

Allergenic proteins are fragmented in low concentrations of sodium hypochlorite

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Summary

Background To facilitate allergen removal from indoor environments, it would be helpful to have household cleaning products that modified allergenic activity. Because NaOCl dissolves proteins in high concentrations and is both capable of killing bacteria and viruses and inactivating viral antigens at somewhat lower concentrations, we explored its effects on Mus m 1 and other indoor allergens.

Objective To examine the ability of NaOCl to reduce the allergenicity of Mus m 1 and other indoor allergens.

Methods Using purified mouse urinary allergen, we examined the effect on protein measured by Coomassie protein assay and on Mus m 1 measured by ELISA. We also examined the effects using SDS/PAGE and Western blots probed with sheep anti-Mus m 1 and with allergic human serum.

Results When NaOCl and Mus m 1 were combined in a molar ratio of 100 : 1, IgE binding to Mus m 1 on Western blot was significantly reduced. At higher NaOCl concentrations the protein appeared to fragment and eventually became undetectable. Fragmentation appeared to be random in that peptides of a wide range of apparent molecular weight were produced. The reaction was complete within 1–2 min at OCl⁻ : pr ratios of greater than 200 : 1 and was optimal at pH 7.4. Immunological activity of other allergens (Fel d 1, Bla g 1, Der p 1) was decreased *in vitro* and dried allergen extracts were removed from surfaces. Adding an extraneous protein, BSA, to NaOCl:Mus m 1 solutions decreased the effect of NaOCl on the allergen.

Conclusions We concluded that NaOCl at concentrations commonly used in household products is capable of dramatically affecting allergenic protein.

Keywords: indoor allergens, Mus m 1, mouse, hypochlorite, peptide bonds, allergenicity

Clinical and Experimental Allergy, Vol. 31, pp. 1086–1093. Submitted 26 July 2000; revised 3 January 2001; accepted 22 January 2001.

Introduction

In order to reduce exposure to indoor environmental allergens, two obvious steps must be taken: remove the source and remove residual allergen that contaminates the home. House dust mite allergen reduction can be accomplished with relatively simple measures. However, in the case of animal and cockroach allergens, it has been found to be difficult to remove them from the home after the source is eliminated. For example, when a cat is

removed from a home, cat allergen declines by 100–1000-fold, but requires a period of 6 months of regular cleaning to do so [1]. In the case of cockroach, allergen concentrations in dust samples decline by no more than 83% immediately after extermination [2], and in homes gradually decline by 80–90% over 6 months of regular cleaning [3]. In both cases it has proven impossible to completely eliminate allergen from settled dust.

Protein denaturants have been suggested as a useful method of rendering residual protein immunologically inactive. To date the only successful treatment is tannic acid, which can reduce allergen by 50–60% [4]. Sodium

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hypochlorite is widely used as a disinfectant in water treatment as well as in hospitals for its ability to render human immunodeficiency virus (HIV) and hepatitis B non-infectious [5]. In higher concentrations it is able to inactivate viral antigens [6]. In higher concentrations still, its ability to dissolve proteins has led to its use as a commercial drain cleaner. We have explored the ability of chlorine bleach to reduce the allergenicity of Mus m 1 and other allergens, and describe the results in the present paper.

Materials and methods

Sodium hypochlorite 5.25% (Clorox®) was obtained commercially and was standardized for free available chlorine by sodium thiosulphate titration [7].

Mouse urinary allergen (Mus m 1) was prepared by the method of Longbottom et al. [8] from pooled urine samples from 10-week-old C57BL/6 J male mice. Urine was dialysed against 0.05 M Tris pH 7.4 at 4° C, then dialysed against deionized water for 4 days at 4° C. The precipitate was lyophilized, then reconstituted in Tris buffer for further purification on a 30 × 100 mm Sephadex G-75 column. Fractions were collected from a peak and examined on 15% SDS PAGE gel. Those that showed minimal contamination with proteins other than 19 kDa (presumed to be Mus m 1) were pooled. On SDS PAGE, this preparation contained a major band at 19 kDa and a band at 16 kDa that was estimated to represent less than 5% of the total protein; on Western blots the minor bands were recognized by sheep polyclonal antibody and human IgE. This purified preparation was dialysed in PBS Tween and found to have a total protein of 5.65 mg/mL by both Lowry and Coomassie (PIERCE, Rockford, IL, USA) methods using a BSA standard curve. The detection limit was 0.1 mg/mL.

Mus m 1 ELISA

Mus m 1 was assayed using the ELISA method of Ohman et al. [9]. Polystyrene microtiter plates (Dynex Technologies Inc., Chantilly, VA, USA) were coated with immunosorbant-purified sheep anti Mus m 1 (kindly supplied by Dr J Ohman) at 4° C overnight and blocked with BSA. A standard curve of purified Mus m 1 and the test samples were added to the plate and incubated for 1 h at 25° C. After a second wash, biotinylated sheep anti-Mus m 1 was added and incubated for an additional hour, then developed with streptavidin peroxidase and read at 405–490 nm. The detection limit was 1 ng/mL.

SDS-PAGE

Fifteen per cent polyacrylamide minigels were prepared according to Laemmli [10]. Proteins were boiled in gel

loading buffer for 4 min then electrophoresed and stained with Coomassie brilliant blue R-250. Gels for Western blot were equilibrated in Tris-glycine, 20% methanol buffer, pH 8.3, and electrophoretically transferred from unstained gels onto 0.22 micro NC membrane (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 2 h in Tris-glycine/methanol buffer. Membranes were blocked with 0.5% gelatin in PBS 0.05% Tween 20 for 30 min then probed with either 1 : 2000 diluted biotinylated sheep anti-Mus m 1 IgG or 1 : 3 diluted human patient serum for 2 h at 25° C. Membranes probed by sheep antibody were developed with streptavidin-horseradish peroxidase, while those probed with human IgE were developed with horseradish peroxidase-labelled Goat anti-human IgE.

Gel fractionation

After digestion at an OCl : pr molar ratio of 160 : 1 for 15 min at 25° C, the reaction mixture was quenched with 20% Na₂SO₃ and fractionated on Superdex-75 HR 10/30 column using PBS pH 7.4 in an FPLC system (Pharmacia, Uppsala, Sweden). Fractions were collected and analysed with a Coomassie protein assay, SDS-PAGE and Western blot probed with sheep anti-Mus m 1.

Results

Effect of chlorine on protein and immunoreactivity of Mus m 1

When MUP was incubated with dilute NaOCl, the protein appeared to fragment. Purified Mus m 1 resolved on SDS-PAGE into a 19-kDa band (Mus m 1) and a 16-kDa band before treatment with NaOCl (Fig. 1a, lane 0). Both Mus m 1 and the 16 kDa protein were recognized on Western blot by sheep anti-Mus m 1 and allergic human serum (Figs 1b and c, lane 0). When mixed with NaOCl at molar ratios of 62 : 1 (1.2 mM NaOCl, 19 (M Mus m 1) both bands became fainter and more diffuse by Coomassie blue stain, while at a slightly higher OCl : pr ratio (123 : 1), protein was almost undetectable. On Western blot the 19 kDa and 16 kDa bands were still detectable at an OCl : pr molar ratio of 62 : 1 when probed with sheep anti-Mus m 1 antibody and human serum, and binding was lost at 123 : 1.

The dose-response relationship was also examined using a Coomassie Plus protein assay and Mus m 1 ELISA to increase detection limits and precision. Mus m 1 allergen effects were determined by diluting Mus m 1 stock with either PBS or NaOCl to create final reaction mixtures of 2.2, 4.5, 8.4 and 16 ng/mL. Figure 2 shows the ELISA final optical density (OD) measured at these starting allergen concentrations. Compared with control values in mixtures incubated with PBS, OD values were decreased when the

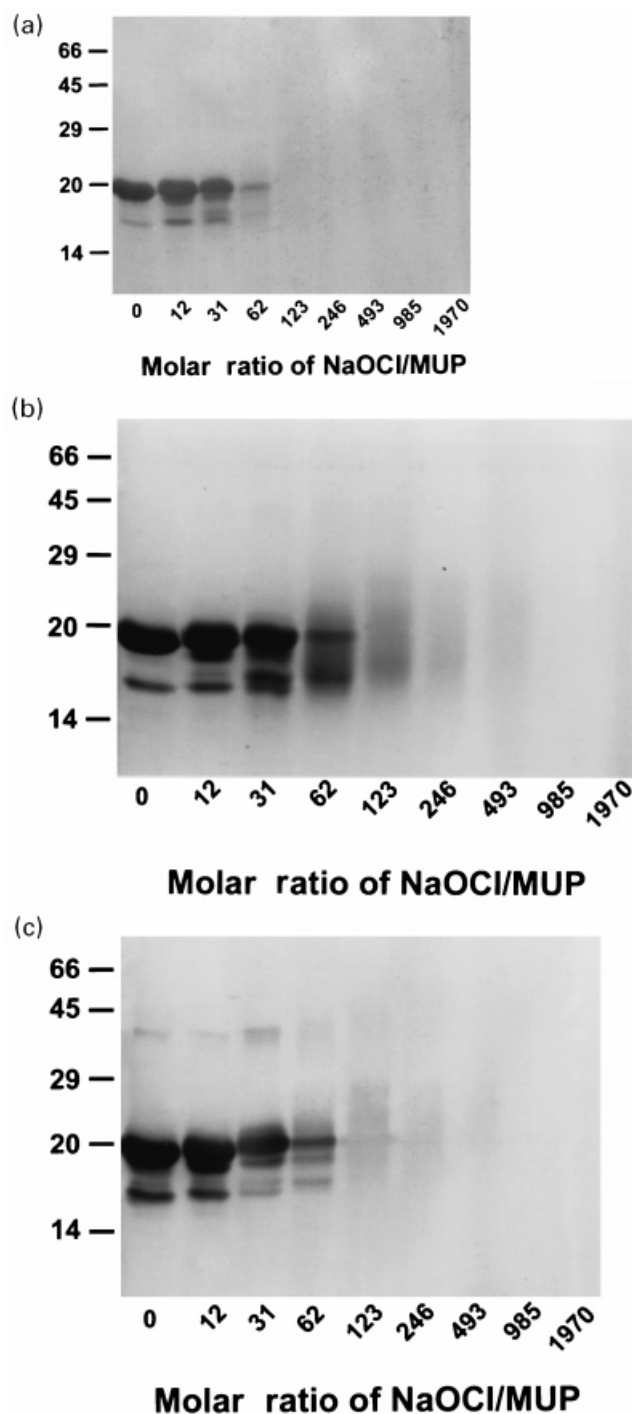


Fig. 1. SDS-PAGE examination of mouse urinary protein untreated and incubated for 25 min with various concentrations of NaOCl. Panel A, stained with Coomassie Blue; panel B Western transfer probed with sheep anti-MUP; panel C, Western transfer probed with human serum containing mouse-specific IgE antibody.

protein was first incubated with NaOCl, then quenched with Na_2SO_3 and assayed. Although the effect was apparent at all concentrations of added protein, there was a clear dose-response relationship, with greater loss with each higher NaOCl concentration. To calculate final allergen concentration, detectable allergen at each of the four test dilutions was multiplied by the appropriate dilution factor. As seen in Fig. 3, detectable protein was lost at an OCl : pr molar ratio of 1970 : 1, while detectable allergen was lost at a molar ratio of 123 : 1. Fifty per cent of measurable protein was lost at an OCl : pr molar ratio of 140 : 1, while 50% of immunoreactive Mus m 1 was lost at a molar ratio of 21 : 1. We concluded that allergenicity was lost at much lower NaOCl concentrations than were required to fragment the protein.

We considered it possible that residual NaOCl interfered with the ELISA assay despite the addition of Na_2SO_3 after the digestion step. To test this question, we incubated an extraneous protein, BSA, with NaOCl at an OCl : pr molar ratio of 1000 : 1, then added Mus m 1 to this solution with and without Na_2SO_3 and assayed with ELISA. In a control (no NaOCl added to BSA), 2.2 ± 0.04 ng/mL Mus m 1 was measured. When the solution of NaOCl-treated BSA was added, the assay was inhibited and Mus m 1 was undetectable. After treatment of the NaOCl:BSA:Mus m 1 solution with 20% Na_2SO_3 , 2.3 ± 0.05 ng/mL was detected. We concluded that Na_2SO_3 used in the experiment fully inhibited the effect of any residual NaOCl.

Kinetics and pH dependence

Loss of immunological activity occurred rapidly. We examined OCl : pr molar ratios of 985 : 1 and 128 : 1. Aliquots were periodically withdrawn, placed in excess Na_2SO_3 , then assayed for Mus m 1. As seen in Fig. 4, Mus m 1 was reduced by 99% at 30 s in OCl : Pr molar ratio of 985 : 1. At a molar ratio of 128 : 1, Mus m 1 was reduced by 88% at 30 s and by 99% at 3 min.

The oxidative activity of aqueous solutions of hypochlorites is strongly pH dependent [11] and the chlorination of amino acids is generally highest at pH 7–9 [12]. We found that the loss of immunoreactivity was also affected by pH (see Table 1). Using an OCl : pr molar ratio of 60 : 1, allergen concentration was significantly lower when incubated at pH 7.4 than at pH 2.5 or at pH 11.0 ($P = 0.0014$, ANOVA). Control solutions that did not contain NaOCl maintained similar concentrations of allergen at all pH ($P = 0.54$; ANOVA), suggesting that the pH effect was not due to an effect on the assay.

Gel fractionation

The smaller protein fragments produced by NaOCl were

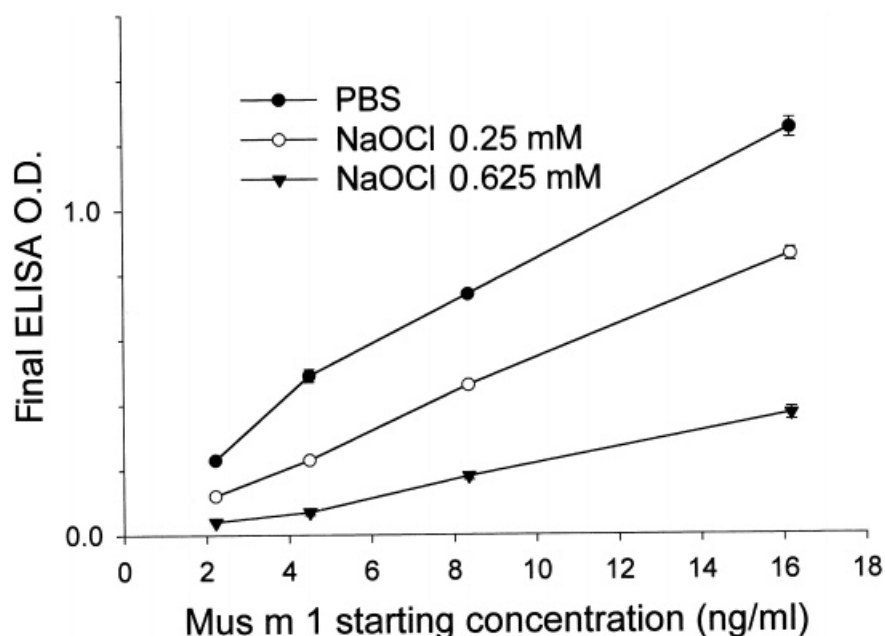


Fig. 2. Final optical density values of solutions ($n = 6$) in which Mus m 1 standard was added at concentrations of 2.2, 4.5, 8.4 and 16 ng/mL. To these solutions were added either PBS, 0.25 mM or 0.625 mM NaOCl. After 10 min the reaction was stopped with 20% Na_2SO_3 . Brackets are drawn at standard error of means.

not well resolved in SDS gels, so we subjected digested protein to gel filtration. As seen in Fig. 5, the protein peak of digested Mus m 1 appeared at fraction 9. On SDS gel, the unfractionated digestate appeared as a diffuse band from 12 to 29 kDa. The densest staining was seen in fraction 9, with fractions 7 and 8 containing primarily larger molecular weight peptides and fractions 10 and 11 containing smaller fragments. On Western blot the higher molecular weight peptides in fractions 7, 8 and 9 bound

sheep anti-Mus m 1, as did the lower molecular weight fragments in fraction 10. Fraction 11, which contained no detectable protein in the SDS gel, was shown to contain intact Mus m 1, represented by a 19-kDa band, in the Western gel. Thus it appeared that digestion at OCl : pr ratio of 160 : 1 produced random fragments of Mus m 1 with molecular weights of 10 kDa and higher, some of which retained immunological activity. In addition, larger peptides with immunological activity were also formed,

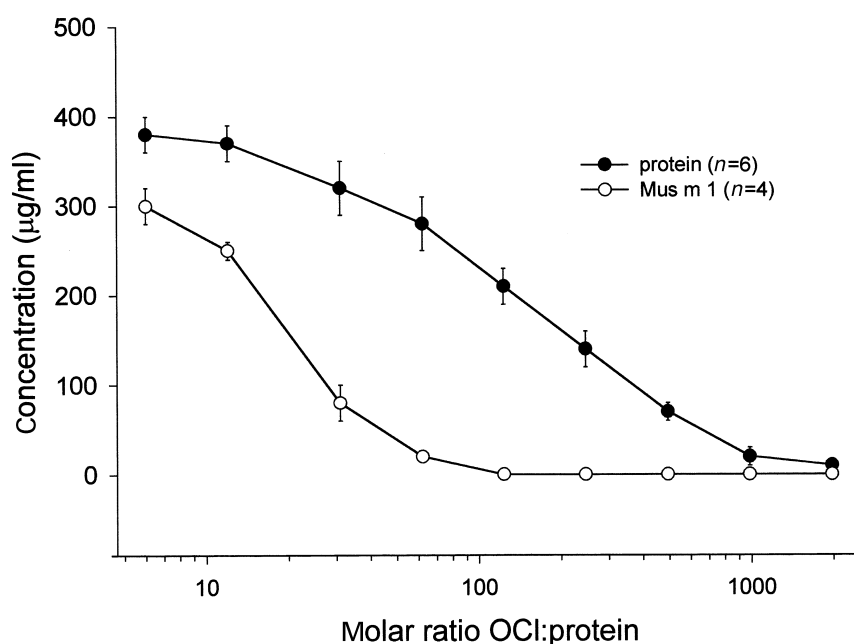


Fig. 3. Dose-response relationship of NaOCl effect on MUP. Molar ratios of NaOCl to protein in the reaction mixture are shown in log scale on the abscissa. Protein concentration in mixtures after 10 min incubation is shown on ordinate in linear scale ($n = 6$). Mus m 1 allergen concentrations in ELISA are shown on ordinate in log scale ($n = 4$). Brackets are drawn at standard error of means.

Table 1. pH effect on digestion of Mus m 1

	pH 2.5	pH 7.4	pH 11.0
MUP + saline	419 ± 28*	380 ± 27	404 ± 22
MUP + NaOCl	12.3 ± 0.4	6.9 ± 0.6	13.3 ± 1.6

MUP mixed with NaOCl at a ratio 60 : 1 or PBS (pH 7.4), 0.03 mM HCl (pH 2.5) or 0.03 mM NaOH (pH 11). Reaction 15 min at 25° C. *n* = 4. *µg/mL (mean (SEM)).

which were probably polymers of the various smaller peptides.

Protein mass

To test the effect of added protein mass on the NaOCl reduction of a target allergen, we combined 0.047–1.5 nM BSA with 0.91 nM Mus m 1 before adding NaOCl. After NaOCl was added, the mixture was incubated at 25° C for 20 min, quenched with Na₂SO₃ and assayed for Mus m 1. As shown in Fig. 6, control aliquots without NaOCl contained 194 µg/mL Mus m 1. Reaction with NaOCl at a molar ratio of 103 : 1 reduced Mus m 1 to 0.09 µg/mL. The addition of 0.19 nM BSA, which lowered the OCl : pr ratio to 85 : 1, reduced the effect on Mus m 1 and the effect on Mus m 1 was almost completely prevented with the addition of enough BSA to raise the OCl : pr ratio to 39 : 1. This differs from the dose–response relationship for Mus m 1 shown in Fig. 2. Thus the results are compatible with a non-specific effect of NaOCl on protein, but it appears that the dose response for the two proteins differs.

Effect on other allergens

The effect of NaOCl was examined in unpurified extracts containing Fel d 1, Bla g 1 and Der p 1. These extracts contained other proteins than the measured allergen so that the extracts containing 20 ng/mL Fel d 1, 39 mU/mL Bla g 1 and 125 ng/mL Der p 1 had total protein concentrations of 1.37, 1.67 and 2.1 µg/mL, respectively. As shown in Table 2, NaOCl reduced the measured concentrations of each allergen, although the molar ratios required were substantially higher than seen with the preparation of purified Mus m 1. For example, when NaOCl was combined with the extract containing Der p 1 in a OCl : Pr ratio of 50 000 : 1, the Der p 1 concentration was reduced from 125 ng/mL to 85 ng/mL and a ratio of 5×10^5 : 1 was required to reduce Der p 1 below the detection limit of the assay (0.5 ng/mL). The need for substantially higher concentrations of NaOCl presumably related to the high concentration of extraneous proteins in the extracts.

Effects on allergens bound to surfaces

As seen in Table 3, allergen concentrations on untreated surfaces did not differ from the surfaces treated with PBS (controls). However, allergens were consistently undetectable on swabs from surfaces exposed to either 43 mM or 186 mM NaOCl.

Discussion

These data demonstrate that sodium hypochlorite fragments allergenic proteins and that this fragmentation reduces binding of IgG and IgE antibodies. Fragmentation occurs

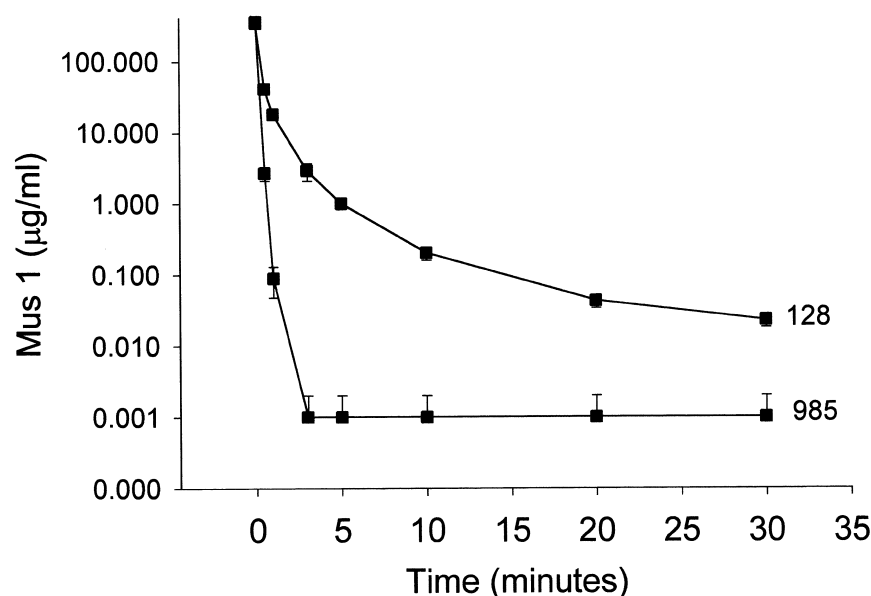


Fig. 4. Kinetics of Mus m 1 allergen loss in NaOCl. At time 0, allergen was mixed with NaOCl solution at a molar ratio of either 128 : 1 or 985 : 1 in several aliquots. At indicated times the reaction was stopped by addition of 20% Na₂SO₃ and Mus m 1 concentrations were measured by ELISA. Allergen concentrations are shown on ordinate in log scale (*n* = 4).

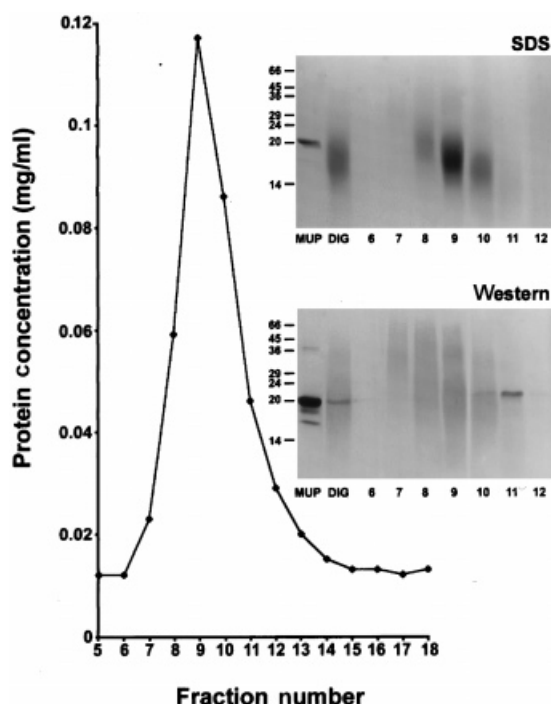


Fig. 5. Gel filtration of digested Mus m 1. MUP and NaOCl at a ratio 160 : 1 was incubated for 15 min at 25° C then stopped with excess Na₂SO₃. The resulting digest was applied to a Superdex-75 HR 10/30 column. Total protein in each fraction was measured using the Coomassie method and is plotted. Inserts are the results of SDS/PAGE gel of the unfractionated digestate and of each fraction, stained for protein in the upper panel and Western blot probed with biotinylated sheep anti-Mus m 1 in the lower panel.

rapidly and at relatively low concentrations of sodium hypochlorite.

These results confirm previous reports with other proteins including albumin [11], fibronectin [12] and IgA [13]. The proposed mechanism of peptide bond fragmentation is through chlorination of amide nitrogen followed by oxidation to imines and hydrolysis of the imine [14,15]. Hawkins and Davies reported that BSA and HSA were fragmented at OCl : Pr ratios greater than 70 : 1, although a fainter, diffuse band could still be demonstrated on SDS gels at ratios of 150 : 1. The reaction could be prevented by antioxidants such as glutathione and ascorbate. In our experiments with the mixture of Mus m 1 and BSA, we found that unexpectedly low molarities of BSA appeared to inhibit the NaOCl effect on Mus m 1. The reaction was significantly inhibited by the addition of 0.19 nM BSA which decreased the total OCl : Pr molar ratio only from 103 : 1 to 85 : 1. As shown in Fig. 2, a similar change in Mus m 1 had no effect on the loss of Mus m 1. The explanation is likely to relate to the differences in mass of the two proteins (BSA 66.3 kDa vs. Mus m 1 18.8 kDa). The number of peptide bonds in BSA (546) is more than threefold greater than in Mus m 1 (180) so that the amide nitrogen available in BSA to react with NaOCl is also greater. In the experiments of Hawkins and colleagues with ribonuclease A (13.6 kDa), the protein was found to fragment at much lower NaOCl : protein ratios than BSA [11]. Given that indoor allergens exist in mixtures with substantial protein concentrations in which allergenic proteins represent a small fraction of the protein content, this difference is likely to have important practical implications.

While this is the first description of the loss of IgE and IgG binding to antigens fragmented by NaOCl, earlier

Fig. 6. Inhibition of Mus m 1 digestion by addition of BSA to the reaction mixture. Figures on ordinate are the final concentrations of reaction components in nM. BSA in the molarity indicated or buffer was mixed with MUP 0.91 nM and the mixtures were incubated with 94 nM NaOCl for 15 min at 25° C, quenched with Na₂SO₃ and assayed for Mus m 1 (*n* = 6).

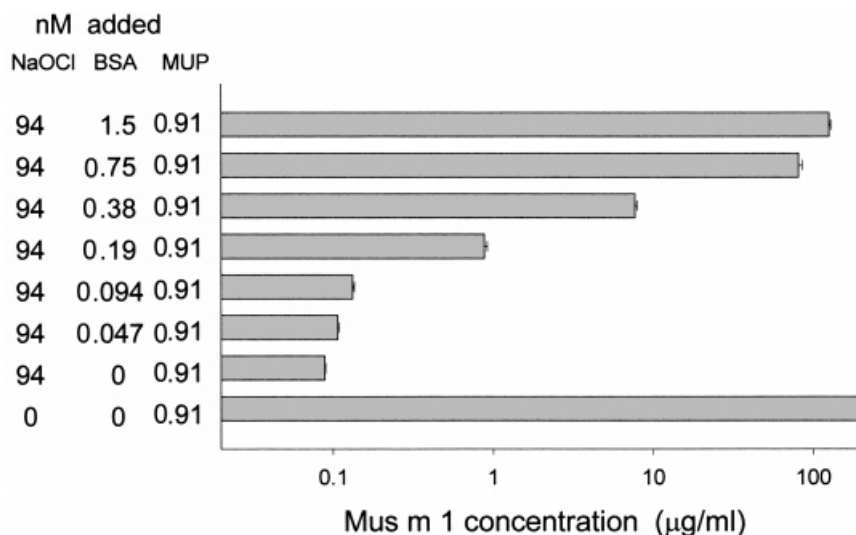


Table 2. The effect of NaOCl on other allergens

	Fel d 1 (ng/mL)*	Bla g 1 (mU/mL)	Der p 1 (ng/mL)
Control	20	39	125
NaOCl 0.025 mM*	13	1.2	ND
NaOCl 0.25 mM	0.3	ND	85
NaOCl 25 mM	ND	BD	BD

*Final concentrations in reaction mixture. BD = below assay detection limits. ND = experiment not done.

reports have described immunological effects. Schulster and colleagues demonstrated that antigenicity of HBsAg was lost after treatment with NaOCl [6]. Our data suggest that antibody binding may be retained in mouse allergen fragments. This is not surprising since the fragmentation should be random, resulting in many fragments that retain all or part of the IgE binding epitope. At the same time, antibody binding appeared to be lost at OCl : Pr ratios that were generally lower than those causing fragmentation. This may relate to modification of amino acid side chains, since in addition to lysine, tyrosine and phenylalanine undergo ring chlorination [11,16], and methionine and cysteine react to form oxyacids, sulphoxides and cystine [15]. In the experiments of Hawkins and Davies, when albumin was reductively methylated, so that 80–85% of lysine residues were blocked, significant fragmentation could be demonstrated at ratios of 25 : 1, suggesting that amino groups in lysine side chains interfered with NaOCl reaction with amide nitrogen. Any of these changes might be expected to affect tertiary structure and antigenicity.

The concentrations of sodium hypochlorite that caused the protein fragmentation are those commonly used in households. For example, in the experiments demonstrating the effects on surface allergens (see Table 3) we found that 186 mM sodium hypochlorite modified surfaces contaminated with allergen so that allergen could no longer be detected in wipe samples from this surface, and that a 43-mM solution would accomplish the same. Household cleaning products that contain 1.8% sodium hypochlorite (257 mM) are sold as disinfectant cleaners in the USA and elsewhere. In addition, liquid household bleach products contain about 5–6% sodium hypochlorite and their labels usually contain directions to make a solution with about 0.3% (43 mM) sodium hypochlorite for cleaning and disinfecting household surfaces by adding about three-quarters cup of product to about a gallon of water. The present data would suggest that this concentration would act on a solution of 1 µM Mus m 1 (18 µg/mL) to render the protein undetectable by protein assay or ELISA within

Table 3. The effect of chlorine bleach on surface allergens

	Mus m 1*	Fel d 1*	Der f 1*	Bla g 1†
<i>Metal</i>				
Baseline	3211 ± 283	2309 ± 142	28 ± 2	656 ± 77
Control	3455 ± 322	2385 ± 115	28 ± 0	656 ± 44
NaOCl 43 mM	BD	BD	BD	BD
NaOCl 186 mM	BD	BD	BD	BD
<i>Benchmark</i>				
Baseline	3096 ± 465	2074 ± 161	22 ± 1	500 ± 16
Control	2520 ± 347	1998 ± 153	22 ± 2	488 ± 78
NaOCl 43 mM	BD	BD	BD	BD
NaOCl 186 mM	BD	BD	BD	BD
<i>Floor Tile</i>				
Baseline	3085 ± 414	2191 ± 185	25 ± 1	533 ± 8
Control	3099 ± 503	1941 ± 305	20 ± 3	437 ± 73
NaOCl 43 mM	BD	BD	BD	BD
NaOCl 186 mM	BD	BD	BD	BD

*Allergen concentrations in extracts from treated surfaces in ng/mL, mean ± SEM. †Allergen concentrations in extracts from treated surfaces in mU/mL, mean ± SEM. BD = below assay detection limits. *n* = 4.

1 min. These effects would be affected in unpredictable ways by extraneous proteins in the solution.

Sodium hypochlorite is also used to clean laundry, although its use is generally limited to white and lightly coloured fabrics since it can fade many colours. Following the label instructions on household bleach products sold in the USA produces a sodium hypochlorite concentration in laundry wash water of about 0.02% (2.6 mM). Our results shown in Figs 1 and 2 suggest that this concentration would eliminate allergenic proteins. In practice, mite allergen has been shown to be effectively removed by laundering without bleach, although less efficiently so in the presence of detergents [17]. Our data suggest that chlorine bleach would contribute to the efficiency of the process.

Acknowledgements

This work was supported by grants from the NIH (ES07527, ES09601), the EPA (R826724) and The Clorox Company.

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