

Effects of Double Mutation at Two Distant IgE-binding Sites in the Three-dimensional Structure of the Major House Dust Mite Allergen Der f 2 on IgE-binding and Histamine-releasing Activity

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Recently, we reported that introduction of mutations that induced conformational changes of the major mite allergen Der f 2 was an efficient strategy to reduce the allergenicity for safer allergen-specific immunotherapy. In this study, we evaluated another strategy, disruption of two independent IgE epitopes without inducing conformational change. We analyzed allergenicities of the wild-type Der f 2, two single mutants with a mutation at either of the two IgE-binding sites (K15A and K77A), and a double mutant with mutations at both of the sites (K15/77A). Purified recombinant forms of Der f 2 expressed in *Escherichia coli* had correct disulfide bonds, equivalent apparent molecular masses of approximately 15 kDa, and similar secondary structures. The mutants of Der f 2 had less IgE reactivities than the wild-type Der f 2 and reduced inhibitory activities for IgE-binding to the wild-type Der f 2. However, the mutations did not significantly reduce histamine-releasing activity.

Key words: mite group 2 allergens; IgE epitopes; tertiary structure; allergen engineering; recombinant allergen

Immediate hypersensitivity is caused by the release of mediators such as histamine from mast cells and basophils activated by aggregation of a complex of the high-affinity IgE receptor (FcεRI) on the cell surface and allergen-specific IgE, crosslinked by

multivalent allergens.^{1,2)} House dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) allergens are major causative factors associated with various allergic diseases such as asthma, rhinitis, and atopic dermatitis.^{3–6)} The major allergens from house dust mite are classified as group 1 (Der f 1 and Der p 1) or group 2 (Der f 2 and Der p 2) allergens.^{7–12)}

Der f 2 is the major group 2 allergen from *D. farinae*,^{13–15)} and consists of 129 amino acid residues with marked sequence similarity to Der p 2,¹⁶⁾ the group 2 allergen from *D. pteronyssinus*. IgE epitopes of Der f 2 and Der p 2 are dependent on the integrity of the conformational structure rather than the contiguous sequence of amino acids.^{17–24)} We located the three disulfide bonds of native Der f 2 (Cys8-Cys119, Cys21-Cys27, and Cys73-Cys78)²⁵⁾ and found that two, Cys8-Cys119 and Cys73-Cys78, are critical to the IgE-binding capacity of Der f 2.²⁰⁾ Site-directed mutagenesis at residues other than cysteine in three parts of the Der f 2 sequence, 1–20, 70–81, and 114–129, showed that ten residues, Asp7, Ala9, Asn10, Lys15, Asp19, Asn71, Ala72, Met76, Lys77, and Ala120, were important to IgE-binding capacity in one serum.²⁶⁾ Referring to the three-dimensional structure of Der f 2 found by NMR, these residues seem to compose at least two distant IgE-binding sites on the surface of the allergen molecules.²⁷⁾

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RAST-EIA, radioallergosorbent test-enzyme immunoassay; CD, circular dichroism

Recombinant allergens with full IgE reactivity are useful for diagnosis and allergen-specific immunotherapy.^{28–33} On the other hand, mutant allergens with reduced allergenicities would be useful for safer and more effective immunotherapy.^{20,22–24,34–36} Recently, we reported that introduction of mutations that induced conformational change of the major mite allergen Der f 2 was an efficient strategy to reduce the allergenicity.^{23,24} In this study, we evaluated another strategy, disruption of two independent IgE epitopes without inducing conformational change. A mutant allergen in which two independent IgE-binding sites are disrupted would be less allergenic than those in which only one of the IgE-binding sites is disrupted. We designed a double mutant of Der f 2 with two mutations, which did not affect the secondary and tertiary structure, at both of the distant IgE-binding sites in the tertiary structure, and compared its allergenicity with those of the wild-type Der f 2 and two single mutants with mutations at one or the other of the two IgE-binding sites.

Materials and Methods

Expression of the wild-type and mutants of Der f 2 in *Escherichia coli*. Expression vectors for the wild-type Der f 2, K15A, and K77A, were constructed as previously described.^{26,28} That for K15/77A was constructed from those for K15A and K77A using the restriction sites for *Cla* I and *Hin* d III.³⁷ The wild-type Der f 2 and the three mutants were expressed in *E. coli* BL21 (DE3) transformed with the expression vectors.

Immunoblot analysis. The method previously described was used with some modifications.²⁰ Briefly, total cellular proteins expressed in *E. coli* BL21 (DE3) transformed with expression vectors of the wild-type and mutants of Der f 2 were put through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electroblotted. The membranes were stained with Coomassie Brilliant Blue, or probed with a combination of a mite-sensitive human allergic serum (1/50 dilution) and horseradish peroxidase-conjugated anti-human IgE (ϵ -specific) antibodies (Biosource International, Camarillo, CA). Blocking, washing, and development were done using BM Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH, Mannheim, Germany).

Purification of the wild-type and mutants of Der f 2. The wild-type and mutants of Der f 2 produced as inclusion bodies in *E. coli* BL21 (DE3) were recovered, solubilized with buffer containing urea, refolded by dialysis against buffer, and purified by anion exchange column chromatography as described.²⁰ The disulfide bond formation of the purified recom-

binant wild-type and mutants of Der f 2 was confirmed correct by peptide mapping as described.²⁵ The purities were checked by SDS-PAGE under non-reducing or reducing conditions.

Gel-filtration analysis. The purified wild-type and mutants of Der f 2 (200 μ l, approximately 100 μ g/ml) were chromatographed by gel filtration on a Superdex 75 HR 10/30 column (1 cm $\times$ 30 cm) (Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min with phosphate-buffered saline (pH 7.4). Absorbance at 280 nm was monitored. Molecular mass standards for gel filtration (Bio-Rad, Richmond, CA) were used for estimation of apparent molecular masses of the recombinant proteins.

Radioallergosorbent test-enzyme immunoassay (RAST-EIA). IgE reactivities of the purified wild-type and mutants of Der f 2 were measured by RAST-EIA using a RAST-FEIA kit (Pharmacia) as previously described.²² Briefly, serum of each allergic patient (1/8–1/4 dilution) was incubated with paper discs coupled with each of the purified antigens in various amounts for three hours at 37°C. Then the IgE that bound to the disc was detected with β -galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence. The sera from patients allergic to house dust mites were kindly provided by Dr. Okudaira, University of Tokyo.

Inhibition assay. The method was as described previously with some modifications.²⁴ Briefly, the serum of each allergic patient (1/2-neat dilution) was incubated with various concentrations of each of the purified wild-type and mutants of Der f 2 for three hours at room temperature, and then the solution was added to a paper disc soaked in wild-type Der f 2. The IgE that bound to discs was detected with β -galactosidase-conjugated anti-human IgE antibodies. The percentage of inhibition was expressed as the relative reduction of the fluorescence intensity in each sample to that when no inhibitors were added.

Histamine release. The method was as described previously except that a Histamine ELISA (ICN, Costa Mesa, CA) was used.²⁰ The percentage of released histamine was calculated as the amount of histamine in the supernatant relative to the total of supernatant and cellular histamine.

Circular dichroism (CD). CD spectra were measured on a JASCO J-700 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) using a 0.1-cm cell. Spectra were recorded at 25°C over 200–250 nm. The concentration of samples used was 114 μ g/ml in 20 mM Tris-HCl, pH 7.4, and 0.9% NaCl.

Results

Design of a double-mutant of Der f 2, K15/77A, with mutations at both of the distant IgE-binding sites in the tertiary structure

The previous analysis of IgE epitopes on Der f 2 using serum from one mite-allergic patient, SN, and analysis of the tertiary structure of Der f 2 by NMR showed that at least two distant IgE-binding sites exist on the surface of the Der f 2 allergen.^{26,27} One includes Asp7, Cys8 Asn10, Lys15, and Asp19, and the other Asn71, Cys73, Cys78, and Lys77. Therefore, as no great reduction in binding to two mouse monoclonal antibodies, 13A4 and 15E11, the recognitions of which are influenced by the conformation of Der f 2, was observed in two mutants, K15A and K77A,^{38,39} we assumed that they have the same conformation as the wild-type Der f 2 except for the side chains of two amino acid residues numbered 15 and 77. From each of the IgE-binding sites, we selected Lys15 and Lys77 (Fig. 1). We considered that a comparison of allergenicities between the double-mutant, K15/77A, the wild-type Der f 2 and the two single-mutants, K15A and K77A, would provide information on the effects of the structural difference in the side chains of amino acid residues.

*Expression of wild-type and mutants of Der f 2 in *E. coli* and immunoblot analysis of their IgE-binding capacities*

Wild-type and mutants of Der f 2 were overexpressed and accumulated as insoluble inclusion bodies in *E. coli* cells. The cells containing inclusion bo-

dies were solubilized in a buffer containing SDS and 2-mercaptoethanol and put through SDS-PAGE (Fig. 2).

Before analysis using refolded and purified recombinant forms of Der f 2, IgE-binding to the proteins electroblotted after SDS-PAGE under reducing conditions was analyzed by immunoblotting (Fig. 2). In six tested patients, K77A and K15/77A showed significantly reduced IgE-binding. K15A showed slightly reduced IgE-binding.

Preparation of purified wild-type and mutants of Der f 2 with correct folding

The correct disulfide bond formations of the refolded and purified wild-type and mutants of Der f 2 were confirmed by peptide mapping (Fig. 3A).²⁵ Each of the purified proteins was detected as a single band on SDS-PAGE under both non-reducing and reducing conditions (Fig. 3B). Under non-reducing conditions, mobilities of the wild-type and mutants of Der f 2 were equivalent. Under reducing conditions, mobilities of K15A and K15/77A were smaller than those of the wild-type Der f 2 and K77A. The mobility under reducing conditions was smaller than that under non-reducing conditions.

Each of the purified wild-type and mutants of Der f 2 was detected as a single peak in gel-filtration analysis (Fig. 4). Apparent molecular masses of the wild-type and mutants of Der f 2 in gel-filtration were approximately 15 kDa. No detectable differences in the secondary structure among the wild-type and mutants of Der f 2 were observed in their CD spectra (Fig. 5).

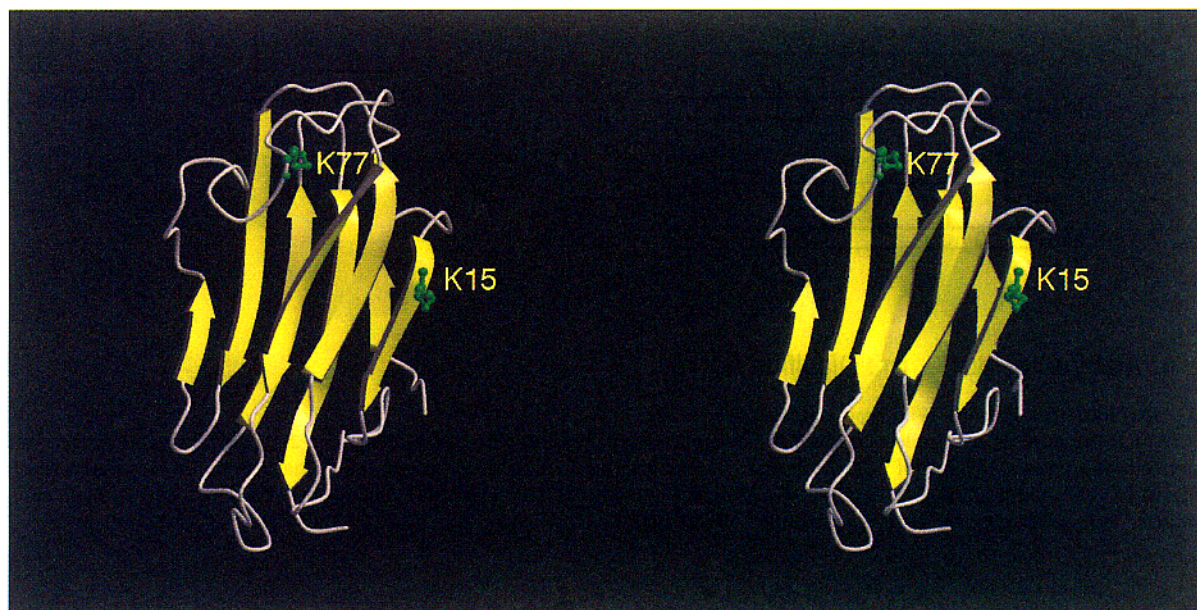


Fig. 1. Ribbon Diagram of the Mean Structure of Der f 2.²⁷

Lys15, Lys77, and β -strands (a', a, b, c, c', e, f, and g) are shown. This figure was produced by the program MOLSCRIPT⁴⁰ and Raster 3D,⁴¹ and is shown in stereo.

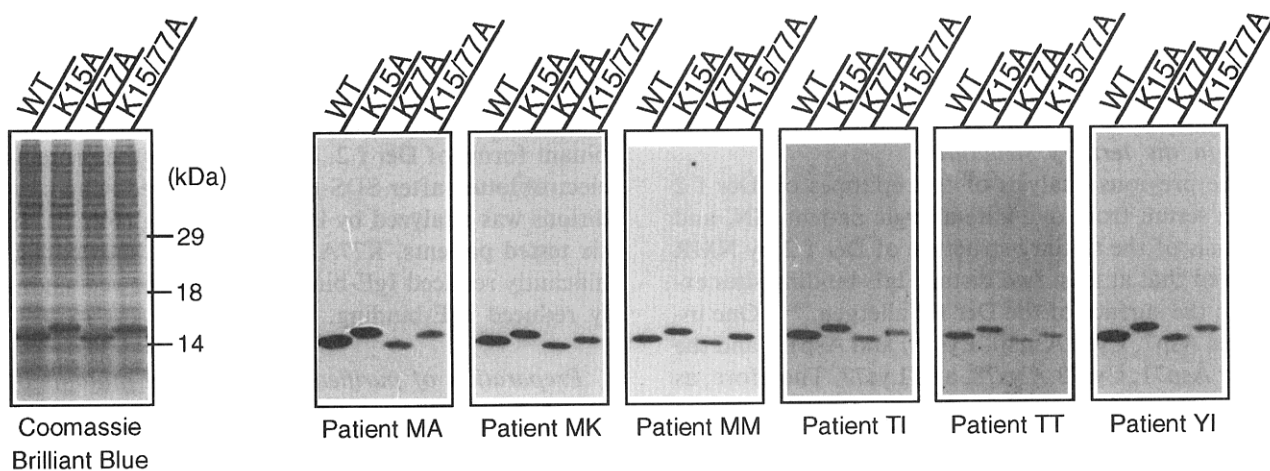


Fig. 2. Expression of the Wild-type and Mutants of Der f 2 in *E. coli* and Immunoblot Analysis for Their IgE-binding Capacities. Total cellular proteins of *E. coli* BL21 (DE3) transformed with the expression vector of the wild-type (WT) and mutants (K15A, K77A, and K15/77A) of Der f 2 were put through SDS-PAGE under reducing conditions and electroblotted. Initials of the patients' names are indicated.

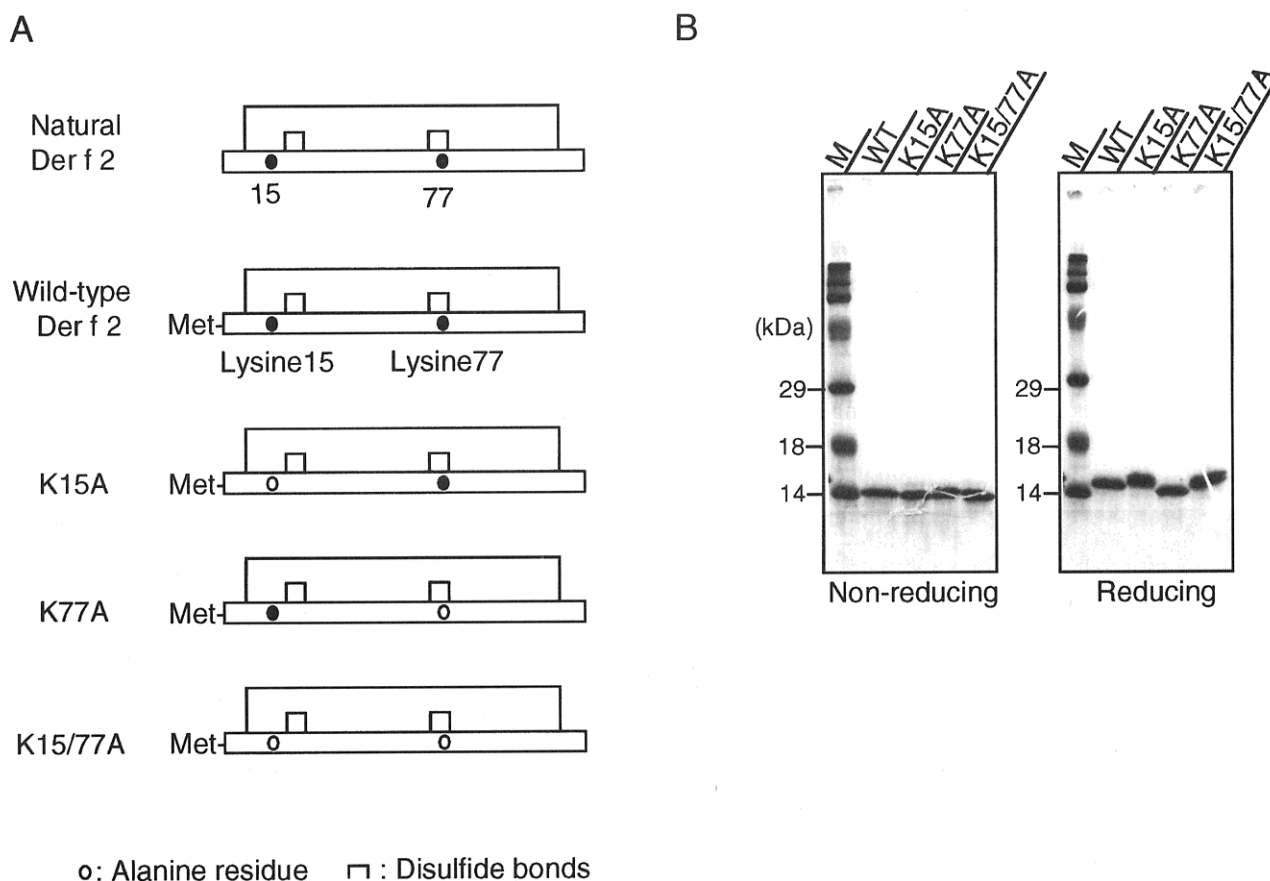


Fig. 3. Refolded and Purified Wild-type and Mutants of Der f 2.

(A) Confirmation of intramolecular disulfide bonds. Schematic representation of natural Der f 2 purified from mite extract and the purified wild-type and mutants of Der f 2 expressed in *E. coli* with a methionine at the first N-terminal residue are shown with three confirmed disulfide bonds (Cys8-Cys119, Cys21-Cys27, and Cys73-Cys78). (B) SDS-PAGE analysis under non-reducing and reducing conditions. WT: wild-type Der f 2. M: molecular weight marker.

IgE-binding capacity of purified wild-type and mutants of Der f 2 in mite-allergic patient sera

The IgE reactivities of the homogeneously purified mutants were compared with that of the wild-type

Der f 2 by RAST-EIA (Fig. 6A). K15A, K77A and K15/77A showed reduced binding in three patients, SN, TT, and HK and no reduction in one patient, TN.

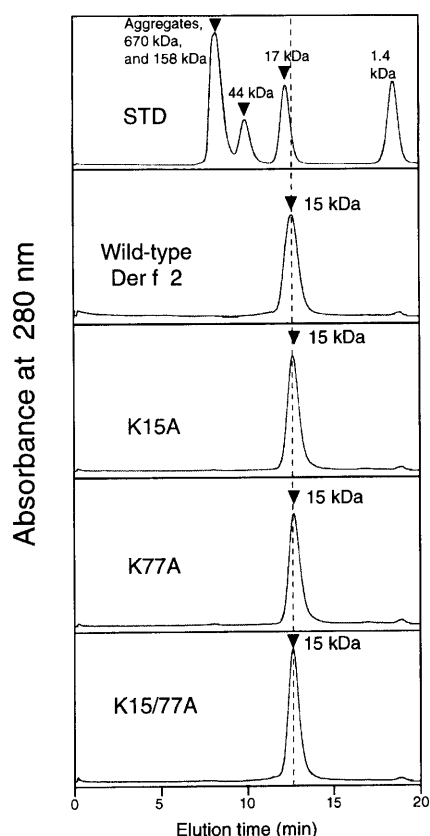


Fig. 4. Elution Profiles in Gel-filtration Analysis of Purified Wild-type and Mutants of Der f 2.

Apparent molecular masses of the peaks of purified wild-type and mutants of Der f 2 are shown with arrows. The elution profile of molecular mass standards is also shown as a control (STD).

The capacities of the mutants to inhibit IgE from binding to the wild-type Der f 2 were also examined using sera of two patients, SN and TT, by inhibition assay (Fig. 6B). The inhibitory effects by the three mutants were reduced compared with that by the wild-type Der f 2. In the patient SN, K15/77A showed the most reduced inhibition followed by K15A at higher concentrations of the inhibitor, 100–1,000 ng/ml. In the patient TT, K77A and K15/77A showed reduced inhibition. The double mutation, K15/77A, increased reduction more than either of the single mutations, K15A and K77A, in the inhibitory activity in the patient SN but not in the patient TT.

Activities of purified wild-type and mutants of Der f 2 to induce histamine release from basophils

Peripheral blood basophils from two mite-allergic volunteers, TT and TU, were stimulated with the wild-type and mutants of Der f 2, and histamine release was measured (Fig. 6C). The threshold concentration of the wild-type and three mutants to stimulate basophils to degranulate was not reduced (approximately 1 ng/ml).

Discussion

In this study, we adopted a rational strategy of allergen engineering based on information from the known tertiary structure of an allergen for the first time. The three mutants, K15A, K77A, and K15/77A, analyzed in this study were selected or designed

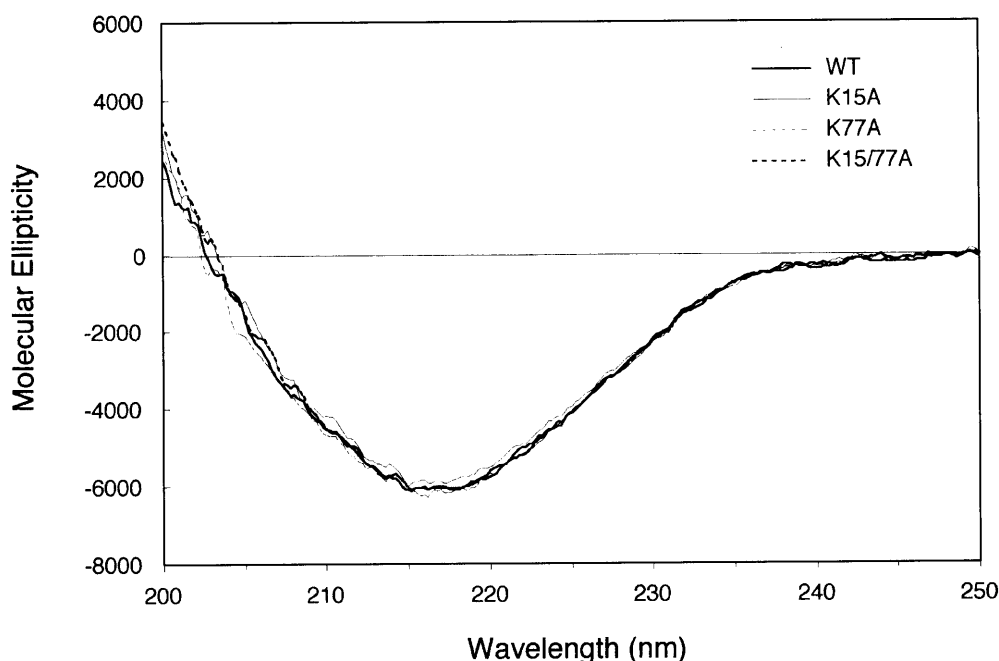
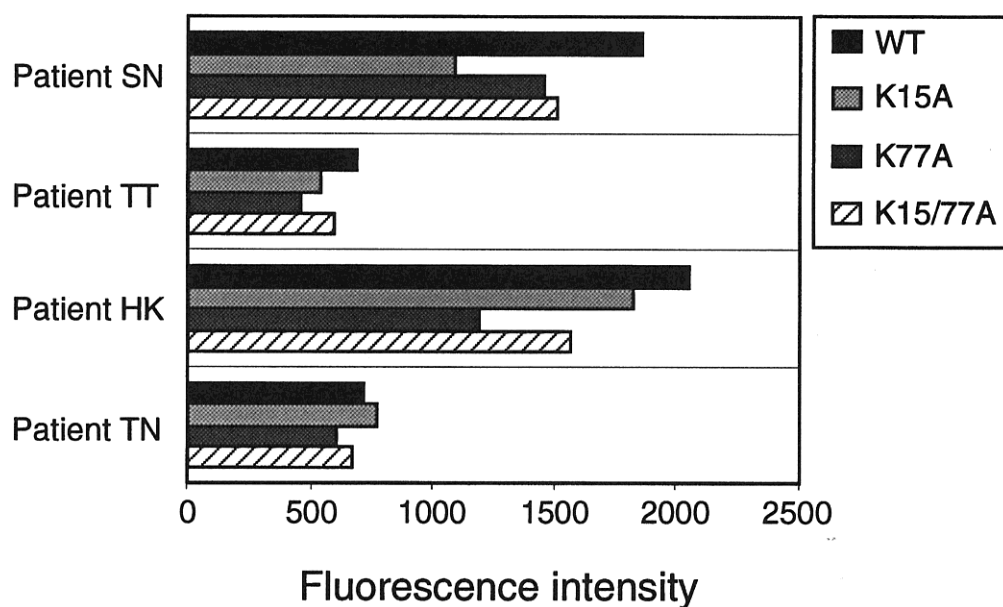


Fig. 5. Circular Dichroism Spectra of the Purified Wild-type and Mutants of Der f 2.

The experimental parameters are: bandwidth 1.0 nm, sensitivity 20 millidegrees, step resolution 0.2 nm/datum, scan speed 50 nm/min, and number of scans 8. Since the protein concentration of K77A had some ambiguity, its spectrum was corrected on the supposition that its concentration was 1.13 times higher than the measurement, respectively. WT: wild-type Der f 2.

A



B

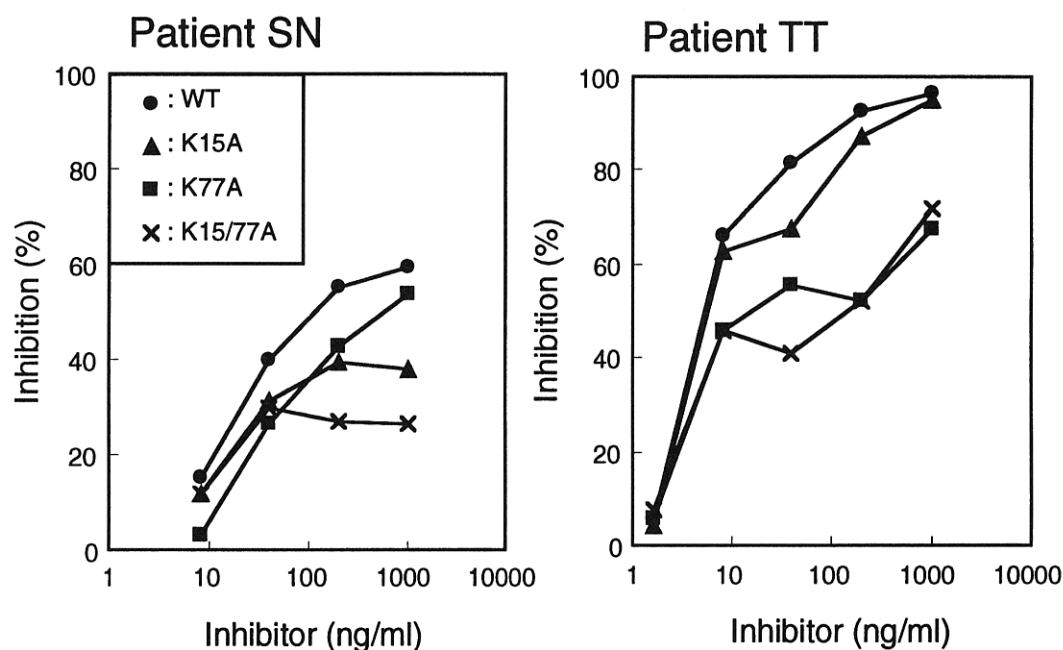


Fig. 6.

to have equivalent structures except in the side chains of amino acid residues. That was confirmed by analysis of the refolded and purified recombinant forms of Der f 2 (Fig. 3A, Fig. 4, and Fig. 5). The mutants of Der f 2 had reduced IgE reactivities compared with the wild-type Der f 2 and reduced inhibitory activities for IgE-binding to the wild-type Der f 2 (Fig. 6A and

B). However, the double mutation of IgE-binding sites, the two of which are three-dimensionally well separated on the surface of the allergen molecule, did not cause a significant reduction in the magnitude of activity to stimulate basophils to degranulate (Fig. 6C).

The results of the binding and inhibition experi-

C

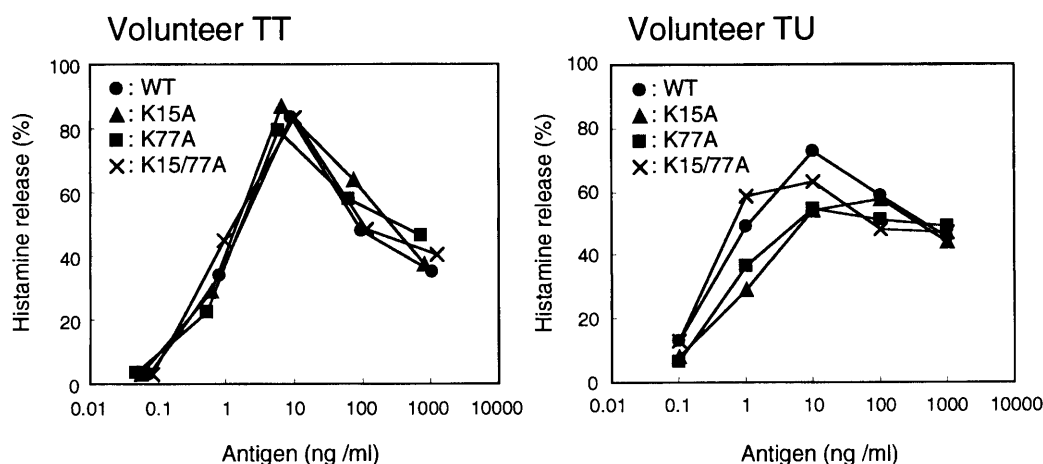


Fig. 6. Allergenicities of Purified Wild-type and Mutants of Der f 2 in Mite-allergic Patients.

(A) IgE-binding profiles of four patients. The concentration of antigens in the immobilization step was 100 ng/ml. Initials of the patients' names are indicated (SN, TT, HK, and TN). (B) Inhibition of IgE-binding to the immobilized wild-type Der f 2 by addition of purified wild-type and mutants of Der f 2. The concentration of the wild-type Der f 2 in the immobilization step was 100 ng/ml. Sera from two patients, SN and TT, were used. (C) Histamine release from basophils stimulated with purified wild-type and mutants of Der f 2. Peripheral blood leukocytes were obtained from two mite-allergic volunteers, TT and TU. WT: wild-type Der f 2.

ments (Fig. 2, Fig. 6A and Fig. 6B) suggest that both Lys15 and Lys77 are involved in IgE-binding and that the number and/or affinity of each epitope including Lys15 and Lys77 is dependent on the individual. The reason why the fluorescence intensities of K15/77A were larger than those of either of K15A or K77A (Fig. 6A) is unclear, although we cannot exclude the possibility that the protein concentration of K15/77A might be a slightly low estimate.

The activity at higher concentrations of each inhibitor is considered to reflect mainly the relative number of IgE that each inhibitor can absorb (Fig. 6B). We considered that there existed a considerable number of IgE molecules recognizing Lys15 in the patient SN and that recognizing Lys77 in the patient TT. Inhibition of IgE-binding to the wild type Der f 2 coupled to a paper disc by addition of soluble wild type Der f 2 was incomplete in the patient SN (approximately 60%). Structures different from soluble Der f 2 might be contained in the population of the decoupled Der f 2 molecules and the serum of the patient SN might contain IgE recognizing such structures.

The magnitude of the histamine-releasing activity of each mutant, K15A, K77A, and K15/77A, is considered similar to or more than 1/10 of that of the wild-type Der f 2 (Fig. 6C). This might result from an insufficient reduction in the number of high-affinity IgE-epitopes in spite of a reduction in the total number of IgE-epitopes (Fig. 6B, Fig. 2, and Fig. 6A).

Ferreira *et al.* reported on a hypoallergenic mutant of the major birch pollen allergen Bet v 1.³⁴⁾ Their strategy is based on the comparative analysis of se-

quences of natural hypoallergenic variants. Therefore, the mutant maintained a secondary structure similar to that of the allergenic wild-type Bet v 1 although their strategy is not based on information from the tertiary structure. The hypoallergenic Bet v 1 mutant included six amino acid substitutions. There might be more unidentified IgE epitopes of Der f 2 and more mutations might be necessary to obtain a large reduction in the allergenicity of Der f 2 as well as Bet v 1.

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