J. Microbiol. Biotechnol. (2017), 27(2), 219–225
https://doi.org/10.4014/jmb.1607.07029

jmb

Combined Treatment on the Inactivation of Naturally Existing Bacteria and *Escherichia coli* O157:H7 Inoculated on Fresh-Cut Kale

Ji Hoon Kang and Kyung Bin Song*

Department of Food Science and Technology, Chungnam National University, Daejeon 34134, Republic of Korea

Received: July 12, 2016
Revised: September 13, 2016
Accepted: October 6, 2016

First published online
October 25, 2016

**Corresponding author*
Phone: +82-42-821-6723;
Fax: +82-42-825-2664;
E-mail: kbsong@cnu.ac.kr

pISSN 1017-7825, eISSN 1738-8872

An aqueous chlorine dioxide (ClO_2) treatment combined with highly activated calcium oxide (CaO) and mild heat was tested for inactivating naturally existing bacteria and *Escherichia coli* O157:H7 inoculated on fresh-cut kale. Kale samples were treated with different concentrations of ClO_2 (10, 30, and 50 ppm), CaO (0.01%, 0.05%, 0.1%, and 0.2%), and mild heat (25°C, 45°C, 55°C, and 65°C) as well with combinations of 30 or 50 ppm ClO_2 and 0.2% CaO at 55°C for 3 min. An increasing concentration of ClO_2 and CaO significantly reduced the microbial population compared with the control. In addition, mild heating at 55°C elicited greater microbial reduction than the other temperatures. A combined treatment of 50 ppm ClO_2 and 0.2% CaO at 55°C reduced the population of naturally existing bacteria on kale by 3.10 log colony forming units (CFU)/g, and the counts of *E. coli* O157:H7 were below the detection limit (1 log CFU/g). In addition, no significant differences in the Hunter color values were evident in any treatment during storage. Therefore, a combined treatment of ClO_2 and active CaO at 55°C can be an effective sanitizing method to improve the microbiological safety of fresh-cut kale without affecting its quality.

Keywords: Kale, *Escherichia coli* O157:H7, chlorine dioxide, calcium oxide

Introduction

Leafy vegetables, such as *Brassica*, are popularly consumed because of their nutritional benefits. *Brassica* vegetables contain a considerable amount of vitamins, phenolic compounds, and antioxidative and anticarcinogenic compounds [1, 2]. Kale (*Brassica oleracea* L. var. *acephala*) is a *Brassica* and has a large amount of vitamin C and glucosinolate [3].

Concern about the hygiene of *Brassica* vegetables exists because they are commonly consumed raw. Foodborne illness outbreaks associated with these vegetables have continually increased owing to their susceptibility to contamination by foodborne pathogens [4, 5]. Therefore, an appropriate sanitizing method is necessary to improve the microbial safety of fresh-cut kale.

A chlorine solution has been widely used to reduce microorganisms and ensure the microbial safety of fresh-cut vegetables [6]. Despite the popular use of a chlorine solution, several EU countries have banned its application in the food industry because of environmental and health concerns related to the production of carcinogenic compounds [7]. Thus, many studies have been performed to identify other sanitizers that can substitute for a chlorine solution. Specifically, chlorine dioxide (ClO_2) [8, 9], ozone [10], organic acid [11], and electrolyzed water [12] have all been used to improve the microbial safety of fresh-cut vegetables. Aqueous ClO_2 treatment has been recognized as a suitable alternative to chlorine solutions because it has a higher oxidative capacity than chlorine, and fewer harmful compounds are formed by reactions with organic molecules [12, 13]. Such considerations have led to the investigation of the effects of ClO_2 alone or combined with other treatments on the inactivation of microorganisms in these vegetables [8, 14, 15].

Other disinfection methods, such as mild heat or natural antimicrobials, have been investigated [16, 17]. A mild heat treatment of 50°C has been reported to enhance the effect of sanitizers applied to vegetables without affecting the quality of the treated produce [16, 18, 19]. Koseki and Isobe [20] also reported that a combined treatment with

electrolyzed water and mild heat more effectively reduced microorganisms than electrolyzed water at room temperature. Based on these results, we hypothesized that the antimicrobial effect of an aqueous ClO_2 solution might be improved when combined with mild heat.

Calcium oxide (CaO) has been studied as a natural antimicrobial [21]. CaO is an environmentally friendly sanitizing agent because it is produced from the conversion of calcium carbonate ($CaCO_3$) in scallop or oyster shells by a heat treatment of more than 700°C [21]. In this study, a highly activated CaO, which was manufactured at more than 1,500°C and an extremely high voltage [22], was used. Highly activated CaO has a purity greater than 99%, strong alkalinity, and no toxicity or side effects compared with CaO produced under different conditions. However, the effect of highly activated CaO on microbial inactivation in fresh-cut vegetables has been barely studied. Therefore, the purpose of this study was to confirm the antimicrobial effect of highly activated CaO and examine the effect of a combined treatment of aqueous ClO_2 and CaO and mild heat on bacterial inactivation in fresh-cut kale as an appropriate sanitizing method for improving the microbiological safety of the vegetable.

Materials and Methods

Bacterial Strains and Culture Preparation

The two strains of *Escherichia coli* O157:H7 (ATCC 43889 and NCTC 12079) used in this study were stored at −70°C in 1 ml of tryptic soy broth (Difco Co., USA) containing 20% glycerol. Each strain of *E. coli* O157:H7 was streaked on tryptic soy agar (Difco Co.), incubated at 37°C for 24 h, and then stored at 4°C before use. A single colony of each *E. coli* O157:H7 strain was transferred to 25 ml of tryptic soy broth with a sterile loop and cultured overnight at 37°C. Each culture was harvested by centrifugation (2,000 ×*g*, 4°C, 15 min) and then washed twice with sterile peptone water (0.1%). The final cell pellets (approximately 7−8 log CFU/g) were resuspended in 0.1% sterile peptone water to retain the standard cell concentration, and suspensions of each *E. coli* O157:H7 strain mixture were used for the inoculation study.

Sample Preparation and Inoculation Study

Fresh kale (*Brassica oleracea* L. var. *acephala*) was purchased from a local market in Daejeon, Korea, stored at 4°C, and then used within 24 h. Damaged kale samples were discarded, and the non-edible part of the kale was cut with a sterile stainless steel blade. The kale leaves (5 ± 0.3 g) were used for the experiment. For the inoculation study, kale samples (10 ± 0.5 g) were placed onto sterile aluminum foil and treated with UV irradiation for 10 min in a laminar flow biosafety hood with UV-C lamps to eliminate naturally existing microorganisms. Sterilized kale leaves (10 ± 0.5 g) were then spot-inoculated with 0.5 ml of an *E. coli* O15:H7 mixture suspension to an initial population of approximately 5−6 log CFU/g, and the inoculated samples were air-dried for 1 h in a laminar flow biosafety hood to allow the attachment of the pathogenic bacteria.

Mild Heat Treatment

Fresh and inoculated kale leaves (10 ± 0.5 g) were immersed in distilled water (DW, 1:20 (w/v)) at various temperatures (25°C, 45°C, 55°C, and 65°C) with gentle agitation for 3 min. After treatment, the samples were air-dried on a laminar flow biosafety hood for 30 min.

Aqueous Chlorine Dioxide Treatment

A high concentration of an aqueous ClO_2 solution was prepared by mixing 1 g of sodium chlorite (80%; Sigma-Aldrich, USA) and 10 ml of 1 N hydrochloric acid in 100 ml of DW, followed by agitating with a stirrer at 25°C for 1 h. The prepared ClO_2 solution was diluted with DW to make different ClO_2 solution concentrations (10, 30, and 50 ppm). The concentration of each ClO_2 solution was determined using the standard iodometry method [8, 23]. Fresh and inoculated kale leaves (10 ± 0.5 g) were submerged in different concentrations of ClO_2 solution (1:20 (w/v)) for 3 min and air-dried as described above.

Calcium Oxide Treatment

A highly activated CaO powder containing more than 99% CaO was obtained from Eco-Biotech (Korea). A CaO solution was prepared by the addition of highly activated powdered CaO (0.02, 0.1, 0.2, and 0.4 g) to 200 ml of DW and agitating with a stirrer for 1 h. The solution was then filtered with a Whatman nylon membrane filter (0.45 μm; GE Healthcare Life Sciences, UK) using a chemically resistant diaphragm vacuum pump (KNF Neuberger, Germany). The treatment procedure was the same as described above.

Combined Treatment

For the combined treatment, fresh and inoculated kale leaves were treated with 30 or 50 ppm ClO_2/0.2% CaO, 30 or 50 ppm ClO_2/mild heat at 55°C, and 30 or 50 ppm ClO_2/0.2% CaO/mild heat at 55°C, respectively. A solution of ClO_2 and CaO was prepared by mixing a 30 or 50 ppm ClO_2 solution with a 0.2% CaO solution to a total volume of 200 ml. For the combined treatment of the ClO_2 solution with mild heat at 55°C, DW was pre-heated to 55°C and a 30 or 50 ppm ClO_2 solution was added to the pre-heated DW. The combined treatment of ClO_2/CaO/mild heat at 55°C was performed as follows. The CaO solution (0.2%) was pre-heated using a hot plate until the target temperature (55°C) was reached, and a 30 or 50 ppm ClO_2 solution was mixed with the pre-heated CaO solution. The prepared solution was used to treat the kale samples, and the treated samples were air-dried for 30 min.

Microbial Enumeration

After treatment, the kale samples (10 ± 0.5 g) were mixed with sterile peptone water (0.1 %, 90 ml) in a sterile stomacher bag and homogenized for 3 min with a Stomacher (MIX 2; AES Laboratoire, France). For the microbial count, 1 ml of the mixture was serially diluted with 9 ml of sterile peptone water and spread on each selective agar plate. Naturally existing bacteria counts were assessed on plate count agar (Difco), and the plate was incubated at 37°C for 48 h. *E. coli* O157:H7 counts were assessed on Sorbitol MacConkey agar (Difco) and incubated at 37°C for 24 h. The results were expressed as the log colony forming units (CFU)/g, and three replications of all microbial counts were performed.

Color Measurement

After treatment, the kale samples were packaged in a low-density polyethylene bag (25 × 30 cm) and stored at 4°C for 7 days. The surface color of the kale leaves was measured using a colorimeter (CR-400, Minolta Chroma Meter; Konica Minolta Sensing, Inc., Japan) at three different locations on the kale surface and repeated in triplicates. The kale samples were placed on a standard white plate and the Hunter color values (L, a, and b) were measured. The L, a, and b values of the standard plate were 96.67, −0.12, and 2.07, respectively.

Statistical Analysis

Data analysis was performed with SAS (ver. 9.4; SAS Institute Inc., USA). All experiments were repeated at least three times, and the results were expressed as the mean ± standard deviation. Duncan's multiple range tests and analysis of variance were used to determine significant differences at $p < 0.05$.

Results and Discussion

Mild Heat Treatment

To investigate the optimal mild heat temperature, fresh and inoculated kale leaves were washed with water at 25°C, 45°C, 55°C, and 65°C. The reduction levels of naturally existing bacteria on kale leaves were 0.41, 0.83, 1.23, and 1.15 log CFU/g after washing with water at 25°C, 45°C, 55°C, and 65°C, respectively, compared with the control (Fig. 1). In addition, the reduction trend of the population of *E. coli* O157:H7 inoculated on kale leaves was similar to that of the naturally existing bacteria; the populations of *E. coli* O157:H7 were reduced by 1.65, 2.26, 2.57, and 2.53 log CFU/g compared with the control (Fig. 1). Both types of microorganisms were significantly reduced after the 45°C and 55°C treatments, whereas no significant difference was seen between 55°C and 65°C. Based on these results, 55°C was chosen as the optimal temperature. Similar results were reported by Lee *et al*. [24]; the reduction of the total aerobic bacteria and *E. coli* O157:H7 on kale leaves

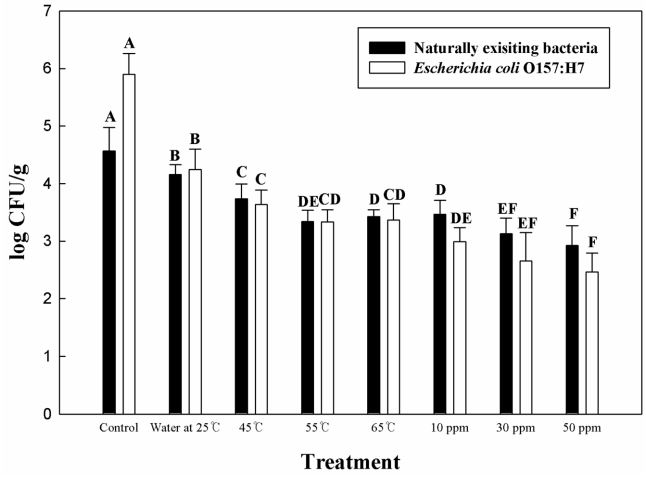

Fig. 1. The effect of different concentrations of aqueous chlorine dioxide and water washing at different temperatures on the inactivation of microorganisms on kale leaves.
Control, no treatment; water at 25°C, water washing at room temperature; 45°C, 55°C, and 65°C, water washing at 45°C, 55°C, and 65°C, respectively; 10, 30, and 50 ppm, aqueous ClO_2 solution of 10, 30, and 50 ppm, respectively. ■: Naturally existing bacteria; □: *E. coli* O157:H7. Each vertical bar represents the standard deviation ($n = 3$). Different capital letters on the bars indicate a significant ($p < 0.05$) difference.

washed with water at room temperature was 0.63 and 1.03 log CFU/g, respectively, compared with the control. These results indicate that water washing without mild heat is insufficient to inactivate microbes. In this study, washing with water under mild heat (45°C, 55°C, and 65°C) reduced the population of both types of microorganisms on the kale leaves by 0.42–0.88 log CFU/g compared with washing with water at 25°C. Similarly, it has been reported that the total bacterial population on cabbage was further reduced by 0.46–0.67 log CFU/g after a mild heat treatment at 40°C and 50°C compared with washing with water at 20°C [25]. However, the inactivation by a mild heat treatment of the microorganisms in fresh-cut vegetables was not powerful because mild heat treatment showed less than a 1 log CFU/g reduction compared with washing with water at room temperature (20–25°C). Therefore, to further enhance microbial inactivation, another sanitizing treatment was necessary.

Treatments of Aqueous ClO_2 and CaO

The antimicrobial mechanism of ClO_2 has been determined to be a direct reaction with microbial amino acids and RNA by penetration through the membrane [26, 27]. The concentration range of the ClO_2 solution was chosen based

Table 1. pH values of different sanitizing solutions.

Treatment	pH
10 ppm ClO_2	3.52 ± 0.01^{G}
30 ppm ClO_2	3.05 ± 0.01^{H}
50 ppm ClO_2	2.83 ± 0.01^{I}
0.01% CaO	11.74 ± 0.01^{F}
0.05% CaO	12.41 ± 0.00^{E}
0.1% CaO	12.70 ± 0.01^{D}
0.2% CaO	12.91 ± 0.01^{A}
30 ppm ClO_2 + 0.2% CaO	12.87 ± 0.01^{BC}
50 ppm ClO_2 + 0.2% CaO	12.86 ± 0.01^{C}

Means in the same column followed by different letters (A-I) are significantly different ($p < 0.05$).

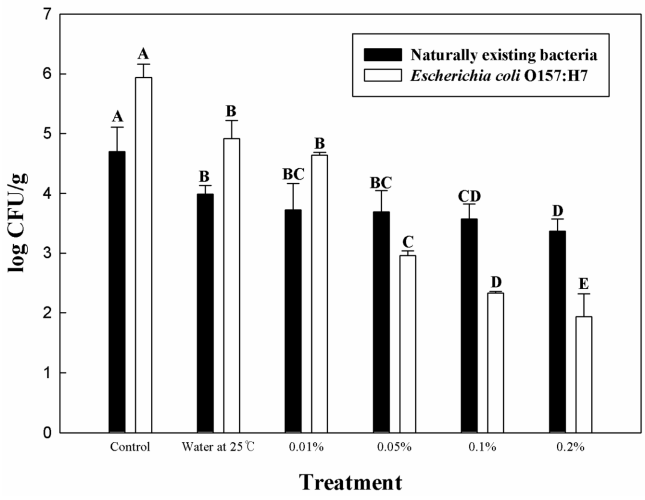

Fig. 2. The effect of different concentrations of aqueous calcium oxide treatment on the inactivation of microorganisms on kale leaves.

Control, no treatment; water at 25°C, water washing at room temperature; 0.01%, 0.05%, 0.1%, and 0.2%, aqueous highly activated CaO solution of 0.01%, 0.05%, 0.1%, and 0.2%, respectively. ■: Naturally existing bacteria; □: *E. coli* O157:H7. Each vertical bar represents the standard deviation ($n = 3$). Different capital letters on the bars indicate a significant ($p < 0.05$) difference.

on previous studies [28, 29]. It has been previously reported that the pH did not affect the antimicrobial activity of aqueous ClO_2 [30]. On the contrary, the antimicrobial effect of a CaO solution has been attributed to the alkaline pH of hydrated CaO [31]. An increasing concentration of CaO caused a pH increase toward a more alkaline condition [21]. We investigated the effect of the highly activated CaO solution at various concentrations (0.01%, 0.05%, 0.1%, and 0.2%) under different pHs on the microbial inactivation in kale leaves (Table 1).

Fresh and inoculated kale leaves were treated with different concentrations of ClO_2 and CaO solutions to determine the optimal concentration of each solution in the combined treatment (Figs. 1 and 2). The reduction of the naturally existing bacteria and *E. coli* O157:H7 on kale leaves increased significantly as the ClO_2 concentration increased from 10 to 50 ppm. For 10, 30, and 50 ppm ClO_2, the bacterial population on the kale leaves was reduced to 3.46, 3.13, and 2.92 log CFU/g for naturally existing bacteria, respectively, and 2.99, 2.66, and 2.46 log CFU/g for *E. coli* O157:H7, respectively, resulting in reductions of 1.11, 1.44, and 1.65 log CFU/g and 2.91, 3.24, and 3.44 log CFU/g, respectively, compared with the control samples (Fig. 1). In a similar study, Lee *et al*. [24] investigated the efficacy of a 50 ppm ClO_2 solution for inactivating the total aerobic bacteria and *E. coli* O157:H7 on kale leaves and found that the populations of the total aerobic bacteria and *E. coli* O17:H7 were reduced by 3.07 and 3.15 log CFU/g, respectively, compared with the control. In addition, the inactivation of bacteria by a 50 ppm of ClO_2 solution in various vegetables and fruits, such as sprouts (broccoli, alfalfa, and clover), iceberg lettuce, red chicory, pak choi, cherry tomatoes, strawberries, and blueberries, has been studied [2, 15, 29, 32–35]. We used combined treatments of a 30 or 50 ppm ClO_2 solution with or without CaO with mild heat at a temperature of 55°C.

A similar pattern of microbial reduction by the CaO treatment was observed (Fig. 2). CaO treatment at 0.01%, 0.05%, 0.1%, and 0.2% reduced the population of naturally existing bacteria and *E. coli* O157:H7 on kale leaves by 0.97, 1.01, 1.13, and 1.33 log CFU/g and 1.30, 2.98, 3.61, and 4.00 log CFU/g, respectively, compared with the control. The reduction of the microorganisms by the 0.01% CaO treatment was similar to that obtained by washing with water at 25°C. In addition, the antimicrobial effect of the CaO treatment on *E. coli* O157:H7 at all concentrations was higher than that for the naturally existing bacteria. Bae *et al*. [36] investigated the antimicrobial effect of a 0.05% CaO treatment against three different pathogens (*E. coli*, *Salmonella* Typhimurium, and *Listeria monocytogenes*) and reported that the inactivation of *E. coli* was the highest (99%) among the three pathogens compared with 70% for *S.* Typhimurium and 45% for *L. monocytogenes*. The antibacterial mechanism of CaO is known to result from the reaction of activated oxygen generated from the CaO and a highly alkaline pH [31]. In line with these results, our results suggest that CaO treatment at a concentration of more than 0.05% inactivated *E. coli* O157:H7 more easily than it does the naturally existing bacteria on kale leaves. The 0.2% CaO solution,

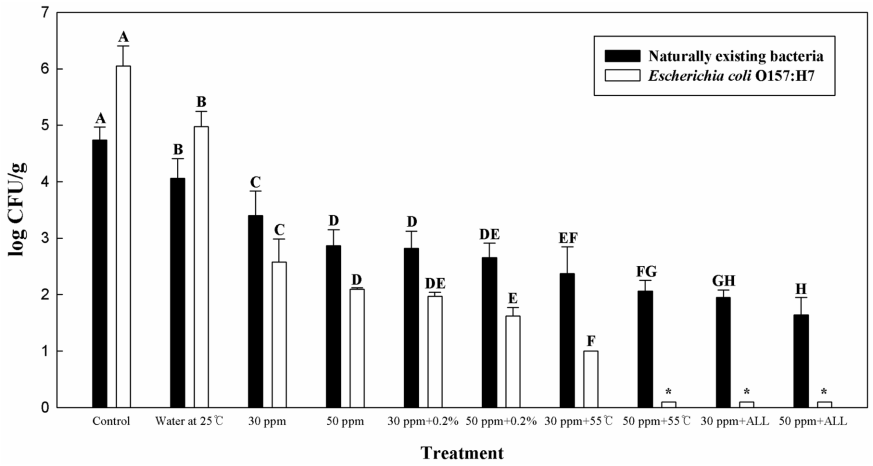

Fig. 3. Combined effects of aqueous chlorine dioxide with calcium oxide or/and mild heat treatment on the inactivation of microorganisms on kale leaves.

Control, no treatment; water at 25°C, water washing at room temperature; 30 and 50 ppm, aqueous ClO_2 solution of 30 and 50 ppm, respectively; 30 ppm+0.2%, combined with 30 ppm ClO_2 and 0.2% CaO; 50 ppm+0.2%, combined with 50 ppm ClO_2 and 0.2% CaO; 30 ppm+ALL, combined with 30 ppm ClO_2 and 0.2% CaO at 55°C; 50 ppm+ALL, combined with 50 ppm ClO_2 and 0.2% CaO at 55°C. ■: Naturally existing bacteria; □: *E. coli* O157:H7. Each vertical bar represents the standard deviation (n = 3). Different capital letters on the bars indicate a significant ($p < 0.05$) difference. *, No detection of microorganisms (below the detection limit, 1 log CFU/g).

which showed a higher reduction of microorganisms on kale leaves than the other concentrations, was chosen for the combined treatment with the ClO_2 solution at 55°C.

Combined Treatment and Color Quality of Kale Leaves

To develop an appropriate sanitizing method for fresh-cut kale, a 30 or 50 ppm ClO_2 solution combined with 0.2% CaO at 55°C was tested on kale leaves. After the combined treatment of a 30 or 50 ppm ClO_2 solution with 0.2% CaO at 25°C, the microbial reduction levels of the naturally existing bacteria and *E. coli* O157:H7 were 1.92–2.09 log CFU/g and 4.08–4.43 log CFU/g, respectively, compared with the control. In addition, the combined treatment of 30 or 50 ppm ClO_2 with mild heat (55°C) reduced the population of the naturally existing bacteria on kale leaves by 2.37–2.68 log CFU/g. Likewise, after the combined treatment of 30 or 50 ppm ClO_2 and mild heat, the population of *E. coli* O157:H7 was lower than with the combined treatment of 30 or 50 ppm ClO_2 and 0.2% CaO. Overall, the combined treatment of 30 or 50 ppm ClO_2 with mild heat (55°C) demonstrated greater microbial reduction than the combination of 30 or 50 ppm ClO_2 and 0.2% CaO. Tango *et al*. [37] reported that a mild heat treatment can weaken the bacteria cell membrane and enable sanitizers to more easily penetrate bacterial cells. Thus, a better antimicrobial effect can be obtained with a combined treatment of ClO_2 and mild heat.

The naturally existing bacterial population on kale leaves treated with a combined treatment of 30 or 50 ppm ClO_2 and 0.2% CaO solution at 55°C was 1.95 and 1.64 log CFU/g, respectively, resulting in a reduction of 2.79 and 3.10 log CFU/g. *E. coli* O157:H7 inoculated on kale leaves was not detected after the combined treatment. These results suggest that the combined treatment of ClO_2 and CaO at 55°C provided the best inactivation of microorganisms on fresh-cut kale.

After the combined treatment of 30 or 50 ppm ClO_2 and 0.2% CaO at 55°C, the Hunter color values (L, a, and b) of the kale leaves were determined (Table 2). The color of leafy vegetables is regarded to be an important quality parameter. No significant differences in the Hunter color values among the treatments during 7 days of storage were found. It has been previously reported that the ClO_2 and CaO treatments did not affect the color quality of vegetables [24, 29, 38]. According to a report by Lee *et al*. [24], no changes in the Hunter color values of romaine lettuce and kale treated with a 50 ppm ClO_2 solution during storage was noted. Kim *et al*. [29] also reported that no color change in iceberg lettuce treated with different concentrations of a ClO_2 solution was observed during storage. Therefore, these results clearly indicate that a combined treatment with ClO_2 and CaO at 55°C is an effective method for inactivating microorganisms on kale leaves without affecting the color quality.

Table 2. Changes in the Hunter color values of kale leaves during storage at 4°C.

Hunter color value	Storage time (day)	Treatment			
		Control[1)]	Water washing at 25°C	30 ppm ClO_2 + 0.2% CaO + MH at 55°C	50 ppm ClO_2 + 0.2% CaO + MH at 55°C
L	0	34.08 ± 1.74$^{B2)a3)}$	34.39 ± 1.96^{Ca}	34.04 ± 1.30^{Ca}	34.78 ± 1.29^{Ba}
	1	34.03 ± 0.99^{Ba}	34.23 ± 1.52^{Ca}	34.09 ± 1.50^{Ca}	34.37 ± 1.57^{Ba}
	3	34.96 ± 1.63^{ABa}	34.97 ± 0.95^{BCa}	35.16 ± 0.77^{Ba}	35.74 ± 0.71^{Aa}
	5	35.50 ± 1.20^{Aa}	35.59 ± 0.73^{ABa}	36.13 ± 0.51^{Aa}	36.04 ± 0.97^{Aa}
	7	35.91 ± 1.53^{Aa}	36.16 ± 1.56^{Aa}	36.58 ± 1.28^{Aa}	36.48 ± 0.93^{Aa}
a	0	-9.32 ± 0.49^{Aa}	-9.63 ± 0.59^{Aa}	-9.70 ± 0.33^{Aa}	-9.59 ± 0.54^{Aa}
	1	-9.46 ± 0.31^{Aa}	-9.59 ± 0.71^{Aa}	-9.70 ± 0.42^{Aa}	-9.84 ± 0.41^{ABa}
	3	-9.78 ± 0.28^{Ba}	-9.88 ± 0.66^{ABa}	-10.13 ± 0.30^{Ba}	-10.12 ± 0.22^{BCa}
	5	-10.04 ± 0.26^{BCa}	-10.36 ± 0.78^{BCab}	-10.52 ± 0.44^{Cb}	-10.42 ± 0.37^{CDab}
	7	-10.26 ± 0.30^{Ca}	-10.63 ± 0.71^{Ca}	-10.58 ± 0.41^{Ca}	-10.56 ± 0.42^{Da}
b	0	10.46 ± 0.36^{Da}	10.99 ± 1.05^{Ba}	10.94 ± 0.57^{Ba}	11.00 ± 0.47^{Ca}
	1	10.51 ± 0.32^{Da}	10.62 ± 0.92^{Ba}	10.93 ± 0.58^{Ba}	11.04 ± 0.45^{Ca}
	3	11.11 ± 0.38^{Cb}	11.78 ± 0.88^{Aa}	12.02 ± 0.47^{Aa}	11.96 ± 0.35^{Ba}
	5	11.65 ± 0.41^{Bb}	12.14 ± 0.68^{Aa}	12.30 ± 0.43^{Aa}	12.31 ± 0.37^{Aa}
	7	12.08 ± 0.31^{Aa}	12.32 ± 0.85^{Aa}	12.40 ± 0.41^{Aa}	12.39 ± 0.28^{Aa}

[1)]No treatment.

[2)]Means in the same column followed by different letters (A-D) are significantly different ($p < 0.05$).

[3)]Means in the same row followed by different letters (a-b) are significantly different ($p < 0.05$).

In conclusion, to develop an appropriate sanitizing method, we examined the effects of a combined treatment of ClO_2 with or without CaO and mild heat on the inactivation of microorganisms on kale leaves. Our results suggest that a combined treatment of ClO_2 and CaO at 55°C is an appropriate method to attain microbiological safety and maintain the color quality of kale leaves. Therefore, this combined treatment can be used on fresh-cut vegetables as a novel sanitizing method. Further study is necessary to examine the antimicrobial effect of highly activated CaO in combined treatments with other chemical sanitizers.

References

1. Fernandes F, DePinho PG, Valentao P, Pereira JA, Andrade PB. 2009. Volatile constituents throughout *Brassica oleracea* L. var. *acephala* germination. *J. Agric. Food Chem.* **57:** 6795-6802.
2. Kim JY, Kim HJ, Lim GO, Jang SA, Song KB. 2010. The effects of aqueous chlorine dioxide or fumaric acid treatment combined with UV-C on postharvest quality of 'Maehyang' strawberries. *Postharvest Biol. Technol.* **56:** 254-256.
3. Korus A, Lisiewska Z. 2011. Effect of preliminary processing and method of preservation on the content of selected antioxidative compounds in kale (*Brassica oleracea* L. var. *acephala*) leaves. *Food Chem.* **129:** 149-154.
4. Kang JH, Song KB. 2015. Inactivation of pre-existing bacteria and foodborne pathogens on perilla leaves using a combined treatment with an organic acid and a surfactant. *Hortic. Environ. Biotechnol.* **56:** 195-199.
5. Warriner K, Huber A, Namvar A, Fan W, Dunfield K. 2009. Recent advances in the microbial safety of fresh fruits and vegetables. *Adv. Food Nutr. Res.* **57:** 155-208.
6. Van Haute S, Sampers I, Holvoet K, Uyttendaele M. 2013. Physicochemical quality and chemical safety of chlorine as a reconditioning agent and wash water disinfectant for fresh-cut lettuce washing. *Appl. Environ. Microbiol.* **79:** 2850-2861.
7. Bull RJ, Reeckgow DW, Li X, Humpage AR, Joll C, Hrudey SE. 2011. Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, *N*-halamines, halonitriles, and heterocyclic amines. *Toxicology* **286:** 1-19.
8. Chun HH, Song KB. 2013. The combined effects of aqueous chlorine dioxide, fumaric acid, and ultraviolet-C with modified atmosphere packaging enriched in CO_2 for inactivating preexisting microorganisms and *Escherichia coli* O157:H7 and *Salmonella typhimurium* inoculated on buckwheat sprouts. *Postharvest Biol. Technol.* **86:** 118-124.
9. Tomás-Callejas A, López-Gálvez F, Sbodio A, Artés F, Artés-Hernández F, Suslow TV. 2012. Chlorine dioxide and chlorine effectiveness to prevent *Escherichia coli* O157:H7 and *Salmonella* cross-contamination on fresh-cut red chard. *Food Control* **23:** 325-332.
10. Miller FA, Silva CLM, Brandão TRS. 2013. A review on ozone-based treatments for fruit and vegetables preservation. *Food Eng. Rev.* **5:** 77-106.

11. Ge C, Bohrerova Z, Lee J. 2013. Inactivation of internalized *Salmonella* Typhimurium in lettuce and green onion using ultraviolet C irradiation and chemical sanitizers. *J. Appl. Microbiol.* **114:** 1415-1424.
12. Keskinen LA, Burke A, Annous BA. 2009. Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves. *Int. J. Food Microbiol.* **132:** 134-140.
13. Beuchat LR, Pettigrew CA, Tremblay ME, Roselle BJ, Scouten AJ. 2004. Lethality of chlorine, chlorine dioxide, and a commercial fruit and vegetable sanitizer to vegetative cells and spores of *Bacillus cereu*s and spores of *Bacillus thuringiensis*. *J. Food Prot.* **67:** 1702-1708.
14. Choi S, Beuchat LR, Kim H, Ryu JH. 2016. Viability of sprout seeds as affected by treatment with aqueous chlorine dioxide and dry heat, and reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* on pak choi seeds by sequential treatment with chlorine dioxide, drying, and dry heat. *Food Microbiol.* **54:** 127-132.
15. Kim YJ, Kim MH, Song KB. 2009. Combined treatment of fumaric acid with aqueous chlorine dioxide or UV-C irradiation to inactivate *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* inoculated on alfalfa and clover sprouts. *LWT Food Sci. Technol.* **42:** 1654-1658.
16. Huang Y, Chen H. 2011. Effect of organic acids, hydrogen peroxide and mild heat on inactivation of *Escherichia coli* O157:H7 on baby spinach. *Food Control* **22:** 1178-1183.
17. Poimenidou SV, Bikouli VC, Gardeli C, Mitsi C, Tarantilis PA, Nychas GJ, Skandamis PN. 2016. Effect of single or combined chemical and natural antimicrobial interventions on *Escherichia coli* O157:H7, total microbiota and color of packaged spinach and lettuce. *Int. J. Food Microbiol.* **220:** 6-18.
18. Dikici A, Koluman A, Calicioglu M. 2015. Comparison of effects of mild heat combined with lactic acid on Shiga toxin producing *Escherichia coli* O157:H7, O103, O111, O145 and O26 inoculated to spinach and soybean sprout. *Food Control* **50:** 184-189.
19. Koseki S, Yoshida K, Kamitani Y, Isobe S, Itoh K. 2004. Effect of mild heat pre-treatment with alkaline electrolyzed water on the efficacy of acidic electrolyzed water against *Escherichia coli* O157:H7 and *Salmonella* on lettuce. *Food Microbiol.* **21:** 559-566.
20. Koseki S, Isobe S. 2007. Microbial control of fresh produce using electrolyzed water. *Jpn. Agric. Res. Q.* **41:** 273-282.
21. Ro EY, Ko YM, Yoon KS. 2015. Survival of pathogenic enterohemorrhagic *Escherichia coli* (EHEC) and control with calcium oxide in frozen meat products. *Food Microbiol.* **49:** 203-210.
22. Jung DK. 2000. Process for producing calcium oxide. Korea Patent 10-0270228.
23. APHA. 1995. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington.
24. Lee JH, Chun HH, Oh DH, Park J, Won M, Song KB. 2012. Sensory and microbiological qualities of romaine lettuce and kale affected by a combined treatment of aqueous chlorine dioxide and ultraviolet-C. *Hortic. Environ. Biotechnol.* **53:** 387-396.
25. Rahman SME, Jin YG, Oh DH. 2010. Combined effects of alkaline electrolyzed water and citric acid with mild heat to control microorganisms on cabbage. *J. Food Sci.* **75:** 111-115.
26. Joshi K, Mahendran R, Alagusundaram K, Norton T, Tiwari BK. 2013. Novel disinfectants for fresh produce. *Trends Food Sci. Technol.* **34:** 54-61.
27. Meireles A, Giaouris E, Simões M. 2016. Alternative disinfection methods to chlorine for use in the fresh-cut industry. *Food Res. Int.* **82:** 71-85.
28. Jin YY, Kim YJ, Chung KS, Won M, Song KB. 2007. Effect of aqueous chlorine dioxide treatment on the microbial growth and qualities of strawberries during storage. *Food Sci. Biotechnol.* **16:** 1018-1022.
29. Kim YJ, Lee SH, Park J, Park J, Chung M, Kwon K, *et al*. 2008. Inactivation of *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes* on stored iceberg lettuce by aqueous chlorine dioxide treatment. *J. Food Sci.* **73:** 418-422.
30. Benarde MA, Israel BM, Olivieri VP, Granstrom ML. 1965. Efficiency of chlorine dioxide as a bactericide. *Appl. Environ. Microbiol.* **13:** 776-780.
31. Sawai J. 2011. Antimicrobial characteristics of heated scallop shell powder and its application. *Biocontrol Sci.* **16:** 95-102.
32. Chun HH, Kang JH, Song KB. 2013. Effects of aqueous chlorine dioxide treatment and cold storage on microbial growth and quality of blueberries. *J. Korean Soc. Appl. Biol. Chem.* **56:** 309-315.
33. Kim HJ, Song HJ, Song KB. 2011. Effect of combined treatment of aqueous chlorine dioxide with ultraviolet-C on the quality of red chicory and pak choi during storage. *J. Korean Soc. Food Sci. Nutr.* **40:** 245-252.
34. Kim YJ, Kim MH, Song KB. 2009. Efficacy of aqueous chlorine dioxide and fumaric acid for inactivating pre-existing microorganisms and *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes* on broccoli sprouts. *Food Control* **20:** 1002-1005.
35. Song HJ, Choi DW, Song KB. 2011. Effect of aqueous chlorine dioxide and UV-C treatment on the microbial reduction and color of cherry tomatoes. *Hortic. Environ. Biotechnol.* **52:** 488-493.
36. Bae DH, Yeon JH, Park SY, Lee DH, Ha SD. 2006. Bactericidal effects of CaO (scallop-shell powder) on foodborne pathogenic bacteria. *Arch. Pharm. Res.* **29:** 298-301.
37. Tango CN, Mansur AR, Oh DH. 2015. Fumaric acid and slightly acidic electrolyzed water inactivate gram positive and gram negative foodborne pathogens. *Microorganisms* **3:** 34-46.
38. Sawai J, Shiga H, Kojima H. 2001. Kinetic analysis of the bactericidal action of heated scallop-shell powder. *Int. J. Food Microbiol.* **71:** 211-218.