoon in the vial. The resultant preparation of minced TSA blocks was used as in the previous study (Sakai et al., 2012). A portion (0.1 ml) of the heated or irradiated spore suspension and then 0.2, 0.5, 0.8 or 1.0 g of spice powder was put into the vial containing minced TSA blocks, and the resulting spice concentrations were 0.04, 0.10, 0.16, 0.20 g/ml, respectively. The vial was firmly fastened with a cap and then kept warm in an incubator at 30°C for 30 min before being set in the calorimeter unit. For the measurement of metabolic heat, a multiplex calorimeter employing the heat conduction principle was used. In using the calorimetric system and its operation techniques, we followed the report of Takahashi (1996).

**Data analysis of calorimetry**

From the calorimetric output signal, which is derived from the amount of heat evolved during the development from spore, the growth curves were depicted, using the previously published algorithm (Antoce et al., 1996) and “BPCL24ch” analysis software. The defined $g(t)$ curve was depicted based on these signals, and then converted to the $f(t)$ curve reflecting the actual heat evolution (Antoce et al., 1996). The $f(t)$ curve can be denoted by the following equation:

$$f(t) = N_0 A e^{\mu t} + N_0 B$$

In this equation, $N_0$ is the initial cell number per g of the whole sample, $\mu$ is the growth rate constant (m⁻¹), and $A$ and $B$ are constants. The $\mu$ value was calculated from the $f(t)$ curve at the initial logarithmic phase. In addition, the level of heat evolution between 3 and 30 % of the height of the peak in the $f(t)$ curve was set as the value of “$A$” (Antoce et al., 1996). $\mu$ and the growth retardation time ($t_o$) were determined from the $f(t)$ curve.

To evaluate growth characteristics, the ratios of the values of $\mu$ and $t_o$ for the sample containing the spice powder ($\mu$ and $t_o(i)$; “$i$” as the concentration of the spice powder) to those values for the sample without the spice powder ($\mu$ and $t_o(0)$; “0” as no spice powder at all), namely, $\mu/\mu_0$ and $t_o(0)/t_o(i)$, were used. A decreasing $\mu/\mu_0$ ratio indicates an increased inhibition on the bacterial growth compared to control sample without spices. Also a reduced $t_o(0)/t_o(i)$ ratio indicates that exponential phase is delayed as a result of extinction or damage. These two ratios were used to determine whether the inhibitory effects of spice powders were enhanced by spore pretreatment with heat or gamma-irradiation.

**RESULTS**

**Survival of B. subtilis spores after heat treatment and gamma irradiation**

The survival of B. subtilis ATCC6633 spores after heat treatment at 100°C and 60Co gamma-irradiation was examined before the growth inhibitory effects of spice powders on the heat-treated and gamma-irradiated spores were investigated. The resultant survival curves are shown in Figs. 1 A and B, respectively.

In case of the heat treatment, an upward convex curve was obtained in the early phase. On the other hand, the survival curve for the gamma-irradiation was linear. A 90% reduction in survival from the initial level was elicited at 8.0 min of heat exposure or at 1.9 kGy of radiation. When B. subtilis spores were heated at 100°C for 5 min, the reduction in the survival rate was approximately 50%, which was similar to that obtained for 1.0 kGy irradiation (40% reduction). In the following experiments, the above conditions inducing approximately equivalent survival rates were adopted to evaluate the enhancement of the inhibitory activity of spice powders by heat treatment and gamma-irradiation.
Inhibitory effects of spice powders on the development from spore after heat treatment

It was examined whether the inhibitory effects of spice powders would be enhanced by the heat pretreatment of spores. All spice powders used in this experiment were in advance gamma-irradiated at a dose of 20 kGy to sterilize them.

Spores heated at 100°C for 5 min were mixed with different spice powders and minced TSA in a vial, and heat evolution in the mixture was monitored by calorimetry. After that, $g(t)$ curves and then $f(t)$ curves were obtained from the resultant thermograms as described earlier (Fig.2).

Figure 3 shows the results of calorimetry for the inhibitory effects of the powders of paprika (A), red pepper (B) and black pepper (C) of the growth development from spore heated at 100°C for 5 min, as evaluated with the ratios of $\mu/\mu_0$ and $t_h(0)/t_h(\theta)$ described earlier.

When heated spores were exposed to paprika powder, both ratios were significantly decreased with an increase in the concentration of added paprika powder, compared with untreated spores. For the spores treated with 0.1 mg/ml paprika powder, the $\mu/\mu_0$ was 0.25 times lower than unheated spores, and the addition of 0.2 g/ml paprika powder inhibited the development of heated spores completely (Fig.3(A)-a). Fig.3(B)-a shows a similar tendency for red pepper powder, and the $\mu/\mu_0$ of heated spores together with 0.2 g/ml of red pepper powder was 0.25 times lower than that of unheated spores. In addition, results for $t_h(0)/t_h(\theta)$, show that the value of heated spores became small depending on the increase in the quantity of the addition of paprika and red pepper powder (Fig.3(A)-b and (B)-b). Figure 3(A) and (B) showed that the inhibitory effect of paprika powder was enhanced effectively by the heat pretreatment. Similar results were also obtained with red pepper powder, although the degree of enhancement was smaller. In the case of black pepper powder, these ratios decreased markedly even for the unheated spores (Fig.3 (C)-a and b). Thus, this means that, in contrast to paprika powder and red pepper powder, black pepper powder had no enhancing activity. Sage, oregano and thyme powders completely suppressed spore growth even at the lowest concentration tested of 0.04 g/ml (data not shown). Therefore, it was not possible to analyze the effects of these powders further.

**Inhibitory effects of spice powders on the development from spore after gamma irradiation**

The inhibition of development from spore by spice powders after 60Co gamma-irradiation was also examined. The suspension of spores irradiated at 1.0 kGy was put into a vial containing the tested spice and minced TSA block, and then the vial was cultured for the analysis of growth patterns by calorimetry.

The results presented in Fig.4 show that the both ratios of $\mu/\mu_0$ and $t_h(0)/t_h(\theta)$ upon exposure to paprika powder were reduced similarly regardless of irradiation. Similar patterns were also observed for red pepper and black pepper powder. Ultimately, no substantial enhancement of growth inhibition by these spice powders appeared for irradiated spores.

To compare quantitatively the inhibitory effects of different spice powders on both the heated and irradiated spores, the concentrations of each spice required for a 50% reduction in the $\mu/\mu_0$ ratios and required for doubling the $t_h(0)/t_h(\theta)$ ratio were determined. The results are summarized in Table 1. The data indicate numerically that the inhibitory effects of paprika and red pepper were enhanced by heat treatment, but not by irradiation.

**DISCUSSION**

The calorimetric system, in which the developmental behaviors of heated or irradiated *B. subtilis* spores were evaluated in this study, is based on the heat evolution during the development from spore. However, since the heat evolution during spore germination and outgrowth periods is substantially undetectable, the heat detected here is considered to be derived from the subsequent vegetative growth. The inhibitory effect on the germination and outgrowth should be reflected by the $t_h$ value, but that on the vegetative growth affects both values of $\mu$ and $t_h$. Using these growth parameters, it was found that the inhibitory effects of paprika and red pepper powder on the development from *B. subtilis* spore were enhanced by heat injury (Fig.3(A) and (B)) and that effect of black pepper powder was not enhanced (Fig.3
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...on the other hand, paprika is derived from the selective breeding of red pepper in order to reduce the levels of pungent compounds such as capsaicin (Musfirho, 2013). Therefore, it is suggested that the effect of paprika powder is unlikely to be due to capsaicin, although we have not yet identified any ingredient compound respon-

\( \text{FIG. 3.} \) The ratios of \( \mu / \mu_0 \) (a) and \( t_0(0)/t_0(0) \) (b) for the inhibition by spices of the development from heated spores of \( B. \ subtilis \). Spices used were paprika (A), red pepper (B) and black pepper (C). Circles connected with a solid line, unheated; squares connected with a broken line, heated at 100°C for 5 min. Values with bars are the mean±S.D. obtained from three independent experiments.

(C)). On the other hand, gamma-irradiation did not enhance the inhibitory effects of any spice powder used in this study (Fig.4). It is suggested that these data have reproduced the phenomenon that appeared in the study by Furuta et al. (2010). In addition, from these results, the inhibitory effect on the development from spore was confirmed to vary with the spice powder.

Red pepper contains capsaicin, which damages the cell membrane and induces osmotic stress in Saccharomyces cerevisiae (Kurita et al., 2002). On the other hand, paprika is derived from the selective breeding of red pepper in order to reduce the levels of pungent compounds such as capsaicin (Musfirho, 2013). Therefore, it is suggested that the effect of paprika powder is unlikely to be due to capsaicin, although we have not yet identified any ingredient compound respon-
supposedly present in red pepper and paprika powder used here may be chemically distinct. The fact that the ratios of two key parameters, $\mu_i/\mu_0$ and $t_{o}(0)/t_{o}(i)$, used in this study were decreased when the spores were treated with either spice powder, indicates the powerful inhibitory effects of both spices.

With black pepper, the above two ratios were decreased much more dramatically than those of paprika and red pepper. The inhibitory action of black pepper

Paprika (Capsicum annuum L.) is a perennial belonging to the Solanaceae family. It has been reported that the water extract of paprika seeds inhibits the growth of S. cerevisiae cells (Yajima et al., 1996). Since the paprika powder used in our experiment does not include seeds, it is suggested the fruit of paprika also contains some antibacterial compounds. In consideration of these points, the growth inhibitory compounds supposedly present in red pepper and paprika powder used here may be chemically distinct.

FIG. 4. The ratios of $\mu_i/\mu_0$ (a) and $t_{o}(0)/t_{o}(i)$ (b) indicating the inhibitory effects by spices on the development of irradiated spores of B. subtilis. Spices used were paprika (A), red pepper (B) and black pepper (C). Circles connected with a solid line, not irradiated; triangles connected with a broken line, gamma-irradiated at 1.0 kGy. Values with bars are the mean±S.D. obtained from three independent experiments.
The main active component of oregano oil is carvacrol and thujone and camphor, thyme contains thymol and a.

...et al., 2008; Roussenova, 2011... These results suggest that the mechanisms of action of some active compound(s) in black pepper on the heated spores are different from those in red pepper and paprika powder.

Our findings on the inhibitory effects of the essential oils in spices enhanced by the heat pretreatment are consistent with results in the previous reports. Haberbeck et al. (2012) indicated that for Bacillus coagulans spores that essential oils from oregano amplified the sensitivity to heat treatment. Periago et al. (2006) suggested that for B. megaterium, the damage caused by the preheating treatment to cell membranes and the denaturation of proteins was enhanced by carvacrol and thymol, and that inhibitory effects of those essential oils correlated with the lag phase of B. megaterium spore growth.

On the contrary to heat treatment, the inhibitory effects of spice powder were not enhanced by pretreatment with gamma-irradiation in our study (Fig.4). Gamma-ray irradiation mainly DNA and the damaged DNA is thought to be repaired during the outgrowth and the early stage of the subsequent vegetative growth in spores. In such gamma-ray damaged spores, the germination system is considered to be unaffected and still functional (Gould et al., 1968; Hayashi et al., 1995). Because it was thought that the target of the gamma-ray irradiation in spores was different from that of spice compounds, the inhibitory effect of spice powder might not be enhanced in suppressing the development of irradiated spores.

Our results obtained with calorimetry in minced blocks of TSA may provide beneficial information for practical...
use of spice powders in solid and heterogenous foods. In the future, there should be further studies on how essential oils from spice powder inhibit spore germination, outgrowth and subsequent vegetative growth processes after heating.

REFERENCES


