

Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding

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Summary

Background Group 1 allergens from mite faeces, Der f 1 and Der p 1, are the most significant in-door allergens. Therefore, they are the most important component in the standardization of house dust mite extract for diagnosis and allergen-specific immunotherapy (AIT). Although their cDNAs have been cloned, efforts to prepare biologically active recombinant forms in expression systems using bacteria or yeast have failed.

Objective Our purpose is to establish an efficient system to prepare recombinant Der f 1 (rDer f 1), identical in quality to native Der f 1.

Methods The preproforms of Der f 1 and a mutant N53Q, whose consensus motif for N-glycosylation was disrupted, were expressed in yeast *Pichia pastoris*. Cysteine protease activity and IgE reactivity were analysed using synthetic substrates and by RAST-EIA, respectively.

Results The proforms of the two rDer f 1 molecules were efficiently secreted into culture medium. Their prosequences were removed autocatalytically by dialysis against acidic buffer. Although the wild-type rDer f 1 was more highly glycosylated than native Der f 1, N53Q had almost the same apparent molecular weight as native Der f 1 on SDS-PAGE. Both the protease and IgE binding activities of the mature rDer f 1 molecules were the same as those of native Der f 1, whereas the proforms had no or markedly reduced activities.

Conclusion The efficient system to prepare active rDer f 1s established in this study is useful for diagnosis and standardized AIT for house dust mite allergy. Furthermore, the system would be a tool for analysis of IgE epitopes, determination of tertiary structure, allergen engineering for safer and more effective AIT, resolving the relation between the enzymatic activity and pathogenesis, and the development of therapeutic inhibitors.

Keywords: recombinant allergen, mite group 1 allergens, *Dermatophagoides farinae*, cysteine protease, IgE reactivity, *Pichia pastoris*

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Introduction

Standardization of allergen extract used for diagnostic testing and for AIT is important to the safety, efficacy, accuracy, and simplicity in the use of extracts from

different manufacturers or lots, and consequently, the allergen standardization programme has been conducted [1,2]. However, complete quality control of native allergen extracts is difficult because the quality of an extract varies among manufacturers and the composition can change as a result of degradation of component allergens during storage. To solve this problem, efforts have been made to isolate cDNAs for various allergens and to produce

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homogeneous recombinant allergens with the same properties as native ones on a large scale by a recombinant DNA technique [3–5].

House dust mite of *Dermatophagoides* species (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) is the most important causative factor associated with various allergic diseases among all the in-door allergens [6,7]. Group 1 (Der f 1 and Der p 1) and group 2 (Der f 2 and Der p 2) allergens are considered the major house dust mite allergens [8]. We have already established methods to refold a recombinant Der f 2, the group 2 allergen from *D. farinae*, expressed in *Escherichia coli* to achieve full IgE reactivity [9]. The technology has been applied to the analysis of IgE epitopes [10–12], allergen engineering for safer and more effective AIT by mutagenesis of the allergen [12–15], and the determination of the tertiary structure of the allergen by NMR [16]. On the other hand, attempts to prepare recombinant group 1 allergens with the same properties as the native types in expression systems of *E. coli* have failed and the analysis of their IgE epitopes has not been complete [17–19]. Chua *et al.* reported the affinity-purification of recombinant Der p 1 (rDer p 1), which was expressed in yeast *Saccharomyces cerevisiae*, using a monoclonal antibody [20]. Although the rDer p 1 expressed in *S. cerevisiae* bound more frequently to IgE than did that expressed in *E. coli*, it had an additional 28 amino acid residues at the N-terminus and clearly differed from native Der p 1. Therefore, there is a great demand for clinical and research use of recombinant forms of group 1 allergens with the same properties as the native types.

Group 1 allergens, which belong to the papain-like cysteine protease family, are mainly found in mite faeces [8–23]. The cDNAs coding for group 1 allergens and their isoforms have been cloned [24–27]. They have the prosequence and one consensus motif for N-glycosylation. Recently, the cysteine protease activity of the house dust mite group 1 allergens has been proposed to be involved in the pathogenesis of allergy [28–30]. Molecular modelling of Der p 1 on the basis of the tertiary structures of papain, actinidin, and papaya proteinase Ω , of the cysteine protease family was performed [31]. However, the structure of group 1 allergens has not been determined with X-ray crystallography or NMR because biologically active recombinant proteins have not been available in large enough amounts.

Shoji *et al.* and ourselves reported the preparation of recombinant Der f 1 (rDer f 1) that retained the IgE binding activity of native Der f 1 by an expression system using baculovirus infected insect SF9 cells and an *in vitro* activation process [32]. In the present study, we developed a much more efficient system to prepare rDer f 1 molecules in quantity using a methylotrophic yeast *Pichia pastoris* secretion system and demonstrated a mature protease

activity and IgE reactivity of recombinant Der f 1 and a mutant whose consensus motif for N-glycosylation is disrupted.

Methods

Cloning of cDNA coding for Der f 1

The *D. farinae* λ gt11 cDNA library was constructed as described in our previous report [33,34]. Rabbit anti-Der f 1 serum was used for plaque immunoscreening along with the Super Screen Immunoscreening System (Amersham International). The cDNAs of positive clones were sequenced.

Construction of vectors encoding cDNA for wild-type Der f 1 and its mutant

The vector for expression in the methylotrophic yeast *P. pastoris* encoding the pro-Der f 1 fused to a *P. pastoris* PHO1 signal sequence [35] was constructed by insertion of the PCR-amplified cDNA fragment of 1.0kbp generated by digestion with *Xho* I and *Bam* H I to the *Xho* I/*Bam* H I site of pHIL-S1 (Invitrogen, San Diego, CA, USA) containing the *HIS4* gene [36]. The vector for expression in *P. pastoris* encoding for a mutant pro-Der f 1, whose consensus sequence for the N-glycosylation site is disrupted (N53Q), fused to a PHO1 signal sequence, was constructed by site-directed mutagenesis.

Transfection of P. pastoris with vectors encoding cDNA for wild-type Der f 1 or its mutants

The method was as described previously [37]. Briefly, the expression vectors encoding cDNA for wild-type Der f 1 or its mutant N53Q were linearized by digestion with *Bgl* II and introduced to *P. pastoris* GS115 cells by electroporation. Transfectants in which the locus of the genomic alcohol oxidase gene (*AOX1*) was homologously recombined with the linearized fragment containing cDNA for rDer f 1 and the *HIS4* gene were selected by their ability to grow on histidine-deficient agar plates but grow slowly on agar plates containing minimum medium and methanol. Southern hybridization using Der f 1 cDNA as probe was performed to confirm the correct homologous recombination [38].

Expression of the wild-type Der f 1 and its mutant in P. pastoris

The method of expression in *P. pastoris* was as described previously [37] with minor modifications. Briefly, the transfectant of *P. pastoris* GS115 cells was cultured at

30 °C in 5 mL of YPD (1% yeast extract, 2% peptone, and 2% glucose). The overnight culture was inoculated to 500 mL of YPD and cultured until the optical density at 600 nm reached 6–8 (36–48 h). The cells were pelleted by centrifugation, resuspended in 50 mL of BMMY (100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acids, $4 \times 10^{-5}\%$ biotin, 1% methanol), and cultured for an additional 48–72 h with addition of methanol to the culture (1% (v/v)) every 24 h to keep continuous the expression of rDer f 1. The supernatant was harvested by centrifugation.

In vitro activation of the wild-type Der f 1 and its mutant

The supernatant containing each of the rDer f 1 molecules was dialysed 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 dialysed against PBS, whereas the mutant N53Q was dialysed against 20 mM Tris–HCl (pH 8.0), purified by anion exchange column chromatography using DEAE-Sepharose or Q-Sepharose (Pharmacia, Uppsala, Sweden), and then dialysed against PBS. The samples were concentrated to an appropriate concentration with Centricon 10 (Amicon, Beverly, MA, USA).

N-glycanase treatment

N-glycanase treatment of mature wild-type Der f 1, which was prepared by processing of the proform secreted from *P. pastoris*, was performed with the use of a Glycopeptidase F De-N-glycosylation Set (Takara, Japan).

Protein sequencing

The N-terminal amino acid sequences of the rDer f 1 molecules were determined with a 473-A protein sequencer (Applied Biosystems, Foster City, CA, USA).

Immunoblot analysis

Expression and processing of the rDer f 1 molecules were confirmed by SDS-PAGE and immunoblotting with a combination of a mouse anti-Der f 1 peptide monoclonal antibody (mAb), 12F1, and an HRP-conjugated anti-mouse IgG antibody (Ab) (Bio-Rad, Richmond, CA, USA) or with that of a rabbit anti-Der f 1 serum and an HRP-conjugated anti-rabbit (Fab')₂ fragment (Amersham, Arlington Heights, IL) as described [11].

Detection of cysteine protease activity

Cysteine protease activity was measured as described previously [23] with minor modifications. Briefly, samples

pre-incubated with 10 mM cysteine at 37 °C for 10 min were diluted fivefold with 20 mM substrate solution (100 mM phosphate buffer, pH 6.0) and incubated at 37 °C for 30 min. The reaction was stopped by addition of 10% acetic acid solution at twice the volume of the reaction mixture. The fluorescence of aminomethylcoumarine released from substrate was measured at excitation, 380 nm, and emission, 460 nm, on a fluorometer. To obtain an appropriate fluorescence intensity within the measurement limitation of the fluorometer, dilutions were subjected to measurement and the virtual values before the dilution were calculated. Succinyl-Ala-Pro-Ala-Tyr-MCA (Suc-APAY-MCA) (Peptide Institute, Osaka, Japan) or Butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA) (Peptide Institute) was used as substrate.

RAST-EIA

The method was as described previously [10]. Briefly, serum of each allergic patient was incubated with paper discs coupled with each of the purified native and recombinant Der f 1 molecules in various amounts. Then, the IgE that bound to the disc was detected with a β -galactosidase-conjugated anti-human IgE Ab by measuring the absorbance at 420 nm.

Results

Cloning of cDNA encoding for Der f 1

The cDNAs encoding for Der f 1 were cloned by plaque immunoscreening. The sequence of the longest cDNA clone covered the complete open reading frame (ORF) encoding the preproenzyme form of Der f 1. The nucleotide sequence of the ORF of the clone was identical with that previously reported by Dilworth *et al.* [25] except for six bases (codons for amino acid residues, 41, 103, 145, 151, 184 and 198). 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). The amino acid residues at positions of 103 and 184 were glutamine and valine, respectively, in our isoform, whereas they were arginine and asparagine in that reported by Dilworth *et al.* The nucleotide and deduced amino acid sequences for mRNA for preproenzyme of Der f 1 have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB034946.

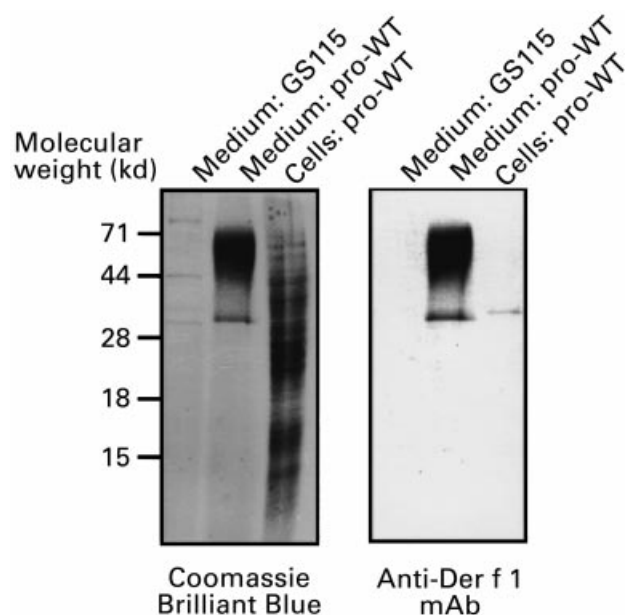


Fig. 1. Expression of the proform of wild-type rDer f 1 in methylotrophic yeast *Pichia pastoris*. Culture supernatant of *P. pastoris* GS115 cells (Medium: GS115), that of GS115 cells transfected with the expression vector of the proform of wild-type rDer f 1 (Medium: pro-WT), or cellular proteins of the transfectant (Cells: pro-WT) were subjected to SDS-PAGE (16% acrylamide) and electroblotted. The membranes were stained with Coomassie Brilliant Blue, or probed with murine anti-Der f 1 monoclonal antibody 12F1.

Expression of wild-type Der f 1 in methylotrophic yeast *P. pastoris*

The proform of wild-type Der f 1 was expressed as a fused form with a signal sequence of *P. pastoris* alkaline phosphatase (PHO1). As shown in Fig. 1, proteins for a smear band of approximately 45–70 kD and a band of approximately 30 kD, which were recognized with anti-Der f 1 mAb, were secreted into culture medium efficiently. On the other hand, much less protein reactive to the mAb was accumulated in the cells than secreted into medium.

In vitro processing of wild-type Der f 1 and its mutant whose consensus sequence for N-glycosylation is disrupted (N53Q)

By treatment of the supernatant in acidic conditions, the bands for the proform of wild-type Der f 1 (Fig. 2a, pro-WT) were shifted to a lower molecular weight (Fig. 2a, WT) on SDS-PAGE. The N-terminal sequences of the secreted proteins were determined to start from –59 or –78, downstream from the putative signal-cleavage site in the prosequence of Der f 1. Those of processed proteins were determined to start from –2 or 1, two-residues

upstream from or at the N-terminus of native Der f 1 (Fig. 3). By treatment with N-glycanase for digestion of asparagine-linked oligosaccharide, the bands for the acid-treated protein were shifted to a mobility near that for native Der f 1 (Fig. 2a, WT (De-N-glycosylated)).

As the wild-type rDer f 1 expressed in *P. pastoris* was more highly glycosylated than native Der f 1, we generated a variant of Der f 1 with a mutation of an asparagine in the consensus motif for N-glycosylation at the 53rd amino acid residue to a glutamine (N53Q). The proform of N53Q expressed in *P. pastoris* (Fig. 2b, pro-N53Q) was efficiently secreted into culture supernatant and processed to be of almost the same molecular weight as native Der f 1 by treatment in acidic conditions (Fig. 2b, N53Q). The N-terminal sequences of the proform of N53Q were determined to start from –59 or –78. Those of the processed N53Q were determined to start from –2 or 1 (Fig. 3).

The concentration of the proforms secreted into culture was 100–200 mg/L, and 25–50 mg of the mature forms was recovered from 1 L of culture.

Cysteine protease activities of wild-type Der f 1 and N53Q expressed in *P. pastoris*

Treatment in acidic conditions and removal of the prosequence resulted in matured cysteine protease activities of the two processed rDer f 1 molecules, wild-type and N53Q (Fig. 4). However, those of the proenzymes were weak and immature.

IgE-binding activity of wild-type Der f 1 and N53Q

Patient IgE-binding capacities of rDer f 1 molecules with or without the prosequence were determined by RAST-EIA. The processed rDer f 1 and N53Q showed the same IgE binding activity as native Der f 1 (Fig. 5). On the other hand, the IgE-binding capacity of the proenzymes was much less than that of the native and processed rDer f 1 molecules.

Discussion

We cloned a cDNA for a complete preproform of a new isoform of Der f 1, the major house dust mite group 1 allergen from *D. farinae*. The differences between our isoform and that reported by Dilworth *et al.* in the cDNA and amino acid sequences are not unexpected because allelic polymorphism is a common feature of the house dust mite group 1 allergens [26,27]. The system to produce biologically active rDer f 1 molecules includes expression in a yeast secretion system and *in vitro* maturation of the inactive prosequence-associated rDer f 1 molecules to prosequence-removed biologically active forms.

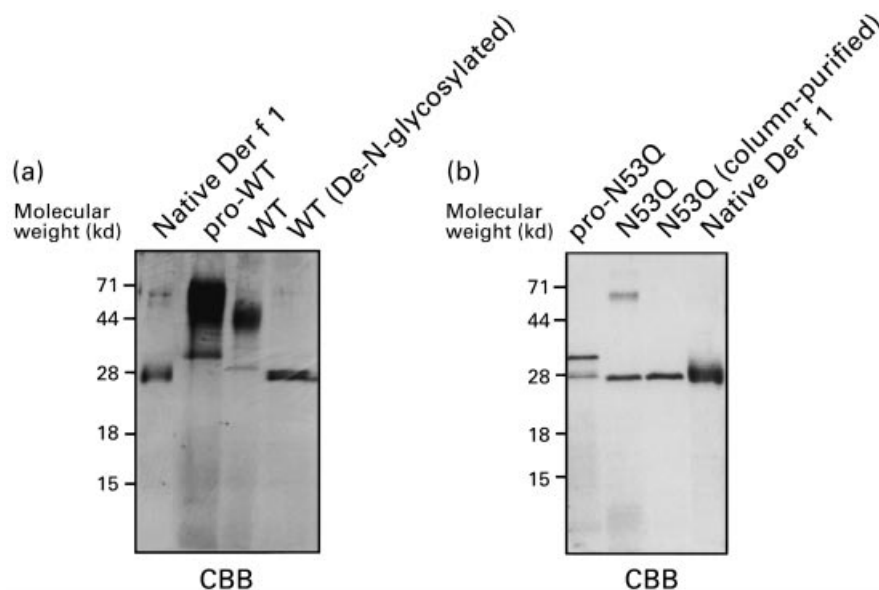


Fig. 2. (a) *In vitro* processing of wild-type rDer f 1. Native Der f 1 purified from mite extract (Native Der f 1), culture supernatant of *P. pastoris* GS115 cells transfected with the expression vector of the proform of wild-type rDer f 1 (pro-WT), culture supernatant after activation by dialysis against acidic buffer (WT), and WT deglycosylated with N-glycosydase (WT [De-N-glycosylated]), were subjected to SDS-PAGE (16% acrylamide) and stained with Coomassie Brilliant Blue. (b) *In vitro* processing of a mutant Der f 1 whose consensus sequence for N-glycosylation is disrupted (N53Q). Culture supernatant of *P. pastoris* GS115 transfected with the expression vector of the mutant (pro-N53Q), that after activation by dialysis against acidic buffer (N53Q), N53Q purified with an anion exchange column (N53Q (column-purified)), and native Der f 1 purified from mite extract (Native Der f 1), were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

The wild-type rDer f 1 expressed in *P. pastoris* was more highly glycosylated and of higher molecular weight than native Der f 1 (Fig. 2a). To obtain another rDer f 1 closer to native Der f 1 in molecular weight, we generated a mutant Der f 1 whose consensus sequence for N-glycosylation is disrupted. This mutant, N53Q, expressed in *P. pastoris*, is closer in molecular weight on SDS-PAGE to native Der f 1 (Fig. 2b). In *P. pastoris*, the presence or absence of the oligosaccharide connected to Asn53 did not affect the processing (Fig. 2 and Fig. 3) or the restoration of enzymatic activity and IgE reactivity (Fig. 4 and Fig. 5).

The prosequence of protease is considered to help correct folding of the precursor suitable for the mature folding of the prosequence-removed active protease, and to keep the protease inactive until transfer and exposure to the sites or circumstances where the protease should be sorted and function. The house dust mite major group 1 allergens, Der f 1 and Der p 1, are mainly found in mite faeces and considered digestive enzymes in mites [8,22,39]. The rDer f 1s were secreted into culture medium after the cleavage of the *P. pastoris* PHO1 signal sequence. After autocatalytic removal of the prosequence in acidic conditions, the cysteine protease activity and reactivity to IgE were restored simultaneously (Fig. 4 and Fig. 5). We concluded that removal of the prosequence under appropriate conditions

was essential for activation of rDer f 1 molecules and that after the process, rDer f 1 molecules folded to become the structural equivalent of native Der f 1, biologically active in terms of both showing enzymatic activity and being recognized by IgE. We assume that the presence of the prosequence is essential for the proenzyme to fold to an intermediate structure competent to conduct the mature folding, i.e. the prosequence functions as an 'intramolecular chaperone' [40].

IgE epitopes of group 1 allergens are highly dependent on the tertiary structure of the allergen [41]. Moreover, the conformational IgE epitopes are denatured irreversibly by heating, pH-treatment, treatment with 6 M guanidine, or reduction and alkylation [39–42]. We assume that without the prosequence, the denatured structure of the group 1 allergens is at a more stable energy state than the correctly folded native structure. Chua *et al.* reported that rDer p 1 expressed in *S. cerevisiae* was not secreted into medium but accumulated in cells in an insoluble form [20]. The insoluble fraction was solubilized with urea and rDer p 1 was affinity-purified from the urea-denatured fraction with a mAb. The purified rDer p 1 showed higher-frequency binding of IgE than did that expressed in *E. coli*. We assume that the folded rDer p 1 fraction might have been concentrated by the affinity

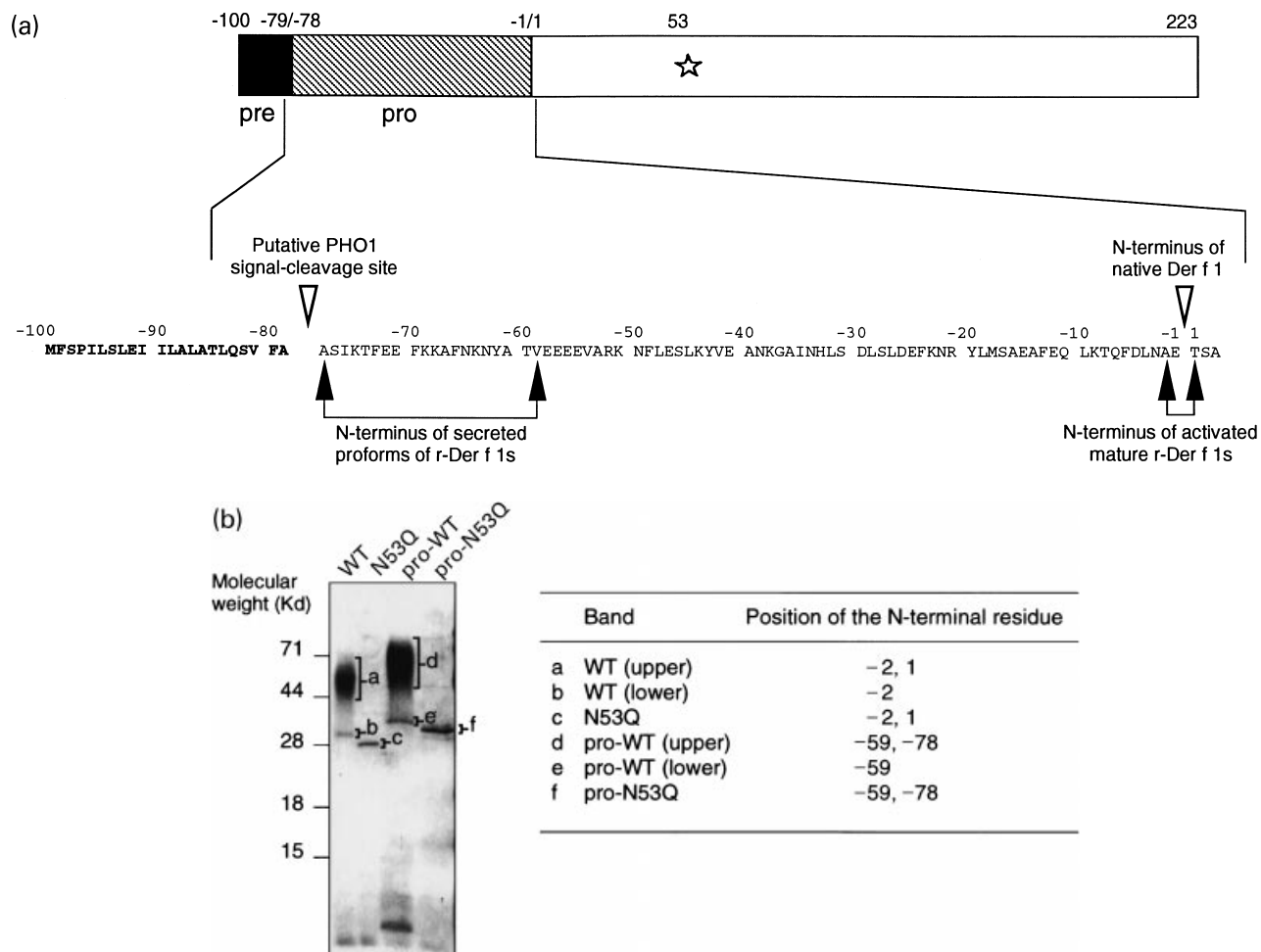


Fig. 3. N-terminal amino acid residues of secreted proforms and mature forms of wild-type rDer f 1 and its mutant N53Q. (a) Schematic representation of N-terminal amino acid residues of secreted proforms and mature forms of rDer f 1 molecules. The amino acid sequence of *P. pastoris* PHO1 is typed in bold [35]. (■) represents the signal sequence of PHO1; (▨) represents the putative prosequence on Der f 1; (□) shows the mature sequence of Der f 1 and (☆) signifies the N-glycosylation motif. (b) The bands on SDS-PAGE used for determination of N-terminal amino acid residues of secreted proforms (pro-WT and pro-N53Q) and mature forms (WT and N53Q) of the rDer f 1 molecules.

purification. However, it had additional 28 amino acid residues at the N-terminus and clearly differed from the native Der p 1. The protease activity of rDer p 1 was not confirmed. Therefore, the development of biologically active rDer f 1 molecules in the present study is an important step in improving the standardization of mite allergen extract using recombinant allergen. Furthermore, this expression and maturation system achieved a very high efficiency of production (proforms: 100–200 mg/L, mature forms; 25–50 mg/L) and would be suitable for production on a large scale. The rDer f 1 molecules, which are assisted in folding by the prosequence, would be useful also for epitope analysis, allergen engineering [4,12,43,44] for improved AIT, and determination of tertiary structure by X-ray crystallography or NMR [16,45].

The cysteine protease reactivity of Der p 1 appears not only to facilitate passage of its own and other allergens across the epithelium, gain access to immune cells including T cells and B cells, and modulate their function, but also to subvert the IgE network and the balance of Th1/Th2 through cleaving CD23 on B cells and CD25 on T cells [28–30]. If this is true, inhibition of the cysteine protease activity of the house dust mite group 1 allergens might be a novel and reasonable strategy for therapy of allergic diseases as proposed by Shakib *et al.* [28]. Therefore, rDer f 1 with cysteine protease activity would be useful for both analysis of the relation of the enzymatic activity of allergen with the pathogenesis of allergy, development of therapeutic inhibitors, and allergen engineering targeting the specific protease activity of group 1 allergens.

Recombinant forms of group 1 allergens with the same

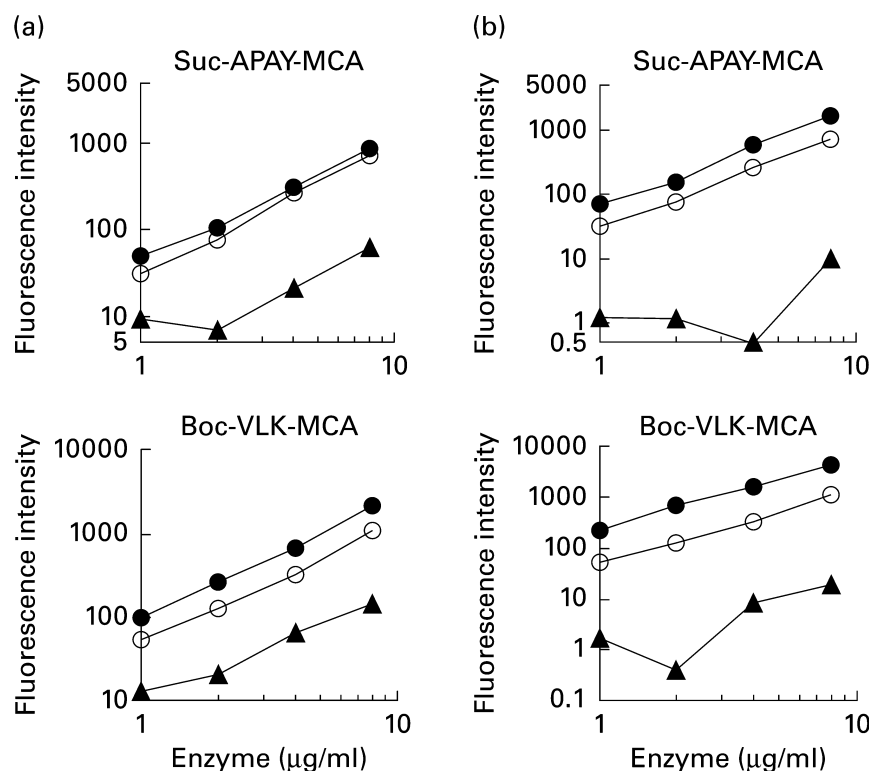


Fig. 4. Cysteine protease activities of recombinant wild-type rDer f 1 and N53Q, and their proforms expressed in *Pichia pastoris*. (a) Comparison of cysteine protease activities of wild-type rDer f 1 (●), its precursor (▲) and native Der f 1 (○). (b) Comparison of cysteine protease activities of N53Q (●), its precursor (▲) and native Der f 1 (○). Suc-APAY-MCA and Boc-VLK-MCA were used as substrate. The values calculated by subtracting the background of the substrate from the original values are shown.

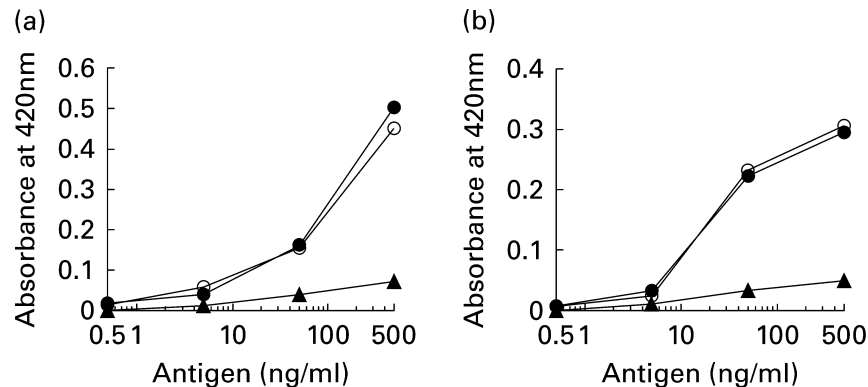


Fig. 5. IgE binding activity of recombinant wild-type Der f 1, N53Q, and their proforms analysed by RAST-EIA. (a) IgE binding activity of wild-type Der f 1 and its proform. Native Der f 1 (○), wild-type rDer f 1 expressed in *P. pastoris* (●), and its precursor (▲) were subjected to RAST-EIA. (b) IgE binding activity of N53Q and its proform. Native Der f 1 (○), N53Q expressed in *P. pastoris* (●), and its precursor (▲), were subjected to RAST-EIA.

properties as the native types will be the basis of various studies. The activated rDer f 1 molecules in this study are considered to be folded and biologically functional. This system would be applicable also to prepare an active recombinant form of Der p 1, another major house dust mite group 1 allergen from *D. pteronyssinus*. IgE-binding to the rDer f 1 molecules in a larger patient population and precise analysis of the protease activity should be addressed in further study.

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