Visible Light-Induced Bactericidal Species Generated From Silver-Loaded Hydrogen Zirconium Phosphate Active Against *Escherichia coli* K12

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Received 3 August 2000/Accepted 16 December 2000

Hydroxyl radical (OH•) was produced under visible light as an oxidized product and superoxide anion (O2•-) as a reduced product from silver-loaded hydrogen zirconium phosphate [Ag1-xHxZr2(PO4)3] suspension in air. These radicals showed the electron spin resonance (ESR) spectra of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) adducts, and usually only DMPO-OH can be obtained because of the rates of reaction between DMPO and OH• and O2•-. The ESR spectrum of DMPO-O2•- emanated in the presence of I- because I- is a trapping reagent of OH•. I- acted first as an inhibitor and finally as did the bactericidal species in the bactericidal activity against *Escherichia coli*. I- formed from the reaction between OH• and I-. This indicated that OH• was a bactericidal species. OH• produces (CH3)2CHOH and CH3CHOH from ethyl alcohol and isopropyl alcohol because the redox potential of OH• is much more positive than those of the alcohols. Those radicals showed stronger bactericidal activities than OH•. We propose that the strong reducing species has higher bactericidal activity against *E. coli* than the strong oxidizing species.

Key words: Silver-loaded hydrogen zirconium phosphate/Bactericidal species/Superoxide radical/C-centered radical/ESR.

INTRODUCTION

Many studies on bactericidal chemical species against *E. coli* have been reported. Silver (I) ion and OH• are known to be typical bactericidal species among the so-called inorganic bactericidal agents. Silver (I) ion gradually emanates from a silver supported zeolite and clay into an aqueous solution (Ohashi and Oya, 1993; Yamamoto et al., 1991). OH• is generated from a TiO2 semiconductor suspension by UV light irradiation (Kikuchi et al., 1997).

Kourai et al. reported that silver-loaded hydrogen zirconium phosphate [Ag1-xHxZr2(PO4)3] exhibited a strong bactericidal activity against *E. coli* K12 W3110 during white light irradiation, and that the bactericidal activity was reduced by the addition of L-cystein (Kourai et al., 1993 and 1994). The structure of this material had ZrO6 octahedron and PO4 tetrahedron (Goodenough et al., 1976; Hagman et al., 1968). The H+ or Na+ bound to the Zr atom was ion-exchanged by various amounts of Ag+ (Ag content, 3 - 11 %, w/w). The resulting Ag1-xHxZr2(PO4)3 was dispersed in an aqueous solution, and OH• was detected in the suspension during light irradiation by the spin trap method of ESR (Miyoshi et al., 1998), suggesting that the photo-generated OH• may be a bactericidal species.

In this study, we used the spin-tapping technique of ESR measurement and investigated the radical
species produced from Ag₁₋xHₓZr₂(PO₄)₃ suspension under visible light. We will also discuss its bactericidal action with its reactivity against E. coli in the presence of I⁻, isopropyl alcohol and ethyl alcohol as an OH⁻ trapping reagent.

MATERIALS AND METHODS

Chemicals

Silver-loaded hydrogen zirconium phosphate [Ag₁₋xHₓZr₂(PO₄)₃] (Ag content, 3, 3.7, 7, and 11 %, w/w) and NH₄Zr₂(PO₄)₃ were provided by Toagosei Co., Ltd. DMPO (LM-9130), 6-hydroxypurine (hypoxanthine) and xanthine oxidase (XOD) obtained from cow's milk were purchased from Labotec Co., Ltd. KI, isopropyl alcohol and ethyl alcohol were purchased from Wako Pure Chemical Co., Ltd. These reagents were used without further purification.

Determination of the minimum bactericidal concentration

Minimum bactericidal concentration (MBC) and bactericidal activity (log MBC⁻¹) were measured according to a previous paper (Kourai et al., 1994). Except when noted, E. coli K12 W3110 was employed for the experiment. Bacteria were inoculated into 5 ml of L-broth (Bacto tryptone 1%, w/v, yeast extract 0.5%, w/v, NaCl 0.5%, w/v, pH 7.2), and incubated at 37 ºC for 18 h. One ml of this culture was added to 100 ml of nutrient broth (Bacto beef extract 0.3%, w/v, Bacto peptone 0.5%, w/v, Difco Laboratories, Detroit, MI, USA) and incubated for 1.5 h (early exponential-phase) or 18 h (stationary-phase). Cells were harvested, washed and suspended in ice-cooled sterilized water (Maeda et al., 1999). E. coli K12 W3110 (early exponential-phase cell) suspension was diluted to give OD₆₆₀ = 0.001 and was used. Ag₁₋xHₓZr₂(PO₄)₃ powder (Ag content, 3, 3.7, 7, and 11 %, w/w) was suspended at the concentration of 10 mg/ml. Ten suspensions were prepared by serially diluting the suspension by 1.25 times one after another with water, 0 - 2.0 mM KI, 0 - 200 mM ethyl alcohol, or 0 - 300 mM isopropyl alcohol aqueous solution. Each suspension (0.5 ml) was mixed with 0.5 ml of the above diluted E. coli W3110 suspension (OD₆₆₀ = 0.001). They were incubated on a reciprocal flask shaker (130 rpm) at 37 ºC for 30 min under irradiation from a 500 W Xe lamp with 400 or 500 nm interference and UV-37 cutoff filters (Toshiba Glass Co., Ltd.).

The MBC of OH⁻ was determined to be 0.32 mM as follows. 0.5 ml of E. coli W3110 suspension (OD₆₆₀ = 0.001) and 0.25 ml of H₂O₂ aqueous solution were cultured in 0.25 ml of the diluted Fe²⁺ aqueous solution without light irradiation. The concentrations of H₂O₂ and Fe²⁺ aqueous solutions were chosen to be equal and their final concentrations were changed from 0.16 mM to 0.4 mM. OH⁻ was obtained by Fenton's reaction between H₂O₂ and Fe²⁺. After being incubated at 37 ºC for 24 h, the bacterial growth was observed by visual inspection and MBC was determined.

ESR measurement of the photoirradiated Ag₁₋xHₓZr₂(PO₄)₃ suspension

ESR spectra were obtained on a JEOL TE-300 (X-band frequency) spectrometer operated at room temperature. Data processing was performed using a JEOL ESPRIT-425 data system attached to the ESR spectrometer. A quartz oblique cell (JEOL DATUM LC 12) for an aqueous solution was used. DMPO (ca.9M) was used as a spin-trapping reagent. Ag₁₋xHₓZr₂(PO₄)₃ powder (Ag content, 3, 3.7, 7, and 11 %, w/w) or NH₄Zr₂(PO₄)₃ powder was suspended at the concentration of 1 mg/ml in the distilled water. DMPO (20 μl) was added to 400 or 200 μl of the suspensions. Furthermore, in the experiment of the addition of I⁻, 5 μl of 0 - 9.2 mM KI aqueous solution was added to 225 μl of the above suspension so that it contained 5 μl of 10 mM KCl aqueous solution. After that suspension was introduced to the cell, their ESR spectra were recorded during irradiation with a 500 W Xe lamp with 400 or 500 nm interference and UV-37 cutoff filters (Toshiba Glass Co., Ltd.).

Photoirradiation of Ag₁₋xHₓZr₂(PO₄)₃ separated from E. coli suspension

E. coli was separated from the Ag₁₋xHₓZr₂(PO₄)₃ suspension using dialysis film. E. coli suspension was put into the dialysis tube (width 2.5 cm, an average pore size 2.4 nm, Sanko Co., Ltd.) and the dialysis tube was put into the Erlenmeyer flask containing Ag₁₋xHₓZr₂(PO₄)₃ suspension or the distilled water. After those Erlenmeyer flasks were irradiated using a white light at 30ºC for 30 min, the E. coli suspension was inoculated on the agar plate and incubated at 37 ºC for 18 h. The formed colonies were counted as a residual cell number.

RESULTS AND DISCUSSION

Visible light induced radical species produced in the Ag₁₋xHₓZr₂(PO₄)₃ suspension
FIG. 1. ESR spectra of DMPO adducts in irradiated 1 mg/ml Ag₁₋ₓHₓZr₂(PO₄)₃ (Ag content, 11%, w/w) suspension in the absence of I⁻ (A) and the presence of 0.1 mM I⁻ (B). (C) presents the mixed spectrum simulated by the spectra of DMPO-OH and DMPO-O₂⁻. Light source: 500 W Xe lamp with 400 nm interference and UV-37 cut-off filters. Irradiation time: 21 min.

ESR spectrum of DMPO loaded Ag₁₋ₓHₓZr₂(PO₄)₃ suspension showed a typical peak pattern of 1:2:2:1 and aΗ = aΝ = 1.5 mT (Fig. 1 A). The peak intensity increased with the irradiation time (Miyoshi et al., 1998). In the presence of I⁻, the signal intensity of DMPO-OH decreased with the concentration of I⁻ as shown in Fig. 2. Finally, the ESR spectrum was different from that of DMPO-OH as shown in Fig. 1 B. That spectrum depicted in Fig. 1B was analogous with the combination of the calculated ESR spectra of DMPO-OH and DMPO-O₂⁻ as shown in Fig. 1C, suggesting the formation of DMPO-O₂⁻. Then, the signal in Fig. 1B disappeared by adding 13.5 unit of superoxide dismutase (SOD) (Miyoshi et al., 1998). The trapping-mechanism of OH⁺ and O₂⁻ by DMPO can be explained by the following reaction scheme.

\[
DMPO + OH⁺ \rightarrow DMPO-OH
\]

(In the presence of I⁻)

\[
OH⁺ + I⁻ \rightarrow OH⁻ + 1/2 I₂
\]

\[
DMPO + O₂⁻ \rightarrow DMPO-O₂⁻
\]

where the rate constants \( k_1, k_2, k_3 \), are respectively \( 3.4 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \), \( 1.0 \times 10^{10} \text{ M}^{-1} \text{s}^{-1} \), and \( 16.9 \text{ M}^{-1} \text{s}^{-1} \) (Buxton et al. 1988). The very different reaction rates between \( k_1 \) and \( k_3 \) show us that only the ESR spectrum of DMPO-OH can be observed in the photo-irradiated Ag₁₋ₓHₓZr₂(PO₄)₃ suspension. Furthermore, only in the presence of I⁻ can O₂⁻ be trapped with DMPO, because the reaction rate of \( k_2 \) was larger than that of \( k_1 \), as shown in Fig. 1B.

Figure 3 shows the relation between the Ag content in Ag₁₋ₓHₓZr₂(PO₄)₃ and the generation rate of DMPO-OH. As shown in Fig. 3, the generation rate of DMPO-OH increased with the content of Ag in Ag₁₋ₓHₓZr₂(PO₄)₃. Ag⁺ seems to play an important role in the production of OH⁺ and O₂⁻. In the previ-
ous paper, we proposed that Ag⁺ is in the reduced condition in the lattice because of the O⁻ around Ag⁺ (Miyoshi and Leyasu et al., 1998). Also, the photoelectrochemical properties of Zr₂(PO₄)₃ showed a photocathodic current (Miyoshi and Leyasu et al., 1998). Thus, the charge separation effectively occurred in the presence of Ag⁺. Henglein reported that the Ag⁰ colloid indicated an electron pool effect (Henglein, 1979). Therefore, Ag⁺ in the lattice of Ag₁₋ₓHₓZr₂(PO₄)₃ would act as an effective charge separator.

\[
\text{FIG. 4. (A) Effect of Ag content on the bactericidal activity of Ag₁₋ₓHₓZr₂(PO₄)₃ against E. coli K12 W3110. The bactericidal activity is represented by log MBC⁻¹, in which MBC means the minimum bactericidal concentration. As shown in Fig. 4A, the bactericidal activity increased with an increase of Ag content. Figure 4B shows the relation between log MBC⁻¹ and the generation rate of DMPO-OH (Data from Fig. 3). A linear relationship was observed with the Ag content of 3.7 - 11% (w/w). However, according to the above equations (4), (5) and (6) and the discussion, O₂⁻ is also generated with OH⁻. We will discuss how these radical species acted against E. coli. It is known that the life time of OH⁻ (200 μs) is much shorter than that of O₂⁻ (5s) (Saito and Matsuura, 1990). We counted the residual cell numbers of E. coli via dialysis film (average pore size, 2.4 nm) under visible light irradiation. The cell numbers with or without Ag₁₋ₓHₓZr₂(PO₄)₃ are summarized in Table 1. The cell numbers were approximately the same values. This result led to the conclusion that the bactericidal species were neither O₂⁻ nor free Ag⁺ released from Ag₁₋ₓHₓZr₂(PO₄)₃ and it indicated the bactericidal action occurred on the surface of Ag₁₋ₓHₓZr₂(PO₄)₃. Moreover, we tried to measure the bactericidal activity of Ag₁₋ₓHₓZr₂(PO₄)₃ against E. coli W3110 in the presence of I⁻, which is an OH⁻ trap reagent as shown in Fig. 5. Initially, the bactericidal activity decreased from 0 to ca. 0.4 mM I⁻, but from ca. 0.4 to 1 mM I⁻ it increased, again. The initial}

**Bactericidal activity of visible light-induced radicals**

Figure 4A shows the effect of Ag content on the bactericidal activity of the photoirradiated Ag₁₋ₓHₓZr₂(PO₄)₃ against E. coli K12 W3110. The log MBC⁻¹ represents the bactericidal activity of Ag₁₋ₓHₓZr₂(PO₄)₃ against E. coli; MBC means the minimum bactericidal concentration. As shown in Fig. 4A, the bactericidal activity of the photoirradiated Ag₁₋ₓHₓZr₂(PO₄)₃ increased with an increase of Ag content. Figure 4B shows the relation between log MBC⁻¹ and the generation rate of DMPO-OH (Data from Fig. 3). A linear relationship was observed with the Ag content of 3.7 - 11% (w/w). However, according to the above equations (4), (5) and (6) and the discussion, O₂⁻ is also generated with OH⁻. We will discuss how these radical species acted against E. coli. It is known that the life time of OH⁻ (200 μs) is much shorter than that of O₂⁻ (5s) (Saito and Matsuura, 1990). We counted the residual cell numbers of E. coli via dialysis film (average pore size, 2.4 nm) under visible light irradiation. The cell numbers with or without Ag₁₋ₓHₓZr₂(PO₄)₃ are summarized in Table 1. The cell numbers were approximately the same values. This result led to the conclusion that the bactericidal species were neither O₂⁻ nor free Ag⁺ released from Ag₁₋ₓHₓZr₂(PO₄)₃ and it indicated the bactericidal action occurred on the surface of the Ag₁₋ₓHₓZr₂(PO₄)₃. Moreover, we tried to measure the bactericidal activity of Ag₁₋ₓHₓZr₂(PO₄)₃ against E. coli W3110 in the presence of I⁻, which is an OH⁻ trap reagent as shown in Fig. 5. Initially, the bactericidal activity decreased from 0 to ca. 0.4 mM I⁻, but from ca. 0.4 to 1 mM I⁻ it increased, again. The initial

**TABLE 1. Viable cell numbers in H₂O with or without Ag₁₋ₓHₓZr₂(PO₄)₃.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Residual cell number (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>without Ag₁₋ₓHₓZr₂(PO₄)₃</td>
<td>4.2×10⁶</td>
</tr>
<tr>
<td>with Ag₁₋ₓHₓZr₂(PO₄)₃</td>
<td>5.5×10⁶</td>
</tr>
</tbody>
</table>

Initial cell number : 1.2×10⁶ cells/ml
Effect of I- on the bactericidal activity of Ag$_{1-x}$H$_x$Zr$_2$(PO$_4$)$_3$ against E. coli K12 W3110. The bactericidal activity is represented by log MBC$^{-1}$, in which MBC is the minimum bactericidal concentration of KI. The log-phase cell suspension (10$^6$ cells/ml) containing Ag$_{1-x}$H$_x$Zr$_2$(PO$_4$)$_3$ (Ag content, 11%, w/w) was treated with KI under white light irradiation with a 200 W incandescent bulb for 30 min at 30°C on a reciprocal shaker (130rpm). The distance between the flask and the bulb was 15cm.

The decrease of log MBC$^{-1}$ is due to the elimination of OH• by I-, because the MBC of OH• against E. coli is ca. 0.32 mM, and this value well agreed with the concentration of I$^-$ at the bottom portion of log MBC$^{-1}$. The re-increase of log MBC$^{-1}$ was suggested to be due to the bactericidal action of I$_2$ (= 25 μM) is less than that of OH• (0.32 mM). These suggested that OH• would be the bactericidal species in the photogenerated Ag$_{1-x}$H$_x$Zr$_2$(PO$_4$)$_3$ suspension. This is also supported from the previous result: when L-cystein was added to the photolysed NOVARON (Ag$_{1-x}$H$_x$Zr$_2$(PO$_4$)$_3$, Ag, 3%, w/w) suspension, the bactericidal activity of NOVARON against E. coli W3110 was inhibited during light irradiation (Kourai et al., 1994).

Bactericidal activities of ethyl alcohol and isopropyl alcohol radicals formed by OH•

The bactericidal activity of the radicals, not OH• as an oxidizing radical ($E_{\text{redox}} = +1.8$ V vs NHE), was investigated. CH$_3$C•OH and (CH$_3$)$_2$C•OH are well-known reducing radicals and they are generated from ethyl alcohol and isopropyl alcohol by the oxidation of OH•. Both radicals have the same redox potential ($E_{\text{redox}} = -1.5$ V vs NHE) (Farhataziz and Rodgers, 1987). The reaction constants of $k_4$ and $k_5$ were 1 × 10$^4$ M$^{-1}$ s$^{-1}$ (Buxton et al., 1988). The reaction rate constants of $k_4$ and $k_5$ show us that those alcohols can immediately trap OH• and produced C-centered radicals as described below.

\[
\text{OH}• + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3\text{C}•\text{HOH} + \text{H}_2\text{O} \quad (7)
\]

\[
\text{OH}• + (\text{CH}_3)_2\text{CHOH} \rightarrow (\text{CH}_3)_2\text{C}•\text{OH} + \text{H}_2\text{O} \quad (8)
\]
Figure 6 shows the bactericidal activity of Ag1-xH2Zr2(PO4)3 against E. coli K12 W3110 in the presence of ethyl alcohol (A) and isopropyl alcohol (B). The bactericidal activity increased with an increase of their alcohol concentrations. These increases in the bactericidal activity were observed well below the concentration of their MBC values (the MBC of ethyl alcohol is 5.4 M and that of isopropyl alcohol is 2.0 M). The presence of those alcohols showed a strong bactericidal activity compared with that of OH• alone (see Fig. 6A and 6B), suggesting that CH3C•OH and (CH3)2C•OH exhibited bactericidal activity.

At the same time, as shown in Fig. 6A and B, the bactericidal activity of CH3C•HOH is ten times greater than that of CH3C•OH. That difference in the bactericidal activity seemed to be due to the stability of CH3C•HOH. The stabilization contributed to the difference of electron donating effects between H3C• and H for the radical on C, CH3C•OHOH and (CH3)2C•OH exhibited bactericidal activity.

Finally, CH3C•HOH or (CH3)2C•OH would give a more effective damage against E. coli than the oxidizing radical of OH•.

ACKNOWLEDGEMENT

We thank Toagosei Co., Ltd. for supplying of the zirconium phosphate and silver-loaded samples (Ag content, 3, 3.7, 7, and 11%, w/w) used in this work.

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