Transactions of the Materials Research Society of Japan 24 [4] 557-560 (1999)

Utilization of heated shell powder in biocontrol

Hirokazu Shiga, Jun Sawai* and Hiromitsu Kojima

Department of Applied Chemistry, Kanagawa Institute of Technology;
1030 Shimo-Ogino, Atsugi, Kanagawa 243-0292, Japan
**Fax: 81-462-91-3193, *e-mail: sawai@chem.kanagawa-it.ac.jp*

The shell powder of scallop (*Patinopecten yessoensis*) was used as the test material and heated at 100 to 1000°C. The powder heated at temperatures of 700°C or higher showed a bactericidal action on both *Escherichia coli* and *Staphyloccocus aureus*. An increase in heating temperature enhanced the bactericidal action of the shell powder. Although the heated powder slurry has a high pH value, it had the bactericidal action greater than NaOH solution at the same pH value as the slurry. Furthermore, from the result of Ames test, the heated shell powder was not mutagenic. These results suggested that the heated shell powder slurry had an antibacterial factor apart from the alkaline effect and did not damage DNA directly in its action mechanism.

Key words: shell, bactericidal action, antibacterial activity, mutagenicity, calcium oxide

1. Introduction

It is well known that calcium plays an important role in human body, and calcium preparation such as some kind of shell powders has been used to supply mineral. The main component of these shell powders is calcium carbonate ($CaCO_3$), which changes to calcium oxide (CaO) by heat treatment. CaO powder exhibits a strong antibacterial activity (1) and shows an efficacy against the spores of *Bacillus subtilis* having high resistivities to thermal sterilization and antibacterial agents (2). Therefore, the application of the heated shell powder to food processing is expected not only to supply mineral but also to prevent microbial pollution. However, there is very few study on the bactericidal action concerned in the shell powder. In this work, scallop (*Patinopecten yessoensis*) was used as the material and an investigation was made to determine the influence of heating temperature of the shell powder on bacteria.

Moreover, the relationships of the antibacterial activity and safety of the heated shell powders have not been cleared yet. Mutagens act on DNA and injure of the base sequences. Many mutagens are known to be carcinogens. And so, the shell powder was tested for its mutagenicity by the Ames test with the tester strains of *Salmonella typhimurium* TA98 and TA100.

2. Material and Methods

2.1 Shell powder slurry

The shell powder of scallop (*Patinopecten yessoensis*) was used as the test material. The shell powder was heated at 100 to 1000°C in air for 1 h. The mean particle size of the heated shell powder was approximately 5 μm. The powder slurry was prepared by suspending with physiological saline.

2.2 Investigation of bactericidal action

Escherichia coli 745 and *Staphylococcus aureus* 9779, which were stored in the Tokyo Metropolitan Laboratory of Public Health, were cultured in Brain Heart Infusion broth (Difco) at 37°C for 20 h. The culture was suspended with physiological saline (NaCl 0.85%) to yield a final bacterial concentration of approximately 10^8 CFU/ml (CFU: Colony Forming Unit).

The powder slurry of 20 ml in a vial with an inner diameter of 32 mm was agitated by a magnetic stirrer at 37°C, and a 0.1 ml bacterial suspension was added to the slurry. The initial bacterial concentration was about 2×10^6 CFU/ml. The bacterial suspension was removed at intervals and diluted by physiological saline. The diluted sample was pour-plated with Nutrient agar (Eiken chemicals) and Standard Method agar (Eiken chemicals) for *E. coli* and *S. aureus*, respectively. The agar plates

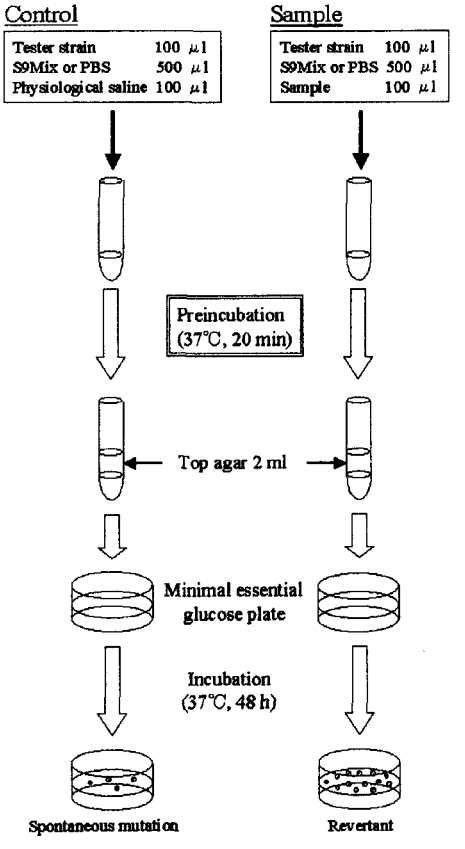

Figure 1 Experimental procedure of Ames test

Table 1 Bactericidal action of shell powder heated at different temperatures

Time [min]	Survival ration [-] CaO (0.25 mg/ml)	1000℃ (0.25 mg/ml)	900℃ (0.75 mg/ml)	800℃ (0.75 mg/ml)	700℃ (10 mg/ml)	Shell powder (10 mg/ml)
0	1.00	1.00	1.00	1.00	1.00	1.00
1	6.56×10^{-1}	6.88×10^{-1}	7.05×10^{-1}	6.02×10^{-1}		
3	7.97×10^{-2}	7.17×10^{-2}	2.69×10^{-2}	1.04×10^{-1}		
5	$< 10^{-6}$	$< 10^{-6}$	7.45×10^{-4}	8.72×10^{-3}	1.97×10^{-1}	
10	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	4.61×10^{-3}	9.65×10^{-1}
15	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$	5.48×10^{-5}	8.44×10^{-1}

Table 2 Comparison of bactericidal action between shell powder heated at 1000℃ and NaOH solution.

Time [min]	Survival ratio [-] Heated shell pH12.0	NaOH pH12.0	Heated shell pH11.5	NaOH pH11.5
0	1.00	1.00	1.00	1.00
1	4.85×10^{-1}	7.68×10^{-1}	9.26×10^{-1}	1.00
2	7.60×10^{-2}	6.38×10^{-1}	6.88×10^{-1}	1.11
3	2.43×10^{-2}	6.94×10^{-1}	5.00×10^{-1}	9.67×10^{-1}
4	2.49×10^{-4}	6.23×10^{-1}	3.44×10^{-1}	9.97×10^{-1}
5	$< 10^{-6}$	5.21×10^{-1}	2.11×10^{-1}	8.93×10^{-1}

were incubated at 37°C for 48 h, and number of colonies was enumerated.

2.3 Mutagenicity test

Salmonella typhimurium TA98 and TA100, which were stored in the Tokyo Metropolitan Laboratory of Public Health, were used as the tester strains. The tester strains were incubated in nutrient broth No.2 (Oxoid) at 37°C for 10 h. The shell powder heated at 1000°C was used for the mutagenicity test.

Mutagenicity test was carried out by the preincubation method (3). Figure 1 shows the experimental procedure. A mixture of the shell powder slurry, the tester strain culture and 0.1 M phosphate buffer (pH 7.0) or S9 mix (4) (Oriental Yeast) was preincubated in a water bath shaker at 37°C for 20 min. Then, a top agar (5) was added to the mixture. This compound was poured onto a minimal essential glucose plate containing 1.5% agar and 2% glucose in a Vogel-Bonner medium (5). The plates were incubated at 37°C. The number of revertant colonies was counted after 48 h.

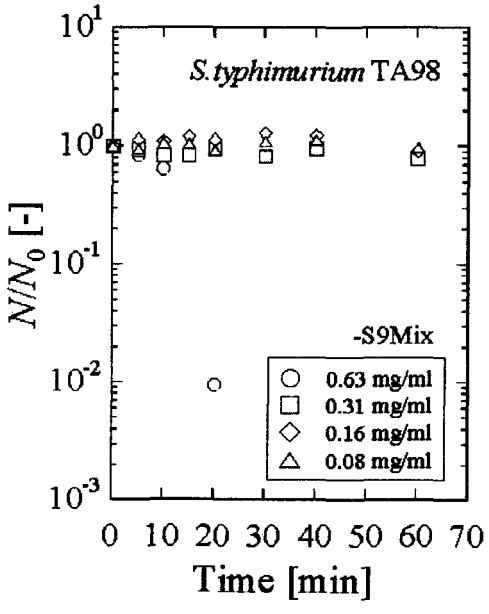

Fig.2 Variation of the number of viable cells of *S.typhimurium* TA98 in heated shell powder slurry.

Table 3 Maximum concentration of the powder slurry in Ames test.

Strain	Maximum concentration of shell Powder slurry [mg/ml]	
	Heated	Unheated
TA98	3.13×10^{-1}	2.5
TA100	6.25×10^{-1}	5.0

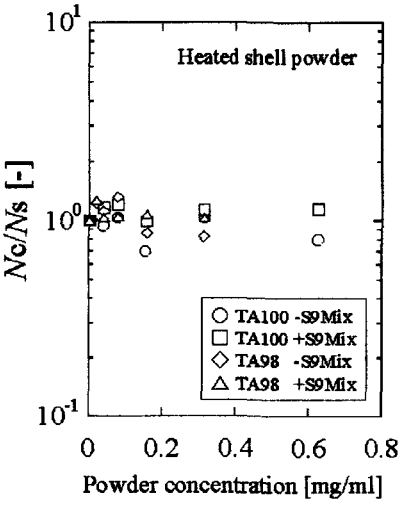

Fig.3 Effect of heated shell powder on the number of revertants *S.typhimurium* TA98 and TA100

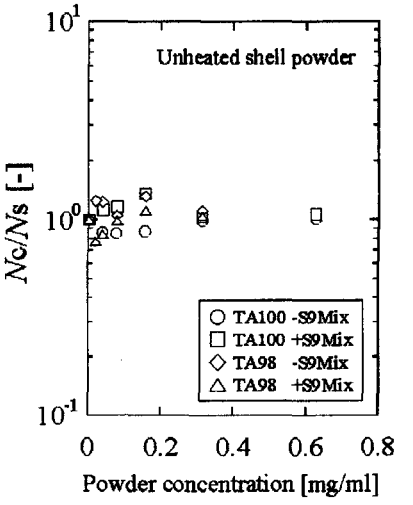

Fig.4 Effect of unheated shell powder on the number of revertants *S.typhimurium* TA98 and TA100

3. Results and Discussion

3.1 Bactericidal action of shell powder

Table 1 exhibits the bactericidal action of the shell powder heated at different temperatures for *E. coli.* The powder heated at temperatures of 700℃ or higher showed a bactericidal action against *E. coli*, and an increase in heating temperature enhanced the bactericidal action. The unheated shell powder did not decrease the survival ratio of *E. coli.* The bactericidal action of the shell powder heated at 1000℃ was almost equal to that of CaO powder and was greater than that of three times concentration of the shell powder heated at 900℃ or 800℃. The bactericidal action of heated shell powder slurry originated from the conversion from $CaCO_3$ in heated shell to CaO by heat treatment. Because the heated shell powder slurry had a high pH value, the alkaline effect might be one of its antibacterial factors. The bacte ricidal action of the heated shell powder slurry was compared with that of NaOH solution with the same pH value as the slurry. As shown in Table 2, the shell powder heated at 1000℃ had the bactericidal action more strongly than NaOH solution. This result suggested that the heated shell powder obviously had the other antibacterial factors except the alkaline effect. Sawai *et al.* reported that the production of superoxide anion, which is one of active oxygen species, was observed from the CaO powder slurry by chemiluminescence analysis (6). Studies are under way to examine the contribution of active oxygen to the bactericidal action of the heated shell powder slurry.

2.2.2 Ames test

Figure 3 shows the results of the mutagenicity test for the heated shell powder. The ordinate (N_c/N_s) is the ratio of number of revertants to that of spontaneous revertants of *S. typhimurium* TA98 or TA100. When the value of N_c/N_s for a test substance is over 2.0, the substance is considered to be mutagenic.

The S9 fraction is the supernatant obtained from centrifuging mammalian rat liver homogenates at 9,000 ×g, and contains metabolic activating enzymes. Many mutagens are activated through in vivo metabolism, and the active forms damage DNA and cause mutation (4) (e. g. Benzo[a]pyrene). However, the tester strains lack metabolic enzymes. For efficient detection of a wide variety of mutagens requiring metabolic activation, S9 is used to activate the mutagens. Coenzymes, buffered solutions and so on are also added to S9, which is called S9 mix (3, 5). In the Ames test, the result for both cases, with and without S9 mix, are needed.

For the heated shell powder, the values of N_c/N_s were almost unit in the presence and the absence of metabolic activation by the addition of S9 mix. *S. typhimurium* TA98 can detect a mutation of frame shift type, and *S. typhimurium* TA100 can detect a mutation of base pair substitution type. The unheated shell powder was not also mutagenic (Fig. 4). The shell powder was not mutagenic to the strains of TA98 and TA100, even the case that it

was heated and became antibiotic. From these results, because mutagenicity is an ability to injure DNA in cells, it was suggested that the heated shell powder did not damage DNA directly,

References

1. J. Sawai, H. Igarashi, A. Hashimoto, T. Kokugan and M. Shimizu, *J. Chem. Eng.Japan*, **28**, 288-293 (1995)
2. J. Sawai, H. Igarashi, A. Hashimoto, T.Kokugan and M. Shimizu, *J. Chem. Eng. Japan*, **28**, 556-561 (1995)
3. T. Yahagi, *Protein, Nucleic acid and Enzyme.*, **20**, 1178-1189 (1975)
4. D. M. Maron and B. N. Ames., *Mutation Res.*, **113**, 173-215 (1983)
5. H. V. Mailling, *Mutation Res.*, **13**, 425-429 (1971)
6. J. Sawai, E. Kawada, F. Kanou, H. Igarashi, A. Hashimoto, T.Kokugan and M. Shimizu, *J. Chem. Eng. Japan*, **29**, 627-633 (1996)

(Received December 10, 1998; accepted April 14, 1999)