



Der p 1 is the primary activator of Der p 3, Der p 6 and Der p 9 the proteolytic allergens produced by the house dust mite *Dermatophagoides pteronyssinus*

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ABSTRACT

Background: The enzymatic activity of the four proteases found in the house dust mite *Dermatophagoides pteronyssinus* is involved in the pathogenesis of allergy. Our aim was to elucidate the activation cascade of their corresponding precursor forms and particularly to highlight the interconnection between proteases during this cascade.

Methods: The cleavage of the four peptides corresponding to the mite zymogen activation sites was studied on the basis of the Förster Resonance Energy Transfer method. The proDer p 6 zymogen was then produced in *Pichia pastoris* to elucidate its activation mechanism by mite proteases, especially Der p 1. The role of the propeptide in the inhibition of the enzymatic activity of Der p 6 was also examined. Finally, the Der p 1 and Der p 6 proteases were localised via immunolocalisation in *D. pteronyssinus*.

Results: All peptides were specifically cleaved by Der p 1, such as proDer p 6. The propeptide of proDer p 6 inhibited the proteolytic activity of Der p 6, but once cleaved, it was degraded by the protease. The Der p 1 and Der p 6 proteases were both localised to the midgut of the mite.

Conclusions: Der p 1 in either its recombinant form or in the natural context of house dust mite extracts specifically cleaves all zymogens, thus establishing its role as a major activator of both mite cysteine and serine proteases.

General significance: This finding suggests that Der p 1 may be valuable target against mites.

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1. Introduction

The house dust mite *Dermatophagoides pteronyssinus* is the primary source of allergens that are involved in the development of allergy leading to asthma, rhinitis or atopic dermatitis. Today, more than 20 groups of house dust mite (HDM) allergens have been identified, among which groups 1, 3, 6 and 9 correspond to secreted proteases [1,2]. The proteolytic

activity of these allergens has been shown to enhance the development and the chronicity of allergy either by cleaving receptors, leading to the activation of innate or adaptive immunity, or by increasing the permeability of the airway epithelium, promoting allergen penetration [3–6].

These proteases are expected to be involved in biological processes in the mite. Indeed, Der p 1 [7,8] and Der f 3, the homolog of Der p 3 in *Dermatophagoides farinae* [9], are localised to the gut of mites and to HDM faecal pellets, suggesting that they play a role in digestion. Similar to other digestive proteases, Der p 1, Der p 3, Der p 6 and Der p 9 are synthesised as inactive precursors to control enzyme activation. These zymogens are termed proDer p 1, proDer p 3, proDer p 6 and proDer p 9. Investigating the activation mechanisms of these zymogens is crucial for understanding the pattern of allergenic proteases in the environment.

The activation of Der p 1, a cysteine protease from the papain family, has been widely characterised. The Der p 1 propeptide (80 amino acids) plays an essential role in zymogen folding (as an intramolecular chaperone) and in the inhibition of its own enzymatic activity. ProDer p 1 was

Abbreviations: AMC, 7-amido-4-methylcoumarin; AMg, anterior midgut; DAPI, 4',6-DiAmidino-2-PhenylIndole; E-64, L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane; FP, faecal pellet; FRET, Förster Resonance Energy Transfer; HDM(e), house dust mite (extracts); Hg, hindgut; nDer p 6, natural Der p 6; pDp6, proDer p 6; PMg, posterior midgut; rDer p 1, recombinant Der p 1; rDer p 6, recombinant Der p 6; SBTI, soybean trypsin inhibitor

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shown to activate itself through a multistep mechanism at an acidic pH [10].

Dumez et al. [11] have shown that the short propeptide (11 amino acids) of Der p 3, a serine trypsin-like protease, is mainly involved in inhibition of the protease. However, once proDer p 3 is activated by Der p 1 through intermolecular cleavage of the propeptide, the latter loses its inhibitory capacity.

The activation mechanisms of the two other serine proteases, Der p 6 (chymotrypsin-like) and Der p 9 (collagenase), are still unknown. The proDer p 6 propeptide (34 amino acids) is somewhat different from chymotrypsinogen in that the arginine that normally occurs before the trypsin cleavage site is replaced by a glycine [12].

Comparison of the propeptide C-terminal sequences from the mite zymogens highlights the similarity of sequences that correspond to the cleavage specificity by Der p 1 (Table 1) [13]. These observations suggest the existence of a particular activation cascade for the mite proteases. Unlike the major HDM allergens Der p 1 and Der p 2, Der p 6 is poorly characterised. This allergen was first identified as a chymotrypsin-like protease (provisionally named DP5) by Yasueda et al. [14] and is expected to account for a large portion of the measurable proteolytic activity in HDM extracts [11]. While the contribution of serine proteases to anti-mite IgE binding is considered low [15], the global prevalence of anti-group 6 IgE varies between less than 10% to 65% [14,16–19], most probably because of the variability observed in the preparation of natural allergens [20–22]. Therefore, obtaining recombinant allergen Der p 6 for RAST determination and diagnosis would be of interest to accurately evaluate the involvement of the protease in mite allergy.

Our objectives were to elucidate the specific activation cascade of the four mite zymogens based on the Förster Resonance Energy Transfer (FRET) method. In parallel, we confirmed the activation mechanism of the proDer p 6 zymogen for which recombinant protein is not yet available, by producing it in the yeast *Pichia pastoris*. The roles of the propeptide in inhibiting the enzymatic activity of Der p 6 and in its activation were elucidated. Finally, we determined the immune co-localisation of the proteases in the gut of the mite *D. pteronyssinus*.

2. Methods

2.1. Chemicals

N-Succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (AMC) acetate, Boc-Gln-Ala-Arg-AMC acetate salts, Boc-Gln-Ala-Arg-p-nitroanilide (pNA), N-succinyl-Ala-Ala-Pro-Phe-pNA and N-succinyl-Ala-Ala-Pro-Leu-pNA were purchased from Bachem (Babendorf, Switzerland). The cysteine protease inhibitor L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) and soybean trypsin inhibitor (SBTI) were obtained from Sigma (Saint Louis, USA). A polyclonal anti-proDer p 6 antibody (rabbit) was manufactured by Eurogentec (Liège,

Belgium) using recombinant proDer p 6 and was purified with protein A HiTrap (GE healthcare, Saint Louis, USA).

2.2. Mites and extracts

D. pteronyssinus was reared in the Earth and Life Institute (UCL, Belgium). They were reared at 20 °C and 75% humidity as previously described [23]. In some rearing boxes, mites were starved until death. Afterwards, rearing boxes contained the bodies of the dead mites, the exuviae (resulting from the successive moults) and the faeces produced by mites *D. pteronyssinus* culture extracts were obtained as previously described [11].

2.3. Solid-phase synthesis of S1–S9 peptides

The S1, S3, S6 and S9 peptides correspond to the proDer p 1, proDer p 3, proDer p 6 and proDer p 9 zymogen activation sites, respectively (Fig. 1 in supplemental data). N- α -Fmoc amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany), and 2,4-Dinitrophenyl (Dnp)-L-Proline and Dnp-L-Valine were obtained from Sigma (Saint Louis, USA), and Dnp-L-Leucine came from TCI Europe (Zwijndrecht, Belgium). The Glu and Thr side chains were protected with a t-butyl derivative; Lys was protected with an N-epsilon-allyloxycarbonyl group; and Asn was protected with a trityl group. The substrates were prepared following a solid-phase peptide synthesis strategy on a PS3 automated peptide synthesiser (Protein Technologies, Inc., Tucson, AZ) using N-(7-Methoxycoumarin-4-yl)acetyl (AMC)-N'-Fmoc-ethylenediamine-MBP-AM resin (Novabiochem, Darmstadt, Germany), as previously described [11]. The side chain-protecting groups were removed, and the peptides, which were cleaved from the resin using trifluoroacetic acid (TFA)/anisole/water (6 ml; 10:1:1) during a 3-hour incubation at room temperature, were precipitated with diethyl ether.

2.4. Characterisation of S1–S9 peptides

The purity of each peptide (50 μ l, 500 μ M) was assessed via analytical HPLC performed on a Nucleodur C18 column (250 \times 4.6 mm, 5 μ m, Macherey-Nagel, Düren, Germany). The HPLC chain consisted of a pump (Waters 600) and a UV detector (PDA Waters 996, Hilford, Massachusetts, USA). The absorbances at 323 nm and 374 nm, corresponding to the AMC and Dnp groups, respectively, were constantly recorded. The solvents consisted of aqueous and 0.1% acetonitrile (V/V) TFA solutions. Elution was carried out for 30 min at a flow rate of 1 ml/min using a linear gradient from 0 to 75% acetonitrile/TFA. Each peptide (100 μ l, 50 μ M) was then characterised via electrospray ionisation mass spectrometry (ESI-MS, TSQ 7000 Thermoquest Finnigan, positive ionisation, 4.5 kV).

2.5. FRET measurements

Unglycosylated recombinant mature Der p 1 (rDer p 1) was obtained from proDer p 1 produced in *P. pastoris* as described previously [10,11]. The reaction mixtures contained 8 μ M substrate (S1–S9) and rDer p 1 (0.04 μ M for S3 and S6 and 2.32 μ M for S1 and S9) or HDM extract (1 mg/ml) in polybuffer (mixture of 50 mM Tris, phosphate, citrate, acetate and KCl containing 1 mM DTT and 1 mM EDTA, adjusted to pH 7.4), with or without the addition of the cysteine protease inhibitor L-trans-epoxysuccinyl-leucylamido (4-guanidino)butane (E-64, 100 μ M) or the serine protease inhibitor soybean trypsin inhibitor (SBTI, 10 μ M). The time course of hydrolysis was monitored for 300 s in an LS 50 B fluorimeter (PerkinElmer Life Sciences) with excitation and emission wavelengths of 328 and 393 nm, respectively.

Table 1

Activation sites of *Dermatophagoides pteronyssinus* zymogens. The underlined residues correspond to the specificity of Der p 1 determined by [13]. The arrow indicates the putative cleavage site of the propeptide. The bold sequences correspond to the S1, S3, S6 and S9 peptides.

Zymogen	C-terminal sequence of propeptide	N-terminal sequence of protease
proDer p 1	. . K T Q F D F D <u>L N A E</u>	<u>T N A C S I N G</u> . .
proDer p 3	N P I L P A S <u>P N A T</u>	<u>I V G G E K A L</u> . .
proDer p 6	. . M M N D N S S <u>P I A G</u>	<u>V I G G Q D A A</u> . .
proDer p 9	T R N I <u>P L G</u>	<u>I V G G S N A S</u> . .

2.6. Identification of the S1–S9 peptide cleavage sites

The peptides (500 μ M) were incubated with rDer p 1 (2.32 μ M), rDer p 3 (0.23 μ M) or HDM extract (1 mg/ml) in 50 mM polybuffer at pH 7.4, with or without the addition of either E-64 (100 μ M) or SBTI (10 μ M), for 16 h at 25 °C. The fragments obtained after the various treatments were separated via analytical HPLC as described above and analysed via ESI-MS.

2.7. Kinetic parameters of rDer p 1 and HDM extracts

To control the proportionality of the FRET response to substrate utilisation and to avoid the inner filter effect [24], calibration was performed by completely hydrolysing the four substrates at concentrations ranging from 0.5 to 40 μ M using rDer p 1 (0.04 μ M for S3 and S6 and 2.32 μ M for S1 and S9). Hydrolysis was monitored via fluorimetry as described above for 180 min at 25 °C. FRET remained linear up to an 8 μ M product concentration. For determination of the kinetic parameters of rDer p 1 (0.04 or 2.32 μ M) and the HDM extract (1 mg/ml), the hydrolysis rates were measured for increasing substrate concentrations (0 to 8 μ M), and the data were analysed according to the Henri-Michaelis–Menten equation.

2.8. Expression of recombinant proDer p 6 in *P. pastoris*

The proDer p 6 gene sequence, optimised for expression in *P. pastoris*, was synthesised by GeneArt (Life Technologies, Paisley, UK). The gene was inserted in a pPIC9K (Invitrogen, Paisley, UK) vector that had been previously digested with the *Sna*BI and *Avr*II restriction enzymes (Promega, Madison, USA).

The *P. pastoris* SMD1168 strain (Invitrogen, Paisley, UK) was transformed through electroporation with the expression vector, which had been linearised by the *Pme*I restriction enzyme. Transformants from pPIC9K carrying the HIS4 gene were grown in histidine-deficient medium (RDB). Clones with multiple integrated copies were selected using increasing geneticin concentrations (0.25 to 1 mg/ml, Invitrogen, Paisley, UK).

For expression of proDer p 6, *P. pastoris* was grown in buffered glycerol complete medium (BMGY) for 24 h at 28 °C to an A_{600} value of ~25. The culture was then centrifuged for 15 min at 10000 g, and the pellet was resuspended in buffered methanol complex medium (BMMY) (0.5% methanol) for 2 days at 28 °C to measure protein expression. The culture was subsequently centrifuged for 15 min at 10000 g, and the supernatant was conserved at –20 °C.

2.9. Purification of recombinant proDer p 6 and of recombinant and native Der p 6

The yeast culture supernatant was filtered through a 0.45 μ m filter (Millipore, Darmstadt, Germany) and dialysed overnight at 4 °C against 20 mM Tris–HCl pH 7.5 (buffer A). The solution was then stirred with 200 ml of QXL streamline matrix (GE Healthcare, Saint Louis, USA) that had been equilibrated with buffer A at 4 °C. The matrix was washed with buffer A and packed into a column (GE Healthcare, Saint Louis, USA). Bound proteins were eluted with buffer A containing 0.5 M NaCl. After SDS-PAGE analysis, fractions containing proDer p 6 were pooled and dialysed overnight at 4 °C against buffer A. The protein was then precipitated by the addition of 60% ammonium sulphate at 4 °C. After centrifugation, the pellet was resuspended in 3 ml of buffer A and dialysed overnight at 4 °C. The solution was then loaded onto a Sephacryl S-100 HR column (120 ml) that had been previously equilibrated with buffer A, and proteins were eluted according to molecular weight. The fractions containing proDer p 6 were pooled and stored at –20 °C.

The recombinant proDer p 1 protease (N-64A, N52A), fused with a C-terminal six-histidine sequence, was produced in *P. pastoris* and

then purified and activated to a mature protease as described previously [10]. This enzyme was incubated with MagneHis™ Ni-Particles (Promega, Madison, USA) overnight at 37 °C, and the particles with coated Der p 1 were washed with PBS containing 1 mM DTT and EDTA prior to incubation with proDer p 6 for 8 h at 37 °C. After the activation of proDer p 6, the particles were removed, and pure rDer p 6 was stored at –20 °C.

To purify natural Der p 6, HMD extracts (4 g suspended in 40 ml of PBS) were dialysed overnight at 4 °C against 20 mM Tris–HCl at pH 7.5 (buffer A). The solution was then loaded onto a Q-HP HiTrap column (GE Healthcare, Saint Louis, USA) equilibrated with buffer A. Bound proteins were eluted with buffer A containing 1 M NaCl. The fractions containing Der p 6 were pooled and loaded onto a Benzamidine Sepharose 4 Fast Flow column (GE Healthcare, Saint Louis, USA) that had been equilibrated with buffer A containing 0.5 M NaCl. The proteins were eluted with 100 mM sodium acetate, pH 4, and then dialysed overnight at 4 °C against buffer A. The solution was next loaded onto a heparin column (GE Healthcare, Saint Louis, USA) that had been equilibrated with buffer A. Elution was performed with buffer A containing 1 M NaCl. The concentration of the obtained proteins was estimated using the BCA assay (Thermo Scientific, Rockford, USA). SDS-PAGE analysis and protease activities were assayed at each stage of purification as previously described [11].

2.10. N-terminal sequencing

The proDer p 6 and rDer p 6 proteins were sequenced in an Applied Biosystems 476A protein sequencer based on Edman degradation. The samples were analysed via SDS-PAGE, followed by electrophoretic transfer to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany).

2.11. In vitro activation of recombinant proDer p 6

The proDer p 6 zymogen (3.5 μ M) was incubated at 37 °C for 90 min with either rDer p 1 (0.1 μ M) or HDM extract (20 μ g/ml) in 50 mM polybuffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA. The E64 inhibitor (100 μ M), SBTI (10 μ M) and/or the Der p 1 propeptide (10 μ M, produced and purified as previously described [10]) were also added to the HDM extract prior to incubation with proDer p 6. The samples were analysed via SDS-PAGE, and Der p 6 activity was measured in 20 mM Tris, pH 7, using the Ala-Ala-Pro-Phe-AMC substrate (200 μ M). The time course of hydrolysis was monitored for 300 s in an LS 50 B fluorimeter (PerkinElmer Life Sciences) with excitation and emission wavelengths of 360 and 480 nm, respectively. The kinetics of hydrolysis were reported as the time course of AMC (μ M) release, using an AMC (Sigma, Saint Louis, USA) standard curve with concentrations ranging from 0 to 15 μ M.

2.12. Der p 6 enzymatic activity measurements and determination of kinetic parameters

For the determination of natural or recombinant Der p 6 (80 nM) kinetic parameters, the rate of the hydrolysis of increasing AAPF-AMC (Bachem, Babendorf, Switzerland) concentrations (0 to 400 μ M) was measured as described above, and the data were analysed according to the Henri-Michaelis–Menten equation.

The optimum pH for rDer p 6 (0.1 μ M) was also determined in 50 mM polybuffer at pH 4 to 11 with the same substrate (400 μ M).

2.13. Determination of the role of the propeptide

2.13.1. Inhibition of recombinant Der p 6 by its propeptide

The peptide corresponding to the full-length propeptide of proDer p 6 (34 amino acids), was purchased from JPT Peptide Technologies GmbH (Berlin, Germany). rDer p 6 activity (50 nM) was measured at 37 °C in 20 mM Tris pH 7 and using increasing concentrations of the

peptide (0 to 60 μM) in the presence of the AAPF-AMC substrate at different concentrations (25 to 100 μM). The inhibition model was determined with a Hanes plot, and the inhibition constant, K_i , was obtained using the following equation:

$$v_i = V \times S / (K_M \times (1 + I/K_i) + S)$$

where v_i , V , S , I , K_M and K_i represent the initial rate of product release ($\mu\text{M/s}$), the maximal rate of product release ($\mu\text{M/s}$), the concentration of the substrate or inhibitor (μM), the K_M of the enzyme for its substrate (μM) and the inhibition constant (μM), respectively.

2.13.2. Mass spectrometry analysis of proDer p 6 propeptide degradation

The proDer p 6 prosequence (3 μM) was incubated with either rDer p 6 or rDer p 1 (50 nM) in 20 mM Tris, pH 7, and aliquots were collected at different times. The reaction was stopped with 0.1% TFA, and the peptides were desalted using Ziptips C18 (Millipore, Darmstadt, Germany). The peptides were then suspended in 50/50 H_2O /Acetonitrile (v/v), 0.1% formic acid solution. ESI-MS analyses were performed on a Q-TOF Ultima Global mass spectrometer (Micromass, now Waters, Manchester, UK). The ESI source was operated in positive ion mode at a capillary voltage of 3 kV. The source block temperature was 100 $^\circ\text{C}$, the desolvation gas (N_2) temperature was 200 $^\circ\text{C}$ and the source pressure was 2.8 mbar. The RF Lens 1 voltage was set to 100 V. All mass spectra were acquired over the 90–3000 m/z range and were processed with MassLynx 4.1 software (Waters).

2.14. Immunolocalisation of Der p 1 and Der p 6

Mites were collected with a brush under a Nikon SMZ645 stereomicroscope (Nikon Belux, Brussels, Belgium) and then incubated overnight in a solution of 2% formaldehyde and 0.05% Triton X-100 in PBS at 4 $^\circ\text{C}$. After three washes in PBS, the mites were incubated in a 30% sucrose solution for 1 h at room temperature and then oriented in 7.5% agar, 15% sucrose. The mites were embedded in OCT compound (VWR International, Poole, England), and incubated at -70°C in an isopentane bath. Cryosections (14–16 μm thick) were obtained by means of a cryostat (Microm HM 560, Prosan) and collected on glass slides. For immunolabeling, cryosections were dried at 30 $^\circ\text{C}$ before being fixed in 2% formaldehyde. After three washes in PBS, the sections were soaked for 30 min at 37 $^\circ\text{C}$ in 10% normal goat serum (Sigma, St Louis, USA) in PBS, and after immersed with 1:750 anti-Der p 6 and 1:1000 anti-Der p 1 (5H8, Indoor biotechnologies, USA) in PBS with 5% goat serum. They were washed in PBS, and immersed for 35 min at 37 $^\circ\text{C}$ in a PBS solution containing 1:250 goat anti-rabbit Alexa Fluor 594 and goat anti-mouse Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands). The unbound antibodies were removed by three washing cycles in PBS. The slides were immersed in a solution of DAPI (1:50000 dilution; 4',6-DiAmidino-2-Phenylindole; Sigma Aldrich) and washed with PBS. Finally, the sections were mounted with Citifluor AF1 (Laborimpex, Brussels, Belgium). Negative control sections were realized by using normal goat serum instead of the primary antibodies. The sections were observed under an Olympus IX71 confocal microscope (Olympus America Inc.).

3. Results and discussion

The allergenic mite proteases Der p 1, Der p 3, Der p 6 and Der p 9 are synthesised as inactive zymogens that then mature into active enzymes through specific cleavage of their propeptides. As recombinant allergens are increasingly used for the diagnosis and treatment of allergy [21,25], understanding the activation mechanisms of these zymogens represents an essential step for obtaining proteins with enzymatic and folding properties similar to those of their natural counterparts. In the future, elucidation of such activation cascades should provide insight into the critical enzyme(s) involved in the onset of the activation

process and, thus, offer new opportunities for the development of therapeutic drugs.

3.1. Kinetic parameters of S1–S9 peptides cleavage

To explore the proteolytic cascade occurring in mites, we synthesised four peptides, termed S1, S3, S6 and S9 (Fig. S1, supplemental data), which contained the activation sites of proDer p 1, proDer p 3, proDer p 6 and proDer p 9, respectively.

The major allergen rDer p 1 was found to recognise and cleave all of the synthesised substrates (Fig. 1). The K_M values for S3 and S6 hydrolysis by rDer p 1 were similar ($<9 \mu\text{M}$), but we unfortunately could not measure the K_M values for S1 and S9 because of the quenching of fluorescence at high peptide concentrations ($>10 \mu\text{M}$). The catalytic efficiency (k_{cat}/K_M) of rDer p 1 for S1 ($220 \pm 20 \text{ mM}^{-1} \text{ min}^{-1}$) was ~ 7 - and 4 -fold lower than that observed for S3 ($1610 \pm 20 \text{ mM}^{-1} \text{ min}^{-1}$) and S6 ($860 \pm 170 \text{ mM}^{-1} \text{ min}^{-1}$), respectively (Table S1, supplemental data). This observation might be explained by the fact that intermolecular activation of proDer p 3 (S3) and proDer p 6 (S6) by small amounts of Der p 1 was likely to require a higher specificity than the auto-activation of proDer p 1 (S1). Finally, the ~ 7 -fold lower k_{cat}/K_M value of rDer p 1 determined for S9 ($31 \pm 5 \text{ mM}^{-1} \text{ min}^{-1}$) might be due in part to the absence of a proline at the P_4 position and, especially, to the presence of a leucine instead of an alanine at the P_2 position.

This result is consistent with the demonstrated specificity of Der p 1 [13], which exhibits a preference for small hydrophobic residues in the P_2 position (Schechter and Berger's nomenclature [26]). We also showed via mass spectrometry that all of the tested peptides were hydrolysed in presence of HDM extracts (Table 2 and Fig. S2, supplemental data), and the obtained K_M values were similar to those observed using pure rDer p 1 (Table S1, supplemental data). The respective contributions of the cysteine and serine proteases present in HDM extracts to the hydrolysis of S1, S3, S6 and S9 were then determined by performing the reactions in the presence of cysteine (E-64) or serine (SBTI) protease inhibitors. We found that the hydrolysis rates for the four substrates were similar with or without the addition of SBTI (Fig. 1, black and white bars), whereas they were strongly reduced in the presence of E-64 (Fig. 1, grey bars). Nearly complete inhibition by E-64 was observed when using purified rDer p 1.

These experiments demonstrate the high activity of Der p 1 in the cleavage of S1, S3, S6 and S9 and suggest that Der p 1 plays a major role in the activation of the four proDer p zymogens.

3.2. Identification of cleavage sites

We showed that Der p 1 (either recombinant or in HDM extracts) cleaved the presented peptides at the expected sites, i.e., between the $P1$ and $P'1$ positions of the activation sites of the zymogens (Table 2 and Fig. S2, supplemental data). This can be explained by the presence of similar residues at the C-terminal end of the zymogen prosequences, which correspond to the specificity of Der p 1 [13]. These observations validated our methodology by confirming the role of Der p 1 as an auto-catalytic enzyme and in proDer p 3 maturation. More interestingly, this study broadens the established activating role of the major allergen Der p 1 to include all mite protease zymogens. Indeed, the addition of E-64 inhibited the specific cleavage of all of the peptides at the predicted processing sites.

Nevertheless, a small amount of non-specific hydrolysis of S1 was observed when using HDM extracts (Table 2). We showed that the presence of additional peptide fragments was due to the activity of serine proteases, as this phenomenon was totally abolished in the presence of SBTI. These data suggest that whereas Der p 1 is involved in the activation of zymogens, the serine proteases are likely to be responsible for non-specific protease degradations.

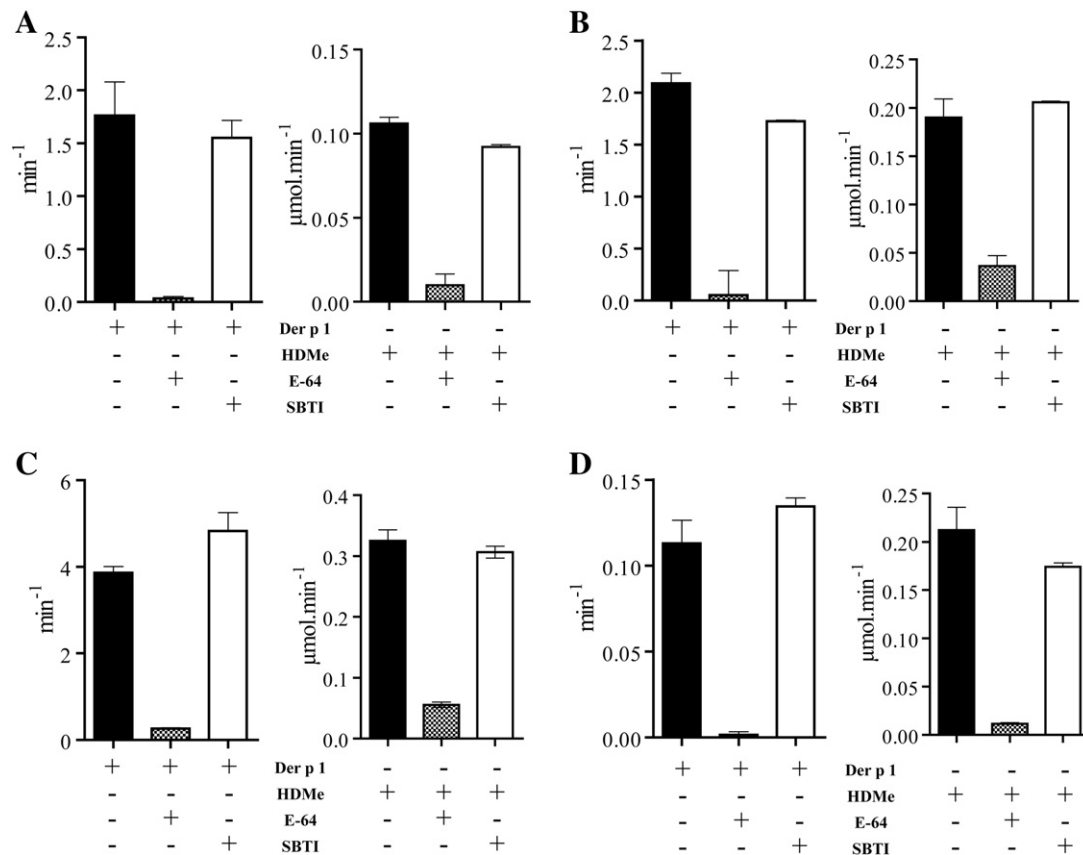


Fig. 1. Cleavage of S1, S3, S6 and S9 substrates. Enzymatic activity of rDer p 1 (0.04 μM or 2.32 μM) or HDM extracts (1 mg/ml) measured with 8 μM (A) S1, (B) S2, (C) S3 and (D) S9 in absence (black) or presence of 100 μM E-64 (grey) or 10 μM SBTI (white).

3.3. Expression, purification and activation of recombinant proDer p 6

We determined the activation mechanism for the recombinant proDer p 6 zymogen. ProDer p 6 was expressed in the culture supernatant of *P. pastoris* after 36 h of methanol induction, and 1 mg of the zymogen was then purified from 1 l of culture. SDS-PAGE analysis (Fig. 2A, lane pDp6) indicated that proDer p 6 was present in two predominant forms with apparent molecular masses of 30 and ~28 kDa. A sequence analysis indicated that these forms corresponded to the entire zymogen (molecular mass of 28834 Da) and to proDer p 6 with the 25 N-terminal residues deleted (proDer p 6': 25872 Da), respectively.

Recombinant proDer p 6 was then incubated in the presence of either rDer p 1 or HDM extracts. SDS-PAGE analysis was performed

(Fig. 2A and B), and the time course of chymotrypsin activity was analysed using AAPF-AMC (Fig. 2G). The two bands (~30 and 28 kDa) corresponding to proDer p 6 and proDer p 6' disappeared over time after the addition of rDer p 1, and a new band corresponding to rDer p 6 appeared (~25 kDa) (Fig. 2B). The expected N-terminal sequence (V₃₅IGGDQ) of rDer p 6 was confirmed. The incubation of proDer p 6 with rDer p 1 or HDM extracts for 1 h led to fully activated Der p 6. The loss of the propeptide was related to an increase in chymotrypsin activity. The maximum activity was reached after 60 min (Fig. 2G).

The same results were obtained following the addition of HDM extracts previously incubated with SBTI (~20 kDa) to proDer p 6, suggesting that serine proteases do not participate in the activation of the zymogen (Fig. 2C). In contrast, the addition of the cysteine protease

Table 2

Identification of cleavage sites of the S1–S9 peptides after hydrolysis by rDer p 1 or HDM extracts. Analytical HPLC analysis of peptides after their incubation with rDer p 1 or HDM extracts in presence or absence of inhibitors was performed followed by the characterization of the different peaks obtained by ESI-MS. The serine protease inhibitor is the Soybean-Trypsin Inhibitor (SBTI) and the cysteine protease inhibitor is the E-64. M⁺ indicates the molecular mass of the peptide. ND is not determined.

Peptides	rDer p 1		HDM extracts		HDM extracts + E-64		HDM extracts + SBTI	
	Fragments	M ⁺	Fragments	M ⁺	Fragments	M ⁺	Fragments	M ⁺
S1	Dnp-LNAE	612	Dnp-LNAE	612	/		Dnp-LNAE	612
	TNA-MCA	563	TNA-MCA	563	/		TNA-MCA	563
			+ Non specific cleavages		Non specific cleavages			
			Dnp-LNAETN	827	Dnp-LNAETN	827		
			AETNA-MCA	763	AETNA-MCA	763		
			ETNA-MCA	691	ETNA-MCA	691		
S3	Dnp-PNAT	569	NA-MCA	462	/		ND	ND
	IVGG-MCA	603	Dnp-PNAT	569	/		ND	ND
S6	Dnp-PIAG	522	IVGG-MCA	603	Dnp-PNATIVGG-MCA	1152	ND	ND
	VIGG-MCA	603	Dnp-PIAG	522	/		ND	ND
S9	Dnp-PLG	451	VIGG-MCA	603	Dnp-PIAGVIGG-MCA	1108	ND	ND
	IVGG-MCA	603	Dnp-PLG	451	/		ND	ND
			IVGG-MCA	603	Dnp-PLGIVGG-MCA	1036		

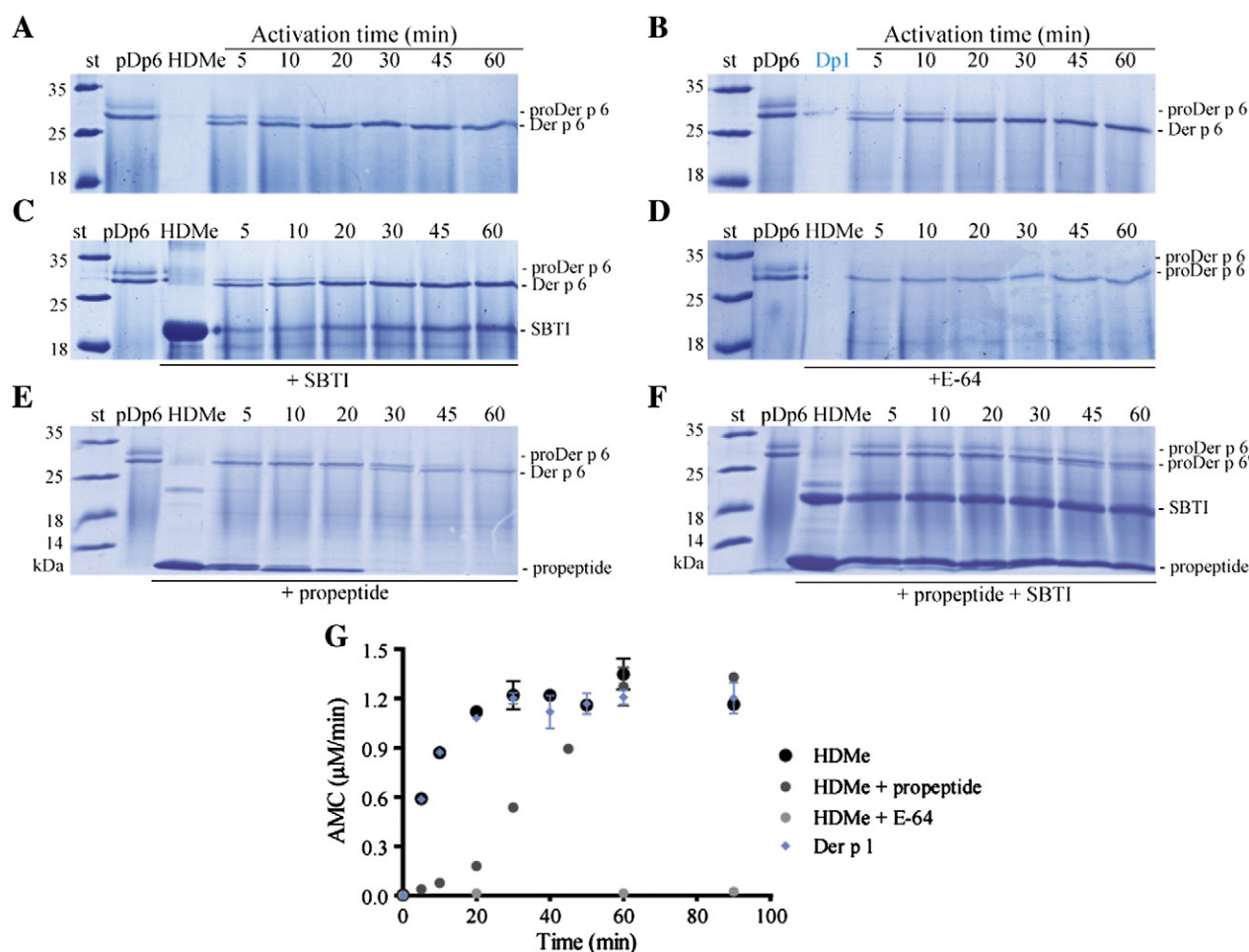


Fig. 2. Inter-molecular activation of proDer p 6 by rDer p 1 or HDM extracts in presence or absence of SBTI, E-64 and/or Der p 1 propeptide. SDS-PAGE analysis: proDer p 6 (pDp6) was incubated at 37 °C in polybuffer with 1 mM DTT/EDTA pH 7.4 with (A) HdMe; (B) rDer p 1; (C) HdMe with SBTI, (D) with E64, (E) with propeptide of Der p 1 or (F) with propeptide and SBTI. (E) rDer p 6 activity for each activation time (0 to 90 min) in presence of rDer p 1 (blue diamond shapes), HdMe extracts (black circles), HdMe with propeptide (dark grey circles) and HdMe extracts with E-64 (light grey circles).

inhibitor (E-64) to rDer p 1 or HdMe extracts totally abolished rDer p 6 production (data not shown and Fig. 2D, respectively) and no chymotrypsin activity was detected (Fig. 2G). Moreover, in the absence of DTT, which enhances the activity of cysteine proteases, very weak activation of proDer p 6 by rDer p 1 or HdMe extracts was observed (Fig. S3, supplemental data).

To further confirm the role of cysteine protease Der p 1 in the activation mechanism of proDer p 6, HdMe extracts were pre-incubated with the highly specific inhibitory propeptide of Der p 1 (K_D 7 nM [10]). The rate of activation of proDer p 6 in the presence of such HdMe extracts was drastically delayed (Fig. 2E and G). It is noteworthy that the gain of proDer p 6 activation after 30 min is due to the degradation of the propeptide of Der p 1 by serine proteases of HdMe extracts [27]. Indeed, the addition of SBTI abolished this phenomenon and no activation could be observed (Fig. 2F), whereas SBTI alone was not able to inhibit the proDer p 6 activation by HdMe extracts (Fig. 2C). Therefore, in the absence of Der p 1 activity, no auto-activation of proDer p 6 could be observed. The intermolecular activation of proDer p 6 confirmed the results of the FRET experiments and the crucial role of the cysteine protease Der p 1.

After activation of proDer p 6 with rDer p 1, rDer p 6 was purified. The catalytic parameters of natural and recombinant Der p 6 were measured using AAPF-AMC and were found to be similar. Indeed, the K_M values were $(120 \pm 10) \mu\text{M}$ and $(40 \pm 10) \mu\text{M}$ and the k_{cat} values were $(0.38 \pm 0.01) \text{ s}^{-1}$ and $(0.38 \pm 0.01) \text{ s}^{-1}$ for nDer p 6 and rDer

p 6, respectively. The optimum pH was determined to be 7, although the protease displayed high activity (more than 90%) between pH 6 and 10 (Fig. S4, supplemental data).

3.4. Determination of the role of the propeptide

The mechanism underlying the maturation of the inactive zymogen proDer p 6 mediated by Der p 1 corresponds to loss of the propeptide and an increase in chymotrypsin activity, suggesting that the propeptide covalently linked to the protease acts as an inhibitor of Der p 6. To explore the role of this propeptide in the inhibition of Der p 6 when it is free in solution, the prosequence was synthesised. We measured the inhibition constant of the propeptide for rDer p 6 using AAPF-AMC as a reporter substrate in either the absence or presence of increasing concentrations of the peptide. The Hanes plot (Fig. S5 supplemental data) obtained using different concentrations of inhibitor shows that the propeptide acts as a competitive inhibitor, with an inhibition constant of $(11 \pm 2) \mu\text{M}$.

Nevertheless, after a 1 minute incubation of the propeptide with rDer p 6, an increase of chymotrypsin activity was observed, indicating that the prosequence could be processed by the protease. To confirm this hypothesis, a mass spectrometry analysis of the propeptide in the presence of rDer p 6 was performed. Fig. 3 shows the results of ESI-MS analysis of the propeptide alone (Fig. 3A) and after a 5 minute incubation with rDer p 6 (Fig. 3C). This analysis was also performed in the

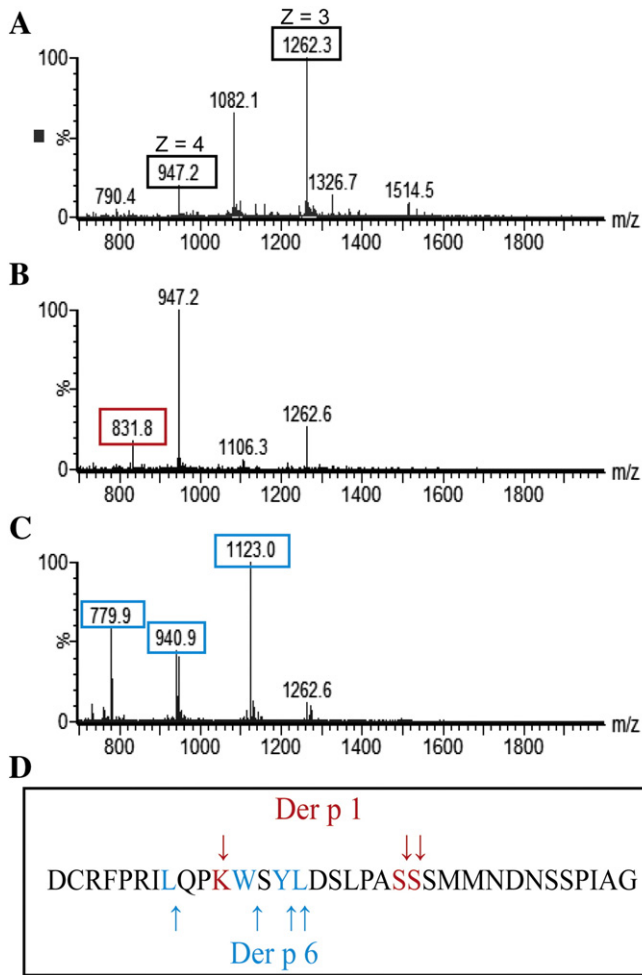


Fig. 3. Mass spectrometry analysis. ESI-QTOF analysis of (A) the peptide corresponding to the propeptide of proDer p 6 (3 μ M) and the peptide after 5 minute incubation with 50 nM (B) rDer p 1 or (C) rDer p 6; (D) represents the propeptide sequence and the arrow indicates the cleavage sites of rDer p 1 (red) and rDer p 6 (blue).

presence of rDer p 1 (Fig. 3B). The peptide was found to be cleaved in several places by the two proteases (Fig. 3D). However, the sites of hydrolysis are different for the two proteases and correspond to their respective cleavage specificities. Indeed, rDer p 6 and rDer p 1 cleaved the peptide after aromatic (tryptophan and leucine) or positively charged (arginine, lysine and serine) residues, respectively.

Here, we demonstrated that the propeptide inhibits the proteolytic activity of Der p 6. However, whereas this inhibition is very stable when the propeptide is covalently linked to the protease, the free propeptide in solution is quickly cleaved by Der p 6 as well as Der p 1, rendering it unable to inhibit the neo-formed Der p 6.

3.5. Immunolocalisation of Der p 1 and Der p 6 in *D. pteronyssinus*

We determined the localisation of Der p 6 and Der p 1 in *D. pteronyssinus*. Examination of coronal sections under a confocal microscope revealed that Der p 6 immunoreactivity is only present in the digestive tract and, more specifically, in the hindgut (Hg) following the terminology of Brody et al. [28]. Indeed, Der p 6 was observed in cells in the absence of ingested material and in the gut lumen (red fluorescence, Fig. 4A and B). Similar localisation patterns have previously been observed for Der f 3, the Der p 3 homologous found in *D. farinae* [9].

Protease Der p 1 was localised in lining cells of the anterior midgut (AMg, yellow fluorescence, Fig. 4C) and in the lumen of the posterior midgut (PMg, Fig. 4D), as previously reported by Thomas et al. [7]. Fig. 4C and D shows that Der p 1 and Der p 6 are co-localised in the hindgut (Hg, orange fluorescence).

Previous observations of the digestive tract of the mite showed that cells lining the anterior midgut, where pH is comprised between 4 and 5, would be gradually detached in the gut lumen and would then be taken in the faecal pellets in the posterior midgut and the hindgut, where pH reaches 6 [28–30]. It is thus likely that the auto-activation of proDer p 1 occurring at acidic pH proceeds in the anterior midgut, thereby providing active protease Der p 1. As the Der p 1 displays a high activity at pH 6 [10], it may activate the proDer p 3 and proDer p 6 zymogens, and most probably proDer p 9, in the hindgut where the serine proteases and Der p 1 were co-localised (Fig. 5) [7,9].

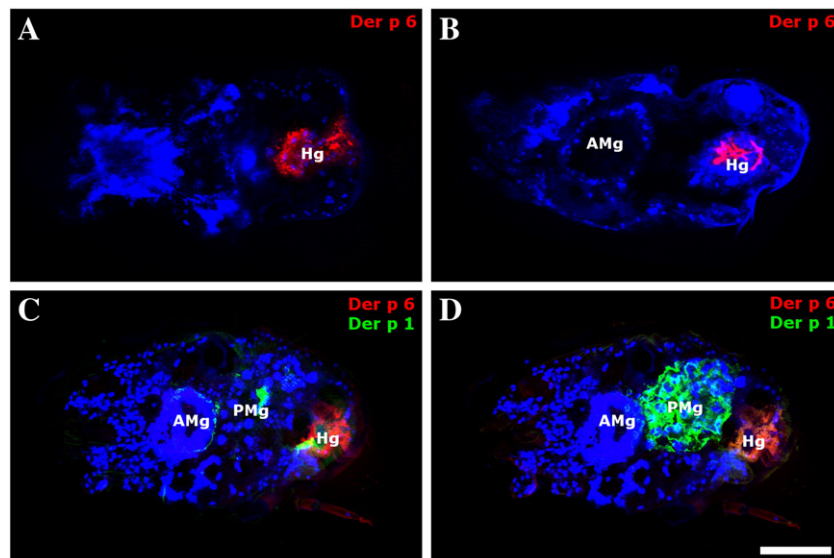


Fig. 4. Immunolocalisation of Der p 1 and Der p 6 in longitudinal sections of *Dermatophagoides pteronyssinus* body. (A–B) Localisation of Der p 6 (red fluorescence) in the posterior midgut and (C–D) co-localisation of Der p 1 (green fluorescence) in the anterior to posterior gut with Der p 6 only detected in posterior part. Mite bodies are in blue fluorescence with DAPI. The scale bar is 50 μ M. AMg, anterior midgut; Hg, hindgut; PMg, posterior midgut.

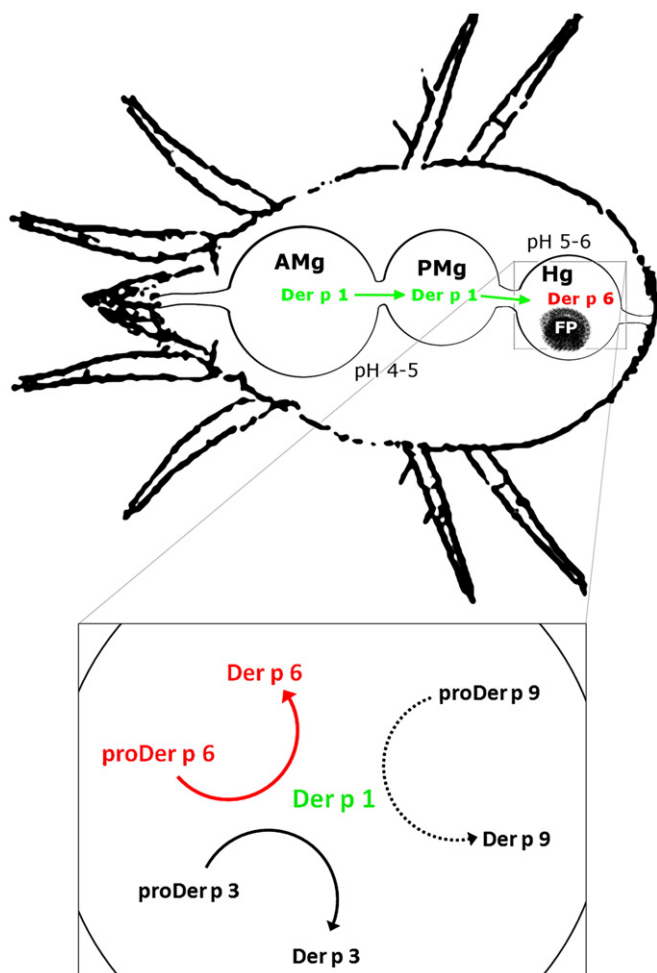


Fig. 5. Proteases activation cascade in the digestive tract of the mite *Dermatophagoides pteronyssinus*. The box represents the activation cascade in the hindgut. AMg, anterior midgut; FP, faecal pellet; Hg, hindgut; PMg, posterior midgut.

4. Conclusion

Our findings demonstrate that Der p 1 is essential for the activation of the mite protease zymogens proDer p 1, proDer p 3, proDer p 6 and probably proDer p 9 (Fig. 5). Moreover, a similar proteolytic cascade might also occur in *D. farinae* and *Euroglyphus maynei*, as the propeptides of the corresponding zymogens in these species exhibit a high degree of similarity with those of *D. pteronyssinus* (Table S2, supplemental data).

According to the critical role of proteolytically active HDM protease allergens in the initiation and in the chronicity of the allergic response, the elucidation of the proDer p 6 maturation process will provide a source of recombinant highly pure and fully active Der p 6 allergen offering new opportunities to deeply characterise its proteolytic specificity and to better define its roles in the activation of innate immune pathways.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.2013.11.017>.

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