Research Note

Sporicidal Kinetics of *Bacillus subtilis* Spores by Heated Scallop Shell Powder

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MS 02-307: Received 12 September 2002/Accepted 3 November 2002

**ABSTRACT**

Scallop shell powder heated at 1,000°C for 1 h exhibited sporicidal action against *Bacillus subtilis* spores. The sporicidal kinetics of this action were analyzed with the use of a nonlogarithmic model. Apparent death rate constants ($k$) were obtained under various conditions. The value of $k$ increased with powder concentration but became constant beyond the concentration representing the solubility of Ca(OH)$_2$. A linear inverse relationship between $k$ and temperature was found, and from this relationship the activation energy required for the death of *B. subtilis* spores in the heated shell powder slurry could be determined.

Although part of a scallop shell is recycled for use as a food additive (i.e., as a Ca supplement) and in plastering and paving materials, most of the shell is regarded as commercial waste. In scallop-harvesting districts, large amounts of shell are heaped near the seaside, creating serious problems such as the emission of offensive odors and soil pollution from the leaching of heavy metals that are contained in the viscera (7, 16). In a previous study (15), heated scallop shell powder was found to exhibit strong antibacterial activity. The main component of scallop shells is CaCO$_3$, whose antibacterial activity is due to the generation of CaO with heat treatment (11). The use of these materials in food processing may therefore not only provide a source of minerals but also prolong the shell lives of foodstuffs. Heated scallop shell powder could be useful as an antibacterial agent, and its use would reduce pollution. Shell powder heated at $\geq$700°C exhibited bactericidal action against vegetative cells of bacteria. The bactericidal action of the powder heated at 1,000°C was comparable to that of pure CaO (15). Moreover, scallop shell powder treatment was found to effectively reduce the aerobic bacterium count in shredded cabbage (14).

Some species of bacteria, such as those of *Bacillus* and *Clostridium*, produce spores in the stationary phase of growth. The spores are capable of long dormancy and are resistant to heat and various chemicals (3). Bacterial spores have caused many serious problems (1, 2, 10). Therefore, the inactivation of bacterial spores is an important step in food processing. In this study, we examined the sporicidal effect of heated scallop shell powder on spores of *Bacillus subtilis* and determined the sporicidal kinetics of this action.

**MATERIALS AND METHODS**

**Preparation of sample.** Powder from scallop (Patinopecten yessoensis) shells was obtained from Yaizu Suisan Kagaku Industry Co., Ltd. (Yaizu, Japan). The powder was heated at 1,000°C in air for 1 h and ground in a ball mill. The mean particle size of the heated shell powder was approximately 5 μm. Scallop shell powder heated at 1,000°C for 1 h contained 70.8% (wt/wt) calcium and 0.16% (wt/wt) magnesium. The elements phosphorus (0.073%, wt/wt), sodium (0.014%, wt/wt), and iron (0.003%, wt/wt) were also present. Silver and copper, which are known to be antibiotic agents, were not detected (<0.01 μg/kg) (15). At a calcium concentration of 70.8% (wt/wt), the shell contains 99% (wt/wt) CaO. Calcium, iron, and phosphorus levels were determined by the permanganate method, the phenanthroline colorimetric method, and the molybdenum blue colorimetric method (17). Other elements were measured by atomic-adsorption analysis.

The powder was mixed with sterile saline (0.85%, wt/vol) to produce a slurry. For the determination of the pH of the slurry, a pH probe was placed into a vial of the slurry, which was agitated in a water bath at 250 rpm until the pH was stable. At any concentration, the heated scallop shell powder slurry reached a stable pH within 10 min. The supernatant was obtained by centrifuging the powder slurry (which had been allowed to sit for 60 min after being prepared) at 4,000 × g for 20 min.

**Test organisms.** *B. subtilis* was purchased from Eiken Kizai Co. Ltd. (Tokyo, Japan). The test bacteria were incubated in brain heart infusion broth (Eiken Chemicals, Tokyo, Japan) at 37°C for 20 h. The culture was spread on a nutrient agar plate. After 10 days of incubation at 37°C, colonies were harvested with the use of a small amount of sterile water and centrifuged at 5,000 × g for 10 min three times. The resulting pellet was resuspended in sterile water containing lysozyme (Sigma Chemical Co., St. Louis, Mo.) at 500 μg/ml and incubated at 37°C for 60 min. The spores were centrifuged three times in sterile water at 5,000 × g for 10 min, resuspended in sterile water, and heated in a water bath at 80°C for 10 min to remove vegetative cells. The final spore suspension was stored at 4°C.
FIGURE 1. Effects of scallop shell powder concentration on viability of *B. subtilis* spores at 60°C. ●, 100 mg/ml; ■, 10 mg/ml; ▲, 1.0 mg/ml; ▼, 0.75 mg/ml; ◇, 0.5 mg/ml; ×, 0.1 mg/ml. Lines indicate values calculated by equation 1.

FIGURE 2. Apparent death rate constant of *B. subtilis* spores in scallop shell powder at 60°C.

cation of the spores was confirmed by staining with methylene blue.

**Viable counts.** Powder slurry (20 ml) was poured into a vial (internal diameter, 32 mm) and agitated with a magnetic stirrer at 250 rpm. The slurry temperature was controlled with the use of a water bath. The spore suspension (100 µl) was aseptically pipetted into the slurry at an initial concentration of approximately 10⁵ CFU/ml. Samples (100 µl) were periodically withdrawn, diluted in sterile saline (0.85%, wt/vol), and pour plated with nutrient agar. The colonies were counted after incubation at 37°C for 24 h. All experiments were carried out in duplicate on three different occasions; the standard error of the average value remained below 10%.

**RESULTS AND DISCUSSION**

**Effect of powder concentration.** A graph of the effects of scallop shell powder concentration on the viability of *B. subtilis* spores is shown in Figure 1. The powder slurry temperature was 60°C. The ordinate is the ratio of the number of spore CFU posttreatment (*N*) to that without treatment (*N₀*). The addition of the heated scallop shell powder decreased the number of viable spores. Spore survival decreased with increasing powder concentration up to 1.0 mg/ml. No additional sporicidal effects were observed above that concentration. At concentrations of ≥1.0 mg/ml, spore survival dropped two orders of magnitude within 20 min. Survival rates for vegetative cells of bacteria treated with shell powder slurry follow first-order reaction kinetics, showing a linear relationship between logarithmic survival ratio and treatment time (15). In contrast, the survival curves for the *B. subtilis* spores became steeper with time. Therefore, the following equation, proposed by King et al. (8), was used for the analysis of death of *B. subtilis* spores by heated scallop shell powder slurry.

\[
\log N_0 - \log N = kt
\]

where *k* and *a* are the apparent death rate constant and an empirical constant, respectively, and are calculated by regression analysis. The optimum value of *a* was determined to be 0.6. The lines drawn on the basis of the obtained parameters (*k* and *a*) were in agreement with the experimental values (Fig. 1).

The calculated *k* value was plotted against the powder concentration (Fig. 2). To determine the dilution coefficient, the equation defining the relationship between *k* and reagent concentration (*C*) (18) was used:

\[
k = \alpha C^n
\]

where *α* is an empirical constant and *n* is the dilution coefficient. The values of *n* were obtained from the slope of the line in Figure 2. The *n* value for *B. subtilis* spores was 3.0 at concentrations of <1.0 mg/ml. However, *k* did not change at powder concentrations of >1.0 mg/ml. The solubility of Ca(OH)₂ at 60°C is 1.08 mg/ml (5), and this almost corresponds to the concentration at which *k* becomes constant. This finding suggests that the soluble compounds in the powder slurry significantly affect the *B. subtilis* spores.
Effect of slurry temperature. Figure 3 shows the effect of slurry temperature on the viability of *B. subtilis* spores at a powder concentration of 100 mg/ml. Sporicidal action increased with increasing slurry temperature, and curves for the spores became steeper with time. The number of spores was reduced with the addition of heated shell powder, even at 25°C. The values of $k$ and $a$ were calculated by equation 1. The optimal value of $a$ is 0.6 across the temperature range. As shown in Figure 3, the lines based on the calculated $k$ and $a$ values were in agreement with the experimental values. Figure 4 shows the relationship between the values of $k$ and the slurry temperature for *B. subtilis* spores (Arrhenius plot). A good linear relationship between log $k$ and the reciprocal of slurry temperature was obtained as follows:

$$k = A \exp[-E_a/(RT)]$$ (3)

where $A$, $R$, and $T$ are the frequency factor, the gas constant, and the reaction temperature, respectively. The values obtained for $E_a$ and $A$ were $5.55 \times 10^4$ s$^{-1}$ and $6.41 \times 10^5$ J/mol, respectively. As indicated by these results, the non-logarithmic model proposed by King et al. (8) describes the sporicidal pattern of *B. subtilis* spores in heated scallop shell powder slurry.

Effect of solute compositions. As shown in Figure 2, the sporicidal action of scallop shell powder became constant at a powder concentration higher than that representing the solubility of Ca(OH)$_2$. The supernatant of the powder slurry exhibited a sporicidal effect almost identical to that of the powder slurry, indicating that the soluble compounds in the powder slurry significantly affect the *B. subtilis* spores. A similar result was obtained for the pure CaO slurry; therefore, this effect is not likely to be the result of an impurity. The main solutes in the slurry were OH$^-$ and Ca$^{2+}$. The pH of saturated slurry at 60°C was 11.8. Alkaline treatment involving NaOH and KOH solutions at pH 11.8 did not reduce the survival of the spores (Fig. 5). The effect of Ca$^{2+}$ was also investigated with the use of CaCl$_2$. Ca$^{2+}$ alone (3.15 $\times$ 10$^{-3}$ mol/liter) did not affect spore survival even at the Ca$^{2+}$ concentration of the solubility of Ca(OH)$_2$. Moreover, the sporicidal effect of the combination of Ca$^{2+}$ and OH$^-$ was markedly less extensive than that of the shell powder supernatant solution.

We previously studied the mechanism of the action of CaO powder against the survival of vegetative cells of bacteria. Because of the high pH of the powder slurry, the alkaline effect is considered a primary factor in antibacterial activity. However, the results of our previous study (13) indicated that other factors, in addition to alkalinity, affect the antibacterial mechanism of CaO. The damage to *Escherichia coli* caused by CaO powder slurry was studied by monitoring change in sensitivity to antibiotics. Although the pH of the CaO powder slurry was high, the changes in antibiotic sensitivity caused by the CaO powder slurry were obviously different from those caused by alkaline treatment. Mendonca et al. (9) stated that the effect of high pH was an all-or-nothing outcome and that high-pH treatment did not damage cells, which grew equally well on both selective and nonselective media. Our results for alkaline treatment (13) agree well with the results of Mendonca et al. (9). For
The generation of active oxygen, such as superoxide anions, in the powder slurry was observed (12). Some sensitivity changes in response to the CaO were consistent with those induced by active oxygen (13). In addition, flow cytometry results obtained by Hewitt et al. (4) support our theory about the bactericidal action of CaO powder. Active oxygen generated from heated shell powder containing CaO as the main component is also related to the mechanism of sporidical action.

In view of generally insufficient calcium intake and an increasing wariness of preservatives in processed foods on the part of the public, the use of scallop shell may not only contribute to the reduction of pollution of heaped shells but also assist in the economical production of healthy processed food. After use in food processing, if the heated shell is discarded as refuse in open air, CaO becomes CaCO$_3$ through the absorption of CO$_2$ from air. In recent years, the development of antimicrobial agents that are very safe for the natural environment has been a goal. Heated scallop shell powder is expected to be used in environmental preservation as well as in food processing. Kawasaki et al. (6) reported that active oxygen species such as H$_2$O$_2$ promoted the germination of spores. The effects of shell powder slurry on the germination of spores is now being investigated.

ACKNOWLEDGMENT

The authors thank Yaizu Suisan Kagaku, Ind. Co., Ltd. (Yaizu, Japan) for the generous donation of shell powders.

REFERENCES


