

Effects of Site-Directed Mutagenesis in the Cysteine Residues and the N-Glycosylation Motif in Recombinant Der f 1 on Secretion and Protease Activity

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Key Words

House dust mite · Recombinant Der f 1 · Site-directed mutagenesis · Secretion · Maturation · Cysteine protease · Disulfide bond · N-glycosylation · *Pichia pastoris* · Insect SF9 cell

Abstract

Background: The group 1 allergens from mite feces, which belong to the papain-like cysteine protease family, are the most significant in-door allergens. In this study, we analyzed the contribution of the cysteine residues and N-glycosylation in Der f 1, the group 1 allergen from *Dermatophagoides farinae*, to secretion and maturation by using systems for expression of recombinant Der f 1 (rDer f 1). **Methods:** The rDer f 1 and its mutants were expressed in yeast *Pichia pastoris* and insect SF9 cells. Secretion of their proforms was checked by SDS-PAGE or immunoblotting. Protease activities of the secreted proform of a mutant and the mature form were compared with that of native Der f 1. **Results:** The proform of a mutant Der f 1, pro-N53Q, whose consensus motif for N-glycosylation was disrupted, was not secreted in insect SF9 cells although secreted in *P. pastoris*. Indirect evidence was obtained to support the disulfide bond formation between Cys4 and Cys118, which were not conserved in papain. A mutant for Cys35 in the catalytic site

of the cysteine protease, pro-C35S/N53Q, was secreted, but the other mutants for cysteines concerning intramolecular disulfide bonds were not secreted in *P. pastoris*. The prosequence of pro-C35S/N53Q was removed by an in vitro activation process. The mature C35S/N53Q showed low protease activity. **Conclusion:** N-glycosylation is essential for secretion in insect SF9 cells but not in *P. pastoris*. Disulfide bonds are essential for secretion in *P. pastoris*. A mutation in the catalytic site, C35S, is not completely critical to removal of the prosequence and protease activity. The findings are useful for future design of recombinant products for application in immunotherapy.

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Introduction

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 [1–3]. Group 1 major allergens of house dust mites, Der f 1 from *D. farinae* and Der p 1 from *D. pteronyssinus*, 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 [4–

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6]. The cDNAs coding for group 1 allergens and their isoforms have been cloned following the cloning of cDNA coding for Der p 1 [7–11]. Group 1 allergens have the pro-sequence, one consensus motif for N-glycosylation and seven cysteine residues: one in the catalytic site and six that might form three intramolecular disulfide bonds.

Recently, the cysteine protease activity of the house dust mite group 1 allergens has been proposed to be involved in the pathogenesis of allergy [12–19]. The proteolytic activity of Der p 1 increases the permeability of the bronchial mucosa, causes disruption of intercellular tight junctions and the epithelial structure, and cleaves α_1 -antitrypsin, which provides innate immunity on mucosal surfaces [13–16]. This suggests that Der p 1 would facilitate the passage of its own and other allergens across the epithelium and gain access to immune cells including T cells and B cells, and modulate their function in allergen presentation, inflammatory cell activation, and chronic tissue injury. Furthermore, the cysteine protease activity has been reported to affect adaptive immunity. Der p 1 cleaves the low-affinity IgE receptor (CD23 or Fc ϵ RII) and the α -subunit of the IL-2 receptor (CD25 or IL-2R) from the cell surface [12, 16–18]. Der p 1 might upregulate IgE synthesis by disrupting the negative feedback signal that was thought to be delivered on the binding of IgE to membrane CD23 on B cells, and bias T cells towards the emergence of a proallergic Th2 by cleavage of CD25 which plays a pivotal role in the development and propagation of Th1 cells. Comoy et al. [19] reported that Th2-type responses to an immunogen were promoted by coimmunization of Der p 1 which retained the cysteine protease activity but not by that of heat-inactivated Der p 1 in mice. Thus, the cysteine protease activity of house dust mite group 1 allergens is considered to have an intrinsic ability to subvert the regulatory process controlling IgE synthesis.

We have cloned cDNA for an isoform of Der f 1 [11] and established systems for expression of recombinant Der f 1 (rDer f 1) in yeast [20], bacteria [21], and insect cells [22]. These rDer f 1 molecules would be useful for diagnosis and allergen-specific immunotherapy (AIT) for house dust mite allergy. Furthermore, mutants of Der f 1 prepared using these systems would be useful for analysis of IgE epitopes and might be applicable for safer and more effective AIT as well as those of Der f 2, the group 2 major allergen from *D. farinae* [23–28]. In this study, we analyzed the effects of mutations to the cysteine residues and the N-glycosylation motif of Der f 1 on secretion and maturation of the allergen using the systems for expression in yeast and insect cells.

Materials and Methods

S-Pyridyl Ethylation of Native Der f 1

Der f 1 was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and electroblotted onto a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue. The band of Der f 1 was scissored out and wetted with methanol. The membrane was soaked in 200 μ l of the reaction buffer (8 M guanidine-HCl, 0.5 M Tris, 0.3% EDTA, 5% acetonitrile) and 1.5 μ l of 4-vinyl-pyridine was added to the tube. The tube was agitated gently for 20 min shielding the light. The membrane collected from the tube was incubated with agitation for 5 min in 100 ml of H₂O, for 5 min in 100 ml of 2% acetonitrile, and then for 5 min in 100 ml of 0.1% SDS. Finally, the membrane was incubated with agitation in 10% acetonitrile for 15 min, dried and subjected to N-terminal amino acid sequencing by a 473A protein sequencer (Applied Biosystems, Foster City, Calif., USA).

Construction of Vectors Encoding cDNA for rDer f 1 Molecules

Construction of the vectors for expression of wild-type Der f 1 and its mutant N53Q in the methylotrophic yeast *Pichia pastoris* encoding the pro-Der f 1 molecules fused to a PHO1 signal sequence were described previously [20]. The vectors for expression of cysteine mutants of N53Q (C4S/N53Q, C32S/N53Q, C35S/N53Q, C66S/N53Q, C72S/N53Q, C104S/N53Q, C118S/N53Q, C32/72S/N53Q, and C66/72S/N53Q) were constructed by site-directed mutagenesis of the vector for pro-N53Q. The vector for expression in insect SF9 cells encoding for pro-N53Q was constructed by site-directed mutagenesis of a vector for pro-Der f 1 described previously [22]. The DNA sequences were confirmed using a 373A DNA sequencer (Applied Biosystems).

Transfection of P. pastoris with Vectors Encoding cDNA for rDer f 1 Molecules

The method was as described previously [20]. Briefly, the expression vectors 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 the rDer f 1 and a *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. PCR using genomic DNA prepared from the transfectant cells as templates was performed to confirm introduction of the expression vectors to the yeast chromosome.

Expression of rDer f 1 Molecules in P. pastoris and in Insect SF9 Cells

The method of expression in *P. pastoris* was as described previously [20]. The method of expression in insect SF9 cells was as described previously [22].

In vitro Activation of a Mutant Der f 1, C35S/N53Q

The method was as described previously [20]. The supernatant containing pro-C35S/N53Q was dialyzed against 100 mM acetate buffer (pH 4.0) at 4°C for 48 h. After this activation process, the mutant Der f 1, C35S/N53Q, was dialyzed against 20 mM Tris-Cl (pH 8.0), purified by anion exchange column chromatography using DEAE-Sepharose or Q-Sepharose (Pharmacia, Uppsala, Sweden), and then dialyzed against PBS. The sample was concentrated to an

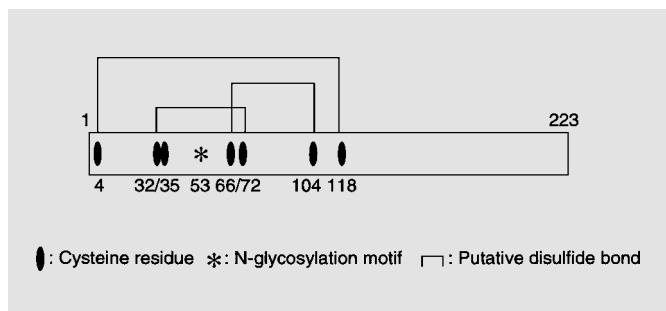


Fig. 1. Schematic representation of native Der f 1. The N-glycosylation motif, cysteine residue in the catalytic site, and three putative intramolecular disulfide bonds are shown.

appropriate concentration with Centricon 10 (Amicon, Beverly, Mass., USA).

Immunoblot Analysis

Secretion of the wild-type Der f 1 and N53Q in the expression system of insect cells was confirmed by SDS-PAGE and immunoblotting with a combination of a rabbit anti-Der f 1 serum and an HRP-conjugated anti-rabbit (Fab')₂ fragment (Amersham, Arlington Heights, Ill., USA) as described [20, 29].

Detection of Cysteine Protease Activity

Cysteine protease activity was measured as described previously [20, 21]. Butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA) (Peptide Institute, Osaka, Japan) was used as substrates.

Results

Intramolecular Disulfide Bonds in Native Der f 1

Given that five cysteine residues, 32, 35, 66, 72 and 104 (numbering in Der f 1), were conserved between Der f 1 and papain, we consider that Cys35 in Der f 1 is in the catalytic site and two intramolecular disulfide bonds are formed between Cys32 and Cys72 and between Cys66 and Cys104 in Der f 1 as well as in papain [8, 30]. The other two cysteine residues of Cys4 and Cys118 are not conserved in papain. Although we tried to digest native Der f 1 with several proteases to confirm whether these two cysteine residues form a disulfide bond, the efficiency of digestion was too low to perform peptide mapping to determine the location of disulfide bonds. Then, another method described in Materials and Methods was applied. Native Der f 1, whose free cysteine residue(s) was labeled with 4-vinyl pyridine, was subjected to peptide sequencing of the N-terminal residues. No significant peak of the labeled cysteine was observed at the fourth cycle of Edman digestion (data not shown). This suggests that

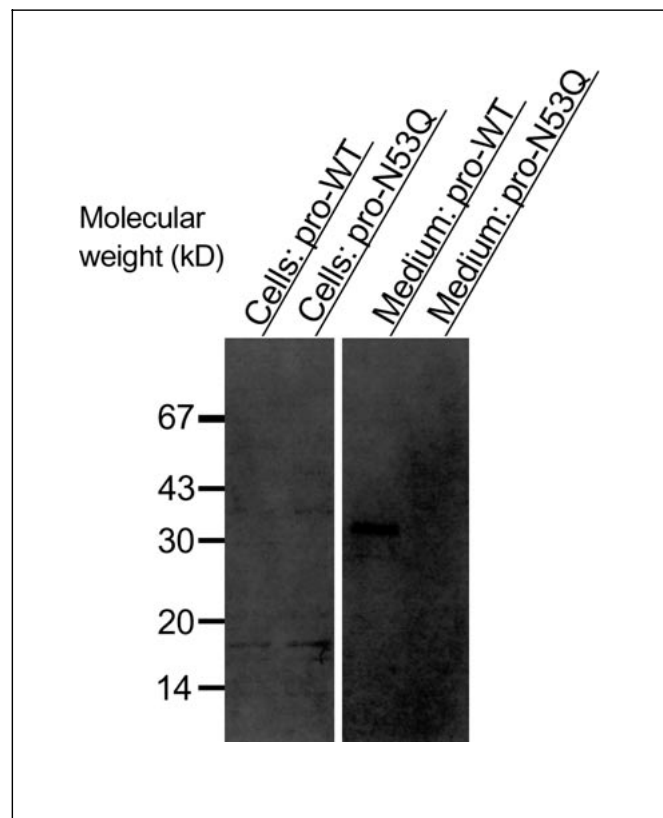


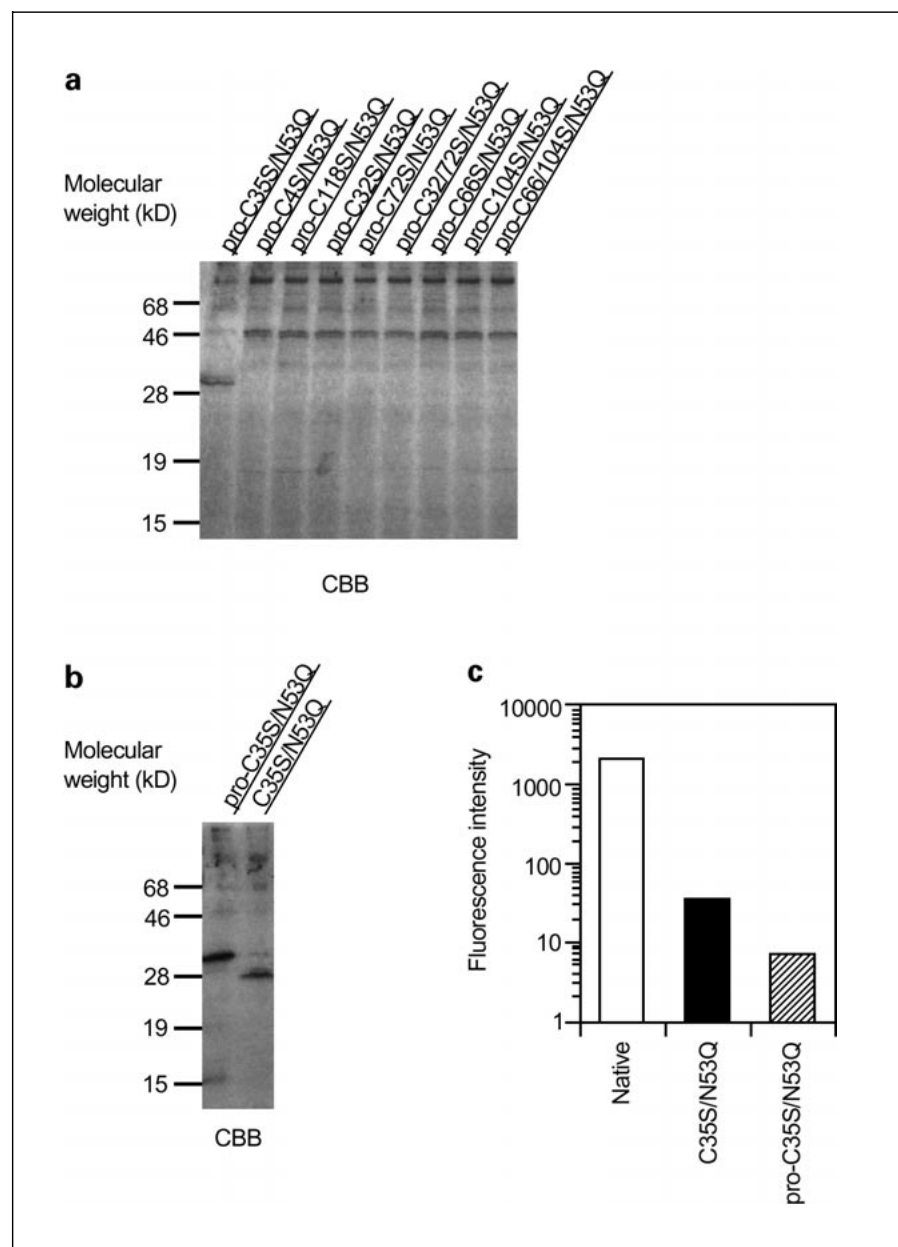
Fig. 2. Secretion of proforms of wild-type Der f 1 and N53Q expressed in insect SF9 cells. Total cellular proteins of SF9 cells transfected with the expression vector of the proform of wild-type Der f 1 (Cells: pro-WT), those transfected with the expression vector of the proform of N53Q (Cells: pro-N53Q), proteins secreted into culture medium from SF9 cells transfected with the expression vector of the proform of wild-type Der f 1 (Medium: pro-WT), and those transfected with the expression vector of the proform of N53Q (Medium: pro-N53Q) were subjected to SDS-PAGE (16% acrylamide) and electroblotted. The membranes were probed with rabbit anti-Der f 1 serum.

Cys4 in the native Der f 1 was free. Therefore, we assumed that the third intramolecular disulfide bond was formed between Cys4 and Cys118 (fig. 1).

Expression of Wild-Type Der f 1 and N53Q in Insect SF9 Cells

Secretion of proforms of wild-type Der f 1 and N53Q expressed in insect SF9 cells was checked by immunoblot analysis of the culture supernatant. Wild-type pro-Der f 1 was secreted to culture medium of baculovirus-transfected insect SF9 cells, but pro-N53Q was not (fig. 2).

Fig. 3. Secretion of proforms of cysteine mutants of Der f 1 expressed in *P. pastoris* and in vitro activation of a cysteine mutant, C35S/N53Q, in which the cysteine residue in the catalytic site of the enzyme was disrupted. **a** Secretion of proforms of nine cysteine mutants of N53Q (pro-C35S/N53Q, pro-C4S/N53Q, pro-C118S/N53Q, pro-C32S/N53Q, pro-C72S/N53Q, pro-C32/72S/N53Q, pro-C66S/N53Q, pro-C104S/N53Q, and pro-C66/104S/N53Q) expressed in *P. pastoris*. Culture supernatant of *P. pastoris* GS115 cells transfected with the expression vectors was subjected to SDS-PAGE (16% acrylamide) and stained with Coomassie Brilliant Blue. **b** In vitro processing of a cysteine mutant of N53Q, C35S/N53Q. Culture supernatant of *P. pastoris* GS115 cells transfected with the expression vector of the proform of C35S/N53Q (pro-C35S/N53Q) and that after activation by dialysis against acidic buffer (C35S/N53Q) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. **c** Comparison of protease activities of native Der f 1 (Native), the mature C35S/N53Q, and its precursor (pro-C35S/N53Q). Boc-VLK-MCA was used as a substrate. The values calculated by subtracting the background of the substrate from the original values are shown. The values are averages of duplicated experiments. The concentration of the proteins was 16 µg/ml.



Expression of Nine Cysteine Mutants of N53Q in P. pastoris and in vitro Activation of a Mutant C35S/N53Q

Secretion of proforms of cysteine mutants of Der f 1 expressed in yeast *P. pastoris* was checked by SDS-PAGE analysis of the culture supernatants. A proform of C35S/N53Q (apparent molecular weight: 35 kD) was efficiently secreted into culture medium, but those of the other eight cysteine mutants, C4S/N53Q, C32S/N53Q, C66S/N53Q, C72S/N53Q, C104S/N53Q, C118S/N53Q, C32/72S/

N53Q, and C66/104S/N53Q, were not (fig. 3a). No band was detected with an anti-Der f 1 mAb, 12F1, in immunoblot analysis of the supernatants of transfectants with vectors for the cysteine mutants except pro-C35S/N53Q (data not shown). The secreted pro-C35S/N53Q was converted to the mature form (apparent molecular weight: 28 kD) by treatment in acidic conditions (fig. 3b). Cysteine protease activity of the processed C35S/N53Q was lower than that of native Der f 1 but significantly higher than that of the proenzyme (fig. 3c).

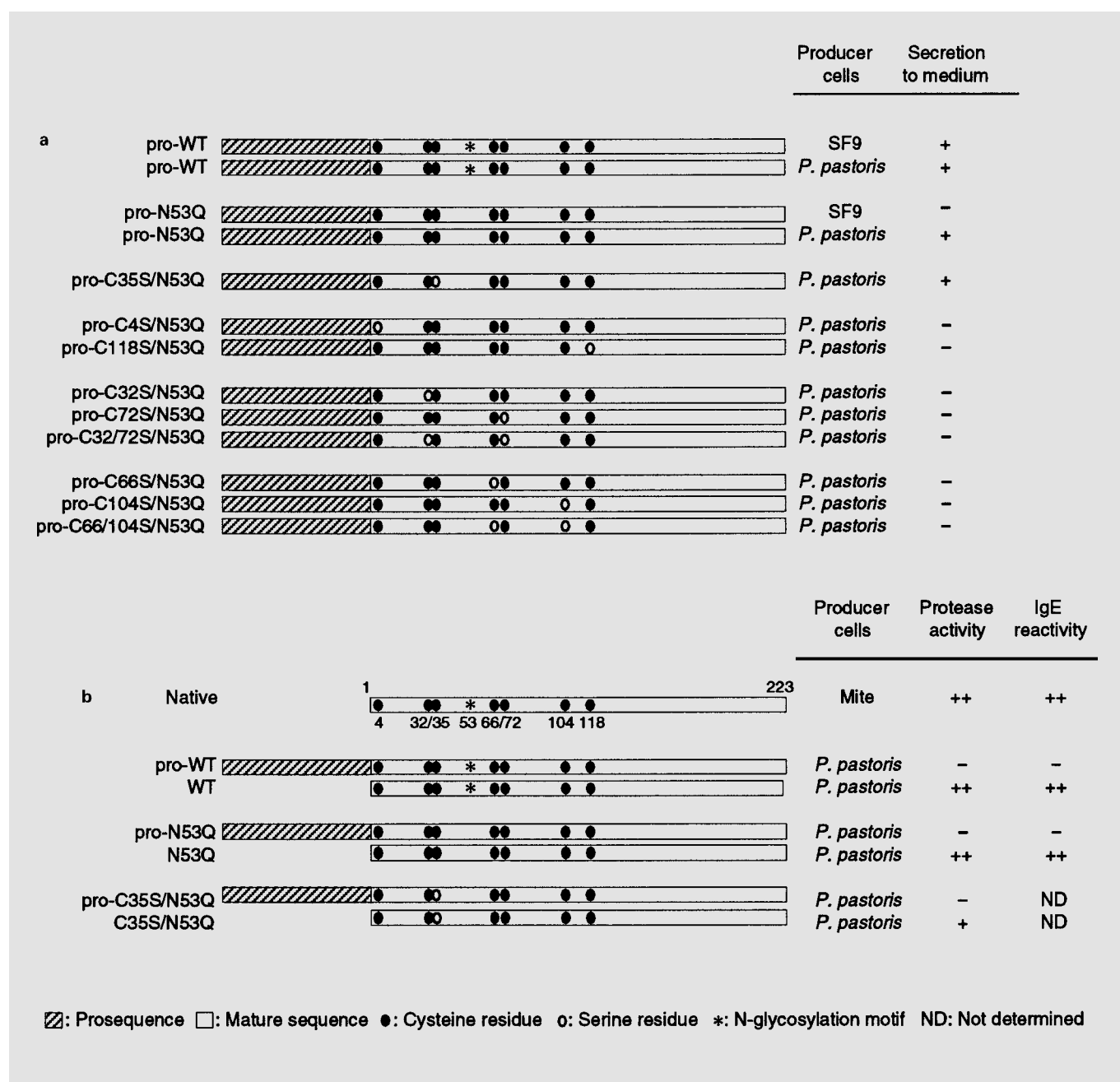


Fig. 4. Schematic representation of variants of Der f 1. The results in figure 2, figure 3, and a previous report [20] are summarized here. **a** Secretion of variants of Der f 1 to culture medium. Proforms of wild-type r-Der f 1 (pro-WT), N53Q (pro-N53Q), and 9 cysteine mutants of N53Q (pro-C35S/N53Q, pro-C4S/N53Q, pro-C118S/N53Q, pro-C32S/N53Q, pro-C72S/N53Q, pro-C32/72S/N53Q, pro-C66S/N53Q, pro-C104S/N53Q, and pro-C66/104S/N53Q) are shown. + = Secreted; - = not secreted. **b** Protease activity and IgE

reactivity of variants of Der f 1. Native Der f 1 purified from mite extract (Native) and r Der f 1 molecules expressed in *P. pastoris*: pro-form of wild-type rDer f 1 secreted to the medium (pro-WT), wild-type rDer f 1 matured by treatment in acidic conditions (WT), pro-form of N53Q secreted to the medium (pro-N53Q), and N53Q matured by treatment in acidic conditions (N53Q), are shown. ++ = Mature activity; + = low activity; - = immature activity.

Discussion

Recently, we reported that the proforms of the wild-type rDer f 1 and N53Q expressed in *P. pastoris* were secreted into culture medium after the cleavage of the signal sequence and that the protease activity and reactivity to IgE of the wild-type and N53Q were restored after autocatalytic removal of the prosequences by an in vitro activation process [20]. The results in the previous report [20] and this study (fig. 2, 3) are summarized in figure 4a concerning secretion and figure 4b concerning activation.

Native Der f 1 is glycosylated [31]. rDer f 1 molecules expressed in yeast and insect cells are considered to have the glycochain whose structure is different from that of native Der f 1. Actually, rDer f 1 expressed in *P. pastoris* showed much larger apparent molecular weight on SDS-PAGE than native Der f 1 because of modification by N-glycosylation [20]. Therefore, a mutant without the glycochain, N53Q, would be useful for allergen standardization for diagnosis and AIT [20]. On the other hand, N-glycosylation was essential for secretion of pro-Der f 1 in insect SF9 cells (fig. 2, 4a). pro-N53Q in insoluble and soluble fractions of extract of the transfected cells was detected by immunoblot analysis (data not shown). The N-glycosylation motif is conserved among house dust mite group 1 allergens [8–11]. N-glycosylation of Asn53 might contribute to secretion in mite cells as it did in insect SF9 cells.

Native Der f 1 had one cysteine residue in the catalytic site (Cys35) and three putative intramolecular disulfide bonds (Cys4-Cys118, Cys32-Cys72, and Cys66-Cys104) (fig. 1). Cys4 and Cys118 are assumed to form a disulfide bond based on molecular modeling [30] and the result in

this study. These intramolecular disulfide bonds could be targets to disrupt the allergenic structure in allergen engineering as well as those in Der f 2 [24, 26, 27, 32]. The catalytic site, Cys35, could be a target to control the protease activity, which is suspected to be related to the pathogenesis of allergy [12–19]. Mutants in which any of the disulfide bond was disrupted were not secreted into culture medium of *P. pastoris* (fig. 3a, 4a). This suggests that intramolecular disulfide bonds are essential for secretion without degradation. The disulfide bond might contribute to the folding of the proform of Der f 1 in endoplasmic reticulum or during transfer. Removal of prosequence of pro-C35S/N53Q and low protease activity of the mature C35S/N53Q are interesting results (fig. 3b, c, 4b). The catalytic mechanism of cysteine protease shares many common features with serine protease [33, 34]. C35S/N53Q might adopt the mechanism of catalysis of serine protease but have low activity because of structural differences between the active sites of the two proteases.

In this study, we analyzed the roles of the cysteine residues and N-glycosylation by using recombinant variants of Der f 1. The findings of this study are useful for future design of recombinant products for application in AIT.

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