

Allergens from *Dermatophagoides* mites with chymotryptic activity

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Summary

A new set of allergens from *Dermatophagoides pteronyssinus* and *D. farinae* (provisionally named DP5 and DF5, respectively) was isolated from the whole culture of mites. The apparent molecular weights of both allergens were shown to be 25 000 on SDS-PAGE under a reducing condition and 27 000 on Sephadex G-75 gel filtration chromatography. Both DP5 and DF5, as well as *Der f* III, possessed proteolytic activity. The results of substrate specificity and susceptibility to various protease inhibitors of DP5 and DF5 strongly suggested that they belonged to the chymotrypsin-like serine protease family. In sera from 88 mite-allergic patients, specific IgE antibodies to DP5 and/or DF5 were detected in only 41% of the sera by radio-allergosorbent test, while 90% and 93% had specific IgE antibodies to *Der p* I and/or *Der f* I and *Der p* II and/or *Der f* II, respectively.

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Introduction

Allergy to house dust mites, *Dermatophagoides pteronyssinus* and *D. farinae*, is one of the most important causes of asthma, allergic rhinitis and atopic dermatitis in many parts of the world [1–5]. Two groups of major allergens, the *Der* I group and the *Der* II group, have been identified and characterized in detail [6,7]. Both groups of mite major allergens have been cloned and sequenced [8–12]. These sequence analyses and subsequent biochemical studies have demonstrated that *Der p* I and *Der f* I were cysteine proteases [13,14]. The third group of *Dermatophagoides* allergens, *Der p* III and *Der f* III, were also shown to be proteases [13,15]. A recent study has demonstrated that *Der p* III belongs to a trypsin-like serine protease [16].

In the present study, we describe a new set of *Dermatophagoides* allergens, provisionally named DP5 and DF5, which represents chymotrypsin-like serine protease activity.

Materials and methods

Mite extracts and purified mite allergens

The whole culture extracts (WCE) of *D. pteronyssinus* and *D. farinae* were prepared as previously described [6,17] and were separated by ammonium sulfate precipitation into three fractions at 0–50%, 50–60% and 60–90% saturation.

Der p I/*Der f* I and *Der p* II/*Der f* II were purified from the ammonium sulfate precipitates of WCE at 0–50% and 60–95% saturation, respectively.

Der p III/*Der f* III was isolated from the ammonium sulfate precipitate of WCE at 60–95% saturation by a combination of Benzamidine-Sepharose affinity chromatography, DEAE-cellulose ion exchange chromatography and Sephadex G-75 gel filtration chromatography according to the method described by Takahashi *et al.* [18].

Isolation of DP5

The ammonium sulfate precipitate at 50–60% saturation obtained from 200 g of the whole culture of *D. pteronyssinus* was dissolved in 30 ml of 0.05 M Tris-HCl, pH 8.0, and dialysed against the same buffer. The sample was applied

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to a DEAE-cellulose (DE-52, Whatman, Springfield Mill, Kent, U.K.) column (1.5 × 20 cm) and eluted first with the same Tris buffer and then with 0.05 M Tris-HCl containing 0.2 M NaCl, pH 7.4. The second eluate was concentrated to 15 ml by pressure filtration through Spectrapor membrane tubing 1 (Spectrum Medical Industries, Los Angeles, U.S.A.) and applied to a Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.6 × 100 cm) equilibrated with 0.05 M ammonium bicarbonate, pH 8.0. The fraction eluted at 390–460 ml was concentrated to 5 ml, dialysed against 0.05 M Tris-acetate containing 0.5 M NaCl, pH 7.5, and applied to a Chelating Sepharose 6B (Pharmacia) column (1.0 × 15 cm), which had been chelated with zinc ion and equilibrated with the same Tris-acetate buffer. The fraction eluted unretarded from the column was concentrated to 2.5 ml and purified by gel filtration chromatography twice on a Sephadex G-75 superfine (Pharmacia) column (1.5 × 100 cm) equilibrated with 0.05 M ammonium bicarbonate. The DP5 thus obtained was further purified by ion-exchange chromatography on a DEAE-cellulose column. DP5 was eluted from the column with a linear NaCl gradient from 0 to 0.2 M in 0.05 M Tris-HCl, pH 7.8.

DF5 was isolated from the WCE of *D. farinae* by the same procedure as already described.

Protease activity

The protease activity of each preparation of the purified allergens and the effect of various protease inhibitors on the protease activity was determined as previously described [19]. In brief, 1 ml of reaction mixture consisting of 0.1 ml of allergen preparation and 0.9 ml of substrate (10 µM) in 0.1 M Tris-HCl, pH 8.0, was incubated at 37°C for 30 min, after which the reaction was terminated by the addition of 2 ml of 15% acetic acid. For the inhibition study, 0.1 ml of allergen preparation, 0.1 ml of protease inhibitor and 0.8 ml of substrate were mixed. The fluorescence of released aminomethylcoumarin was measured with excitation at 380 nm and emission at 460 nm. Specific substrates for trypsin and chymotrypsin, *t*-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA), *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methyl-coumaryl-7-amide (Boc-Val-Leu-Lys-MCA), succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-methyl-coumaryl-7-amide (Suc-Ala-Ala-Pro-Phe-MCA) and succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methyl-coumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) were purchased from Peptide Institute, Inc. (Osaka, Japan).

Radio-allergosorbent test (RAST)

Sera were obtained from mite-allergic patients with asthma who gave a positive skin response to the intra-

dermal skin test with 1:20 000 (w/v) of *D. farinae* extract (Hollister-Stier, Spokane, WA, U.S.A.).

RAST was performed as previously described [6]. CNBr-activated paper disks were coupled with 2 µg/disk of each preparation of the purified allergens. RAST results were expressed in Phadebas RAST units per millilitre (PRU/ml) using the Phadebas RAST Reference System. The coefficient of correlation between IgE antibody levels to (see Fig. 3) the corresponding allergens was obtained using a linear regression analysis after log transformation of the data.

Intradermal skin test

Intradermal skin tests were carried out by injecting 0.02 ml of each allergen preparation in saline containing 0.5% phenol and 0.03% human serum albumin. The allergen concentrations were 1 ng/ml for *Der p* I and *Der p* II and 100 ng/ml for DP5. The skin response was measured after 15 min and weal diameters of 8 × 8–15 × 15 mm and > 15 × 15 mm were regarded as positive (+) and strong positive (++) responses, respectively.

Physicochemical analyses

SDS-PAGE was carried out in 14% polyacrylamide gels under reducing conditions. The gels were stained with Coomassie blue R-250.

N-terminal amino acid sequence analysis of DP5/DF5 was performed on an Applied Biosystems 471-A protein sequencer.

Results

Isolation and physicochemical characterization

One of the key steps for the purification of *Der f* I in our previous study was zinc chelate chromatography on Chelating Sepharose [17]. The *Der f* I-containing fraction obtained from the ammonium sulfate precipitate of WCE at 0–60% saturation was applied to the Chelating Sepharose column equilibrated with Tris-acetate buffer, pH 7.5. *Der f* I was not eluted with the first buffer step but was eluted from the column with the gradient of decreasing pH of the buffer. The unretarded fraction, which contained an undetectable level of *Der f* I, was shown to possess an allergenic activity. Chromatography of this unretarded fraction on Sephadex G-75 yielded a sharp protein peak (referred to as DF5) at an apparent molecular weight of 27 000. In the later experiment, DF5 was found to mainly precipitate at 50–60% saturation of ammonium sulfate, while *Der f* I was at 0–50%. Thus, *Der p* I/*Der f* I and DP5/DF5 were isolated in this study from the ammonium sulfate precipitates at 0–50% and 50–60% saturation, respectively.

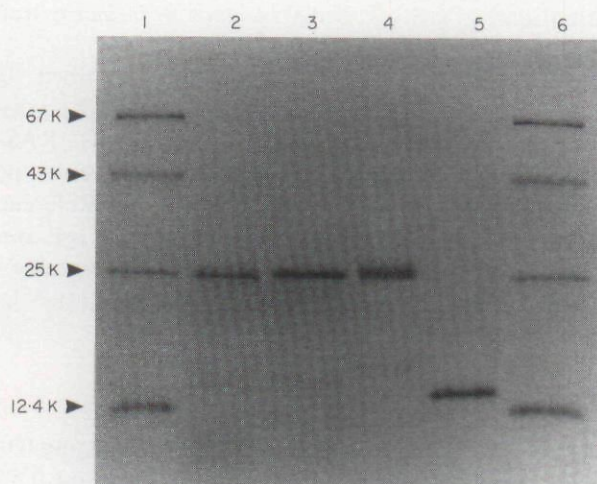


Fig. 1. SDS-PAGE analysis of purified mite allergens. Lanes 1 and 6, molecular weight markers including bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome C; lane 2, DF5; lane 3, DP5; lane 4, *Der p* I; lane 5, *Der f* II. Samples were analysed on a 14% acrylamide gel under reducing conditions.

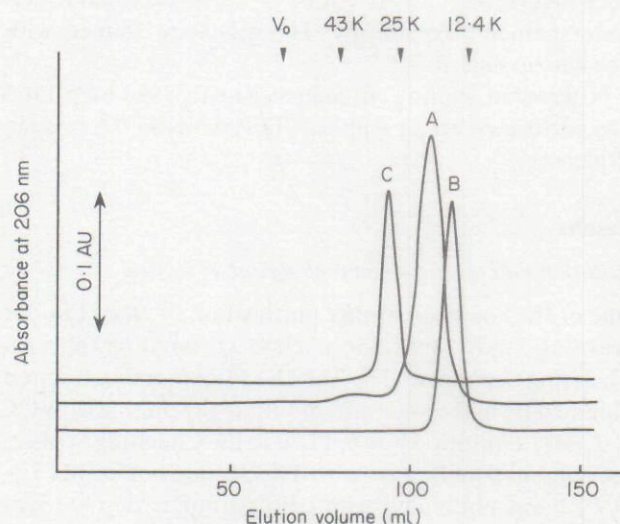


Fig. 2. Elution profiles of purified *Der p* I (A), *Der f* II (B) and DP5 (C) on a Sephadex G-75 superfine column (1.5 × 100 cm). Approximately 50–100 µg of each sample preparation was loaded onto a column and the effluent monitored at 206 nm.

The apparent molecular weights of DP5/DF5 were demonstrated to be 25000 on SDS-PAGE under reducing conditions and 27000 on Sephadex G-75 gel filtration chromatography (Figs 1 and 2). On polyacrylamide gel isoelectric focusing, both DP5 and DF5 showed several bands in the pH range 4–5 (data not shown).

Table 1. Comparison of N-terminal amino acid sequences of *Der p* III/*Der f* III and DP5/DF5

	5										10
<i>Der p</i> III	I	V	G	S	E	K	A	L	A	G	
<i>Der f</i> III	I	V	G	G	V	K	A	L	A	G	
DP5	(A)	I	G	X	Q	(P)	A	A	E	(A)	
DF5	(A)	(V)	G	G	Q	D	A	D	L	A	

	15										20
<i>Der p</i> III	E	X	P	Y	Q	I	S				
<i>Der f</i> III	D	X	P	Y	E	I	S	L	E	V	
DP5	(E)	A	P	F	Q	I	(S)	L	M	K	
DF5	E	A	P	F	Q	I	S	L	L	K	

Residues in parentheses were tentatively identified. Sequence data for *Der p* III and *Der f* III were obtained from Stewart *et al.* [13] and Heymann *et al.* [15], respectively.

Table 2. Substrate specificity in the proteolytic activity of DP5/DF5 and *Der f* III

Substrate	Relative activity (%)		
	DP5	DF5	<i>Der f</i> III
Suc-Ala-Ala-Pro-Phe-MCA	100	100	0
Suc-Leu-Leu-Val-Tyr-MCA	50	52	0
Boc-Phe-Ser-Arg-MCA	2.0	0.7	100
Boc-Val-Leu-Lys-MCA	1.2	0.2	58

The N-terminal amino acid sequences of DP5/DF5 are shown in Table 1 together with those of *Der p* III [13] and *Der f* III [15]. A high degree of homology (70%) was seen between DP5 and DF5 in their 20 N-terminal amino acid residues. They demonstrated no homology with the N-terminal sequences previously reported for *Der p* I/*Der f* I and *Der p* II/*Der f* II [8,15,20]. Although DF5 demonstrated an N-terminal amino acid homology (40%) with *Der f* III [15], DF5 did not significantly react with mouse antiserum to *Der f* III and monoclonal antibody raised against *Der f* III, which were kindly provided by Dr W. R. Thomas (Western Australian Research Institute for Child Health, Perth, Western Australia) and Dr P. W. Heymann (University of Virginia, Charlottesville, VA, U.S.A.), respectively.

To assess the purity of the DP5 and DF5 preparations the concentrations of the two groups of major allergens, *Der p* I/*Der f* I and *Der p* II/*Der f* II, in these preparations

Table 3. Effect of various protease inhibitors on the proteolytic activity of DP5/DF5 and *Der f* III

Inhibitor		% Inhibition		
		DP5	DF5	<i>Der f</i> III
Chymostatin (μ M)	1	98	93	16
	10	100	100	40
Aprotinin (KIU/ml)	1	90	75	100
	10	100	99	100
Leupeptin (μ M)	1	3.4	2.1	n.d.
	10	12	12	n.d.
E-64 (μ M)	1	0	0	10
	10	0	0	12
Iodoacetic acid (μ M)	1	0	2.0	n.d.
	10	0	2.6	n.d.

Suc-Leu-Leu-Val-Tyr-MCA and Boc-Phe-Ser-Arg-MCA were used as substrates for the proteolytic activity of DP5/DF5 and *Der f* III, respectively.
n.d., Not done.

Table 4. Number of positive RAST (≥ 0.35 PRU/ml) against the four groups of *Dermatophagoides pteronyssinus* (*D.p.*) and *D. farinae* (*D.f.*) allergens among the 88 mite-allergic patients

Group	No. of patients with positive RAST (%)		
	<i>D.p.</i>	<i>D.f.</i>	<i>D.p.</i> and/or <i>D.f.</i>
<i>Der</i> I	79	78	79 (89.8)
<i>Der</i> II	82	82	82 (93.2)
<i>Der</i> III	45	37	52 (59.1)
DP5/DF5	34	27	36 (40.9)

were measured by the allergen-specific radioimmunoassays previously reported [21]. Less than 0.1 ng/ml of either *Der p* I/*Der f* I or *Der p* II/*Der f* II was detected in 1 μ g/ml of the DP5 and DF5 preparations, suggesting that the contamination of the two groups of major allergens in the DP5 and DF5 preparations was negligibly low (<0.01%).

Protease activity

DP5/DF5 was found to have proteolytic activity. The substrate specificity and susceptibility to various protease

inhibitors of DP5/DF5 were compared with those of *Der f* III. As shown in Table 2, DP5/DF5 cleaved chymotrypsin-specific substrates, Suc-Ala-Ala-Pro-Phe-MCA and Suc-Leu-Leu-Val-Tyr-MCA, while *Der f* III cleaved trypsin-specific substrates, Boc-Phe-Ser-Arg-MCA and Boc-Val-Leu-Lys-MCA. The susceptibility of the protease activity of DP5/DF5 and *Der f* III to various protease inhibitors is shown in Table 3. The activity of DP5/DF5 was strongly inhibited by a serine protease inhibitor, aprotinin, and a specific chymotrypsin inhibitor, chymostatin [22], but was not affected by leupeptin, which does not react with chymotrypsin-like enzymes [23], and cysteine protease inhibitors, E-64 [24] and iodoacetic acid.

Allergenic activity

IgE antibody levels to each of the corresponding allergens from *D. pteronyssinus* and *D. farinae* in sera from 88 mite-allergic patients were measured by RAST (Table 4). IgE antibodies to DP5 and/or DF5 were detected in 36 of 88 (41%) sera. Among these 36 sera, 25 had IgE antibodies to both DP5 and DF5, and nine and two had IgE antibodies only to DP5 and to DF5, respectively. Linear regression analysis showed a statistically significant correlation between IgE antibody levels to DP5 and DF5 (Fig. 3). However, the coefficient of correlation between IgE antibody levels to DP5 and DF5 ($r=0.796$) was lower than those between IgE antibody levels to *Der p* I and *Der f* I ($r=0.954$), and to *Der p* II and *Der f* II ($r=0.984$).

In another group of patients both intradermal skin tests and RAST were performed with the three groups of *D. pteronyssinus* allergens, *Der p* I, *Der p* II and DP5 (Table 5). A good overall agreement was seen between skin test and RAST results. In this group of patients, eight (44%) and seven (39%) out of 18 patients demonstrated a positive response against DP5 by skin test and RAST, respectively.

Discussion

Recent studies have demonstrated that extracts from *Dermatophagoides* mites contain a variety of hydrolytic enzymes [25], some of which have been shown to be potent allergens, including cysteine proteases (*Der p* I/*Der f* I) [13,14], trypsin-like serine proteases (*Der p* III/*Der f* III) [13,15] and amylase from *D. pteronyssinus* [26]. This study demonstrates the purification and characterization of a new set of allergens from *D. pteronyssinus* and *D. farinae* which, in contrast to *Der p* III/*Der f* III, possess chymotrypsin-like serine protease activity.

DP5 and DF5 isolated from the ammonium sulfate precipitates of WCE at 50–60% saturation were found to

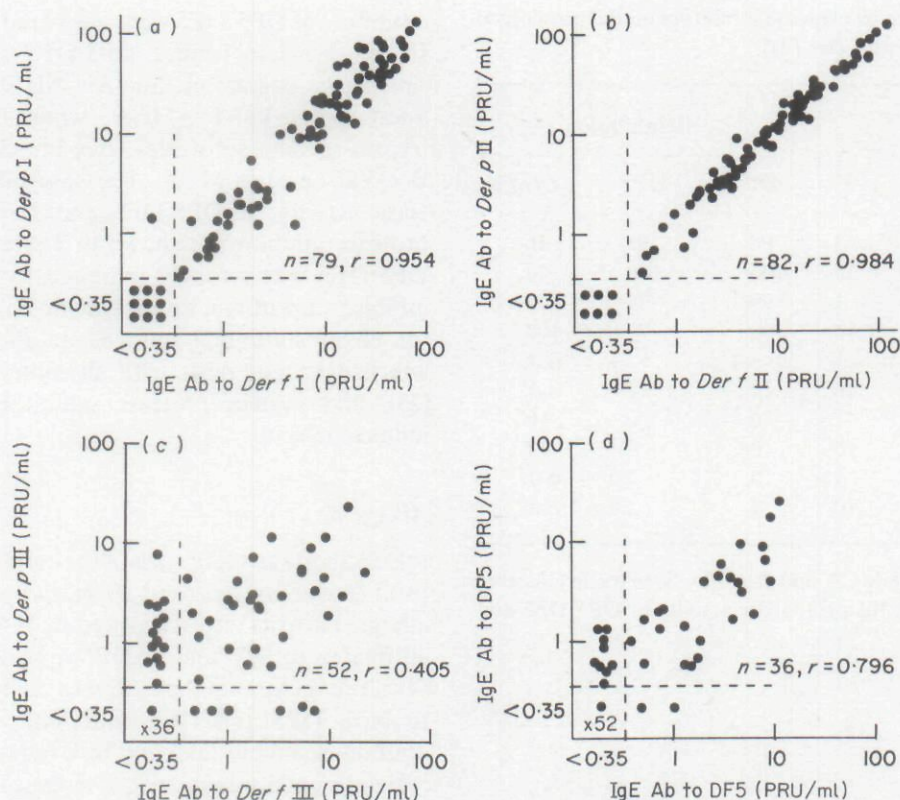


Fig. 3. Comparison of IgE antibody levels to the corresponding allergens between *D. pteronyssinus* and *D. farinae* in sera from 88 mite-allergic patients. (a) *Der p I* vs. *Der f I*; (b) *Der p II* vs. *Der f II*; (c) *Der p III* vs. *Der f III* and (d) DP5 vs. DF5. The coefficient of correlation was calculated with the sera IgE antibody-positive to at least one of the corresponding allergens.

have apparent molecular weights of 25 000 on SDS-PAGE under reducing conditions and of 27 000 on Sephadex G-75 gel filtration chromatography and acidic isoelectric points. These data suggest that DP5 is equivalent to the chymotrypsin-like activity which has been detected in the spent growth medium of *D. pteronyssinus* by Stewart *et al.* [25].

The DP5 and DF5 preparations isolated in this study were confirmed to contain negligible levels of the two groups of major allergens, *Der p I*/*Der f I* and *Der p II*/*Der f II*, by using the allergen-specific radioimmunoassays. However, it could not be denied that these preparations contained significant levels of the third group of allergens, *Der p III*/*Der f III*, since both groups of allergens had very similar apparent molecular weights. Although the concentrations of *Der p III*/*Der f III* in the DP5 and DF5 preparations remain to be determined by the immunochemical quantification method, the contamination of these preparations with *Der p III*/*Der f III* seemed to be less than 0.1% (w/w) of DP5/DF5 from the results of protease activity of the preparations (Table 2). Since the absolute activity of *Der p III*/*Der f III* to cleave trypsin-specific substrates (Boc-Phe-Ser-Arg-MCA and Boc-Val-

Leu-Lys-MCA) was 100–1000 times more potent than that of DP5/DF5 to cleave chymotrypsin-specific substrates (Suc-Ala-Ala-Pro-Phe-MCA and Suc-Leu-Leu-Val-Tyr-MCA), 0.1–1% of contamination with *Der p III*/*Der f III* would cause the same level of tryptic activity as chymotryptic activity in the DP5 and DF5 preparations.

The results of substrate specificity and susceptibility to various protease inhibitors of DP5 and DF5 (Tables 2 and 3) strongly suggested that both DP5 and DF5 belonged to the chymotrypsin-like serine protease family. DP5 and DF5 cleaved N-acetyl-L-tyrosine ethyl ester in addition to the substrates cited in Table 2 (unpublished observation). This specific esterase activity of DP5 and DF5 also confirms that they are chymotrypsin-like serine proteases.

In our RAST data using sera from 88 mite-allergic patients, only 41% of the sera had detectable IgE antibodies to DP5 and/or DF5, while 90% and 93% of the sera were IgE antibody-positive to *Der p I* and/or *Der f I* and *Der p II* and/or *Der f II*, respectively (Table 4). This suggests that DP5 and DF5 are 'minor allergens' of the *Dermatophagoides* mites. In addition, 11 of 36 sera IgE antibody-positive to DP5 and/or DF5 had IgE antibodies

Table 5. Intradermal skin test and RAST with *Der p* I, *Der p* II and DP5

Patient	Intradermal skin test*			RAST (PRU/ml)		
	<i>Der p</i> I	<i>Der p</i> II	DP5	<i>Der p</i> I	<i>Der p</i> II	DP5
IK	++	++	++	17.5	16.1	2.9
TM	++	++	+	110	39.7	2.4
NH	++	++	+	42.0	31.2	0.72
SA	+	+	++	35.7	19.6	3.4
HN	+	+	+	31.0	31.1	1.3
SK	+	++	++	21.5	30.8	7.4
TE	+	+	+	24.2	11.0	3.7
US	+	+	+	33.6	54.0	<0.35
HR	+	+	-	73.9	26.9	<0.35
MH	++	++	-	86.4	48.3	<0.35
HT	++	++	-	13.0	9.9	<0.35
SKo	+	+	-	20.1	33.2	<0.35
SM	+	+	-	19.1	33.9	<0.35
SKe	+	++	-	11.5	6.8	<0.35
IS	++	++	-	2.8	2.2	<0.35
YI	+	-	-	165	0.47	<0.35
KM	+	-	-	1.7	<0.35	<0.35
OT	-	-	-	74.5	105	<0.35
Control (n=3)	-	-	-	<0.35	<0.35	<0.35

* Intradermal skin tests were carried out by injecting 0.02 ml of 1 ng/ml of either *Der p* I or *Der p* II or 100 ng/ml of DP5. The skin response was measured after 15 min and weal diameters of 8 × 8–15 × 15 mm and > 15 × 15 mm were regarded as + and ++ responses, respectively.

specific for one but not for another (Fig. 3), and the coefficient of correlation between IgE antibody levels to DP5 and DF5 was not as high as that between IgE antibody levels to *Der p* I and *Der f* I or to *Der p* II and *Der f* II. These findings may indicate that crossreactivity between DP5 and DF5 in human IgE antibody response is not as intensive as that between *Der p* I and *Der f* I or between *Der p* II and *Der f* II [6].

There are conflicting data regarding the frequencies of IgE antibody reactivity with the group III trypsin-like serine protease allergens (*Der p* III/*Der f* III). Heymann *et al.* [15] reported that *Der f* III reacted with only 16% of sera from 51 mite-allergic patients by the antigen binding assay, while Stewart *et al.* [16] demonstrated that all of the sera from 54 mite-allergic individuals had IgE antibodies to *Der p* III by RAST and suggested *Der p* III to be a major allergen. Our RAST data were not consistent with either of the previous studies. Intermediate results, in which about 60% of sera from 88 mite-allergic patients were IgE antibody-positive to *Der p* III and/or *Der f* III, were obtained. Although the reasons why there is a wide difference in the frequency of reactivity of IgE antibody

with the group III allergens among the studies are unclear, they may be related to the difference in the purity of the allergen preparations used, of the study population or of the methods for the measurement of IgE antibody.

Further investigations on the two groups of serine protease allergens, *Der p* III/*Der f* III and DP5/DF5, will be needed to determine whether these allergens are as important as the group I and group II allergens, or whether the proteolytic activity of these allergens plays a role in the development of sensitization in allergic individuals.

Acknowledgments

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