

Chiharu Nishiyama^a
 Toshifumi Yuuki^a
 Yukiko Usui^a
 Namiko Iwamoto^a
 Yasushi Okumura^a
 Hirokazu Okudaira^b

^a Central Research Laboratories,
 Asahi Breweries Ltd.,

^b Department of Medicine and
 Physical Therapy, Faculty of Medicine,
 University of Tokyo, Japan

Effects of Amino Acid Variations in Recombinant *Der f*II on Its Human IgE and Mouse IgG Recognition

Key Words

*Der f*II
 Amino acid variation
 Disulfide bond
 Antibody
 Stability

Abstract

Amino acid sequencing of the major mite allergen *Der f*II purified from mite body extract revealed that it is a mixture of at least two variants. Substitutions were found only at positions in which amino acid variations were predicted from the nucleotide sequences of three cloned cDNAs. When cDNAs corresponding to the three variants were expressed in *Escherichia coli*, *Der f*II proteins were produced as inclusion bodies. Denaturation and renaturation with urea converted recombinant *Der f*II into a protein with three intramolecular disulfide bonds (Cys8-Cys119, Cys21-Cys27, and Cys73-Cys78), which were identical to those previously identified in native *Der f*II. All the variants, including native *Der f*II, were equally recognized by human IgE antibodies from 14 different sera of mite-allergic patients. These results suggested that recombinant *Der f*II proteins assumed a conformation identical to that of the native *Der f*II and that all the *Der f*II variants acted as allergens. This result also suggested that IgE antibody binds to the region where there were no amino acid variations. The binding ability of the monoclonal antibody (mAb) 18G8 for the variant clones 1, 2, and 11 *Der f*II was almost the same. However, mAb 15E11 bound to clone 1 *Der f*II more efficiently than to clones 2 and 11, whereas mAb 13A4 recognized clone 2 *Der f*II as the most preferable antigen. This suggested that these antibodies recognized the region of amino acid variations. The stability of *Der f*II variants was analyzed by measuring their IgE antibody binding activity after heating, freezing, or acid treatment, and was analyzed by resistance to trypsin digestion. These analyses revealed that clone 2 *Der f*II was the most stable among the *Der f*II variants.

Introduction

*Der f*II is a major allergenic protein of the mite *Derma-tophagoides farinae*, which causes allergic diseases such as asthma, perennial rhinitis, and eczema [1–3]. *Der f*II is

composed of 129 amino acid residues that have no sequence homology with other proteins except for *Der p* II, which is also an allergic protein produced in a similar mite, *Derma-tophagoides pteronyssinus* [4–6]. The physiological function of the group II allergen (*Der f*II and *Der p* II) in the

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Correspondence to: Dr. Chiharu Nishiyama
 Central Research Laboratories, Asahi Breweries Ltd.
 2-13-1, Ohmori-kita, Ohta-ku
 Tokyo 143 (Japan)

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mites is still unknown. However, skin tests and radioallergosorbent test (RAST) analyses revealed that a specific interaction between *Der f*II and human IgE was involved in the allergic diseases [7–13].

As reported previously, three cDNA variants of *Der f*II were highly homologous [4]. Compared with clone I of *Der f*II, clone 2 possesses a single amino acid exchange and clone 11 has four amino acid substitutions. Clarification of the difference in the structure and antigenicity of these *Der f*II variants should provide useful information for the therapy of allergic diseases mediated by this protein. We examined the interaction of these *Der f*II variants with human IgE antibody and mouse monoclonal IgG antibodies. This paper describes the binding of human IgE and mouse monoclonal IgG to the *Der f*II variants.

Materials and Methods

Purification of Der f II from Mite Body Extract

Native *Der f*II was purified from freeze-dried mite body extract, which was supplied by Torii & Co., Ltd., Tokyo, Japan. Four milligrams of *Der f*II was purified from 5 g of mite body extract by means of DEAE-Sephacel, S-Sepharose, and Sephadex G-75 chromatography as described previously [14].

Expression and Purification of Recombinant Der f II

E. coli BL21 [15] cells harboring a pGEMEX1 (Promega Co., Wisc., USA) derivative containing each of the cDNAs for three *Der f*II variants under the control of the T7 promoter (manuscript in preparation), were cultured in 5 ml of LB medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl per 1 liter, pH 7.5) containing 50 µg/ml ampicillin at 30°C. The same medium, 170 ml, was inoculated with 1.7 ml of this preculture in a 500 ml shaking flask. After 2.5 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the T7 promoter. After an additional 4 h incubation, the cells were harvested by centrifugation, resuspended in 6 ml of TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 7.5) and disrupted by sonication. Since *Der f*II proteins were produced as inclusion bodies in this expression system, insoluble *Der f*II was collected by centrifugation at 7,000 *g* for 15 min, and solubilized with 50 ml of 6 M urea containing 20 mM Tris-HCl (pH 8.5), and dialyzed against 20 mM Tris-HCl (pH 8.5) for 12 h to obtain the correctly refolded molecule [16]. The dialysate containing refolded *Der f*II was applied onto a DEAE-Toyopearl column (3.5×9.8 cm, Tosoh Co., Tokyo, Japan) equilibrated with 20 mM Tris-HCl (pH 8.5) and eluted with 20 mM Tris-HCl (pH 8.5) including 50 mM NaCl. The eluate gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Peptide Mapping of Der f II

Purified native or each recombinant *Der f*II was digested with lysendopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described previously [14]. The mixture of peptide fragments was separated by high-performance liquid chromatography (HPLC) using a C8 column (RP-300 100×2.1 mm, Applied Biosystems, Inc.,

Calif., USA) equilibrated with solvent A (0.1% trifluoroacetic acid (TFA)/distilled water). The gradient profile was from 0% solvent B (0.1% TFA/70% acetonitrile) at 0 min to 65% at 45 min and to 100% at 55 min in linear segments and was kept 100% for 5 min. The eluate from this column was monitored by absorbance at 215 nm. Each peptide fragment was sequenced with 473A protein sequencing system (Applied Biosystems).

Binding of Human IgE Antibodies to Der f II

Fifty microliters of the *Der f*II solution containing 0.1 ng–1 µg *Der f*II protein (0.1 M borate buffer, pH 8.0) was soaked on a paper disc (6.0 mm in diameter) activated with BrCN and stored in a test tube (Shionogi & Co., Ltd., Osaka, Japan) overnight at room temperature. The antigen-coupled paper disc was washed once with 0.5 ml of 0.1 M NaHCO₃ and blocked with 0.25 ml of 0.1 M monoethanolamine, pH 9.0, for 3 h at room temperature. After a 3-hour incubation, the blocking buffer was aspirated, then the disk was washed once with 0.5 ml of 0.1 M NaHCO₃ and three times with 0.5 ml of 0.1 M acetate buffer, pH 4.0. Radioallergosorbent test-enzyme immunoassay (RAST-EIA) was then carried out using a RAST-EIA kit (Kabi Pharmacia Diagnostics, Uppsala, Sweden) according to the supplier's instructions. In brief, human serum containing anti-*Der f*II IgE was applied onto the disc. The amount of the IgE antibody bound to *Der f*II, which was fixed on the paper was measured with β-D-galactosidase-labeled anti-human IgE rabbit IgG using *o*-nitrophenyl-β-D-galactopyranoside as a substrate.

Binding of Mouse mAbs to Der f II

Various concentrations of *Der f*II in 100 µl of PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8.1 mM Na₂HPO₄, pH 7.4) were applied to each well of the enzyme-linked immunosorbent assay (ELISA) plate, Immulon 2 (Dynatech Laboratories, Inc., Va., USA), and incubated for more than 2 h at room temperature. After removing the *Der f*II solution, the well was washed with PBS 3 times and blocked with PBS including 1% (w/v) bovine serum albumin (BSA) for 1 h at room temperature. After removing the blocking buffer and washing the wells with PBS including 0.05% (w/v) Tween 20 (PBS-Tween), 100 µl of mouse mAb was applied to the wells at a concentration of 10 µg/ml for 15E11, 5 µg/ml for 13A4, and 2.5 µg/ml for 18G8 and incubated for 2 h or more at room temperature [17]. One hundred microliters of 10 µg/ml biotinylated anti-mouse IgG goat IgG antibody (Vector Laboratories, Inc., Calif., USA) in PBS containing 0.02% (w/v) NaN₃ and 1 mg/ml BSA was then added and incubated for 2 h. The wells were washed with PBS-Tween as described above and incubated with 100 µl of alkaline phosphatase-labeled avidin (Vector Laboratories) containing 100 units of alkaline phosphatase activity in 90 ml PBS/NaN₃-BSA as above for 30 min at room temperature. The enzyme-labeled avidin was removed and the wells were washed with PBS-Tween three times. One hundred microliters of 0.5 mg/ml *p*-nitrophenylphosphate disodium in diethanolamine/HCl (pH 9.8) was added as the substrate and incubated at room temperature. Ten minutes later, the reaction was stopped by adding 100 µl of 5 N NaOH and the absorbance of the solution in each well was measured at 405 nm.

Conformational Stability of Der f II

Heat-stability was measured as the remaining binding ability to human IgE antibody using the RAST-EIA after heating 250 µl of *Der f*II (200 µg/ml of 20 mM Tris HCl, pH 8.5) at 70°C for 30 min. The effect of freeze-thawing on the stability was examined as follows. Five

	10	20	30	40	50
Clone 1	DQVDVKDCANNEIKKVMVDGCHGSDPCIHRGKPFLEALFDANQNTKTA				
Clone 2	-----				
Clone 11	-----				
	60	70	80	90	100
	KIEIKASLDGLEIDVPGIDTNACHFVKCPLVKGQQYDIKYTNWVPKIAPK				
	-----M-----				
	-----M-----A-----				
	110	120	129		
	SENVVVTVKLIGDNGVLACAIATHGKIRD				

	-----V-----A-----				

Fig. 1. The amino acid sequences of *Der f*II deduced from three cDNA clones [4].

hundred microliters of *Der f*II (40 µg/ml of PBS buffer) was frozen at -20°C for 1 h then thawed at 37°C. This cycle was repeated three times, then the IgE antibody binding ability was analyzed using the RAST-EIA. Stability under acidic conditions was examined by dialyzing *Der f*II against 0.3 M acetate buffer pH 5.0, overnight at 4°C. The stability of *Der f*II was evaluated as described above.

Resistance to Digestion with Trypsin

To examine the protease sensitivities, *Der f*II variants (0.2 mg/ml of 14 mM Tris-HCl and 50 mM NaCl, pH 8.5) were incubated with 0.1 µg of trypsin at 37°C and the reaction was stopped by rapid freezing 3, 6, or 9 h later. The amount of *Der f*II protein present in 10 µl of each reaction was analyzed by SDS-PAGE.

Results

Heterogeneity of *Der f*II Purified from *D. farinae*

We used three independent *Der f*II cDNA clones, each of which encodes *Der f*II protein with 4 amino acid exchanges [4] (fig. 1). To examine the variations in *Der f*II proteins purified from mite bodies, we purified *Der f*II (native *Der f*II) from the house-dust mite, *D. farinae*, and examined the heterogeneity of native *Der f*II. As shown in figure 2, lysylendopeptidase-generated peptide fragments of native *Der f*II were mapped and their amino acids were sequenced. These analyses revealed variations in peak 8 and between peaks 6 and 7. Peptide fraction 8 containing amino acid residues from Ala56 to Lys82 possessed both Met and Val residues at position 76 at a ratio of 4/3. As shown in figure 2, peaks 6 and 7 contained the same disulfide-bridged peptides, one from Asp7 to Lys14 and the other from Leu110 to Lys126, which are connected by the disulfide bond between Cys8 and Cys119. Peak 6 had Val and Ala residues at

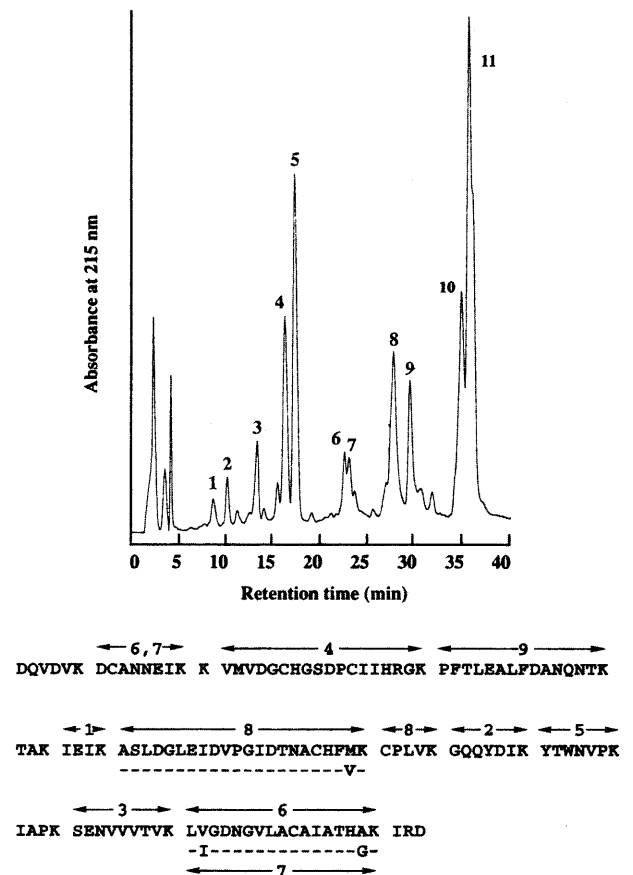


Fig. 2. Peptide map of native *Der f*II digested by lysylendopeptidase. The fragment number is the same as that of the corresponding peak shown in the chromatogram.

positions 111 and 125, respectively, whereas Ile and Gly were detected at the corresponding positions of peak 7. The ratio of Ile/Val at position 111 was about 7/9, and that of Gly/Ala at 125 was almost 1/1. Peak 2 containing the peptide from Gly83 to Lys89 generated by lysylendopeptidase, possessed only an Ile residue at position 88. Peptide mapping and amino acid sequencing of a peptide fragment generated by V8 protease, however, revealed an Ala as well as an Ile residue at position 88 (data not shown). These results showed that at least two variants were produced in *D. farinæ*.

Disulfide Bonds in Recombinant Der f II

The three cDNA clones were expressed in *E. coli* under the control of the T7 promoter and their products were purified as described in Materials and Methods. We examined disulfide bond formation of recombinant *Der f*II proteins prior to their characterization, because the affinity of native *Der f*II to human IgE antibody is remarkably decreased by reduction and alkylation [18]. We have described three disulfide bonds, Cys8-Cys119, Cys21-Cys27, and Cys73-Cys78, in *Der f*II proteins purified from mite bodies [14]. To confirm the formation of the correct disulfide bridges in the recombinant *Der f*II proteins, we constructed peptide maps and sequenced the recombinant *Der f*II proteins from clones 1, 2 and 11 (fig. 3). The HPLC elution profile of each recombinant *Der f*II digested with lysylendopeptidase was principally the same as that of the native protein. Therefore, we assigned the peptide fragments of the recombinants. When the native *Der f*II proteins were digested with lysylendopeptidase, a peak eluted at 24 min containing two peptides connected by a disulfide bond between Cys8 and Cys119, and the peak at 27 min corresponded to two peptides connected by a disulfide bridge between Cys73 and Cys78 [14]. Amino acid sequencing of the peptide fragments of the corresponding fractions of these recombinant *Der f*II proteins proved the formation of the two disulfide-bonds of Cys8-Cys119 and Cys73-Cys78. Other two cysteine residues, both Cys21 and Cys27, are located in the same peptide fragment from Val16 and to Lys33 generated by the peptidase. The formation of the disulfide bond between Cys21 and Cys27 was judged by considering the retention time of the fragment on HPLC. This peptide eluted at 17 min when the disulfide bond was formed, and while it was eluted at 16.5 min under reducing conditions when the two cysteine residues were in a free thiol state. The corresponding peptide fragments of the recombinant were eluted exactly at the same time as that in the disulfide state. These results indicated that all the three disulfide bridges were formed in the recombinant *Der f*II proteins.

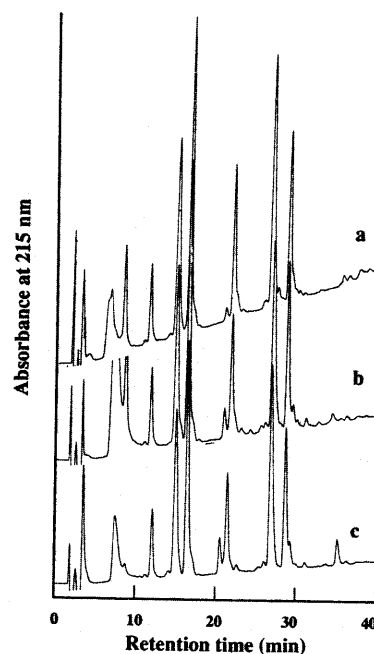


Fig. 3. Chromatogram of recombinant *Der f*II fragments generated by lysylendopeptidase digestion. **a** Clone 1. **b** Clone 2. **c** Clone 11.

Binding Activity of Anti-*Der f* II Human IgE to Native and Recombinant *Der f* II

Analysis using serum from patient with asthma and dermatitis, which contained anti-*Der f*II IgE antibody at a high titer, revealed that the antibody recognized three recombinant *Der f*II proteins with almost equal efficiency (fig. 4). Similar results were noted with native *Der f*II protein, which is a mixture of more than two variants. Furthermore, when 13 other sera were used, there were no differences among the *Der f*II proteins tested.

Binding Activity of Anti-*Der f* II mAb to Three *Der f* II Variants

We examined the effect of the amino acid variation on *Der f*II recognition by the mAbs, 15E11, 13A4, and 18G8 [17]. Figure 5 shows dose response profiles of *Der f*II proteins against peroxidase activity which is proportional to the amount of mAb bound to *Der f*II protein. The mAb 15E11 had the highest specificity for clone 1. The mAb 15E11 also bound the other two *Der f*II clones, clone 2 and clone 11, but with lower efficiency; the specificity for clone 2 was higher than that for clone 11. There were no such large differences between the three recombinant *Der f*II proteins when the

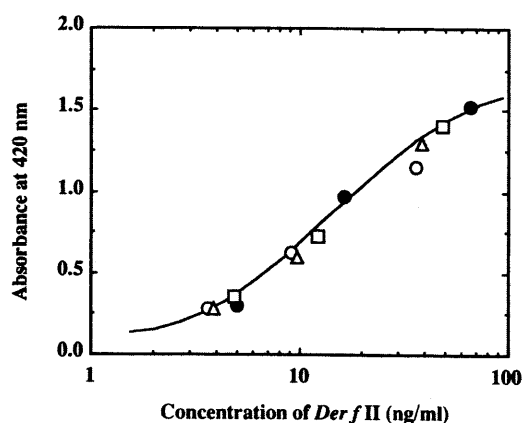


Fig. 4. Binding activity of anti-*Der fII* human IgE to native and three types of recombinant *Der fII*. ● = Native *Der fII*; ○ = clone 1; △ = clone 2; □ = clone 11.

other two mAb were used. However, antibody 13A4 might have higher specificity for clone 2 *Der fII*.

Conformational Stability of *Der fII* Variants

Human IgE antibody binding activity to the three *Der fII* variants was measured after heating, freezing, or acid treatment. As shown in figure 6, these *Der fII* variants retained the IgE binding ability after these procedures. Among the *Der fII* variants, clone 2 *Der fII* was the most stable. We next examined the trypsin-sensitivity of *Der fII* variants. Clone 2 was again the most stable (fig. 7). After 9 h, most of clone 2 *Der fII* remained without any degradation, although half of clone 11 *Der fII*, for example, was lost. By quantitation of the *Der fII* protein in SDS-PAGE in figure 7 by densitometry, remaining amounts of *Der fII* proteins after 9 h trypsin digestion were 71, 82, and 31% for clone 1, clone 2, and clone 11, respectively.

Discussion

Der fII has been purified from house-dust mite, *D. farinae*, as one of major mite allergen [1–3]. When the purified native *Der fII* was analyzed by SDS-PAGE, it migrated as a single band of molecular weight 14,000 (data not shown). However, it was separated into two bands by isoelectric focusing, and two close peaks were eluted by HPLC (data not shown). These results suggested that at least two types of *Der fII* are produced in house-dust mite. On the other hand, we have found three cDNA clones encoding *Der fII* proteins which have amino acid exchanges at maximally 4 re-

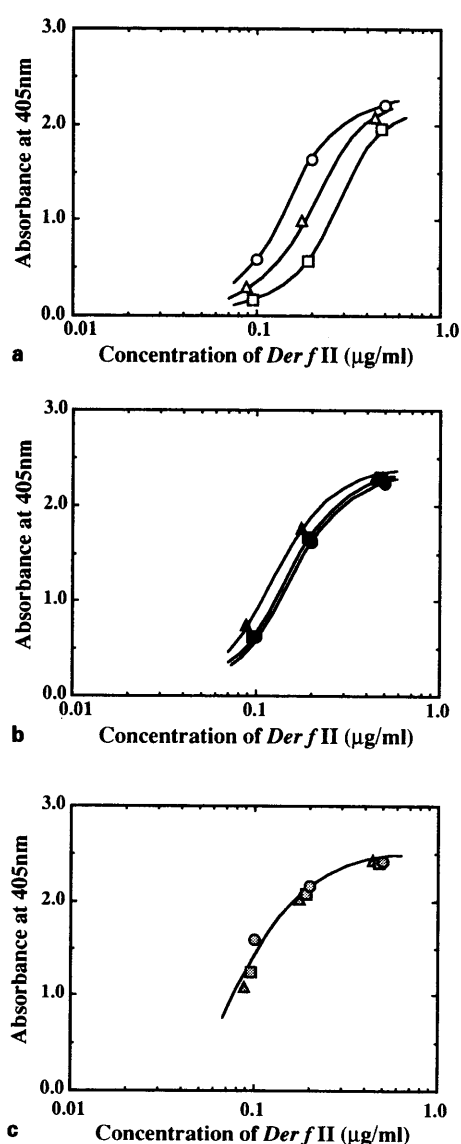


Fig. 5. Binding activity of anti-*Der fII* mouse mAbs to three variants of recombinant *Der fII*. **a** Binding activity of mAb 15E11 to each recombinant *Der fII*. ○ = Clone 1; △ = clone 2; □ = clone 11. **b** Binding activity of mAb 13A4 to clone 1 (●), clone 2 (▲), and clone 11 (■). **c** Binding activity of mAb 18G8 to clone 1 (○), clone 2 (△), clone 11 (□).

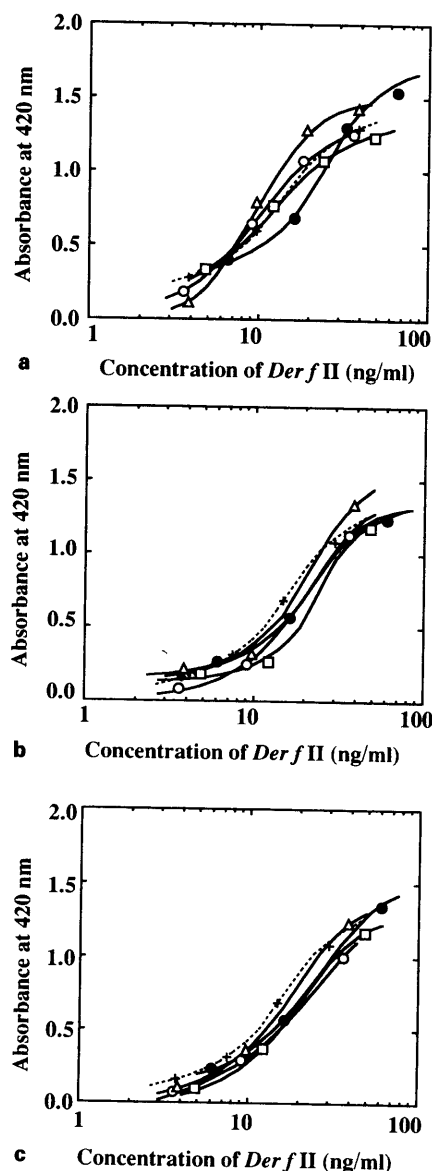


Fig. 6. Remaining binding activity of anti-*Der fII* human IgE to native and recombinant *Der fII* after heating, freezing, or acid treatment. +=Native *Der fII* without any treatment as a control. ●=native; ○=clone1; △=clone2; □=clone11 under each condition. **a** Heat. **b** Freezing. **c** Acid treatment.

sidues out of 129 each [4] (fig. 1). To clarify the heterogeneity, all the lysylendopeptidase- and V8 protease-generated peptides of native *Der fII* purified from *D. farinae* were collected with HPLC and sequenced by protein sequencer. This analysis showed at least two variants in which amino acid variations were found at positions 76, 88, 111 and 125. According to amino acid sequences of *Der fII* deduced from cDNA clones (fig. 1), clone 1 has Val, Ile, Ile, and Gly at 76, 88, 111, and 125, respectively, and clone 2 has Met at residue 76, the sole difference between clone 1 and 2. Clone 11 has Met, Ala, Val, and Ala, at each position. The polymorphism identified in this study coincided well with the amino acid variations among the three cDNA clones.

Der fII purified from the dust mite was heterologous. Therefore, the effects of each variation upon, for example, IgE antibody binding leading to allergy, or the structural stability may be difficult to analyze with native *Der fII*. On the other hand, since we have three independent cDNA clones for *Der fII*, the effect of the variation of *Der fII* was examined with recombinant *Der fII* protein produced by an *E. coli* expression system. All variants of recombinant *Der fII* were confirmed to form three disulfide bonds, which was suggested to be important for correct folding and binding to IgE antibody. We also found that each recombinant *Der fII* variant had a CD spectrum profile identical to that of native *Der fII* (data not shown). Consequently, we concluded that recombinant *Der fII* took the same three-dimensional structure as native *Der fII*. Then, human IgE binding to three variations of recombinant and native *Der fII* were examined using RAST-EIA and no differences were found in binding ability between IgE and each antigenic protein. Furthermore, when the allergenicity of recombinant and native *Der fII* was analyzed by the Prick test, all the *Der fII* proteins induced an allergic response to the same extent (data not shown). From these results, we speculate that the human IgE antibodies mainly bound to *Der fII* region(s) where there were no variation in the amino acid sequence. At present, it is also possible that even though the IgE bound directly or close to the region showing variations, such variations might have no effect on the IgE binding. To clarify the effects of amino acid sequence on IgE binding ability further, site-directed mutagenesis and deletion analyses of *Der fII* protein are now in progress.

Dose response curves in figure 5a suggested that the monoclonal antibody 15E11 distinguished the difference in amino acid sequence between the three recombinant *Der fII* proteins. Using monoclonal antibody 15E11 as the solid-phase antibody and alkaline phosphatase labeled mAb 13A4 as the secondary (detection is better) antibody [19], the binding of recombinant *Der fII* from clones 2 and 11 was

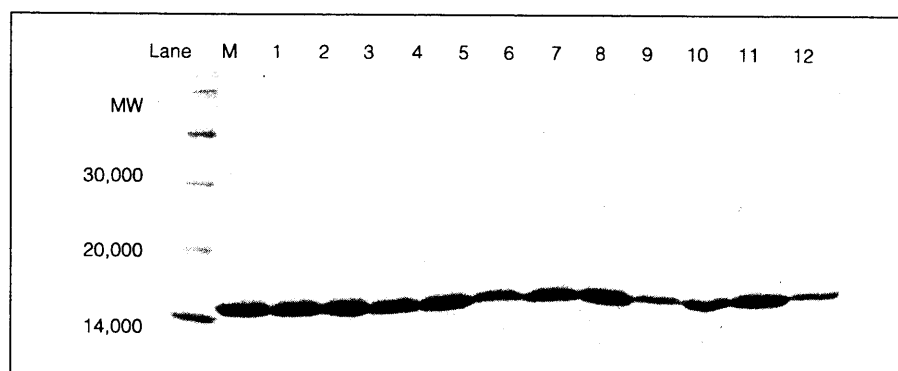


Fig. 7. Trypsin-sensitivity of three *Der fII* variants. Lane M: molecular weight marker; lanes 1, 2 and 3, clones 1, 2 and 11, respectively, before trypsin digestion; lanes 4, 5 and 6, clones 1, 2 and 11, respectively, after digestion for 3 h; lanes 7, 8 and 9, clones 1, 2 and 11, respectively, after digestion for 6 h; lanes 10, 11 and 12, clones 1, 2 and 11, respectively, after digestion for 9 h. Relative amounts of remaining *Der fII* calculated by densitometry were 100, 100, 100, 99, 102, 55, 82, 91, 36, 71, 82, and 31% for lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12, respectively.

reduced to 1/2 and 1/5 of the activity of clone 1. Since no such great difference in the recognition by 13A4 between these three *Der fII* clones was observed (fig. 5b), we concluded that the difference in amino acid sequence between the *Der fII* clones affected the recognition of *Der fII* protein by 15E11. Amino acid sequencing of *Der fII* revealed that Val residue at position 76 of clone 1 was exchanged for Met in clone 2. Since clone 1 had higher binding efficiency to mAb 15E11, the Val residue might play an important role for recognition by the antibody. In addition, mAb 15E11 recognizes only *Der fII* but not *Der p II* in which the 76th amino acid is Met. These results suggested that the mAb recognizes *Der fII* at the region around the 76th position. Consistent with this, Western blotting of several *Der fII* proteins with various deletions verified that the epitope of *Der fII* for mAb 15E11 was between the 67th and 90th residues (data not shown). On the other hand, there was a small but obvious difference in the binding to mAb 15E11 between clones 2 and 11. The amino acid sequences of both *Der fII* variants differed at the 88th residue (Ile in clone 11 and Ala in clone 2) between 67th and 90th residues. This suggests that mAb 15E11 recognized the central portion of *Der fII* in amino acid sequence, which contained the 76th and 88th amino acid residues. Determination of the three-dimensional structure of *Der fII* is apparently required to further clarify the mechanism of the mAb recognition, the allergic function of *Der fII*, and the therapy of the allergic diseases mediated by this protein.

The native *Der fII* fraction contains at least two variants and two close peaks eluted at 36.5 and 37.0 min from HPLC (unpublished data). When native *Der fII* was digested with

lysylendopeptidase and analyzed by HPLC, the decreasing rate of the non-digested form which eluted at 36.5 min was significantly larger than that of non-digested *Der fII* which eluted at 37.0 min. Consistent with this, stable recombinant *Der fII*, clone 1 and clone 2, eluted at 37.0 min, while susceptible clone 11 *Der fII* eluted at 36.5 min. This observation emphasized that native *Der fII* is a mixture of at least two variants with a higher and a lower stability, and that clone 1 and/or clone 2 are the components with the higher stability and clone 11 is the component showing the lower stability. This study also revealed that only a few amino acid exchanges, 4 amino acids out of 129 among the three *Der fII* clones, alters the stability. Determination of the three-dimensional structure is obviously required to further characterize their stability.

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