

## The Efficiency of Disinfection of Acidic Electrolyzed Water in the Presence of Organic Materials

Toshihiro OOMORI, Takumi OKA, Tooru INUTA, and Yoji ARATA

Water Research Institute, Sengen 2-1-6, Tsukuba 305-0047, Japan

Acidic electrolyzed water (acidic EW), which is prepared by the electrolysis of an aqueous NaCl solution, has recently become of great importance for disinfection in a variety of fields, including medicine, the food industry and agriculture. In a previous paper we showed that: 1) acidic EW is a mixture of hypochlorite ion, hypochlorous acid and chlorine, depending upon the pH; 2) hypochlorous acid is primarily responsible for disinfection in the case of *Escherichia coli* K12 and *Bacillus subtilis* PCI219, both in clean culture media. In practice, however, the use of acidic EW is in many cases severely hampered due to the presence of a variety of non-selective reducing agents. In view of the salient nature of acidic EW, it is therefore strongly urged to establish an optimum way to use acidic EW in a variety of systems. The present paper is the first report on our attempt along this line in order to characterize the nature of the chemical changes that the bactericidal activity of the acidic EW deteriorates in the presence of organic materials, which include amino acids and proteins.

(Received August 16, 1999; Accepted January 6, 2000)

The use of chlorine for disinfection dates back to 1847, when Semmelweis ordered hand-washing with chlorinated water before deliveries.<sup>1</sup> It was later realized that aqueous chlorine solution is highly effective in preventing streptococcal infection. In recent years it has become a common practice to use acidic electrolyzed water (acidic EW) prepared by the electrolysis of an aqueous NaCl solution for disinfection. Acidic EW has strong bactericidal activity, and has been used in medical applications, such as in the prevention of methicillin resistant *Staphylococcus aureus* (MRSA) infections. It has also become useful in the food and agricultural industry. It has recently been established in our laboratory that in the case of *Escherichia coli* (*E. coli*) K12 and *Bacillus subtilis* PCI219, the bactericidal activity of acidic EW was virtually identical to that of a hypochlorous acid solution at the same pH value.<sup>2</sup> We thus concluded that the bactericidal activity of acidic EW is correlated with the concentration of hypochlorous acid.

Chlorine species that exist in aqueous medium and are available for disinfection are generally called *available chlorine* for disinfection. It is known that in aqueous media chlorine exists in three different chemical species, *i.e.*, Cl<sub>2</sub>, HClO, ClO<sup>-</sup>, depending upon the pH. The concentration of the unreacted mixture of Cl<sub>2</sub>, HClO and ClO<sup>-</sup> is called *free available chlorine*. It has, however, been known that the bactericidal activity of free available chlorine is strongly influenced by the concentration of non-specific reducing agents that coexist in the medium. This indicates that we must be careful in quantitating the effective concentration of free available chlorine in the medium in order to achieve the maximum effect of acidic EW for disinfection.

Free available chlorine, as determined around neutral pH, is known to be depleted through oxidation-reduction reactions with a variety of inorganic and organic materials.<sup>3</sup> Furthermore, it can react with ammonia and amino acids to form *N*-chloro compounds. The total amount of this form of reacted chlorine is generally termed *combined available chlorine*.<sup>3</sup> Combined

available chlorine is much lower in bactericidal activity than the free form.<sup>3-6</sup>

In the present report we show that the concentration of free available chlorine is influenced by the presence of reducing agents in order to characterize the optimal condition for disinfection by acidic EW. Three methods are commonly used for quantitating the available chlorine concentration in aqueous media: iodometric titration, the *o*-tolidine method and the *N,N*-diethyl-*p*-phenylenediamine (DPD) method. The iodometric titration method is unable to distinguish free available chlorine from combined available chlorine. The *o*-tolidine method can distinguish these two forms, but only with poor accuracy. The DPD method is the most selective method for assaying these two forms. This method is based on the oxidation of an indicator (DPD) to colored species. In the absence of iodide ion, free available chlorine reacts with DPD reagent to produce a red color. A subsequent addition of iodide ion acts catalytically to cause combined available chlorine to produce color.<sup>7</sup>

In practical usage, acidic EW generally has to be used in the presence of organic materials, which potentially react with free available chlorine and change it to the combined form. Therefore, in the presence of organic materials the expected bactericidal activity of acidic EW is not simply proportional to the *total available chlorine*, which is the sum of the free and combined forms, as obtained from iodometric titration. Clearly, for efficient disinfection, an efficient way must be established.

Although the effects of organic materials on acidic EW have been reported by Tanaka *et al.*,<sup>8</sup> there has been no discussion reported on how the organic materials mediate the conversion of the free to the combined form of chlorine in acidic EW.

In the present study we examined the effects of some organic materials on available chlorine in acidic EW in order to clarify the conversion process of the free available chlorine to the combined form. In this study a variety of organic materials were added to acidic EW, and their effects on the free and

combined available chlorine concentrations were measured. The bactericidal activity of the combined available chlorine was also compared with the free form of chlorine in acidic EW. On the basis of the obtained results, we discuss several points which are potentially important for the practical use of acidic EW.

## Experimental

### Reagents

All chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan), except for trans-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid monohydrate (Kanto Chemicals, Tokyo, Japan). All chemicals were of reagent grade with the exception of sodium hypochlorite solution (practical grade). Milli Q-grade water (Millipore, MA, USA) was used in all experiments.

### Determination of the available chlorine concentration

The concentration of the available chlorine was determined by iodometric titration<sup>9</sup> and/or the DPD method.<sup>10</sup> The concentrations of the individual free and combined available chlorine species were determined by the DPD method.

### Acidic EW

An aqueous NaCl solution (0.1%) was electrolyzed (6.0 V/10.2 A) at ambient temperature using an electrolysis instrument (Daikin, Osaka, Japan). Acidic EW was diluted with 0.1% NaCl to an available chlorine concentration of 50 ppm and adjusted to pH 5.0 with NaOH. Freshly prepared acidic EW was used without delay in all experiments.

### The effects of organic materials

A nutrient broth (Nutrient broth "Eiken", Eiken Chemical, Tokyo, Japan), proteose peptone (Proteose peptone NO. 3, Difco Laboratories, MI, USA), glycine, glucose, sucrose and corn oil (commercial product) were used. Each concentration of these materials was 100 mg/l. This value was set to approximate the residual levels of organic materials after imperfect washing before the practical use of acidic EW.

All of the sample solutions, except for corn oil, were adjusted to pH 5.0 using a NaOH solution, and diluted to a concentration of 10 g/l. These solutions were autoclaved at 121°C for 15 min. Then, 1 ml of each solution was added to 99 ml of acidic EW (pH 5.0, 50 ppm Cl<sub>2</sub>) and left for 0 to 180 min at ambient temperature. Then, an aliquot of each solution was quickly diluted 10–50 times, and the free and combined available chlorines were measured using the DPD method. In the case of corn oil, 10 mg of corn oil was dispersed in 100 ml of acidic EW (pH 5.0, 50 ppm Cl<sub>2</sub>) using a homogenizer for 1 min to form an emulsion. After this procedure, the available chlorine was measured as mentioned above. In order to maintain the emulsion state, the sample solution was dispersed every 1 h. To correct the background absorbance due to being in an emulsion state, the absorbance of corn oil dispersed in Milli Q water was also measured. The available chlorine was calculated after subtracting the background values from the measured absorbance observed using the acidic EW containing corn oil.

Cow's milk, minced meat and chopped cabbage were also used with the practical usage of acidic EW in mind. The milk and minced meat were used at concentrations of 5000 mg/l and 1000 mg/l, respectively, since 100 mg/l was too low concentration in this experiment. The available chlorine in these samples was measured as mentioned above. Cabbage was cut into fine strips after removing its core, and then washed for 2 min with tap

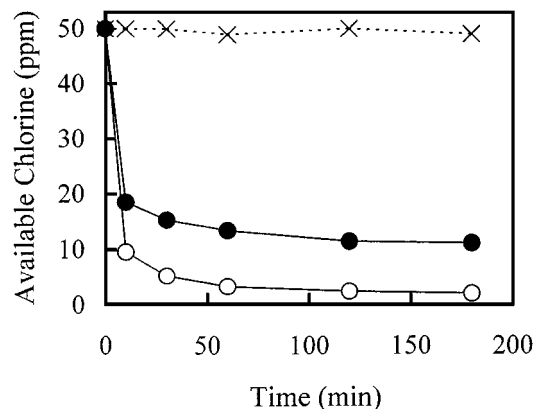


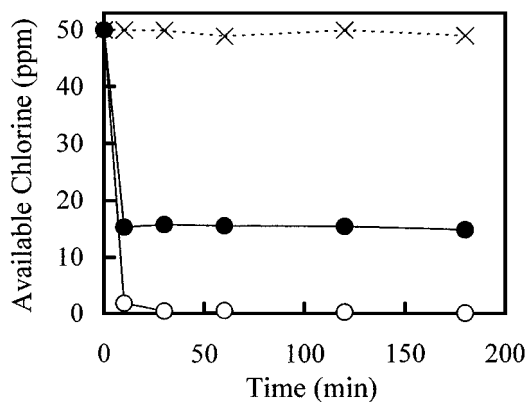
Fig. 1 Effect of nutrient broth on the available chlorine concentration in acidic EW. Nutrient broth was added to acidic EW (pH 5.0, 50 ppm Cl<sub>2</sub>) to give a concentration of 100 mg/l and left for 0 to 180 min at ambient temperature. The free and combined available chlorine concentrations were measured using the DPD method. The chlorine detection limit was 0.5 ppm. The values represent the means of duplicate experiments. Symbols: ○, free available chlorine; ●, total (free + combined) available chlorine; ×, control.

water and drained. Twenty grams of the cabbage strips were then soaked in 200 ml of acidic EW and stirred with an electric stirrer at ambient temperature. Although in practical usage the bactericidal treatment of chopped cabbage is usually about 5–10 min, in our experiments the available chlorine was measured over the range 0 to 180 min in the same manner as mentioned above. As a control, the available chlorine was also measured in acidic EW samples stirred in the same way as the cabbage and meat samples; the milk sample was not stirred.

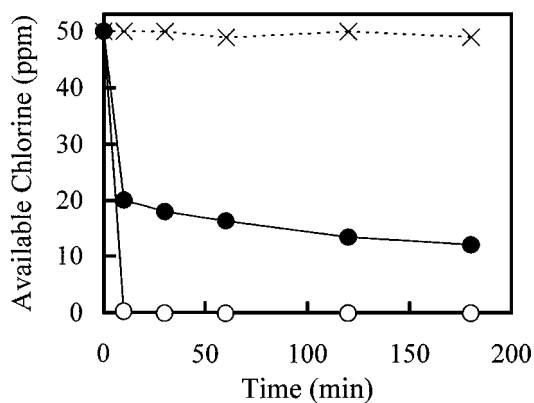
### Assay of bactericidal activity

*E. coli* K12 strain was obtained from the Institute for Fermentation Osaka (Osaka, Japan). *E. coli* cells were propagated in a nutrient broth "Nissui", Nissui Pharmaceutical, Tokyo, Japan) for 14–16 h at 37°C. The culture at the end of the exponential phase/beginning of the stationary phase was centrifuged at 2600 × *g* for 10 min at 4°C, and the resulting pellet was washed with a sterilized 0.85% NaCl solution 4 times. Finally, this pellet was re-suspended in a sterilized 0.85% NaCl solution. Prior to the bactericidal activity assay, the absorbance of the cell suspension was measured at 600 nm and diluted with a sterilized 0.85% NaCl solution so that the range of cell number was 3–6 × 10<sup>8</sup> cfu/ml in all of the bactericidal assays.

Before starting the bactericidal assays, the acidic EW was examined with the DPD method in order to confirm that all available chlorine was present in the free form. Each of the nutrient broth, proteose peptone and glycine was autoclaved at 121°C for 15 min and mixed with acidic EW (pH 5.0, 50 ppm Cl<sub>2</sub>) in order to achieve a concentration of 100 mg/l. These solutions were left for about 3 h at ambient temperature, during which time the concentration of free available chlorine was observed to become below the detection limit (<0.05 ppm) of the DPD method. These acidic EW/organic material samples were then diluted to appropriate concentrations with 0.1% NaCl and adjusted to pH 5.0 with NaOH. Then, 0.9 ml of acidic EW containing free available chlorine or combined available chlorine was vigorously mixed with 0.1 ml of an *E. coli* suspension (3–6 × 10<sup>8</sup> cfu/ml) in sterilized 1.5 ml centrifuge tubes. The range of available chlorine concentration was 0–8



A



B

Fig. 2 Effects of proteose peptone (A) and glycine (B) on the available chlorine in acidic EW. Proteose peptone or glycine were added to acidic EW to give a concentration of 100 mg/l. The free and combined available chlorine concentrations were measured. Symbols: ○, free available chlorine; ●, total (free + combined) available chlorine; ×, control.

ppm. This mixture was left at ambient temperature for 2 min. Then, 0.1 ml of a neutralizing agent, which consisted of 50 mM potassium dihydrogenphosphate and 50 mM sodium thiosulfate adjusted to pH 7.2 using NaOH solution, was added to this mixture with vigorous mixing in order to quench the acidic EW. Then, a small aliquot (0.1 ml) of this neutralized suspension was transferred to a nutrient broth agar (Nutrient agar "Nissui", Nissui Pharmaceutical, Tokyo, Japan) plate. The plates were incubated for 24–48 h at 37°C and the number of colonies counted.

## Results

### *The effects of organic materials on acidic EW*

The time course of the free and combined available chlorines of acidic EW in the presence of nutrient broth is shown in Fig. 1. The free available chlorine quickly disappeared upon the addition of the nutrient broth. It has been shown that the detectable available chlorine mostly exists as the combined form in this solution. The concentration of the total available chlorine decreased from the initial concentration. The results obtained using proteose peptone (A) and glycine (B) are shown in Fig. 2. Similarly, the free available chlorine in these solutions quickly disappeared upon the addition of these materials. Most

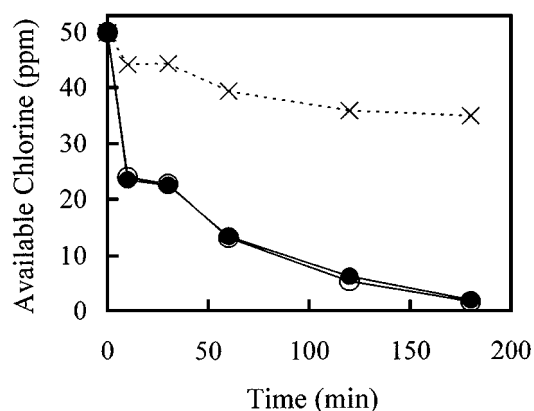


Fig. 3 Effect of corn oil on the available chlorine in acidic EW. Ten milligrams of corn oil was dispersed in 100 ml of acidic EW using an homogenizer to form an emulsion at ambient temperature. This sample was redispersed every hour. The free and combined available chlorine concentrations were calculated after subtracting the emulsion background values as described in the text. As a control, the available chlorine concentration was measured in an acidic EW sample homogenized in the same way. The chlorine detection limit was 1 ppm. Symbols: ○, free available chlorine; ●, total (free + combined) available chlorine; ×, control.

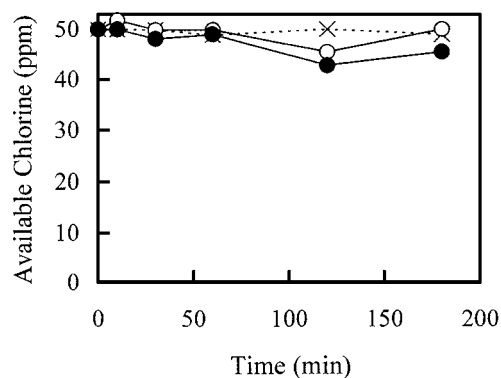


Fig. 4 Effect of glucose on the available chlorine in acidic EW. Glucose was added to acidic EW to give a concentration of 100 mg/l. The free and combined available chlorine concentrations were measured. Symbols: ○, free available chlorine; ●, total (free + combined) available chlorine; ×, control.

of the available chlorine exists in the combined form in these solutions, and the total available chlorine was also decreased.

The result of adding corn oil to acidic EW is shown in Fig. 3. This result was different from those shown in Figs. 1 and 2. In this sample, the available chlorine was decreased with time; chlorine in the combined form was not detected. The result obtained for glucose is shown in Fig. 4, which clearly shows that glucose did not react with available chlorine under these conditions. Even if the glucose concentration was increased to 1000 mg/l, it still did not react. Sucrose gave the same result as in the case of glucose (data not shown).

The results obtained for milk and minced meat are shown in Fig. 5. These results were similar to those given in Figs. 1 and 2. The result obtained using fine strips of chopped cabbage is shown in Fig. 6. The free available chlorine quickly disappeared within 10 min, and no significant production of the combined form was observed under these conditions. However, the level of available chlorine was maintained above 40 ppm

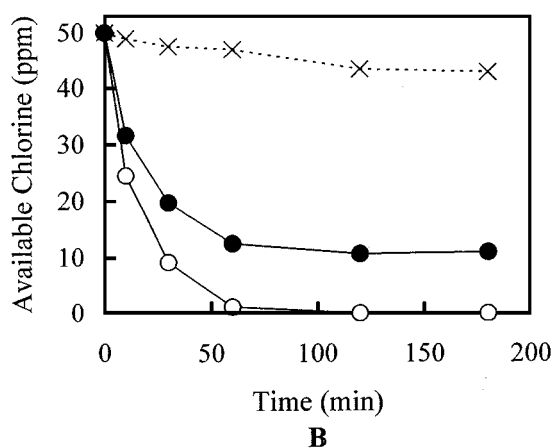
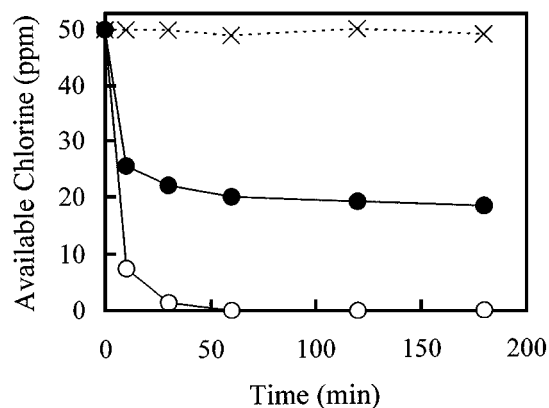


Fig. 5 Effects of milk (A) and minced meat (B) on the available chlorine in acidic EW. Milk was added to acidic EW to give a concentration of 5000 mg/l at ambient temperature. The free and combined available chlorine concentrations were also calculated after subtracting the background values as described in the text. Five hundred milligrams of minced meat was mixed with 500 ml of acidic EW to give a concentration of 1000 mg/l. Then it was stirred with an electric stirrer at ambient temperature. The free and combined available chlorine concentrations were also measured. As a control, the available chlorine concentration was measured in an acidic EW samples stirred in the same way. The chlorine detection limit was 1 ppm. Symbols: ○, free available chlorine; ●, total (free + combined) available chlorine; ×, control.

when the same experiment was carried out using larger strips of chopped cabbage (data not shown).

#### Bactericidal activities of free and combined available chlorines in acidic EW

As shown in Figs. 1 and 2, the free available chlorine in acidic EW was quickly transformed into the combined form when nutrient broth, proteose peptone or glycine was added. Milk and minced meat also gave similar results as shown in Fig. 5. To determine the bactericidal activity of these combined available chlorines in acidic EW, three typical organic materials (*i.e.*, nutrient broth, proteose peptone or glycine) were chosen and added. This experiment was repeated 5 times; a typical result is shown in Fig. 7. The free available chlorine was found to have stronger bactericidal activity than the combined forms. Free available chlorine at a concentration of 2 ppm was sufficient to disinfect (*i.e.*, below the detection limit; <10 cfu/ml) an *E. coli* population of the order of  $10^7$ . However,

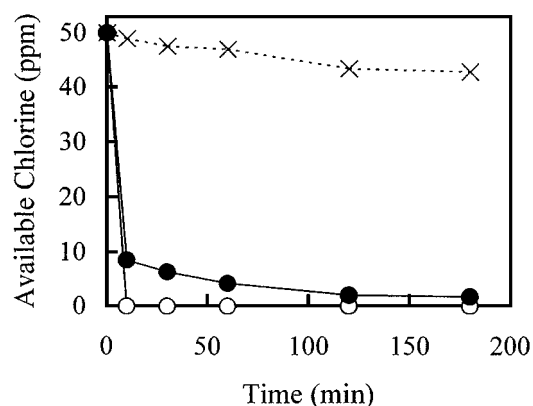


Fig. 6 Effect of chopped cabbage on the available chlorine concentration in acidic EW. The cabbage was chopped into fine strips and washed with tap water. Twenty grams of the cabbage was soaked in 200 ml of acidic EW and stirred at ambient temperature. The free and combined available chlorine concentrations were measured. As a control, the available chlorine concentration was measured in an acidic EW sample stirred in the same way. The chlorine detection limit was 0.5 ppm. Symbols: ○, free available chlorine; ●, total (free + combined) available chlorine; ×, control.

even at 8 ppm, combined available chlorine, formed by the addition of glycine, was unable to disinfect, bringing it below the detection limit. In the case of the nutrient broth or proteose peptone,  $10^3 - 10^4$  *E. coli* remained in the presence of 8 ppm combined available chlorine. In addition, the effect of sucrose on the bactericidal activity of acidic EW was examined under the same condition. It did not have any effect on the bactericidal activity of acidic EW at concentrations up to 100 mg/l (data not shown).

## Discussion

In the present study we examined the effects of organic materials on acidic EW. It has been demonstrated that in the presence of amino acids or proteins containing materials the free available chlorine in acidic EW was quickly transformed into *N*-chloro compounds (*i.e.*, combined available chlorine). This result is quite consistent with a previous study concerning an aqueous chlorine solution.<sup>3</sup> It was also observed that some of the available chlorine was removed through oxidation-reduction reactions with a variety of materials (*e.g.*, proteins, vitamins, lipids and minerals *etc.*). In the case of amino acids, some of the available chlorine was consumed by reactions which generated carbon dioxide, ammonia, aldehydes and nitriles.<sup>11</sup> Milk and minced meat were examined with the practical use of acidic EW in mind. They were also found to quickly generate the combined available chlorine. In the case of corn oil, although the available chlorine was decreased, the combined available chlorine was not detected.

It has been reported that available chlorine reacts with the carbon-carbon double bonds in lipids.<sup>12</sup> It is likely that a similar reaction proceeds in the presence of corn oil that consists of a number of unsaturated fatty acid chains. It has also been reported that available chlorine reacts with sugar under drastic conditions at a high concentration of chlorine for a long reaction time.<sup>12</sup> However under the present conditions, glucose and sucrose did not react at all with available chlorine in acidic EW. In the case of chopped cabbage, the amount of remaining available chlorine depended on the surface area of the cabbage.

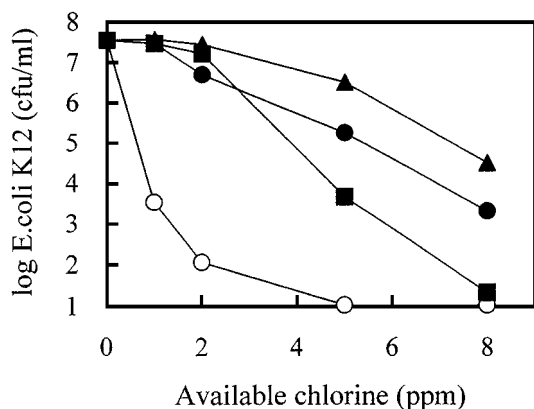


Fig. 7 Bactericidal activities of free and combined available chlorines in acidic EW in samples containing different organic materials. Combined available chlorine in acidic EW was prepared by mixing each organic material (*i.e.*, nutrient broth, proteose peptone and glycine) with acidic EW. We confirmed that in each sample the free available chlorine was below the detection limit ( $<0.05$  ppm). The acidic EW containing the free or combined available chlorine was vigorously mixed with *E. coli* suspension ( $3.6 \times 10^8$  cfu/ml) for 2 min. Then 0.1 ml of neutralizing agent was added to quench the acidic EW. The range of available chlorine concentration was 0–8 ppm. This suspension was transferred to nutrient broth agar plates. The plates were incubated for 24–48 h at  $37^\circ\text{C}$  and the number of colonies counted. The values represent typical result from five replicate experiments. Symbols:  $\circ$ , free available chlorine;  $\bullet$ , nutrient broth combined available chlorine;  $\blacktriangle$ , proteose peptone combined available chlorine;  $\blacksquare$ , glycine combined available chlorine.

This suggests that the quick disappearance of the available chlorine is related to oxidation-reduction reactions between the available chlorine and organic substances like vitamin C that possibly originates from the cross-section of the chopped cabbage.

We have also shown using *E. coli* that there is a significant difference in bactericidal activity between the free and combined available chlorines in acidic EW. Combined available chlorines had much lower bactericidal activity than the free form at an identical available chlorine concentration. In the case of glycine, the combined available chlorine had slightly stronger bactericidal activity than in the cases of nutrient broth and proteose peptone. Although the properties of free and combined available chlorines have been examined using aqueous chlorine solution,<sup>3-6</sup> nothing has been reported on the results obtained examined using acidic EW.

Acidic EW is a convenient and economical material for bacterial disinfection. In practical use for food industry, agriculture and medicine, the presence of organic materials potentially results in the formation of chlorine primarily present in combined forms, which have lower bactericidal activity. In such cases, the traditional iodometric titration for the

quantitation of active chlorine would certainly overestimate the bactericidal activity, since it measures the total available chlorine irrespective of the free and combined available chlorines. Thus, in practical usage, it is crucial to always take the reactions between the available chlorine in acidic EW and the organic species present into serious consideration. In the presence of larger amounts of organic materials it should be necessary to increase the total available chlorine by increasing the concentration of active free chlorine in acidic EW. For practical purposes, washing of materials prior to disinfection would help the efficiency of disinfection by removing soluble organic matter that would otherwise reduce the amount of free available chlorine and thus lowers the bactericidal activity of acidic EW.

### Acknowledgement

We thank Dr. W. S. Price for his help in editing the manuscript.

### References

1. R. Porter, "The Greatest Benefit to Mankind. A Medical History of Humanity", 1997, W. W. Norton & Company, 369-370.
2. S. Nakagawara, T. Goto, M. Nara, Y. Ozawa, K. Hotta, and Y. Arata, *Anal. Sci.*, **1998**, *14*, 691.
3. G. C. White, "Handbook of Chlorination and Alternative Disinfectants", 3rd ed., 1992, Van Nostrand Reinhold, New York, 184-240.
4. C. T. Butterfield, *J. Amer. Water Works Assoc.*, **1948**, *40*, 1305.
5. T. H. Feng, *J. Water Pollut. Control Fed.*, **1966**, *38*, 614.
6. R. D. Sung, "Effects of Organic Constituents in Wastewater on the Chlorination Process", Ph. D. Thesis, Univ. of Calif., Davis, CA, 1974.
7. A. T. Palin, *J. Amer. Water Works Assoc.*, **1957**, *49*, 873.
8. H. Tanaka, Y. Hirakata, M. Kaku, R. Yoshida, H. Takemura, R. Mizukane, K. Ishida, K. Tomono, H. Koga, S. Kohno, and S. Kamihara, *J. Hosp. Infect.*, **1996**, *34*, 43.
9. American Public Health Association, American Water Works Association, and Water Environment Federation, "Standard Methods for the Examination of Water and Wastewater", 18th ed., 1992, American Public Health Association.
10. Pharmaceutical Society of Japan, "Standard Methods of Analysis for Hygienic Chemists" (in Japanese), 1990, Kanehara, Tokyo.
11. H. Tan, A. C. Sen, W. B. Wheeler, J. A. Cornell, and C. I. Wei, *J. Food Sci.*, **1987**, *52*, 1706.
12. M. Y. Fukayama, H. Tan, W. B. Wheeler, and C. I. Wei, *Environ. Health Perspectives*, **1986**, *69*, 267.