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ORIGINAL ARTICLE

# Allergic Potency of Japanese Cedar Pollen Cry j 1 Is Reduced by a Low Concentration of Hypochlorous Acid Generated by Electolysis

Tetsuya Terada[1], Yoko Akimoto[1,2], Sawako Hyo[1], Daisuke Suzuki[3], Kazuhiko Tanaka[2] and Hiroshi Takenaka[1]

## ABSTRACT

**Background:** Although Japanese cedar (Cryptomeria japonica) pollinosis has developed into a health problem, few methods eradicate indoor allergens completely. In a recent study, however, the effectiveness of inactivation with sodium hypochlorite (NaOCl) treatment was revealed. Therefore, the present study aimed to elucidate the ability of chlorine bleach (NaOCl) to reduce the immunogenicity of the major allergenic protein of Japanese cedar (Cry j 1).

**Methods:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis, Western blotting, enzyme-linked immunosorbant assay, and skin testing were carried out in 7 individuals.

**Results:** The allergenic protein was undetectable using sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining at a sodium hypochlorite/allergenic protein molar ratio of 457. Western blotting with human sera showed the same dose-dependent efficacy. The immunogenicity of the purified protein and cedar pollen was also demonstrated on enzyme-linked immunosorbant assay to be reduced by sodium hypochlorite treatment in a dose- and time-dependent manner. Moreover, sodium hypochlorite-treatment inhibited the skin test response to the protein in all 7 individuals.

**Conclusions:** Hypochlorous acid generated by electrolysis is an effective method for significantly reducing the immunogenicity of Cry j 1.

## KEY WORDS

Cry j 1, human, hypochlorous acid, immunogenicity, Japanese cedar pollinosis

## *INTRODUCTION*

Pollen allergens are thought to be a risk factor for pollen allergy only outdoors. However, pollen grains originating from allergenic plants reach the indoor environment, and it is difficult to remove residual allergen that contaminates the home. Frequent indoor cleaning eliminates the largest particles but may not have a particularly great effect on particles of submicronic size. Moreover, vacuum cleaning actually resuspends settled particles in the air.

Although there are effective methods of reducing exposure to dust mite allergen,[1] few effective methods exist for eradication of animal and cockroach allergens.[2-4] Protein denaturants have therefore been suggested as a useful method of rendering residual protein immunologically inactive.

NaOCl is widely used as a disinfectant in water treatment in hospitals, laboratories, schools, prisons, and homes. Its widespread use reflects its ability to kill a broad spectrum of microorganisms in a concentration that presents little toxicity to individuals.[5,6]

Several studies have demonstrated that NaOCl also reduces the detection of protein antigens by ELISA.[7]

[1]Department of Otorhinolaryngology, Osaka Medical College, [2]Clinical Pharmacy and Clinical Pharmacokinetics, Osaka University of Pharmaceutical Sciences, Osaka and [3]R & D H.Q. Human Ecology Research Center, SANYO Electric Co., Gunma, Japan.
T.T. and Y.K. contributed equally to this work.
Correspondence: Tetsuya Terada, Department of Otorhinolaryngology, Osaka Medical College, 2–7 Daigaku-cho, Takatsuki, Osaka 569–8686, Japan.
Email: tetsu@yc4.so–net.ne.jp
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Antigen levels also fell when allergen-contaminated smooth surfaces were wiped with NaOCl solution.[7] Furthermore, Matsui *et al.* found that low concentrations of NaOCl reduced detection of cat antigen in solution.[8]

Together, these studies demonstrate the ability of NaOCl to reduce indoor allergens. Periera *et al.* hypothesized that the mechanism for this effect was a result of chlorination of amide nitrogen followed by protein oxidation to an imine with further hydrolysis, thus denaturing allergenic proteins.[9,10]

Recently, electrolyzed NaCl solution has become popular for sanitation in the medical and food industries in Japan.[11] It is known that electrolyzed NaCl solution contains hypochlorous acid and hypochlorous ions, and also has a strong disinfectant effect for various microbes.[12] These findings encouraged us to test the potential effects of hypochlorous acid generated by electrolysis on the allergen protein of Japanese cedar pollen. The purpose of this study was to test the ability of chlorine bleach to reduce the immunogenicity of Cry j 1, the major allergenic protein of Japanese cedar pollen.

## *METHODS*

Hypochlorous acid solution was generated from saline solution by electrolysis with an electrical current of 1.4 mA. Subsequently, the objective free chlorine concentration was adjusted by dilution.

Free chlorine concentrations were determined to be >2.00 ppm by the Pocket Colorimeter Analysis System and SwifTest DPD Free Chlorine Reagent Dispenser (Hach Company, CO, USA).

Cry j 1 (Hayashibara, Okayama, Japan) and cedar pollen extracts (Torii Pharmaceutical, Tokyo, Japan) were incubated for 1 hour with increasing concentrations of NaOCl and analyzed by means of SDS-PAGE, Western blotting, and ELISA.

### SDS-PAGE AND WESTERN BLOTTING

SDS-PAGE was conducted according to the method of Laemmli. Four percent to 20% precast polyacrylamide gels (Daiichi Pure Chemicals, Tokyo, Japan) were used for protein electrophoresis. Samples were analyzed under reducing conditions by boiling them in gel-loading buffer containing β-mercaptoethanol for 2 minutes, electrophoresing at 30 mA for 1 hour, and visualizing with silver stain (Biorad). For Western blotting, the separated proteins were then transferred onto polyvinilydene difluoride membranes (Immobilon P, Millipore) using 110 mV constant for 1 hour. The membranes were blocked with 2.5% nonfat milk in TTBS (tris-buffered saline and 0.05% Tween-20, pH 7.4) for 1 hour, before incubating with human sera. Human sera were obtained from three volunteers with Japanese cedar pollinosis for Western blot. After washing with TTBS 3 times for 5 minutes each, the blots were incubated with anti human IgE antibody (Asahi Breweries, Tokyo, Japan) for 3 hours at room temperature. Finally, after washing, the membrane was detected by goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (Biorad). Protein bands were visualized on chemiluminescence film using ECL plus Western blotting detection regent (Amersham Biosciences, UK).

### ELISA

Polystyrene microtiter plates (Dynex Technologies, VA, USA) were coated with monoclonal anti-Cry j 1 mAb013, diluted with coating buffer to 2 μg/mL, at 4℃ overnight. The plates were washed 3 times and then blocked with 1% BSA for 30 minutes at room temperature (RT). The test samples, along with a standard curve, were added to the plate at RT overnight. The plates were washed 3 times, and then monoclonal anti-Cry j 1 mAb053 conjugated with horseradish peroxidase was added to the wells and incubated for 2 hours at RT and developed with ortho-phenylendiamine in 0.1 mol/L citrate-0.2 mol/L phosphate buffer at pH 7.2, containing 0.01% hydrogen peroxide. Plates were read at 490 nm.

### SKIN TEST

After approval of the human research protocol by the Research Committee at Osaka Medical College, we studied the effect of NaOCl on Cry j 1 skin test extracts (contains 0.3 μg/ml Cry j 1, Torii Pharmaceutical). We enrolled and screened 7 subjects who reported a clinical diagnosis of Japanese cedar pollen allergy. Only those in whom Cry j 1 skin test reactivity could be verified were included in the remainder of the study. These remaining subjects were skin tested with untreated Cry j 1 extracts and those prepared from solutions treated with NaOCl. Skin tests were measured by the same observer. The diameter of the wheal and flare response was recorded for each extract. A positive skin test result was one with a minimum wheal diameter of 9 mm or a minimum flare diameter of 20 mm.

## *RESULTS*

### EFFECT OF NaOCl ON PROTEIN CONTENT OF Cry j 1

Cry j 1 was incubated with increasing concentrations of NaOCl and analyzed using SDS-PAGE and silver staining. Untreated Cry j 1 was detected as two bands around 40–45 kD after silver staining. Silver-stained protein was preserved at lower NaOCl/Cry j 1 molar ratios (between 45 and 112) but began to dissipate as the molarity of NaOCl was increased to a ratio of between 223 and 457, and was undetectable at an NaOCl/Cry j 1 molar ratio of 457 (Fig. 1). These results were confirmed in repeated protein gel analyses.

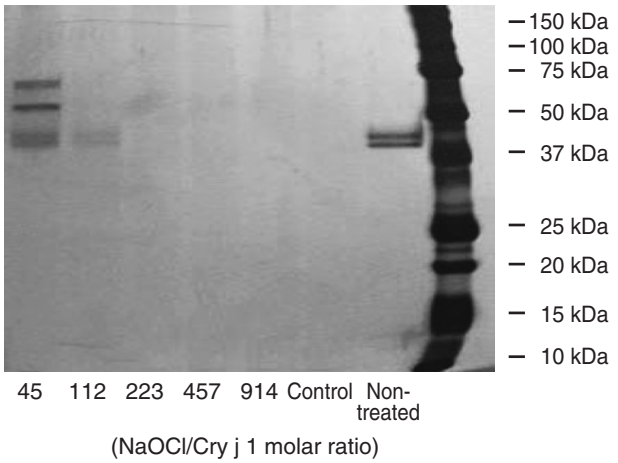


**Fig. 1** Effect of NaOCl on Cry j 1. SDS-PAGE and silver staining of NaOCl-treated purified Cry j 1. NaOCl amount is expressed as NAOCl/Cry j 1 molar ratio. Cry j 1 begins to disappear at an NaOCl/Cry j 1 ratio of 112, and no protein is detected at a molar ratio of 914.

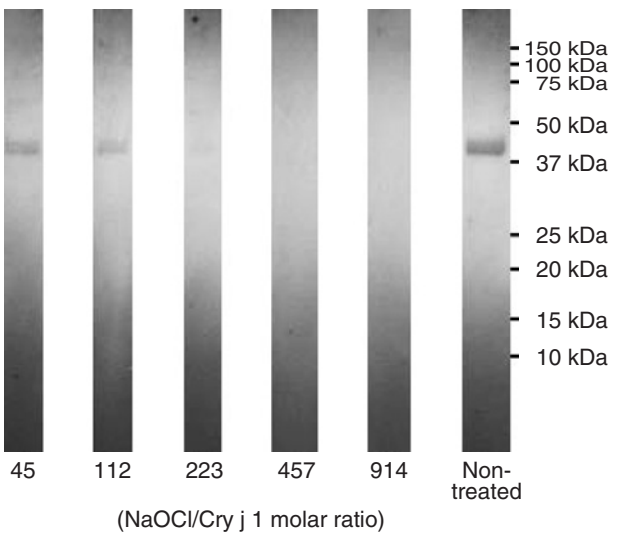


**Fig. 2** Effect of NaOCl on Cry j 1 as shown by Western blotting. Western blotting of NaOCl-treated Cry j 1. The detection antibodies are human anti-Cry j 1 IgE and human anti-IgE mAb. A distinct loss of antibody binding occurs at an NaOCl/Cry j 1 ratio of 223. No antibody binding is detected at a molar ratio of 457.

## EFFECT OF NaOCl ON IMMUNOGENICITY OF Cry j 1

After treatment with increasing concentrations of NaOCl, Cry j 1 was analyzed using Western blotting with human sera. Antibodies in the sera recognized bands around 40–45 kD (Fig. 2). This banding decreased at a NaOCl/protein molar ratio of 223, was almost absent at a molar ratio of 457, and was undetectable at a molar ratio of 914.

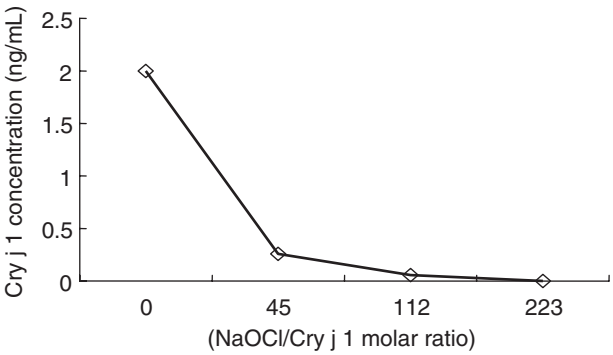


**Fig. 3** Effect of NaOCl on Cry j 1 immunogenicity. ELISA of NaOCl-treated purified Cry j 1. The concentration of Cry j 1 is expressed in final optical density values of solution. NaOCl is expressed in the NaOCl/Cry j 1 molar ratio. Decreases in the quantity of Cry j 1 detected are seen at an NaOCl/Cry j 1 molar ratio of 45, and no Cry j 1 is detected at 223.

NaOCl-treated purified Cry j 1 protein was also analyzed using ELISA with anti-Cry j 1 mAb013 to quantify the effect of NaOCl on the immunogenicity of Cry j 1. Results verified the qualitative decrease in immunogenicity that was seen on Western blots. Anti-Cry j 1 antibody binding decreased with increasing concentrations of NaOCl; there was considerable loss of antibody binding at a molar ratio of 45, and very little binding occurred at an NaOCl/Cry j 1 molar ratio of between 112 and 223 (Fig. 3).

## EFFECT ON CEDAR POLLEN EXTRACT

The effect of NaOCl on Cry j 1 skin test extracts was also examined. Qualitatively similar results were found using ELISA. About 80% of measurable allergen was lost at an NaOCl/protein molar ratio of $24 \times 10^3$, and all of the allergen was lost at a molar ratio of $84 \times 10^3$ (Fig. 4A). Much higher concentrations of NaOCl were required to reduce antibody binding to Cry j 1 than those required for degradation of the purified allergen because cedar pollen extract contains large amounts of glycerol which consumes NaOCl.

In addition, we examined the time-response relationship of the inactivation effect of NaOCl. Cry j 1 was reduced by 100% at 5 minutes with an NaOCl/protein molar ratio of $11 \times 10^3$ (Fig. 4B).

## SKIN TEST RESPONSE

We compared results of skin tests conducted with NaOCl-treated extract and those conducted with untreated extract. All 7 individuals who reported that they were allergic to Cry j 1 had positive skin test reactions to 200 JAU/mL pollen extract allergen (Fig. 5 A). NaOCl-treatment with a protein molar ratio of $11 \times 10^3$ failed to inhibit positive reaction to the extract (Fig. 5B), but a protein molar ratio of $419 \times 10^3$ com-

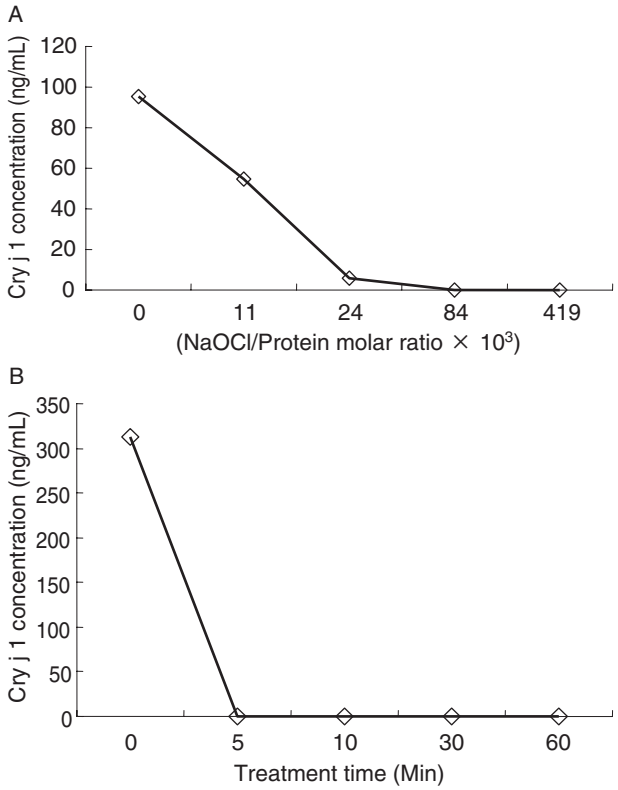


**Fig. 4** Effect of NaOCl on cedar pollen extract. **A**) Dose relationship of NaOCl to concentration of Cry j 1 in cedar pollen extract. The concentration of Cry j 1 within the solution is expressed in final optical density values of solution. Decreases in the quantity of Cry j 1 detected are seen at an NaOCl/protein molar ratio of $11 \times 10^3$, and no Cry j 1 could be detected at $84 \times 10^3$. **B**) Treatment time relationship of NaOCl effect on cedar pollen extract. The concentration of Cry j 1 within the solution is expressed in final optical density values of solution. Cedar pollen extract was treated by hypochlorous acid at an NaOCl/protein molar ratio of $84 \times 10^3$ at several different times. No Cry j 1 could be detected at 5 minutes.

pletely inhibited the reaction in all 7 individuals (Fig. 5C).

## *DISCUSSION*

In this report, we have demonstrated that NaOCl reduces immunogenicity of Cry j 1. Similar effects were found for cedar pollen extract, although higher concentrations of NaOCl were required to achieve the same reduction. Importantly, our results demonstrated that NaOCl fragments allergenic proteins and that this fragmentation reduces binding of human IgE antibody. Fragmentation occurs rapidly and at relatively low concentrations of NaOCl.

On Western blotting using human antibody, bands disappeared at the treatment dose of NaOCl. However, the dose of NaOCl that was required to significantly reduce immunogenicity was less than that required to degrade the protein. This finding suggests that NaOCl affects the IgE epitopes of Cry j 1 at lower concentrations than those required to break peptide bonds. Previous studies[7] examining the effects of NaOCl on purified Mus m 1 (Mouse urinary allergen) demonstrated findings similar to our results: antibody binding by Mus m 1 was lost at a NaOCl/protein molar ratio of 100, whereas protein was fragmented at a molar ratio of 1000. The molar ratios that resulted in similar effects on Cry j 1 were comparable: an NaOCl/Cry j 1 ratio of 223 was required to substantially decrease antibody recognition, and an NaOCl/Cry j 1 molar ratio of 914 was required to fragment the protein. This is not surprising since the fragmentation should be random, resulting in many fragments that retain all or part of the IgE binding epitope.

The core sequences of the Cry j 1 protein, 332GNATPQLTKN341 and 103NGGPCVFIKRVS114, have been considered important for IgE binding.[13] Human IgE reacted with the former sequence, of which the second residue was glycosylated, and this conformational structure is necessary in effective binding between the IgE and Cry j 1.[14] In the present study, the Cry j 1 epitope we detected on Western blotting might have been 103NGGPCVFIKRVS114, since we destroyed the conformational amino acid sequence of the Cry j 1 protein by thermal denaturation.[15]

When the Cry j 1 is treated with NaOCl, the allergen is no longer detected by ELISA or by skin testing (Fig. 5). This supports our findings that NaOCl can block IgE binding in vitro and also inhibit skin test reactivity in humans. NaOCl's inhibition of skin reactivity to Cry j 1 is also likely due to the ability of HOCl to reduce the binding of IgE.

These results confirm previous reports with other antigens including Mus m1, mold, Fel d 1, and Bla g 1. Periera *et al*. hypothesized that the mechanism behind this effect was the chlorination of amide nitrogen followed by protein oxidation to an imine with further hydrolysis, thus denaturing allergenic proteins.[9]

Although immunologic and biochemical protein studies examining the effects of NaOCl have been performed, this is the first published report of the effect of NaOCl on the biologic potency of an allergen as measured by human skin test reactivity. NaOCl-treatment inhibited the skin test response to Cry j 1 in all 7 of the individuals tested. We confirmed that cedar pollen extract treated with NaOCl does not induce an immediate phase allergic response on skin prick testing in cedar pollinosis volunteers. Notably, intradermal injection of HOCl failed to elicit local allergic reactions greater than those for saline controls.

Not surprisingly, the biologic potency of Cry j 1, as measured by skin testing, was diminished when

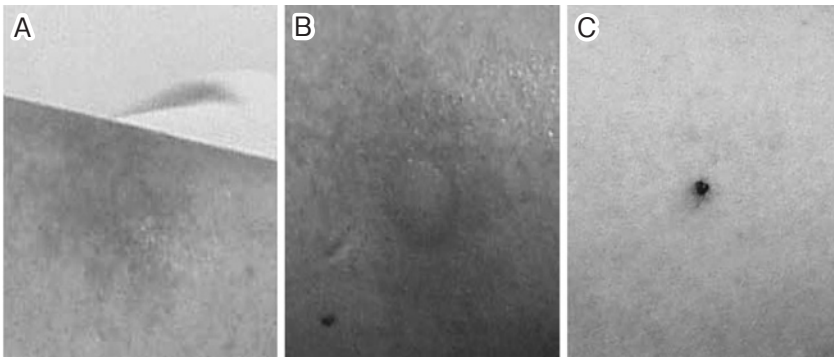


**Fig. 5** Skin test responses. Biological activity of NaOCl-treated cedar pollen extract was evaluated by skin testing 7 Cry j 1-sensitive subjects. Positive allergic reaction was observed with untreated cedar pollen extract at 200 JAU/mL (**A**), and NaOCl-treated at HOCl/Cry j 1 molar ratio of $11 \times 10^3$ resulted in the same reaction (**B**). All subjects had a negative response at $419 \times 10^3$ (**C**).

treated with NaOCl. As increasing doses of NaOCl were used to treat Cry j 1, skin test reactivity decreased significantly.

This study provides important evidence for the following principle: NaOCl not only destroys the antigen but also inhibits its antigenic properties. On the basis of these findings, additional research is warranted. This experiment was conducted in a laboratory setting, and therefore it is difficult to draw conclusions about concentrations that would be sufficient for reducing the immunogenicity of Cry j 1 in a home environment. Field studies should be conducted to determine optical application procedures, including application method, concentration, duration, and frequency of treatment in relation to duration of antigen inhibition. Studies conducted in pollen-contaminated homes may help us establish methods applicable to everyday life, as well as safety and benefits of antigen inactivation performed either with or without NaOCl.

This experiment was only on the use of the residual chlorine contained in tap water. We reported that using only tap water and the electrode board decreased immunogenicity of Cry j 1 without using the hypochlorite as the chemical reagent. And also, an air purifier using low concentrations of hypochlorous acid might be useful for reducing immunogenicity in home environment.

In conclusion, this study revealed that NaOCl is an effective method for significantly reducing the immunogenicity of Cry j 1, the most common allergenic protein in Japanese cedar pollen. Certainly, the reduction in allergic potency of Cry j 1 suggests that low concentrations of hypochlorous acid could be an effective allergen removal or allergen modification agent.

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