Maturation of the activities of recombinant mite allergens Der p 1 and Der f 1, and its implication in the blockade of proteolytic activity

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Abstract **Recombinant pro-Der p 1 expressed in yeast *Pichia pastoris* was convertible into the prosequence-removed mature Der p 1 with full activities of cysteine protease and IgE-binding with or without N-glycosylation of the mature sequence as well as pro-Der f 1. The active recombinant variants will be the basis for various future studies. The major N-terminus of pro-Der p 1 with low proteolytic activity was the putative signal-cleavage site, while that of pro-Der f 1 contained not only the equivalent site but also 21 residues downstream, and pro-Der f 1 retained significant activity. Contribution of the N-terminal region of the Der p 1 prosequence including an N-glycosylation motif on effective inhibition of proteolytic activity of pro-Der p 1 was suggested.**

Key words: Recombinant major house dust mite group 1 allergen; Maturation; Prosequence; *N*-Glycosylation; Cysteine protease; IgE-binding

1. Introduction

House dust mites of the *Dermatophagoides* species (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are the most important causative factor associated with various allergic diseases among all the indoor allergens [1–3]. Group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) allergens are considered the major house dust mite allergens [4–7]. IgE-binding activity for both group 1 and group 2 allergens is detected in the majority of sera from patients allergic to house dust mites. Group 1 allergens, which belong to the papain-like cysteine protease family, are mainly found in mite feces and are thought to function as digestive enzymes in the intestine of mites [8–10]. IgE-binding to the group 1 allergens is highly dependent on the tertiary structure of the allergens [5,11,12]. Recently, the cysteine protease activity of a group 1 allergen from *D. pteronyssinus*, Der p 1, has been proposed to be involved in the pathogenesis of allergy [13–17]. Therefore, enzymatically and immunologically active recombinant group 1 allergens with structures equivalent to the natural ones will be useful tools for diagnosis, standardized allergen-specific immunotherapy (AIT) [18–20], and various studies, including the analysis of conformation-dependent IgE epitopes [21–27], determination of tertiary structure [28], allergen engineering for safer and more effective AIT [18–20,23–26,29], resolving the relationship between the enzymatic activity and pathogenesis [13–17,30], and so on.

The cDNAs for the mite group 1 allergens and their isoforms have been cloned [31–34]. They encode the preprosequence at the N-terminus of the mature sequence. Recently, we have developed efficient systems to prepare recombinant forms of the group 1 allergen from *D. farinae*, Der f 1, which is expressed abundantly in methylotrophic yeast *Pichia pastoris* [35,36] and bacteria [37], and demonstrated their mature protease activity, IgE reactivity, and an apparent molecular weight equivalency to that of natural Der f 1. On the other hand, preparation of recombinant mature Der p 1 with equivalent properties in terms of activities and molecular weight has not been reported, although enzymatically inactive pro-Der p 1 was reported by some researchers [38–40], and highly glycosylated mature Der p 1 with enzymatic and allergenic activities but with a broad apparent molecular weight much higher than natural Der p 1 was reported recently [41].

We show that recombinant proforms of Der p 1 as well as those of Der f 1 expressed in yeast *P. pastoris* with or without N-glycosylation of the mature sequence are convertible into the prosequence-removed mature forms with full cysteine protease activity. IgE reactivities of the recombinant mature forms were also equivalent to those of natural ones. This is the first report that demonstrates the preparation of recombinant mature Der p 1, which has no yeast-derived N-glycosylation and retains an apparent molecular weight equivalency to natural Der p 1, with full enzymatic and IgE-binding activities. Moreover, we compare the properties of the recombinant pro- and mature forms of the two homologous allergens Der p 1 and Der f 1 and discuss the role of the N-terminal region of the prosequence in the maturation and inhibition of proteolytic activity of the recombinant proforms of the house dust mite group 1 allergens.

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2. Materials and methods

2.1. Cloning of cDNA coding for prepro-Der p 1 and prepro-Der f 1

The total RNA from cultured mites was prepared, and the cDNA fragments of prepro-Der p 1 and prepro-Der f 1 were synthesized and amplified by reverse transcription- polymerase chain reaction (RT-PCR). The PCR primers were designed on the basis of previously described cDNA sequences [33,34]. The cDNA fragments were subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA, USA) or pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands) and sequenced by a DNA sequencer (ABI Prism 377, Applied Biosystems, Foster City, CA, USA).

2.2. Construction of expression vectors encoding prepro-Der p 1, prepro-Der f 1, and their mutants

A *Bam*HI site and a *Not*I site were added to the 5′ and 3′ termini, respectively, of each open reading frame (ORF) for prepro-Der p 1 and prepro-Der f 1 by PCR. Vectors for expression in the methylotrophic yeast *P. pastoris* encoding the prepro-Der p 1-WT and prepro-Der f 1-WT, each of which has the intact wild-type sequence, were constructed by insertion of the PCR-amplified cDNA fragments digested with *Bam*HI and *Not*I to the *Bam*HI/*Not*I site of pPIC3.5 (Invitrogen). Expression vectors encoding for mutants whose N-glycosylation motifs within the mature sequences are disrupted, prepro-Der p 1-N52Q and prepro-Der f 1-N53Q, were constructed by site-directed mutagenesis. It was confirmed that the inserted sequences encoded the correct amino acid sequences by DNA sequencing.

2.3. Expression of prepro-Der p 1, prepro-Der f 1, and their mutants in P. pastoris

The method was as described previously [35] with minor modifications. Briefly, the expression vectors were introduced into *P. pastoris* GS115 cells by electroporation. Transfectants in which the locus of the genomic alcohol oxidase gene was homologously recombined with the linearized vector DNA were selected. The transfectants were cultured at 30°C in 5 ml of YPD. Four milliliters of the one or two overnight full-growth culture was inoculated in 400 ml of BMGY and cultured for 24 h. The cells were pelleted by centrifugation, resuspended in 80 ml of BMMY and cultured for an additional 72–96 h with addition of methanol to the culture every 24 h to maintain the continuous expression of the recombinant products. The culture supernatant was harvested by centrifugation. The culture supernatants, to which a 1/5-volume of 0.5 M Tris–HCL (pH 9.0) containing 0.1% NaN_3 was added, were concentrated by ammonium sulfate-precipitation. The concentrations of ammonium sulfate were 80%-saturation for pro-Der p 1-WT and pro-Der f 1-WT, and 50%-saturation for pro-Der p 1-N52Q and pro-Der f 1-53Q. The precipitates were dissolved in 50 mM Tris–HCL (pH 9.0) containing 0.01% NaN_3 at 1/20 volume of the original culture supernatants.

2.4. Maturation of pro-Der p 1, pro-Der f 1, and their mutants, and purification of the pro- and mature forms

The method was as described previously [35] with minor modifications. Briefly, the supernatants concentrated by ammonium sulfate-precipitation were dialyzed against 100 mM sodium acetate buffer (pH 4.0) for 3 h at room temperature and an additional 24–48 h at 4°C. After this activation process, the recombinant products were subjected to anion exchange column chromatography after dialysis against a buffer for the chromatography. Purities of the fractions of NaCl-gradient elution (0–400 mm) were checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The fractions containing pure recombinant proteins were mixed, aliquoted, and stored at −80°C. Protein concentrations were determined by the Bradford's procedure using a protein assay kit (Bio-Rad, Richmond, CA, USA) with bovine IgG (Bio-Rad) as the standard. The recombinant proforms of Der p 1 and Der f 1 were purified by the same methods as the mature forms but without the activation process of dialysis against the acidic buffer.

2.5. Protein sequencing

The method was as described previously [42,43]. The samples were subjected to SDS–PAGE and electroblotted onto membranes. Before the SDS–PAGE, the samples were boiled under reducing conditions with dithioerythritol in the sample buffer, and then the cysteine residues were alkylated [43]. The membranes were stained with Coomassie Brilliant Blue (CBB). The visualized bands were subjected to N-terminal amino acid sequencing using protein sequencers G1005A (Hewlett Packard, Palo Alto, CA, USA) and/or Procise 492cLC (Applied Biosystems).

2.6. Purification of natural Der p 1 and Der f 1

Natural Der p 1 and Der f 1 were purified from the whole culture extracts from house dust mites, *D. pteronyssinus* and *D. farinae*, respectively, as described previously by Yasueda et al. [5,6]

2.7. Measurement of proteolytic activity

Cysteine protease activity was measured as described previously [44] with modifications. Assays were conducted in reaction buffer (50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA) with or without 1 mM dithiothreitol (DTT) at 37°C. Final concentrations of the recombinant or natural allergens were 100 nM. The substrate butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-Gln-Ala-Arg-MCA) (Peptide Institute, Osaka, Japan) was used at 0.1 mM. The fluorescence of aminomethylcoumarin released from the substrate was measured at an excitation of 355 nm and emission of 460 nm on a fluorometer, Fluoroskan Ascent (Labsystems, Vantaa, Finland).

2.8. Radioallergosorbent test-enzyme immunoassay (RAST-EIA)

IgE reactivities were measured by radioallergosorbent test-enzyme immunoassay (RAST-EIA) using a RAST-EIA Kit (Pharmacia, Peapack, NJ, USA) as previously described [24]. Briefly, serum of each allergic patient (1/8 dilution) was incubated with paper discs coupled with the allergens. Then, the IgE that bound to the disc was detected with β-galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence at an excitation of 355 nm and emission of 460 nm on the fluorometer. The mite-allergic patients' sera were kindly provided by Dr. Hirokazu Okudaira, Tokyo University.

3. Results

3.1. Cloning of cDNAs encoding for prepro-Der p 1 and prepro-Der f 1

The cDNAs encoding for prepro-Der p 1 and prepro-Der f 1 were cloned by RT-PCR. Polymorphism was found in the nucleotide sequences of the ORF of the clones. The amino acid sequence of the prepro-Der p 1 clone selected for expression in this study was identical with that previously reported as 'clone (c)' by Chua et al. [33] except for one residue (−95 in the signal sequence). The amino acid residue at position −95 was threonine in this isoform of prepro-Der p 1, whereas it was valine in 'clone (c)' reported by Chua et al. The amino acid sequence of the prepro-Der f 1 clone selected for expression in this study was identical with that previously reported by Yasuhara et al. [34].

3.2. Preparation of purified recombinant and natural types of Der p 1 and Der f 1

The prepro-Der p 1 and prepro-Der f 1 with original mite amino acid sequences (Fig. 1A), prepro-Der p 1-WT and prepro-Der f 1-WT, and their mutants whose N-glycosylation motif within the mature sequences was disrupted by the substitution of an asparagine residue in the motif to a glutamine residue, prepro-Der p 1-N52Q and prepro-Der f 1-N53Q, were expressed. The proforms were secreted into culture medium, and processed to mature forms by the methods described in Materials and methods. Processing of the proforms was checked by SDS–PAGE before the anion exchange chromatography. Essentially, all the allergen was processed during dialysis against the acidic buffer. The amounts of purified recombinant products per 1 l of BMMY medium were 80–400 mg of pro-Der p 1-WT and pro-Der f 1-WT, 60–210 mg of mature Der p 1-WT and Der f 1-WT, 15–70 mg of pro-Der p 1-N52Q and pro-Der f 1-N53Q, and 10–40 mg of mature Der p 1-N52Q and Der f 1-N53Q.

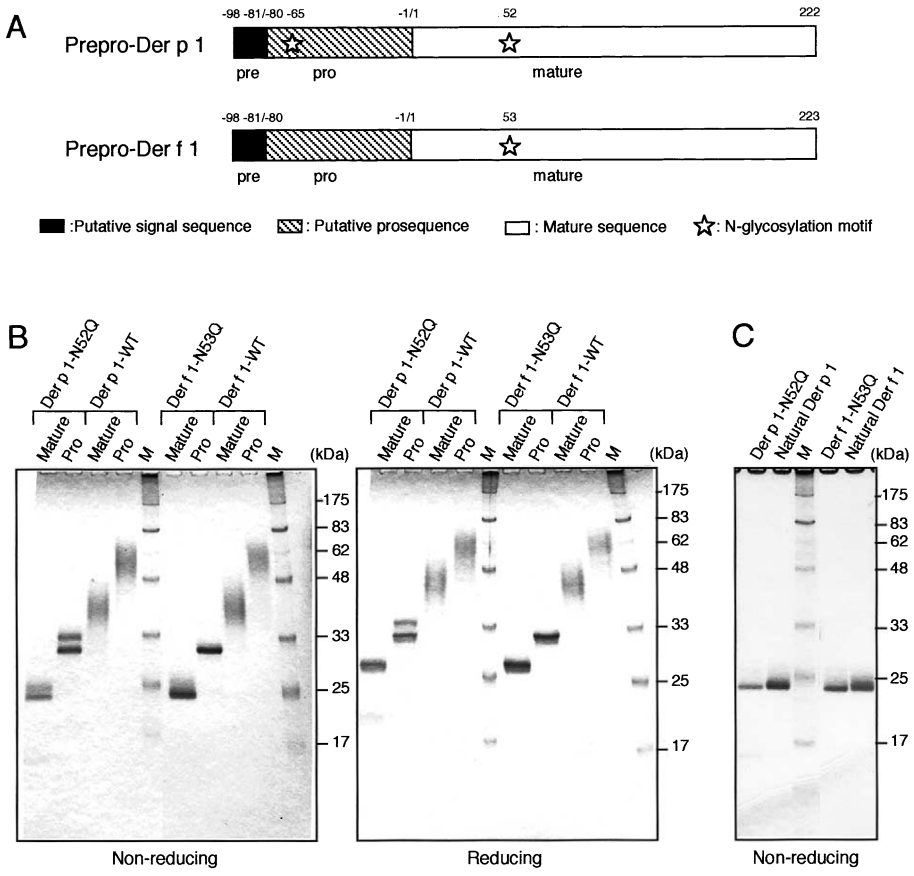


Fig. 1. Recombinant and natural types of the major house dust mite group 1 allergens Der p 1 and Der f 1. A: Schematic representation of prepro-Der p 1 and prepro-Der f 1 expressed in this study, which have the original mite amino acid sequences. B: Purified recombinant forms of Der p 1 and Der f 1. Proforms were purified from concentrated supernatants of yeast *P. pastoris* by column chromatography. Mature forms were prepared from the proforms in concentrated supernatants by dialysis against an acidic buffer and purified by column chromatography. They were subjected to SDS–PAGE (15–25%) and stained with CBB. Non-reducing and reducing: samples were boiled in the absence and presence of 2-mercaptoethanol in the sample buffer, respectively. C: Comparison of recombinant mature forms without N-glycosylation, Der p 1-N52Q and Der f 1-N53Q, and natural types of Der p 1 and Der f 1. Natural types of Der p 1 and Der f 1 were purified from mites. Mature: mature form; pro: proform; M: molecular weight marker; N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence.

The results of SDS–PAGE of purified recombinant forms of Der p 1 and Der f 1 are shown in Fig. 1B. Two bands were observed in the lane for pro-Der p 1-N52Q. The bands for recombinant proteins with an N-glycosylation motif in the mature sequence showed as smears (Der p 1-WT, pro-Der p1-WT, Der f 1-WT, and pro-Der f 1-WT). Mobilities of the mature forms under reducing conditions were significantly less than those under non-reducing conditions. Mobilities of the proforms under reducing conditions were equivalent to or slightly less than those under non-reducing conditions. Approximate apparent molecular weights based on the mobilities of the bands on the SDS–PAGE under non-reducing conditions were 24 kDa for mature Der p 1-N52Q and Der f 1-N53Q, 31 kDa and 33 kDa for pro-Der p 1-N52Q, 31 kDa for pro-Der f 1-N53Q, 35–45 kDa for mature Der p 1-WT and Der f 1-WT, and 50–70 kDa for pro-Der p 1-WT and pro-Der f 1-WT. Those under reducing conditions were 27 kDa for mature Der p 1-N52Q and Der f 1-N53Q, 32 kDa and 34 kDa for pro-Der p 1-N52Q, 32 kDa for pro-Der f 1-N53Q, 40–50 kDa for mature Der p 1-WT and Der f 1-WT, and 50–70 kDa for pro-Der p 1-WT and pro-Der f 1-WT.

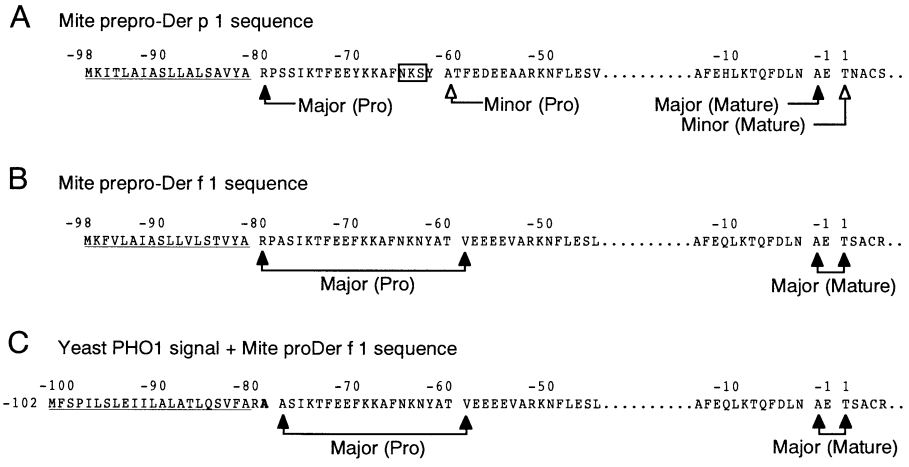
The mobilities on SDS–PAGE of mature Der p 1-N52Q and Der f 1-N53Q were compared with those of natural Der p 1 and Der f 1 (Fig. 1C). The mobilities of the recombinant mature forms without the N-glycosylation expressed in yeast were equivalent to those of the natural allergens purified from mites.

3.3. *N-terminal sequencing of recombinant pro- and mature forms of Der p 1 and Der f 1*

The samples of purified recombinant pro- and mature forms of Der p 1 and Der f 1 were electrophoresed and electroblotted, and the bands visualized by CBB-staining were subjected to N-terminal sequencing. The results are summarized in Fig. 2.

The N-terminal sequences of the four mature forms (Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT) were determined to start from −2 or 1, two-residues upstream from or at the N-terminus of the natural ones (Fig. 2A,B). However, the ratios of the peaks corresponding to these two sequences in the four recombinant variants were different from one another (Fig. 2D). The contents of the peaks corresponding to the sequence starting from the N-terminus of the natural ones were only trace amounts in Der p 1-N52Q and minor amounts in Der p 1-WT.

The N-terminal sequences of the two proforms of Der p 1



D

Recombinant mature form	N-terminus (% ratio) -2	1
Mature Der p 1-N52Q	100 %	Trace
Mature Der p 1-WT	95-100 %	0-5 %
Mature Der f 1-N53Q	85 %	15 %
Mature Der f 1-WT	65-70 %	30-35%

E

Recombinant proform	N-terminus (% ratio) -80	-61	-59
Pro-Der p 1-N52Q	100 %	Trace	
Pro-Der p 1-WT	90-95 %	5-10 %	
Pro-Der f 1-N53Q	60-70 %		30-40 %
Pro-Der f 1-WT	25-35 %		65-75 %

Fig. 2. N-terminal amino acid sequencing of purified recombinant pro- and mature forms of the major house dust mite group 1 allergens Der p 1 and Der f 1. Schematic representation of N-terminal amino acid residues of recombinant pro- and mature forms of Der p 1 in this study (A), Der f 1 in this study (B), and Der f 1 in our previous report [35] (C), and approximate molecular ratios of variations in the different N-terminal sequences of the mature forms (D) and the proforms (E) are shown. In A and B, mite-derived original amino acid sequences were used. In C, yeast *Pichia*-derived PHO1 signal sequence was fused to the mite prosequence. The bold type indicates mutation as a result of introducing a restriction site, and the sequence in the previous report [35] is revised here. Underlined: putative signal sequence for secretion; boxed: N-glycosylation motif. In D and E, the ratios were approximately estimated from the peak heights of HPLC in the protein sequencing. N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence; Trace: peaks corresponding to the sequence starting from the N-terminus were detected as only a trace.

(pro-Der p 1-N52Q and pro-Der p 1-WT) were determined to mainly start from −80, which was just after the putative signal-cleavage site in the original mite prepro-Der p 1 sequence (Fig. 2A,E). Peaks corresponding to the sequence starting from −61, which was 19 residues downstream from the putative signal-cleavage site and 4 residues downstream from the N-glycosylation site in the prosequence of Der p 1, Asn (−65), were also observed but there were only trace amounts in pro-Der p 1-N52Q and minor amounts in pro-Der p 1-WT. The N-terminal sequences for the two major bands for pro-Der p 1-N52Q corresponding to 31 kDa and 33 kDa on SDS–PAGE under non-reducing conditions, or 32 kDa and 34 kDa under reducing conditions, were identical.

The N-terminal sequences of the proforms of Der f 1 (pro-Der f 1-N53Q and pro-Der f 1-WT) were determined to start from −80, which was just after the putative signal-cleavage site in the original mite prepro-Der f 1 sequence, and also from −59, which was 21 residues downstream from the putative signal-cleavage site (Fig. 2B,E).

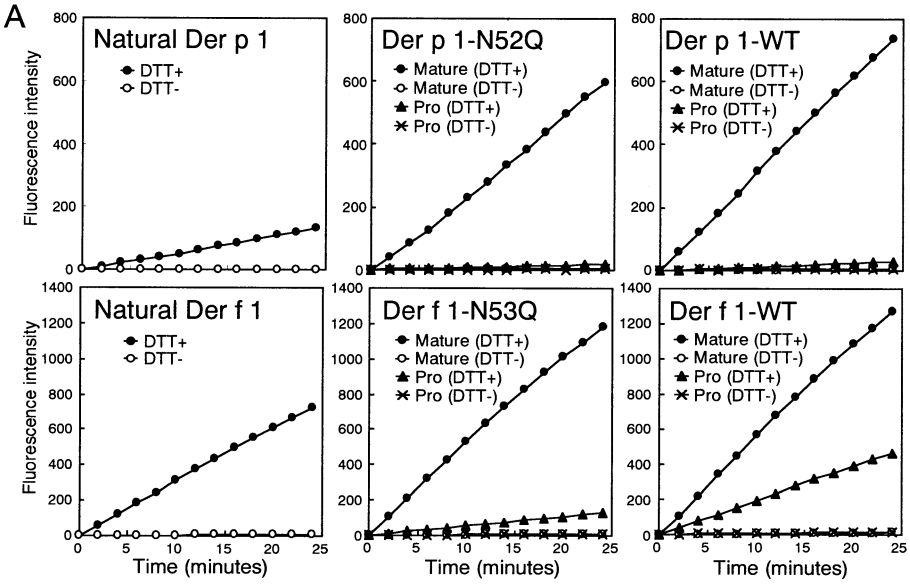
3.4. *Cysteine protease activities of natural and recombinant forms of Der p 1 and Der f 1*

The proteolytic activities of a total of ten variants of purified natural forms and recombinant pro- and mature forms of Der p 1 and Der f 1 were analyzed in the presence or absence of DTT, which activates the catalytic site of cysteine proteases (Fig. 3A). The natural allergens (natural Der p 1 and natural Der f 1) and the four recombinant mature forms (Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT) retained their activities in the presence of DTT but did not in the absence of DTT. Those of the two proforms of Der p 1 (pro-Der p 1-N52Q and pro-Der p 1-WT) were very low in the presence of DTT and not detectable in the absence of DTT. The two proforms of Der f 1 (pro-Der f 1-N53Q and pro-Der f 1-WT) retained higher protease activities than the proforms of Der p 1, although they were immature compared with their mature forms in the presence of DTT (Fig. 3B), and showed no activities in the absence of DTT.

3.5. *IgE-binding of natural and recombinant mature forms of Der p 1 and Der f 1*

Patient IgE-binding profiles of recombinant mature forms of Der p 1 and Der f 1 were determined by RAST-EIA (Fig. 4). In sera from two mite-allergic patients, the recombinant mature Der p 1 (Der p 1-WT and Der p 1-N52Q) and Der f 1 (Der f 1-WT and Der f 1-N53Q) with or without the N-glycosylation showed IgE-binding curves equivalent to those of natural Der p 1 and Der f 1, respectively (Fig. 4A). Using thirty or twenty-seven individual sera from mite-allergic patients, IgE-binding to recombinant Der p 1-N52Q or Der f 1-N53Q, which lacked the yeast-derived N-glycosylation, was compared with that of natural Der p 1 or Der f 1 purified from mites, respectively (Fig. 4BC). Close correlations of IgE-binding between natural Der p 1 and recombinant Der p 1-

A



B

Recombinant	Relative proteolytic activity to mature Der f 1-WT (%)
Mature Der p 1-N52Q	47
Mature Der p 1-WT	58
Mature Der f 1-N53Q	93
Mature Der f 1-WT	100
Pro-Der p 1-N52Q	1
Pro-Der p 1-WT	2
Pro-Der f 1-N53Q	10
Pro-Der f 1-WT	37

Fig. 3. Proteolytic activities of purified recombinant and natural types of the major house dust mite group 1 allergens Der p 1 and Der f 1. A: Time courses of digestion of Boc-Gln-Ala-Arg-MCA. The values are calculated by subtracting the background of the substrate, and the initial values from the original values are shown. B: Relative proteolytic activities of the recombinant proforms to that of Der f 1-WT. The values are calculated from fluorescence intensities in the presence of DTT at 24 min. N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence; mature: mature form; pro: proform; DTT+ and DTT-: the reaction was performed in the presence and absence of DTT, respectively.

N52Q ($R^2 = 0.9805$, $P < 0.0001$) (Fig. 4B) and between natural Der f 1 and recombinant Der f 1-N53Q ($R^2 = 0.9877$, $P < 0.0001$) (Fig. 4C) were observed.

4. Discussion

The first reports of activation of a recombinant house dust mite group 1 allergen were made by Shoji et al. [45,46], in which recombinant pro-Der f 1 secreted by insect SF9 cells was converted to active prosequence-removed Der f 1 by dialysis against an acidic buffer. Recently, we also demonstrated that proforms of Der f 1 secreted by yeast *P. pastoris* cells matured to the enzymatically active mature forms [35]. However, concerning Der p 1, recombinant forms of pro-Der p 1 secreted by insect *Drosophila* cells [38] and mammalian CHO cells [39] were not convertible to enzymatically active mature forms and, very recently, van Oort et al. [40] reported that pro-Der p 1 secreted by *P. pastoris* with yeast-derived heterogeneous sugar chains was not spontaneously processed, while Jacquet et al. [41] reported the activation of the highly glycosylated form. Some molecular mechanisms might underlie such discrepancies between the results in these trials to obtain active recombinant forms of Der f 1 and Der p 1. Therefore, the purposes of our study were not only to establish a system for the preparation of active Der p 1 with properties equivalent to natural Der p 1, but also to provide some insights into the molecular mechanisms in processing, activation, and/or inhibition of maturation of the proforms of the two house dust mite group 1 allergens by comparing the differences in properties among the recombinant products.

We used completely mite-derived amino acid sequences of prepro-Der p 1 and prepro-Der f 1 for the expression in this study (Figs. 1A, 2A,B), while a *Pichia*-derived signal sequence was used in our previous report [35] for secretion of pro-Der f 1 (Fig. 2C). The N-glycosylation of the wild-type mature sequences of both Der p 1 and Der f 1 in *Pichia* cells leads to heterogeneous glycosylation (Fig. 1B, Der p 1-WT, Der f 1-WT). Pro-Der p 1-N52Q without N-glycosylation in the mature sequence showed two major bands on SDS–PAGE (Fig. 1B, Der p 1-N52Q, Pro). This is considered a result of differential modifications of the N-glycosylation motif in the Der p 1 prosequence (Figs. 1A, 2A), which does not exist in the Der f 1 prosequence, on the basis of the results of N-terminal sequencing (Fig. 2E), SDS–PAGE showing a single band for the mature form (Fig. 1B, Der p 1-N52Q, Mature), and analysis of the amino acid compositions (data not shown). Signifi-

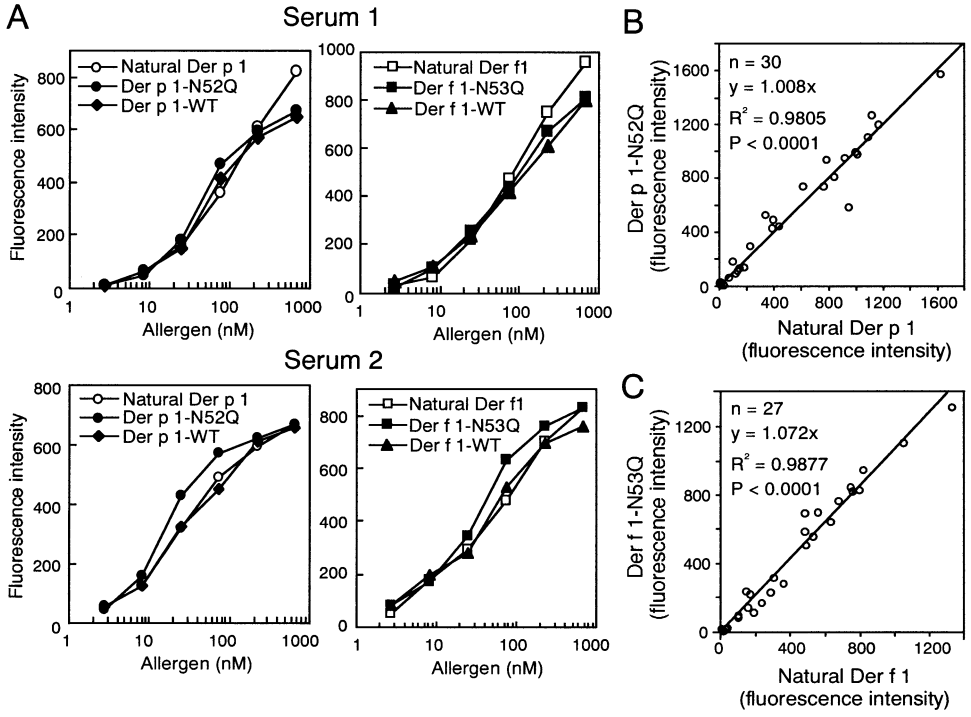


Fig. 4. IgE-binding activities of recombinant mature forms and natural types of the major house dust mite group 1 allergens Der p 1 and Der f 1. Paper discs to which allergen was coupled were incubated with diluted mite-allergic patients' sera and IgE that bound to the discs was detected with β-galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence. A: IgE-binding profiles of recombinant mature forms with and without yeast-derived N-glycosylation, and natural types of Der p 1 and Der f 1 in two sera. B: Correlation between the IgE reactivity of recombinant mature Der p 1-N52Q without yeast-derived N-glycosylation and natural Der p 1. Thirty sera were tested. The concentration of the allergens in the immobilization step was 75 nM. C: Correlation between the IgE reactivity of recombinant mature Der f 1-N53Q without yeast-derived N-glycosylation and natural Der f 1. Twenty-seven sera were tested. The concentration of the allergens in the immobilization step was 25 nM. N52Q and N53Q: mutation of Asn in the N-glycosylation motif within the mature sequence to Gln; WT: wild-type sequence. The values calculated by subtracting the background of the paper discs without coupling of allergen from the original values are shown.

cantly higher mobility of the mature forms on SDS–PAGE under non-reducing conditions than under reducing conditions is considered a result of the formation of intramolecular disulfide bonds of Der p 1 and Der f 1 [36] (Fig. 1B, Mature). In the mature forms, almost all of the prosequence was removed (Fig. 2A,B,D), and both cysteine protease (Fig. 3A) and IgE-binding (Fig. 4) activities were fully retained. The activated recombinant mature forms are considered to be folded and biologically functional. To obtain recombinant proteins more closely resembling natural ones, we generated mutants in which the N-glycosylation motif within the mature sequence was disrupted. The mutants showed no smear bands on SDS–PAGE (Fig. 1B, Der p 1-N52Q, Der f 1-53Q) and the mature forms of the mutants retained an apparent molecular weight equivalent to the natural ones (Fig. 1C). These recombinant mature house dust mite group 1 allergens with the same properties as the natural types will be useful tools for diagnosis, standardized AIT [18–20], and various other studies, as well as the major house dust mite group 2 allergen from *D. farinae*, Der f 2, which we have investigated as a model allergen for allergen engineering [21–29].

All the visible bands stained after electroblotting were subjected to N-terminal sequencing and variation in the N-terminal sequences existed in the purified recombinant samples (Fig. 2A,B,D,E). Interestingly, the proforms of Der p 1 and Der f 1 differed in the N-terminal sequences (Fig. 2A,B,E) and the proteolytic activity (Fig. 3). While the N-termini of the proforms of Der p 1 (pro-Der p 1-WT and pro-Der p 1-N52Q) mainly started just after the putative signal-cleavage site, the proforms of Der f 1 (pro-Der f 1-WT and pro-Der f 1-N53Q) had those starting not only from the equivalent site but 21 residues downstream (Fig. 2A,B,E), and retained higher activity than the proforms of Der p 1 (Fig. 3). The ratios of partial digestion before position −61 in pro-Der p 1 or −59 in pro-Der f 1 correlated with the proteolytic activities of the proforms (Fig. 2E, Fig. 3B). These results raised two questions. Firstly, why did the difference in the ratio of the partial digestion correlate with the enzymatic activity of the proforms? Secondly, why did the proforms of Der f 1 show higher ratios of partial digestion than those of Der p 1?

The prosequence of protease is considered to help in correct folding of the precursor suitable for mature folding of the prosequence-removed active protease, and to maintain the protease inactive until transfer and exposure to sites or situations where the protease could be sorted and function. As an answer to the first question, we consider that the N-terminal regions of Der p 1 and Der f 1 have a role in blocking the proteolytic activity effectively and/or that removal of the 18 or 20 amino acid sequence in the prosequences of Der p 1 or Der f 1 leads to partial activation of the proforms. This consideration correlates well with recent reports by two groups on the maturation of pro-Der p 1 with the yeast-derived heterogeneous sugar chain secreted by *P. pastoris*, although their results were different from each other, i.e., van Oort et al. [40]

reported that the pro-Der p 1-WT with N-terminus just after the putative signal-cleavage site was mainly obtained and not spontaneously processed, while Jacquet et al. [41] reported that the pro-Der p 1-WT with N-terminus of 19 residues downstream of the putative-cleavage site was obtained and successfully processed to the active mature form. Our results showing that pro-Der p 1-N52Q and pro-Der p 1-WT with the N-terminus just after the putative signal-cleavage site were mainly obtained and processed to the active mature forms was intermediate between those by the two groups. The differences among the three studies could be attributable to differences in procedures for maturation and/or origins of the signal sequences fused to the proforms for secretion.

As an answer to the second question, we suspect that N-glycosylation of the Der p 1 prosequence might be a reason for the higher ratios of partial digestion in pro-Der f 1 than in pro-Der p 1. Interestingly, the minor N-terminus of the proforms of Der p 1 in this study, Ala (−61), is only 4 residues downstream from the N-glycosylation site in the Der p 1 prosequence, Asn (−65) (Fig. 1A, Fig. 2A). Therefore, digestion near Asn (−65) might be inhibited by the sugar chain attachment in pro-Der p 1 differing from pro-Der f 1. Effects of the sugar chain in the prosequence on folding and/or interaction between the prosequence and mature portion might also be possible. This hypothesis could also explain the difficulty in preparing enzymatically active recombinant mature Der p 1 compared to Der f 1 in the previous reports [38–40]. Molecular mechanisms of processing, activation, and/or inhibition of maturation of the proforms should be analyzed in further studies.

In this study, we demonstrated for the first time the preparation of recombinant mature Der p 1, Der p 1-N52Q, which has no yeast-derived N-glycosylation and retains an apparent molecular weight equivalent to natural Der p 1, with full enzymatic and IgE-binding activities. The recombinant mature house dust mite group 1 allergens prepared in this study with the same properties as the natural types will be the basis of various studies. Moreover, by comparing the properties of the recombinant pro- and mature forms of the two homologous allergens Der p 1 and Der f 1, we suggested the contribution of the N-terminal region of the Der p 1 prosequence including the N-glycosylation site, Asn (−65), on effective inhibition of proteolytic activity in pro-Der p 1. This region should be a target candidate for the manipulation of proteolytic activities of recombinant variants. Such variants are considered useful as allergen vaccines because the proteolytic activity of Der p 1 has been proposed to be involved in the pathogenesis of allergies [13–17,30]. The findings in this study implicate a method for effective blockade of the harmful proteolytic activity of the major house dust mite group 1 allergens.

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