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Inactivation of Indoor Environmental Allergy-Related Substances by Ozonated Water *In Vitro*

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Abstract: Allergic diseases are thought to be caused by a combination of acquired immune activation, excessive activation of innate immunity, and disruption of epithelial barrier function due to scratching, proteases, and more. The removal and inactivation of allergy-related substances in indoor environments are considered effective for reducing allergic disease symptoms. The typical allergens in Japan are Japanese cedar pollen and house dust mites (HDM), and together with bacteria and fungi, HDM are the main sources of proteases in indoor environments. We investigated the inactivating effects of ozonated water on these substances *in vitro* in terms of allergenicity, inflammation induction in epithelial and immune cell lines (i.e., HaCaT, A549, and RAW 264 cells), and protease activity. We observed that ozonated water inactivated the Japanese cedar pollen allergen Cry j1 and the HDM allergen Der f1, the innate immune activator lipoteichoic acid from the bacterium *Staphylococcus aureus*, and proteases from *S. aureus*, HDM, and the fungus *Alternaria* in an ozone concentration-dependent manner. In all experiments, ozonated water at 7.5 mg/L significantly inactivated allergy-related substances compared to the untreated group ($p < 0.01$). The comparison of the effects of ozonated water treatment and thermal treatment at 80 °C revealed that ozonated water treatment is superior to thermal treatment in terms of both effectiveness and reaction time. Together, our findings demonstrate that ozonated water can inactivate allergy-related substances in the indoor environment. The management of indoor environments using ozonated water can thus be expected to contribute to the alleviation of symptoms and suppression of allergic diseases.

Keywords: Ozonated Water; Allergic Disease; Indoor Environment; Inactivation; Allergy-Related Substances

1. Introduction

The number of individuals in Japan with allergic diseases such as hay fever and perennial rhinitis is increasing every year, and about half of the country's population now suffers from some form of allergic disease [1]. Medication is an obvious way to improve allergy symptoms, but treatments such as desensitization therapy, cleaning the indoor

environment, and avoiding allergens are also recommended. Allergy-related substances in indoor environments in Japan include pollen, house dust mites (HDM), fungi, bacteria, pet hair, pollen, and air pollutants, and yellow sand entering from the outside air. Since HDM, fungi, and bacteria are not only generated indoors but also originate in human life itself, individuals who are suffering from an allergic disease should improve their indoor environments.

Allergic diseases have traditionally been considered diseases created by the activation of the acquired immune system, but in 2020, it was proposed that allergic diseases are actually diseases of excessive type 2 inflammation created by orchestration between the body's innate and acquired immune systems [2]. In other words, allergic diseases are thought to involve an activation of acquired immunity, excessive activation of innate immunity, and disruption of epithelial barrier function. In short, not only allergens but also pathogen-associated molecular patterns (PAMPs) [3] and proteases [4] are considered allergy-related substances. The removal or inactivation of these allergy-related substances in the environment is thus expected to prevent or alleviate the symptoms of allergic diseases.

Ozone has the advantages of exhibiting strong oxidizing power and having few problems concerning its persistence. In its gaseous state, ozone has the disadvantage of being toxic to the respiratory system, but ozonated water is safe to use because extremely low levels of ozone volatilize from the surface of the ozonated water [5]. In addition, ozone molecules can generate free hydroxyl radicals (·OH) in water, and these have stronger oxidizing power than ozone. Moreover, ozonated water is much easier to handle than ozone gas. Taking advantage of these features, ozonated water is already being used for disinfection in water purification plants [6] and in medical equipment for hand washing [7] and endoscope disinfection [8], and it is expected to be applied in other fields.

Ozonated water has been used for a long time in dental and oral medicine [9], but research on the improvement of allergic diseases using ozonated water is limited to animal experiments [10] and clinical studies [11] on atopic dermatitis (AD). These reports focus on the bactericidal effect on live Staphylococcus species or the restoration of microbial diversity. In other words, the effect of ozonated water on allergens, PAMPs, and proteases, which are allergy-related substances, is unknown.

In this study, we selected characteristic allergy-related substances in indoor environments in Japan and investigated their inactivation by ozonated water *in vitro*.

2. Materials and Methods

2.1. Ozonated Water

Ozonated water was prepared by an electrolytic ozone generator and diamond electrodes, with autoclaved ultrapure water produced by Milli-Q Reference (Merck, Darmstadt, Germany) as the raw water. After the measurement of the ozone concentration with a dissolved ozonated water meter (model OZM-300, Suisei Kogyo, Hyogo, Japan), the ozone concentration was adjusted by dilution with sterile ultrapure water (Milli-Q water).

2.2. Allergy-Related Substances

The Japanese cedar pollen allergen Cry j1 was purchased from BioDynamics Laboratory Inc. (Tokyo). The recombinant Der f1, the major allergen 1 of *Dermatophagoides farina*, was purchased from Asahi Food and Healthcare (Tokyo). Lipoteichoic acid (LTA) from *Staphylococcus aureus*, an agonist of Toll-like receptor 2 (TLR2), was purchased from InvivoGen (San Diego, CA). V8 protease (V8) from *S. aureus* was purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). *Dermatophagoides pteronyssinus* feces extract (Dpf) was purchased from Biostir (Osaka, Japan), and *Alternaria* extract (Alt) was purchased from the Institute of Tokyo Environmental Allergy (Tokyo).

2.3. Cell Lines and Cell Culture Reagents

Cells of the human epithelial keratinocyte cell line HaCaT were obtained from CosmoBio (Tokyo). Cells of the human lung epithelial cell line A549 and the mouse macrophage-like cell line RAW264 were obtained from RIKEN BioResource Center (Ibaraki, Japan). Dulbecco's modified Eagle's medium (DMEM) (high glucose) and penicillin-streptomycin solution were obtained from Fujifilm Wako Pure Chemicals. Fetal bovine serum (FBS) was obtained from Biowest (Nuaillé, France).

2.4. Treatment of Allergy-Related Substances with Ozonated Water

Prior to the reaction, each of the allergy-related substances was dissolved in Milli-Q water. In 1.5-mL tubes, various concentrations of ozonated water and allergy-related substance solutions were mixed at a ratio of 9:1 and reacted at 25 °C under shaking in a thermomixer (Eppendorf 5355; Eppendorf, Saxony, Germany) at 300 rpm. The reaction was stopped by adding 0.5% sodium thiosulfate solution or FBS-containing medium to the tube. The concentrations in the ozonated water were 1 μg/mL for the allergens and 10 or 100 μg/mL for other allergy-related substances. As a comparison experiment, V8 was treated in Milli-Q water at 80 °C with the same reaction time and stirring conditions.

2.5. Measurement of Antigenicity by ELISA

The antigenicity of Cry j1 and Der f1 was evaluated with commercial enzyme-linked immunosorbent assay (ELISA) kits (Institute of Tokyo Environmental Allergy). Each assay was performed according to the manufacturer's instructions. The absorbance was read at the wavelength 450 nm using a microplate reader (model 680XR, Bio-Rad, Hercules, CA). Residual antigenicity was calculated as 100% of the value for each allergen in Milli-Q water treatment (O_3: 0 mg/L).

2.6. Cell Culture and Cell Stimulation Experiments

HaCaT cells, A549 cells, and RAW 264 cells were maintained in DMEM containing high glucose (4,500 mg/L), sodium pyruvate (110 mg/L), and L-glutamine (584 mg/L), supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. The cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere.

In cell-stimulation experiments, cells were incubated in DMEM containing high glucose, sodium pyruvate, and L-glutamine, supplemented with 1% FBS and antibiotics [12]. HaCaT cells or A549 cells were seeded in 24-well plates at a density of 3.0×10^5 cells/well and pre-incubated at 37 °C for 24 h. RAW 264 cells were seeded in 24-well plates at a density of 6.0×10^5 cells/well and pre-incubated at 37 °C for 2 h. The cells were then stimulated with LTA or V8 for an additional 24 h. After stimulation, the culture supernatants were collected and stored at −20 °C until assayed.

2.7. Cell Viability Assay

For the measurements of cell viability, we used the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was read at the wavelength of 450 nm using the 680XR microplate reader. The cell viability ratio was calculated by the following formula: Cell viability (%) = (average of treated group/average of control group) × 100.

2.8. Measurements of the Levels of Inflammatory Markers in the Culture Supernatants

ELISA kits (BioLegend, San Diego, CA) were used to measure the protein levels of interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-alpha (TNF-α) in the culture supernatants. The assays were performed according to the manufacturer's instructions. The absorbance was read at 450 nm using the 680XR microplate reader. The nitric oxide (NO) concentration in the culture supernatants was measured with a Griess reagent system (Promega, Fitchburg, WI). The absorbance was read at 540 nm using the 680XR microplate reader.

2.9. Measurement of Proteolytic Activity Using Peptidyl-MCA Substrate

Protease activity was measured as described by Takai et al. [13] with some modifications. Briefly, 50 μL of enzyme solutions were incubated with or without 50 μL of 2 mM dithiothreitol (DTT) (Fujifilm Wako Pure Chemicals) for 5 min at 37 °C. Additional incubation with 50 μL of 100 μM enzyme substrate was conducted in the reaction buffer for 30 min at 37 °C on a 150-μL scale. The reaction was carried out in a 96-well microplate (Sumilon MS-8496K; Sumitomo Bakelite, Tokyo). The enzyme substrates used were benzyloxycarbonyl-Leu-Leu-Glu-MCA (Z-LLE) for V8 and butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA) for Dpf and Alt. These enzyme substrates were obtained from Peptide Institute (Osaka, Japan). The fluorescence of 7-amino-4-methylcoumarin (AMC) released from the peptidyl-MCA substrates was measured at 365-nm excitation and 465-nm emission on a fluorome-

ter (Multimode Detector DTX 800, Beckman Coulter, Brea, CA).

2.10. Evaluation of the Protease Activity Using a Cross-Linked Gelatin Thin Film (cGTF)

Cross-linked gelatin thin film (cGTF) was prepared as described [14]. Briefly, gelatin from porcine skin (APH-200, Nitta Gelatin, Osaka, Japan) was dissolved in a reaction buffer (25 mM Tris–HCl, pH 6.8) at 60 °C for 1 h under shaking to prepare a 2%(w/v) gelatin solution. After cooling down to 45 °C, glutaraldehyde (GA) (Fujifilm Wako Pure Chemicals) was added to the gelatin solution along with these reagents, and then the solution was added to a 100-mm-diameter petri dish for the formation of a crosslinked film. This reaction was carried out on a clean bench to avoid contamination with environmental proteases. The thickness of the cGTF was held to approximately. 10 µm. The final concentrations of the reagents were 0.025% for GA and 2 mM for DTT.

One µL of the sample solutions was dropped onto a cGTF, incubated in a moist box (15 × 23 × 14 cm) containing 100 mL of water at 37 °C for 16 h, and kept at 4 °C for 30 min. The cGTF dish was then stained with an ice-cold Biebrich scarlet solution (Fujifilm Wako Pure Chemicals) for 5 min at room temperature. After being washed with ice-cold methanol, the cGTF dish was dried at room temperature for 1 h.

2.11. SDS-PAGE and Silver Staining

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were performed to investigate the effect of ozonated water on V8 protease molecules. The untreated (Milli-Q water treatment), ozonated, or heated V8 protein samples were dissolved in sample loading buffer (Wako Pure Chemicals) and then loaded onto a 12% SDS–PAGE gel (Bio-Rad Laboratories, Berkeley, CA). After electrophoresis, the V8 proteins in the gel were visualized with a silver staining kit (Cosmo Bio) according to the manufacturer's protocol.

2.12. Statistical Analysis

The results were examined using Student's t-test after confirming equal variance using the F-test, and are presented as the mean ± standard deviation (SD) (n = 3 or 4). Statistical significance is denoted as $*p < 0.05$ or $**p < 0.01$.

3. Results

3.1. Inactivation of Allergens by Ozonated Water

The antigenic changes in the Japanese cedar pollen allergen Cry j1 and in the HDM allergen Der f1 by ozonated water treatment are illustrated in **Figure 1**. The concentration of both allergens was 1 mg/L. We observed that allergenicity was almost inactivated at ozone concentrations >0.5 mg/L for Cry j1 (**Figure 1a**) and >1 mg/L for Der f1 (**Figure 1b**).

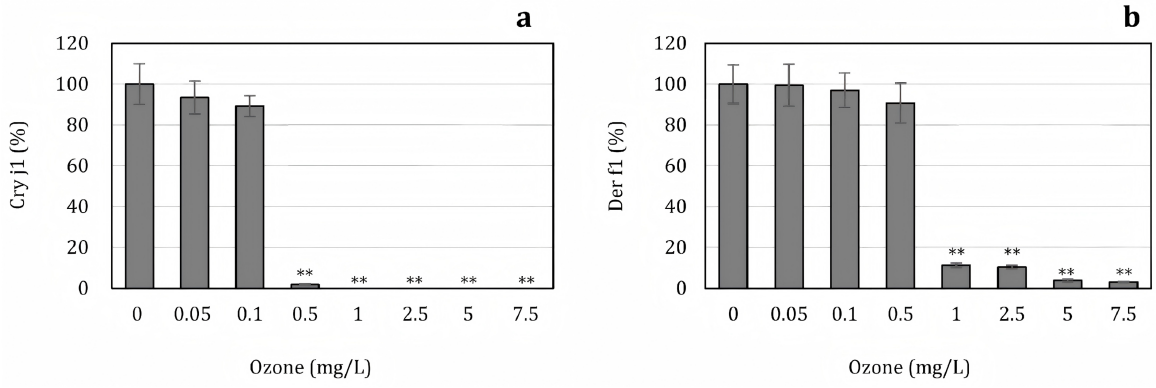

Figure 1. The inactivation of allergens by ozonated water. Cry j1 (**a**) or Der f1 (**b**) were treated with ozonated water for 30 min at 25 °C with shaking at 300 rpm. Both allergen concentrations in the ozonated water were 1 mg/L. Residual antigenicity was calculated as 100% of the value for each allergen in Milli-Q water treatment (O_3: 0 mg/L). The results are mean ± standard deviation (SD) (n = 3), by Student's *t*-test.

Note: ** $p < 0.01$ vs. the Milli-Q water treatment.

3.2. Inactivation of Lipoteichoic Acid (LTA)-Induced Innate Immune Activation by Ozonated Water

The changes in innate immune activation by *S. aureus*-derived LTA after treatment with ozonated water are shown in **Figure 2**. The LTA concentration during the ozonated water treatment was 100 mg/L. The activation of innate immunity by LTA was examined with cells of the human skin keratinocyte cell line HaCaT, the human lung epithelial-derived cell line A549, and the mouse macrophage-like cell line RAW264. The concentration of inflammatory markers in the culture supernatants was evaluated as an indicator. The LTA concentrations in culture medium during cell stimulation experiments were 10 mg/L for the HaCaT and A549 cells and 1 mg/L for RAW264 cells.

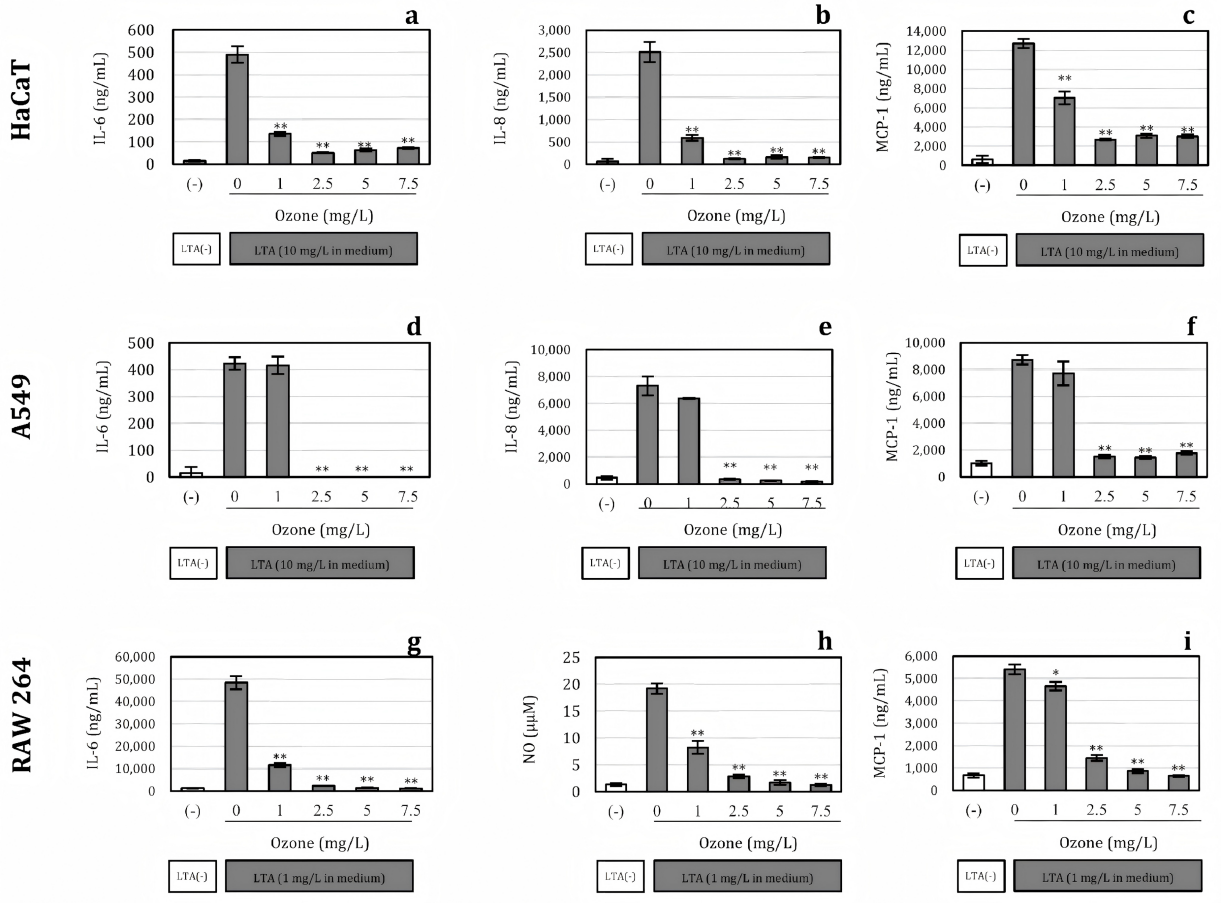

Figure 2. The inactivation of LTA-induced innate immune activation by ozonated water. LTA was treated with ozonated water for 30 min at 25 °C with shaking at 300 rpm. The LTA concentration during ozonated water treatment was 100 mg/L. HaCaT cells (**a–c**), A549 cells (**d–f**), and RAW 264 cells (**g–i**) were stimulated by ozone-treatment (O_3: 1, 2.5, 5, 7.5 mg/L) or Milli-Q water treatment (O_3: 0 mg/L) LTA for 24 h. The LTA concentrations in the culture medium during the cell stimulation experiments were 10 mg/L for the HaCaT and A549 cells and 1 mg/L for the RAW264 cells. The concentrations of inflammatory mediators in the supernatants of these cells were evaluated. The concentrations of inflammatory markers were normalized by cell numbers. The data are mean ± SD (n = 4), by Student's *t*-test.

Note: * $p < 0.05$, ** $p < 0.01$ vs. the Milli-Q water treatment.

When the cell viability in the absence of LTA was set as 100%, the cell viability of the LTA-added group in each cell line was as follows: in the HaCaT cells, the cell viability was 98.6% in the untreated LTA group (Milli-Q-water treatment) and 85.9% in the ozonated LTA group (O_3: 7.5 mg/L). In the A549 cells, the cell viability was 99.3% in the untreated LTA group and 100.5% in the ozonated LTA group. In the RAW264 cells, the cell viability was 98.3% in the untreated LTA group and 98.7% in the ozonated LTA group. The concentrations of IL-6, IL-8, MCP-1, and nitric oxide (NO) were normalized by the cell numbers. The evaluations of the IL-6, IL-8, and MCP-1

concentrations in the culture supernatants revealed that ozonated water significantly suppressed the LTA-induced inflammation in HaCaT cells and A549 cells at ozone concentrations >1 mg/L (**Figure 2a–c**) and >2.5 mg/L (**Figure 2d–f**), respectively. In the case of RAW 264 cells and IL-6, NO, and MCP-1 in the culture supernatants, the LTA-induced inflammation was significantly suppressed by ozonated water at concentrations ≥1 mg/L (**Figure 2g–i**).

3.3. Inactivation of Proteases by Ozonated Water

The changes in the protease activity of V8 from *S. aureus*, the HDM feces extract Dpf, and the fungi extract Alt after ozonated water treatment are depicted in **Figure 3a–c**. We observed that V8, Dpf, and Alt, in that order, were more likely to be inactivated by ozonated water and were dependent on both the ozone and protease concentrations for inactivation. At 100 mg/L and 10 mg/L V8, the ozone concentrations required to halve the protease activity were 5 mg/mL and 1 mg/L, respectively (**Figure 3a**). At 100 mg/L and 10 mg/L Dpf, the ozone concentrations required to halve the protease activity were 7.5 mg/mL and 2.5 mg/L, respectively (**Figure 3b**). At 100 mg/L Alt, the protease activity was not halved even at an ozone concentration of 7.5 mg/L, and at 10 mg/L Alt, the ozone concentration required to halve the activity was 5 mg/L (**Figure 3c**).

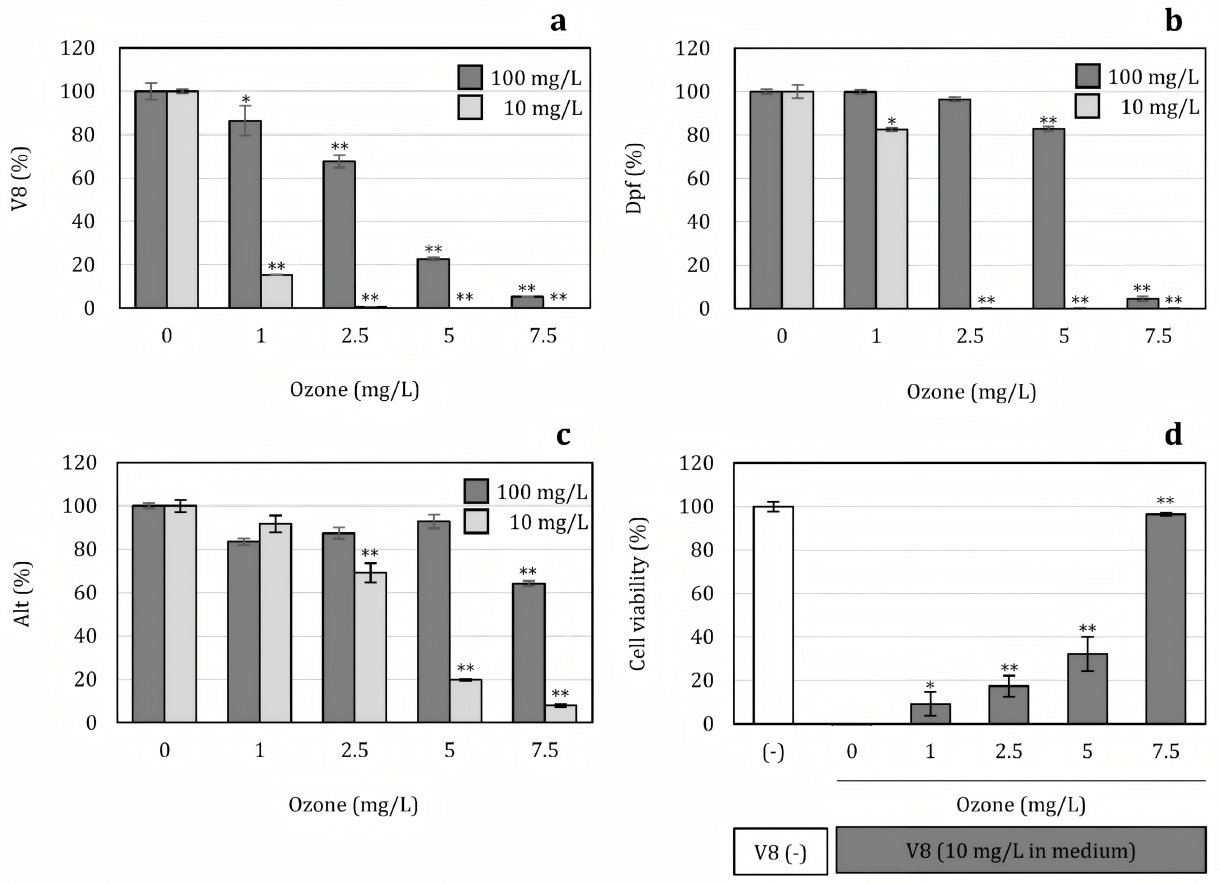

Figure 3. The inactivation of proteases by ozonated water. V8, DPF, and Alt were each treated with ozonated water for 30 min at 25 °C with shaking at 300 rpm. The concentrations of these reagents in ozonated water were 100 and 10 mg/L. The protease activities of V8 (**a**), Dpf (**b**), and Alt (**c**) were evaluated using MCA as the substrate. The cytotoxicity of V8 (**d**) was evaluated by measuring the cell viability in HaCaT cells. The V8 concentration during the ozonated water treatment was 100 mg/L, and the V8 concentration in the culture medium during HaCaT cell stimulation was 10 mg/L. After 24-h stimulation, the viability of the HaCaT cells was evaluated based on the intracellular dehydrogenase activity. Residual protease activity was calculated as 100% of the value for each protease in Milli-Q water treatment (O_3: 0 mg/L). The cell viability in the absence of V8 was calculated as 100%. The results are mean ± SD (A: n = 4, B: n = 3, C: n = 3 (100 mg/L) or 5 (10 mg/L), D: n = 4).

Note: * $p < 0.05$, ** $p < 0.01$ vs. the Milli-Q water treatment, by Student's *t*-test.

Because we observed that V8 exerted a cytotoxic effect on HaCaT cells, we investigated the effect of ozonated water on V8's cytotoxic effect. This effect was inhibited in an ozone concentration-dependent manner, and the effect was almost inhibited when the ozone concentration of 7.5 mg/L was used at 100 mg/L of V8 (**Figure 3d**).

3.4. Comparison of Ozonated Water Treatment and 80 °C Heat Treatment for V8 Protease

The differences in the protease activity of V8 by ozonated water treatment and heat treatment (wet condition) are shown in **Figure 4a,b**. The heat treatment of 100 mg/L V8 (Milli-Q water, 80 °C, 30 min) was maintained approximately. 75% protease activity, whereas the ozonated water treatment (O_3: 7.5 mg/L, 25 °C, 30 min) nearly eliminated the protease activity (**Figure 4a**). After the treatment, we dropped 100 ng of V8 onto weakly cross-linked gelatin film (cGTF) and kept the film in 100% humidity at 37 °C for 16 h. Both the heat-treated and untreated (Milli-Q water, 80 °C, 30 min) V8 degraded cGTF, but ozonated V8 did not, which is the same result as that obtained with the Milli-Q water (V8-free) (**Figure 4b**). Visualization of V8 molecules by SDS-PAGE and silver staining showed that the band density of V8 molecules decreased in an ozone concentration-dependent manner by ozonated water treatment, and the band almost disappeared at 7.5 mg/L. On the other hand, the band of V8 molecules heated at 80 °C showed a decrease in density compared to the untreated one (Milli-Q water treatment), but was still visible (**Figure 4c**). The time required for sufficient V8 inactivation by ozonated water was 1 min (**Figure 4d**).

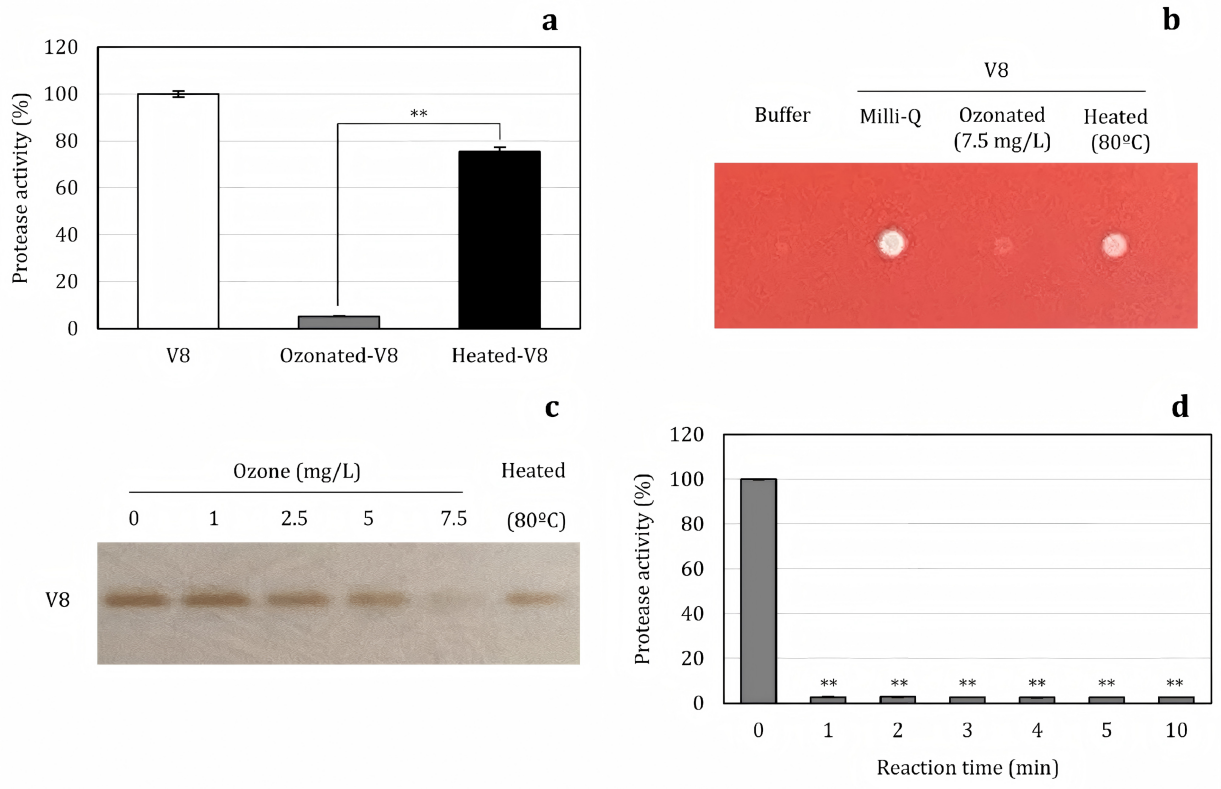

Figure 4. The comparison of ozonated water treatment and 80 °C heat treatment for V8 protease. V8 was treated with ozonated water (O_3: 7.5 mg/L) at 25 °C or with Milli-Q water at 25 °C or 80 °C for 30 min with shaking at 300 rpm. The V8 concentration was 100 mg/L. After the treatment, the protease activities of these V8 samples were examined by using MCA substrate (**a**) and cGTF (**b**). The V8 molecules were visualized by SDS-PAGE and silver staining (**c**). The time required for V8 inactivation by ozonated water (O_3: 7.5 mg/L, 25 °C, 300 rpm) was investigated (**d**). The residual protease activity was calculated as 100% of the value in the Milli-Q water treatment (O_3: 0 mg/L). The data are mean ± SD (A: n = 4, C: n = 3).

Note: ** $p < 0.01$ between the ozonated water treatment and the heat treatment (**a**) and between the ozonated water treatment and Milli-Q water treatment (**d**), by Student's *t*-test.

4. Discussion

Allergic diseases are now recognized as public health problems that have a significant impact on low- and middle-income countries in addition to high-income countries [15]. Although various medications comprise the most common response to allergy symptoms, there are other ways to reduce allergy symptoms that individuals themselves can apply in their daily lives, such as keeping the indoor environment clean [1] and consuming foods that alleviate allergy symptoms [16,17]. With regard to foods, we have been studying the usefulness of lactic acid [18] and *Citrus jabara* fruit peel [19–21], and in the present study, we focused on cleaning the indoor environment; specifically, we investigated the inactivation effect of ozonated water on allergy-related substances *in vitro*. As allergens, the Japanese cedar pollen allergen Cry j1 and the HDM allergen Der f1, which are the main causes of allergic disease in Japan [22], were examined, and the results of our experiments demonstrated that ozonated water inactivated the allergenicity of both Cry j1 and Der f1 in an ozone concentration-dependent manner (**Figure 1**).

Excessive activation of innate immunity is also associated with the development of allergic diseases. For example, *S. aureus* is closely related to atopic dermatitis [23]. LTA, a PAMP from *S. aureus*, is known as an agonist of TLR2 [24]. LTA has been used as an allergy-related substance to activate innate immunity. Our present analyses revealed that in skin keratinocyte HaCaT cells, lung epithelial A549 cells, and macrophage-like RAW264 cells, the addition of Milli-Q water-treated LTA induced the production of inflammatory mediators, and that the pre-treatment of LTA with ozonated water suppressed this production in an ozone concentration-dependent manner (**Figure 2**).

Disruption of epithelial barrier function is also thought to be involved in allergic disease, and proteases are thought to be involved in this process [25]. Since bacteria, HDM, and fungi are known main sources of proteases in indoor environments [25,26], we examined V8 from the bacterium *S. aureus*, Dpf (a fecal extract of HDM), and the fungal extract Alt. V8 is a purified product, whereas Dpf and Alt are crude products. Although differences in the effect for each protease were observed, ozonated water inactivated these protease activities in an ozone concentration-dependent manner (**Figure 3a–c**). The differences in these effects may be due to the differences between the purified and crude products. The crude products contained many foreign substances, which may have depleted ozone molecules. V8 also exerted cytotoxic effects on HaCaT cells, but ozonated water suppressed the cytotoxic effects in an ozone concentration-dependent manner (**Figure 3d**).

We used V8 and a 30-minute treatment time to investigate the differences between ozonated water treatment (O_3: 7.5 mg/L) and heat treatment (wet condition, 80 °C), and the results demonstrated that approx. 75% of the protease activity remained, and gelatine film (cGTF) was also degraded in the heat treatment, whereas with the ozonated water treatment, the protease activity was almost inactivated, and gelatine membranes were not degraded (**Figure 4a,b**). Because gelatin is derived from dermal collagen, the results obtained for cGTF (**Figure 4b**) suggest that V8 damages the skin, but ozonated water inhibits it. Visualization of V8 molecules by SDS-PAGE and silver staining showed that ozone-treated water (7.5 mg/L) caused almost complete degradation of V8 molecules, whereas heat treatment did not (**Figure 4c**), which is thought to have resulted in the significant difference in protease activity. In addition, the time required for sufficient V8 inactivation by ozonated water was only 1 min (**Figure 4d**). These results demonstrate that the present ozonated water treatment is superior to the thermal treatment in terms of both effectiveness and reaction time.

The inactivation of allergy-related substances by ozonated water is thought to be caused by oxidative reactions involving both ozone molecules and hydroxyl radicals in water [27]. The reaction of ozone molecules is highly selective, resulting in some substances being highly reactive and others being less reactive. On the other hand, hydroxyl radicals generated by the decomposition of the ozone molecule are less selective and highly reactive. Oxidation of peptides and proteins by ozone in water occurs mainly at tyrosine, tryptophan, histidine, cysteine, and methionine residues [28]. The rates of oxidation of these amino acids by ozone are very rapid, with half-lives mostly in the range of milliseconds to tens of seconds (O_3: 1 mg/L, pH 8). Oxidation of proteins results in a change in their folding ability and tertiary structures. Since allergens and proteases are proteins, the loss of allergenicity and protease activity caused by ozonated water in this experiment is presumed to be due to the abovementioned oxidation mechanism. In this study, V8 protease activity was almost completely inactivated within one minute after exposure to 7.5 mg/L ozonated water (**Figure 4d**), which is consistent with the reaction rate of ozone oxidation described above. Comparison of V8 protease activity (**Figure 3a**) and V8 molecular bands (**Figure 4c**) after ozonated water treatment suggests that protease inactivation occurs at lower concentrations than the decrease in V8 molecular band density,

as can be clearly seen at 5 mg/L. This suggests that the inactivation of V8 protease activity by ozone-treated water is caused by both changes in the three-dimensional structure of the molecule and the degradation of the molecule, and that structural changes occur at lower concentrations than degradation.

Clinical studies and animal experiments on ozonated water have been conducted in the fields of dental and oral medicine and allergic diseases [9–11]. Regarding the relationship between ozonated water and allergic diseases, Kaneki et al. demonstrated that ozonated water at concentrations of 3 and 11 mg/L exhibits dual bactericidal and anti-inflammatory effects in mice with AD [10]. In the present study, ozonated water (1, 2.5, 5, and 7.5 mg/L) inactivated allergy-related substances such as allergens, PAMPS, and proteases, suggesting that inactivation of allergy-related substances also contributed to improvement in mice with AD. Therefore, the results of this study are considered to support the application of ozonated water to AD. Clinical and microbial effects of ozone-nanobubble water irrigation in patients with periodontitis have also been reported [29]. *Porphyromonas gingivalis*, a representative causative bacterium of periodontal disease, is known to induce inflammation by stimulating Toll-like receptors and by its protease [30]. Therefore, the inactivating effects of ozonated water on PAMPs and proteases demonstrated in this study may also contribute to the use of ozonated water in dental and oral medicine.

The ozone concentrations in the ozonated water that we used herein (1, 2.5, 5, and 7.5 mg/L) are similar to or lower than the ozone concentrations (7 ± 3 ppm) in medical equipment that is designed for hand hygiene at medical facilities [7]. Regarding the safety of ozonated water on skin, this product's safety was observed at 4 mg/L in a study using a three-dimensional cultured epidermal model of human keratinocytes [31], and no cytotoxicity was observed at 0.25–8 mg/L in a monolayer culture model of human keratinocytes and fibroblasts [32]. Those studies' authors reported that ozonated water was safer than ethanol, benzalkonium chloride, chlorhexidine, and other chemicals at the concentrations tested. Regarding hand disinfection, ozonated water has been reported to be as effective as or more effective than alcohol [33,34].

The use of ozonated water in indoor environments requires consideration of its safety for the human body. A systematic review of the risks of ozonated water to human skin found no significant dermatological risks reported, but, since the longest treatment period was only six weeks, further short- and long-term trials are recommended [35]. However, since ozonated water is used in dental and oral medicine [9,29] and for the treatment of hand infections [7,33,34], and since direct exposure to the human body is not anticipated, the risk of using ozonated water in indoor environments is considered low. Thus, in terms of both the inactivation effect and safety, ozonated water could be used to inactivate allergy-related substances in indoor environments.

In summary, our findings demonstrate that ozonated water can inactivate allergy-related substances in indoor environments. The management of indoor environments using ozonated water may contribute to the alleviation of allergic disease symptoms and the suppression of the onset of allergic diseases. The application we propose is an ozonated water sprayer, which has already been developed and is commercially available. Although it is affected by conditions such as temperature and raw water quality, it is capable of supplying ozonated water at a concentration equivalent to that used in this experiment. In actual use in indoor environments, the biggest factor inhibiting the inactivation effect of ozonated water is considered to be organic substances other than allergen-related substances. This problem can be overcome by supplying ozonated water that exceeds the amount of ozone consumed by these substances, because, in actual use, the effect depends on the amount of ozone supplied. Regarding the time required for the inactivation of allergen-related substances, as mentioned above, the reaction rates of ozone and hydroxyl radicals in water are both extremely fast, so it is not expected that the time required for the inactivation of allergen-related substances will be a significant issue in actual use. However, to achieve clinical efficacy, detailed evaluations of actual use in indoor environments are necessary, and these remain future challenges.

5. Conclusions

Ozonated water caused the inactivation of allergens, PAMPs, and proteases that are characteristic of allergy-related substances in indoor environments *in vitro*. These effects of ozonated water may contribute to the alleviation of symptoms and the suppression of the onset of allergic diseases. However, further research is needed to verify this in actual use.

Author Contributions

Conceptualization: Y.M., Y.T., K.E., K.B., M.T., and F.F.; Methodology: Y.M., Y.T., and K.E.; Experimental work: Y.M., Y.T., and K.E.; Writing—original draft preparation: Y.M.; Writing—review and editing: Y.M., Y.T., K.E., K.B., M.T., and F.F.; Supervision: F.F.; Project administration: Y.M., Y.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

This study, titled "Inactivation of Indoor Environmental Allergy-Related Substances by Ozonated Water *in vitro*", did not involve any experiments on humans or animals conducted by the authors. The research does not include any identifiable personal or clinical information. Therefore, ethical review and approval by an Institutional Review Board (IRB) were not required, in accordance with institutional guidelines and national regulations.

Informed Consent Statement

This study did not involve human participants, human data, or human tissue. Therefore, informed consent was not required. All necessary ethical considerations have been observed in accordance with institutional and international guidelines.

Data Availability Statement

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare no conflict of interest.

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