Antimicrobial Activity of Peracetic Acid Preparation in the Presence of Various Compounds

ANTIMICROBIAL ACTIVITY OF PERACETIC ACID PREPARATION IN THE PRESENCE OF VARIOUS COMPOUNDS.

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Abstract

It is known that peracetic acid (PAA) is one of the few powerful antimicrobial agents available for use as a sporicidal agent. In this study, the antimicrobial action of a commercial preparation of PAA is characterized and the effects of chemicals on the action are investigated using *Escherichia coli* cells. The antimicrobial activity of the PAA preparation used in this study was markedly higher under high temperatures and low pHs. However, such a high activity of PAA preparation was strictly inhibited in the presence of a chemical, such as some amino acids, metal salts, and metal chelating agents. By the quantitative analysis of PAA and H_2O_2, it was also observed that these inhibitory chemicals had a strong influence on decomposition of PAA. Although manganese sulfate, and *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA) had a slight inhibitory effect on the PAA antimicrobial action, these chemicals decomposed PAA, suggesting that these chemicals might reduce cell activity to the PAA resistance. Furthermore, our results indicate that the H_2O_2 contained in the PAA preparation has a slight effect on the antimicrobial action of the PAA preparation.

1. Introduction

Recently, contamination by microbial cells, including pathogens, is one of the greatest problems for human health. As technologies of advanced mass production and long-term preservation develop, risk of contamination through invasion by microbial cells in various systems and products are is becoming more common. If microbial cells are contaminated, serious incidents, such as food poisonings, result. To avoid these incidents, sterilization systems - disinfectants and germicides - are being developed and are now widely employed.

Two type cells (vegetative form and spore form) exist in microbial cells. Vegetative cells have active growth, whereas spores are dormant cells which survive in inconclusive environments for cell growth. As, spores are resistant to several stresses, such as heat, ultra-violet light, and many of disinfectants, it is necessary to construct effective spore-inactivation systems for both the food industry and the medical institutions.

The application of antimicrobial agents is one type of effective antimicrobial treatment[1]. Compared with other treatments, this method is cost-effective and broadly available.

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However, the antimicrobial activities of antimicrobial agents are influenced by environmental conditions, such as temperature, pH, chemical compounds and so on. Almost all antimicrobial agents are also ineffective for bacterial spores. These disadvantages could not be surmounted, but antimicrobial chemicals are still useful because of their other properties.

Peracetic acid preparation, which is a mixture of peracetic acid, acetic acid, hydrogen peroxide, and water, is a powerful oxidant, and possesses strong antimicrobial and sporicidal properties[2]. Peracetic acid is unstable and its decomposition products are non-toxic acetic acid, oxygen and water. This property facilitates its application as an environmental disinfectant, although peracetic acid preparation at a high concentration has a strong acrid odor and is explosive[3]. Actually, the peracetic acid preparation is utilized as a disinfectant for waste water effluents and for cleaning equipment surfaces in the food and medical industries[4]. However, the instability of peracetic acid is one of its disadvantages, for its activity is rapidly halted by contaminated chemicals[5]. In fact, some organic compounds and metal ions of copper, iron, and manganese decompose peracetic acid to acetic acid. In this study, the antimicrobial properties of the peracetic acid preparation and the inhibitory effects of the other chemicals are evaluated.

2. Materials and Methods

2.1 Bacterial strain and growth conditions.

Escherichia coli strain OW6[6] was used as an indicator strain for the antimicrobial activity of the PAA preparation. Cells were aerobically cultivated at 37 ℃ in an EM9 medium, consisting of 7 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 2 g of glucose, and 1 g of casamino acid per liter. Vegetative cells were cultivated until an optical density of approximately 0.3 at 650 nm. They were collected by centrifugation at 6,000 x *g* at 4 ℃ for 5 min and then washed in 50 mM citrate buffer (pH6.0). Washed cells were resuspended in a fresh citrate buffer and used for an antimicrobial activity analysis. EM9 plate containing 1.5 % agar and spread with 120 $U \cdot ml^{-1}$ catalase (ECM9 plate) was used for measurement of the number of viable cells after the PAA treatment.

2.2 Antimicrobial activity of the PAA preparation

As a basic protocol, PAA preparation at 5.0 μM was added to the citrate buffer containing 1 x 10^7 cells and incubated at 37 ℃ for 20 min while shaken. the chemicals, L-alanine, L-cysteine·HCl, L-histidine, L-methionine, L-tryptophan, yeast extract, bovine serum albumin (BSA), $FeSO_4 \cdot 7H_2O$, $FeCl_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $MnSO_4 \cdot 5H_2O$, Ethylenediamine-*N,N,N',N'*-tetraacetic acid disodium salt dihydrate (EDTA·2Na), *O,O'*-Bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA), 2,2'-dipyridyl, and *o*-phenanthroline · H_2O, were added to this reaction solution. Cell suspensions at a volume of 100 μl were sampled at appropriate times and were suspended in a 0.9-ml stop solution, consisting of sodium thiosulfate (at a final concentration of 0.5%) and 50 mM potassium

phosphate buffer (pH7.0), and then incubated for 5 min at 37 ℃ to stop PAA reaction. Viable cells in the resultant cell suspension were cultivated on ECM9 plates at 37 ℃ for 1 day, after which the suspension was diluted with a 50mM potassium phosphate (pH7.0) buffer. Survival rates were calculated from colony numbers on the ECM9 plates.

2.3 Decomposition of PAA in the presence of chemicals.

In a 5.0 mM PAA preparation containing 50 mM citrate buffer (pH6.0), chemicals at appropriate concentrations were mixed and incubated for 20 min at 37 ℃. A portion of the reaction solution was immediately utilized to measure concentrations of PAA and H_2O_2, as described below.

2.4 Measurement of concentrations of PAA and H_2O_2

Concentrations of PAA and H_2O_2 in a PAA preparation were measured by the methods reported by Pinkernell *et al.*[7]. In a PAA preparation of 100 μl, 20 mM methyl *p*-tolyl sulfide (MTS) of 100 μl and 300 μl MilliQ water were mixed and incubated for 10 min at 25 ℃. 400 μl acetonitrile and 10 mM triphenylphosphine (TPP) were subsequently mixed in the resultant solution and incubated for 30 min at 25 ℃. Methyl *p*-tolyl sulfoxide (MTSO) was synthesized from the reaction of MTS and PAA, and triphenylphosphine oxide (TPPO) was synthesized from reactions of TPP and H_2O_2. MTSO, TPPO, MTS, and TPP were separated with a reverse phase column (TSK-GEL Octyl 80Ts, particle size ϕ 5.0 μm, 4.6 mm I.D. x 150 mm; TOSOH Corporation, Tokyo, Japan) installed in a Hitachi LaChrom HPLC system (Hitachi High-Technologies Corporation, Tokyo, Japan) and detected at an absorbance of 225 nm. The flow rate was 1.0 $ml \cdot min^{-1}$. These compounds were eluted by 40 % acetonitrile for 3 min and subsequently 100 % acetonitrile for 3 min. Concentrations of PAA and H_2O_2 were calculated from peak areas of MTSO and TPPO, respectively. In this method, it was possible to measure PAA at 0.07-6.0 mM and H_2O_2 at 0.07-11.6 mM.

2.5 Chemicals

The PAA preparation consisting of 38.3 % PAA, 5.6 % H_2O_2, 39.4 % acetic acid, 16.5 % water, and 0.5% sulfuric acid, was donated by Mitsubishi Gas Chemistry Company, Inc. (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich Japan K. K. (Tokyo, Japan).

3. Results

3.1 Antimicrobial properties of PAA preparation

The antimicrobial activity of PAA was examined with vegetative cells ($1x10^7$ cfu/ml) of *E. coli* OW6 in 50 mM citrate buffer (pH7.0). 6.8 μM PAA indicated a strong antimicrobial activity and killed generally six-order cells for 20 min, but 3.5 μM PAA indicated no antimicrobial activity for vegetative cells (Fig. 1). These results indicate that antimicrobial activity of PAA strictly depends on its concentration. Furthermore, the dependency of PAA

activity on temperature and pH was also evaluated (Fig. 2 and Table 1). Its activity markedly increased under high temperature and acidic conditions.

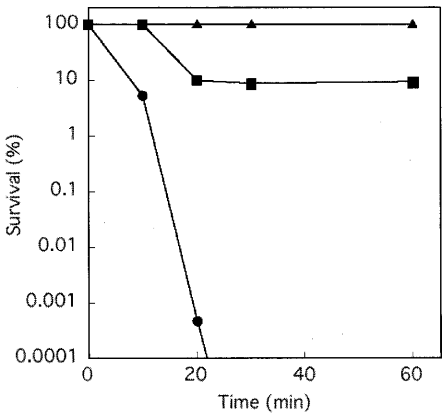

Fig.1 The antimicrobial activity of PAA preparation to *E. coli* cells. Cells were treated with PAA preparation at 3.5 (closed triangles), 5.0 (closed squares), and 6.9 μM (closed circles) at 37 ℃.

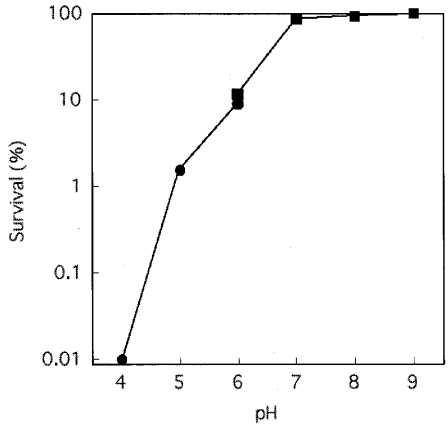

Fig.2 Effect of pHs on the antimicrobial activity of the PAA preparation. Cells were treated with 10.5 μM PAA preparation at 37 ℃ for 5 min. PAA preparations were prepared in 50 mM citrate buffer (closed circles) and potassium phosphate buffer (closed squares).

Table 1. Effects of temperatures on the PAA action[a)]

Temperature (℃)	Survival (%)
0	104
20	84
37	0.74
42	<0.00018

a) Cells were treated with 17.0 μM PAA solution for 5 min.

3.2 Antimicrobial activity of PAA preparation is inhibited by metal ions

It is known that contamination of metal ions destabilizes PAA[8)]. Therefore, we examined how ferrous, manganese, and magnesium ions influenced the PAA antimicrobial activity (Fig. 3). The ferrous ion at 1 mM strongly inhibited the antimicrobial activity of the PAA preparation. Manganese and magnesium ions at 1 mM partially inhibited the PAA antimicrobial activity. The chloride ion also inhibited the PAA activity (data not shown).

3.3 Antimicrobial activity of a PAA preparation is inhibited by organic compounds

The effects of amino acids, proteins, and metal chelating agents on the antimicrobial

activity of the PAA preparation were examined. Although L-alanine and L-histidine at 5mM did not influence the antimicrobial activity of a 5.0 mM PAA preparation, L-methionine, L-cycteine, and L-tryptophan at 5.0 mM strongly inhibited its activity (Fig. 4 and data not shown). Yeast extract and BSA at 0.1% also inhibited the activity of PAA (data not shown). EGTA and 2,2'-dipyridyl at 5 mM inhibited the activity, but EDTA and *o*-phenanthroline had only a slight inhibitory effect on the antimicrobial activity of the PAA preparation (Fig. 5). We are very interested in the antithetical effects of *o*-phenanthroline and 2,2'-dipyridyl for the PAA activity, because these compounds are both ferrous chelating agents. It suggests that metal chelating activity is not involved in the inhibitory effect on the PAA antimicrobial activity.

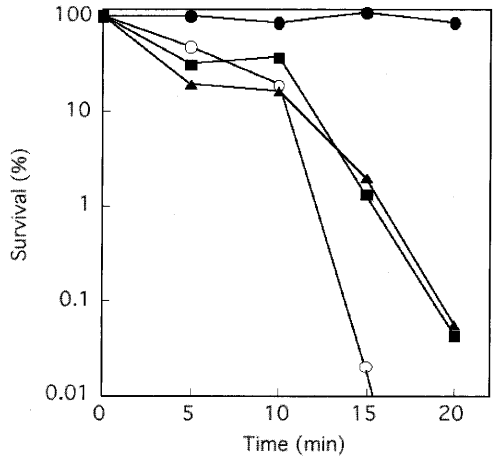

Fig.3 Effect of metal ions on the antimicrobial activity of the PAA preparation. Cells were treated with 5.0 μM PAA preparation at 37 ℃ in the presence of $FeSO_4$ (closed circles), $MgSO_4$ (closed squares), and $MnSO_4$ (closed triangles) at 1 mM, and in the absence of contaminated chemicals (open circles).

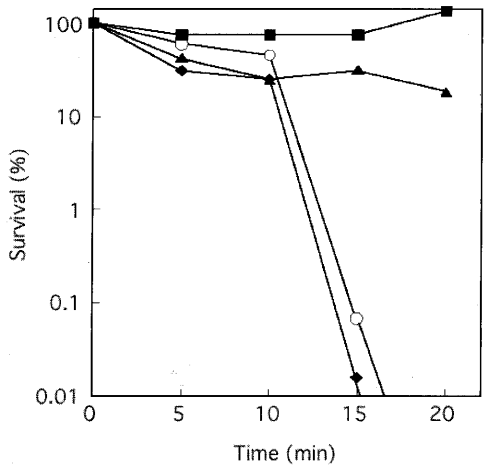

Fig.4 Effect of amino acids on the antimicrobial activity of the PAA preparation. Cells were treated with 5.0 μM PAA preparation at 37 ℃ in the presence of L-alanine (closed diamonds), L-methionine (closed squares), and L-tryptophan (closed triangles) at 5 mM, and in the absence of contaminated chemicals (open circles).

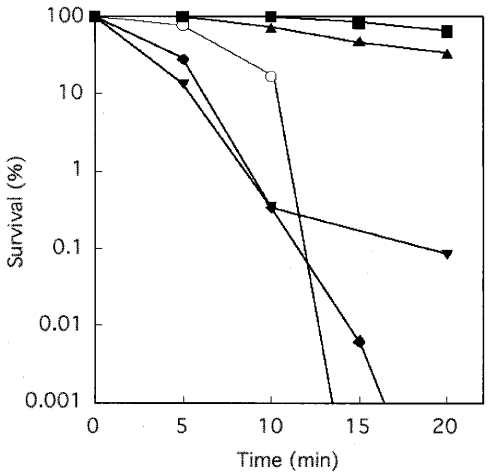

Fig.5 Effect of metal-chelating agents on the antimicrobial activity of the PAA preparation. Cells were treated with 5.0 μM PAA preparation at 37 ℃ in the presence of 2,2'-dipyridyl (closed diamonds), EGTA (closed squares), *o*-phenanthroline (closed triangles), and EDTA (closed inverse triangles) at 5 mM, and in the absence of contaminated chemicals (open circles).

3.4 PAA decomposition by chemicals

It is been known that many compounds, such as ferrous ion and organic compounds, decompose PAA and it has been suggested that the apparent inhibition of antimicrobial activity of PAA results from decomposition of PAA[9]. However, some chemicals had slight or partial inhibitory effects for antimicrobial activity of PAA. Furthermore, PAA preparation also contains H_2O_2, although it is supposed that the H_2O_2 contained in PAA preparation has little influence on the antimicrobial activity of PAA preparation because of the markedly lower antimicrobial activity of H_2O_2 over that of PAA. Then, we measured a concentration of PAA and H_2O_2 in PAA preparation in the presence of the chemical compounds, amino acids, proteins, metal-chelating agents, and metal salts used in this study.

To facilitate the measurement of concentrations of PAA and H_2O_2, 5.0 mM PAA suspended in 50 mM citrate buffer (pH6.0) was used for the PAA stability test. In 5.0 mM PAA preparation, 5.40±0.20 mM PAA and 0.80±0.30 mM H_2O_2 were measured by HPLC analysis. It was also shown that the detection limits of PAA and H_2O_2 were both at 0.07 mM. Furthermore, PAA and H_2O_2 in 5.0 mM PAA preparation were confirmed to be stable for at least 40 min without other chemicals (data not shown).

L-alanine and L-histidine at 10 mM had no destabilizing effect on PAA and H_2O_2 in 20-min incubation at 37 ℃ (Table 2). However, The presence of L-tryptophan, L-cycteine, and

Table 2. Effect of chemicals on the stability of 5.0 mM PAA solution.[a]

Chemicals		Remaining content (%)			
		PAA		H_2O_2	
		1 min	20 min	1 min	20 min
L-alanine	0.1 mM	NT[b]	NT	NT	NT
	10	97.4	98.6	92.2	124
L-histidine	0.1	NT	NT	NT	NT
	10	98.6	92.0	107	114
L-tryptophan	0.1	NT	96.2	129	103
	10	96.2	14.1	99.5	93.2
L-cysteine	0.1	88.2	94.4	93.1	128
	10	ND[c]	8.0	161	170
L-methionine	0.1	93.8	96.0	87.4	125
	10	ND	2.5	108	122
$FeSO_4$	0.1	104	94.3	117	93.1
	10	ND	ND	55.3	69.8
$MgSO_4$	0.1	93.1	103	90.3	119
	10	98.6	105	112	148
$MnSO_4$	0.1	106	34.2	95.2	50.3
	10	104	ND	106	29.9
EDTA	0.1	98.2	99.4	101	97.0
	5.0	34.8	3.9	137	94.8
EGTA	0.1	99.6	101	109	113
	5.0	107	26.9	268	132
o-phenanthroline	0.1	98.9	95.1	106	106
	5.0	97.6	24.1	87.5	102
2,2'-dipyridyl	0.1	99.0	92.8	120	85.6
	5.0	99.9	84.8	131	103

a) 5.0mM PAA was incubated with chemicals st 37℃
b) Not tested.
c) Not detected.

L-methionine at 10 mM led to the decomposition of PAA, although concentrations of H_2O_2 were slightly variable. In metal salts at 10 mM, ferrous sulfate and manganese sulfate led to decomposition of PAA and H_2O_2, but magnesium sulfate had no destabilizing effect on PAA and H_2O_2. In metal-chelating agents, EDTA, EGTA, and *o*-phenanthoroline led to decomposition of PAA, but 2,2'-dipyridil decomposed PAA and H_2O_2, only slightly. These results indicate that addition of destabilizing agents to PAA has inhibited the antimicrobial activity of PAA and H_2O_2, except for some exceptional compounds, such as manganese sulfate, magnesium sulfate, and EGTA.

4. Discussion

In this study, we have examined the antimicrobial activity of PAA preparation contaminated in the presence of a compound. Some compounds, several amino acids, metal salts and metal-chelating agents, strongly inhibited the PAA activity, but common function for the inhibition among these compounds is unclear. It was demonstrated, however, that the compounds which inhibited the PAA activity had PAA decomposition activity, although it is unclear how these compounds actually decompose PAA. These results indicate that the stabilization of PAA is one of the most important conditions for antimicrobial activity of a PAA preparation.

The antimicrobial activity of a PAA preparation was markedly influenced by temperature and pH, and showed a higher activity at a high temperature and low pH. These phenomena have already been reported by other investigators[10, 11]. But it is unclear why the dependencies on temperature and pH have been observed. It is presumed that the reaction of PAA to microbial cells is induced by higher temperature, similar to other antimicrobial agents, and that the permeability of cells to PAA is increased by low pH arising from the structural property of PAA. It is supposed that the predominant species is the dissociated acidic form under alkaline conditions and this hardly penetrates cells because PAA has a pK_a of 8.2. Additionally, PAA might be unstable under alkaline conditions.

In this study, we have also examined the inhibitory effects of chemicals and qualitatively identified their destabilizing effects on PAA activity. However, it is still unclear if the intensity of their inhibitory effects correlates with the PAA decomposition activity. We suspect that it is important to improve sensitivities of PAA and H_2O_2 detections and to analyze the kinetics of PAA decomposition reactions in detail.

Manganese sulfate and EGTA had slight or partial inhibitory effects on the antimicrobial activity of the PAA preparation, but had decomposition activities in PAA. These observations were contradictory to our hypothesis described above. In the case of EGTA, the activity of EGTA in the PAA decomposition was relatively low, compared with those of other inhibitory chemicals. This suggests that low decomposition activity slightly influenced the antimicrobial activity of the PAA preparation and that competition between the PAA decomposition and attack on cells may be important for the expression of PAA's antimicrobial activity. However, the decomposition activity of manganese sulfate in PAA was enough high, compared with those of other inhibitory chemicals. It is known that the

manganese ion is an important physiological material and influences several cell activities, such as superoxide stress and ethanol stress responses in the bacterial cells[12, 13]. The Manganese ion might sensitize cells to PAA.

Magnesium sulfate also slightly inhibited the PAA action. However, this compound had no decomposition activity in PAA, unlike to manganese sulfate. The magnesium ion also influences bacterial cell activities and exists around the bacterial cell surface. It is supposed that the manganese ion might decrease the permeability of PAA and reduce the PAA sensitivity to bacterial cells[14].

Our results in this study indicate that PAA is a powerful antimicrobial agent under a condition without compounds, and it is necessary to clean up or remove chemicals before PAA treatment. Furthermore, the development of stabilizers for PAA preparation will improve the antimicrobial activity of PAA and expand its application area, as well as disinfection of waste water treatment.

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