DETECTION OF ACTIVE OXYGEN GENERATED FROM CERAMIC POWDERS HAVING ANTIBACTERIAL ACTIVITY

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In order to elucidate the antibacterial mechanisms of magnesium oxide (MgO), calcium oxide (CaO) and zinc oxide (ZnO), the generation of active oxygen from these ceramic powder slurries was examined by oxygen electrode analysis and chemiluminescence analysis. Hydrogen peroxide (H_2O_2) generated from the ZnO powder slurry was detected using the oxygen electrode. Active oxygen from the MgO and CaO powder slurries was not detected by the oxygen electrode analysis. Chemiluminescence analysis could detect the generation of active oxygen from three kinds of powders. The luminescence response of the CaO powder slurry was markedly strong. The chemiluminescence responses of the CaO and MgO powder slurries are due to the superoxide anion (O_2^-). The order of the strength of luminescence response was CaO, MgO, and ZnO, which agreed with that of the antibacterial activity of these powders. These results suggested that active oxygen species generated from the ceramic powders were associated with their antibacterial activities.

Introduction

Ceramics which possess antibacterial activity have recently received much attention as new antibacterial materials (Hiyama *et al.*, 1995; Isshiki *et al.*, 1993; Katsui *et al.*, 1994; Mine *et al.*, 1995; Yamanaka *et al.*, 1995). In the previous work (Sawai *et al.*, 1995a), we have evaluated the antibacterial activities of ceramic powder slurries by measurement of conductance changes with bacterial metabolism and growth (conductance method). Magnesium oxide (MgO) and calcium oxide (CaO) powder slurries acted upon both gram-positive and negative bacteria in a bactericidal manner. On the other hand, the zinc oxide (ZnO) powder slurry acted in a bacteriostatic manner and exhibited antibacterial activity against gram-positive bacteria stronger than gram-negative bacteria. These three ceramic powders have been effective against the spores of *Bacillus subtilis*, which have a high resistance to heat and antibacterial agents. (Sawai *et al.*, 1995b). In addition, the particle size and heating temperature of the ceramic powders (MgO, CaO and ZnO) have influenced the antibacterial activity of their slurries (Sawai *et al.*, 1996).

However, the antibacterial mechanisms of these ceramic powders have been not understood as yet. MgO, CaO, and ZnO have been used as catalysts in various reactions and found to show a remarkable activity as solid base catalysts (Dent and Kokes, 1969; Hattori *et al.*, 1975; Iizuka and Tanabe, 1975). It was reported that these reactions originated in the electron donating nature of the surface of these ceramics (Baird and Lunsford, 1972). The existence of superoxide anion (O_2^-) on the surfaces of the MgO and CaO was observed (Iizuka, 1973; Iizuka and Tanabe, 1975).

Generally, O_2^-, hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical ($\cdot OH$) are called active oxygen species (Nakano and Sakurai, 1996). They are very reactive and powerful oxidizing agents. The relationships between active oxygen and inflammation (Shiokawa, 1987), carcinogenesis (Kasai and Nishimura) or phenomena of aging (Harman, 1984) have recently been a focus of intense research interest. On the other hand, in immune reactions of the living body, active oxygen species play an important role to prevent the infection of pathogenic bacteria (Arai, 1992; Farr and Kogoma, 1991).

In this work, we have conjectured that active oxygen species, such as H_2O_2 and O_2^-, were generated on the surfaces of the MgO, CaO and ZnO powders, and that the active oxygen acted against bacteria as one of the factors of their antibacterial mechanism.

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Hence, we have tried to detect the active oxygen generated from the antibacterial ceramic powders using an oxygen electrode and a chemiluminescence method. The relation between active oxygen and antibacterial activity of the ceramics has been studied.

1. Materials and Methods

1.1 Oxygen electrode analysis

1.1.1 Preparation of ceramic powder slurry MgO, CaO and ZnO (Kishida Chemicals Co., Ltd.) were used. The ceramic powders were heated at 453 K for 20 min, and suspended with physiological saline. The mean particle size of the MgO, CaO and ZnO powders were 3.6, 2.7 and 2.6 μm, respectively (Sawai *et al.*, 1995b)

1.1.2 Preparation of enzyme solution A ready-made catalase solution for oxygen electrode analysis (5000 units/ml: Oriental Electric Co., Ltd.) was used. Superoxide dismutase (SOD) from *Bacillus* sp. was purchased from Wako Pure Chemical Industries, Ltd. SOD solution was prepared with 0.5 M phosphate buffer to yield a concentration of 0.7 mg/ml.

1.1.3 Measurement of active oxygen **Figure 1** illustrates the apparatus of the oxygen electrode (Oritector Model-III; Oriental Electric Co., Ltd.). A ceramic powder slurry of 2 ml was poured into a cell. In the case of the addition of SOD, 20 μl of the SOD solution was pipetted into the cell together with the slurry. The cell was sealed with a rubber stopper. While stirring, nitrogen gas was passed through the cell to get rid of O_2 dissolved in the slurry. The catalase solution of 20 μl, from which dissolved oxygen was removed in advance, was injected with a microsyringe through the rubber stopper. O_2 generated from the decomposition of H_2O_2 in the slurry was measured by the oxygen electrode. Experiments were carried out at 303 K.

1.2 Chemiluminescence analysis

1.2.1 Chemicals Phenyl-10-methyl-acridinium-9-carboxylate fluorosulfonate (PMAC; Dojindo Laboratories Co., Ltd.) was used as a luminescent compound for the chemiluminescence analysis. In the case of the chemiluminescence analysis, catalase from bovine liver (Wako Pure Chemical Industries Co. Ltd.) was used. The solutions of PMAC, catalase, and SOD were prepared with distilled water passing through an ion exchange resin. The concentrations of both enzyme solutions were 0.1 mg/ml. The ceramic powders were also suspended with the distilled water.

1.2.2 Measurement of chemiluminescence response The chemiluminescence response induced by active oxygen generated from the ceramic powders was detected by using Micro Plate Reader LUMINOUS Model CT 9000D (Toyo Sokki Co., Ltd.). **Figure 2** illustrates the schematic diagram of the apparatus.

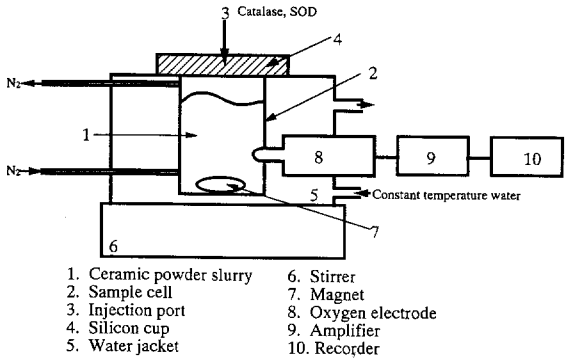

Fig. 1 Scheme of apparatus of oxygen electrode

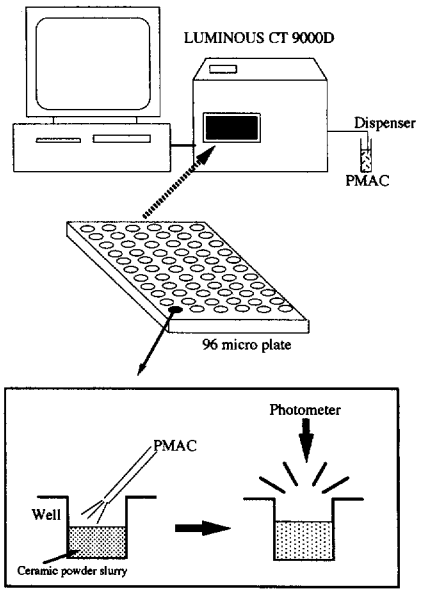

Fig. 2 Scheme of apparatus of chemiluminescence method

For measurements, a microplate with 96 individual wells was used. A ceramic powder slurry of 100 μl was pipetted into the well, and the microplate was set in the apparatus. The chemiluminescence reaction was initiated by the addition of 50 μl of PMAC to the slurry from the dispenser, and the chemiluminescence response was recorded. When the effects of catalase or SOD on the chemiluminescence response were examined, an enzyme solution of 25 μl was added to the well from the dispenser before the addition of PMAC. 0.5 μg per well of solution of PMAC was prepared. Experiments were carried out at 303 K.

2. Results and Discussion

2.1 Detection of active oxygen by oxygen electrode analysis

An oxygen electrode can detect dissolved oxygen, but not measure directly active oxygen such as H_2O_2 and O_2^-. These active oxygen species become

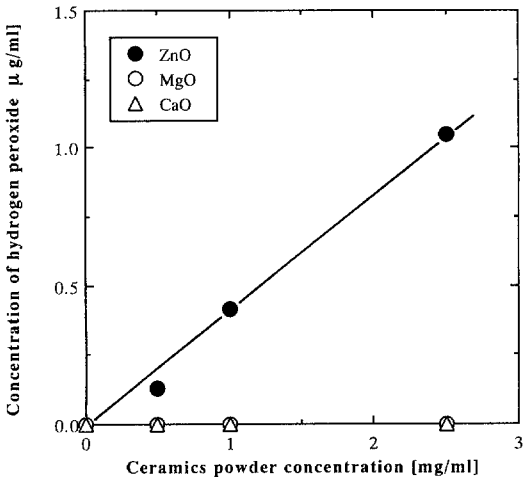

Fig. 3 Generation of H_2O_2 from ZnO powders slurry (oxygen electrode)

detectable by using enzymes counteracting active oxygen. As shown in Eq. (1), catalase catalyzes the decomposition of H_2O_2 into H_2O and O_2. If H_2O_2 is present in a sample, the oxygen generated by the addition of catalase is measured using the oxygen electrode. The concentration of H_2O_2 was obtained from the concentration of O_2 detected by the oxygen electrode analysis.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2} O_2 \qquad (1)$$

SOD is added to detect O_2^-. SOD catalyzes the dismutation between O_2^- and O_2^- as shown in Eq. (2). In this reaction, hydrogen ion in solution is used. Therefore, the detection of O_2^- by the oxygen electrode requires two steps; first, SOD is added to the sample, and next, the generated H_2O_2 is decomposed into H_2O and O_2 by the addition of catalase. Then, O_2 generated from H_2O_2 is detected using the oxygen electrode.

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad (2)$$

Figure 3 shows the results of measurements of H_2O_2 generation from the MgO, CaO and ZnO powder slurries by the oxygen electrode analysis. H_2O_2 was detected only from the ZnO powder slurry. The generation of H_2O_2 increased linearly with increasing slurry concentration. When SOD was added before catalase, the generation of H_2O_2 did not change (data not shown). It was considered that there is slight or no generation of O_2^- from the ZnO powder slurry. H_2O_2 is widely used as a bactericide. The ZnO powder slurry exhibited a bacteriostatic action on gram-positive and negative bacteria (Sawai *et al.*, 1995a). H_2O_2 generated from the ZnO powders will contribute to the antibacterial activity of the slurry.

In both cases of the MgO and CaO powder slurries, the generation of H_2O_2 and O_2^- was not observed. The MgO and CaO powder slurries are highly alkaline, with pH 10.4 and 12.6, respectively. It was impossible to adjust the pH values of the MgO and CaO powder slurries to the neutral range, even when the MgO and CaO powder slurries were prepared with phosphate buffer. If bulk pH values of the slurries are able to reduce, a region of high pH would exist near the surfaces of the powders. Because of inactivation of catalase and SOD by the high pH of these slurries, the active oxygen would not be detected by the oxygen electrode analysis.

2.2 Detection of active oxygen by chemiluminescence analysis

Analysis using chemiluminescence reactions has been widely and intensively studied as a highly sensitive ultramicroanalysis in recent years. In particular, chemiluminescence analysis is employed in the measurement of ability for production of active oxygen species in macrophages and leukocytes as a biological defense of the living body (Allen and Loose, 1976; Bahior *et al.*, 1973; Ushijima and Nakano, 1980). In this work, we used PMAC as a luminescent compound and tried to detect the active oxygen generated from the ceramic powders (MgO, CaO and ZnO) by chemiluminescence analysis. PMAC luminesces with O_2^- and H_2O_2 at pH range of neutral to alkaline.

Figure 4 shows the time courses of chemiluminescence responses of the MgO, CaO and ZnO powder slurries when PMAC was added once to the slurries. The ordinate, CPM, represents chemiluminescence intensity, which is counts of photometer per second. The oxygen electrode analysis could not detect the generation of active oxygen from the MgO and CaO powder slurries. However, for the chemiluminescence analysis, chemiluminescence responses from the MgO and CaO powder slurries were observed, which indicated a possibility of generation of active oxygen from these powders. There were differences in the patterns of the chemiluminescence responses of three powder slurries. The chemiluminescence response of the CaO powder slurry peaked immediately after the addition of PMAC to the slurry. On the other hand, a long response time was observed for the ZnO powder slurry. The decreases in chemiluminescence responses of the MgO and CaO are likely due to the disappearance of active oxygen and inactivation of PMAC by high pH of their slurries. Therefore, it is impossible that generation of active oxygen from the antibacterial ceramics is compared using integration values along each CPM curve as shown in Fig. 4. Hereafter, we determined to use the CPM values at the peak of chemiluminescence responses as a lumi-

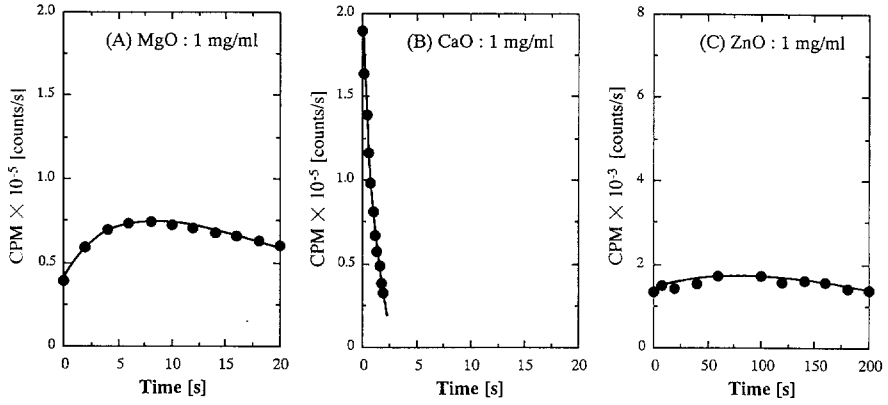

Fig. 4 PMAC-evoked chemiluminescence responses of ceramic powder slurries; (A) MgO, (B) CaO, (C) ZnO

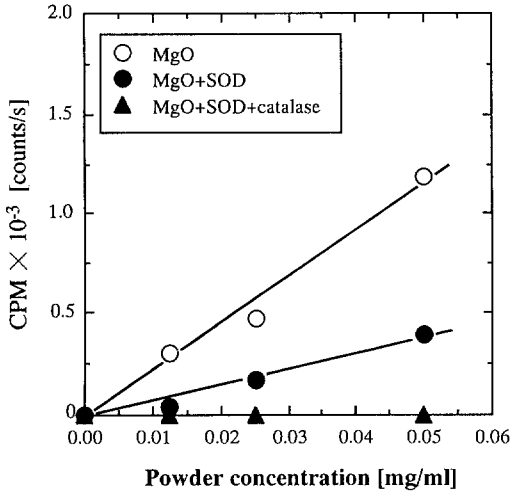

Fig. 5 Effect of enzyme counteracting active oxygen on PMAC-evoked chemiluminescence responses of MgO powder slurry

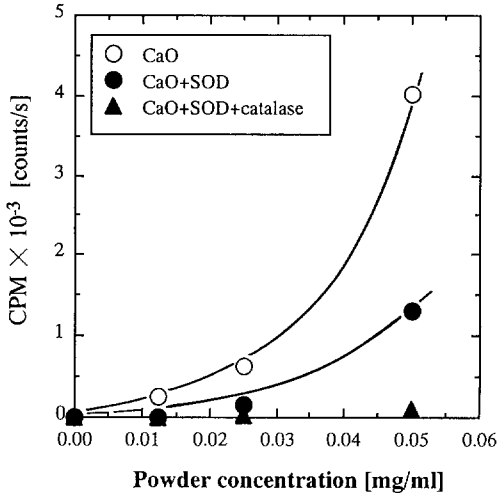

Fig. 6 Effect of enzyme counteracting active oxygen on PMAC-evoked chemiluminescence responses of CaO powder slurry

nescence intensity (MgO: 10 s, CaO: 1 s, ZnO: 10 s, integral time: 1 s).

The order of the strength of chemiluminescence response is CaO, MgO and ZnO. In the previous work (Sawai *et al.*, 1995a), we have already examined the antibacterial activities of these ceramic powders. The order of the strength of the antibacterial activity was consistent with that of the chemiluminescence response. Also, α-Al_2O_3 exhibited no antibacterial activity. There was no chemiluminescence response for α-Al_2O_3 powder slurry (data not shown). Therefore, active oxygen generated from the MgO, CaO and ZnO powders will take some part in growth inhibition of bacteria.

Increases in powder concentrations enhanced the chemiluminescence responses (**Figs. 5, 6** and **7**). In the case of chemiluminescence analysis, it is necessary to investigate whether or not the luminescence reaction really originates in the active oxygen

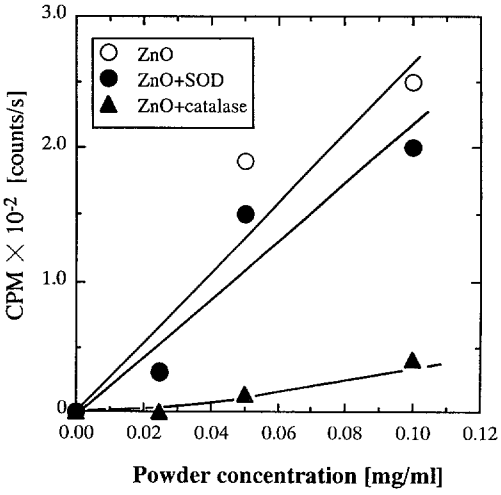

Fig. 7 Effect of enzyme counteracting active oxygen on PMAC-evoked chemiluminescence responses of ZnO powder slurry

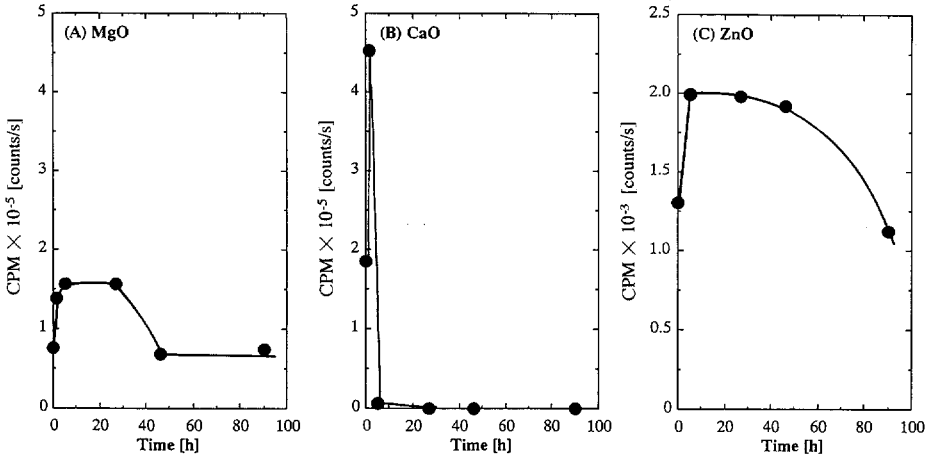

Fig. 8 Duration of generation of active oxygen from ceramic powder slurry; (A) MgO, (B) CaO, (C) ZnO

species. In order to prove that the luminescence responses were due to active oxygen species generated from the ceramic powders, the effects of the enzymes counteracting active oxygen (catalase and SOD) on the chemiluminescence response were examined.

For the MgO and CaO powder slurries, the addition of SOD remarkably inhibited their chemiluminescence responses (Figs. 5 and 6). Although the inhibitory effect of SOD was not necessarily complete, the chemiluminescence responses for the MgO and CaO decreased by approximately one-half. As shown in Eq. (2), SOD catalyzes dismutation between O_2^- and O_2^-, and H_2O_2 is produced. PMAC reacts not only with O_2^-, but also with H_2O_2, and evokes a luminescence response. The chemiluminescence responses of the MgO and CaO powder slurries were completely inhibited by the addition of both SOD and catalase. If the chemiluminescence intensity of one molecule of O_2^- is the same as that of H_2O_2, a large part of the PMAC-evoked chemiluminescence responses for the MgO and CaO would reflect the generation of O_2^- from these powders. Also, the reaction of Eq. (2) spontaneously proceeds even without SOD. The rate of reaction strongly depends on the concentration of hydrogen ion in solution. The rate of spontaneous dismutation of O_2^- is extremely suppressed in the highly alkaline range (Saito and Matsugo, 1988). O_2^- is more stable than H_2O_2 in the alkaline range. The pH values of the MgO and CaO powder slurries were 10.4 and 12.6, respectively (Sawai *et al.*, 1995a). Though some H_2O_2 exists naturally in the slurries, most would result from the dismutation of O_2^-. PMAC would react with H_2O_2 resulted from the dismutation of O_2^- and evoke the luminescence response. Therefore, it is natural to suppose that the MgO and CaO powders generate O_2^-. Unlike the ZnO powder slurry, the MgO and CaO powder slurries exhibit bactericidal action (Sawai *et al.*, 1995a). It was considered that O_2^- contributes to the bactericidal action of the MgO and CaO powders.

Figure 7 illustrates the result of the ZnO powder slurry. The chemiluminescence response for the ZnO was markedly inhibited by the addition of catalase but not SOD. This result indicated that the ZnO powder generated H_2O_2 and little O_2^-. Similar results were obtained between the chemiluminescence analysis and the oxygen electrode analysis.

We examined how long the generation of active oxygen from these powders continued. When the chemiluminescence responsefinished after one addition of PMAC, it was considered that most active oxygen in the powder slurry disappeared. After the first addition of PMAC, PMAC was added to the slurry a number of times, and variation of the chemiluminescence responses was observed. For the MgO and ZnO powder slurries, the chemiluminescence responses appeared even after PMAC was added 6 times up to 90 h. The generation of active oxygen from these powders remained for a long time (**Figs. 8**(A) and (C)). On the other hand, the chemiluminescence response of the CaO powder slurry markedly decreased when PMAC was added 3 times up to 5 h (Fig. 8(**B**)). However, even the decreased response of the CaO powder slurry after 5 h (about 6.0×10^3 counts/s) was larger than that of the ZnO powder slurry.

2.3 Active oxygen and antibacterial activity of ceramic

Oxygen as well as water is one of the most important substances for a lot of living creatures. However, it is also well known that the action of oxygen is often extremely harmful to all living creatures. Ionizing radiation, some agents, and some reducing agents destroy membranes, enzymes and nucleic acids in cells, and their ability in the

presence of oxygen is higher than that in the absence of oxygen (Gomez and Sinskey, 1975; Tanooka, 1978). Furthermore, under the condition that active oxygen species, such as H_2O_2, O_2^-, $^1O_2$ and $\cdot OH$, were generated, enzymes and nucleic acids in bacteria and macrophages were injured and destroyed (Sawada and Yamazaki, 1974). The antibacterial ceramic powders (MgO, CaO and ZnO) generated active oxygen species, such as H_2O_2 and O_2^-. These active oxygen species are one of the factors of antibacterial mechanism of the ceramics.

For the MgO and CaO powder slurries, active oxygen could not be detected using the oxygen electrode. For the oxygen electrode analysis, oxygen dissolved in slurries and enzyme solution was removed by passing nitrogen gas. On the other hand, for the chemiluminescence analysis, the removal of dissolved oxygen was not carried out, and the chemiluminescence responses caused by active oxygen species can be observed. Therefore, dissolved oxygen in the slurries possibly acts as a source of active oxygen. O_2^- basically resulted from the one-electron reduction of O_2. The reaction of the oxygen will be concerned with reducing sites which are active sites on the surfaces of these ceramic powders (Baird and Lunsford, 1972). However, when the oxygen electrode analysis was performed without the removal of dissolved oxygen in the slurries, serious errors easily appeared. Further work is needed to determine the role of dissolved oxygen in generation of active oxygen from the antibacterial ceramics.

In the previous work (Sawai *et al.*, 1996), we reported that the antibacterial activity of the MgO, CaO and ZnO powder slurries decreased with an increase in heating temperature of the powders. It is well known that hydroxyl groups exist on the surfaces of metallic oxides and that the hydroxyl groups play an important role as an acid or a base in various reactions. The hydroxyl groups will be removed by heat treatment. The decrease in the antibacterial activity of the ZnO powder slurry was much larger than those of the MgO and CaO powder slurries. In the case of the ZnO powder slurry, the generation of H_2O_2 was observed even under the condition that dissolved oxygen in the slurries was removed by passing nitrogen gas. The production of H_2O_2 from the ZnO powder slurry would be concerned with the hydroxyl groups on the surfaces.

H_2O_2 is relatively permeable to the cell membrane of bacteria (Saito and Matsugo, 1988). On the other hand, O_2^- is not permitted to diffuse into the membrane (Kobayashi, 1988). However, O_2^- is in equilibrium as shown in the following reaction.

$$O_2^- + H^+ \rightleftharpoons HO_2\cdot \qquad (3)$$

When O_2^- captures a hydrogen ion in solution, hydroperoxyl radical ($HO_2\cdot$) is produced. $HO_2\cdot$ is much more reactive than O_2^- and able to penetrate the cell membrane like H_2O_2 (Asada, 1987; Farr and Kogoma, 1991). Moreover, important reactions of O_2^- and H_2O_2 are to form the $\cdot OH$ which is the most known potent oxidant (Bucker and Martin, 1981). An especially significant source of $\cdot OH$ is the reaction of H_2O_2 in the Fenton reaction (Farr and Kogoma, 1991). These reactions are catalyzed with intercellular or extracellular transition metallic ions. The effects of eluate including active oxygen and metallic ions on bacterial growth are now being examined in further detail.

We have already reported that the MgO, CaO and ZnO powder slurries exhibited antibacterial activity against the spores of *B. subtilis*, which have high tolerance to heat treatment and antibacterial agents (Sawai *et al.*, 1995b). Especially, the killing effect of the CaO powder slurry on the spores was markedly strong. And the supernatants of the ceramic powder slurries promoted the germination of bacterial spores. In that work, it was considered that the effect was due to dissolved metallic ions. However, in this work, the generation of active oxygen species (H_2O_2 and O_2^-) from the powders was proved. Kawasaki *et al.* (1970) studied the bactericidal action of H_2O_2 against the spores of *B. subtilis*. During treatment with H_2O_2, it became clear that remarkable chemical and physical changes occurred in the spore coat and that dipicolinic acid was released from the spores. The release of dipicolinic acid is one of the changes which take place in a spore at the point of germination (Sykes, 1970). Maeda and Koga (1981) indicated that denaturation of the spore coat triggered off the spore germination in heat activation. Considering these reports, the promotion of spore germination by the ceramic powder slurry will be concerned with not only dissolved metallic ions, but also active oxygen generated from the ceramic powders.

Moreover, we previously investigated damaged parts in bacteria by far-infrared irradiation and thermal conductive heating on the basis of changes in sensitivity of *Escherichia coli* to antibiotics (Sawai *et al.*, 1995c, 1995d). Studies are under way to examine damaged parts in bacteria in the ceramic powder slurries by the same method.

Conclusion

In this work, we conjectured that active oxygen was generated from the ceramics. Active oxygen was detected by using the oxygen electrode analysis and the chemiluminescence method. The following results and conclusions were obtained.

1) The generation of H_2O_2 from the ZnO powder slurry was observed by the oxygen electrode.

2) Active oxygen generated from the MgO, CaO and ZnO powder slurries was detected by the chemiluminescence method. For the MgO and CaO powder slurries, the generation of O_2^- was observed.

3) The order of the strength of chemiluminescence response was CaO, MgO and ZnO, which agreed with that of the strength of antibacterial activity. This result suggested that active oxygen generated from these powder slurries was one of the factors of their antibacterial mechanism.

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