Activation mechanism of recombinant Der p 3 allergen zymogen; contribution of cysteine protease Der p 1 and effect of propeptide glycosylation

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Running title: proDer p 3 activation mechanism

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The trypsin-like protease Der p 3, a major allergen of the house dust mite *Dermatophagoides pteronyssinus* is synthesized as a zymogen, termed proDer p 3. No recombinant source of Der p 3 has been described yet and the zymogen maturation mechanism remains to be elucidated. The Der p 3 zymogen was produced in *Pichia pastoris*. We demonstrated that the recombinant zymogen is glycosylated at the level of its propeptide. We showed that the activation mechanism of proDer p 3 is intermolecular and is mediated by the house dust mite cysteine protease Der p 1. The primary structure of the proDer p 3 propeptide is associated with a unique zymogen activation mechanism, which is different from those described for the trypsin-like family and relies on the house dust mite papain-like protease Der p 1. This is the first report of a recombinant source of Der p 3 (rDer p 3), with the same enzymatic activity as the natural enzyme and trypsin. Glycosylation of the propeptide was found to decrease the rate of maturation. Finally, we showed that rDer p 3 is inhibited by the free modified prosequence TP1R.

House dust mite allergens have been shown to be causative factors of allergic manifestations such as atopic dermatitis, perennial rhinitis or bronchial asthma. More than 80% of patients suffering of allergic asthma are positive to mite extracts and have large amounts of IgE specific for mite allergens. In Europe, the most prevalent species of house dust mites are *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) and *Dermatophagoides farinae* (*D. farinae*), depending on relative humidity and temperature. Not less than 23 groups of allergens were identified from extracts of these species (http://www.allergen.org) (1,2).

Although the group 1 and 2 allergens were extensively studied, those from group 3 were poorly characterized. In *D. pteronyssinus*, the allergen of this group has been first identified by Stewart *et al.* (3) as a trypsin-like protease termed Der p 3. The binding of IgE from sera of allergic patients to Der f 3, a *D. farinae* protease homologous to Der p 3, appears to depend on the purity of the allergen, the tested patient populations and the sensitivity of the technique (4). Whereas the frequency of IgE reactivity measured towards Der p 3 was approximately 70-80%, suggesting that Der p 3 is a major allergen (5), a recent study determined a lower allergenic potency similar to mite allergen groups 8 and 10 (6). On the basis of both sequence comparisons and enzymatic studies, Der p 3 has been classified among the trypsin-like proteases from the S1A serine proteases family. Two other serine proteases with chymotryptic and collagenolytic activities and showing 36% and 76% of identity
with Der p 3, were also identified in *D. pteronyssinus* extracts and were termed Der p 6 and Der p 9 respectively (7-9).

The trypsin-like protease Der p 3 displays 47% of identity with salmon trypsin and 45% with bovine trypsin (3,10,11). All residues involved in the catalytic activity (i.e. the catalytic triad His 51, Asp 96 and Ser 196), in the substrate specificity, and the six cysteines engaged in disulphide bridges formation, are highly conserved (10). In addition to their allergenic properties, the enzymatic activity of group 3 allergens has been shown to enhance the inflammatory process, either by activating lung epithelial cells through cleavage of the protease-activated receptor 2 (PAR-2), or by proteolytic processing of proteins C3 and C5 leading to formation of anaphylatoxins C3a and C5a. These peptides are known to act directly on small blood vessels, smooth muscles, mast cells and peripheral blood leukocytes (12,13). Analysis of Der p 3 cDNA reveals that the protease is synthesized as a prezymogen (preproDer p 3) formed by a signal peptide of 18 amino acids, an N-terminal propeptide of 11 amino acids and a domain of 232 amino acids (10). After cleavage of the signal peptide, the zymogen (proDer p 3) matures into a protease of 232 residues (Der p 3) that cleaves peptide bonds after arginine or lysine residues.

Maturation of the zymogen is associated with cleavage and release of the inhibitory propeptide, leading to the desinhibition of the proteolytic activity. Cleavage of the propeptide implies the recognition of a specific activation site, either by another protease (intermolecular mechanism) or by the catalytic domain of the zymogen itself (intramolecular mechanism) (14). Although the proDer p 3 activation process is still unknown, maturation of the human trypsinogen has been widely described and can occur according to different mechanisms. First of all, human trypsinogen is known to be activated by an intermolecular mechanism, in which the duodenal enterokinase, a transmembrane serine protease, can cleave the zymogen into active trypsin (15). An alternative mechanism, associated with hypercalcemia, involves an intramolecular processing and leads to pancreatitis (16,17).

In trypsin-like proteases, the length of the propeptide varies between 4 and 24 amino acids. The motif commonly found at the C-terminal extremity of propeptide is a polyaspartyl sequence followed by a lysine residue (-DDDDK<sub>P1</sub>) at P1 position (18) according to the Schechter and Berger nomenclature (19) for the description of protease subsites. Surprisingly, this polyaspartyl sequence is not conserved in the Der p 3 propeptide (Fig. 1). Furthermore, it contains a potential N-glycosylation site at position P3 and, most interestingly, a threonine at its C-terminal extremity instead of a residue such as lysine or arginine, reported to be associated with removal of the pro-region, suggests a unique activation mechanism (10,20).

Natural Der p 3, like the other mite proteases Der p 6, Der p 9 and Der p 1, another mite protease, is more abundant in faecally enriched dust mite extracts than in body extracts, suggesting their implication in mite digestion (3,21,22). Der p 1 is a *D. pteronyssinus* cysteine protease, which is also synthetized as an inactive zymogen termed proDer p 1. The Der p 1 zymogen is able to recognize its own C-terminal propeptide extremity (-LNAE<sub>P1</sub>) and can autoactivate at acidic pH (23).

Unfortunately, the quantities of purified Der p 3 allergen from the faecal pellets are very low (10), probably due to autolysis or degradation by other proteases during the purification process, as indicated by the low molecular mass products of Der p 3 found in dust extracts (3). In order to study the implication of Der p 3 in allergy, it is thus really important to develop strategies for producing a pure and active recombinant form of the allergen. To date, only two studies reported the expression of proDer f 3 or mature Blo t 3 as fusion proteins with glutathione-S-transferase in *Escherichia coli* (*E. coli*). The authors suggested that the proteins did not exhibit the appropriate conformation indicating a possible implication of the propeptide in the correct folding of the protease (20,24).

In this study, we report the production of correctly folded, N-glycosylated and inactive recombinant Der p 3 zymogen in *Pichia pastoris*. With the use of recombinant Der p 1 (rDer p 1) and house dust mite (HDM) extracts, we highlighted a particular activation mechanism of the zymogen, depending on the house dust mite cysteine protease Der p 1. We have determined the impact of proDer p 3 propeptide glycosylation in the activation kinetics. Finally, we have explored the interaction of the enzyme with its prosequence in terms of inhibition of the enzymatic activity.
Experimental procedures

Chemicals- N-p-Tosyl-Gly-Pro-Arg 7-amido-4-methylcoumarin (MCA) and N-t-Boc-Phe-Ser-Arg-MCA were purchased from Sigma (Saint-Louis, Missouri, USA), Boc-Ile-Glu-Gly-Arg-MCA acetate, Boc-Gln-Ala-Arg-MCA acetate salts, Boc-Gln-Ala-Ala-Arg-p-nitroanilide (pNA), N-succinyl-Ala-Ala-Pro-Phe-pNA and N-succinyl-Ala-Ala-Pro-Leu-pNA were purchased from Bachem (Buttendorf, Switzerland). The cysteine protease inhibitor, L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64), the trypsin inhibitor, 4-amidinophenylmethanesulfonyl fluoride hydrochloride (p-APMSF) and general serine proteases inhibitor, Soybean Trypsin Inhibitor (SBTI), were obtained from Sigma (Saint-Louis, Missouri, USA). Unglycosylated recombinant mature Der p 1 (rDer p 1) was obtained from P. pastoris SMD1168 strain with the pPICZ α expression vector (Invitrogen, Groeningen, The Netherlands) previously restricted with the same enzymes.

Expression of Recombinant proDer p3 in Pichia pastoris- Expression plasmid pPIC9K contained the proDer p 3 gene (A. Jacquet, unpublished work) cloned downstream from the S. cerevisiae α factor signal peptide. P. pastoris SMD1168 strain was transformed with expression vector pPIC9K-proDer p 3 by electroporation. Transformants carrying the HIS4 gene were grown on histidine-deficient medium (RDB). Clones with multiple integrated copies were further selected for resistance to increasing Geneticin (G418, Gibco, Paisley, Scotland) concentrations (0.25-3 mg/ml). For production of proDer p 3, the most resistant clone was grown in 400 ml of Buffered Glycerol-complex Medium (BMGY) at 30 °C up to an A600 value of 2 to 6. The culture was then transferred into 3 L of BMGY in a 5 L Sartorius fermentor and grown during 24 hours at 30 °C. During the next 12 hours, glycerol feed rate was regulated by following the dissolved oxygen level. At an A600 value of approximately 100, culture was induced with methanol to a final concentration of 0.5 %. During 24 hours, the methanol feed rate was regulated to maintain a dissolved oxygen level of minimum 30 %. Culture was then centrifuged at 13000 g during 20 min and the supernatant was stored at -20 °C.

After electroporation of P. pastoris SMD1168 strain with the pPICZα expression vector containing the proDer p 3 (N9Q) sequence, transformants were selected on Yeast extract Peptone Dextrose (YPD) medium containing Zeocine (50 µg/ml) (Invitrogen). In the pPICZα, the sequence of interest is also cloned downstream from the S. cerevisiae α factor signal peptide. The expression of the mutant was then tested in 100 ml of BMGY at 28 °C up to an A600 value of approximately 1. The culture was centrifuged during 10 min at 5000 g. The pellet was resuspended into 100 ml of Buffered Methanol-complex Medium (BMMY, 0.5 % methanol) for

Construction of the proDer p 3 (N9Q) expression vector- The N9Q proDer p 3 mutant was constructed by PCR from the proDer p 3 sequence using primers introducing the restriction sites for EcoR I and Xba I, respectively 5’-ATC-GAA-TTC-AAT-CCG-ACC-ATG-TGT-GGC-GGC-GAA-AAA-GCA-CTG-3’ and 5’-CGA-TTG-GAT-TGA-ATC-TAA-ACG-TAG-CCA-GTG-ATC-TAG-AA-T-A-3’. The amplified fragment was cloned into pGEM-T easy vector (Promega, Madison, USA). The presence of the N9Q mutation was verified by DNA sequencing. The proDer p 3 (N9Q) sequence was isolated by digestion of the pGEM-T easy vector with EcoR I-Xba I and was subsequently cloned into the pPICZα vector (Invitrogen, Groeningen, The Netherlands) previously restricted with the same enzymes.

Expression of Recombinant proDer p3 in Escherichia coli- The expression plasmid pET 15b containing proDer p 3 sequence (A. Jacquet, unpublished work) was transformed into E. coli Origami™ 2(DE3) cells (Novagen, Nottingham, UK). The transformants were selected on Luria-Bertani agar plates containing ampicillin (100 µg/ml) at 37 °C. For expression, transformants were grown in a LB solution containing ampicillin (100 µg/ml) at 37 °C. The cultures were induced with 0.5mM IPTG and samples were collected after 2, 4 and 16 hours. The samples were centrifuged at 4000 g during 20 min and the supernatant (soluble fraction) and the pellets (insoluble fraction) were analysed by SDS-PAGE.

Construction of the proDer p 3 (N9Q) expression vector- The N9Q proDer p 3 mutant was constructed by PCR from the proDer p 3 sequence using primers introducing the restriction sites for EcoR I and Xba I, respectively 5’-ATC-GAA-TTC-AAT-CCG-ACC-ATG-TGT-GGC-GGC-GAA-AAA-GCA-CTG-3’ and 5’-CGA-TTG-GAT-TGA-ATC-TAA-ACG-TAG-CCA-GTG-ATC-TAG-AA-T-A-3’. The amplified fragment was cloned into pGEM-T easy vector (Promega, Madison, USA). The presence of the N9Q mutation was verified by DNA sequencing. The proDer p 3 (N9Q) sequence was isolated by digestion of the pGEM-T easy vector with EcoR I-Xba I and was subsequently cloned into the pPICZα vector (Invitrogen, Groeningen, The Netherlands) previously restricted with the same enzymes.
the expression at 28 °C during 3 days. The culture was centrifuged at 10000 g during 10 min and the supernatant stored at -20 °C.

Purification of Recombinant WT proDer p 3 and Der p 3- 1 liter of the culture supernatant containing proDer p 3 was filtered through a 0.45 µm filter (Millipore, Billerica, USA) and dialysed overnight at 4 °C against a 20 mM ethanolamine buffer pH 9.5 (buffer A). The solution was then stirred with 200 ml of Qstreamline exchanger (Amersham Biosciences, GE Healthcare, Uppsala, Sweden) equilibrated with buffer A at 4 °C. The exchanger was washed with buffer A and then packed into a 250 ml column (Amersham Biosciences, GE Healthcare, Uppsala, Sweden). Bound proteins were eluted stepwise with buffer A added with 1 M NaCl. After SDS-PAGE analysis, fractions containing proDer p 3 were pooled and dialysed at 4 °C against a 20 mM ethanolamine buffer pH 9 (buffer B). Solution was then loaded onto a Q-HP sepharose column (60 ml) (2.6x10 cm, Amersham Biosciences, GE Healthcare, Uppsala, Sweden) equilibrated with buffer B. The flowthrough containing proDer p 3 was dialysed at 4 °C against 20 mM sodium citrate pH 6.5 (buffer C) before purification on an S-HP sepharose column (25 ml) (1.6x10 cm, Amersham Biosciences, GE Healthcare, Uppsala, Sweden) equilibrated with buffer C. The proDer p 3 in the flowthrough was concentrated by ultrafiltration (cut off: 10 kDa) and stored at -20 °C. The concentration of proDer p 3 was estimated by the BCA assay (Pierce, Rockford, USA).

After activation of proDer p 3 by the rDer p 1 protease, mature rDer p 3 was isolated by a fourth purification step with a 1 ml MonoQ column (0.5x5 cm, Amersham Biosciences, GE Health-care, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl pH 8.5 buffer (buffer D). Elution was performed with a linear gradient of buffer D added with 1 M NaCl over 10 column volumes. Fractions containing Der p 3 activity were pooled and dialysed against 20 mM sodium acetate pH 4 before storage at -20 °C.

Western blot analysis- Mutant N9Q and purified proDer p 3, deglycosylated proDer p 3, rDer p 3 and rDer p 1 were denatured at 100 °C in the presence of denaturing buffer and separated by SDS-PAGE (15%). The proteins were electroblotted onto a polyvinylidine difluoride (PVDF) membrane. Immunoblot analyses using polyclonal anti-Der p 3 and anti-Der p 1 antibodies at dilutions of 1/2000 and 1/2500 respectively were carried out and mouse antibodies were detected with BCIP and NTB by using rabbit alkaline phosphatase-conjugated antimouse antibodies.

N-terminal sequencing- The proDer p 3 zymogen, mature rDer p 3 and bands resulting from activation of proDer p 3 by the house dust mite extracts, rDer p 1 and rDer p 3 were sequenced on an Applied Biosystems 476A protein sequencer (Applied Biosystems), based on Edman degradation. Samples were analysed by SDS-PAGE followed by electrophoretic transfer onto a PVDF (polyvinylidine difluoride) membrane (Millipore, Billerica, USA).

Autoactivation of Recombinant glycosylated and unglycosylated proDer p 3 by pH and Ca^{2+}- ProDer p 3 (65 µM final concentration in 300 µl final volume) was incubated at 37 °C for 16 hours in 50 mM polybuffer (mix of 50 mM Tris, citrate, CAPS and potassium chloride) adjusted to desired pH ranging 2 to 12 with NaOH 1 M or HCl 1 M. Assays were analysed by SDS-PAGE and rDer p 3 enzymatic activity was measured as described below.

To analyze the putative autoactivation of proDer p 3 in the presence of Ca^{2+} ions, the zymogen (65 µM final concentration) was incubated at 37 °C in 50 mM polybuffer (pH 8) in the presence of increasing CaCl_{2} concentrations (0 to 20 mM). After periods of time ranging from 15 minutes to
12 hours, aliquots were withdrawn and the rDer p 3 activity was measured. The different samples were analysed by SDS-PAGE.

House dust mite (HDM) extracts activity measurements- The different enzymatic activities of the extracts (125 µg/ml) were measured by following the hydrolysis of Boc-Gln-Ala-Arg-pNA, N-succinyl-Ala-Ala-Pro-Phe-pNA and N-succinyl-Ala-Ala-Pro-Leu-pNA 1mM in Tris-HCl 20 mM pH 7.8 or in PBS pH 7.4 containing 1 mM DTT and 1 mM EDTA during 1 hour at 25 °C in a PowerWave spectrophotometer (λ = 410 nm) (Bio-Tek Instruments, Inc., Winooski, USA). The hydrolysis of Boc-Gln-Ala-Arg-pNA by rDer p 3 (20 nM) and rDer p 1 (100 nM) in Tris-HCl 20 mM pH 7.8 and in PBS pH 7.4 containing 1 mM DTT and 1 mM EDTA respectively were used as controls.

Activation of Recombinant glycosylated proDer p 3 by house dust mite extracts (HDM), rDer p 1 and rDer p 3- ProDer p 3 (150 µM) was incubated at 37 °C for several periods of time with rDer p 1 (1 µM) in 20 mM Na citrate buffer pH 6.5 containing 1 mM DTT and 1 mM EDTA, with rDer p 3 (0.05-0.6 µM) in 20 mM Tris-HCl pH 7.8 or with HDM extracts (2mg/ml) in PBS pH 7.4 containing 1 mM DTT and 1 mM EDTA respectively were used as controls.

Der p 3 enzymatic activity measurements and kinetic parameters determination- Hydrolysis of 10 µM substrate (N-p-Tosyl-Gly-Pro-Arg-MCA, N-t-Boc-Phe-Ser-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA, Boc-Gln-Ala-Arg-MCA, Asn-Ala-Thr-ACC or Asn-Ala-Arg-ACC, see below) by rDer p 3 (325 pM) in 50 mM polybuffer pH 8.5 at 37 °C was followed during 200 seconds in a Perkin Elmer fluorimeter LS 50 B with excitation and emission wavelengths of 380 and 460 nm respectively. Kinetics of hydrolysis were reported as the time-course of MCA (µM) released, using a MCA (Sigma, Saint-Louis, Missouri, USA) standard curve with concentrations ranging from 0 to 1.8 µM. For determination of the rDer p 3 kinetic parameters, the rate of hydrolysis of increasing substrate concentrations (0 to 300 µM) was measured and the data analyzed according to the Henri-Michaelis-Menten equation.

Glycosylated and unglycosylated proDer p 3 activation kinetics- The enzymatic test was adapted from the experimental procedure described for processing of Procathepsins L, B and S (25,26). Glycosylated and unglycosylated proDer p 3 (130 nM) were activated at 37 °C in the presence of increasing concentrations of rDer p 1 (0 to 17 nM) in 50 mM phosphate buffer pH 7.4, containing 150 mM NaCl, 1 mM DTT and 1 mM EDTA. rDer p 3 enzymatic activity was followed continuously, by measuring hydrolysis of Boc-Ile-Glu-Gly-Arg-MCA 10 µM during 30 minutes. Data obtained from activation of glycosylated and unglycosylated proDer p 3 were then fitted to equation (1) (27-31) using the Grafit software Version 5.0.10 (Erithacus Software Ltd.) and the first pseudo-order rate constants (k_{obs}) were calculated for each rDer p 1 concentration.

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P = v_{ss} t + \frac{v_{ss} - v_0}{k_{obs}} (e^{-k_{obs} t} - 1) \quad \text{(Eq.1)}
\]

where, \(P\), \(v_0\), \(v_{ss}\) correspond respectively to the amount of MCA produced (µM), the initial rate for product release (µM/s) and the steady-state rate for product release (µM/s).

Solid phase synthesis of Asn-Ala-Thr-ACC, Asn-Ala-Arg-ACC, NPILPASPNNAT (WT) and NPILPASPNAR (TP1R) peptides- The peptides were prepared by the solid-phase peptide synthesis strategy on a PS3 automated peptide synthetizer (Protein Technologies, Inc, Tucson, USA) using N-α-fluorenylmethoxy carbonyl (Fmoc)-based chemistry on 4-Methylbenzhydryl resin or Fmoc-L-Arg(Pbf)/Fmoc-L-Thr(tBu)-ACC resin amide resin (supplemental data, Fmoc-L-Arg(Pbf)/Fmoc-L-Thr(tBu)-ACC resin synthesis). The 4-Methylbenzhydryl resins (Fmoc-L-Arg(Pbf)-4-Methylbenzhydryl resin and Fmoc-L-Thr(tBu)-4-Methylbenzhydryl resin) were purchased from CBL, Baltimore, Maryland, USA. N-α-Fmoc amino acids (0.4 mmol) were purchased from Iris Biotech GmbH, Marktredwitz, Germany. The side chains of Ser and Thr were protected with the t-butyl derivative (tBu), Arg was protected with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group and Asn was protected with the trityl (Trt) group.
and activating reagents, respectively. Fmoc deprotection at each step was carried out using 20% piperidine/DMF (12 ml). The peptides attached to the resin beads were washed subsequently with DMF, EtOH and dichloromethane (DCM). The side-chain protecting groups were removed and the peptides cleaved from the resin using trifluoroacetic acid/anisole/water (6 ml; 10:1:1) during 3 hours at room temperature. The uncharged resin was separated from the solution by filtration. The crude peptides were precipitated with Et2O and were then purified by reverse-phase HPLC using a semi-preparative C18 column (10X250 mm, 10 µm XTerra Prep RP18, Waters, Milford, Massachusetts, USA) to obtain approximately 50 mg of each peptide. The solvents consisted of an aqueous 0.1% (V/V) trifluoroacetic acid solution and acetonitrile. The elution was carried out in 20 min at a flow rate of 4 ml/min by using a linear gradient from 0 to 40% acetonitrile. Each peptide was characterized after purification by mass spectrometry (ESI-MS, TSQ 7000 Thermoquest Finnigan, positive ionization, 4.5 kV) and the purity was assessed by analytical HPLC. The HPLC chain consisted of a pump (Waters 600) and a UV detector (PDA Waters 996, Milford, Massachusetts, USA), the absorbance at wavelengths between 198 and 400 nm being constantly recorded. The analytical HPLCs were performed on an XTerra RP18 column (150X4.6 mm, 3.5 µm, Waters, Milford, Massachusetts, USA).

Recriment Der p 3 inhibition by its propeptide The model of the Der p 3 protease was established by using the program CPPhmodels 2.0 Server (32) and was based on the available crystallographic structures of trypsin.

Results

Expression of recombinant proDer p 3 Expression of Der p 3 zymogen in Escherichia coli (E. coli) yielded inclusion bodies at all tested temperatures (20, 28 and 37 °C), whereas a soluble form could be obtained in Pichia pastoris (P. pastoris) culture supernatants. To determine whether solubilized E.coli-expressed proDer p 3 was correctly folded, the recombinant allergen was analyzed by infrared spectroscopy and its secondary structure content was compared with that of natural Der p 3 in the amide I and II regions which are highly sensitive to the secondary structure content of proteins. The secondary structure of proDer3 was shown to be different from that of natural Der p 3 in the amide I and II regions (supplemental data, Fig. S1). Particularly, the recombinant protein showed an increase content of antiparallel β pleated sheet. This phenomenon, commonly observed for aggregated and thermally denatured proteins, confirmed that recombinant proDer p3 did not adopt the Der p 3 appropriate folding, most probably because of imperfect denaturation/refolding steps.

In contrast to the zymogen produced in bacteria, the spectra of natural Der p 3 and P. pastoris expressed-proDer p 3 were highly similar in shape and position of their maximum absorbance (1642 cm⁻¹), suggesting that secreted proDer p 3 adopted a fold similar to that of the mature Der p 3 (supplemental data, Fig. S1).

This secreted zymogen could be purified to homogeneity, yielding approximately 120 mg of proDer p 3 per liter of culture supernatant. N-terminal sequencing (YVNPILP-5-) revealed that the expected sequence was preceded by two additional residues (Tyr and Val), resulting from the SnaB I cloning site in the pPIC-9K plasmid. SDS-PAGE (Fig. 2A, lane 1) indicated that proDer p 3 migrates as a broad band of
approximately 40 kDa. It contrasts with the molecular mass of 26327 Da calculated on the basis of the zymogen sequence and suggests the occurrence of large post-translational modifications on the recombinant allergen, likely N-glycosylations because of the presence of one putative N-glycosylation site in the prosequence (-N$_9$AT-). Staining of the electrophoresis gel with the Gel Code Glycoprotein Staining Kit$^\text{®}$ (Fig. 2B), showed that the 40 kDa band corresponding to the proDer p 3 carried glycosylations. Moreover, incubation of the zymogen for 6 h with N-glycosylase F yielded a sharp band at 29 kDa (Fig. 2A, lane 2). Furthermore, analysis by mass spectrometry indicated that deglycosylated proDer p 3 appears as a single peak with the expected molecular mass of 26320 Da.

To confirm that the sugar chain is carried by the propeptide -N$_9$AT-, the asparagine residue at position P3 was replaced by a glutamine by site directed mutagenesis. The N$_9$Q mutant was also expressed in P. pastoris as a secreted soluble form. As shown by western-blotting (Fig. 2C), the N$_9$Q mutant migrated as a single band of approximately 29 kDa, undistinguishable from that corresponding to the N-glycosylase F treated zymogen.

Absence of autoactivation of recombinant proDer p 3- In analogy with the autocatalytic mechanism of trypsinogen maturation (16,17), both glycosylated and deglycosylated proDer p 3 were incubated at different pH (ranging from 2 to 12) and in the presence of increasing Ca$^{2+}$ concentrations (0 to 20 mM), but no effect was observed. Indeed, no decrease of the molecular mass and no rDer p 3 activity could be detected suggesting that maturation of proDer p 3 did not follow an autocatalytic process. This observation suggests an intermolecular activation mechanism of proDer p 3 in which the activity of a mature protease could be involved.

Intermolecular activation mechanism of recombinant proDer p 3- Upon incubation at pH 6.5 in the presence of the mite cysteine protease rDer p 1 (25 kDa), both glycosylated and deglycosylated proDer p 3 were activated, yielding a single band of approximately 29 kDa on SDS-PAGE, with the expected N-terminal sequence of Der p 3 protease (I$_{12}$VGGEKALAG). With glycosylated zymogen, incubation in the presence of rDer p 1 led to the expected molecular mass decrease of approximately 11 kDa, corresponding to the glycosylated propeptide removal (Fig. 3A). Aliquots taken at regular time intervals and analyzed by SDS-PAGE (Fig. 3A) indicate that the propeptide is completely removed within two hours. This proteolytic cleavage of the propeptide is accompanied by an increase of the catalytic activity of the protease (Fig. 3B), as monitored by hydrolysis of the fluorescent tetrapeptic substrate, IEGR-AMC, specific of trypsin-like proteases (not cleaved by rDer p 1 in these conditions) and previously described as a specific substrate for the natural Der p 3 (Fig. 3B).

Propeptide removal, as followed by SDS-PAGE (Fig. 3A) and zymogen activation, as measured by substrate hydrolysis (Fig. 3B), seem to occur over the same time scale, suggesting that intermolecular activation of proDer p 3 occurs in one single step, corresponding to the loss of the entire propeptide.

Utilisation of rDer p 1 previously incubated with 100 µM E-64, an inhibitor of cysteine proteases, did not result in activation of proDer p 3 (supplemental data, Fig. S2A). Indeed, even after 6 hours of contact, neither propeptide nor Der p 3 activity could be detected (data not shown). This demonstrates the essential role of the rDer p 1 cysteine protease activity in the maturation process of proDer p 3.

In the course of the proDer p 3 activation process, a minor band of approximately 19 kDa was also detected (Fig. 3A). It is characterized by the A$_{117}$VGLP N-terminal sequence and corresponds to the hydrolysis of -NAK$_{114}$A$_{115}$VGLP- in the mature recombinant Der p 3 (rDer p 3). Interestingly, a corresponding degradation site was previously reported for human trypsin (\textit{-NAR$_{117}$V$_{118}$STIL-}) (33). To determine the sensitivity of rDer p 3 to hydrolysis, pure rDer p 3 was incubated in 50 mM polybuffer pH 8.5 at 37 °C in presence or absence of rDer p 1 and its enzymatic activity was monitored after different times ranging from 0 to 240 hours. Figure 3C shows that, total inactivation of rDer p 3 occurs within 240 min in these conditions. rDer p 1 appears to have a minor role in this phenomenon, as seen by the inactivation of rDer p 3 in the absence of the cysteine protease. The predominant role of Der p 3 in its own degradation was firstly confirmed by the addition of 100 µM E-64 10 min after starting proDer p 3 activation in the presence of rDer p 1. SDS-PAGE analysis (supplemental data, Fig. S2B) shows that upon addition of E-64, rDer p 1 is totally inhibited (\textit{i.e.} the maturation process of proDer p 3 is stopped), whereas both proDer p 3 and Der p 3...
bands diminish continuously in intensity, leading to low molecular mass degradation fragments. Furthermore, the increase in rDer p 3 activity measured for the first 10 min of the experiment was stopped and a progressive decrease followed, due to Der p 3 autolysis (Fig. 3B). Secondly, after the activation of proDer p 3 by rDer p 1 in the presence of 10 mM benzamidine, a reversible inhibitor of trypsin-like proteases, a larger Der p 3 activity was recorded after 5000-fold dilution of the samples (Fig. 3B) and proteolysis of the active Der p 3 was decreased (supplemental data, Fig. S2C).

The possibility of inter-molecular activation of proDer p 3 (150 µM) by rDer p 3 (after its purification, see below) was also tested. Upon SDS-PAGE, rDer p 3 appeared to process proDer p 3 into two major bands of 29 kDa and 19 kDa but more slowly than rDer p 1 (Fig. 3A’). Whereas the 19 kDa degradation band corresponds to the previously identified fragment of Der p 3 (A_{18}LAG-), the 29 kDa band has the A_{18}LAG-N-terminal sequence which follows the -GEK_{17}- residues in the Der p 3 sequence. As expected, rDer p 3 is not able to recognize the Thr at the C-terminal extremity of the proDer p 3 propeptide but cleaves 6 residues downstream lysine 17. No Der p 3 activity could be measured however, suggesting that the truncated form of Der p 3 (A_{18}LAG-) obtained directly from the maturation of thezymogen under these conditions is not active (Fig. 3B).

ProDer p 3 activation by house dust mite (HDM) extracts- Several proteases with different specificities were identified in the HDM extracts, including the cysteine protease Der p 1 of the papain-like family, the trypsin-like Der p 3, the chymotrypsin-like Der p 6 and the elastase/collagenase-like Der p 9 (3,7-9,11,22). Both cysteine and serine protease activities were detected in the HDM extracts (Table I) with specific substrates showing a predominant chymotrypsin-like activity in the extracts. The QAR-pNA substrate was used to measure cysteine (Der p 1, (34)) and trypsin-like (Der p 3, (35)) activities, AAPF-pNA, the chymotrypsin-like activity of Der p 6 (but it is also cleaved by Der p 9, (9,11)) and AAPL-pNA, the elastase activity of Der p 9 (11). The activities of rDer p 1 and rDer p 3 were used as controls. Table I shows that 100 µM E-64 inhibits specifically rDer p 1, leading to a decrease of the QAR-pNA hydrolysis in HDM extracts. Indeed, in these conditions, only the contribution of Der p 3 (trypsin-like protease) is detected with this substrate. The specific inactivator of trypsin-like proteases, APMSF (200 µM) inhibits only the Der p 3 activity whereas the general inhibitor of serine proteases SBTI (10 µM) does not affect Der p 1. Only chymotrypsin-like and elastase activities could be measured in the presence of a combination of E-64 and APMSF.

The contribution of the different HDM extracts enzymatic activities to the proDer p 3 maturation was explored. To determine specifically the role of the cysteine protease, trypsin-like and chymotrypsin-like/elastase activities, glycosylated proDer p 3 was incubated (5 to 60 min) with HDM extracts previously inhibited with E-64, APMSF/SBTI or APMSF/E-64. The incubation of proDer p 3 with uninhibited HDM extracts leads to its complete processing after 15 min (Fig. 4A) into a band of 29 kDa with the Der p 3 N-terminal sequence (I_{12}VGGE-) and in several fragments. In the presence of E-64, the proDer p 3 cleavage was very limited and led to the formation of low proportions of truncated and inactive Der p 3 (A_{18}LAG-) (Fig. 4A). These observations confirm the essential role of the cysteine protease activity in the activation of proDer p 3 into a mature and active protein. The addition of APMSF and SBTI to the HDM extracts did not impair the maturation of proDer p 3 into Der p 3 (I_{12}VGGE-) and further proteolysis was very limited (Fig. 4B) as shown for the maturation of proDer p 3 by rDer p 1 in the presence of benzamidine (see above). These observations indicate that the serine proteases are more involved in Der p 3 inactivation than in its formation. Moreover, upon incubation of proDer p 3 with HDM extracts previously treated with APMSF and E-64, only degradation bands were formed thus excluding the involvement of chymotrypsin-like or elastase activities in the formation of active Der p 3 (Fig. 4B). The N-terminal sequences of Der p 3 or degradation bands obtained in the different proDer p 3 activations experiments are summarized in the Table II.

Effect of glycosylation on proDer p 3 maturation rate- Increase of the catalytic activity of rDer p 3 during its intermolecular activation by rDer p 1 could be monitored in real time (see experimental procedures) (Fig. 5A). Under the experimental conditions described in Figure 5, proDer p 3 displayed a very low activity, which remained constant over the time scale of the experiment (≤ 30 min). A similar phenomenon was reported with
trypsinogen (36). In contrast, the rate of product formation (AMC) increased during the maturation of proDer p 3 and the rate of the activation process increased with the concentration of rDer p 1 (Fig. 5A and 5B). For each proDer p 3 maturation curve, the constant velocities of rDer p 3 reached were different indicating that the real steady state, corresponding to complete activation of thezymogen, was not reached. This phenomenon could be correlated with the degradation of rDer p 3 after its activation until a steady state between the two processes was reached. For these reasons, the results were only interpreted in a qualitative manner. The kinetics were fitted to equation 1 to give pseudo first order rate constants (k_{obs}) values. These values were then plotted as a function of the rDer p 1 concentration (Fig. 5C). The observation that k_{obs} increase linearly with the rDer p 1 concentration suggests an intermolecular activation process. Moreover, in the absence of rDer p 1, extrapolation of the k_{obs} values confirm that no intramolecular process occurs (i.e. k_{obs} = 0). Data, in Fig. 5C, indicate that the non-glycosylated proDer p 3 is activated faster than its glycosylated counterpart. This can be explained by the situation of the N-glycosylation site in the propeptide of proDer p 3 zymogen, only three residues upstream the activation cleavage site, which could thus well represent a steric hindrance to rDer p 1.

Recombinant Der p 3, a trypsin-like protease-

Following activation in the presence of rDer p 1, recombinant Der p 3 was purified to homogeneity, using a high resolution Mono Q column. Western-blot analysis (Fig. 6) using anti-Der p 1 and anti-Der p 3 antibodies confirmed that the rDer p 1 protease could be completely removed from the preparation (lanes 2 and 2'). Furthermore, N-terminal sequencing resulted in the expected Der p 3 sequence (I_{12}VGGEKALAG-), with no significant amount of contaminant.

The pH activity profile of rDer p 3 was determined between pH 2 and 12, in a 50 mM polybuffer, using IEGR-AMC (10µM) as substrate. Figure 7 shows that Der p 3 retains activity between pH 5 and 11, with a maximum at pH \( \approx 8.5 \); a similar behaviour has been reported for trypsin and natural Der p 3 (11,35). In addition, the percentage of activity recovered after readjusting the pH value to 8 (using 100 mM polybuffer, pH 8) was measured for all samples incubated in the pH 2-12 range (Fig. 7). Complete return of activity could be achieved for rDer p 3 samples incubated for 1 hour at pH values ranging from 4 to 11.

The catalytic parameters of rDer p 3 were measured with four different synthetic substrates (Table III). In agreement with the behaviour reported for trypsin (35), rDer p 3 shows little specificity for residues in P2 and P3 positions. Interestingly, its catalytic efficiency is approximately 50 fold higher than that of the rDer p 1 protease measured with the substrate QAR-AMC. We synthesized the NAT-ACC substrate corresponding to the C-terminal extremity of the proDer p 3 propeptide and NAR-ACC as control to highlight the potential recognition of its own propeptide by Der p 3. Whereas the catalytic efficiencies of rDer p 3, measured with the QAR-AMC and NAR-ACC are similar, the NAT-ACC substrate was not cleaved by the protease. This observation is in agreement with a specific trypsin-like activity (i.e. specific cleavage after Arg or Lys (35)) and corroborates the absence of the release of the I_{12}VGGE- Der p 3 form after cleavage of proDer p 3 by rDer p 3.

Specific inhibition of rDer p 3 by the wild-type (WT) and modified (T_{P1}R) propeptides-

Initial rate measurements in the presence of increasing concentrations (up to 5 mM) of the wild-type propeptide indicated only very poor inhibition of the enzyme activity (inhibition constant (K_{i}) >> 5 mM). The single substitution of Thr by Arg at the C-terminal extremity of the propeptide (T_{P1}R mutant) was sufficient, however, to dramatically enhance the inhibitory properties of the peptide. The Hanes plot suggests that the T_{P1}R behaves as a non competitive inhibitor (Fig. 8), with dissociation constants K_{i} (for the rDer p 3-T_{P1}R complex, EI) and K'_{i} (for the rDer p 3-IEGR-AMC- T_{P1}R complex, ESI) of (1,2 ± 0,2) mM and (1,4 ± 0,2) mM respectively. The difference between these values is probably non significant and this indicates that the ternary rDer p 3-substrate-T_{P1}R complex (ESI) formed should be as stable as rDer p 3-T_{P1}R (EI). The linearity of the Dixon plots (1/v vs I) showed that this ternary complex was not catalytically competent (data not shown) but the binding mode of the propeptide analogue to the active enzyme remains undetermined.

Discussion

To date, the trypsin-like allergen Der p 3 was poorly characterized, most probably because the Der p 3 content of whole mite cultures is very low (50 µg / g exhausted mite cultures). In
addition, the purified protease isolated from house dust mite extract is highly unstable due to its degradation (3,10). Consequently, production of recombinant Der p 3 is highly desirable.

The present study reports the successful production of recombinant proDer p 3 in *Pichia pastoris*. By site-directed mutagenesis and glycosidase F treatment, we clearly demonstrated that proDer p 3, produced in yeast, is glycosylated at the level of the potential N-glycosylation site present in the propeptide.

Infrared spectroscopy clearly detected important differences at the level of the overall secondary structures between renatured *E.coli* proDer p 3 and natural Der p 3, as judged by the increase of the β-sheet content in proDer p 3. It must be pointed out that transformation of α-helical structure into intermolecular antiparallel β-sheet is usually observed in aggregated proteins (37,38).

We provided evidences that the Der p 3 precursor form produced in *P.pastoris* adopted the global fold of trypsin-like proteases. As expected, thezymogen was inactive indicating that its propeptide could act as an inhibitor of the protease domain. Indeed, many proteases are synthesized as inactivezymogens. This allows a spatially and temporally controlled activation of the proteases and avoids uncontrolled digestions (14). During the course of evolution of its sequence, the trypsin ArgP1- or LysP1- residues have been selected in the propeptide probably to permit an autoactivation mechanism of trypsinogen. In vertebrates, this intramolecular activation phenomenon has decreased with the appearance of a repetition of two to four Asp residues preceding the P1 amino acid. This motif is associated with the recognition of the propeptide by the enterokinase, a duodenal serine protease which can activate trypsinogen (18). Moreover, these acidic residues permit the control of the trypsinogen autoactivation in defined conditions such as in presence of Ca$^{2+}$ which can neutralize the carboxylate negative charges (18). This mechanism occurs in humans affected by pancreatitis due to hypercalcemia. In this pathology, a high Ca$^{2+}$ concentration (> 1 mM) leads to a premature autoactivation of the trypsinogen associated with an uncontrolled digestion of the pancreatic cells (16,17,39). Our results indicate that proDer p 3 cannot be activated in presence of Ca$^{2+}$ and/or by incubation at different pH. The lack of a polyaspartyl motif in its prosequence and the replacement of the ArgP1- or LysP1- residue by a threonine residue at the C-terminal extremity of the propeptide (Fig. 1) can explain our observations. Indeed, the trypsin-like proteases are known to specifically cleave peptide bonds after arginine or lysine residues (18,35). In addition, the expression of the recombinant homologous proDer f 3 in fusion with glutathione S transferase was previously described by Nishiyama et al. In this study, proDer f 3 activation was unsuccessful suggesting that another protease present in dust mites could be responsible for thezymogen activation (20).

The mite Der p 1 cysteine protease belonging to the papain-like protease family, abundant in mite faeces, could be co-localized with the proDer p 3 zymogen during the digestion of the mite. We evaluated the putative maturation of proDer p 3 under the action of rDer p 1 in *in vitro*. Under our experimental conditions, the incubation of recombinant proDer p 3 with rDer p 1 led to mature rDer p 3 (Fig. 3A). This activation mechanism occurred in one step. It corresponded to the recognition and cleavage of the propeptide of proDer p 3 as seen by N-terminal sequencing which revealed only the sequence corresponding to the mature Der p 3 form (I$_{12}$VGGEKALAG-). The enzymatic activity of rDer p 3 released, after increasing times of incubation with rDer p 1 (Fig. 3B), was correlated to the molecular mass changes (Fig. 3A). This proDer p 3 maturation was totally abolished in the presence of E-64, suggesting an essential role of the cysteine protease activity in the intermolecular activation of thezymogen. Moreover, we demonstrated that the Der p 1 activity in the HDM extracts was necessary to process proDer p 3 into the mature and active form (I$_{12}$VGGEK-) (Fig. 4A). Another band of approximately 19 kDa corresponding to a degradation of rDer p 3 appeared during the activation of proDer p 3. It corresponds to a cleavage of the peptide bond between the Lys 114 and Ala 115 residues in the Der p 3 sequence – NAK$_{114}$A$_{115}$VGLP-. This proteolysis of Der p 3 was not influenced by the presence of E-64 whereas it was significantly decreased by the addition of benzamidine (Fig. 3B and supplemental data, Fig. 5B and 5C), demonstrating the role of the protease Der p 3 in its own degradation. A similar site of degradation is observed with human trypsin (NAK$_{114}$V$_{115}$STIL-) and could control the half-life of the active enzyme (33,40). Indeed, a natural R117H mutant of human trypsin seems to be involved in hereditary pancreatitis (40).
structure model of Der p 3 (Fig. 9) indicates that the –NAR$^{117}$- and –NAK$^{114}$- degradation sites, in the trypsin and Der p 3 respectively, are situated on the surface of the proteins and are thus accessible to degradation by proteases. The K$^{114}$H mutation in Der p 3 could likely abolish this degradation.

The incubation of proDer p 3 with rDer p 3 clearly indicated that the protease did not recognize the Thr at the C-terminal extremity of its own propeptide but processed the zymogen into two major forms (Fig. 3A') : a nearly complete form, A$^{115}$VGLP- and the degradation fragment A$^{115}$VGLP-. This observation correlates with the specificity of the trypsin-like proteases (see below). The Der p 3 form devoid of the 6 N-terminal residues, previously identified with the natural enzyme (3), was shown to be inactive (Fig. 3B). X-ray structures of trypsin and trypsinogen indicate that the proteins display approximately 85% of structural identity (41-43). These studies show that upon activation of trypsinogen by removal of its propeptide, the new N-terminal Ile16 folds into a pocket and establishes a salt bridge with Asp194 liberating the S1 binding site and the oxyanion hole. More recent investigations demonstrated the essential role of the Ile16 in the stabilization of the catalytic domain for the activity of trypsin (44,45). The lack of activity of the deleted form of Der p 3 obtained after zymogen cleavage by the protease itself could thus be explained by the fact that an N-terminal Ala18 is unable to replace the N-terminal Ile12 to ordering the catalytic domain of the protease.

Other proteases have been identified in the HDM extracts. Der p 6 has been described as a chymotrypsin-like protease which can cleave substrates containing Phe or Tyr in the P1 position (7,8). Der p 9 cleaves SA$_2$PL-pNA and SA$_2$PF-pNA, like the cathepsin G, but also type III collagen (9,11). We explored the potential specific roles of Der p 6 and Der p 9 in the activation mechanism of proDer p 3 by incubation in the presence of the HDM extracts previously treated with APMSF/E-64. We showed that these compounds respectively and specifically inhibit the trypsin-like and cysteine protease activities in the HDM extracts leaving intact the chymotrypsin-like and elastase activities (Table I). The action of these serine proteases on proDer p 3 resulted in the appearance of degradation bands without formation of Der p 3 (Fig. 4B) confirming the essential role of Der p 1 in the activation of proDer p 3 but also excluding the participation of Der p 6 and Der p 9 in this mechanism. However, a recent study described that, like Der p 3, Der p 9 cleaves the PAR-2 receptor of lung epithelial cells after Arg36 (13) suggesting a trypsin-like specificity of Der p 9. In this case, a protease with the same specificity as Der p 3 should cleave proDer p 3 yielding the same major and inactive forms (A$^{115}$LAGE- and A$^{115}$VGLP-). A study on the recombinant Der p 9 should be necessary to further clarify its specificity.

In consequence, we can conclude that the cysteine protease Der p 1 is probably the major activator of proDer p 3 in the house dust mite D. pteronyssinus, whereas the serine proteases (Der p 3, Der p 6 and Der p 9) are involved in the degradation phenomenon.

The intermolecular activation mechanism of proDer p 3 by the cysteine protease Der p 1 could represent one important step of a potential protease activation cascade which could occur in D. pteronyssinus. This proteolytic cascade, depicted in Figure 10, could imply the initial intramolecular maturation of the Der p 1 zymogen under acidic conditions, followed by proDer p 3 maturation, through Der p 1 action. In turn, Der p 3 could be involved both in the mite digestive function and in its own inactivation. The maturation of the proDer p 3 zymogen by the Der p 1 protease could be explained by the specificity of the protease previously studied by Harris et al. (46). Indeed, they showed that Der p 1 was very specific for a P2 alanine residue, a P4 proline residue and polar amino acids in P1 and P3 positions. These observations fit the sequence of the C-terminal extremity of the proDer p 3 propeptide (Fig. 1). Moreover, the proDer p 1 and proDer p 3 zymogens belonging to the papain-like and trypsin-like families have conserved residues at the extremity of their prosequences, -LNAE$_{P1}$ and -PNAT$_{P1}$ respectively. From these results and in vivo observations, the involvement of Der p 1 in the maturation of the proDer p 3 zymogen during digestion in the house dust mite appears to be quite likely. In humans, activation of trypsinogen by a papain-like protease was observed in a type of pancreatitis. In this pathology, cathepsin B, a lysosomal cysteine protease, prematurely activates trypsinogen in trypsin in the pancreas leading to the cell lysis (47-49).

The enzymatic test used to measure the intermolecular activation of proDer p 3 by the rDer p 1
protease clearly showed that no intramolecular activation occurred during the proDer p 3 processing and that the propeptide glycosylation decreased the maturation rate. Unfortunately, in the dust mite extracts, the natural Der p 3 enzyme is only present in its mature form making the study of the potential propeptide glycosylation of the naturalzymogen impossible. However, we observed that, in vitro, the non glycosylated proDer p 3 (the N9Q mutant) was more sensitive to degradation. It has also been shown that the dust mite proDer p 1 precursor contains two potential sites of N-glycosylation, one in the propeptide and one in the mature enzyme, and that the mature protease is glycosylated in its natural and recombinant forms (23,50).

Glycosylation of the propeptide decreases the rate of autoactivation of proDer p 1 (23,50) as well as the rate of proDer p 3 activation by Der p 1. It is very likely that the two phenomenons are correlated since, in both propeptides, the glycosylation site is only three residues away from the cleavage site. This could constitute an additional protection against attacks by other proteases or premature activation in the mite.

The optimum activity pH of rDer p 3 is approximately 8.5 which is in agreement with previous studies on trypsin and natural Der p 3 (11,35). The specificity of rDer p 3 was also explored for the P1, P2 and P3 residues using six different synthetic substrates (IEGR-AMC, GPR-AMC, FSR-AMC, QAR-AMC, NAR-ACC and NAT-ACC). Our data indicate that the residues in P2 and P3 positions have little effect on the activity as demonstrated for trypsin (35). Indeed, the specificity pocket of trypsin-like proteases rests on the presence of an aspartate residue in the P1 binding site which in consequence shows a strong preference for the basic side chains of arginine and lysine (35,42). Indeed, rDer p 3 was not able to cleave the NAT-ACC substrate. This is in agreement with the observed processing of proDer p 3 by rDer p 3 which does not cleave its zymogen after the C-terminal residue of the propeptide (Thr11) but after the first lysine (Lys17) in the Der p 3 domain.

We finally investigated the interaction between the mature rDer p 3 and its free prosequence. We observed that, even at a propeptide concentration of 5 mM, no major decrease of protease activity could be detected. This result could be explained by local differences in the secondary structures of the zymogen and the mature protease. Indeed, L. Gombos et al (51) showed that the maturation of trypsinogen resulted in a small irreversible conformation change in the protease domain without affecting the secondary structures content. A similar event is likely to occur during the maturation process of proDer p 3 and the absence of strong inhibition by the propeptide could be correlated with a modification of the interactions between the protease and its propeptide after hydrolysis of the Thr11-Ile12 bond. A replacement of Thr by Arg at the C-terminal extremity of the propeptide enhanced its affinity for rDer p 3. Interestingly, while no autoactivation mechanism was observed for the proDer p 3 zymogen and for its homologous proDer f 3, Nishiyama et al. showed that the proDer f 3 Tp3R mutant was activated during the fermentation process (20). Therefore, the C-terminal extremity of the proDer p 3 propeptide is really important for the zymogen inhibition and activation mechanisms. Complementary studies with different propeptide mutants would be interesting to determine the amino acids which control this interaction.

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The abbreviations used are: Blot 3, Blomia tropicalis protease, homologous to Der p 3; rDer p 1, recombinant Der p 1; rDer p 3, recombinant Der p 3; E-64, L-trans-epoxysuccinyl-leucylamido (45-64) pepstatin A; HPLC, high pressure liquid chromatography; SEA, small extracellular antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.
guanidino) butane; p-APMSF, 4-amidinophenylmethanesulfonyl fluoride hydrochloride; SBTI, Soybean Trypsin Inhibitor; WT, wild-type; Boc, t-Butyloxycarbonyl; CAPS, 3-(Cyclohexylamino)-1-propanesulfonic acid; IPTG, Isopropyl-β-D-thiogalactopyranoside; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NTB, Nitroblue Tetrazolium; MCA, Methoxy-coumarin-acetic acid; DTT, Dithiotreitol; EDTA, Ethylene diamine tetra acetic; DMF, Dimethylformamide; Fmoc, 9-Fluorenylethoxycarbonyl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; rBu, tert-butyl; Trt, Trityl.

**Figure legends**

Fig 1. PreproDer p 3 sequence. The 18 residue signal peptide is in bold type. The propeptide is underlined and the protease sequence is in italic. Residue numbering starts at the N-terminal residue (Asn) of the propeptide. The arrows indicate the cleavage sites of the signal peptide (1) and of the propeptide (2). The dotted arrow indicates the site where cleavage yields an inactive protein.

Fig 2. Deglycosylation and staining of glycosylated proDer p 3. (A) Glycosylated proDer p 3 (200 µM) before (lane 1) and after (lane 2) a 6 h incubation with N-glycosylase F (1 U per 5 nmol of proDer p 3), in 20 mM citrate buffer, pH 6.5, at 37 °C. (B) Staining of glycosylated and deglycosylated proDer p 3 (200 µM) with the Gel Code Glycoprotein Staining Kit® (Pierce) according to the manufacturer. (C) Immunodetection after western-blotting of proDer p 3 (1 µg, lane 1), deglycosylated proDer p 3 (1 µg, lanes 2) and mutant proDer p 3 N9Q (lane 3, 25 µl culture supernatant) using anti-proDer p 3 polyclonal antibody. St, Unstained or Prestained Protein Molecular Weight Marker (Fermentas GmbH, St Leon-Rot, Germany).

Fig 3. Inter-molecular activation of glycosylated proDer p 3 by rDer p 1 (A) or rDer p 3 (A’). St, Unstained Protein Molecular Weight Marker (Fermentas); *, rDer p 3 degradation band (A115VGLP). (B) For each activation time (0 to 1440 min) of proDer p 3 by rDer p 1 ( ), rDer p 3 ( ), rDer p 1 with addition of 100 µM E-64 after 10 min ( ) and rDer p 1 with addition of 10 mM benzamidine ( ), aliquots were diluted 5000-fold and the rDer p 3 activity was measured using IEGR-AMC as substrate (10µM). (C) After an incubation of rDer p 3 (2.3 µM) in a 50 mM polybuffer pH 8.5 at 37 °C during increasing times (0 to 12 hours) in presence of rDer 1 (0.6 µM) or not, respectively in black and white, the relative activity (%) of rDer p 3 was measured in 1000-fold diluted aliquots using the IEGR-AMC substrate (10 µM). Standard deviations were < 10 %.

Fig 4. Inter-molecular activation of glycosylated proDer p 3 by HDM extracts. (A, B) SDS-PAGE (15 %) analysis; 150 µM glycosylated proDer p 3 (lanes A and A’) was incubated at 37 °C, in 20 mM Na-citrate buffer (pH 6.5) with 1 µM rDer p 1 (lane B) or in 20 mM Tris-HCl (pH 7.8) with 0.2 µM rDer p 3 (lane B’). St, Unstained Protein Molecular Weight Marker (Fermentas); *, rDer p 3 degradation band (A115VGLP). (B) For each activation time (0 to 1440 min) of proDer p 3 by rDer p 1 ( ), rDer p 3 ( ), rDer p 1 with addition of 100 µM E-64 after 10 min ( ) and rDer p 1 with addition of 10 mM benzamidine ( ), aliquots were diluted 5000-fold and the rDer p 3 activity was measured using IEGR-AMC as substrate (10µM). (C) After an incubation of rDer p 3 (2.3 µM) in a 50 mM polybuffer pH 8.5 at 37 °C during increasing times (0 to 12 hours) in presence of rDer 1 (0.6 µM) or not, respectively in black and white, the relative activity (%) of rDer p 3 was measured in 1000-fold diluted aliquots using the IEGR-AMC substrate (10 µM). Standard deviations were < 10 %.

Fig 5. Continuous assay for the glycosylated and deglycosylated proDer p 3 maturation by rDer p 1. Substrate hydrolysis (10 µM IEGR-AMC) versus time curves related to the activation of glycosylated (A) and deglycosylated (B) proDer p 3 (130 nM) by increasing concentrations of rDer p 1 (0, 3, 4, 6.4, 8.5 and 17 nM) in 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, 1 mM DTT and 1 mM EDTA at 37 °C. Curves fitted to equation 1 are shown in gray. (C) Variation of the first pseudo order rate constants (kobs) measured in A (open circles, standard deviations were between 0 and 20 %) or B (black circles, standard deviations < 2 %) with rDer p 1 concentration (nM).

Fig 6. Analysis of rDer p 3 purity. Immunodetection of purified proDer p 3 as control (1 µg, lanes 1 and 1’), rDer p 3 after its purification (1 µg, lanes 2 and 2’) and purified rDer p 1 as control (0.5 and 3 µg for lanes 3,3’ and 4,4’ respectively, molecular mass; 25kDa) by western-blotting using anti-Der p 3 (A) or anti-rDer p 1 (B) polyclonal antibodies. St, Prestained Protein Molecular Weight Marker (Fermentas).
Fig 7. pH activity profile of rDer p 3. Enzymatic activity was measured by using 10 µM IEGR-AMC as substrate. The relative activity (%) of rDer p 3 after a one hour incubation in 50 mM polybuffer, pH ranging from 2 to 12, at 37 °C, is shown in black. The activity recovered after adjusting the pH back to 8 for 1 hour at 37 °C is represented in white.

Fig 8. Hanes plot of the inhibition of rDer p 3 (0.6 nM) by the T_P1R peptide. Substrate concentration (IEGR-AMC)/initial hydrolysis rate vs substrate concentration (10, 30, 50, 75 µM) in the presence of increasing concentrations of T_P1R peptide; 0 ( ), 1.25 ( ), 2.5 ( ) and 5 mM ( ) in 50 mM polybuffer (pH 8.5) at 37 °C.

Fig 9. Der p 3 model structure and human cationic trypsin X-ray structure. (A) Model of Der p 3. The –NAK_115− degradation site is in orange. (B) X-ray structure of human cationic trypsin (2R9P). The –NAR_117− autolysis site is in green.

Fig 10. Proposed proteolytic cascade occurring in the D. pteronyssinus house dust mite. The intermolecular activation of the Der p 3 zymogen by the Der p 1 protease, the autoactivation of proDer p 1 at acidic pH and the degradation of Der p 3 in Der p 3** (A_115VGLP…) are represented by solid arrows. The involvement of Der p 3 in cleavage of its own zymogen in inactive Der p 3* (A_18LAGE…) and degradation (Der p 3**) is suggested by dotted arrows.

Fig S1. FITR spectra of Der p 3 and ProDer p 3. Infrared absorbance spectra in the amide I (1600-1700 cm⁻¹), amide II (1500-1600 cm⁻¹) and amide II’ (1420-1500 cm⁻¹) regions after deuteration of the different proteins. Spectra are normalized at full scale on the amide I band. a: natural Der p 3, b: P. pastoris proDer p 3 and c: E. coli proDer p 3.

Fig S2. Inter-molecular activation of glycosylated proDer p 3 by rDer p 1. SDS-PAGE (15 %) analysis; 150 µM glycosylated proDer p 3 (lanes A, A’, A”) was incubated at 37 °C, in 20 mM Na-citrate buffer (pH 6.5) with 1 µM rDer p 1 (lanes B, B’, B”) in the presence of 100 µM E-64 (A), 100 µM E-64 added after 10 minutes of activation (B) or 10 mM benzamidine (C). St, Unstained Protein Molecular Weight Marker (Fermentas); *, rDer p 3 degradation band (A_115VGLP).
Table I. Enzymatic activities of rDer p 1, rDer p 3 and HDM extracts in presence of PBS pH 7.4 containing 1 mM EDTA and 1 mM DTT or in Tris-HCl 20 mM pH 7.8. The activities can be mainly attributed to the various proteins as follows trypsin-like : Der p 3, cysteine protease : Der p 1, chymotrypsin-like : Der p 6 and elastase-like : Der p 9. See also the text.

| Substrates | rDer p1 | rDer p3 | Trypsin/Cys protease | Chymotrypsin | Elastase |
|------------|---------|---------|----------------------|--------------|---------|
| QAR-pNA    | 4.5 ± 0.4 | 3.4 ± 0.1 | 3.27 ± 0.02 | 6.2 ± 0.4 | 1.1 ± 0.1 |
| E-64       | 0.06 ± 0.06 | 3.4 ± 0.1 | 1.7 ± 0.1 | 5.8 ± 0.3 | 1.04 ± 0.04 |
| APMSF      | 4.4 ± 0.7 | 0.28 ± 0.01 | 1.64 ± 0.04 | 5.3 ± 0.2 | 1.01 ± 0.01 |
| SBTI       | 4.9 ± 0.1 | 0.00 ± 0.00 | 1.44 ± 0.01 | 0.01 ± 0.00 | 0.00 ± 0.00 |
| APMSF + SBTI | 5.1 ± 0.4 | 0.00 ± 0.00 | 1.6 ± 0.3 | 0.014 ± 0.002 | 0.004 ± 0.006 |
| APMSF + E-64 | 0.01 ± 0.00 | 0.22 ± 0.07 | 0.07 ± 0.00 | 5.3 ± 0.3 | 0.98 ± 0.00 |
| APMSF + SBTI + E-64 | 0.01 ± 0.00 | 0.003 ± 0.004 | 0.02 ± 0.01 | 0.02 ± 0.00 | 0.1 ± 0.1 |

Table I. Dumez et al.
Table II. N-terminal sequence analysis of the activation tests of proDer p 3 incubated with rDer p 1, rDer p 3 or HDM extracts.

| Proteases            | N-terminal sequences of Der p 3  | Trypsin-like protease activity of Der p 3 |
|----------------------|----------------------------------|------------------------------------------|
|                      |                                  | yes                                      |
| rDer p 1             | I_{12}VGGEKAL                    | no                                       |
|                      | A_{115}VGLP                      | no                                       |
| rDer p 3             | A_{18}LAGECP                     | no                                       |
|                      | A_{115}VGLP                      | no                                       |
|                      | other degradation bands          | no                                       |
| HDM extracts         |                                  | yes                                      |
|                      | degradation bands                | no                                       |
| HDM extracts + E-64  |                                  | no                                       |
|                      | degradation bands                | no                                       |
| HDM extracts + APMSF + SBTI |                  | yes                                      |
|                      | degradation bands                | no                                       |
| HDM extracts + APMSF + E-64 |     | no                                       |

Table II. Dumez et al.
Table III. Kinetic parameters for rDer p 3 and rDer p 1 in the presence of 50 mM polybuffer, pH 8.5 or PBS, pH 7.4 containing 1 mM EDTA and 1 mM DTT, respectively.

| Substrate       | $K_m$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (10$^5$ M$^{-1}$s$^{-1}$) |
|-----------------|------------|----------------------|----------------------------------------|
| Boc-IEGR-AMC    | 27 ± 1     | 17.3 ± 0.2           | 6.4 ± 0.3                              |
| Boc-GPR-AMC     | 14 ± 1     | 12.4 ± 0.2           | 8.9 ± 0.7                              |
| Boc-FSR-AMC     | 11 ± 1     | 9.9 ± 0.3            | 9 ± 0.9                                |
| Boc-QAR-AMC     | 33 ± 3     | 8.6 ± 0.2            | 2.6 ± 0.2                              |
| Boc-QAR-AMC     | 450 ± 80   | 2.3 ± 0.2            | 0.05 ± 0.01                            |
| NAR-ACC         | 190 ± 30   | 70 ± 3               | 3.7 ± 0.6                              |
| NAT-ACC         | n.c.*      | n.c.*                | n.c.*                                  |

* not cleaved

Table III. Dumez et al.
Figure 1. Dumez et al.
Figure 2. Dumez et al.
Figure 3. Dumez et al.
Figure 4. Dumez *et al.*
Figure 5. Dumez et al.
Figure 6. Dumez et al.
Figure 7. Dumez et al.
Figure 8. Dumez et al.
Synthesis of 7-N-(Fluorenylmethoxycarbonyl) aminocoumarin-4-acetic acid

To a 500 ml round-bottom flask were added 7-aminocoumarin-4-acetic acid (1) (10 g, 45.6 mmol), 320 ml tetrahydrofuran (THF)/H₂O (4/1), Fmoc.Cl (14 g, 54.1 mmol, Bachem, Budendorf, Switzerland) and Na₂CO₃ (11.6 g, 109.4 mmol). The reaction was stirred for 4 hours at room temperature and then the pH was adjusted to 4, by addition of HCl. The organic phase was washed with ethyl acetate (EtOAc) (3 x 300 ml), H₂O pH 2 (3 x 300 ml) and brine solution (3 x 300 ml). The resulting organic phase was dried with MgSO₄ and filtered. The filtrate was concentrated to afford a yellow precipitate of 7-N-(Fluorenylmethoxycarbonyl) aminocoumarin-4-acetic acid (Fmoc-ACC) (6.6 g) (33%). The analytical data (1H NMR and 13C NMR) were in good agreement with literature values (1).

Synthesis of Fmoc-ACC-resin

Fmoc protected ACC was then linked to a Rink amide AM resin (CBL, Baltimore, Maryland, USA), following the synthesis described by Maly (1). To a 250 ml round-bottom flask were added Rink amide AM resin (10.8 g, 7 mmol) and N,N-dimethylformamide (DMF, 110 ml). The mixture was stirred for 30 minutes at room temperature and then filtered. Resin was collected in a 250 ml round-bottom flask, a 20% solution of piperidine in DMF (110 ml) was added and the reaction mixture was stirred for 30 minutes. The deprotected resin was filtered and washed with DMF (3 x 100 ml). Resin, Fmoc-ACC (6 g, 13.6 mmol), N-hydroxybenzotriazole (HOBt) (3.8 g, 13.6 mmol, Iris Biotech GmbH, Marktredwitz, Germany) and DMF (110 ml) were added in a round-bottom flask of 250 ml followed by diisopropylcarbodiimide (DICI) (2.15 ml, 13.6 mmol, Sigma-Aldrich, Saint-Louis, Missouri, USA). The mixture was stirred overnight, then filtered and washed with DMF (3 x 100 ml), THF (3 x 100 ml) and MeOH (3 x 100 ml) to afford 11.64 g of Fmoc-ACC-resin. The substitution level of Fmoc-ACC-resin was 0.45 mmol/g as determined by Fmoc analysis (2).

Coupling of Fmoc-L-Thr(tBu)OH or Fmoc-L-Arg(Pbf)OH on the Fmoc-ACC-resin (1)

To a 25 ml round-bottom flask were added Fmoc-ACC-resin (1 g, 0.52 mmol) and DMF (10 ml). The mixture was stirred for 30 minutes and then was filtered. Fmoc-ACC-resin was incubated with piperidine 20% in DMF (10 ml) for 30 minutes. The ACC-resin was filtered, washed with DMF (3 x 10 ml) and then agitated with an Fmoc-amino acid (Fmoc-L-Thr(tBu)OH or Fmoc-L-Arg(Pbf)OH, 2.6 mmol), DMF (6 ml), 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) (1 g, 2.6 mmol, Novabiochem, Darmstadt, Germany) and 2,4,6-collidine (360 µl, 2.6 mmol, Alfa Aesar, Lancashire, England) during 24 hours. The Fmoc-L-Thr(tBu)- and Fmoc-L-Arg(Pbf)-ACC-resins were filtered and washed with DMF (3 x 10 ml). The coupling levels for Fmoc-L-Thr(tBu)- and Fmoc-L-Arg(Pbf)-ACC-resins were respectively 0.40 mmol/g and 0.29 mmol/g as determined by Fmoc analysis (2). A second coupling step was necessary for Thr and Arg.

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Supplemental data Fmoc-L-Thr/Arg-ACC-resins synthesis. Dumez et al.
Supplemental data Figure S1. Dumez et al.
| kDa | St | A | B | 5 | 10 | 20 | 60 | 120 | 240 | 360 |
|-----|----|---|---|---|----|----|----|-----|-----|-----|
| ... |    |   |   |   |    |    |    |     |     |     |

A + 1 μM rDer p 1 + 100 μM E-64

B + 1 μM rDer p 1 + 100 μM E-64

C + 1 μM rDer p 1 + 10 mM Benzamidine

Supplemental data Figure S2. Dumez et al.
Activation mechanism of recombinant Der p 3 allergen zymogen; contribution of cysteine protease Der p 1 and effect of propeptide glycosylation

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