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Decontamination of Lettuce Using Acidic Electrolyzed Water

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ABSTRACT

The disinfectant effect of acidic electrolyzed water (AcEW), ozonated water, and sodium hypochlorite (NaOCl) solution on lettuce was examined. AcEW (pH 2.6; oxidation reduction potential, 1140 mV; 30 ppm of available chlorine) and NaOCl solution (150 ppm of available chlorine) reduced viable aerobes in lettuce by 2 log CFU/g within 10 min. For lettuce washed in alkaline electrolyzed water (AlEW) for 1 min and then disinfected in AcEW for 1 min, viable aerobes were reduced by 2 log CFU/g. On the other hand, ozonated water containing 5 ppm of ozone reduced viable aerobes in lettuce 1.5 log CFU/g within 10 min. It was discovered that AcEW showed a higher disinfectant effect than did ozonated water significantly at $P < 0.05$. It was confirmed by swabbing test that AcEW, ozonated water, and NaOCl solution removed aerobic bacteria, coliform bacteria, molds, and yeasts on the surface of lettuce. Therefore, residual microorganisms after the decontamination of lettuce were either in the inside of the cellular tissue, such as the stomata, or making biofilm on the surface of lettuce. Biofilms were observed by a scanning electron microscope on the surface of the lettuce treated with AcEW. Moreover, it was shown that the spores of bacteria on the surface were not removed by any treatment in this study. However, it was also observed that the surface structure of lettuce was not damaged by any treatment in this study. Thus, the use of AcEW for decontamination of fresh lettuce was suggested to be an effective means of controlling microorganisms.

Numerous microorganisms capable of causing disease in humans have been isolated from fresh fruits and vegetables *(3)*. Currently, washing with tap water is the recommended means for consumers to reduce microbial contamination on the surface of raw fruits and vegetables. Although washing fresh produce in running tap water may have some effectiveness in removing soil and other debris, it should not be relied on to remove the surface microorganisms. Tap water has a limited or no effect on microorganisms that occur at populations ranging from 10^3 to 10^9 CFU/g on fresh produce *(2, 5, 13)*.

Chlorination has long been widely used for the decontamination of foods. However, washing and chlorine or ozone treatment cannot completely remove or inactivate microorganisms in or on fresh produce *(1, 2, 5, 13, 18)*. The effectiveness of a disinfectant treatment is probably dependent on the ability of the active disinfecting agent to make contact with the target microorganisms. Therefore, microorganisms that are embedded in tissue will be protected from chemicals, such as chlorine, that have little penetrating power *(15)*.

There have been reports on the antimicrobial and antiviral activities of acidic electrolyzed water (AcEW) produced by the electrolysis of an aqueous sodium chloride solution using an instrument in which anode and cathode are separated by a membrane to form two compartments *(6, 14)*. Using this technique in the electrolysis of dilute sodium chloride solutions is not new. However, the application of this technique to areas other than chlorine production, such as agricultural, water treatment, and food sanitation, is new. Recently, the efficacy of AcEW as a disinfectant for fruits and vegetables has been reported *(7–9)*.

The objectives of this study were to confirm the disinfectant effect of AcEW on lettuce. As a comparison, we also investigated the effect of ozonated water containing 5 ppm of ozone and sodium hypochlorite (NaOCl) solution containing 150 ppm of available chlorine. Moreover, the differences in the reduction of microorganisms on the surface of and embedded in the whole of lettuce were examined. Then, the surface structure of lettuce and microorganisms on the surface of lettuce were observed by a scanning electron microscope (SEM). From these studies, we confirmed the disinfectant characteristics of AcEW for lettuce and the possibility of using AcEW as a disinfectant for lettuce.

MATERIALS AND METHODS

Lettuce. Lettuce was purchased at a local supermarket in Tsukuba, Ibaraki, Japan. The outer leaves were removed and discarded. The remaining leaves were then cut into 5 by 5-cm squares for decontamination experiments and microscopic observation.

Comparison of the disinfectant effect of AcEW, ozonated water, NaOCl solution, and tap water on lettuce. Lettuce (100 g) was soaked in each solution (2,000 ml) of AcEW, ozonated water, and tap water for a fixed time. Flow rate was set at 1,000 ml/min. As a comparison, lettuce (100 g) was soaked in NaOCl solution (2,000 ml) containing 150 ppm of available chlorine for a fixed time. Treatment time was set at 1, 5, and 10 min. A flow-type electrolysis apparatus (ROX-20TA, HOSHIZAKI, Nagoya, Japan) was used to prepare the electrolyzed water. The current

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passing through the electrolysis apparatus and voltage between the electrodes were set at 14 A and 18 V, respectively. AcEW was prepared in the anode side of an electrolytic cell, and alkaline electrolyzed water (AlEW) was prepared in the cathode side. The pH of the tested solution was measured with a pH meter (D-22, Horiba, Kyoto, Japan). The oxidation reduction potential (ORP) was measured with an ORP meter (HM-60V, TOA Electronics Ltd., Tokyo, Japan). The initial concentration of the available chlorine in the AcEW, tap water, and NaOCl used in this study was quantified by spectrophotometric analysis at 530 nm using *N,N*-diethyl-*p*-phenylendiamine *(17)*. A flow-type ozone-generating apparatus (MCX-2000, SILVER REED, Tokyo, Japan) was used to prepare the ozonated water. The concentration of ozone in the ozonated water used in this study was quantified by spectrophotometric analysis at 254 nm. Viable aerobic bacteria in or on the lettuce were enumerated as an index of the disinfectant effect.

Comparison of the disinfectant effect on the surface of and in the whole lettuce. Lettuce (100 g) was soaked in running each solution (2,000 ml) of AcEW, ozonated water, and tap water for 10 min. Flow rate was set at 1,000 ml/min. As a comparison, lettuce (100 g) was disinfected by soaking in the NaOCl solution (2,000 ml) containing 150 ppm of available chlorine for 10 min. Another sample of lettuce (100 g) was washed by soaking in running AlEW (2,000 ml) for 1 min and then disinfected by soaking in running AcEW (2,000 ml) for 1 min.

Aerobic bacteria, coliform bacteria, molds, and yeasts on the surface of and in the whole lettuce were enumerated as index of the disinfectant effect.

Enumeration of microorganisms. Aerobic bacteria, coliform bacteria, molds, and yeasts were enumerated by general procedures. To enumerate surface microorganisms, the surface of lettuce (both sides) was swabbed with sterile absorbent cotton (approximately 0.1 g) containing 1 ml of 0.85% sodium chloride solution. The swabbed area was 50 cm^2. The absorbent cotton was then washed with 50 ml of sterile 0.85% sodium chloride solution in a sterile polyethylene bag and was pummeled with a Stomacher for 2 min at high speed. Wash fluid was serially diluted, and 1 ml was mixed with approximately 15 ml of each medium. Microorganism counts were expressed by log CFU/cm^2.

To enumerate the microorganisms within lettuce, lettuce (10 g) was combined with 90 ml of sterile 0.85% sodium chloride solution in a sterile polyethylene bag and was pummeled with a Stomacher for 2 min at a high speed. Then, wash fluid was serially diluted, and 1 ml was mixed with approximately 15 ml of each medium. Microorganism counts were expressed by log CFU/g.

Media for the various microorganisms included standard methods agar (Nissui, Tokyo, Japan) for total viable aerobic bacteria, violet red bile agar (Difco Laboratories, Detroit, Mich.) for coliform bacteria, and YM agar (Difco) plus 0.1 g/liter of chloramphenicol (Wako, Osaka, Japan) for molds and yeasts. All pour plates were duplicated and incubated at 35°C except for molds and yeasts, which were incubated at 25°C. Violet red bile agar plates were read after 24 h and aerobic bacteria plates after 48 h. Mold and yeast plates were read after 72 h and confirmed after 120 h.

Microscopic observation. Lettuce was immersed in 2.5% glutaraldehyde prepared in a 0.1 M phosphate buffer and then left for 2 h at room temperature. For further processing, samples were washed twice with buffer alone and then freeze-dried. The dried samples were coated with a thin layer of gold in a model JFC-1500 sputtering unit (JEOL, Tokyo, Japan). Digital images were collected using a personal computer integrated with a model LV-5600 SEM (JEOL) operated in the secondary electron imaging mode.

TABLE 1. *Physicochemical properties of tested solutions*[a]

Solution	pH	ORP (mV)	ACC or OC (ppm)
AcEW	2.6 ± 0.1	1,140 ± 7	30.3 ± 3.1
Ozonated water	6.6 ± 0.1	1,256 ± 28	5.1 ± 0.3
NaOCl	9.3 ± 0.2	638 ± 18	155.2 ± 5.8
Tap water	7.0 ± 0.1	414 ± 25	0.3 ± 0.1
AlEW	11.4 ± 0.1	−870 ± 12	—

[a] Results are mean ± SD ($n = 5$). ORP, oxidation reduction potential; ACC, available chlorine concentration; OC, ozone concentration; AcEW, acidic electrolyzed water; NaOCl, sodium hypochlorous; AlEW, alkaline electrolyzed water.

Statistical analysis. All trials were replicated five times. Photomicrographs were selected to represent typical results. Reported plate count data represented the mean value ± SD obtained from five independent trials, each of these values being obtained from duplicated samples. Significant differences in plate count data were established by least significant difference at the 5% level of significance.

RESULTS

Changes in viable aerobic bacteria count on the lettuce. The physicochemical properties of the tested solution are shown in Table 1. The pH values of AcEW, ozonated water, NaOCl solution, and tap water were 2.6, 6.6, 9.3, and 7.0, respectively. The ORP values of AcEW and ozonated water were high, such as 1,140 and 1,256 mV, respectively. On the other hand, AlEW showed a very low ORP value, such as −870 mV. The ORP values of the NaOCl solution and tap water were 638 and 414 mV, respectively. The available chlorine concentrations of AcEW and NaOCl were 30.3 and 155.2 ppm, respectively. The ozone concentration in the ozonated water was 5.1 ppm.

The changes in viable aerobic bacteria populations on lettuce treated with various solutions are shown in Figure 1. Treatment with AcEW containing 30 ppm of available chlorine showed a higher disinfectant effect than did treatment with ozonated water containing 5 ppm of ozone. NaOCl solution containing 150 ppm of available chlorine showed an effect equal to AcEW and ozonated water within 5 min. Although NaOCl solution showed a higher disinfectant effect than did ozonated water after 10-min soaking, AcEW showed a disinfectant effect equal to the NaOCl solution. Tap water did not show a disinfectant effect within 10 min. In every treatment except for tap water, viable aerobic bacteria populations were reduced by about 1 log CFU/g or more within 1 min. However, prolongation of treatment time did not increase reduction ratio. AcEW reduced more aerobic bacteria than did ozonated water in all treatment times under these study conditions. The difference between these two treatments after 10-min soaking was about 0.6 log CFU/g, and this difference was significant at $P < 0.05$.

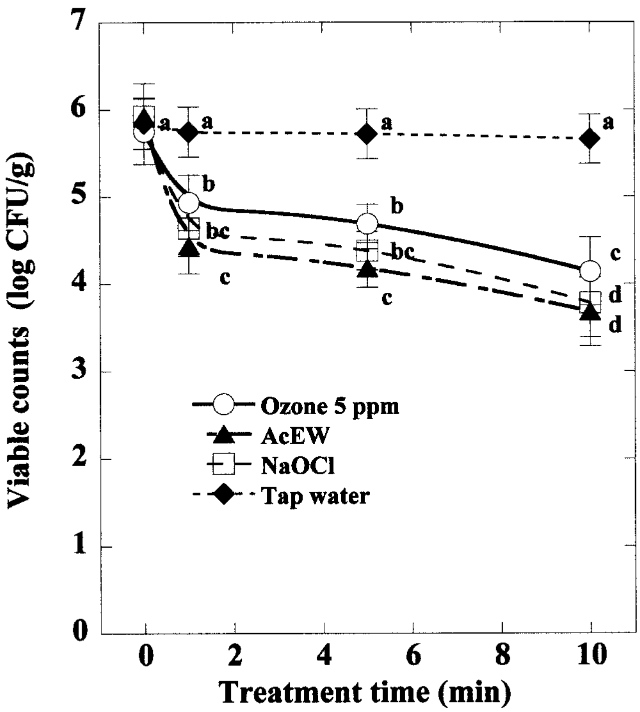

FIGURE 1. *Changes of viable aerobic bacteria counts in the lettuce treated with ozonated water containing 5 ppm ozone, acidic electrolyzed water (AcEW) containing 30 ppm available chlorine, NaOCl solution containing 150 ppm available chlorine and tap water. Results are means ± SD,* n *= 5. Values with different letters differ significantly at* P *< 0.05.*

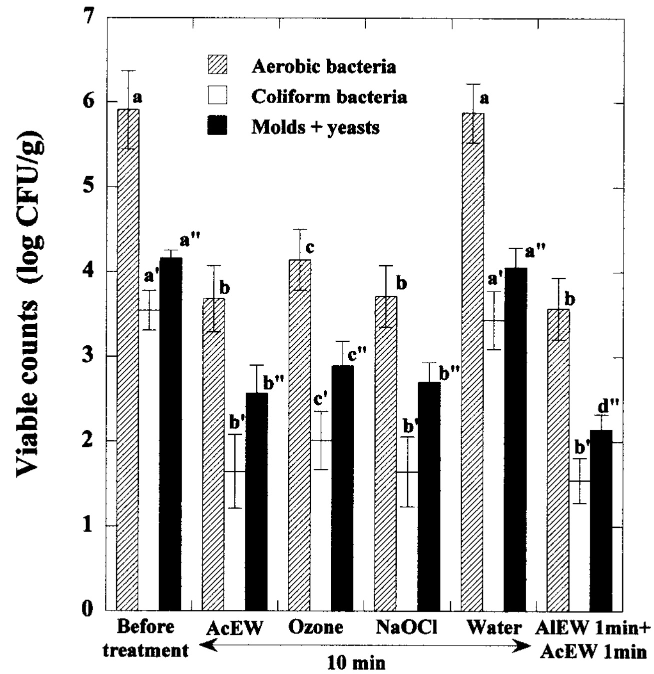

FIGURE 2. *Comparison of disinfectant effect on the lettuce treated with acidic electrolyzed water (AcEW), ozonated water, NaOCl, and tap water for 10 min, and washed with alkaline electrolyzed water (AlEW) for 1 min and then treated with acidic electrolyzed water (AcEW) for 1 min. Results are means ± SD,* n *= 5. Values with different letters in the same medium differ significantly at* P *< 0.05.*

Comparison of the disinfectant effect of AcEW, ozonated water, and NaOCl solution on the surface of and in the whole lettuce. The microorganisms embedded in the lettuce treated with various solutions were enumerated (Fig. 2). AcEW and the NaOCl solution reduced the viable aerobic bacteria in the lettuce by 2 log CFU/g within 10 min. This result agrees with our past experiment *(9)*. For lettuce washed with AlEW for 1 min and then disinfected with AcEW for 1 min (this treatment is referred to as 1 + 1 treatment), viable aerobic bacteria in the lettuce were reduced by 2 log CFU/g. On the other hand, treatment with ozonated water reduced the viable aerobic bacteria in the lettuce by 1.5 log CFU/g within 10 min. Tap water did not decrease aerobic bacteria in the lettuce.

Coliform bacteria populations were reduced to less than 10^2 CFU/g by all treatments except for tap water. There were little differences in the disinfectant effect among the treatments. Although the difference of disinfectant effect between AcEW and NaOCl solution was not significant, the effect of ozonated water was smaller than the other two treatments significantly at $P < 0.05$. Tap water did not decrease coliform bacteria in the lettuce.

Mold and yeast populations were reduced by 1.5 log CFU/g by the treatment with AcEW, NaOCl, and 1 + 1. Treatment with ozonated water reduced molds and yeasts by about 1 log CFU/g. Thus, the disinfectant effect of ozonated water on molds and yeasts was fewer than that of the other three treatments. This difference was significant at P

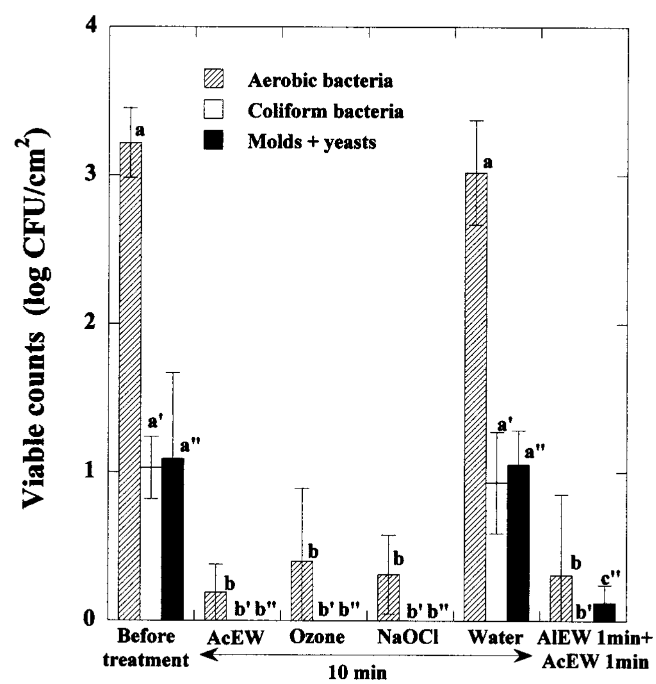

FIGURE 3. *Comparison of disinfectant effect on the surface of lettuce treated with acidic electrolyzed water (AcEW), ozonated water, NaOCl and tap water for 10 min, and washed with alkaline electrolyzed water (AlEW) for 1 min and then treated with acidic electrolyzed water (AcEW) for 1 min. Results are means ± SD,* n *= 5. Values with different letters in the same medium differ significantly at* P *< 0.05.*

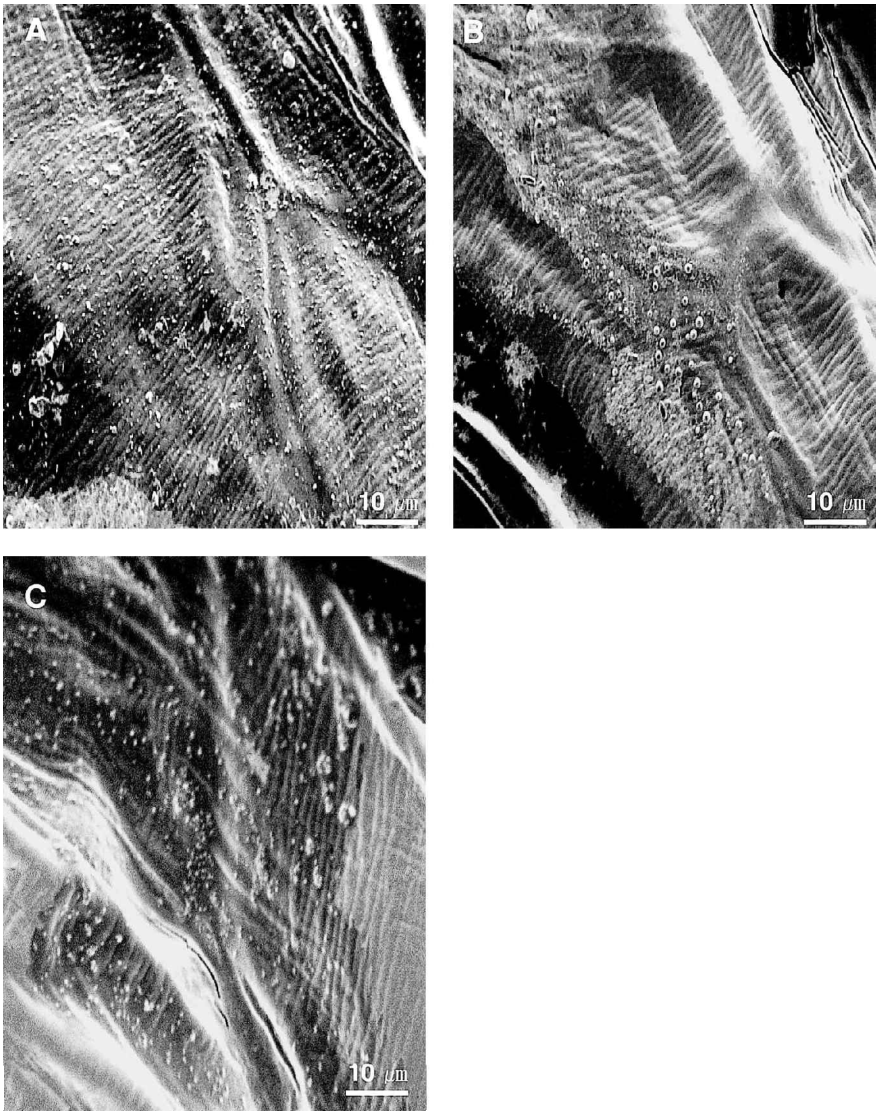

FIGURE 4. *Scanning electron micrographs of bacteria (A) and spores (B) on the surface of untreated lettuce, and the bacteria on the surface of the lettuce treated with tap water (C).*

< 0.05. Tap water did not decrease molds and yeasts in the lettuce.

The microorganisms on the surface of lettuce treated with various solutions were enumerated by swabbing the surface of lettuce (Fig. 3). On the surface of the untreated lettuce, the viable aerobic bacteria population was found to be about 10^3 CFU/cm^2. Coliform bacteria, molds, and yeasts on the surface of lettuce were about 10^1 CFU/cm^2. Viable aerobic bacteria populations were reduced to less than 10^1 CFU/cm^2 by all treatments in this experiment. No coliform bacteria populations were detectable after each treatment. Mold and yeast populations were not detectable after treatment with AcEW, NaOCl solution, and ozonated water. After 1 + 1 treatment, the populations of viable molds and yeasts were less than 10^1 CFU/cm^2. Tap water did not decrease the microorganisms on the surface of lettuce.

Visualization of microorganisms on lettuce. The surfaces of the lettuce were observed by SEM for microorganisms and surface structure. The surfaces of the untreated lettuce and the lettuce treated with tap water are shown in Figure 4. There were many bacteria widely distributed on the surface of lettuce. Most of the observed microorganisms were bacteria. No yeasts and molds were observed. However, what appeared to be bacterial spores are apparent in Figure 4B. Figure 4C shows that tap water could not remove the surface bacteria. Figure 5A through 5D show the surface of lettuce after treatment with AcEW, ozonated water, NaOCl solution, and 1 + 1 treatment, respectively. No bacteria are seen on the surface of lettuce in Figure 5A through 5D. No differences in disinfectant effect were seen among all treatments. Moreover, the surface structure was not damaged by all treatment. However, there were spores of bacteria on the surface of the lettuce treated with AcEW (Fig. 6A), ozonated water (Fig. 6B), and NaOCl (Fig. 6C). Figure 6D shows the biofilm on the surface of lettuce treated with AcEW.

DISCUSSION

AcEW is produced by the electrolysis of a dilute sodium chloride solution, and it contains (among other things) hypochlorous acid (HOCl), a disinfectant very effective at low pH *(12)*. At a low pH, HOCl is a very weak acid that undergoes virtually no hydrolysis compared with the much

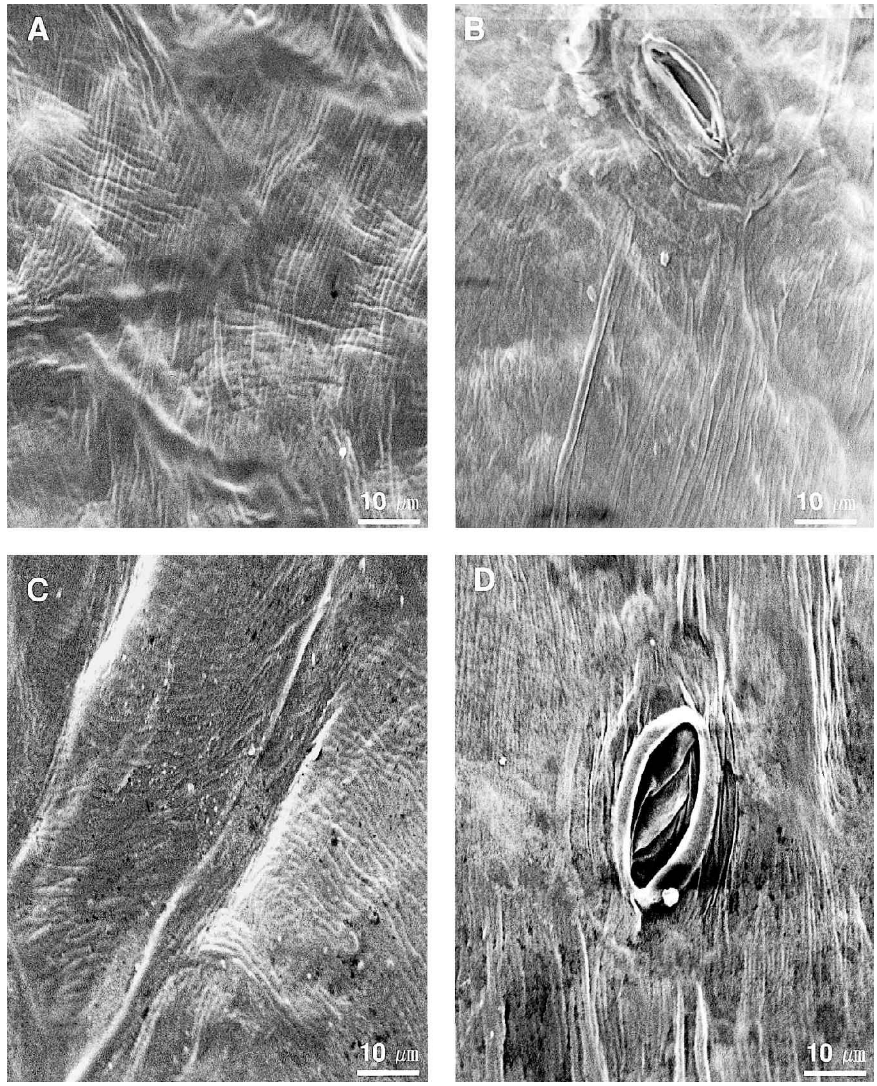

FIGURE 5. *Scanning electron micrographs of the surface on the lettuce treated with acidic electrolyzed water (AcEW) (A), ozonated water (B) and NaOCl (C) for 10 min, and washed with alkaline electrolyzed water (AlEW) for 1 min and then treated with AcEW for 1 min (D).*

less effective hypochlorite ion (OCl^-). Also, it was suggested that during the electrolysis of dilute sodium chloride solution a redox reaction occurs, producing many reactive and toxic compounds, such as ozone, and very highly reactive and short-lived radicals, such as ˙O, ˙Cl, and ˙OH, in the anode chamber *(4, 16).* These compounds and radicals contribute to the disinfectant effect of AcEW.

Ozone is a strong oxidant and disinfectant. Ozone can react with contaminants directly as molecular ozone (O_3) or indirectly as ozone-derived free radicals such as ˙OH and ˙H_2O. Ozone has been demonstrated to be useful for the inactivation of microorganisms *(11, 20)* and postharvest storage of fruits *(1, 18).*

In our study, the disinfectant effect of AcEW, ozonated water, and NaOCl solution on lettuce was first confirmed to establish the effect of treatment time. There has been no comparison of the disinfectant effect of AcEW and ozonated water on fresh produce. Both solutions greatly reduced the number of aerobic bacteria on the lettuce as soon as decontamination started. However, the prolongation of decontamination time did not indicate an increase in disinfectant effect. In addition, AcEW showed a stronger disinfectant effect than did ozonated water for all treatment times. These results can explained by the participation of the free radicals derived from HOCl or ozone. Although AcEW is considered to contain various chemical species, ozonated water contains only ozone. The greater action of AcEW against microorganisms, therefore, might be due to the multiple effects of various chemical species.

Kim et al. *(8)* noted that the ORP would be the primary factor affecting microbial inactivation. If the ORP is the main factor of antimicrobial activity, a higher ORP kills more microorganisms. However, the higher ORP of ozonated water did not show higher disinfectant effect than AcEW, which showed a lower ORP than ozonated water. Thus, the higher ORP did not indicate a higher disinfectant effect. Moreover, we had confirmed that the ORP of AcEW was not main factor of antimicrobial activity *(10).* We had concluded the main factor of antimicrobial activity of AcEW was available chlorine. Available chlorine, mainly HOCl, produces ˙OH, a radical that acts on microorganisms. Ozone produces ˙OH, too. Antimicrobial activity is considered the oxidation of ˙OH; therefore, the more ˙OH produced, the better the antimicrobial activity. A high concentration of HOCl or ozone is needed to produce more ˙OH. From this point, HOCl was found in higher concentrations

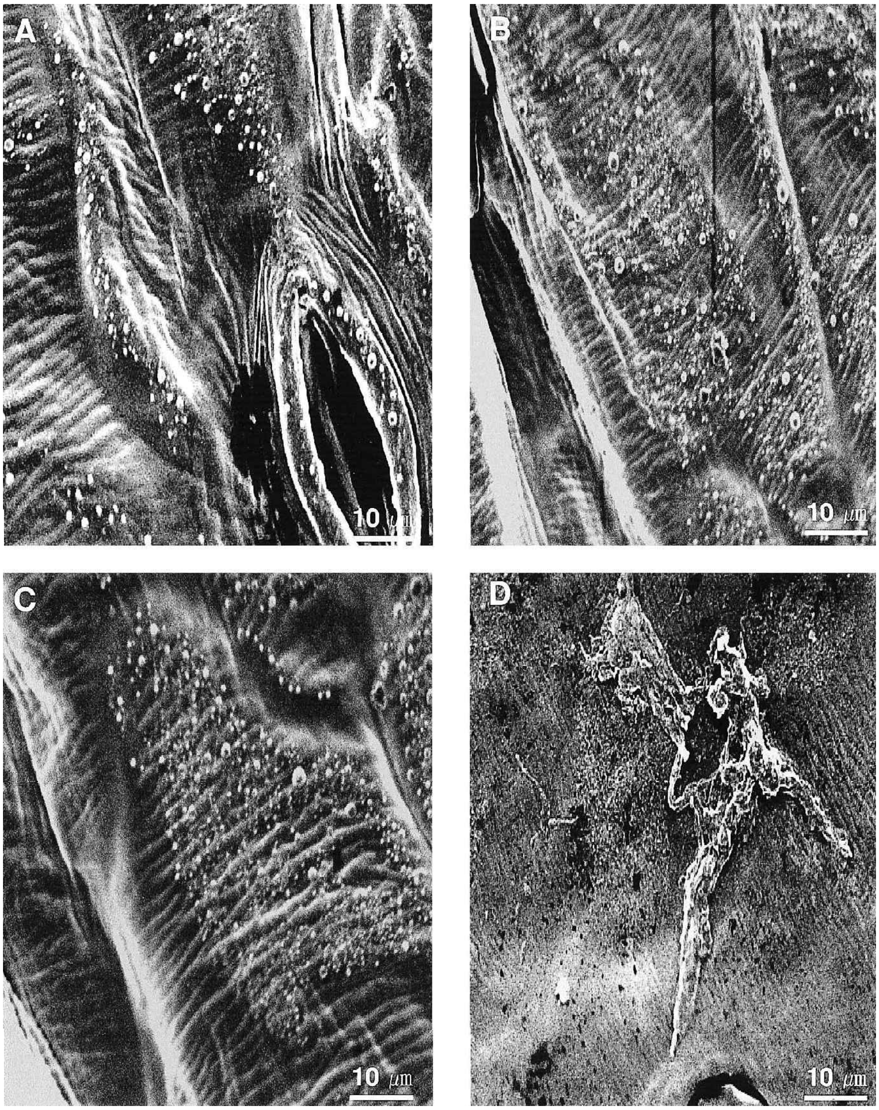

FIGURE 6. *Scanning electron micrographs of residual spores on the surface of lettuce treated with acidic electrolyzed water (AcEW) (A), ozonated water (B), and NaOCl solution (C). Biofilms occurred on the lettuce treated with AcEW (D).*

than ozone in this study, so HOCl could produce more ·OH. Therefore, it was considered that AcEW showed the better disinfectant effect than ozonated water.

AlEW has a high pH (more than 11) and a very low ORP (below −800 mV). When lettuce was washed in AlEW for 1 min and then disinfected with AcEW for 1 min (1 + 1 treatment), the disinfectant effect on aerobic bacteria, molds, and yeasts was larger than that for the treatment of lettuce by soaking in AcEW or ozonated water for 10 min. AlEW is considered to act like a dilute sodium hydroxide aqueous solution. Thus, it would act like a surface-active agent against the surface of lettuce, so the microorganisms on the surface would be disinfected easily with AcEW. If the initial populations of microorganisms were low (approximately 10^3 CFU/g), such as that of coliform bacteria, the effect of pretreatment with AlEW would be small. Because AcEW has a strong disinfectant effect, the small number of microorganisms would be disinfected even without pretreatment with AlEW.

A swabbing test has not previously been used to determine the microorganisms on the surface of lettuce. After decontamination, microorganism populations on the lettuce were less than 10^1 CFU/cm^2. This indicates that the microorganisms on the surface of lettuce were easily disinfected with AcEW, ozonated water, and NaOCl solution. The residual microorganisms embedded in the whole lettuce were either inside the cellular tissue, such as the stomata, or making the biofilm on the surface of lettuce *(15).*

According to the results of SEM observations, the surface structures of lettuce were not damaged by all treatments. This result suggests that the quality of the lettuce surface is not adversely affected by treatment with AcEW, ozonated water, and NaOCl. Active forms of chlorine, such as HOCl, and ozone rapidly diminish on contact with organic material. Thus, the efficacy of chlorine and ozone in killing microorganisms on the surface of raw produce such as lettuce is markedly less than in disinfectant systems free of organic matter, such as those used in the standard Association of Official Analytical Chemists procedure. However, in this study, microorganisms, except spores on the surface of lettuce, were removed. The spores of bacteria, such as *Bacillus subtilis,* are known to require 5 min to be sterilized in vitro *(19).* The spores of bacteria would adhere to the lettuce surface. Therefore, spores were not easily disinfected on the surface of lettuce. Moreover, biofilms were occurring on the surface of lettuce, which could not be disinfected with AcEW, ozonated water, or NaOCl. Killing the bacteria inside the biofilm is difficult. Thus, residual bacteria were detected even after treatment with AcEW and ozonated water.

The disinfectant effect of AcEW will be applicable for many fields, such as food factory hygiene, medicine, and agriculture. Therefore, results of this study could contribute to food sanitation on cut vegetable production. Despite cut or noncut lettuce, we confirmed that the disinfectant effect was not changed (data not shown). Thus, we considered these results adaptable for home use and vegetable packing. Moreover, using AcEW in the home or restaurant kitchen just before eating fresh vegetables could prevent food poisoning. In many cases, when pathogenic microorganisms increase at some level, food poisoning occurs. Therefore, decreasing the number of initial microorganisms on the fresh produce would prevent the outbreak of food poisoning. In this article, aerobic bacteria, coliform bacteria, molds, and yeasts were reduced by some disinfectant to the level of 10^3, 10^1, and 10^2 CFU/g, respectively. Thus, the possibility of food poisoning will decrease and food safety will be preserved.

AcEW is a new disinfectant technique for fresh produce. We are focusing on the prevention of food poisoning and the prolongation of fresh produce shelf life by using AcEW. In this article, we have confirmed the decontamination characteristics of AcEW. Since AcEW, like ozone, readily becomes inert, it is thought to leave little residue. Therefore, AcEW has a less adverse impact on the environment. From these features and the results of this study, it is suggested that AcEW has great potential as the disinfectant of choice for fresh produce.

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