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Molecular Biological Mechanism of Plasma Sterilization in Liquid with the Reduced pH Method

低pH法によるプラズマ液中殺菌の分子生物学的メカニズム

Satoshi Ikawa and Katsuhisa Kitano
井川　聡[1)], 北野　勝久[2)]

1)Technology Research Institution of Osaka Prefecture
2-7-1, Ayumino, Izumi, Osaka 594-1157, Japan
大阪府立産業技術総合研究所 〒594-1157　大阪府和泉市あゆみ野2-7-1
2)CAMT, Graduate School of Engineering, Osaka University
2-1 Yamadaoka, Suita, Osaka, 565-0871, Japan
大阪大学工学研究科　原子分子イオン制御理工学センター〒565-0871　大阪府吹田市山田丘2-1

Atmospheric pressure plasma jet with low gas temperature has been applied to bacterial suspension to sterilize bacteria in liquid. Successful method of bacterial inactivation in liquid was developed by the acidification of bacterial suspension (called "Reduced pH method"). Plasma treated *Escherichia coli* maintained some physiological activity such as aerobic glucose metabolism, whereas proliferation function was completely inactivated. Quantitative PCR analysis revealed plasma treatment in liquid causes extremely little or no DNA damages as seen by UV irradiation. On the other hand, some protein molecule in plasma treated *E. coli* showed the increase in its molecular weight. These results suggests that the mechanism of plasma sterilization in liquid is due to the chemical modification of bacterial proteins, which leads to physiological inactivation of the proteins.

1. Introduction

Although many studies of plasma sterilization against dried target of bacteria were carried out, not many studies against wet target, like liquid or animal tissue. For medical application of cold atmospheric pressure plasma, however, development of the method for bacterial inactivation in liquid is necessary. Typically, bacterial inactivation in whole of the liquid is hardly achieved by plasma application to the surface of the liquid, because components of plasma, electron and excited ions can not penetrate into the liquid. Successful method of bacterial inactivation in liquid was developed by acidification of bacterial suspension (called "Low pH method") [1]. It is considered that strong bactericidal activity of "Reduced pH method" is brought by hydroperoxy radical (HOO•) generated from the association of hydrogen ion (H^+) and superoxide anion radical (O_2^-•), as shown in Fig 1. Oxygen molecules included in atmospheric gas are excited to produce O_2^-• at the interface of plasma and atmospheric gas, then O_2^-• diffuses into an acidic liquid that allows association of O_2^-• with H^+ to generate HOO• [2]. As the nature without electric charge, HOO• can diffuse into bacterial cytosol via hydrophobic cell membrane, therefore HOO• shows extremely stronger bactericidal activity than negative charged O_2^-• [3].

As well as the investigations of active species generated with plasma discharge, to elucidate the mechanism to affect target bacterial composition such as proteins or nucleic acids, that is very important ensuring safety in plasma medicine. In the most of studies in plasma sterilization, damages of plasma exposed bacteria are estimated by only electron microscopic observation. However, surface observation presents almost no information about molecular mechanism of bactericidal activity. In this study, we examined the damages of plasma exposed bacteria by using molecular biological methods to elucidate the molecular mechanism of bacterial inactivation in liquid by plasma exposure.

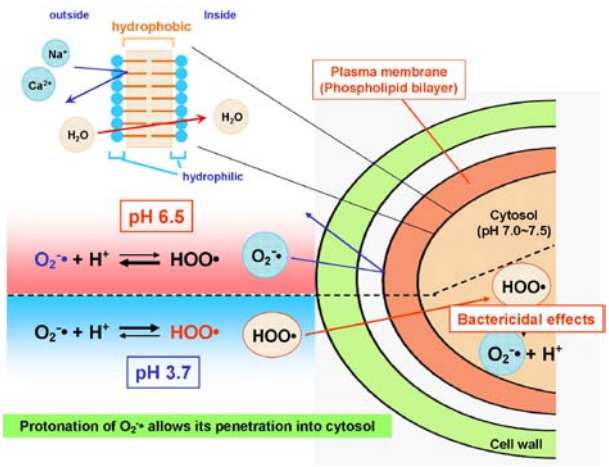

Fig. 1 Scheme for the model of O_2^-• permeation through the cytoplasmic membrane.

2. Result and discussion

To examine bacterial inactivation in liquid, atmospheric pressure cold plasma jet called LF (low-frequency) jet was used [4]. When *Escherichia coli* suspended in acidic buffer (pH 3.7) was exposed to LF jet for 10 min, no colony was obtained on LB agar plate. However, microscopic observation of this suspension proved no morphological change in the bacterial cells, suggesting bacterial inactivation was resulted from physiological damages on cytoplasmic membrane or in cytosol. To examine the physiological activity on cytoplasmic membrane of plasma expoesd *E. coli*, WST assay, a method for detection of aerobic metabolism of glucose, was carried out. Results show that plasma exposed *E. coli*, completely inactivated colony formation ability, maintain the metabolic activity for about 40% comparing with intact cells. This fact suggests that plasma treated bacteria were inactivated in proliferative function while maintaining other physiological functions. A similar state of impaired cells is found at sterilization by ultraviolet (UV) irradiation causing DNA damage. Thus, a quantitative PCR was performed to compare the effect on DNA molecule of plasma exposure with that of UV irradiation. As shown in Fig. 2, UV-irradiation to *E. coli* resulted in decrement of DNA amplification, depending on the irradiation period, it was confirmed that the accumulation of DNA damage. On the other hand, plasma exposure to *E. coli* had no effect on DNA amplification. These results indicate that a molecular mechanism of bacterial inactivation by plasma exposure is not due to the damage on DNA brought by UV light.

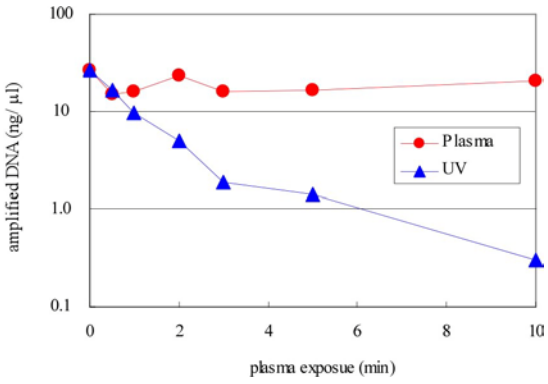

Fig. 2 Quantitative PCR of plasma exposed and UV irradiated *E. coli*.

Finally, the effect of plasma exposure to proteins in a bacterial cell was examined. Total proteins of plasma treated *E. coli* were analyzed by SDS - polyacrylamide gel electrophoresis, visualizing each protein to individual band separated in its molecular weight. When *E. coli* was exposed to plasma at lower pH, distinct band shift indicating increase in molecular weight was found at some protein molecule, whereas no shift was observed in plasma exposure at neutral pH, at which no bactericidal activity is seen. This suggests that distinct band shift is correlated with bactericidal activity. Increase in the molecular weight of protein is considered to be due to chemical modification in amino acid residues, such as oxidation or nitration. It is considered that excessive or critical chemical modification of amino acid residues leads to physiological inactivation of bacterial protein, thereby the proliferation function is inactivated by plasma exposure. Additional investigation of proteins from plasma treated bacteria is expected to be elucidated the molecular mechanism of plasma sterilization in liquid.

In addition to the measurement of the generated active species and the physicochemical reactions in liquid, the affects of plasma exposure to human body for hemostasis, disinfection, wound healing and so on in plasma medicine should be achieved by a similar technique.

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