

Glycosylation of Recombinant Proforms of Major House Dust Mite Allergens Der p 1 and Der f 1 Decelerates the Speed of Maturation

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Key Words

Recombinant major house dust mite group 1 allergen · Cysteine protease · N-glycosylation · Prosequence · Maturation speed

Abstract

Background: The efficient manufacture of recombinant Der p 1 and Der f 1 has been an important bottleneck in the study of house dust mite allergies and the development of applications for allergen engineering. While Der f 1 has only one N-glycosylation motif in the mature sequence, Der p 1 has two motifs, one in the prosequence and the other in the mature sequence. To test whether inefficient maturation of a recombinant Pro-Der p 1 versus Pro-Der f 1 is due to N-glycosylation, the maturation speed of N-glycosylation motif mutants was compared. **Methods:** Expression vectors for the mutants, in which the motif in the Der p 1 prodomain was disrupted or a motif was created within the Der f 1 prodomain, were constructed by site-directed mutagenesis of preproforms with or without the motif within the mature portion. Culture supernatants of yeast *Pichia pastoris* transfectant cells containing proforms were buffer exchanged by gel filtration and incubated for maturation. Samples

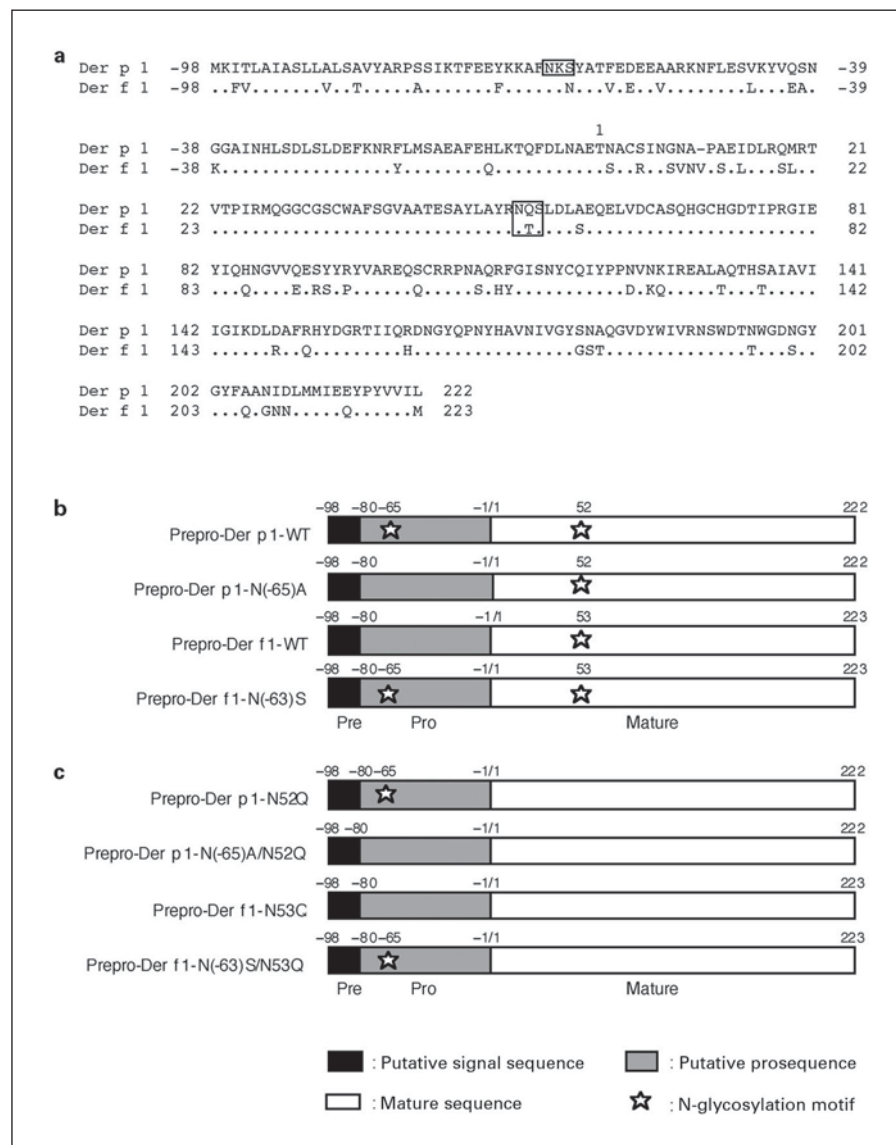
from the reactions were collected every 20 min and subjected to electrophoresis. The maturation speed was compared based on the band densities of the pro- and mature forms. **Results:** Disruption of the motif in the mature portion decreased the productivity and accelerated the maturation. Maturation was also accelerated by disruption of the other motif in the Der p 1 prodomain and slowed down by introduction of the motif into the Der f 1 prodomain. **Conclusions:** Maturation systems using Pro-Der p 1 without the prodomain glycosylation are useful for the efficient preparation of a recombinant mature allergen. In addition, these results demonstrated that the maturation of cysteine protease could be controlled through glycosylation of the prodomain.

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Introduction

House dust mites of two species, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, are a major source of allergens associated with allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis [1]. Group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) allergens derived from house dust mites are ma-

Fig. 1. Schematic representation of preproforms of recombinant Der p 1 and Der f 1 expressed in *P. pastoris*. **a** Amino acid sequences of preproforms of Der p 1 [30] and Der f 1 [31]. N-glycosylation motifs (N-X-S/T) are boxed. **b** Recombinant preproforms with an N-glycosylation motif within the mature sequence. **c** Recombinant preproforms in which the N-glycosylation motif within the mature sequence was disrupted. WT = Wild-type sequence; N(-65)A = a mutation that disrupted the N-glycosylation motif within the prosequence of Der p 1; N(-63)S = a mutation that created an N-glycosylation motif within the prosequence of Der f 1; N52Q and N53Q = mutations that disrupted the N-glycosylation motif within the mature sequences of Der p 1 and Der f 1, respectively; Pre = signal peptide; Pro = propeptide; Mature = mature form.



major allergens in terms of the frequency of patients sensitized, the amount of specific immunoglobulin E (IgE), and levels in mite extract [2]. Group 1 allergens, Der p 1 and Der f 1, belong to the papain-like cysteine protease family, and their cysteine protease activity has been suggested to be involved in the pathogenesis of allergies [3–11].

The cDNAs of Der p 1 and Der f 1 encode signal peptides of 18 amino acid residues responsible for secretion, propeptides of 80 residues, and a mature sequence of 222 residues in Der p 1 and 223 residues in Der f 1 [12, 13]. Mature Der p 1 and Der f 1 have 82% sequence identity with each other, and while Der f 1 has only one N-glyco-

sylation motif in the mature sequence, Der p 1 has two motifs, one in the prosequence and the other in the mature sequence (fig. 1a). There is some controversy about whether the natural allergens are glycosylated [14, 15].

The efficient manufacture of recombinant Der p 1 and Der f 1 has been an important bottleneck in the study of house dust mite allergies and the development of applications for allergen engineering. Some researchers reported no conversion from precursor forms of Der p 1 to mature forms in insect and yeast expression systems without the aid of heating, enzymatic deglycosylation, or addition of proteolytically active natural Der p 1 [15, 16]. We recently reported on efficient systems that can be used to pro-

duce recombinant mature Der f 1 and Der p 1 with allergenicity and proteolytic activity [10, 17–20] through acid treatment of the proforms expressed in yeast *Pichia pastoris*. Here, to test whether the inefficient maturation of Pro-Der p 1 is due to N-glycosylation and, if so, to know which of the two N-glycosylation sites in Pro-Der p 1 contributes to the inhibition of the maturation, N-glycosylation motif mutants of Pro-Der p 1 and Pro-Der f 1 were expressed in a yeast system, and their maturation speed was compared.

Materials and Methods

Establishment of Yeast Transfectant Cells for the Expression of Prepro-Der p 1, Prepro-Der f 1, and Their Mutants

Expression vectors for preproforms of Der p 1-N(-65)A, Der p 1-N(-65)A/N52Q, Der f 1-N(-63)S, and Der f 1-N(-63)S/N53Q were constructed by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, Calif., USA) of the preproforms of Der p 1-WT, Der p 1-N52Q, Der f 1-WT, and Der f 1-N53Q [19] (fig. 1). The method used to establish yeast *P. pastoris* GS115 cells transfected with the expression vectors was previously described [19].

Expression and Maturation of Prepro-Der p 1, Prepro-Der f 1, and Their Mutants

Expression of the preproforms in *P. pastoris* was carried out as previously described [19]. Culture supernatants containing secreted proforms [19] were recovered, buffer exchanged by gel filtration using NAP-5 columns (Amersham Biosciences, Piscataway, N.J., USA), equilibrated with 100 mM sodium acetate buffer (pH 4.0), and incubated at room temperature for maturation. Samples from the reactions were collected every 20 min and immediately boiled for 10 min in a buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 2-mercaptoethanol. These samples were then subjected to SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue (CBB) or silver. The speed at which the proforms matured was compared based on the band densities of the pro- and mature forms.

Homology Modeling

The tertiary structure of Pro-Der p 1 was modeled on the basis of the crystal structure of a mutant of Pro-Der p 1 with mutations of C34A/N52D/V124A and additional 10 amino acid residues including a His-tag at the C-terminus (1XKGA), which was recently submitted to the Protein Data Bank database by Meno et al., by using the automated comparative protein modeling server Swiss-Model.

Results

Expression of N-Glycosylation Motif Mutants of Pro-Der p 1 and Pro-Der f 1

While preparing recombinant mature Der p 1 and Der f 1 using the yeast expression system, we recognized that

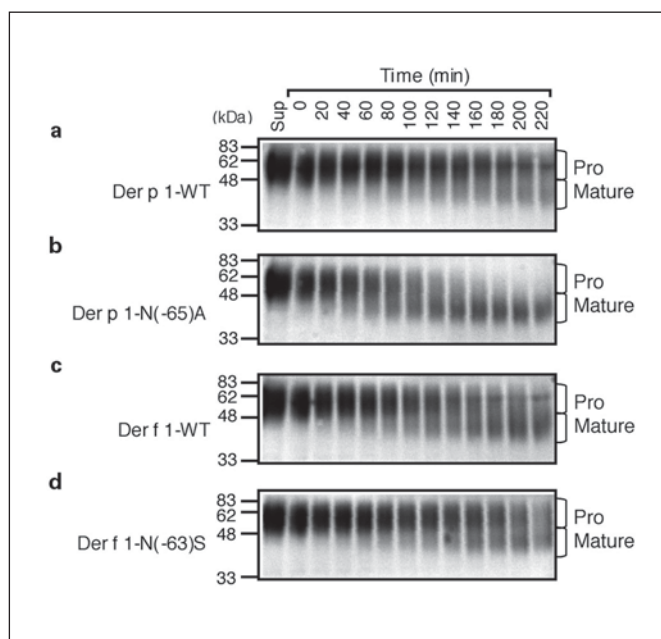


Fig. 2. Maturation of proforms of recombinant Der p 1 and Der f 1 with an N-glycosylation motif within the mature sequence. The preproforms shown in figure 1b were expressed in *P. pastoris*. Culture supernatants containing the proforms were buffer exchanged by gel filtration using columns equilibrated with an acidic buffer and incubated at room temperature. The collected samples were then subjected to SDS-PAGE and the gels were CBB stained. The incubation times after the buffer exchange are indicated. Sup = Culture supernatant before gel filtration; Pro = proform; Mature = mature form.

Pro-Der p 1-WT matured slower than Pro-Der f 1-WT. The reason for this might be similar to the reason why there is no or inefficient maturation from Pro-Der p 1 in insect and yeast expression systems [15, 16]. To evaluate the contribution of N-glycosylation, which occurs in the Der p 1 prodomain but not in the Der f 1 prodomain (fig. 1a), to the speed of maturation, we constructed expression vectors for mutants with and without the prodomain glycosylation (fig. 1b). Der p 1-WT and Der f 1-WT with wild-type sequences are modified by yeast-derived hyperglycosylation at the N-glycosylation site within the mature sequence. From this, Der p 1-N52Q and Der f 1-N53Q without hyperglycosylation were generated to obtain recombinant proteins with molecular sizes equivalent to natural Der p 1 and Der f 1 in previous studies [18, 19]. As well as the expression vectors for mutants with the N-glycosylation motif in the mature sequence (fig. 1b), vectors for mutants without the motif were constructed (fig. 1c).

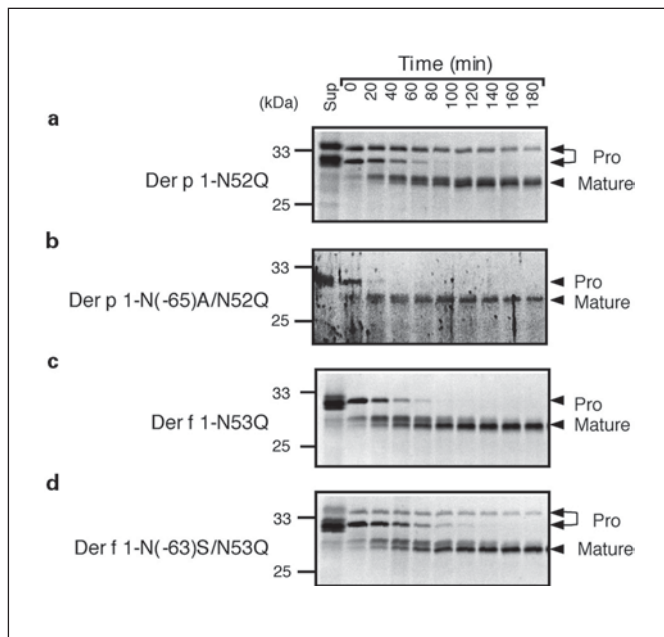


Fig. 3. Maturation of proforms of recombinant Der p 1 and Der f 1 without an N-glycosylation motif within the mature sequence. The preproforms shown in figure 1c were expressed in *P. pastoris*. Culture supernatants containing the proforms were buffer exchanged by gel filtration using columns equilibrated with an acidic buffer and incubated at room temperature. The collected samples were subjected to SDS-PAGE, and the gels were then silver stained. The incubation times after the buffer exchange are indicated. Sup = Culture supernatant before gel filtration; Pro = proform; Mature = mature form.

All recombinant proforms were secreted into the culture supernatant of *P. pastoris* (fig. 2, Sup; fig. 3, Sup). On SDS-PAGE, bands associated with the recombinant forms were smeared when the mature portions were glycosylated (fig. 2, Sup). Two bands were detected for each of Pro-Der p 1-N52Q and Pro-Der f 1-N(-63)S/N53Q (fig. 3a, d, Sup), and the ones with a lower molecular weight showed similar mobility with Pro-Der p 1-N(-65)A/N52Q and Pro-Der f 1-N53Q, respectively (fig. 3b, c, Sup), suggesting that the upper bands were more extensively modified by N-glycosylation at Asn(-65).

Effect of Disruption of the N-Glycosylation Motif in the Mature Portion

Disruption of the N-glycosylation motif in the mature portion decreased the productivity of the proforms (fig. 2, Sup: CBB stained; fig. 3, Sup: silver stained), and accelerated the maturation (fig. 2, 3).

N-Glycosylation of the Prosequences Decelerated the Maturation

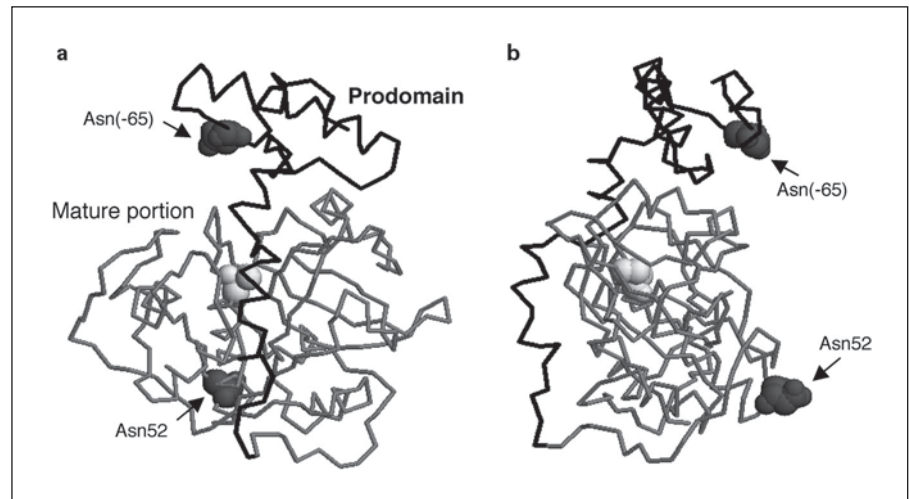
To achieve a simultaneous start to the reaction and simultaneous samplings, simultaneous gel filtration with individual columns was applied for exchange of the culture supernatants to the acidic buffer as an alternative to dialysis in the previous studies [18–20]. Disruption of the N-glycosylation motif within the Der p 1-WT prosequence caused a significant acceleration of maturation in Pro-Der p 1-N(-65)A (fig. 2a, b), and most of Pro-Der p 1-N(-65)A was converted into the mature form within 180 min (fig. 2b). Introduction of the motif into the Der f 1-WT prosequence slowed down the maturation in Pro-Der f 1-N(-63)S (fig. 2c, d). Bands for the proforms except for Der p 1-N(-65)A remained even after 220 min (fig. 3a, c, d). In terms of speed of maturation, the proforms rank as follows: Der p 1-N(-65)A > Der f 1-WT > Der f 1-N(-63)A > Der p 1-WT.

Disruption of the N-glycosylation motif of the Der p 1-N52Q prosequence caused a significant acceleration of maturation in Pro-Der p 1-N(-65)A/N52Q (fig. 3a, b), and most of Pro-Der p 1-N(-65)A/N52Q was converted into the mature form within 20 min (fig. 3b). Introduction of the motif into the Der f 1-N53Q prosequence slowed the maturation in Pro-Der f 1-N(-63)S/N53Q (fig. 3c, d), and most Pro-Der f 1-N53Q was converted within 80 min (fig. 3c). Most of the lower bands corresponding to Pro-Der p 1-N52Q and Pro-Der f 1-N(-63)S/N53Q were converted within 80 and 120 min, respectively, while the upper bands remained even after 160 min (fig. 3a, d). In terms of speed of maturation, the proforms rank as follows: Der p 1-N(-65)A/N52Q > Der f 1-N53Q > Der f 1-N(-63)S/N53Q > Der p 1-N52Q. Although the mature forms were detected as doublet bands in the first half of the incubation, the upper band was gradually converted to the lower band of the completely matured form, suggesting that the upper band corresponded to an intermediate product with a truncated prosequence.

Modeling of the Tertiary Structure of Pro-Der p 1

The Swiss-Model server modeled the tertiary structure of Pro-Der p 1 except N-terminal 3 amino acid residues of the prodomain (fig. 4). In the model, the Der p 1 prosegment consisted of an N-terminal domain structure similar to other cathepsin L-like cysteine proteases [21]. Asn(-65), which corresponds to the N-glycosylation site of the prodomains of Der p 1-WT, Der p 1-N52Q, Der f 1-N(-63)S, and Der f 1-N(-63)S/N53Q, was located in the C-terminal portion of the first helix (α_1).

Fig. 4. Homology modeling of the tertiary structure of Pro-Der p 1. **a** Front view. **b** View from the right side of **a**. N-terminal 3 amino acid residues of the prodomain are not displayed. The prosegment and mature Der p 1 are shown as backbone models in black and gray, respectively. N-glycosylation sites corresponding to Asn(-65) of the prodomain of Der p 1-WT, Der f 1-N(-63)S, Der p 1-N52Q, Der f 1-N(-63)S/N53Q, and Asn52 of Der p 1 or Asn53 of Der f 1 in the mature portion are shown as space-filling models in dark gray. The catalytic center, Cys34, is shown as a space-filling model in light gray. These figures were produced using the RasMol program [32].



Discussion

Disruption of the N-glycosylation motif in the mature portion decreased the productivity of the proforms and accelerated the maturation. Yasuhara et al. [20] reported that enzymatically deglycosylated Der f 1-WT showed a molecular weight identical to Der f 1-N53Q and natural Der f 1, and Jacquet et al. [22] reported that enzymatically deglycosylated Der p 1-WT showed a molecular weight identical to natural Der p 1. On SDS-PAGE, bands associated with Der p 1-WT and Der f 1-WT were smeared, suggesting heterogeneity in the size of the sugar chain. As the N-glycosylation site in the mature portion is located on the surface far from the catalytic site and the prosegment (fig. 4, Asn52), we assume that the glycochain attached to these residues sterically hinders the access of proteolytically activated molecules of Der p 1, Der f 1, and/or *Pichia*-derived proteases to the proforms, avoids aggregation, and/or affects intracellular translocation. We considered that the decrease in total band density during maturation (fig. 2) is according to removal of the prodomain and degradation by activated mature forms or yeast-derived protease in the supernatant.

The tendency for the disruption in Der p 1 or the introduction into Der f 1 of the N-glycosylation motif within the prosequence to speed up or slow down maturation was similar between those with (fig. 2) and those without (fig. 3) the other glycosylation motif within the mature sequence. Particularly, the deceleration of the Der p 1 maturation by the prodomain glycosylation was clearly demonstrated (fig. 2a, b; fig. 3a, b) and more effective than

that of the Der f 1 maturation (fig. 2d, c; fig. 3d, c). However, less efficiency of glycosylation in the prosequence of Pro-Der f 1-N(-63)S/N53Q (fig. 3d) than of Pro-Der p 1-N52Q (fig. 3a) suggests that the deceleration of Der f 1 maturation might be greater if the prodomain of Der f 1 is modified as efficiently as Der p 1. Prodomains of the papain-like cysteine protease family block proteolytic activity and assist in the correct folding of the mature portions [23–26]. Although α_1 does not interact with the mature portion (fig. 4) [21], it contributes to the inhibition of enzymatic activity [21] and to the maintenance of the global structure of the prodomain [27]. We assume that the initial step of maturation is a cleavage within the prosequence by activity of *Pichia*-derived protease activity in the culture supernatant, or by weak activity of Pro-Der p 1 and Pro-Der f 1. Glycosylation of Asn(-65) in the C-terminus of α_1 of Pro-Der p 1 [fig. 4, Asn(-65)] might stabilize the domain structure of the prosegment and/or prevent the initial generation of a partially deleted intermediate [19] by protecting the cleavage site from attack by Der p 1 or other proteases in the culture supernatant. As far as we know, such inhibition of the maturation of the precursor of cysteine proteases by modification of the prodomain has not been reported so far.

Our results obtained using the N-glycosylation motif mutants indicate that deglycosylation of both the mature portion and the prodomain contributes to efficient maturation. Jacquet et al. [15] reported almost no maturation of Pro-Der p 1 expressed in insect cells without heating, and van Oort et al. [16] reported that *Pichia*-expressed Pro-Der p 1-WT achieves maturation by deglycosylation

with endoglycosidase in the presence of SDS after heating or addition of natural Der p 1. The absence of any spontaneous maturation in their reports might be attributable to the effect of N-glycosylation in addition to differences in culture conditions and maturation conditions from the other systems achieving maturation of Pro-Der p 1-WT, i.e. our *Pichia* system with a prolonged period for the maturation of more concentrated samples even achieved the maturation of Pro-Der p 1-WT as well as Pro-Der p 1-N52Q [19], and Jacquet et al. [22] also reported maturation of *Pichia*-expressed Pro-Der p 1-WT. Pro-Der p 1-N(-65)A (fig. 2b) and Pro-Der p 1-N(-65)A/N52Q (fig. 3b) without the N-glycosylated prodomain have the advantage of maturing faster than Pro-Der p 1-WT (fig. 2a) and Pro-Der p 1-N52Q (fig. 3a).

Der p 1-N52Q and Der f 1-N53Q are considered to be useful for studies in vitro and in vivo as alternatives to natural Der p 1 and Der f 1, because they exhibit similar molecular sizes, secondary structures, and allergenicities as their natural counterparts [18, 19], have enzymatic activity to cleave human protein substrates in vitro, have IgE-eliciting activity in vivo [10], and are considered free from the yeast-derived N-linked glucan-specific IgG antibodies detectable in sera of healthy individuals [28]. IgE-binding affinity and basophil histamine-releasing activity of the proforms are lower than those of the mature recombinant and natural Der p 1 and Der f 1 [18]. Therefore, in spite of the reduction in productivity upon deglycosylation of the mature portion, the yields of which were

mentioned previously [19], the maturation system starting with Pro-Der p 1-N(-65)A/N52Q is useful in the preparation of unglycosylated mature Der p 1 as an alternative to Pro-Der p 1-N52Q, in which the upper band of the precursor remained immature (fig. 3a).

In summary, by comparing the speed of maturation of eight variants of the N-glycosylation motifs of Pro-Der p 1 and Pro-Der f 1 expressed in *P. pastoris*, we found a contribution of the N-glycosylation motif in the mature portion to the enhancement of productivity and inhibition of maturation, and a contribution of the N-glycosylation of the prodomain to the inhibition of the maturation process. The findings in this study will be useful for designing efficient systems for the preparation of recombinant Der p 1 and Der f 1 as allergen vaccines for allergen-specific immunotherapy [29], and, in addition, include an interesting topic concerning maturation of papain-like cysteine proteases.

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