Why Food-Poisoning Bacteria Attached to Shredded Cabbage Are Not Efficiently Disinfected by Sodium Hypochlorite (NaClO)

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Received February 8, 2012; Accepted February 26, 2013; Online Publication, June 7, 2013

The aim of this study was to determine why food poisoning bacteria attached to cut cabbage are not efficiently disinfected by sodium hypochlorite (NaClO). Pretreatment of shredded cabbage with diethyl ether definitely decreased the survival numbers of Escherichia coli O157:H7 and Salmonella spp. after disinfection with 100 ppm of NaClO. The density of E. coli O157:H7 at the cut edge of a cabbage section was larger than that on the surface. The residual ratio of attached bacteria at the cut edge after NaClO disinfection was significantly higher than that on the surface. Microscopical observation indicated that the cut edge of shredded cabbage pretreated with diethyl ether was almost closed, resulting in a decrease in bacterial infiltration. Pretreatment of shredded cabbage with a higher concentration of NaClO to penetrate it more deeply significantly decreased the numbers of surviving bacteria after NaClO disinfection. Based on these results, we concluded that the bacteria attached to cut cabbage were not efficiently disinfected by NaClO, because not enough NaClO deeply infiltrated into the cut edges, and hence not enough came in contact with the bacteria.

Key words: cabbage; sodium hypochlorite; disinfection; Escherichia coli O157; Salmonella spp.

Vegetables are important foods supplying vitamins, minerals, and dietary fibers. Consumer desire for minimally processed vegetables has increased recently, and people have many chances to eat fresh vegetables. As consumption of fresh produce increases, concerns about microbial contamination rise. Many studies have been undertaken to estimate the contamination of vegetables and fruits by microorganisms.1–3 Vegetables are regarded as microbiologically safer than other unprocessed foods, such as meat, poultry, milk, eggs, and seafood. Although improvements in agronomic practices, processing, preservation, and distribution have made it possible to supply fresh produce of high quality to consumers all year round, outbreaks of food-borne disease due to consumption of fresh vegetables have recently increased.4–6 In 2006, an outbreak of Escherichia coli O157:H7 occurred due to fresh spinach and lettuce in United States,7,8 and in 2011 an outbreak of E. coli O104:H4 occurred due to fresh sprouts in the European Union.6 Prevention and decontamination methods to minimize the risk of microbial infection are therefore greatly to be desired.

The application of sodium hypochlorite (NaClO) in water is widely used in the disinfection of vegetables,2–10 but the antimicrobial effect of NaClO on microbes attached to vegetables is limited. For example, 100–200 ppm of NaClO decreased the numbers of E. coli O-157:H7, Salmonella spp., and Staphylococcus aureus attached to cut lettuce or shredded cabbage only by log 2 or log 3, although the minimal inhibitory concentrations of NaClO against these microbes were 0.1–0.2 ppm in vitro.11,12 Similar results showing that NaClO diminished the number of bacteria attached to vegetables only by log 2 or 3 have been reported.13–15 Several reasons are proposed for the inefficiency of NaClO against bacteria attached to vegetables.16 It is well-known that the bactericidal activity of NaClO is lost when it is reacted with organic substances.17 Nevertheless, less than 100 ppm of NaClO dramatically decreased bacterial numbers even in cabbage juice.15 The wax layer of vegetable surfaces inhibits the penetration of NaClO into plant tissues and affects the numbers of adsorbed bacteria.18 When bacteria form biofilms, they are not easily killed by disinfectants such as NaClO,19,20 but it takes several days to produce biofilms. The physical structure of vegetables is pointed out as another important factor in disinfection of bacteria. Bacteria located deep in stomata and cut edges are not considered to be easily killed.21–24 In those studies, however, quantitative analysis was not done sufficiently. In the present study, we examined the major reason why bacteria attached to shredded cabbage are not efficiently sterilized by NaClO. We used cabbage as the case in point, because cabbage is one of the popular vegetables for eating without heating or cooking in Japan. Shredded cabbage is often eaten with breaded pork cutlets or fried meat. We used E. coli O157:H7, Salmonella spp., and Staphylococcus aureus as pathogens. E. coli O157:H7 and Salmonella spp. are Gram-negative motile rods with flagella. E. coli O157:H7 and Salmonella spp. are carried by ruminants in the gastrointestinal tract and are shed in the feces. Non-composted
Bacterial cells were collected from 1 mL of fermented broth by centrifugation at 10,000 × g for 5 min. They were washed twice with a sterile phosphate buffered saline (PBS) and then resuspended in sterile PBS. The final concentration of bacteria was adjusted to about 10^5–10^6 colony-forming units (CFUs)/mL with PBS.

**Materials and Methods**

**Cabbage samples.** Cabbage (*Brassica oleracea* L. var. *capitata* L.) was purchased from a retail shop in Tokyo and used in experiments within 24 h. Two or three outer leaves and the core were removed, and the remaining leaves were sliced with a hand slicer (Aikogyo, Shizuoka, Japan) at a width of about 1.5 mm. The shredded cabbage was washed twice with tap water and drained with a drainer for home use.

**Pretreatment of shredded cabbage.** Pretreatment with solvents was done as follows: shredded cabbage (about 10 g) was immersed in 100 mL of acetone, ethanol, hexane, or diethyl ether, and left for 30 min. The solvent was drained off, and then each sample was dried for 1 h under vacuum. As a control, shredded cabbage immersed in water for 30 min was dried similarly. Pretreatment with boiling or freeze-drying was done as follows: shredded cabbage was left in boiling water for 10 min, or was frozen at −20 °C and freeze-dried with Freezvac-1C (Tozai Tsusho, Tokyo).

**Bacterial cultures and preparation of inocula.** *E. coli* O157:H7 C-13 which is resistant to ampicillin, *Salmonella Enteritidis* NBRC3313, *Salmonella Typhimurium* DT104 C-13, *Staphylococcus aureus* C-29 (SEA^+^), and *S. aureus* C-1 (SEB^+^), kept in our laboratory were used. These strains were subcultured on heart infusion (HI) agar. Each strain was incubated at 37 °C for 16–18 h in 2 mL of HI broth. After incubation, 20 μL of bacterial suspensions of *E. coli* O157:H7 C-13 and *Salmonella Enteritidis* NBRC3313, and of *S. aureus* FDA209P, *S. aureus* C-29, and *S. aureus* C-1 were mixed and used. The shredded cabbage (about 10 g) was immersed in 100 mL of each bacterial inoculum at 4 °C for 1 h without shaking, and then the inoculum was fully drained off.

**Preparation of disinfectants.** A NaClO solution containing 10, 20, and 50 μg of available chlorine per mL (100, 200, and 500 ppm) was prepared by mixing sodium hypochlorite (Kanto Chemical, Tokyo) with reverse osmosis (RO) water. The concentration of available chlorine was determined by the iodine titration method. The pH was adjusted to 6.0 with 1 M HCl.

**Procedures for disinfection.** An inoculated sample (10 g) was immersed in 100 mL of NaClO solution (100 ppm, pH 6.0) at room temperature for 10 min, and then was fully drained off. After the samples were rinsed 3 times with 100 mL of sterile RO water, the bacterial population of the cabbage was enumerated. Decreased rates of bacteria were counted as −log[(CFU after treatment)/(CFU before treatment)]. For pretreatment with a high concentration of NaClO, the shredded cabbage (10 g) was immersed twice in a 200 or 500 ppm NaClO (pH 6.0) solution for 10 min and rinsed twice with water. The bacteria were inoculated, and then the inoculated samples were disinfected as described above.

**Microbiological analysis.** The samples (about 10 g of shredded cabbage each) were homogenized in 100 mL of sterile PBS with Masticator PS (GSI Creos, Tokyo). Appropriate dilutions were made with PBS, and then 1.0 mL of a diluted sample was mixed with 10 mL of a selective medium. HI agar supplemented with 100 μg/mL of ampicillin, Xylose Lysine Desoxycholate (Merck, Darmstadt, Germany), and Mannitol Salt Agar (Eiken Chemical, Tokyo) were used as selective media for *E. coli* O157:H7, *Salmonella* spp., and *S. aureus*, respectively. Inoculates agar plates were incubated at 37 °C for 1–2 d, and then CFUs were enumerated.

**Estimation of the bacterial density of a cabbage section.** Two methods (Fig. 1) were used for the estimation of bacterial densities or bacterial numbers per cm² on the surface and the cut edge of a cabbage. In method I, bacteria were inoculated onto a 7 × 7 cm cabbage section. After disinfecting it with NaClO (100 ppm, pH 6.0), a 5 × 5 cm section was cut out from the disinfected 7 × 7 cm section.
The bacterial numbers of the cut-out 5 × 5 cm section and the remaining section were counted. From the data, the bacterial densities of the surface and the cut edge were estimated. The thickness of a cabbage section was assumed to be 0.035 cm (five samples on average). In method II, bacteria were inoculated onto it. The inoculated bacteria were killed with NaClO disinfection. The ratio of decrease of attached bacteria was examined. First the effect of pretreatment of cabbage with solvents on disinfection with NaClO was examined. In general, the surface of a plant is covered with a hydrophobic layer. We assumed that solvent treatment changed the hydrophobicity and the structure of plant surface and had an influence on NaClO disinfection. The ratio of decrease of E. coli O157:H7 due to NaClO treatment increased significantly when cabbage was pretreated with hexane or diethyl ether (Table 2), while pretreatment with ethanol and with acetone had no influence on NaClO treatment. The ratio of decrease of Salmonella spp. similarly increased when cabbage was pretreated with diethyl ether. In the case of S. aureus, the ratio of decrease was not changed by the ether pretreatment. As it was supposed that cabbage cells died under pretreatment with diethyl ether, we examined the effect of being alive or dead on disinfection with NaClO. Shredded cabbage was boiled or freeze-dried, which was assumed to result in the death of cabbage cells. Boiling and freeze-drying of cabbage did not raise the ratio of decrease of the inoculated bacteria due to NaClO treatment (Table 3). Although we did not know why pretreatment with ether did not change the ratio of decrease of S. aureus due to NaClO treatment or why pretreatment by freeze-drying tended to reduce its ratio of decrease, cabbage cells being alive

### Results and Discussion

**Effect of pretreatment of cabbage on the disinfection of bacteria by NaClO treatment**

As shown in Table 1, bacterial numbers were significantly more decreased by NaClO treatment than by rinsing with water, but the rate of decrease was only log 2–3 levels, and a fair number of bacteria survived. To examine the reason many bacteria survived after treatment with NaClO, cabbage was chemically and physically treated before being inoculated with bacteria, and then the disinfection efficiency of NaClO against the attached bacteria was examined. First the effect of pretreatment of cabbage with solvents on disinfection with NaClO was examined. In general, the surface of a plant is covered with a hydrophobic layer. We assumed that solvent treatment changed the hydrophobicity and the structure of plant surface and had an influence on NaClO disinfection. The ratio of decrease of E. coli O157:H7 due to NaClO treatment increased significantly when cabbage was pretreated with hexane or diethyl ether (Table 2), while pretreatment with ethanol and with acetone had no influence on NaClO treatment. The ratio of decrease of Salmonella spp. similarly increased when cabbage was pretreated with diethyl ether. In the case of S. aureus, the ratio of decrease was not changed by the ether pretreatment. As it was supposed that cabbage cells died under pretreatment with diethyl ether, we examined the effect of being alive or dead on disinfection with NaClO. Shredded cabbage was boiled or freeze-dried, which was assumed to result in the death of cabbage cells. Boiling and freeze-drying of cabbage did not raise the ratio of decrease of the inoculated bacteria due to NaClO treatment (Table 3). Although we did not know why pretreatment with ether did not change the ratio of decrease of S. aureus due to NaClO treatment or why pretreatment by freeze-drying tended to reduce its ratio of decrease, cabbage cells being alive

### Table 1. Disinfection Efficiency of NaClO against Escherichia coli O157:H7, Salmonella spp., and Staphylococcus aureus Attached to Shredded Cabbage

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Treatment</th>
<th>Bacterial numbers before treatment (log CFU/g)</th>
<th>Bacterial numbers after treatment (log CFU/g)</th>
<th>Ratio of decrease (−log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O157:H7</td>
<td>Water</td>
<td>5.46</td>
<td>4.27</td>
<td>1.19 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>NaClO</td>
<td>5.46</td>
<td>3.02</td>
<td>2.45 ± 0.17*</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>Water</td>
<td>5.29</td>
<td>4.54</td>
<td>0.74 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>NaClO</td>
<td>5.29</td>
<td>2.63</td>
<td>2.66 ± 002*</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Water</td>
<td>5.63</td>
<td>4.35</td>
<td>1.28 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>NaClO</td>
<td>5.63</td>
<td>3.22</td>
<td>2.31 ± 0.06*</td>
</tr>
</tbody>
</table>

Inoculated samples were immersed in treatment solution, NaClO (100 ppm, pH 6.0) or RO water for 10 min at room temperature. Samples were then rinsed with RO water 3 times. *Significant difference against the same bacteria treated with RO water (p < 0.05, n = 3).

### Table 2. Effects of Pretreatment of Shredded Cabbage with Solvents on NaClO Disinfection against Escherichia coli O157:H7, Salmonella spp., and Staphylococcus aureus

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Pretreatment</th>
<th>Bacterial number before treatment (log CFU/g)</th>
<th>Bacterial number after treatment (log CFU/g)</th>
<th>Decrease ratio (−log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O157:H7</td>
<td>Water (control)</td>
<td>5.47</td>
<td>3.24</td>
<td>2.23 ± 0.19*</td>
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<tr>
<td></td>
<td>Ethanol</td>
<td>5.16</td>
<td>2.93</td>
<td>2.24 ± 0.15*</td>
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<tr>
<td></td>
<td>Acetone</td>
<td>5.32</td>
<td>2.87</td>
<td>2.45 ± 0.15*</td>
</tr>
<tr>
<td></td>
<td>Diethyl ether</td>
<td>5.32</td>
<td>0.49</td>
<td>4.83 ± 0.83*</td>
</tr>
<tr>
<td></td>
<td>Hexane</td>
<td>5.19</td>
<td>0.43</td>
<td>4.75 ± 0.75*</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>Water</td>
<td>5.41</td>
<td>3.21</td>
<td>2.20 ± 0.10*</td>
</tr>
<tr>
<td></td>
<td>Diethyl ether</td>
<td>5.41</td>
<td>1.09</td>
<td>3.22 ± 0.50*</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Water</td>
<td>5.37</td>
<td>3.07</td>
<td>2.35 ± 0.41*</td>
</tr>
<tr>
<td></td>
<td>Diethyl ether</td>
<td>5.37</td>
<td>3.04</td>
<td>2.33 ± 0.15*</td>
</tr>
</tbody>
</table>

Shredded cabbage was pretreated with a solvent, and then bacteria were inoculated onto it. The inoculated bacteria were killed with NaClO (100 ppm, pH 6.0, 10 min). Values not labeled with the same letter are significantly different for a given microorganism (p < 0.05, n = 3).
or not did not appear to be critical for the inefficiency of NaClO, at least against *E. coli* O157:H7 and *Salmonella* spp. These results suggest that the hydrophobicity and the structure of cabbage surfaces and cut edges as changed by solvent treatment had a crucial role in the survival of bacteria.

**Comparison of bacteria attached to the surface and to the cut edge of cabbage samples**

We compared the bacterial numbers attached to the surface and to the cut edge, and then examined whether bacteria attached to the cut edge were more difficult to be killed by NaClO treatment than bacteria attached to the surface. Two methods (method I and method II, Fig. 1) were used for estimation of bacterial densities or bacterial numbers per cm² on the surface and at the cut edge of a cabbage section. As shown in Table 4, the density of *E. coli* O157:H7 at the cut edge (10⁷–10⁸ CFU/cm²) was definitely greater than that on the surface (10³–10⁴ CFU/cm²), although the values obtained by two methods were different. Takeuchi and Frank found that *E. coli* O157:H7 preferentially attached to the cut edge of lettuce as compared to the surface. Our data support their results. The disinfection efficiencies of NaClO against the bacteria attached to the surface and to the cut edge were then compared. The bacterial density to the surface decreased by less than 1% by NaClO treatment (average of method I and method II), while that at the cut edge decreased only by about 6% (average of method I and method II). This result clearly indicates that it was more difficult to kill the bacteria attached to the cut edge than those attached to the surface.

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**Observation of cabbage by electron microscope**

We found that pretreatment of cabbage with diethyl ether significantly increased the efficiency of NaClO against *E. coli* O157:H7 and *Salmonella* spp. This suggests that changes in the hydrophobicity and the structure of the surface and the cut edge of cabbage are a principal factor in the survival of bacteria. We found that more bacteria were attached to or in the cut edge than to the surface, and that the bacteria attached to or in the cut edge were more difficult to kill with NaClO. To explain these findings, we observed cabbage samples. Shredded cabbage samples on which *E. coli* O157:H7 cells were inoculated and which were subsequently rinsed with water were observed (Fig. 2). Most bacteria on the surface of cabbage were washed out by the water (Fig. 2A1 and A2), although small numbers of bacteria remained (Fig. 2A3). Some clumps of bacteria in veins and at cut edges remained after rinsing with water (Fig. 2B2, B3, C2, and C3). Some of these bacteria appeared to survive after treatment with NaClO. Micrographs of *Salmonella* spp. and *S. aureus* attached to cabbage sections are shown in Fig. 3 and Fig. 4, respectively. Clumps of *Salmonella* spp. cells were observed at or in stomata and cut edges (Fig. 3A1 and B1), and some of them remained after rinsing with water (Fig. 3A2 and B2), and appeared to survive after treatment with NaClO. Compared to *E. coli* O-157:H7 and *Salmonella* spp., more cells of *S. aureus* appeared to remain on the surface after rinsing with water (Fig. 4A2). Although we do not know the reason, this observation might have some relation to the fact that pretreatment with diethyl ether was not effective for
S. aureus as compared to E. coli O157:H7 and Salmonella spp. (Table 2). Takeuchi and Frank found that E. coli O157:H7 inoculated onto cut lettuce were located mainly 73.5 ± 16.0 µm below the surface of the cut tissue using a confocal scanning laser microscope, and that most E. coli O157:H7 cells (68.3%) that had penetrated 30–40 µm from the damaged tissue surface remained viable after chlorine treatment, although the cells on the surface survived least (25.2%) and the cells entering the stomata showed an intermediate survival rate (45.6%).

Burnett and Beuchat also found that E. coli O157:H7 attached to the subsurface structure of apples were better protected against inactivation by chlorine than cells located on exposed surfaces. Our observation of cabbage sections treated with diethyl ether indicated that the cut edge was almost closed (Fig. 5B). This indicates that pretreatment of shredded cabbage with diethyl ether prevented the infiltration of bacteria into a deep area of the cut edge. This explains why pretreatment with diethyl ether increased the efficiency of NaClO against E. coli O157:H7 and Salmonella spp. No significant change in the surfaces treated with diethyl ether was observed (Fig. 5D). The change in the hydrophobicity of the surface by ether treatment did not appear to be important for disinfection with NaClO. The section of dried cut cabbage was partly closed (Fig. 5C), which suggests that a drying process to remove ether as well as direct action of ether is necessary to close the section.

Effect of pretreatment of shredded cabbage with a high concentration of NaClO on the disinfection efficiency of NaClO against inoculated bacteria

Combining our results, we concluded that bacteria attached to shredded or cut cabbage were not completely killed because a sufficient amount of NaClO did not deeply infiltrate into cut edges and did not come into contact with the bacteria. To confirm this, we examined the effect of pretreatment of shredded cabbage with a higher concentration of NaClO on the disinfection efficiency of NaClO against subsequently inoculated bacteria. Shredded cabbage was pretreated with 200 ppm
or 500 ppm of NaClO and rinsed with water to remove the NaClO. In this pretreatment, some NaClO appeared to remain in a deeper part of the cut cabbage even after rinsing with water. After pretreatment, E. coli O157:H7 was inoculated into the cabbage sample, which was then sterilized with 100 ppm of NaClO. Pretreatment with NaClO improved the disinfection efficiency of 100 ppm NaClO, and no bacteria were detected in the cabbage pretreated with 500 ppm NaClO (Table 5). In this experiment, the detection limit for bacteria was about 10 CFU/g sample. Even when the cabbage was treated with water, the residual bacteria were significantly less in number than in the cabbage treated with 100 ppm NaClO. When Salmonella spp. or S. aureus was similarly added to the pretreated cabbage with 200 or 500 ppm NaClO, the disinfection efficiency of 100 ppm NaClO improved (Table 5). These results suggest that bacteria infiltrating into a deeper part of a cut edge were killed with the remaining NaClO in the cut edge.

Combining the results described above, we conclude that the reason food-borne disease bacteria attached to cut cabbage were not efficiently disinfected with NaClO was that a sufficient amount of NaClO did not infiltrate deeply into the cut edges and did not come into contact with the bacteria. It is necessary in order to improve the efficiency of NaClO disinfection of fresh produce to contrive physical and chemical methods promoting the penetration of NaClO.

Acknowledgments

We thank Dr. Tatsuro Maeda of Nisshin Seifun Group (Tokyo) for taking micrographs.

References