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Effects of Aqueous Ozone Combined with Organic Acids on Microflora Inactivation in the Raw Materials of *Saengsik*

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Abstract This study was conducted to determine the effects of microorganism inactivation using 3 ppm of aqueous ozone (AO), 1% citric acid, 1% lactic acid, and 1% acetic acid alone, as well as the combinations of AO and organic acid, for washing the raw materials of *saengsik* (carrot, cabbage, glutinous rice, barley) with or without agitation. The combination of AO and 1% of each organic acid significantly inactivated spoilage bacteria in both the vegetables and the grains ($p<0.05$). However, in the glutinous rice, no inhibitory effects were shown for total aerobic bacteria by using water, ozone, or the combination of AO with citric acid or lactic acid, without agitation. Microbial inactivation was enhanced with agitation in the grains, whereas dipping (no agitation) treatments showed better inhibitory effects in the vegetables than in the barley, suggesting that washing processes should take into account the type of food material.

Keywords: aqueous ozone, water, organic acid, washing

Introduction

Recently, consumer demand for minimally processed fresh-like foods, that are convenient, safe, and healthy, has led to a remarkable increase in the variety of products available. *Saengsik* is defined as a processed food made from cereal, vegetable, and animal ingredients, and is dehydrated and made into powder or granules, or other forms such as bars, paste, gel, or sol. It is a convenient food since it can be consumed as is, without further preparation such as dissolving it in water, milk, or another type of beverage (1). The market for *saengsik* has been increased remarkably with consumer interest since the later 1990s.

However, potential safety problems resulting from spoilage or pathogenic bacteria have been increased within raw and minimally processed foods, because quite often these foods are not thoroughly treated to eliminate harmful microorganisms (2). Ozone treatments are promising as a means for treating waste and drinking water in order to remove undesirable contaminants (3). Ozone is a strong oxidant, capable of reacting with a wide variety of contaminants in water and food materials. Ozone has been declared generally recognized as safe (GRAS) by an expert panel for use in food processing, and a petition for its approval as a direct food additive, for the treatment, storage, and processing of foods in the gas and aqueous phases, was accepted in 2001 (4). Restaino *et al.* (5) reported that ozone effects can vary depending upon pH, temperature, relative humidity, and the amount of organic matter surrounding cells. Previously, ozone's effectiveness was evaluated for inactivating different harmful bacteria, including mesophilic and psychotrophic bacteria (6) and *Escherichia coli* O157:H7 (7, 8), and obtained considerable reductions in both lettuce and apples. Additionally, several studies have reported on *saengsik*, such as its microflora during processing and in final products, methods for reducing natural microorganisms in *saengsik*, and hazard analysis and critical control points (HACCP) for its raw materials (1, 9, 10).

The aim of this study was to investigate the microorganism reductions within the raw materials (vegetables and grains) of *saengsik* during a pre-washing step using aqueous ozone, various organic acids alone, and organic acids combined with aqueous ozone, with or without agitation.

Materials and Methods

Raw materials The raw materials for *saengsik*, including grains (glutinous rice and barley) and vegetables (carrots and cabbage), were purchased at supermarkets in Chunchon, Korea. All samples were already trimmed, peeled, and washed, and were kept at refrigeration temperature.

Ozone generation Three ppm of aqueous ozone (AO) was produced on site by an electrochemical process using an ozone generator (GW-1000, Youlchon, Seoul, Korea). During the preparation of the AO, concentration was monitored continuously using a dissolved ozone monitor (model A 15/64; ATI Inc., Collegeville, PA, USA). This particular model was an on-line monitoring system designed for the continuous measurement of ozone gas in solution.

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Washing treatments for the raw materials of *saengsik* Two experimental washing treatments were used to determine the effects of water, food grade organic acids (citric, lactic, and acetic), AO alone, and AO in combination with organic acids on the raw materials of *saengsik*. Unwashed samples were used as controls. Each 200 g of vegetable (carrot or cabbage) and 200 g of grain (glutinous rice or barley) was placed into a separate sterile plastic bag (Whirl-Pak; Nasco, Janesville, WI, USA) containing 400 mL of AO alone, or AO combined with organic acid, and submerged for 3, 5, or 10 min (vegetables), or 6 hr (grains), with or without agitation using a shaker (100 rpm, 22°C). The washing solutions consisted of distilled water, AO alone (3 ppm), or the combination of 3 ppm AO and 1% citric acid (Dae-Jung Chemical, Korea), 1% lactic acid (Tedia, Fairfileld, OH, USA), or 1% acetic acid (Yakuri Pure Chemical, Kyoto, Japan). After washing, the samples were drained and surface dried using sterile paper towels.

Microbiological analysis The control and washed samples were analyzed for total aerobic bacteria (TAB) and yeast and mold (YM) using plate count agar (PCA, BD Difco, Sparks, MD, USA) and acidified (10% tartaric acid) potato dextrose agar (PDA, BD Difco) plates, respectively (11). For each sample, a 10 g portion of control or sample was placed into a sterile Whirl-Pak filter bag (Nasco, Fort Atkinson, WI, USA), and mixed with 90 mL of 0.1% sterile peptone water. The mixture was homogenized using a stomacher (Seward stomacher 400, London, UK) for 2 min at room temperature. Dilutions were made and 1 mL of each appropriate dilution was plated in duplicate. The PCA (CFU/g) plates were incubated at 35°C for 24 hr, and the PDA plates were incubated at 25°C for 48 hr. TAB and YM were counted from the plates that had 25 to 250 colonies.

Statistical analysis The experiment was replicated 2 times and each sample was plated in duplicate, resulting in four observations per mean. The reported plate count data represent means±standard deviations obtained from 4 observations. All data were subjected to analysis of variance; then the Turkey test was conducted with the least square means used to determine the significant differences ($p<0.05$). All statistical procedures were performed with the Statistical Analysis System, Version 9.1 (SAS Institute, Cary, NC, USA).

Results and Discussions

The initial (control) numbers of total aerobic bacteria (TAB)

Table 1. Effects of aqueous ozone, organic acids, or combined ozone with organic acids on the numbers of TAB and YM in carrots

Treatment time	Treatment	TAB (log_{10} CFU/g)		YM (log_{10} CFU/g)	
		No agitation	Agitation	No agitation	Agitation
3 min	Control	4.53$^{a1)}$	4.53^{a}	3.82^{a}	3.82^{a}
	Water	4.27^{b}	4.26^{b}	3.89^{a}	3.93^{a}
	Citric Acid (CA)	3.02^{d}	3.32^{e}	3.06^{b}	2.47^{c}
	Lactic Acid (LA)	3.45^{c}	3.69^{c}	3.12^{b}	2.77^{b}
	Acetic Acid (AA)	3.01^{d}	3.58^{d}	2.91^{b}	2.78^{b}
	Ozone	2.73^{e}	3.64^{cd}	2.60^{b}	3.41^{a}
	Ozone+CA	2.39^{f}	3.26^{e}	ND^{d}	3.10^{a}
	Ozone+LA	2.97^{d}	2.60^{f}	2.05^{c}	3.17^{a}
	Ozone+AA	2.49^{f}	2.57^{f}	2.14^{c}	2.37^{c}
5 min	Control	4.53^{a}	4.53^{a}	3.82^{a}	3.82^{a}
	Water	3.97^{b}	4.20^{b}	3.75^{a}	3.72^{a}
	Citric Acid (CA)	2.84^{c}	2.96^{d}	2.63^{b}	2.20^{b}
	Lactic Acid (LA)	2.93^{c}	3.36^{c}	2.70^{b}	2.41^{b}
	Acetic Acid (AA)	2.85^{c}	2.99^{d}	2.44^{b}	2.47^{b}
	Ozone	2.33^{e}	2.90^{de}	2.45^{b}	2.73^{b}
	Ozone+CA	2.27^{f}	2.85^{e}	ND^{c}	2.68^{b}
	Ozone+LA	2.60^{d}	2.44^{f}	ND^{c}	2.78^{b}
	Ozone+AA	2.43^{e}	2.31^{g}	ND^{c}	2.31^{b}
10 min	Control	4.53^{a}	4.53^{a}	3.82^{a}	3.82^{a}
	Water	3.18^{b}	4.04^{b}	3.68^{a}	3.64^{a}
	Citric Acid (CA)	2.14^{d}	2.86^{d}	2.38^{b}	2.06^{b}
	Lactic Acid (LA)	2.69^{c}	3.00^{c}	2.35^{b}	2.17^{b}
	Acetic Acid (AA)	2.75^{c}	2.90^{cd}	2.29^{b}	2.23^{b}
	Ozone	2.15^{d}	2.56^{e}	2.14^{b}	2.59^{b}
	Ozone+CA	2.11^{d}	2.56^{e}	ND$^{c2)}$	2.32^{b}
	Ozone+LA	2.19^{d}	2.21^{f}	ND^{c}	2.51^{b}
	Ozone+AA	2.01^{d}	2.16^{f}	ND^{c}	2.08^{b}

[1)]Means with different letters in the same column are significantly different ($p<0.05$).
[2)]No colonies detected.

Table 2. Effects of aqueous ozone, organic acids, or combined ozone with organic acids on the numbers of TAB and YM in cabbage

Treatment time	Treatment	TAB (log_{10} CFU/g)		YM (log_{10} CFU/g)	
		No agitation	Agitation	No agitation	Agitation
3 min	Control	5.00[a1)]	5.00[a]	4.90[a]	4.90[a]
	Water	3.91[b]	3.67[b]	3.82[b]	4.25[b]
	Citric Acid (CA)	3.27[c]	2.26[d]	ND[d]	ND[e]
	Lactic Acid (LA)	2.17[d]	ND[e2)]	ND[d]	ND[e]
	Acetic Acid (AA)	2.11[d]	ND[e]	2.04[c]	2.45[d]
	Ozone	3.64[b]	3.69[b]	3.32[c]	3.57[c]
	Ozone+CA	2.07[d]	2.44[c]	2.20[c]	2.38[d]
	Ozone+LA	2.14[d]	ND[e]	3.01[c]	ND[e]
	Ozone+AA	2.06[d]	ND[e]	2.59[c]	3.14[c]
5 min	Control	5.00[a]	5.00[a]	4.90[a]	4.90[a]
	Water	3.48[b]	3.21[b]	ND[c]	3.92[b]
	Citric Acid (CA)	2.74[c]	ND[e]	ND[c]	ND[e]
	Lactic Acid (LA)	ND[d]	ND[e]	ND[c]	ND[e]
	Acetic Acid (AA)	ND[d]	ND[e]	ND[c]	2.27[d]
	Ozone	2.74[c]	2.94[c]	3.09[b]	2.85[c]
	Ozone+CA	ND[d]	2.24[d]	ND[c]	2.09[d]
	Ozone+LA	ND[d]	ND[e]	ND[c]	ND[e]
	Ozone+AA	ND[d]	ND[e]	ND[c]	2.06[d]
10 min	Control	5.00[a]	5.00[a]	4.90[a]	4.90[a]
	Water	2.89[b]	2.89[b]	ND[c]	3.48[b]
	Citric Acid (CA)	2.30[c]	ND[d]	ND[c]	ND[d]
	Lactic Acid (LA)	ND[d]	ND[d]	ND[c]	ND[d]
	Acetic Acid (AA)	ND[d]	ND[d]	ND[c]	ND[d]
	Ozone	2.06[c]	2.24[c]	2.23[b]	2.11[c]
	Ozone+CA	ND[d]	2.04[c]	ND[c]	ND[d]
	Ozone+LA	ND[d]	ND[d]	ND[c]	ND[d]
	Ozone+AA	ND[d]	ND[d]	ND[c]	2.06[c]

[1)]Means with different letters in the same column are significantly different ($p<0.05$).
[2)]No colonies detected.

were 4.53±0.01, 5.0±0.08, 2.41±0.03, and 4.41±0.03 log_{10} CFU/g, in the carrots, cabbage, glutinous rice, and barley, respectively. Yeast and mold (YM) numbers were 3.82± 0.68, 4.90±0.04, 3.93±0.01, and 3.36±0.03 (log_{10} CFU/ g) in the carrots, cabbage, glutinous rice, and barley, respectively (Table 1 and 2). In general, for most treatments, the inactivation of TAB and YM in the vegetables increased as the washing time increased from 3 to 10 min (Table 1 and 2). Also in the vegetables, TAB inactivation increased significantly by all treatments with increasing treatment time from 3 to 10 min, regardless of agitation ($p<0.05$). In the carrots, the water, 1% citric acid (CA), 1% lactic acid (LA), 1% acetic acid (AA), and AO treatments alone decreased TAB populations by 1.35, 2.39, 1.84, 1.78, and 2.38 log_{10} CFU/g, respectively, when treated for 10 min without agitation.

When AO was combined with CA, LA, or AA, TAB populations decreased by 2.42, 2.34, and 2.52 log_{10} CFU/ g, respectively, under the same treatment condition as in the carrots. The effects of the combined treatments of AO with CA, and AO with AA, were significantly ($p<0.05$) greater than the individual treatments regardless of agitation. However, in the carrots, neither the combination of AO with CA, nor CA alone, showed significant inactivation effects with agitation ($p>0.05$) (Table 1). For YM populations in the carrots, the water, CA, LA, and AA treatments significantly decreased populations by 0.18, 1.76, 1.65, and 1.59 log_{10} CFU/g (10 min with agitation), respectively ($p<0.05$).

Organic acids are used as GRAS disinfectants to inhibit bacterial growth in vegetables (12-14). Dipping produce in citric or ascorbic acid solutions prior to packaging reduces bacteria during storage. Organic acids have antimicrobial properties that are related to their amounts of undissociated acid (15, 16). The antimicrobial effects of organic acids depend on the dissociation constant (pKa) of the acid. In contrast to strong acids, weak organic acids such as acetic and citric acid are only partially undissociated. The degree of undissociation is directly related to the antimicrobial activity; that is, more undissociation results in greater antimicrobial activity, as the undissociated acid can penetrate the cell membrane and lower the internal pH of the cell (16).

The combination treatments of AO and organic acids for 5 or 10 min with agitation caused complete inactivation of YM populations (3.82 log_{10} CFU/g) in the carrots, where no colonies were found. The effects of the combined treatments on YM were more pronounced without agitation than with agitation when the treatment time was longer than 5 min. In contrast, the organic acid treatments (CA or LA) alone

resulted in higher inactivation with agitation. Water or AA alone had no effect on YM inactivation in the carrots, regardless of time and agitation.

Similar results were obtained for cabbage, but the inactivation effects were much greater in the carrots than in the cabbage. The water and AO treatments alone significantly decreased TAB populations by 2.11 and 2.94 log CFU/g, respectively, when the cabbage was treated for 10 min without agitation ($p<0.05$). The organic acids alone showed higher inhibitory effects than the AO treatment, and caused 5 log reductions of TAB regardless of agitation, except for the treatment of CA with agitation (5 and 10 min). Due to this, we tested AO combined with LA and AA although the inhibitory effects of the combinations were not clear. However, the combined treatment of AO and CA with agitation showed a synergistic effect against the TAB populations in cabbage, as compared to each treatment alone ($p<0.05$). Similar to TAB, in the case of YM, the organic acid effects were more pronounced than those of AO regardless of time and agitation. The organic acids alone inactivated YM below the detection limit (with the exception of AA), thus the effects of the combination treatments are unknown.

In the grains, as compared to the control, AO alone did not have any antimicrobial effects against TAB. The antimicrobial effects of AO combined with the organic acids were enhanced with agitation, and resulted in significant decreases in TAB in the glutinous rice, as compared to the treatments without agitation ($p<0.05$) (Fig. 1).

Also in the grains, the effects of AO alone, and AO combined with the organic acids on YM were more pronounced with agitation. The AO and organic acid (LA and AA) combined treatments with agitation, significantly

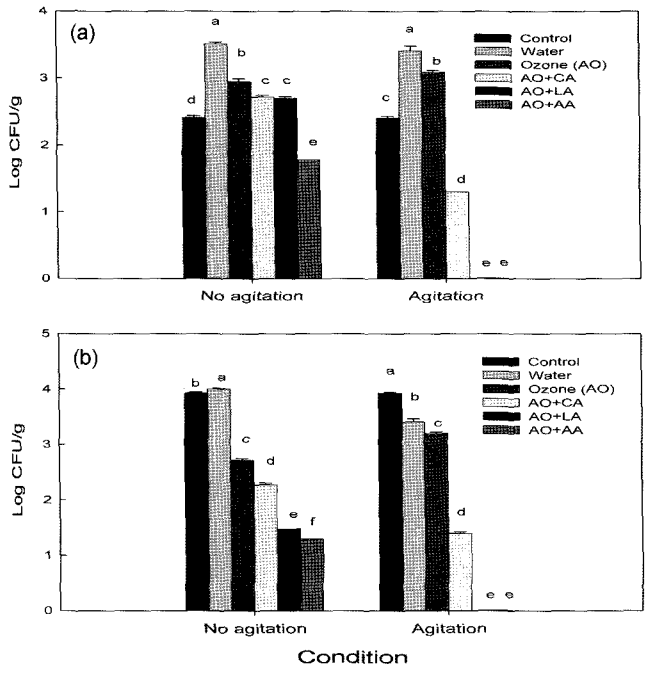

Fig. 1. Effects of aqueous ozone, or ozone combined with organics acid, on the number of TAB (a) and YM (b) in glutinous rice during 6 hr of treatment.

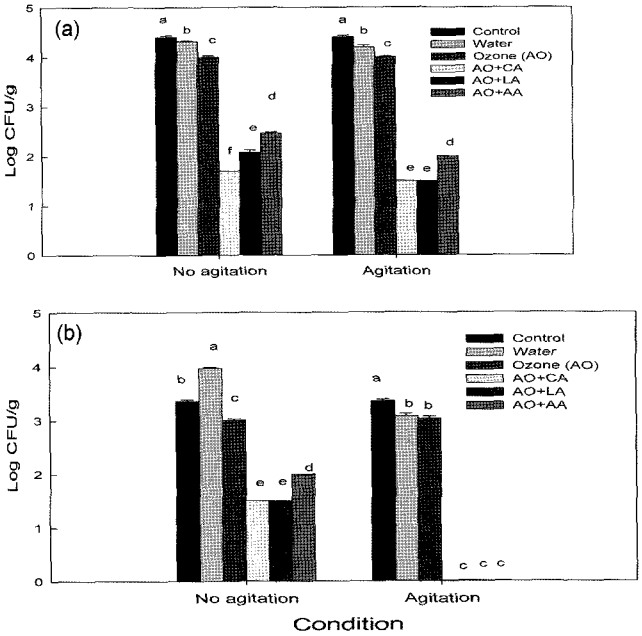

Fig. 2. Effects of aqueous ozone, or ozone combined with organic acid, on the numbers of TAB (a) and YM (b) in barley during 6 hr of treatment.

decreased YM by 3.93 $\log_{10}$ CFU/g ($p<0.05$). Figure 2 shows the inhibitory effects of the treatments against TAB and YM for barley during 6 hr with or without agitation. The numbers of TAB and YM in the barley were significantly decreased by AO as well as by AO combined with organic acids, regardless of agitation ($p<0.05$). The antimicrobial effects were significantly more pronounced in the combination treatments than in AO alone, regardless of agitation.

Studies have been reported the antimicrobial effects of AO for inactivating spoilage microorganisms in vegetables. Singh *et al.* (17) reported that an ozone water treatment with 10 min of immersion and agitation resulted in a 1.2 log reduction of *E. coli* O157:H7 in romaine lettuce. A combination treatment of 3 ppm ozone with 1% citric acid showed a higher antimicrobial effectiveness than either treatment of 3 ppm ozone or 1% citric acid alone in *enoki* mushrooms (*Flammulina veluptipes*) (18). In addition, Yoo (19) reported that different results were observed using a combination of 3 ppm ozone with various organic acids in lettuce and mushrooms, depending on the type of organic acid, microorganism, and treatment time. When using the combination of AO and CA, our results against TAB were consistent with these findings in carrots and cabbage, and in barley against TAB and YM. In the glutinous rice, the combination of AO and organic acid had much more antimicrobial effectiveness against YM than it did against TAB.

Khadre *et al.* (20) reported that the antimicrobial properties of ozone are associated with its hydroxyl radicals reacting with intracellular enzymes, nucleic material, or components such as cell envelopes, spore coats, and viral capsides. Others have reported using ozone to inactivate *E. coli* O157:H7 in apple cider and orange juice, and *Salmonella* Typhymurium on beef carcass

surfaces (21, 22). A high concentration of AO may result in a significant inactivation of microorganisms. However, it may also contribute to changing the organoleptic qualities of foods. Therefore, when using AO in foods, relatively lower ozone concentrations should be applied and treatment time and food type need to be taken into account.

Our data show the effectiveness of using AO combined with various organic acids for washing the raw materials of *saengsik*; although further studies may be needed before it can be used as a modified antimicrobial substance for these raw materials, our study has demonstrated that the combination of AO with organic acid has the potential for commercial application.

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