

# Maturation of *Pichia pastoris*-derived recombinant pro-Der p 1 induced by deglycosylation and by the natural cysteine protease Der p 1 from house dust mite

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The mature cysteine protease from *Dermatophagoides pteronyssinus*, Der p 1, is a major house dust mite allergen. Its enzymatic activity has been shown to have pro-inflammatory effects that could also negatively influence efficacy of allergen-specific immunotherapy. The aim of this study was to express recombinant pro-Der p 1 (rpro-Der p 1) in the yeast *Pichia pastoris* and to study its maturation. Expression was achieved at a concentration ranging from 45 mg·L<sup>-1</sup> (methanol-induced expression) to 168 mg·L<sup>-1</sup> (constitutive expression). No significant spontaneous maturation of the secreted proenzyme was observed. rpro-Der p 1 with a sequence-based molecular mass of 34 kDa was hyperglycosylated by the yeast, migrating at 50–60 kDa on SDS/PAGE. Compared with its natural counterpart (nDer p 1), the recombinant proenzyme demonstrated decreased IgE reactivity, resulting in a 30-fold lower capacity to induce histamine release from human basophils. Decreased immunoreactivity was also shown by competitive RIA and

sandwich ELISA with Der p 1-specific antibody reagents. CD spectra of rpro-Der p 1 and nDer p 1 revealed significant structural differences. Deglycosylation of rpro-Der p 1 with endoglycosidase H resulted in a decrease in apparent molecular mass from 50 kDa to 34 kDa, but did not affect nDer p 1. On removal of N-glycans from rpro-Der p 1, which harbours two putative N-glycosylation sites in both propeptide and mature sequence, the mature rDer p 1 appeared. This suggests that hyperglycosylation hampers spontaneous maturation. Maturation of the recombinant pro-enzyme was also achieved by addition of the active natural cysteine protease, nDer p 1. In conclusion, high-level expression of rpro-Der p 1 in *P. pastoris* results in a stable hypoallergenic proenzyme with potential for use in allergen-specific immunotherapy.

**Keywords:** allergy; Der p 1; house dust mite; pro-allergen; yeast.

Group 1 allergen produced by the house dust mite *Dermatophagoides pteronyssinus* (Der p 1) has been described as an aeroallergen with a molecular mass of ≈ 27 kDa, carried (mostly) on mite faeces [1–3]. It is a glycoprotein with cysteine protease activity and is able to cleave human CD25 and CD23 [4–7]. This activity enhances total and specific IgE production in mice immunized with proteolytically active Der p 1 [8–10]. Protease activity of Der p 1 has also been reported to increase the permeability of the human respiratory epithelium [11]. The structure of Der p 1 was determined by comparative modeling with papain, actinidin and papaya proteinase  $\Omega$  of the cysteine proteinase family [12], and epitopes responsible for binding to IgE and IgG could be identified [13–15].

To produce a fully reactive recombinant version of Der p 1, several expression systems have been tested. An *Escherichia coli*-derived recombinant (as fusion protein) [16] showed < 50% of the IgE-binding activity of that of the

natural allergen. Expression of Der p 1 in the yeast *Saccharomyces cerevisiae* revealed high IgE reactivity, although clear differences from the natural allergen were demonstrated [17]. Recently, the precursor form of Der p 1 produced in *Drosophila* and mammalian cells has been characterized [18,19]. Although enzymatically inactive, it was claimed to have similar IgE reactivity to that of the natural allergen, even though the prosequence was still attached. This contrasts with results obtained for pro-Der f 1 expressed in the baculovirus system, where cleavage of the prosequence was necessary to obtain a fully IgE-reactive recombinant [20]. The autocatalytic processing of pro-Der f 1 was achieved by incubation in acidic pH as described for other cysteine proteases [21]. Jacquet *et al.* [18] described rpro-Der p 1 autocatalytic processing by incubation at 60 °C, acidic pH and addition of up to 20 mM cysteine. Maturation of up to 80% was reported, which did not improve over time [18]. Expression of Der f 1 and Der p 1 in *Pichia pastoris* was recently reported by Yasuhara *et al.* [23] and Best *et al.* [24,25], respectively. Maturation of rpro-Der f 1 was shown to be induced by dialysis against pH 4.0, resulting in complete IgE-binding capacity and biological activity. Best *et al.* [24,25] reported spontaneous maturation of both rpro-Der f 1 and rpro-Der p 1 during induced and constitutive expression in *P. pastoris*.

In our study, secretory expression and immunochemical characterization of the precursor form of Der p 1 in

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Abbreviations: Endo H, endoglycosidase H; RAST, radioallergosorbent assay; YPD, yeast extract peptone.

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*P. pastoris* is reported. Methanol-induced expression in two different strains (SMD1168H and GS115) and constitutive expression in strain X-33 were obtained. Partial cleavage of the prosequence was achieved spontaneously after deglycosylation or by incubation with nDer p 1.

## MATERIALS AND METHODS

### Cloning and sequencing of mature and pro-Der p 1

From a house dust mite  $\lambda$ gt11 library (kindly provided by W. R. Thomas, Princess Margaret Children's Medical Research Foundation, Perth, Australia) cDNAs of mature and pro-Der p 1 were obtained by PCR (Table 1). Subsequently, they were cloned into pPICZ $\alpha$ A (and pGAPZ $\alpha$ A) in-frame with the secretion peptide (Table 1). DNA sequences were determined by automated sequencing (Applied Biosystems) using the DYEnamic<sup>TM</sup> ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech Inc) according to the manufacturer's instructions. Sequence primers were used as described in Table 1.

### Expression in *P. pastoris*

Pro-Der p 1 was expressed in *P. pastoris* strain SMD1168h (PEP4 mutant, deficient in protease A, his4+), GS115 (his4+) or X-33 (wild-type), and mature Der p 1 only in SMD1168h. Transformation was performed as described by the manufacturer (Invitrogen, San Diego, CA, USA). Positive clones were selected from yeast extract/peptone/dextrose medium (YPD plates) containing zeocine (100  $\mu$ g mL<sup>-1</sup>) as a selection marker. Selected clones were inoculated in YPD with zeocine and grown overnight at 29 °C. Cells transformed with pPICZ $\alpha$ A were then transferred to buffered glycerol-complex medium for 24 h, after which they were centrifuged (glycerol inhibits expression) and transferred to expression medium (buffered methanol-complex medium, pH 5.0) at  $D_{600} = 10$  (SMD1168h) or  $D_{600} = 1$  (GS115) for methanol-induced expression. After 96 h, the supernatant was harvested.

For constitutive expression, cells containing pro-Der p 1 in pGAPZ $\alpha$ A remained in YPD medium after inoculation of a single colony from a YPD/zeocine plate. From an overnight culture, 0.225 mL was transferred to inoculate 125 mL YPD medium as described by the manufacturer (Invitrogen). After 96 h the supernatant was harvested.

### Purification of (recombinant) allergens

Recombinant pro-Der p 1 was purified from culture supernatant by affinity chromatography with Sepharose-coupled monoclonal antibody against nDer p 1 [26]. After the

Der p 1 had been allowed to bind, the column was washed with NaCl/P<sub>i</sub> and subsequently eluted with 50% ethylene glycol/5 mM lysine, pH 11. Purity was assessed by SDS/PAGE/silver staining (Novex, San Diego, CA, USA). nDer p 1 was affinity-purified from spent medium extract [2% (w/v) in NaCl/P<sub>i</sub>/0.01% poly(ethylene glycol) 6000/0.01% sodium azide (CSL, Melbourne, Australia)]. Protein concentrations were determined using the BCA method as described by the manufacturer (Pierce, Rockford, IL, USA).

### SDS/PAGE and immunoblotting

Proteins were separated by SDS/PAGE (4–12%) (Novex) as described by the manufacturer, and silver-stained according to the ExcelGel protocol (Amersham Pharmacia Biotech, Uppsala, Sweden). Western blotting was performed by transferring the proteins on to nitrocellulose membrane as described by the manufacturer (Novex). Subsequently, the blots were blocked with NaCl/P<sub>i</sub>/1% BSA and incubated overnight with polyclonal rabbit anti-(Der p 1) Ig. After being washed, the blots were incubated overnight with <sup>125</sup>I-labeled sheep anti-(rabbit IgG) Ig (CLB) and exposed to an autoradiographic film (Eastman Kodak Company, Rochester, NY, USA).

### Radio Allergo Sorbent test (RAST)

RAST was performed as described previously [27]. Briefly, both natural and recombinant proteins were coupled to CNBr-activated Sepharose 4B (250  $\mu$ g of allergen per 100 mg of Sepharose; Amersham Pharmacia Biotech). The Sepharose was resuspended to 2 mg mL<sup>-1</sup> in NaCl/P<sub>i</sub>/0.3% BSA/0.1% Tween-20, 250  $\mu$ L of which was incubated with 50  $\mu$ L human serum. After incubation overnight, unbound material was washed away, and 50  $\mu$ L <sup>125</sup>I-labeled sheep anti-(human IgE) Ig (CLB) was added. After incubation overnight and a wash, bound radioactivity was measured in a  $\gamma$  counter. The results were expressed as IU mL<sup>-1</sup>, which were calculated from a standard curve of serial dilutions of a human/mouse chimeric IgE antibody directed to Der p 2 and Sepharose-coupled rDer p 2 [28]. A result greater than 0.30 IU mL<sup>-1</sup> was regarded as positive.

### Radiolabeling

Radiolabeling of purified Der p 1 samples ( $\approx 25$   $\mu$ g) with <sup>125</sup>I (37 MBq) was performed by the chloramine-T method. Radiolabeled allergen and free iodine were separated by size-exclusion chromatography (ACA 54) (Life Technologies, BioSeptra SA Cergy-Saint-Christophe, France).

**Table 1.** Primers used for PCR, cloning and sequencing of mature and pro-Der p 1.

| Primer name                                               | Primer sequence                                |
|-----------------------------------------------------------|------------------------------------------------|
| pPICZ $\alpha$ A 5' cloning primer pro-Der p 1            | 5'-GGGCTCGAGAAAA-GACGTCCATCATCGATCAAACTTTTG-3' |
| pPICZ $\alpha$ A 3' cloning primer mature and pro-Der p 1 | 5'-GGGGAGCTCTTAGAGAATGACAACATATGG-3'           |
| pPICZ $\alpha$ A 5' cloning primer mature Der p 1         | 5'-GGGCTCGAGAAAAGAACTAACGCCTGCAGTATCAAT-3'     |
| Sequence primers used for vector pPICZ $\alpha$ A         | 5'AOX, 3'AOX and $\alpha$ -factor primer       |

### Competitive RIA

In a competitive RIA [29,30], 50  $\mu\text{L}$  rabbit anti-(Der p 1) (1 : 2500) [26] was preincubated for 2 h at room temperature with 50  $\mu\text{L}$  of serial dilutions of the inhibitor (rpro-Der p 1, nDer p 1, mite extract, or *Pichia* culture supernatants), before addition of 250  $\mu\text{L}$  Protein A–Sepharose (2  $\text{mg}\cdot\text{mL}^{-1}$ ), and 50  $\mu\text{L}$   $^{125}\text{I}$ -labeled nDer p 1. After overnight incubation (end-over-end rotation at room temperature), samples were washed, and bound radioactivity was counted. For the uninhibited value, polyclonal antibody was preincubated with  $\text{NaCl}/\text{P}_i/0.3\%$  BSA/0.1% Tween-20 instead of allergen. All tests were performed in duplicate.

### Der p 1 ELISA

A Der p 1 ELISA was obtained from Indoor Biotechnologies (Cardiff, UK) and carried out according to the manufacturer's instructions, except for the substrate system, which was modified for 3,3',5,5'-tetramethylbenzidine usage. Consequently, color development was initiated by adding 100  $\mu\text{L}$  3,3',5,5'-tetramethylbenzidine (10  $\text{mg}\cdot\text{mL}^{-1}$ ) in sodium acetate, pH 5.5, and 10  $\mu\text{L}$  3%  $\text{H}_2\text{O}_2$ . The reaction was stopped by adding 2 M  $\text{H}_2\text{SO}_4$ , after which the absorbance was measured at 450/540 nm. All tests were performed in duplicate.

### In vitro histamine-release assays

White blood cells were isolated from blood of a nonallergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere [31,32]. Subsequently, cells were resensitized with patients' sera ( $n = 6$ ) that tested positive (RAST) on Der p 1. Histamine release was performed with purified natural and recombinant Der p 1 (0.1  $\text{ng}\cdot\text{mL}^{-1}$  to 10  $\mu\text{g}\cdot\text{mL}^{-1}$ ). Liberated histamine was measured by the fluorimetric method essentially as described by Siraganian [33]. The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Center under project number: MEC97/030.

### Endoglycosidase H (Endo H) cleavage of recombinant pro-Der p 1

One volume of protein ( $\approx 5 \mu\text{g}$ ) was combined with 1 vol. 100 mM ammonium acetate, pH 5.5, and a final concentration of 0.2% SDS, which was incubated for 10 min at 80 °C. Subsequently, 1.5 mU Endo H (Boehringer, Mannheim, Germany) was added and incubated at 37 °C overnight. Endo H is active on N-linked oligosaccharides of glycopeptides/proteins and cleaves only high-mannose structures and hybrid structures (AcNeu-Gal-GlcNAc). The results were analyzed by SDS/PAGE (silver staining), immunoblot with rabbit anti-(Der p 1) Ig, and concanavalin A binding.

### Glycan analysis

Natural Der p 1, recombinant pro-Der p 1 (SMD1168h and X-33) and Endo H-treated rpro-Der p 1 (SMD1168 h) were electroblotted on to nitrocellulose membrane and then incubated overnight in  $\text{NaCl}/\text{Tris}/0.1\%$  Tween 20. Subse-

quently the blot was incubated with concanavalin A (25  $\mu\text{g}\cdot\text{mL}^{-1}$ ; Sigma, St Louis, MO, USA) in  $\text{NaCl}/\text{Tris}/0.1\%$  Tween 20, containing 1 mM  $\text{MgCl}_2$  and 1 mM  $\text{CaCl}_2$  for 90 min. After a wash with  $\text{NaCl}/\text{Tris}/0.1\%$  Tween 20, containing 1 mM  $\text{MgCl}_2$  and 1 mM  $\text{CaCl}_2$ , the membrane was incubated with horseradish peroxidase for 60 min (50  $\mu\text{g}\cdot\text{mL}^{-1}$ ; Sigma) [34]. The bands were visualized with one tablet of diaminobenzidine in aqua dest (10 mg diaminobenzidine tetrahydrochloride; Kem-En-Tec, Copenhagen, Denmark). The reaction was started with 40  $\mu\text{L}$  30%  $\text{H}_2\text{O}_2$ .

Further glycan analysis was carried out with the DIG Glycan Differentiation Kit (Roche Diagnostics GmbH, Mannheim, Germany) using the following lectins: *Galanthus nivalis* agglutinin, *Sambucus nigra* agglutinin, *Maackia amurensis* agglutinin, peanut agglutinin, and *Datura stramonium* agglutinin. The Der p 1 samples were dot-blotted or electroblotted on nitrocellulose after separation by SDS/PAGE.

### Circular dichroism

In CD experiments, ellipticity measurements were performed with nDer p 1 (740  $\mu\text{g}\cdot\text{mL}^{-1}$  and 370  $\mu\text{g}\cdot\text{mL}^{-1}$ ) and rpro-Der p 1 (300  $\mu\text{g}\cdot\text{mL}^{-1}$ ) dissolved in 10 mM Tris/EDTA buffer, pH 7.5. The proteins were measured in a 0.05-mm cuvette and subjected to 20 cycles with a resolution of 0.2 nm and a speed of 20  $\text{nm}\cdot\text{min}^{-1}$ . The spectra were calculated after subtraction of the blank (spectra obtained with 10 mM Tris/1 mM EDTA, pH 7.5). Both spectra were also corrected with respect to concentration and number of amino acids. The percentages of  $\alpha$  helices,  $\beta$  sheets and random structures were interpreted from known reference spectra.

### Autoprocessing of rpro-Der p 1

Purified rpro-Der p 1 ( $\approx 37 \mu\text{g}\cdot\text{mL}^{-1}$ ; Pierce), was dialyzed for 2 days against 0.2 M sodium acetate, pH 4.0, which was reported to induce autocatalyzed cleavage of the prosequence in case of Der f 1 [20]. Alternatively, purified recombinant pro-Der p 1 ( $\approx 100 \mu\text{g}\cdot\text{mL}^{-1}$ ) was applied to a PD-10 column (Sephadex G-25, bed vol. 9.1 mL; Amersham Pharmacia Biotech AB) equilibrated in 50 mM sodium acetate, pH 4.0, to exchange buffer. Cysteine was added to a concentration of 20 mM, and the sample was incubated at 60 °C for 1.5 h [18]. The effect of SDS (0.05–0.2%) under these conditions was also studied. Samples were analyzed by SDS/PAGE.

### Proteolytic processing with nDer p 1

$^{125}\text{I}$ -Labeled rpro-Der p 1 (2  $\mu\text{L}$ ) was incubated with nDer p 1 ( $\approx 1 \mu\text{g}$ ) at room temperature or 37 °C in  $\text{NaCl}/\text{P}_i$ , pH 7.4, or sodium acetate, pH 5.5, for 4 h in a final volume of 20  $\mu\text{L}$ . Incubation was ended by the addition of reducing sample buffer. Samples were analyzed by autoradiography after separation by SDS/PAGE on Excel gel (8–18%) (Amersham Pharmacia Biotech).

nDer p 1 was coupled to Sepharose (400  $\mu\text{g}$  nDer p 1 per 100  $\text{mg}^{-1}$  Sepharose) and taken up in  $\text{NaCl}/\text{P}_i$  at 32  $\text{mg}\cdot\text{mL}^{-1}$ . rPro-Der p 1 (6  $\mu\text{g}$ , volume 34  $\mu\text{L}$ ) was incubated with 100  $\mu\text{L}$  of this solid phase at room

temperature for times ranging from 2 to 72 h. Supernatant was harvested after centrifugation and analyzed by SDS/PAGE/silver staining (Novex).

### N-Terminal sequencing

rpro-Der p 1 was separated by SDS/PAGE (4–12% gel; Novex) and electroblotted on poly(vinylidene difluoride) membrane. The blot was stained with Coomassie R-250 (Bio-Rad, Hercules, CA, USA) in 50% methanol. The band corresponding to rpro-Der p 1 was excised and sequenced on a PerkinElmer/Applied Biosystems 476A gas-phase sequencer (Edman degradation).

### Sera

Sera ( $n = 198$ ) with specific IgE antibodies against house dust mite allergens ( $> 0.3 \text{ IU} \cdot \text{mL}^{-1}$ ) were used for RAST analysis.

### Statistical analysis

RAST results for natural and recombinant proDer p 1 were compared by Spearman rank correlation and Student's *t*-test after log transformation. Responses in Der p 1 ELISA and competitive RIA were compared by parallel-line analyses.

## RESULTS

### Sequence analyses of mature and pro-Der p 1

cDNAs of mature and pro-Der p 1 were picked up by PCR from a  $\lambda$ gt11 *D. pteronyssinus* cDNA library. All clones had identical sequences (81E, 124A, 136S, 149A and 215E) with those published by Chua *et al.* [15]. Of the six reported polymorphisms, only one was observed, being either a tyrosine or a histidine at position 50. The clone containing polymorphism 50Y was selected for expression, because T-cell responses to peptides containing 50H were decreased compared with peptides containing 50Y [35].

### Expression of mature and pro-Der p 1 in *P. pastoris* strain SMD1168h

Both cDNAs were cloned into pPICZ $\alpha$ A and transformed to *Pichia* strain SMD1168 h. Mature Der p 1 was not expressed at a detectable level ( $< 1 \text{ ng} \cdot \text{mL}^{-1}$ ) as judged by competitive RIA. Pro-Der p 1 expression resulted in a final yield of  $55 \text{ mg} \cdot \text{L}^{-1}$  (competitive RIA) [29]. Affinity purification of rpro-Der p 1 gave a final purification yield of 15%.

nDer p 1, rpro-Der p 1 and Endo H-treated rpro-Der p 1 were separated by SDS/PAGE (4–12% gel) and silver stained (Fig. 1A). rpro-Der p 1 with a theoretical molecular mass of 34 kDa migrated as a broad band of  $\approx 50 \text{ kDa}$  without any detectable mature Der p 1 at the level of nDer p 1 (25 kDa). Endo H treatment resulted in a shift from 50 kDa to  $\approx 34 \text{ kDa}$ , being similar to the theoretical molecular mass of rpro-Der p 1. This implies that the high molecular mass of rpro-Der p 1 was caused by glycosylation. In addition, at least two weaker bands of lower molecular mass appeared on Endo H treatment, one with molecular mass identical with that of nDer p 1. The

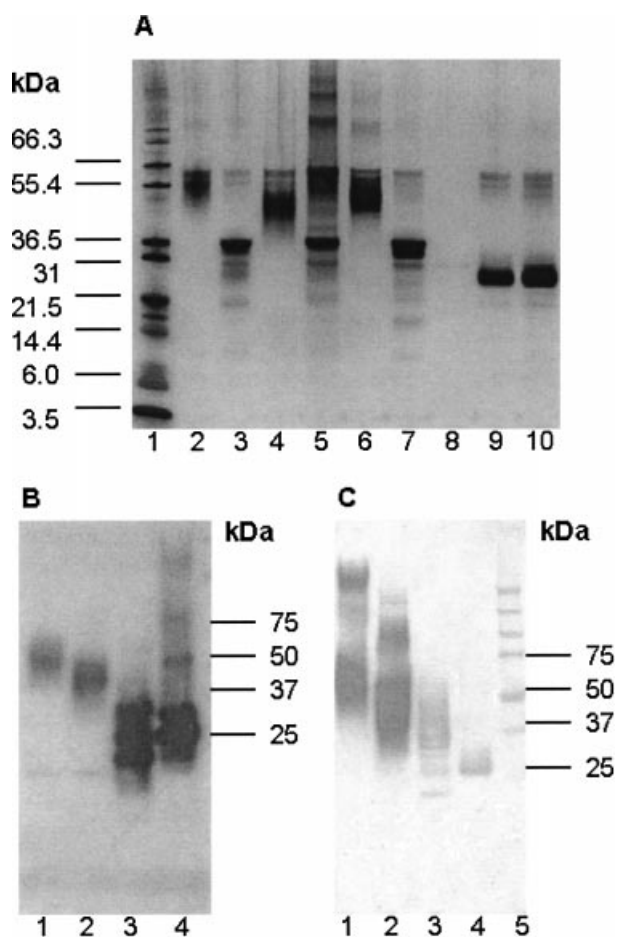


**Fig. 1.** (A) SDS/polyacrylamide gel (silver stained), (B) immunoblot with rabbit anti-(Der p 1) Ig, and (C) concanavalin A blot. (A) Lane 1, Mark 12 protein ladder (Novex); lane 2, rpro-Der p 1 (X-33); lane 3, Endo H-treated rpro-Der p 1 (X-33); lane 4, rpro-Der p 1 (SMD1168h); lane 5, rpro-Der p 1 Endo H-treated (SMD1168h); lane 6, rpro-Der p 1 (GS115); lane 7, rpro-Der p 1 Endo H-treated (GS115); lane 8, Endo H (control); lane 9, nDer p 1; lane 10, nDer p 1 (Endo H treated). (B) Lane 1, rpro-Der p 1 (X-33); lane 2, rpro-Der p 1 (SMD1168 h); lane 3, Endo H-treated rpro-Der p 1 (SMD1168h); lane 4, nDer p 1. (C) Lane 1, rpro-Der p 1 (X-33); lane 2, rpro-Der p 1 (SMD1168h); lane 3, Endo H-treated rpro-Der p 1; lane 4, nDer p 1; lane 5, prestained, broad-range precision ladder (Bio-Rad).

other band of  $\approx 20 \text{ kDa}$  was also present in nDer p 1. Immunoblot analysis with rabbit antibodies against Der p 1 confirmed the Der p 1 nature of all three bands (Fig. 1B). Endo H treatment did not affect nDer p 1, suggesting the absence of N-linked glycosylation (at least the absence of N-linked glycans for which Endo H has specificity).

Blot analysis with concanavalin A confirmed the hyperglycosylation of rpro-Der p 1 (Fig. 1C). Concanavalin A staining almost completely disappeared on Endo H treatment. Concanavalin A staining of nDer p 1 was weak but significant. Of the different lectins tested with rpro-Der p 1 and nDer p 1 on dot blot, only peanut agglutinin gave a positive reaction with nDer p 1 (not shown). This suggests the presence of O-glycans on nDer p 1, which were not present on the recombinants. These glycans have been

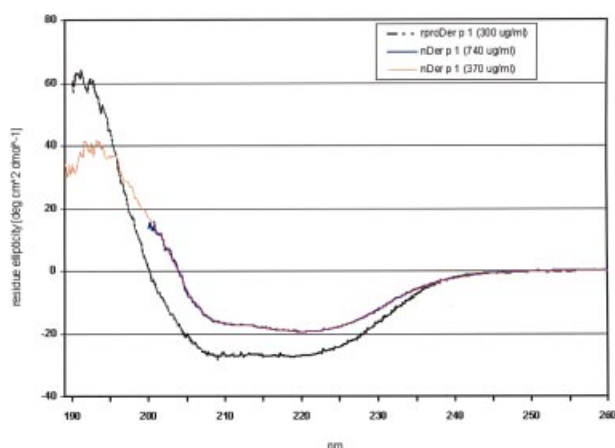


**Fig. 2.** CD spectrum of nDer p 1 vs. rpro-Der p 1. Spectra obtained with  $740 \mu\text{g mL}^{-1}$  and  $370 \mu\text{g mL}^{-1}$  nDer p 1 are represented by blue and red lines, respectively. rpro-Der p 1 ( $300 \mu\text{g mL}^{-1}$ ) is represented by the dashed and dotted line.

described as having a core disaccharide galactose  $\beta(1-3)$  *N*-acetylgalactosamine which forms the core unit of O-glycans (except in yeast glycoproteins).

### N-Terminal sequencing and CD spectra

N-Terminal sequencing was performed on rpro-Der p 1 to investigate whether inefficient cleavage of the yeast secretion peptide could also be involved in the higher apparent molecular mass observed on SDS/PAGE. Sequencing revealed that the recombinant proenzyme starts with the correct sequence (RPSSIKTFEE) and that no signal peptide was left attached [15]. Analysis of the CD spectra resulted in the following predictions for the secondary structures of nDer p 1 and rpro-Der p 1: 50%  $\alpha$  helical and 50%  $\beta$  pleated sheets compared with an  $\alpha/\beta$  combination with 30% random coil, respectively (Fig. 2).

### IgE reactivity (RAST and histamine-release assays)

Patients allergic to house dust mites were tested in a RAST ( $n = 198$ ) for IgE-specific antibodies against nDer p 1 and rpro-Der p 1 (not shown). IgE binding to rpro-Der p 1 showed significant correlation with that to nDer p 1 [ $R_s = 0.9077 (+0.8774 \text{ to } +0.9308)$ ,  $p_s < 0.01$ ]. However, binding to nDer p 1 was twice as potent than to the recombinant protein (2.2 mean ratio; 95% confidence interval 2.0 to 2.4). Endo H treatment did not alter the results significantly ( $n = 14$ ; not shown), although it cannot be excluded that SDS treatment and low pH (pH 5.5) during deglycosylation masked a possible favorable effect on the IgE binding of rpro-Der p 1.

In histamine-release assays, six mite allergic sera were used to test the ability of the pro-allergen compared with nDer p 1 to induce histamine release ( $0.1 \text{ ng mL}^{-1}$  to  $10 \mu\text{g mL}^{-1}$ ). The recombinant pro-allergen showed a greatly decreased biological activity. A 25% histamine release was achieved with  $2 \text{ ng mL}^{-1}$  nDer p 1, whereas the recombinant required a concentration of  $60 \text{ ng mL}^{-1}$ . In addition, the mean maximum release was 31% for rpro-Der p 1 compared with 41% for nDer p 1 (Fig. 3). No

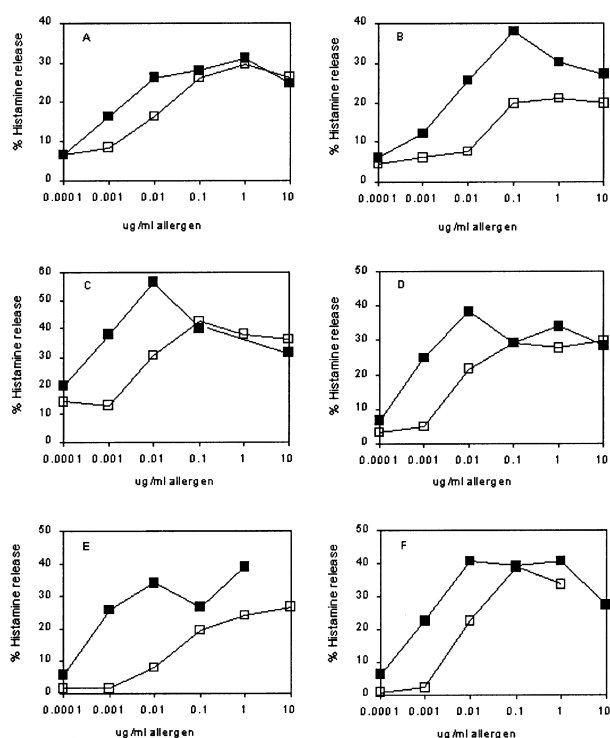


**Fig. 3.** Histamine-release assays with six Der p 1 allergic patients. (A–F) represent patients 1 to 6. (■) Release induced with nDer p 1; (□) release induced by rpro-Der p 1. Concentration of the allergen ranged from  $0.1 \text{ ng mL}^{-1}$  to  $10 \mu\text{g mL}^{-1}$ . Histamine release induced by rpro-Der p 1 was significantly lower than that induced by nDer p 1, varying from a factor of 10 (A) to a factor of 100 (E).

significant release ( $< 3\%$ ) from stripped cells was detected (data not shown).

### Major allergen tests (competitive RIA, sandwich ELISA)

Affinity-purified nDer p 1 and rpro-Der p 1 were also compared in a competitive RIA with  $^{125}\text{I}$ -labeled nDer p 1. nDer p 1 was 9.2-fold more efficient as an inhibitor than rpro-Der p 1 (Fig. 4).

Comparison of nDer p 1 and rpro-Der p 1 in a sandwich ELISA with two Der p 1-specific monoclonal antibodies resulted in much smaller differences. Here, the recombinant was only 2.5-fold less potent (Fig. 5).

### Expression of pro-Der p 1 in *Pichia* strain GS115 and X-33

As no mature Der p 1 spontaneously appeared in the protease-deficient strain SMD1168h, expression was performed in a nonprotease-deficient strain, GS115 ( $45 \text{ mg L}^{-1}$ ). Again no mature protein was detected (Fig. 1). The molecular mass of GS115-produced rpro-Der p 1 was even slightly higher than of the allergen produced in SMD1168h. On Endo H treatment no significant difference between recombinant products from either strain was observed. Deglycosylated GS115-derived rpro-Der p 1 also migrated at  $\approx 34 \text{ kDa}$  and mature rDer p 1 appeared.

Finally, constitutive expression in strain X-33 ( $168 \text{ mg L}^{-1}$ ) was performed to investigate whether this

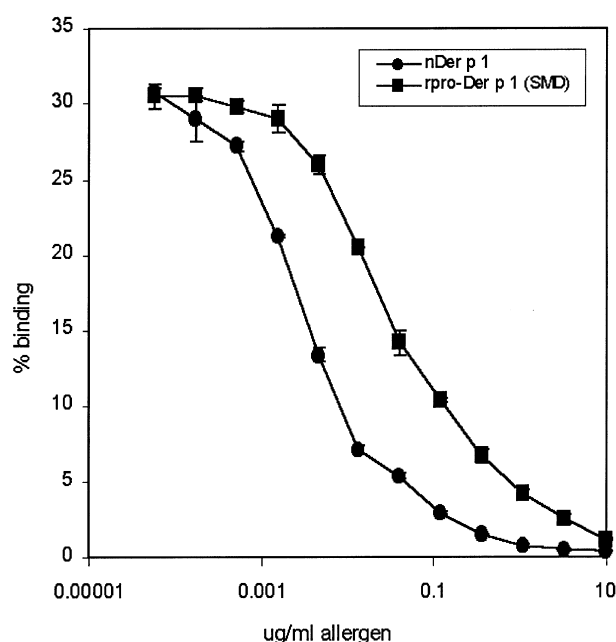


**Fig. 4. Competitive RIA.** rpro-Der p 1 was 9.2 times less effective as an inhibitor than nDer p 1 in a competitive RIA with rabbit anti-(Der p 1) Ig and radiolabeled purified nDer p 1. Error bars show the range between duplicates.

wild-type strain facilitates maturation of Der p 1. Results were, however, essentially identical with those observed for GS115-produced rpro-Der p 1 (Fig. 1). No spontaneous maturation occurred. Only after deglycosylation was some mature Der p 1 detected.

#### Autocatalytic processing of rpro-Der p 1

Methods described for autocleavage of cysteine proteases [21,22] which were performed for rDer f 1 [20] (buffer exchange to pH 4.0) and rpro-Der p 1 [18] [buffer exchange to pH 4.0, addition of cysteine, and heating to 60 °C (with/

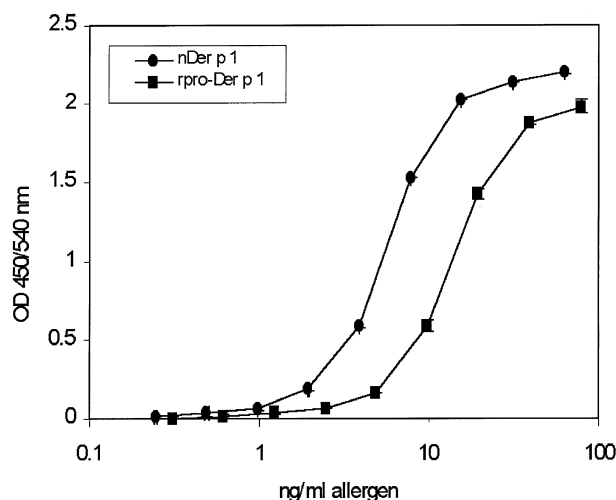


**Fig. 5. Der p 1 ELISA.** rpro-Der p 1 was 2 times less potent in binding to the monoclonal antibodies used in this ELISA than nDer p 1. Error bars show the range between duplicates.

without SDS)] did not result in maturation of the recombinant pro-allergen (data not shown).

#### Proteolytic cleavage of recombinant pro-Der p 1

As autocatalytic cleavage was not achieved, enzymatically active natural Der p 1 was evaluated as a tool to induce maturation of rpro-Der p 1. Incubation of <sup>125</sup>I-labeled rpro-Der p 1 with crude mite extract and affinity-purified nDer p 1 for 4 h at room temperature did result in dose-dependent cleavage (Fig. 6A). A band with similar molecular mass to that of the prosequence appeared with increasing intensity on addition of increasing doses of nDer p 1. Surprisingly, no clear band of mature Der p 1 was detected, although a smear became visible slightly below the molecular mass of rpro-Der p 1. The approach was repeated with nonradiolabeled rpro-Der p 1. To separate natural and recombinant mature Der p 1, enzymatically active nDer p 1 was immobilized on Sepharose. Then, the Sepharose was incubated with rpro-Der p 1. Time-dependent maturation was observed, with weak but significant appearance of both mature Der p 1 (25 kDa) and the cleaved propeptide (Fig. 6B). The 25-kDa mature band was recognized by rabbit antibodies against nDer p 1, confirming the identity of the 25-kDa band as Der p 1 (not shown). The 10-kDa fragment referred to as the propeptide was also recognized by these polyclonal rabbit antibodies. The total cleavage product was subsequently radiolabeled and separated by size-exclusion chromatography. Four peaks were detected, two of which were again identified as mature Der p 1 and the prosequence, respectively (Fig. 6C).

#### DISCUSSION

In this study, successful high-level expression of recombinant pro-Der p 1 is reported. The recombinant protein proves to be hypoallergenic as it has less than 5% of the biological activity of its natural counterpart, although IgE binding in RAST decreases only twofold. Immunoreactivity as studied by competitive RIA and sandwich ELISA was also effected. The limited decrease in reactivity observed in the sandwich ELISA suggests that both monoclonal antibodies used are relatively insensitive to the structural differences between rpro-Der p 1 and nDer p 1. These discrepancies stress the need to analyze allergenicity of candidate hypoallergenic recombinants not only in IgE-binding tests such as RAST, ELISA, and immunoblot, where allergen saturation is usually reached, but also in biological assays such as histamine-release assays and the skin prick test. Discrepancies between serological and biological activity were also reported in studies on Bet v 1, in which it was shown that some mAbs enhanced IgE binding up to fivefold, without influencing histamine-releasing capacity [36,37]. In the sandwich ELISA, purified nDer p 1 was also compared with a crude *D. pteronyssinus* extract that was calibrated on the WHO standard in international units (not shown). This analysis showed that the conversion factor that is generally used, of 1 IU Der p 1 being equivalent to 0.125 ng, is too high. Our calculations gave similar results as those found by Yasueda *et al.* [38]: 1 IU = 0.05 ng Der p 1.

None of the expression systems used in this study resulted in spontaneous maturation of rpro-Der p 1. To

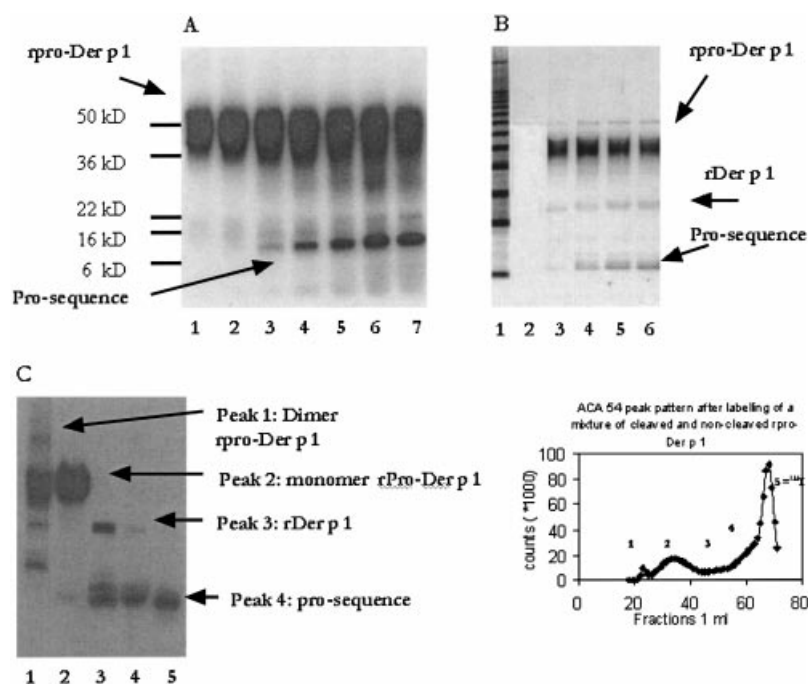


**Fig. 6. Cleavage of rpro-Der p 1 with nDer p 1.** (A) SDS-PAGE/autoradiography. Cleavage of  $^{125}$ I-labelled recombinant pro-Der p 1 facilitated by purified nDer p 1. Lane 1, 0 h rpro-Der p 1; lane 2, +0.37  $\mu$ g nDer p 1; lane 3, +0.74  $\mu$ g nDer p 1; lane 4, +1.48  $\mu$ g nDer p 1; lane 5, +2.96  $\mu$ g nDer p 1; lane 6, +4.44  $\mu$ g nDer p 1; and lane 7, +5.92  $\mu$ g nDer p 1. All incubated for 5 h at room temperature.  $M_r$  compared to SeeBlue Plus 2 pre-stained standards (Novex). (B) SDS-PAGE/silverstaining. rpro-Der p 1 incubated with nDer p 1 coupled to Sepharose. Lane 1, 10 kDa ladder (Life technologies); lane 2, control NaCl/P; lane 3, 2 h incubation; lane 4, 1 night; lane 5, 2 nights; lane 6, 3 nights. (C) SDS-PAGE/autoradiography. rpro-Der p 1 was incubated for 2 nights with Sepharose coupled nDer p 1, subsequently radiolabeled ( $^{125}$ I) and separated by ACA 54 size exclusion chromatography. Five different fractions were analyzed by SDS-PAGE/autoradiography, revealing: lane 1, dimerized rpro-Der p 1; lane 2, monomeric non-cleaved rpro-Der p 1; lane 3, mature rDer p 1; lane 4, containing both mature rDer p 1 and pro-peptide; lane 5, pro-peptide.  $M_r$  compared to SeeBlue Plus 2 pre-stained standards (Novex).

the best of our knowledge, we have copied the conditions for expression that were claimed to result in spontaneous maturation by Best *et al.* [24]. The only difference is that they optimized codon usage for expression in *Pichia*. It seems unlikely that codon usage can be at the basis of differences in post-translational processing. The lack of induction of maturation of rpro-Der p 1 after dialysis to pH 4.0 observed in our study contrasts with observations reported by Yasuhara *et al.* [23] for rpro-Der f 1. The main difference between their approach and ours is that in the present study maturation was attempted with affinity-purified rpro-Der p 1 whereas Yasuhara *et al.* directly used *Pichia* culture medium containing the proenzyme. Possibly yeast-derived proteases facilitated the maturation process.

Both the propeptide and the mature sequence of Der p 1 contain a putative N-glycosylation site, although Jacquet *et al.* have reported that only the asparagine in the propeptide is glycosylated [18]. In accordance with this, lack of detectable N-linked glycans on the mature natural allergen was implicated by the observation that Endo H treatment (cleaving off high-mannose and hybrid N-glycans) did not affect nDer p 1. In contrast, Endo H treatment of our rpro-Der p 1 resulted in a shift of  $\approx 20$  kDa in apparent molecular mass on SDS/PAGE. From these results, it cannot, however, be concluded whether this is a result of cleavage of N-glycans from one or both glycosylation sites present in the sequence of pro-

Der p 1. The insensitivity of nDer p 1 to Endo H does not mean that the original claim that nDer p 1 is a glycoprotein is incorrect [1]. Analysis with several lectins revealed that nDer p 1 most likely carries O-linked glycans with a core disaccharide galactose  $\beta(1-3)$  N-acetylgalactosamine that forms the core unit of O-glycans (except in yeast glycoproteins). Endo H treatment did have a strong effect on rpro-Der p 1. On removal of N-glycans, spontaneous maturation was observed. These data suggest that hyperglycosylation of rpro-Der p 1 in *P. pastoris* might be an important factor in preventing maturation. The results with Endo H support the hypothesis that a large high-mannose structure on the pro-allergen could block cleavage of the propeptide. Maturation was also observed when the recombinant proenzyme was incubated with its enzymatically active natural counterpart. This process was, however, still far from efficient. Cleavage of radiolabeled rpro-Der p 1 did not result in any detectable mature rDer p 1. Cleavage was, however, occurring because the propeptide was clearly detected. When the enzymatic cleavage was repeated with nonradiolabeled rpro-Der p 1 and nDer p 1 immobilized on Sepharose, mature rDer p 1 was detected. Most likely, the mature part of rpro-Der p 1 is not efficiently substituted with  $^{125}$ I in the presence of the propeptide, in contrast with the recombinant mature Der p 1 after removal of the propeptide.

In summary, enzymatically inactive rpro-Der p 1 with significantly decreased IgE-binding capacities was produced

at high expression levels in *Pichia*. Both the lack of enzymatic activity and the hypoallergenic character make this recombinant a potential safe candidate for application in allergen-specific immunotherapy. To further evaluate the potential of this approach, future investigations must examine whether naturally occurring human cysteine proteases could transform hypoallergenic rpro-Der p 1 into biologically active mature Der p 1.

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