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Cloning and expression in *Escherichia coli* of cDNA encoding house dust mite allergen Der f 3, serine protease from *Dermatophagoides farinae*

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Abstract Der f 3 is one of the allergens produced by house dust mite *Dermatophagoides farinae* showing serine protease activity. Based on its amino acid sequence, a cDNA clone encoding Der f 3 was isolated from a cDNA library of *D. farinae*. Sequencing analysis of the clone revealed the presence of an open reading frame of 780 bp, which encodes a mature protein of 232 amino acids with 27 amino acids of pre-pro sequence at the N-terminus. When proDer f 3 was produced in *Escherichia coli* as a fused protein with glutathione-*S*-transferase, the fused protein was accumulated as inclusion bodies. The protein purified with 8 M urea and glutathione-affinity column chromatography, however, did not show protease activity. When an arginine residue was introduced at the C-terminus of the pro-region in place of threonine, removal of the pro-region to produce an active mature protease was observed. The specificity and the activity of this recombinant protease were almost the same as those of native Der f 3.

Key words: Der f 3; Allergen; Serine protease; cDNA; Sequence

1. Introduction

Dermatophagoides species are known as house dust mite, which produce many allergens that cause allergic diseases, such as atopic dermatitis and asthma [1–3]. Several allergenic proteins which reacted with human IgE antibody, resulting in triggering the allergic symptoms, have been isolated from two *Dermatophagoides* species, *D. farinae* and *D. pteronyssinus* [1–3]. Der f 3 isolated from *D. farinae* [4] is one of these allergenic proteins belonging to the group-3 allergens. Although its physiological role in mites is still unknown, it has been shown that Der f 3 is a serine protease with trypsin-like activity [5–8], which can activate the kallikrein–kinin system involved in various allergic reactions in human plasma [9,10]. Over several years allergenic properties of Der f 3 have been examined. However, various extents of reactivity to IgE (16–100%) were reported [4–7]. To measure the exact reactivity of the group-3 allergens to IgE antibodies, it is required to establish a system to produce Der f 3 in large quantity. Furthermore, a high expression system for allergens may assure analyzing the mechanism of allergic response as well as diagnosis and treatment of IgE-mediated allergy disorders. In case of Der f 2, one of the major house dust mite allergens, for example, we have succeeded in the elucidation of human IgE epitopes for Der f 2, by using the *Escherichia coli* expression system for the allergen [11,12]. By using the expression system, it will also be possible to examine the involvement of the trypsin-like serine protease activity of Der f 3 in allergic diseases. We here report the isolation of Der f 3 cDNA obtained from *D. farinae* and the production of Der f 3 in *E. coli*.

2. Materials and methods

2.1. Construction of the D. farinae λgt11 cDNA library

The mRNA of *D. farinae* was purified from a culture of *D. farinae* as described in our previous report [13]. cDNA was synthesized with this mRNA and cloned to λgt11 phage DNA using Complete rapid cloning system (Amersham). In vitro packaging of the phage DNA with *D. farinae* cDNA and transformation of *Escherichia coli* Y1090 was carried out with Giga pack II packaging extract (Stratagene).

2.2. Isolation of Der f 3 cDNA clone from the cDNA library

A DNA fragment was amplified by PCR with the following synthetic oligonucleotides as primer and the cDNA library as the template. One oligonucleotide 5′-GGGGATCCATTGTTGGTGGTGTTAAAGCA-3′ was designed from the N-terminus amino acid sequence of native Der f 3 [6] containing a *Bam*HI site at its 5′-end, and the other was from a sequence of λgt11, 5′-GGTGGCGACGACTCCTGGAGCCCG-3′. PCR was performed by repeating the thermal cycle (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) 25 times with a thermal cycler RC-700 (Astec, Japan). About 1000 bp of the PCR fragment was amplified. After digestion with *Bam*HI plus *Eco*RI, the DNA fragment was subcloned into plasmid, pBluescript II, and introduced into *E. coli* DH5α. The resulting plasmid was purified with QIAGEN plasmid Midi-kit (QIAGEN) and digested with *Bam*HI plus *Eco*RI. The resultant DNA fragment was labeled with ECL direct nucleic acid labeling system (Amersham) and used as the probe for plaque hybridization. The plaque cDNA library was plated on a 90 mm petri dish at about 10,000 pfu per dish and transferred onto the Hybond-N+ membrane filter (Amersham) according to the supplier's instruction. Hybridization was performed with ECL detection system (Amersham). One positive clone was obtained from about 50,000 plaques and isolated by the secondary screening. The phage DNA was purified from this positive clone using QIAGEN Lambda Midi kit (QIAGEN).

2.3. Sequence analysis of Der f 3 cDNA

The phage DNA with Der f 3 cDNA sequence was digested with *Eco*RI plus *Cla*I and subcloned into pBluescript II. The DNA sequence of Der f 3 cDNA was verified by the dideoxy-chain termination method [14] using an Applied Biosystems 370A sequencer.

2.4. Construction of expression plasmids

To introduce a *Bam*HI site at upstream of the pro-region and *Hin*dIII site just downstream of the stop codon for Der f 3, PCR was performed using the cDNA of Der f 3 as template, and the following synthetic oligonucleotides as primers: one (primer A) is 5′-AGTGGATCCACACCGATTCTTCCATCA-3′, and the other (primer B) is 5′-CGAAGCTTACTGTGAACGTTTTGATTCAAT-3′. The reaction condition of the PCR is the same as described above. After digestion with *Bam*HI plus *Hin*dIII, the amplified DNA fragment was subcloned into the expression plasmid pGEX-4T-2 (Pharmacia Biotech). The resulting plasmid was designated pGEX/proDer f 3. In this construction, proDer

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f 3 was produced as a fusion protein (GST-proDer f 3) composed of glutathione-*S*-transferase and proDer f 3 protein linked with a dipeptide (Gly–Ser). Another expression plasmid pGEX/T(-1)R-proDer f 3, which has almost an identical construction to that of pGEX/proDer f 3 but with replacement of the codon (ACT) for Thr at the C-terminus of the pro-region by CGT for Arg, was constructed by site-directed mutagenesis using PCR as the similar method described above. Two synthetic oligonucleotides 5′-GCGGATCCACACCGATTCTTCCA-TCATCACCAAATGCACGT-3′ for introducing the mutation, and 5′-CGAAGCTTACTGTGAACGTTTTGATTCAAT-3′ which is the same as primer B used for the construction of the pGEX/proDer f 3, was used as the primer for PCR. The resulting PCR fragment was digested with *Bam*HI plus *Hin*dIII and subcloned into pGEX-4T-2. ProDer f 3 T(−1)R was produced in *E. coli* BL21 harboring the resulting plasmid, pGEX/T(−1)R-proDer f 3, as inclusion bodies as the same as above.

2.5. Production and purification of recombinant fusion protein

Overnight culture of *E. coli* BL21 carrying pGEX/proDer f 3 grown in L-broth containing 50 μg/ml of ampicillin, was diluted to 100-fold with the same medium and further cultured for 2 h at 30°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM to induce the *tac* promoter. After an additional 4 h incubation at 30 °C, the *E. coli* cells were harvested by centrifugation, resuspended in the buffer containing 100 mM Tris-HCl and 10 mM EDTA (pH 9.0), and disrupted by sonication. Since GST-proDer f 3 was produced as inclusion bodies, the fused protein was precipitated by centrifugation at 7000 × *g* for 15 min. This insoluble protein was solubilized with 8 M urea containing 20 mM Tris-HCl (pH 9.0) and dialyzed against 20 mM Tris-HCl (pH 9.0) for renaturation for 4 h. After successive dialysis against PBS [11] for 2 h, the solution containing GST-proDer f 3 was applied onto a column of glutathione-Sepharose 4B equilibrated with PBS and eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione. The GST-proDer f 3 thus purified was digested with thrombin (1 U per mg protein) for more than 2 h at room temperature to produce proDer f 3 with additional two amino acids at N-terminus. Purified proDer f 3 was obtained by collecting passing fraction through the successive glutathione-Sepharose 4B column chromatography.

2.6. Electrophoresis and Western blotting

The protein samples were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membrane (Bio-Rad) for Western blotting analysis. After blocking with PBS containing 0.05% (w/v) Tween 20 for 1 h at room temperature, the membrane was incubated with allergic patient sera diluted to 1/4 by 0.05% Tween-PBS, overnight at 4°C. After washing three times with 0.05% Tween-PBS, horseradish peroxidase-conjugated goat anti-human IgE antibody (Cappel) diluted to 1/1000 by 0.05% Tween-PBS was applied to the membrane as secondary antibody. After incubation for more than 5 h at room temperature, the membrane was washed with PBS as the same as above, and 30 mg of 1-chloro-4-naphtol resolved in 10 ml of methanol and 50 ml of PBS containing 0.03% H_2O_2 was applied onto the membrane as substrate.

2.7. Measurement of protease activity

Protease activity of wild-type or mutant Der f 3 was measured using synthetic substrates. Forty μM of synthetic substrate (Boc-Q-G-R-MCA, Boc-F-S-R-MCA, Boc-V-L-K-MCA, Suc-L-L-V-Y-MCA, or Suc-A-A-P-F-MCA) in 1 ml of 50 mM of Tris-HCl, pH 8.0 was incubated at 37°C after addition of an appropriate amount of wild-type or mutant Der f 3. The amount of 7-amino-4-methylcoumarin (AMC) released from synthetic substrates was determined by measuring the fluorescence at 380 nm for excitation, and at 460 nm for emission on a fluorometer (Hitachi S-2000 fluorescence spectrophotometer, Tokyo).

2.8. PCR-based cloning of Der f 3 cDNA

To examine the polymorphism of Der f 3 in dust mite, PCR was again performed using *D. farinae* cDNA library as template, and two primers, primers A and B, used in the construction of expression plasmid for wild-type Der f 3. The DNA fragment amplified by PCR in the same conditions as above was subcloned into pBluescript II, and the nucleotide sequence of Der f 3 clones were determined.

3. Results and discussion

3.1. Cloning and sequence of Der f 3 cDNA

Based on N-terminal amino acid sequence of Der f 3 already reported, an oligonucleotide was synthesized. When PCR was carried out together with another oligonucleotide having the sequence for a part of λgt11, a single DNA fragment of about 1000 bp was amplified. Nucleotide sequencing revealed that the amplified fragment contained the sequence which corresponds to the amino acid sequence for Der f 3. We therefore used this fragment as a probe for plaque hybridization to clone Der f 3 cDNA. A single Der f 3 clone was obtained from about 50,000 clones of *D. farinae* cDNA library by plaque hybridization. The nucleotide sequence and the amino acid sequence deduced from the nucleotide sequence was shown in Fig. 1. The cDNA was 1043 bp in length and contained a single open reading frame of 780 bp which started from ATG (nt 46) and terminated at stop codon TGA (nt 823). The N-terminal amino acid sequence

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                                                ATTTTCCAAATTAACAAATAAATCGTATATTGAAATCG    38

                                            -20
AACCAAG ATG ATG ATT TTA ACC ATT GTC GTG TTA TTG GCT GCA AAC ATT    87
        Met Met Ile Leu Thr Ile Val Val Leu Leu Ala Ala Asn Ile

            -10                                     -1  1
TTG GCC ACA CCG ATT CTT CCA TCA TCA CCA AAT GCA ACT ATT GTT GGT   135
Leu Ala Thr Pro Ile Leu Pro Ser Ser Pro Asn Ala Thr Ile Val Gly

                        10
GGT GTG AAA GCA CAA GCC GGT GAT TGT CCA TAT CAA ATT TCA TTG CAA   183
Gly Val Lys Ala Gln Ala Gly Asp Cys Pro Tyr Gln Ile Ser Leu Gln

 20                                 30
TCA AGC AGC CAT TTT TGT GGT GGT AGT ATC CTG GAT GAA TAT TGG ATC   231
Ser Ser Ser His Phe Cys Gly Gly Ser Ile Leu Asp Glu Tyr Trp Ile

                40                                      50
TTG ACC GCT GCA CAT TGT GTC AAT GGA CAA TCA GCA AAA AAA CTT TCA   279
Leu Thr Ala Ala His Cys Val Asn Gly Gln Ser Ala Lys Lys Leu Ser

                            60
ATT CGT TAC AAT ACT CTT AAA CAT GCA TCT GGT GGT GAA AAG ATT CAA   327
Ile Arg Tyr Asn Thr Leu Lys His Ala Ser Gly Gly Glu Lys Ile Gln

        70                                      80
GTG GCG GAA ATT TAT CAA CAT GAA AAT TAT GAT AGC ATG ACT ATC GAT   375
Val Ala Glu Ile Tyr Gln His Glu Asn Tyr Asp Ser Met Thr Ile Asp

                        90
AAT GAT GTT GCA TTG ATA AAA CTC AAA ACA CCA ATG ACA TTG GAT CAA   423
Asn Asp Val Ala Leu Ile Lys Leu Lys Thr Pro Met Thr Leu Asp Gln

100                                 110
ACA AAT GCT AAA CCC GTA CCA TTA CCA GCA CAA GGA TCA GAT GTA AAA   471
Thr Asn Ala Lys Pro Val Pro Leu Pro Ala Gln Gly Ser Asp Val Lys

                120                                     130
GTT GGT GAT AAA ATT CGT GTT TCT GGT TGG GGT TAT CTT CAG GAA GGA   519
Val Gly Asp Lys Ile Arg Val Ser Gly Trp Gly Tyr Ler Gln Glu Gly

                            140
AGT TAT TCA TTA CCA TCG GAA TTA CAA CGT GTT GAT ATT GAT GTT GTA   567
Ser Tyr Ser Leu Pro Ser Glu Leu Gln Arg Val Asp Ile Asp Val Val

        150                                     160
TCA CGT GAA CAA TGT GAC CAA TTA TAT TCA AAA GCA GGC GCC GAT GTT   615
Ser Arg Glu Gln Cys Asp Gln Leu Tyr Ser Lys Ala Gly Ala Asp Val

                        170
AGT GAA AAT ATG ATT TGC GGC GGT GAT GTC GCT AAT GGT GGT GTT GAT   663
Ser Glu Asn Met Ile Cys Gly Gly Asp Val Ala Asn Gly Gly Val Asp

180                                 190
TCA TGT CAA GGT GAT TCT GGC GGA CCA GTT GTT GAT GTT GCC ACT AAA   711
Ser Cys Gln Gly Asp Ser Gly Gly Pro Val Val Asp Val Ala Thr Lys

                200                                     210
CAA ATT GTT GGT ATT GTT TCA TGG GGT TAT GGT TGT GCA CGT AAA GGT   759
Gln Ile Val Gly Ile Val Ser Trp Gly Tyr Gly Cys Ala Arg Lys Gly

                            220
TAT CCA GGT GTC TAT ACA CGT GTT GGT AAT TTT GTC GAT TGG ATT GAA   807
Tyr Pro Gly Val Tyr Thr Arg Val Gly Asn Phe Val Asp Trp Ile Glu

        230
TCA AAA CGT TCA CAG TGA ATGATAAAATGGCAACAACGGTCACAATGGCACGATAAT   864
Ser Lys Arg Ser Gln Trm

ACACCCTTACGTGCAATCATTATGTTTTTTATAATTTTCTTTTTTCTTCTATCTCTTTATTTT   927
TTATCGAAAGTAAAAAAACAGCAAAATCAATATTGACTTAAGAGAATTGATTTTTGTTTCGAA   990
AAATAAAATTGAATGGACAACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA          1043
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Fig. 1. Nucleotide and deduced amino acid sequences of Der f 3 cDNA. The nucleotide sequence data reported in this paper is registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number D63858.

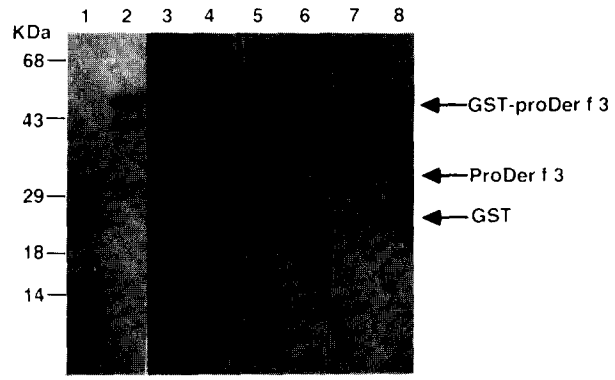

Fig. 2. IgE-binding ability of recombinant proDer f 3 analyzed by Western blotting. Lanes 1, 3, 5, and 7, GST-proDer f 3 digested with thrombin; lanes 2, 4, 6, and 8, GST-proDer f 3 fused protein. Lanes 1 and 2, Coomassie brilliant blue staining. IgE antibodies used are: lanes 3 and 4, pooled patient sera A; lanes 5 and 6, pooled patient sera B; lanes 7 and 8, serum from an atopic patient.

of the first 20 amino acids of mature Der f 3 already determined was found in the deduced amino acid sequence from the 28th to the 47th of the open reading frame, suggesting that Der f 3 was produced as a precursor with a N-terminal extension of 27 amino acids. Der f 3 is known to have a trypsin-like serine protease activity [5–8]. Pancreatic trypsin was produced as a precursor with a N-terminal extension of over 20 amino acids [15,16]. N-terminal part 15 amino acids of the extension serves as the signal sequence for secretion and C-terminal part 6–9 amino acids of the extension is known as pro-segment in trypsin. N-terminal portion (1st to 16th) of the extension of Der f 3 protein had high hydrophobic amino acid sequence, which is a typical feature of signal sequence for secretion. We assume that the N-terminal extension of Der f 3 was divided into two functional parts, pre-sequence (1st to 16th) for secretion and pro-sequence (17th to 27th) probably for folding as will be described below. For exact understanding of the role of the N-terminal segment further study will be required. By alignment of the amino acid sequence of the trypsin family, the catalytic-triad of Der f 3, which is essential for the enzyme activity, was assigned to His-40, Asp-85 and Ser-185. One hundred and seventy bp downstream of the termination codon, the sequence AATAAA is present, which may serve as a poly(A) signal to the 3′ terminus of the mRNA for Der f 3 [17].

3.2. Human IgE binding ability of recombinant Der f 3

The binding ability of recombinant proDer f 3 to human IgE antibodies from patients sera was studied by Western blotting analysis. The proDer f 3 expressed as a fusion protein with GST actually bound to human IgE antibodies from every serum used as shown in Fig. 2. When similar analysis was carried out with the preparation of GST-proDer f 3 treated with thrombin, the human IgE antibodies reacted with proDer f 3 portion, while only negligible binding was observed to the GST portion.

3.3. Protease activity of recombinant Der f 3

As described above, Der f 3 is known to be a trypsin-like serine protease [5–8]. Therefore, the protease activity and substrate specificity could become an index for correct refolding of Der f 3 protein from inactive inclusion bodies. When either of refolded GST-proDer f 3-fused protein or proDer f 3 portion purified from refolded GST-proDer f 3-fused protein was used, no protease activity was observed, although both recombinant proDer f 3 proteins showed IgE-binding activity. Trypsin or kallikrein, which has the protease activity to cleave the peptide bond of C-terminal side of Arg and/or Lys, have Arg or Lys residues at the C-terminal end of its own pro-segment, and was therefore activated by an autocatalytic process to remove the pro-region in intra- and/or inter-molecule manner [18]. On the other hand, despite that native Der f 3 was shown to have Arg-and Lys-specific cleavage activity [6,7], a C-terminal residue of the pro-segment of proDer f 3 is Thr. Pro-sequence of protease is known to have a role in correct folding. Furthermore, pro-sequence can be regarded as a specific inhibitory segment maintaining the protease in an inactive form, which protects the cell from the attack by the protease under unfavorable conditions. Therefore, it was probable to think that the failure of proDer f 3 in showing protease activity resulted from inability to cleave the Thr(−1)-Ile-1 peptide bond due to less specificity to this peptide bond. We, therefore, replaced Thr(−1) by Arg. The mutant Der f 3 containing T(−1)R replacement was produced as a fusion protein like the case for wild-type in a form of inclusion bodies in *E. coli* cells. During the refolding process by dialysis against Tris-HCl buffer to remove urea, proteolytic activity to remove the N-terminal pro-region was observed, while such processing was not found with GST-wild-type proDer f 3. This processing was inhibited by the

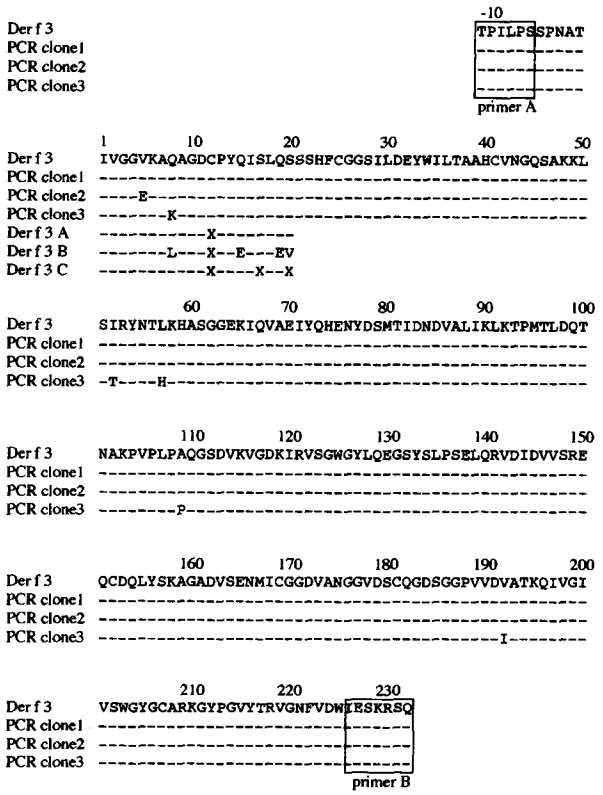

Fig. 3. Amino acid sequence variation in Der f 3. The amino acid residues which differ from those deduced from the nucleotide sequence determined with the cDNA were indicated. Der f 3 A, B, and C indicate the N-terminal amino acid sequences of Der f 3 reported in other publications, [10], [4], and [6], respectively. The nucleotide sequences of primers A and B are shown in section 2.

Table 1
Comparison of protease activity between recombinant Der f 3 and native Der f 3

Synthetic substrates	Relative protease activity (%)			
	Recombinant Der f 3 T(−1)R[a]	Native Der f 3		
		A[a]	B[a]	C[b]
Boc-Q-G-R-MCA	100	105	N.D.	100
Boc-F-S-R-MCA	100	100	100	N.D.
Boc-V-L-K-MCA	50	50	58	43
Suc-L-L-V-Y-MCA	2	30	0	0
Suc-A-A-P-F-MCA	0	0	0	N.D.

Protease activity was measured with the method reported previously [9]. Relative protease activities, A, B, and C, of native Der f 3 purified from house dust mite were quoted from three different references, [6], [7], and [10], respectively.
[a] Protease activities with Boc-F-S-R-MCA were expressed as 100%.
[b] Protease activities with Boc-Q-G-R-MCA were expressed as 100%.
N.D., not determined.

addition of *p*-aminobenzamidine or leupeptine, which is known as a serine protease inhibitor (data not shown). Renatured GST-proDer f 3 T(−1)R fraction was then applied onto a DEAE-Toyopearl column and a major peak was collected. When the pooled fractions were subjected to SDS-PAGE, the fraction was found to contain only two proteins, one was GST and the other was the protein cross-reactive to sera from allergic patients to house dust mite (data not shown). When the N-terminal 20 amino acid sequence of the latter protein was determined by automated Edman degradation, a sequence identical to that of mature Der f 3 was obtained, indicating that the replacement at position −1 caused the correct processing to produce mature Der f 3. With this preparation containing mature Der f 3, the relative protease activity of Der f 3 was determined using synthetic substrates (Table 1). The substrate specificity of recombinant Der f 3 was almost the same as that of native Der f 3. Since we have not yet obtained pure recombinant Der f 3 preparation because of further degradation during the refolding process, quantitative characterization of the enzyme activity of the recombinant Der f 3 has not yet been performed. However, based on the densitometric estimation of the amount of purified recombinant Der f 3, recombinant Der f 3 has specific activity almost identical to that of native Der f 3 (data not shown). This study showed that proDer f 3 did not have the ability to produce mature enzyme. This may suggest that another protease which convert proDer f 3 to the active form is present in dust mite.

3.4. Polymorphism of Der f 3

We obtained a single clone of Der f 3 cDNA as described above. The amino acid sequence of 20 residues at N-terminus deduced from the nucleotide sequence was identical to that of the native Der f 3 reported by Ando et al. [6] and Kohmoto et al. [10] but was somewhat different to that by Heymann et al. [4] as shown in Fig. 3. Since polymorphism is found in other house dust mite allergens, Der p 1 [19] and Der f 2 [11,13], the amino acid variation in the reported sequences might be due to polymorphism. To examine the possibility, we tried to clone other-type Der f 3 cDNAs by PCR. Although PCR clones directing the amino acid sequence reported by Heymann was not isolated, three different clones for Der f 3 were obtained. The amino acid sequences deduced from the nucleotide sequences were shown in Fig. 3. We designated these clones as PCR clone-type 1, -type 2, and -type 3. Among 7 PCR-clones sequenced, 1 was classified to type 1, 5 were type 2, and one was type 3. The amino acid sequence of PCR-clone-type 1 is identical to that of cDNA we first isolated. The amino acid sequence of PCR-clone-type 2 has a single amino acid replacement at the 5th residue. On the other hand, PCR-clone-type 3 has amino acid variations at five positions, which is identical to that deduced from the nucleotide sequence of PCR-amplified Der f 3 DNA fragment used as the probe for the first plaque hybridization. In the case of Der f 2, which also shows polymorphism, a mite has a single Der f 2 gene (unpublished result). Therefore, we at present speculate that each mite has only one gene for Der f 3. To clarify this possibility, further study will be obviously required. Amino acid variations found in this study may give a clue to clarify the epitopes to IgE antibodies of this allergen. In every clone, the amino acid sequence at the −1 position is Thr. Furthermore, Der p 3 which is also a group-3 allergen produced by *D. pteronyssinus* has Thr at position −1 [20]. Tryptase, a trypsin-like serine protease, is produced as a precursor with a N-terminal pro-segment. This precursor is known to be converted to an active form by another protease which cleaves the peptide bond of Gly–Ile at the pro-mature junction [21]. From these observations, we assume that group-3 allergens are converted to an active form from the precursor by the mechanism mediated by some other factors.

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