Antimicrobial Characteristics of Heated Eggshell Powder

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Eggshells have high bioavailability and can be used as a source of calcium. The main component is CaCO₃, which, when heated, is converted to CaO. Seashells are also mainly composed of CaCO₃ and previously found to exhibit antimicrobial activity after being heated. In this study, heated eggshell powder (HESP) was found to have antimicrobial activity against bacterial vegetative cells, fungi and bacterial spores. Parameters, such as the minimum inhibitory concentration, were determined with kinetic analysis using an indirect conductimetric assay. Moreover, HESP was able to kill the Bacillus subtilis spores. There were no significant differences in the activity between HESP, heated scallop-shell powder and pure CaO. The MIC values for HESP against bacteria and fungi were 0.29-0.43 and 1.3-1.5 mg/mL, respectively. Against B. subtilis spores, a reduction of two orders of magnitude of viability was confirmed following 20 min of treatment at 10 mg/mL at 60 °C. The active oxygen generated from the HESP slurry was examined with chemiluminescence. The intensity of this increased with increasing concentrations of the HESP slurry. This suggests that HESP could be used as a natural antimicrobial agent. Although a high pH is the main contributor to this antimicrobial activity, active oxygen species generated from HESP are likely to be the main antimicrobial agents.

Key words: Eggshell / Scallop-shell / Calcium oxide / Antimicrobial activity / Bacillus subtilis spore.

INTRODUCTION

The yield of chicken eggs in Japan was 2,522 thousand tons in 2013 according to statistics from the Ministry of Agriculture, Forestry and Fisheries. Annual consumption per person was about 330 eggs—one of the highest globally. Furthermore, eggs are also used in industrial and medical products and in supplements, and the eggshell and its membrane are used for catalysts (Gao and Xu, 2012) and defluoridation (Lunge et al., 2012), respectively.

Eggshells have high bioavailability and a source of calcium. Kushimi et al. (1999) showed that the solubility of eggshell calcium in the stomach is significantly higher than calcium carbonate (CaCO₃). Eggshell Ca could also be effective at preventing bone loss after an ovariectomy (Omi and Ezawa, 1998).

The main component of eggshells is CaCO₃, which is converted to CaO when heated. Seashells are also mainly composed of CaCO₃ and have exhibited antimicrobial activity after being heated (Sawai, 2011; Kubo et al., 2013; Watanabe et al., 2014). Recently, heated seashell powders, such as those from scallops and oysters, have been effective as disinfection and decontamination agents for food (Asada et al., 2001; Bari et al., 2002; Mehmetoglu, 2011; Bodur and Mehmetoglu, 2012). However, there are no similar reports for heated eggshell powder (HESP).

The HESP Ca preparations are slightly soluble, so conventional methods, such as the halo test and turbidometry, are unsuitable for use in the determination
of the minimum inhibitory concentration (MIC). Therefore, in this study, the antimicrobial activity of HESP was analyzed with the indirect conductimetric assay (Sawai and Yoshikawa, 2004) and compared with that of the heated scallop-shell powder (HSSP).

In this study, the sporidical activity of HESP against *B. subtilis* was also investigated. This is because some species of bacteria, such as *Bacillus* and *Clostridium*, produce spores in their stationary phase of growth. These are capable of long dormancy and are resistant to heat and various chemicals (Popham et al., 1995). Bacterial spores have caused many serious problems in food processing and medical treatment, leading to food poisoning and infection (Welt et al., 2003; Kennedy, 2004). Therefore, the inactivation of bacterial spores is of great interest.

**MATERIALS AND METHODS**

**Sample preparation**

Eggshell powder for food additives (Kewpie Co., Tokyo, Japan) was heated to 700-1000 °C in air for 1 h and then ground using a planetary ball mill to a mean particle size of approximately 5 µm. The HESP was then immediately stored in a desiccator. A slurry was prepared by suspending this in sterile saline (0.85% w/v). Scallop-shell powder (Soy-com Co. Ltd., Atsugi, Japan) was also heated at 1000 °C for 1 h, ground to approximately 5 µm and prepared as a powder slurry. The HESP phases were analyzed by X-ray diffractometry (XRD; MiniFlex, Rigaku Co., Tokyo, Japan).

**Microorganisms**

*Escherichia coli* NBRC 3301, *Staphylococcus aureus* NBRC13276, *Saccharomyces cerevisiae* NBRC1950, *Candida albicans* NBRC 1060 and *Rhizopus stolonifer* NBRC 4781 were obtained from the National Institute of Technology Evaluation Biological Resource Center (Kazusa, Japan). *E. coli* and *S. aureus* were stored at -80 °C. They were thawed and incubated in nutrient broth (Eiken Chemicals, Tokyo, Japan) for 20 h at 37 °C. The culture was then suspended in sterile saline at approximately 10⁶ colony forming units (CFU)/mL. The bacterial suspension was kept in ice water until use.

Yeasts and filamentous fungi were subcultured on Potato Dextrose Agar (PDA: Eiken Chemicals) slants at 25 °C for 7 and 10 days, respectively, and then stored at 4 °C. Yeasts were inoculated on PDA plates and incubated at 25 °C for 7 days. The colonies were suspended in 0.05% Aerosol-OT (AOT) solution (di-2-ethylhexyl sodium sulfosuccinate; Nacalai Tesque, Inc., Kyoto, Japan) to give a microbial concentration of approximately 10⁶ CFU/mL. Filamentous fungi were inoculated on PDA slants and incubated at 25 °C for 10 days. The spores were collected by adding 10 mL of 0.05% AOT solution to a test tube with slants. The concentration of spores collected was measured by a PA2000 particle analyzer (Erma Inc., Tokyo, Japan) and adjusted to approximately 10⁶ spores/mL.

*B. subtilis* ATCC6633 was incubated in nutrient broth (Eiken Chemicals) at 37 °C for 20 h. The culture was spread on a nutrient agar (Eiken Chemicals) plate. After incubation for 10 days at 37 °C, the colony was harvested using a small amount of sterile water and centrifuged at 5,000 × g for 10 min. This was repeated three times. The resultant pellet was resuspended in sterile water containing lysozyme (Sigma, St. Louis, MO, USA) 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 and heated at 80 °C for 10 min in a water bath to remove vegetative cells. The final spore suspension was stored at -20 °C. Identification of the spores was confirmed by staining with methylene blue (Hoffman et al., 1977).

**Evaluation of HESP antimicrobial activity against vegetative bacteria cells and fungi by indirect conductimetric assay**

The indirect conductimetric assay detects CO₂ produced by microorganisms as the electric conductivity of an absorbent changes (Owens et al., 1989). The key advantage of this method is its applicability to highly turbid samples, such as food and insoluble materials (Bolton, 1990; Sawai and Yoshikawa, 2004). Electric conductivity was measured using a RABIT™ (Rapid Automated Bacterial Impedance Technique) system (Don Whitley Scientific Ltd., Shipley, UK), which includes a display, computer, printer and incubator module.

A glass tube (inner well) was placed into a RABIT™ sample tube with paired electrodes at the bottom, and conductivity was measured. A two-fold concentration of nutrient broth (1.75 mL) and the powder slurry (1.75 mL) were poured into the glass tube, and the bacterial suspension (0.1 mL) with vegetative cells was added. A two-fold concentration of Dextrose Peptone Broth (Eiken Chemicals) (1.75 mL) was used as the growth medium for fungi. The broth (1.75 mL), HESP slurry (1.75 mL) and yeast or fungal spore suspension (0.1 mL) were added. A solution of 0.01 M NaOH (1.5 mL) was poured between the glass and sample tubes. The sample tube was sealed tightly with silicone cap and set in the incubator module. The change in conductivity of the NaOH solution was monitored for 72 h at 37 and 25 °C for bacteria and fungi, respectively.
HESP treatment against *B. subtilis* spores
The HESP slurry (20 mL) was poured into a vial (internal diameter 32 mm) and agitated using a magnetic stirrer at 250 rpm. The slurry temperature was maintained at 37 or 60 °C using a water bath. The spore suspension (0.2 mL) was pipetted into the slurry at an initial concentration of approximately 10⁵ CFU/mL. Samples (0.1 mL) were periodically taken from the slurry, diluted in sterile saline and poured into a plate with nutrient agar (Eiken Chemicals). Colonies were counted following incubation at 37 °C for 24 h.

Measurement of active oxygen
Active oxygen species generated from the HESP were detected by chemiluminescence, as described by Kohtani et al. (2008). A Mithras LB09470 microplate reader (Berthold Technologies, Bad Wildbad, Germany) was used for measurements. HESP slurry (0.1 mL) was pipetted into 96 well microplates, and chemiluminescence was initiated on addition of 0.05 mL of 7 mM luminol (Nacalai Tesque, Kyoto, Japan). This was then recorded by the microplate reader. To examine the effects of antioxidative enzymes, 0.025 mL of 0.1 mg/mL superoxide-dismutase (SOD) or 0.1 mg/mL catalase solution (Wako Pure Chemical Industries, Osaka, Japan) was added to the wells from the dispenser prior to the addition of luminol.

Statistical analysis
All experiments were carried out at least in duplicate on three different occasions. Data points with bars represent means ± standard error. Analysis of significant difference was compared by one-way ANOVA using IBM SPSS Statistics. The probability level interpreted as statistically significant was *P* < 0.05.

**RESULTS**

Characteristics of HESP
Fig.1 shows the eggshell powder heated at different temperatures. The color of unheated powder was white. The powder heated at 700 °C was gray and became white in color as the temperature rose over 900 °C.

Fig.2 shows the XRD analysis of HESP. A CaCO₃ peak was observed at 700 °C. Large peaks of CaO appeared at 800 °C, and at more than 900 °C the CaO peak became sharp and the CaCO₃ peak markedly became weak. The CaO content of the HESP and HSSP prepared at 1000 °C was estimated from chelatometric titration results to be 98.5 and 99.0%, respectively.

Indirect conductimetric assay
Fig.3 shows the effect of HESP concentration on the conductivity of NaOH solution for *S. cerevisiae* NBRC1950. The conductivity began to decrease after approximately 5 h and had decreased by 10% after approximately 8 h. The conductivity curves shifted towards a longer decline period as the concentration increased. A conductivity change was not observed at greater than 2.0 mg/mL. In a previous study, there was a correlation between microbial growth and change in electrical conductivity (Sawai et al., 2002a; 2002b).

The applicability of an indirect conductimetric assay for evaluation of antibacterial activity was also examined. The MIC determined by the indirect method closely correlated with that of the direct assay and the turbidity method (Sawai et al., 2002b). Thus, the delay observed represents the inhibition of *S. cerevisiae*
was set at 60 °C and the concentration at 5 mg/mL. The powder heated at 700 °C did not exhibit a decrease in spore viability for 20 min. The powder heated at 800 °C or above killed the B. subtilis spores. The highest sporicidal activity was observed when the powder was heated at 1000 °C.

NBRC1950 growth with the addition of HESP. Similar measurements were conducted for other fungi and bacteria.

Viability of bacterial spores treated with HESP

Fig.4 shows the sporicidal activity of HESP after being heated at 700-1000 °C against B. subtilis spores. The ordinate is the ratio of CFU of B. subtilis spores post-treatment (\( N_0 \)) divided by pretreatment CFU (\( N_0 \)). This represents the survival ratio. The slurry temperature was set at 60 °C and the concentration at 5 mg/mL.

The B. subtilis spores were incubated for 60 min in 0.1 M NaOH solution (pH 13) to investigate their viability following exposure to a strong base (Fig.5). This caused a reduction in the survival of B. subtilis spores by approximately one order of magnitude. This was almost the same as 0.5 mg/mL HESP (pH 12.4). HESP treatment at 0.7-1.00 mg/mL (pH 12.7) for 1 h decreased the spore viability by two orders of magnitude, and the sporicidal activity was almost constant at a slurry concentration higher than 3 mg/mL. This result shows that HESP could kill bacterial spores.

A comparison of sporicidal activities for heated Ca preparations of HESP and HSSP is shown in Fig.6. The powder concentration was kept constant at 10 mg/mL. There was no significant difference in the sporicidal activity for the Ca preparations and pure CaO. A higher temperature increased the sporicidal activity of Ca preparations markedly. A reduction by two orders of magnitude was observed after 60 min at 37 °C. At 60 °C a further reduction by two orders of magnitude was observed after 20 min.

Active oxygen generated from HESP

Previous studies by our group showed that active oxygen species were generated from CaO (Sawai et al., 1996) and HSSP (Kubo et al., 2013), so we...
The model proposed by Takahashi (1990) was used in the indirect conductimetric assay for quantitative evaluation of the antimicrobial activity of HESP. This was first analyzed using the non-competitive inhibition model. If microbial growth is assumed to follow Michaelis-Menten kinetics, a viable cell $V$ takes up the substrate $S$ to form an intermediate $VS_n$ and produces a new viable cell and a metabolic by-product $P$:

$$V + nS \rightarrow VS_n \rightarrow 2V + P$$  \hspace{1cm} (1)

If an antimicrobial agent is an inhibitor $I$, it inhibits the viable activity of cells by forming the following nonviable states $VIm$ and $VSnIm$:

$$V + mI \rightarrow VIm, \quad VS_n + mI \rightarrow VSnIm$$  \hspace{1cm} (2)

where $m$ is the apparent stoichiometric number of the antimicrobial agent necessary to inhibit the reproduction of new cells. The microbial growth kinetics proposed by Monod (1949) give the following equation from the analysis of equations 1-3:

$$\frac{\mu_i}{\mu_0} = \frac{V}{V + nS} \left(1 + \frac{[I]}{K_d} \right)^{-1}$$  \hspace{1cm} (4)

where $K_c = \frac{[V]}{V + nS}$ is the dissociation constant of the substrate, and $K_{ci} = \frac{[V]}{VS_n}$ and $K_{ci} = \frac{[V]}{VS_n}$ are dissociation constants of the inhibitors. In Eq. 4, $\mu$ is the growth rate constant at the inhibitor concentration of $[I] = i$ and $\mu_0$ represents the maximum growth rate constant, corresponding to that at $[I] = 0$. As the substrate exists in excess concentration in actual measurements, Eq. 4 can be reduced to the following given $K_c \ll [S]$:

$$\frac{\mu}{\mu_0} = \left(1 + \frac{[I]}{K_c} \right)^{-1}$$  \hspace{1cm} (5)

The decrease in growth in the conductimetric assay is given as the time lag with respect to the conductivity curve of the control. As shown in Fig.3, the delay in the growth rate is postulated to be the time lag between $t_0$ and $t_i$ at which the values of the conductivity change reach a defined value $\alpha$ at the HESP powder concentrations zero and $i$, respectively. Here, the value of $\alpha$ is set at $-10\%$. Since the threshold of the conductance curves has occurred in the range of $0 - 5\%$, the points where these changes have been completed are easy to determine as the values of $t_i$.

The conductivity change ($CC$) induced by bacterial cells is assumed to approximate the following:

$$CC(t) \equiv AN_0 \exp \left\{ \mu (t - t_i) \right\}$$  \hspace{1cm} (6)
The values of \( K_a \) and \( m \) are determined by regression analysis on the basis of Eq. 7.

The MIC was determined by the model previously described (Takahashi, 1990). The decrease in growth rate, \((1 - \tau_c / \tau)\), is assumed to be proportional to the antimicrobial agent concentration \( \mu \) to the power of \( b \):

\[
(1 - \tau_c / \tau) = a [\mu]^b,
\]

where \( a \) and \( b \) are constants. Because the MIC corresponds to the agent concentration in the case of \( \tau_c = \infty \), it can be defined as follows:

\[
\text{MIC} = [\mu]_{\infty} = (a/b)^{1/b}
\]

The values of \( a \) and \( b \) can then be determined by regression analysis to obtain MIC values. The calculated values of antimicrobial parameters for HESP against bacterial vegetative cells and fungi are summarized in Table 1.

The MIC calculated for HESP against *S. cerevisiae* (1.5 ± 0.3 mg/mL) was in agreement with the experimental results, as demonstrated by the absence of conductivity change at powder concentrations between 1.0 and 2.0 mg/mL (Fig. 3). Similar results were obtained for other microorganisms and HSSP. Although there was a minimal difference in the MIC for bacteria and fungi, bacteria were more sensitive to HESP than fungi. HESP had a one order smaller value of \( K_a \) against bacteria than fungi. \( K_a \) shows the equilibrium state between \( V_{S_o} \), \( V_{S_b} \), \( m \), and \( I \), and bacterial cells were found to more readily form the nonviable state with HESP than fungal cells. Conversely, no large differences were observed in \( b \), which represents the dependence of antimicrobial activity on agent concentration, and \( m \), which represents the apparent stoichiometric number of the antimicrobial agent necessary to inhibit the reproduction of new cells. In addition, the values for HESP were almost equal to those of HSSP.

**Sporidal activity of HESP**

There are very few studies on the effect of calcium preparation against resistive bacterial spores (Sawai et al., 2003; 2007). Yasue et al. (2014) investigated the sporidal effects of heated dolomite against *Bacillus subtilis* spores. Dolomite is a double salt composed of CaCO₃ and MgCO₃. This activity was only apparent when the dolomite powder was heated to higher than 800 °C and increased as the temperature increased. This temperature corresponds to the generation of CaO, not MgO. The sporidal activity of HESP appeared on heating the eggshell powder at higher than 800 °C (Fig. 4). This is consistent with an increase in CaO peaks in the XRD measurement (Fig. 2). Moreover, the sporidal activity of HESP was almost the same as that of HSSP and pure CaO, even when the temperature changed from 37 to 60 °C. This activity may be because of the generation of CaO. Sawai et al. (2007) suggested that HSSP slurry caused spores to lose their barrier to permeability and killed the spores that lost resistance. A similar event might occur for HESP.

**Active oxygen generated from HESP**

The production of active oxygen species is poorly understood at present. Furthermore, the mechanism by which metal oxides, such as CaO, exert their antimicrobial effects requires clarification. The hydration of CaO, which generates alkaline conditions, is considered to be the primary mechanism of action for HESP, as it also is for HSSP. One possible reason for the high disinfection efficacy of HESP is that the pH of the thin water layer formed around the particles is much higher than that of the equilibrated solution (Sugiyama et al., 1995; Dong et al., 2010). However, the sensitivity changes observed for *E.coli* treated with CaO differed from those treated with NaOH (Sawai et al., 1997; Sawai, 2011). This is consistent with the changes induced by active oxygen treatment (Sawai et al.,

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**TABLE 1. Antimicrobial parameters of HESP and HSSP. Different letters within columns indicate significant difference (P<0.05).**

<table>
<thead>
<tr>
<th>Micro-organisms</th>
<th>HESP</th>
<th>HSSP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( K_a )</td>
<td>( m )</td>
</tr>
<tr>
<td><em>E.coli</em> NBRC3301</td>
<td>0.034 ± 0.020</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td><em>S.aureus</em> NBRC102135</td>
<td>0.015 ± 0.008</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td><em>C.albicans</em> NBRC1950</td>
<td>0.49 ± 0.10</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td><em>S.serevisiae</em> NBRC1067</td>
<td>0.58 ± 0.20</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td><em>R.stronfer</em> NBRC4067</td>
<td>0.36 ± 0.09</td>
<td>2.8 ± 1.4</td>
</tr>
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1997). Thus, such active oxygen species could also largely contribute to the antibacterial activity of CaO. A high pH is the main contributor to the antimicrobial activity of HESP; however, active oxygen species generated from HESP are also likely to contribute.

CONCLUSION

Microbial contamination induced by microorganisms has resulted in various problems in living conditions, public health and industries. As a result, many new antibacterial agents and techniques have been investigated, for example, organic, inorganic and natural antibacterial agents and physical sterilizing methods (Bingshe et al., 2008). Sodium chloride (NaClO) can be used directly or indirectly on food as a sanitizer. However, chemical disinfectants can leave potentially carcinogenic or mutagenic residues, such as haloacetic acids, on the surface of food and vegetables (Lozarova et al., 1998). This study is the first to suggest that heated eggshell powder has the same antimicrobial activity as heated sea shell powder. Eggshells are an abundant resource, so HESP could be developed for use in the control of microbes in food processing, medical treatment and the environment. We are currently conducting research to apply our findings to real foods and the environment.

REFERENCES


Bingshe, X., Wensheng, H., Shuhua, W., Liqiao, W., Husheng, et al., 1998


