

Cloning and Expression of cDNA Encoding the Complete Prepro-Form of an Isoform of Der f 1, the Major Group 1 Allergen from House Dust Mite *Dermatophagoides farinae*

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cDNA clones encoding a major house dust mite allergen, Der f 1, were isolated from a *Dermatophagoides farinae* cDNA library by plaque immunoscreening using rabbit anti-Der f 1 serum. The sequences cover the complete open reading frame encoding the prepro-form. The sequence is different from previously reported cDNA of Der f 1 in six bases and the encoded amino acid sequence is different in two residues. Pro-forms of Der f 1 and its mutant, in which the N-glycosylation motif was disrupted, expressed in *Pichia pastoris* were converted to the mature forms by an *in vitro* activation process and they showed significant IgE-binding. The biologically active rDer f 1 molecules would be useful for diagnostic testing and allergen-specific immunotherapy. In contrast, Der f 1 directly expressed in *Escherichia coli* without the prosequence had very low IgE binding. The hypoallergenic Der f 1 polypeptide could be useful for safer and more effective immunotherapy.

Key words: house dust mite; Der f 1; cDNA; isoform; recombinant allergen

House dust mites of *Dermatophagoides* species (*D. farinae* and *D. pteronyssinus*) are the most important causative factor associated with various allergic diseases among all the indoor allergens.^{1,2)} The major house dust mite allergens are classified into group 1 (Der f 1 and Der p 1) or group 2 (Der f 2 and Der p 2).³⁾ IgE-binding activity for both group 1 and 2 allergens is detected in the majority of sera from patients allergic to house dust mites. Group 1 allergens, Der f 1 and Der p 1, which belong to the papain-like cysteine protease family, are mainly

found in mite feces,^{3,4)} and are the most important allergens clinically and in standardization of mite extract. The cDNA fragments for mite group 1 allergens encode the prepro-sequence at the N-terminal of the mature sequence.^{5–8)} Although cDNA fragments for five isoforms of Der p 1 have been cloned,⁷⁾ only one cDNA⁶⁾ and one partial amino acid sequence of an isoform encoded by a genomic DNA⁹⁾ have been reported for Der f 1. Here, we describe cloning of a cDNA encoding the complete prepro-form of an isoform of Der f 1. Furthermore, we demonstrate that both IgE-reactive rDer f 1 and its hypoallergenic form are able to be prepared.

Materials and Methods

Cloning of cDNA coding for Der f 1. The *D. farinae* lambda gt11 cDNA library was constructed as described in our previous reports.^{10,11)} Rabbit anti-Der f 1 serum, which was kindly provided by Torii Pharmaceutical Co. Ltd., was used for plaque immunoscreening by the use of the Super Screen Immunoscreeing System (Amersham International).

Preparation of rDer f 1 expressed in *P. pastoris*. The methods were described previously.¹²⁾ The vector for expression in the methylotrophic yeast *P. pastoris* encoding the pro-Der f 1 fused to a *P. pastoris* PHO1 signal sequence was constructed by insertion of the polymerase chain reaction (PCR)-amplified cDNA fragment of into pHIL-S1 (Invitrogen, San Diego, CA). The vector encoding for a mutant pro-Der f 1, the consensus sequence of which for the N-glycosyla-

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Abbreviations: rDer f 1, recombinant Der f 1; rDer p 1, recombinant Der p 1; ORF, open reading frame; PCR, polymerase chain reaction; DEAE Sepharose, diethylaminoethyl Sepharose; Q Sepharose, quaternary ammonium Sepharose; PBS, phosphate-buffered saline; DTT, dithiothreitol; RAST-EIA, radioallergosorbent test-enzyme immunoassay; FcεRI, high affinity IgE receptor

tion site is disrupted (N53Q), fused to a PHO1 signal sequence, was constructed by site-directed mutagenesis. The vectors linearized by digestion with *Bgl*/II were introduced into *P. pastoris* GS115 cells by electroporation. Expression was induced by addition of methanol. The supernatant was harvested by centrifugation and dialyzed against 100 mM acetate buffer (pH 4.0) at 4°C for 48 h. After this activation process, the recombinant wild-type Der f 1 was dialyzed against phosphate-buffered saline (PBS), while the mutant N53Q was dialyzed against 20 mM Tris-Cl (pH 8.0), purified by anion exchange column chromatography using diethylaminoethyl (DEAE) Sepharose or quaternary ammonium (Q) Sepharose (Pharmacia, Uppsala, Sweden), and then dialyzed against PBS.

Preparation of rDer f 1 expressed in *E. coli*. The mature form of Der f 1 with an additional methionine at the N-terminus was overexpressed as inclusion bodies in *E. coli* BL21 (DE3). Inclusion bodies were recovered, solubilized, and dialyzed as described¹³ with a modification of the addition of 1% DTT to the buffer for solubilization.

Radioallergosorbent test-enzyme immunoassay (RAST-EIA). The method was as described previously.¹⁴ Briefly, serum of each allergic patient was incubated with paper discs coupled with each of the purified natural and recombinant Der f 1. Then, the IgE that bound to the disc was detected with β -galactosidase-conjugated anti-human IgE antibodies by measuring the absorbance at 420 nm.

Results

Cloning of cDNA encoding for Der f 1

The cDNA fragments encoding for Der f 1 were cloned by plaque immunoscreening. Eight possible candidates were found among approximately 30,000 clones. Four long cDNA clones out of the eight were sequenced (1,081 bp, 1,056 bp, 987 bp, and 981 bp). The sequences of the two longest cDNA clones covered the complete open reading frame (ORF) encoding the prepro-form of Der f 1 (Fig. 1). The other two clones encoded a partial signal peptide, the complete prosequence, and the complete mature sequence. The overlapping sequences of the four positive clones were identical. The nucleotide sequence of the ORF was identical with that previously reported by Dilworth *et al.*⁶ except for six bases (codons for amino acid residues, 41, 103, 145, 151, 184, and 198) (Figs. 1 and 2A). The cDNA encoded the amino acid sequence containing a putative signal sequence from -98 to -81, a putative prosequence from -80 to -1, and a mature sequence from 1 to 223 that was identical with that reported by Dilworth *et al.* except for two residues (103 and 184 in the mature sequence)

(Fig. 2A). A sequence comparison among the prepro-forms of three house dust mite group 1 allergens, Der f 1 from *D. farinae* in this study (Der f 1 A in Fig. 2B), that reported by Dilworth *et al.* (Der f 1 B in Fig. 2B),⁶ Der p 1 from *D. pteronyssinus*,⁷ and Eur m 1 from *Euroglyphus maynei*,⁸ was also made (Fig. 2B). The nucleotide and deduced amino acid sequences for cDNA for prepro-form of Der f 1 cloned in this study have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB034946.

IgE-binding of rDer f 1

Pro-forms of rDer f 1, the wild-type and N53Q, were secreted into the medium of *P. pastoris*. The wild-type Der f 1 has an N-glycosylation motif conserved among mite group 1 allergens (Fig. 2B). In N53Q, the N-glycosylation motif is disrupted by site-directed mutagenesis. The prosequence was removed by the *in vitro* activation (Fig. 3). By N-terminal peptide sequencing, the N-terminal additional methionine of rDer f 1 directly expressed as the mature polypeptide in *E. coli* was confirmed to be removed. IgE-binding capacities of the three rDer f 1 molecules and natural Der f 1 were compared by RAST-EIA (Fig. 4). The mature wild-type Der f 1 and N53Q, which were expressed as the pro-forms in *P. pastoris* and then converted to the mature forms, had IgE-binding equivalent to natural Der f 1. On the other hand, the IgE-binding capacity of the polypeptide of the mature wild-type Der f 1 directly expressed in *E. coli* had low IgE-binding.

Discussion

Allelic polymorphism is a common feature of house dust mite allergens.^{7,8,10,11,15} The cloned cDNA in this study encoded a complete prepro-form, (-98)–223, of an isoform of Der f 1 differing in two amino acid residues from the sequence reported by Dilworth *et al.* (Figs. 1 and 2).⁶ The two changes were within the amino acid sequence of the mature form. Interestingly, the two amino acid residues were identical with those encoded by a partial genomic sequence reported by Kent *et al.* although the predicted amino acid sequence is a partial sequence, (-1)–211.⁹

It was demonstrated that IgE-reactive rDer f 1 was able to be prepared from its precursor encoded by the cDNA cloned in this study (Figs. 3 and 4). N-glycosylation did not affect IgE-binding of rDer f 1, which was expressed as the pro-form in *P. pastoris* and then converted to the mature form. Therefore, the failure of rDer f 1 directly expressed in *E. coli* to bind IgE might result from the absence of the prosequence in the refolding process or because the conditions for refolding were not optimized. Recently, we reported maturation of IgE-binding and protease activity of

1	TTTTTTTTTTTTTTTTTCCATCAAAATTAAAAATTCATCAAAA	ATG	AAA	TTC	GTT	TTG	GCC	ATT	GCC	TCT	70	
		-98	Met	Lys	Phe	Val	Leu	Ala	Ile	Ala	Ser	-90
71	TTG TTG GTA TTG AGC ACT GTT TAT GCT CGT CCA GCT TCA ATC AAA ACT TTT GAA GAA TTC	130										
-89	Leu Leu Val Leu Ser Thr Val Tyr <u>Ala Arg</u>	-70										
		-80										
131	AAA AAA GCC TTC AAC AAA AAC TAT GCC ACC GTT GAA GAG GAA GAA GTT GCC CGT AAA AAC	190										
-69	Lys Lys Ala Phe Asn Lys Asn Tyr Ala Thr Val Glu Glu Glu Glu Val Ala Arg Lys Asn	-50										
		-60										
191	TTT TTG GAA TCA TTG AAA TAT GTT GAA GCT AAC AAA GGT GCC ATC AAC CAT TTG TCC GAT	250										
-49	Phe Leu Glu Ser Leu Lys Tyr Val Glu Ala Asn Lys Gly Ala Ile Asn His Leu Ser Asp	-30										
		-40										
251	TTG TCA TTG GAT GAA TTC AAA AAC CGT TAT TTG ATG AGT GCT GAA GCT TTT GAA CAA CTC	310										
-29	Leu Ser Leu Asp Glu Phe Lys Asn Arg Tyr Leu Met Ser Ala Glu Ala Phe Glu Gln Leu	-10										
		-20										
311	AAA ACT CAA TTC GAT TTG AAT GCC GAA ACA AGC GCT TGC CGT ATC AAT TCG GTT AAC GTT	370										
-9	Lys Thr Gln Phe Asp Leu Asn Ala Glu Thr Ser Ala Cys Arg Ile Asn Ser Val Asn Val	11										
		-1	1									
371	CCA TCG GAA TTG GAT TTA CGA TCA CTG CGA ACT GTC ACT CCA ATC CGT ATG CAA GGA GGC	430										
12	Pro Ser Glu Leu Asp Leu Arg Ser Leu Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly	31										
		21										
		T										
431	TGT GGT TCA TGT TGG GCT TTC TCT GGT GTC GCC GCA ACT GAA TCA GCT TAT TTG GCC TAC	490										
32	Cys Gly Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu Ala Tyr	51										
		41										
491	CGT <u>AAC ACG TCT</u> TTG GAT CTT TCT GAA CAG GAA CTC GTC GAT TGC GCA TCT CAA CAC GGA	550										
52	Arg <u>Asn Thr Ser</u> Leu Asp Leu Ser Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly	71										
		61										
551	TGT CAC GGC GAT ACA ATA CCA AGA GGC ATC GAA TAC ATC CAA CAA AAT GGT GTC GTT GAA	610										
72	Cys His Gly Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln Gln Asn Gly Val Val Glu	91										
		81										
		G										
611	GAA AGA AGC TAT CCA TAC GTT GCA CGA GAA CAA <u>CAA</u> TGC CGA CGA CCA AAT TCG CAA CAT	670										
92	Glu Arg Ser Tyr Pro Tyr Val Ala Arg Glu Gln <u>Gln</u> Cys Arg Arg Pro Asn Ser Gln His	111										
		101										
		Arg										
671	TAC GGT ATC TCA AAC TAC TGC CAA ATT TAT CCA CCA GAT GTG AAA CAA ATC CGT GAA GCT	730										
112	Tyr Gly Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asp Val Lys Gln Ile Arg Glu Ala	131										
		121										
		C										
731	TTG ACT CAA ACA CAC ACA GCT ATT GCC GTC ATT ATT GGC ATT AAA GAT TTG AGA GCT TTT	790										
132	Leu Thr Gln Thr His Thr Ala Ile Ala Val Ile Ile Gly Ile Lys Asp Leu Arg Ala Phe	151										
		141										
791	CAA CAT TAT GAT GGA CGA ACA ATC ATT CAA CAT GAC AAT GGT TAT CAA CCA AAC TAT CAT	850										
152	Gln His Tyr Asp Gly Arg Thr Ile Ile Gln His Asp Asn Gly Tyr Gln Pro Asn Tyr His	171										
		161										
		A										
851	GCC GTC AAC ATT GTC GGT TAC GGA AGT ACA CAA GGC <u>GTC</u> GAT TAT TGG ATC GTA CGA AAC	910										
172	Ala Val Asn Ile Val Gly Tyr Gly Ser Thr Gln Gly <u>Val</u> Asp Tyr Trp Ile Val Arg Asn	191										
		181										
		Asp										
		A										
911	AGT TGG GAT ACT ACC TGG GGT GAT AGC GGA TAC GGA TAT TTC CAA GCC GGA AAC AAC CTC	970										
192	Ser Trp Asp Thr Thr Trp Gly Asp Ser Gly Tyr Gly Tyr Phe Gln Ala Gly Asn Asn Leu	211										
		201										
971	ATG ATG ATC GAA CAA TAT CCA TAT GTT GTA ATC ATG TGA ACATTGAAATTGAATATATTTATTG	1036										
212	Met Met Ile Glu Gln Tyr Pro Tyr Val Val Ile Met ***	223										
		221										
1037	TTTTCAAAATAAAAACAACACTACTCTTGCAGTATTTTCTTTT	1081										

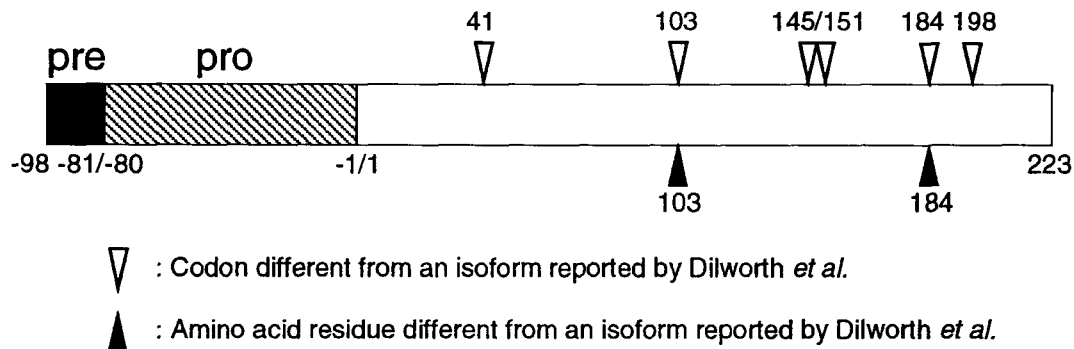
Fig. 1. Sequence Analysis of cDNA for the Isoform of Der f 1.

Nucleotide and deduced amino acid sequences of the cDNA for the isoform of Der f 1 cloned in this study. Positive amino acid residue numbers correspond to the sequence of the natural mature Der f 1 beginning with threonine. Negative sequence numbers refer to the signal sequence and the prosequence of Der f 1. The underlined sequence is the predicted cleavage site for the signal sequence (-81 and -80). The nucleotide and amino acid residues that are indicated above and below the sequences respectively are the residues of an isoform reported by Dilworth *et al.*⁶ different from those in this study. The boxed sequence is a consensus motif for N-glycosylation. Three asterisks indicate a stop codon.

rDer f 1, which was expressed as the pro-form in *E. coli* and then converted to the mature form¹⁶⁾ as well as that expressed in *P. pastoris*.¹²⁾ The prosequence

might be essential for refolding of Der f 1 in these systems. Collins *et al.* reported that none of the pin-coupled Der p 1 or Der f 1 decapeptides was found

A



B

	-98	-90	-80	-70	-60	-50	-40
Der f 1 A	MKFLVLAIASLLVLSTVYAR	PASIKTFEEFKAFNKNYATVEEEEVARKNFLES	LK	YVEAN			
Der f 1 B							
Der p 1	..I.....A..A...S.....Y.....S...F.D..A.....V...QS.						
Eur m 1	..II.....A.....T...P.K.....S.						
	** ***** ** ***** ***** ***** ** * ** ***** ** *						
	-30	-20	-10	1	10	20	
Der f 1 A	KGAINHLSLSDLSLDEFKNRYLMSAEAFEQLKTQFDLNAETSACRINSVNVPS	ELDLRLSRT					
Der f 1 B							
Der p 1	G.....F.....H.....N..S..G-.A.A.I...QM..						
Eur m 1	QF..N.N.....Y..S...SL.....						
	***** ** * ** * ***** ** * ** * ** *						
	30	40	50	60	70	80	
Der f 1 A	VTPIRMQGGCGSCWAFSGVAATESAYLAYRNTSLDLSEQELVDCASQHGCHG	DTPRGIE					
Der f 1 B							
Der p 1							
Eur m 1	S.....M.....A.....N.....						
	***** ** * ** * ***** ** * ** * ** *						
	90	100	110	120	130	140	
Der f 1 A	YIQQNGVVEERSYPYVAREQQCRPN	SQHYGISNYCQIYPPDVKQIREALTQTHT	AI	AVI			
Der f 1 B							
Der p 1	...H...Q.SY.R.....S.....A.RF.....N.NK.....A...S.....						
Eur m 1	Q.HY.....S.H...A.R..LK.....S...SNK..Q.....V...						
	*** ** * ** * ***** ** * ** * ** *						
	150	160	170	180	190	200	
Der f 1 A	IGIKDLRAFQHYDGRITIIQHDNGYQPNYHAVNIVGYGSTQGVYDWIVRNSW	DWTWGD	SGY				
Der f 1 B							
Der p 1	D..R.....R.....SNA.....N...N..						
Eur m 1	N..R.....M.....N.....N...						
	***** ** * ** * ***** ** * ** * ** *						
	210	220					
Der f 1 A	GYFQAGNNLMMIEQYPYVVIM						
Der f 1 B							
Der p 1	...A.NID....E.....L						
Eur m 1	...A.NI.....ML						
	*** * ***** ** *						

..: Identical to Der f 1 A. -: Gap. *: Conserved among the four sequences.

Underlined: Predicted cleavage site for the signal sequence.

□: N-glycosylation motif

Fig. 2. Amino Acid Sequence Analysis of the Isoform of Der f 1.

(A) Schematic representation of differences in nucleotide and deduced amino acid residues between the cDNA sequences of the ORF of the isoform of Der f 1 cloned in this study and that reported by Dilworth *et al.*⁶⁾ (B) Sequence comparison among the prepro-forms of three mite group 1 allergens, Der f 1 from *D. farinae* in this study (Der f 1A), and that reported by Dilworth *et al.* (Der f 1B),⁶⁾ Der p 1 from *D. pteronyssinus*,⁷⁾ and Eur m 1 from *E. maynei*.⁸⁾

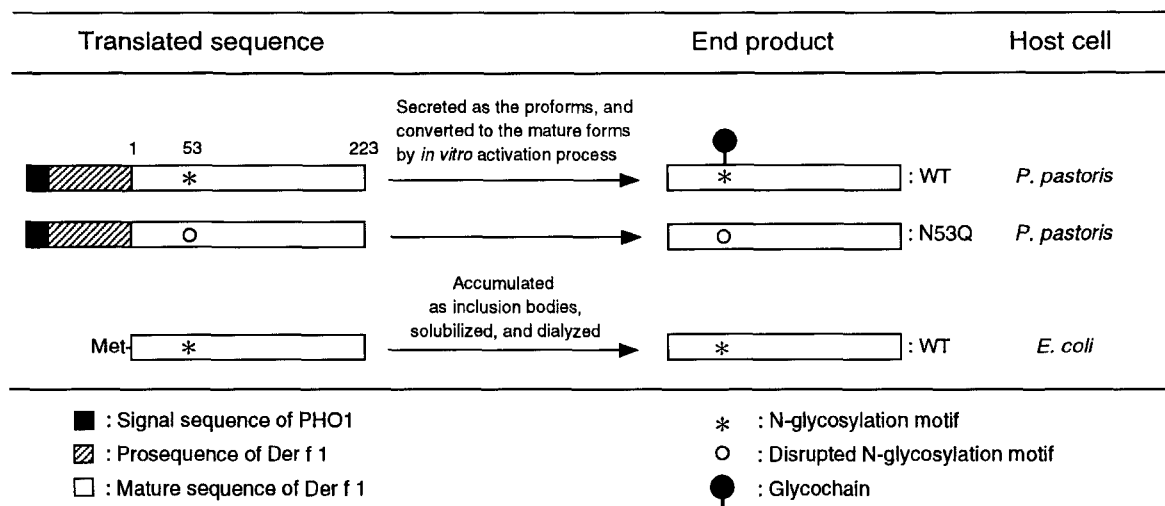
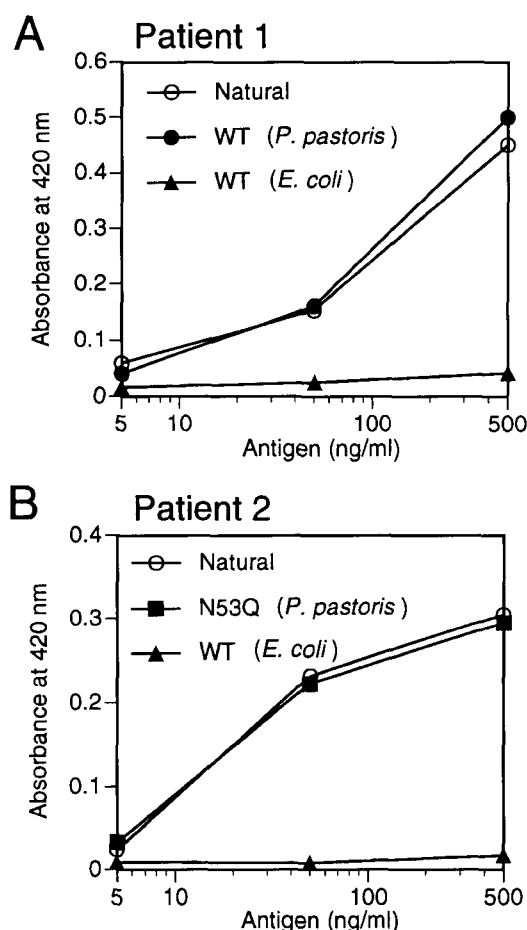


Fig. 3. Schematic Representation of Three Recombinant Types of Der f 1.

Fig. 4. IgE-Binding of Recombinant Der f 1 Expressed in *P. pastoris* or *E. coli*.

(A) IgE-binding of natural Der f 1 (Natural), the wild-type Der f 1 with a glycochain, which were expressed as the proforms in *P. pastoris* and then converted to the mature forms (WT (*P. pastoris*)), and that directly expressed in *E. coli* (WT (*E. coli*)). (B) IgE-binding of natural Der f 1 (Natural), the N53Q without a glycochain, which were expressed as the proforms in *P. pastoris* and then converted to the mature forms (N53Q (*P. pastoris*)), and the wild-type Der f 1 directly expressed in *E. coli* (WT (*E. coli*)).

by the continuous epitope mapping procedure to bind significantly to specific IgE in sera of hypersensitive patients.¹⁷⁾ We considered that the inactive Der f 1 polypeptide directly expressed in *E. coli* in this study was unfolded and that majority of IgE epitopes of Der f 1 were dependent on the conformation of the allergen molecule rather than the primary sequence. Concerning another group 1 allergen, Der p 1, from *D. pteronyssinus*, although Chua *et al.* reported that recombinant Der p 1 (rDer p 1), which was expressed in *S. cerevisiae* and affinity-purified by a monoclonal antibody, showed higher frequency binding of IgE than did that expressed in *E. coli*, the rDer p 1 had additional 28 residues at the N-terminus of the mature sequence.¹⁸⁾

Biologically active recombinant allergens such as the activated rDer f 1 molecules would be useful for diagnostic testing and allergen-specific immunotherapy.²²⁾ However, a major problem with allergen-specific immunotherapy is the risk of an anaphylactic reaction. Therefore, modified allergens with reduced IgE-binding capacities have been developed.²²⁻²⁷⁾ Ideally, the modified allergens do not crosslink the complex of allergen specific IgE and high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils, nor induce the release of mediators from the cells. The Der f 1 polypeptide directly expressed in *E. coli* in this study has reduced IgE-binding capacity, would not cause anaphylaxis even at high doses, and therefore could be useful for safer and more effective immunotherapy.

The cDNA cloned in this study has been used for development of systems for expression of biologically active recombinant Der f 1 in insect cells,^{19,20)} *Aspergillus oryzae*,²¹⁾ *P. pastoris*,¹²⁾ and *E. coli*.¹⁶⁾ The biologically active rDer f 1 molecules would be useful for diagnostic testing and allergen-specific immunotherapy. On the other hand, the hypoallergenic Der f 1 polypeptide directly expressed in *E. coli* in

this study could be useful for safer and more effective immunotherapy.

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