

Effects of proline mutations in the major house dust mite allergen Der f 2 on IgE-binding and histamine-releasing activity

Toshiro Takai¹, Saori Ichikawa^{2,3}, Hideki Hatanaka², Fuyuhiko Inagaki^{2,4} and Yasushi Okumura¹

¹Bioscience Research and Development Laboratory, Asahi Breweries, Ltd, Ibaraki, Japan; ²Department of Molecular Physiology, Tokyo Metropolitan Institute of Medical Science, Japan; ³Department of Material and Biological Science, Faculty of Science, Japan Women's University, Tokyo, Japan; ⁴Division of Structural Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

Der f 2 is the major group 2 allergen from house dust mite *Dermatophagoides farinae* and is composed of 129 amino-acid residues. Wild-type and six proline mutants of Der f 2 (P26A, P34A, P66A, P79A, P95A, and P99A) expressed in *Escherichia coli* were refolded and purified. Formations of intramolecular disulfide bonds in the purified proteins were confirmed correct. The apparent molecular masses analyzed by gel-filtration were 14–15 kDa. The IgE-binding capacity in the sera of seven mite-allergic patients, inhibitory activity for IgE-binding to immobilized wild-type Der f 2, and activity to stimulate peripheral blood basophils to release histamine in two volunteers were analyzed. P95A and P99A, which slightly differed from the wild-type Der f 2 in their CD spectrum, showed reduced IgE-binding, reduced inhibitory activity, and less histamine-releasing activity than the wild-type. P34A also showed reduced allergenicity. Considering that Pro95, Pro99 and Pro34 are closely located in loops at one end of the tertiary structure of Der f 2, we concluded that these loop regions included an IgE-binding site common to all tested patients. P66A showed reduced IgE-binding in two sera out of seven. P26A and P79A showed no reduced allergenicity. However, in immunoblot analysis after SDS/PAGE under reduced conditions, P79A showed no or markedly reduced IgE-binding while the other mutants showed IgE-binding corresponding to that in the assay using correctly refolded proteins. This suggests that Pro79 is involved in refolding of Der f 2. The findings in this study are important for the understanding of the antigenic structure of mite group 2 allergens and for manipulation of the allergens for specific immunotherapy.

Keywords: mite group 2 allergens; proline mutants; IgE epitope; refolding; allergen engineering.

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 of various allergic diseases such as asthma, rhinitis, and atopic dermatitis [3–5]. The major allergens from house dust mite are classified as group 1 (Der f 1 and Der p 1, M_r 25 000) or group 2 (Der f 2 and Der p 2, M_r 14 000) allergens [6–11].

Der f 2 is the major group 2 allergen from *D. farinae* [12–14], and consists of 129 amino-acid residues with marked homology to Der p 2 [15], the group 2 allergen from *D. pteronyssinus*. Der f 2 and Der p 2 are also homologous to other group 2 allergens from different mite species [16–18], and proteins found in human epididymis (HE1) [19], cow milk (EPV20) [20], and moth trachea (esr16) [21].

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 [22–27]. We determined the location of three disulfide bonds of natural Der f 2 (Cys8–Cys119, Cys21–Cys27 and Cys73–Cys78) [28] and found that two

bonds, Cys8–Cys119 and Cys73–Cys78, are critical to the IgE-binding capacity of Der f 2 in sera of several mite-allergic patients [25]. Site-directed mutagenesis of residues other than cysteine in three parts of the Der f 2 sequence, 1–20, 70–81, and 114–129, revealed that 10 residues, Asp7, Ala9, Asn10, Lys15, Asp19, Asn71, Ala72, Met76, Lys77, and Ala120, were important to IgE-binding capacity in one serum [29]. Smith and Chapman reported that Cys73–Cys78 was critical in Der p 2 in four sera [24] and that a mutant of the sequence 44–46, N44P/Q45P/N46H, showed reduced IgE-binding capacity in two sera and one serum pool from seven patients [30]. However, referring to the three dimensional structures of Der f 2 and Der p 2 that were recently determined by NMR [31,32], the residues analyzed by site-directed mutagenesis so far cover only limited areas of the surface of the allergen molecules. Therefore, further mutational analysis of the house dust mite group 2 allergens would provide new information on IgE-binding sites that were not identified in the previous studies.

Mutant allergens with markedly reduced binding to IgE would be useful for safer and more effective allergen-specific immunotherapy [25,33–37]. Proline residues have been considered to have special significance in the architecture of protein, because (a) the cyclic nature of proline prevents rotation around the N–Cα bond, (b) the peptide backbone has no amino hydrogen as a potential donor in hydrogen bonding, and (c) the difference in steric hindrance between *cis* and *trans* conformations is very small in proline compared with in the other natural amino acids, in which *trans* is much more favorable [38]. Thus, alteration of proline would be a reasonable strategy to obtain mutants with significantly reduced allergenicity.

Correspondence to T. Takai, Bioscience Research and Development Laboratory, Asahi Breweries, Ltd, 1–21, Midori 1-chome, Moriya-machi, Kitasoma-gun, Ibaraki, 302–0106, Japan. Fax: + 81 297 46 1505, Tel.: + 81 297 46 1503, E-mail: toshi-ro.takai@asahibeer.co.jp

Abbreviations: FcεRI; high-affinity IgE receptor; RAST-EIA; radioallergosorbent test-enzyme immunoassay.

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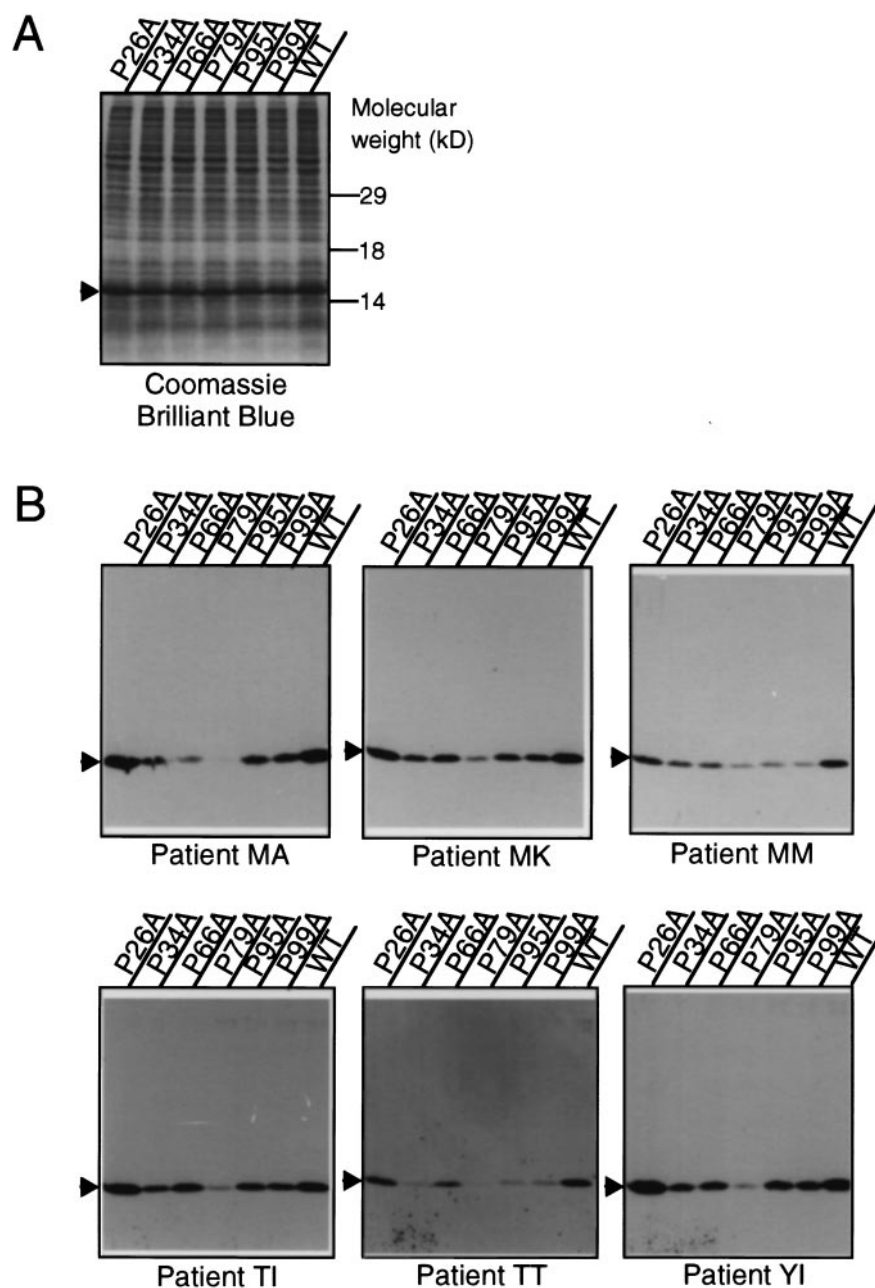


Fig. 1. Expression of wild type and proline mutants of Der f 2 in *E. coli* (A) and immunoblot analysis of their IgE-binding capacities (B). Total cellular proteins of *E. coli* BL21 (DE3) transformed with the expression vector of the wild-type (WT) and six proline mutants of Der f 2 were subjected to SDS/PAGE under reducing conditions and electroblotted. Initials of the patients' names are indicated. An arrow in each panel indicates the location of the bands for the wild-type and proline mutants of Der f 2.

In this study, we analyze six proline mutants with the aim of screening new IgE-binding sites on house dust mite group 2 allergens and obtaining mutants useful for allergen-specific immunotherapy. The structural background of the reduced allergenicity will be discussed on the basis of the tertiary structure of Der f 2.

MATERIALS AND METHODS

Expression of the wild-type and proline mutants of Der f 2 in *Escherichia coli*

Expression vectors for P26A, P34A, P66A, P95A, and P99A were constructed by site-directed mutagenesis. Those for the

wild-type Der f 2 and a mutant, P79A, were constructed as previously described [29,39]. The wild-type Der f 2 and its six proline mutants were expressed in *E. coli* BL21 (DE3) transformed with the expression vectors.

Immunoblot analysis

The method previously described was used with some modifications [25]. 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 subjected to 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

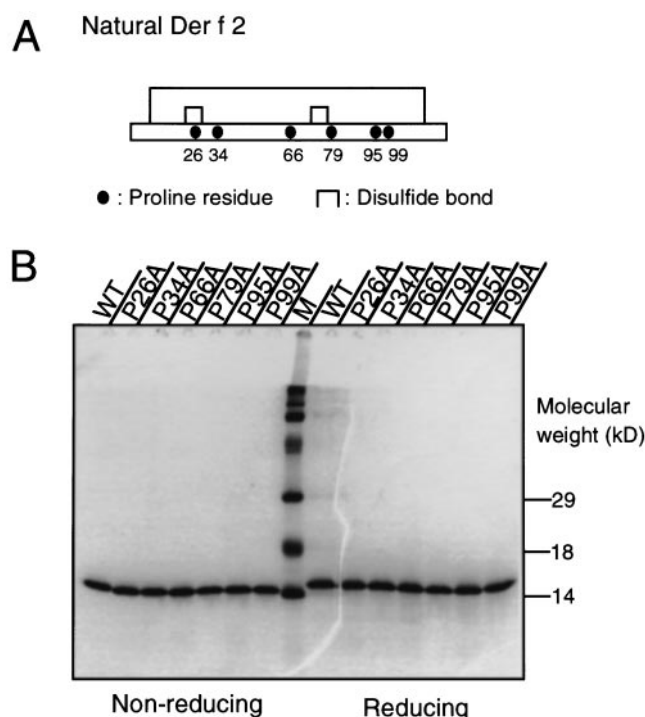


Fig. 2. Refolded and purified wild-type and six proline mutants of Der f 2. (A) Schematic representations of natural Der f 2 with three intramolecular disulfide bonds (Cys8–Cys119, Cys21–Cys27 and Cys73–Cys78). (B) SDS/PAGE analysis under nonreducing and reducing conditions. WT: wild-type Der f 2. M: molecular mass marker.

combination of a mite-sensitive human allergic serum (1/50 dilution) and horseradish peroxidase-conjugated anti-(human IgE) (ϵ -specific) Ig (Biosource International, Camarillo, CA, USA). Blocking, washing, and development were performed with BM Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH, Mannheim, Germany).

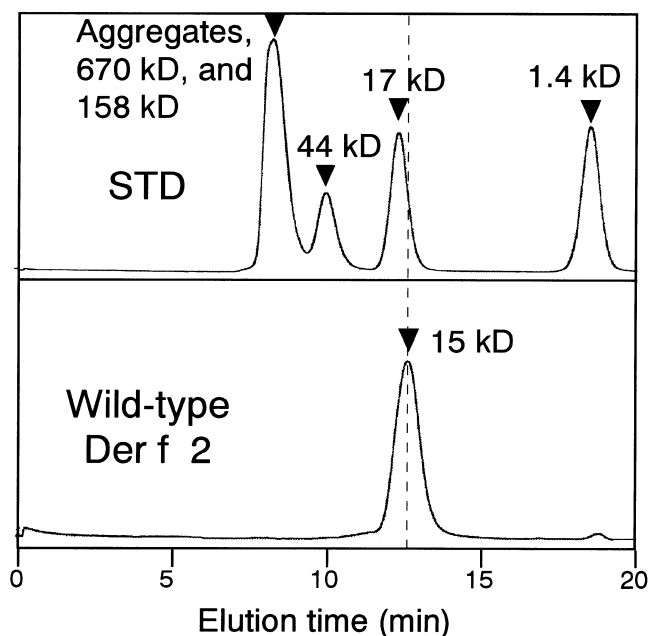


Fig. 3. Elution profile in gel-filtration analysis of purified wild-type Der f 2. The apparent molecular mass of the peak of the purified wild-type Der f 2 is shown with an arrow. The elution profile of molecular mass standards is also shown as a control (STD).

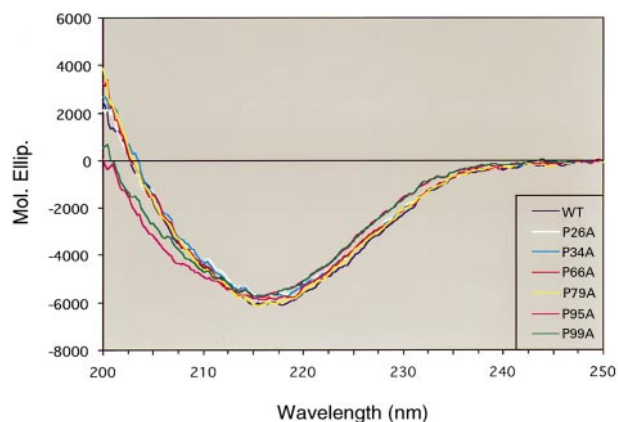


Fig. 4. Circular dichroism spectra of purified wild-type and proline mutants of Der f 2. The experimental parameters are: bandwidth 1.0 nm, sensitivity 20 millidegrees, step resolution 0.2 nm per datum, scan speed 50 nm·min⁻¹, and number of scans 8. As the protein concentrations of P79A and P95A had some ambiguity, their spectra were corrected on the supposition that their concentrations were 1.18 and 1.35 times higher than the measurements, respectively. WT: wild-type Der f 2.

Purification of the wild-type and proline 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 [25,29,39]. The purities were checked by SDS/PAGE under nonreducing or reducing conditions.

Confirmation of correct disulfide bond formation

The disulfide bond formation of the purified recombinant wild-type and mutants of Der f 2 was confirmed correct by peptide mapping as described [28,39]. Briefly, the purified recombinant proteins were digested by lysylendopeptidase and analyzed by HPLC. The peak containing two peptides connected by the Cys8–Cys119 bond and that containing two peptides connected by the Cys73–Cys78 bond were confirmed on the HPLC elution profile. Formation of the Cys21–Cys27 bond was judged by considering the retention time of the peptide containing Cys21 and Cys27.

Gel-filtration analysis

The purified wild-type and mutants of Der f 2 (200 μ L, approximately 100 μ g·mL⁻¹) were subjected to gel-filtration chromatography on a Superdex 75 HR 10/30 (1 cm $\times$ 30 cm) (Pharmacia, Uppsala, Sweden) at a flow rate of 1 mL·min⁻¹ with phosphate buffered saline (pH 7.4). Absorbance at 280 nm was monitored. Molecular mass standards for gel-filtration (Bio-Rad, Richmond, CA, USA) 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 [27]. 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

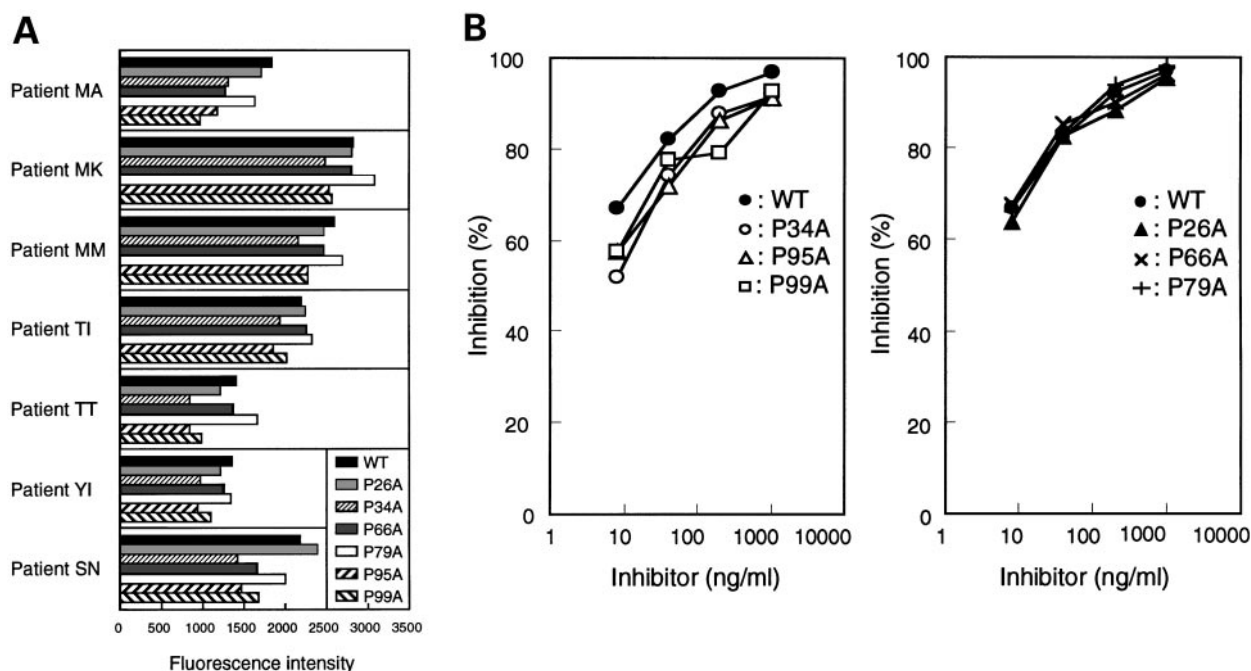


Fig. 5. IgE-binding and inhibitory activities of purified wild-type and proline mutants of Der f 2 in mite-allergic patients. (A) IgE-binding profiles of seven patients. The concentration of antigens in the immobilization step was $100 \text{ ng} \cdot \text{mL}^{-1}$. Initials of the patients' names are indicated. (B) Inhibition of IgE-binding to the immobilized wild-type Der f 2 by addition of purified wild-type and proline mutants of Der f 2. The concentration of the wild-type Der f 2 in the immobilization step was $100 \text{ ng} \cdot \text{mL}^{-1}$. Serum from the patient TT was used.

bound to the disc was detected with β -galactosidase-conjugated anti-(human IgE) Ig by measuring the fluorescence. The sera from patients allergic to house dust mite were kindly provided by H. Okudaira, University of Tokyo.

Inhibition assay

The method was as described previously with some modifications [29]. Briefly, the serum of each allergic patient (1/2–1 dilution) was incubated with various concentrations of each of the purified wild-type Der f 2 and its mutants for three hours at room temperature, and then the solution was added to a paper disc presoaked in wild-type Der f 2. The IgE that bound to discs was detected with β -galactosidase-conjugated anti-(human IgE) Ig. 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, USA) was used [25]. The percentage of released histamine was calculated as the relative amount of histamine in the supernatant to the total of supernatant and cellular histamine.

Circular dichroism

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. Samples were $114 \mu\text{g} \cdot \text{mL}^{-1}$ in 20 mM Tris/HCl, pH 7.4, and 0.9% NaCl.

RESULTS

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

Wild-type and six proline mutants of Der f 2 were over-expressed as insoluble inclusion bodies in *E. coli* cells. The cells containing inclusion bodies were solubilized in a buffer containing SDS and 2-mercaptoethanol and subjected to SDS/PAGE (Fig. 1A).

The reactivities of IgE to the Der f 2 mutants were examined by immunoblot analysis (Fig. 1B). In all tested sera of six patients, P79A showed no or markedly low IgE-binding, three mutants, P34A, P95A, and P99A, showed significantly reduced binding, and P26A showed no reduction. P66A showed markedly low IgE-binding in one patient, MA, and slightly reduced binding in the other five patients, MK, MM, TI, TT, and YI.

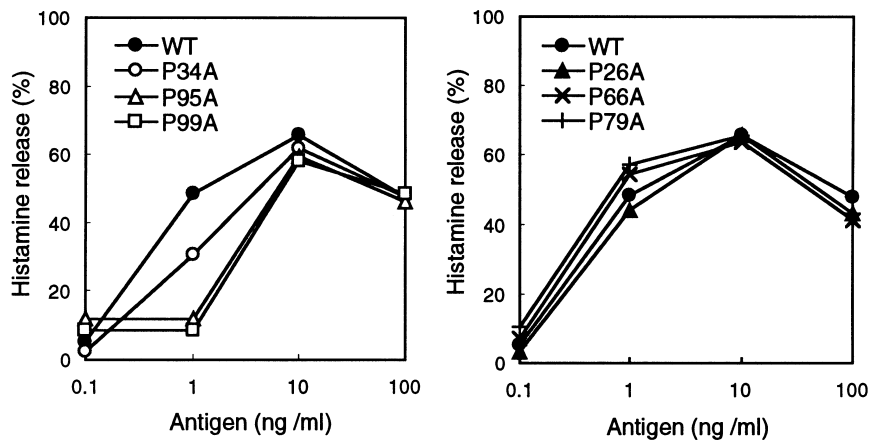
Preparation of purified proline mutants of Der f 2 with correct disulfide bonds

Natural Der f 2 has three intramolecular disulfide bonds (Fig. 2A). The disulfide bond formations of the refolded and purified wild-type and six proline mutants of Der f 2 were confirmed correct by lysylendopeptidase digestion and HPLC. Each of the purified proteins was detected as a single band on SDS/PAGE under both nonreducing and reducing conditions (Fig. 2B). Mobilities of the wild-type and all mutants of Der f 2 were equivalent to each other. The mobility under reducing conditions was less than that under nonreducing conditions.

Gel-filtration analysis of purified proline mutants of Der f 2

The purified wild-type Der f 2 was detected as a single peak in gel-filtration analysis and its apparent molecular mass was

Volunteer TT



Volunteer TU

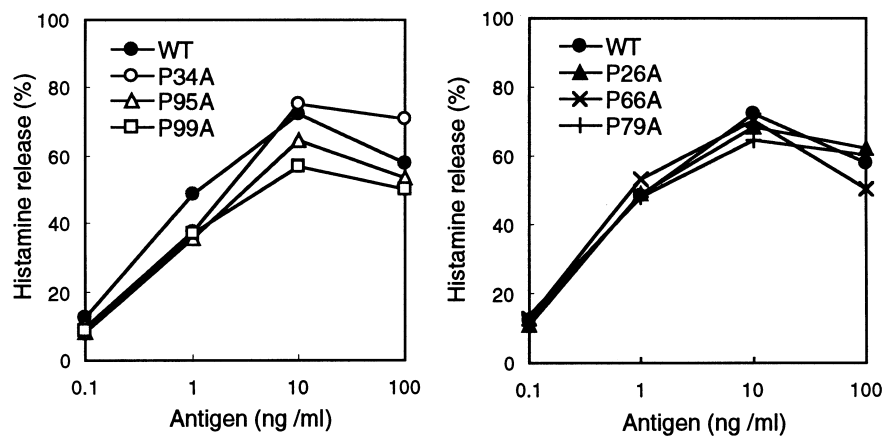


Fig. 6. Histamine release from basophils stimulated with purified proline mutants of Der f 2. Peripheral blood leukocytes were obtained from two mite-allergic volunteers, TT and TU. WT: wild-type Der f 2.

14 kDa (Fig. 3). The purified six proline mutants were also detected as a single peak and their apparent molecular masses were 14–15 kDa (data not shown).

Secondary structures of purified proline mutants of Der f 2

The wild-type Der f 2, P26A, P34A, P66A, and P79A showed a similar CD spectrum (Fig. 4). Two mutants, P95A and P99A, showed slightly different patterns from the wild-type Der f 2.

Reactivities of purified proline mutants of Der f 2 to IgE in patient sera

The IgE reactivities of the homogeneously purified six proline mutants with correct disulfide bonds were compared with that of the wild-type Der f 2 by RAST-EIA (Fig. 5A). In all tested sera of seven patients, three mutants, P95A, P99A, and P34A, showed a reduction and two mutants, P26A and P79A, showed reactivity equivalent to the wild-type Der f 2. P66A showed reduced reactivity in two patients, MA and SN, and reactivity equivalent to the wild-type Der f 2 in the other five patients, MK, MM, TI, TT, and YI.

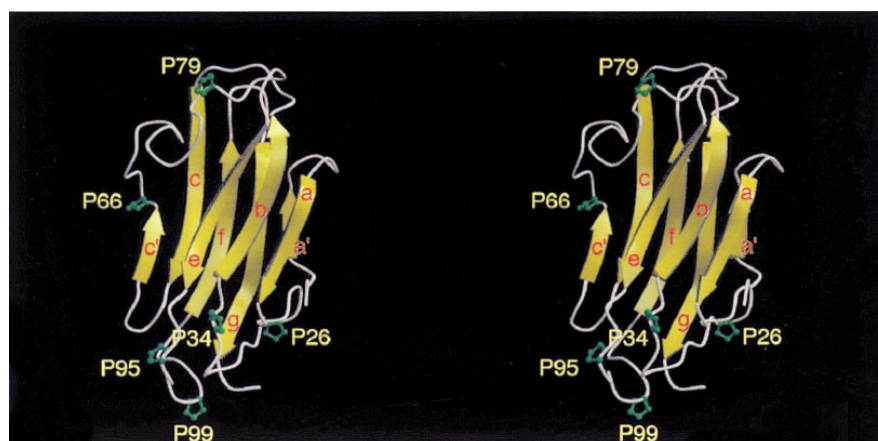
Activities of purified proline mutants of Der f 2 to inhibit IgE-binding to immobilized wild-type Der f 2

The capacities of the mutants to inhibit IgE from binding to the wild-type Der f 2 were also examined using serum of one patient, TT, by inhibition assay (Fig. 5B). The inhibitory effects by three mutants, P34A, P95A, and P99A, were reduced compared with that by the wild-type Der f 2. The inhibitory effects by the wild-type Der f 2, P26A, P66A, and P79A, were equivalent.

Activities of purified proline mutants of Der f 2 to induce histamine release from basophils

Peripheral blood basophils from two allergic volunteers, TT and TU, were stimulated with the wild-type and six proline mutants of Der f 2, and histamine release was measured (Fig. 6). Amounts of 0.1 ng·mL⁻¹ had no effect, and 10 ng·mL⁻¹ was the saturating concentration in all cases. In volunteer TT, at 1 ng·mL⁻¹, histamine release by two mutants, P95A (12 %) and P99A (9 %), was very low and that by P34A (31 %) was lower than that by the wild-type Der f 2 (49 %), P26A (44 %), P66A (55 %) and P79A (58 %). In volunteer TU, at 1 ng·mL⁻¹, histamine release by three mutants, P34A (38 %),

Fig. 7. Ribbon diagram of the mean structure of Der f 2 [31]. The proline residues (P26, P34, P66, P79, P95, and P99) and β -strands (a', a b, c, c', e, f, and g) are shown. This figure was produced by the program MOLSCRIPT [40] and RASTER 3D [41], and is shown in stereo.



P95A (36 %) and P99A (37 %), was lower than that by the wild-type Der f 2 (49 %), P26A (49 %), P66A (53 %) and P79A (48 %).

DISCUSSION

Purified wild-type and all proline mutants of Der f 2 were considered correctly refolded because of the formation of three intramolecular disulfide bonds and reasonable apparent molecular masses estimated with gel-filtration analysis (Fig. 3). Less mobility on SDS/PAGE under reducing conditions than non-reducing conditions is considered a result of the elimination of the disulfide bonds under reducing conditions (Fig. 2B). These recombinant proteins, which were correctly refolded and purified until homogeneous, were used in this study except for immunoblotting.

Three mutants, P95A, P99A, and P34A, showed reduced IgE-binding in all tested patients (Figs 5 and 6 and Fig. 1B). Alterations of Pro95 and Pro99 to alanine affected the secondary structure of Der f 2 (Fig. 4). Pro95, Pro99, and Pro34 themselves and/or some of the surrounding residues were considered recognized by IgE. Pro95, Pro99, and Pro34 are closely located in loop regions at one end of the tertiary structure of Der f 2 as determined by NMR [31] (Fig. 7). Pro95 and Pro99 are located in the loop between the β -strands e and f, and Pro34 is located in the C-terminal region of the loop between the β -strands a and b. The area including the three prolines, Pro95, Pro99, and Pro34, were clearly defined as a common IgE-binding site for the first time in this study.

P26A showed no reduced IgE-binding and P66A showed reduced IgE-binding in two patients, MA and SN, out of seven (Fig. 5 and Fig. 1B). Pro66 itself and/or some of the surrounding residues were considered recognized by IgE in some patients.

Refolded and purified P79A showed no reduction in IgE-binding and histamine-releasing activity (Figs 5 and 6). However, in immunoblot analysis after SDS/PAGE under reduced conditions, P79A showed no or markedly reduced IgE-binding while the other mutants showed IgE-binding corresponding to that in the assay using correctly refolded and purified proteins (Fig. 1B). These results suggest that Pro79, which is next to Cys78 forming a disulfide bonds Cys73–Cys78, plays roles in the refolding of Der f 2. Interestingly, the conformation of Pro79 in Der p 2 was reported as *cis* [32], and it was found independently by using NMR that Pro79 in Der f 2 also adopts the *cis* conformation (S. Ichikawa & H. Hatanaka, unpublished

results). In natural amino acids other than proline, the *trans* conformation is much more favored than the *cis* conformation because of a large difference in steric hindrance between *cis* and *trans*. Therefore, the *trans* in Ala79 substituted for the *cis*-Pro79 might have prevented refolding of P79A from the polypeptide that had been reduced and denatured with 2-mercaptoethanol and SDS. The involvement of *cis*-Pro in the refolding of the allergenic structure is an interesting topic for manipulation of allergens for vaccination.

In this study, we identified an IgE-binding site which included three prolines, Pro95, Pro99, and Pro34, common to all tested patients and suggested that Pro79 had roles in the refolding of the architecture of mite group 2 allergens. The findings in this study are important for the understanding of the antigenic structure of mite group 2 allergens and for manipulation of the allergens for safer and more effective immunotherapy.

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