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. Der p 3 zymogen was produced in Pichia pastoris. Its activation mechanism was monitored by SDS-PAGE and enzymatic activity measurements. The biochemical properties of recombinant Der p 3 were studied and, in particular, the interaction with the free synthetic wild-type and TrpR propeptides was explored in terms of activity inhibition. 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. Further more, 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 TrpR. Conclusions-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 of the house dust mite papain-like protease Der p 1. This is the first report of a recombinant source of Der p 3, with the same enzymatic activity as the natural enzyme and trypsin.

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 of mite allergens. In Europe, the most prevalent species of house dust mites are Dermatophagoides pteronyssinus and D. farinae, depending on relative humidity and temperature. Not less than 16 groups of allergens were identified from extracts of these species (1,2).

Although the allergens of groups 1 and 2 were extensively studied, those from the group 3 were poorly characterized. In Dermatophagoides 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 allergenic properties of Der f 3, a protease homologous to Der p 3 from Dermatophagoides farinae, appear to depend on the purity of the allergen, the populations of patients tested and the sensitivity of the technique used (4). However, the frequency of IgE reactivity towards Der p 3 is approximately 70-80%, suggesting that Der p 3 is a major allergen (5). On the basis of both sequence comparisons and enzymatic studies, Der p 3 has been classified among the S1 serine proteases family, which comprises trypsin-like proteases. Thus, Der p 3 displays 47% of identity with salmon trypsin and 45% with bovine trypsin (3,6,7). Furthermore, all residues involved in the catalytic activity (i.e. the catalytic triad His 51,
Asp 96 and Ser 196) and in the substrate specificity, and the six cysteines involved in disulphide bridges formation, are highly conserved (6). 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 (8,9). Analysis of Der p 3 cDNA reveals that the propeptide is synthesized as a prezymogen (proDer 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 (6). After cleavage of the signal peptide, the zymogen (proDer p 3) is then matured in a propeptide of 232 residues (Der p 3) that cleaves peptide bonds after an arginine or a lysine residue.

Maturation of the zymogen is associated with cleavage and release of the inhibitory propeptide, leading to liberation of the proteolytic activity. Cleavage of the propeptide implies the recognition of a specific activation site, either by another protease (inter-molecular mechanism) or by the catalytic domain of the zymogen itself (intramolecular mechanism) (10). 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 (11). An alternative mechanism, associated with hypercalcemia, involves an intramolecular processing and leads to pancreatitis (12,13).

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 (-DDDDDKp1) at P1 position (14) according to the Schechter and Berger nomenclature (15) 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, more interestingly, the occurrence of a threonine at its C-terminal extremity suggests a unique activation mechanism (6,16).

Natural Der p 3, like 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 (3,17) 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 (-LNAEp1) and can autoactivate at acidic pH (18).

Unfortunately, the quantities of purified Der p 3 allergen from the faecal pellets are very low (6), 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 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 (16,19).

In this study, we report the production of correctly folded, N-glycosylated and inactive recombinant Der p 3 zymogen in Pichia pastoris. We highlight a particular activation mechanism of the zymogen, depending on 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 enzymatic activity inhibition.

**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 and Boc-Gln-Ala-Arg-MCA acetate salts were purchased from Bachem (Buttendorf, Switzerland). Cysteine protease inhibitor E-64 (L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane) was obtained from Sigma (Saint-Louis, Missouri, USA). Unglycosylated mature Der p 1 was obtained from P. pastoris recombinant proDer p 1, as described (18).
Expression of Recombinant proDer p3 in Escherichia coli: The expression plasmid pET 15b containing proDer p3 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), tetracycline (12.5 µg/ml) and streptomycin (50 µg/ml) at 20, 28 and 37°C until an A600 value of 0.5-0.8 was reached. 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, cells were resuspended in Phosphate Buffer Saline (PBS) pH 7.4 and lysed by a cell disrupter (Constant Systems, Daventry, UK). The lysates were centrifuged at 12000 g during 5 min. The supernatants (soluble fraction) and the pellets (insoluble fraction) were analysed by SDS-PAGE.

Construction of the proDer p3 (NqQ) expression vector- The NqQ proDer p3 mutant was constructed by PCR from the proDer p3 sequence using primers introducing the restriction sites for EcoRI and XbaI, respectively 5’-ATC-GAA-TTC-AAT-CCG-ATC-CTG-CCG-GCA-TCC-CCG-CAG-ACC-ATC-GTT-GGC-GGC-GAA-GAA-GCA-CTG-3’ and 5’-CGA-TTG-GAT-TGA-ATC-TAA-ACG-TAG-CCA-GTG-ATC-TAG-AAT-A-3’. The amplified fragment was cloned into pGEM-T easy vector (Promega, Madison, USA). The presence of the NqQ mutation was verified by DNA sequencing. The proDer p3 (NqQ) sequence was isolated by digestion of the pGEM-T easy vector with EcoRI-XbaI and was subsequently cloned into the pPICZα 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 p3 gene (A. Jacquet, unpublished work) cloned downstream from S. cerevisiae α factor signal peptide. P. pastoris SMD1168 strain was transformed with expression vector pPIC9K-proDer p3 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 (418, Gibco, Paisley, Scotland) concentrations (0.25-3 mg/ml). For production of proDer p3, 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 for 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 p3 (NqQ) sequence, transformants were selected on Yeast extract Peptone Dextrose (YPD) medium containing Zeocin (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 the expression at 28 °C during 3 days. The culture was centrifuged at 10000 g during 10 min and the supernatant was stored at -20 °C.

Purification of Recombinant WT proDer p3 and Der p 3- 1 liter of the culture supernatant containing proDer p3 was filtered through a 0.45 µm filter (Millipore, Billerica, USA) and dialysed overnight at 4 °C against a 20 mM ethanalamine buffer (pH 9.5) (buffer A). The solution was then stirred with 200 ml of Qstreamline matrix (Amersham Biosciences, GE Healthcare, Uppsala, Sweden) equilibrated with buffer A at 4 °C. The matrix 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 p3 were pooled and dialysed at 4 °C against a 20 mM ethanalamine buffer (pH 9) (buffer B). Solution was then loaded onto a Q-HP sepharose column (60 ml) (2.6x10cm, Amersham Biosciences, GE Healthcare, Uppsala, Sweden) equilibrated with buffer B. The flowthrough containing proDer p3 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) (1.6x10cm,
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 Der p 1 protease, mature Der p 3 was isolated by a fourth stage of purification with a 1 ml MonoQ column (0.5x5 cm, Amersham Biosciences, GE Healthcare, 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 NoQ 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 polyvinylidene difluoride (PVDF) membrane. Immunoblot analyses using polyclonal anti-proDer 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 anti-mouse antibodies.

**Autoactivation of Recombinant glycosylated and unglycosylated proDer p 3 by pH and Ca2+**

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 N. 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 Ca2+ ions, the zymogen (65 μM final concentration) was incubated at 37 °C in 50 mM polybuffer (pH 8) in the presence of increasing CaCl2 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.

**Activation of Recombinant glycosylated proDer p 3 by rDer p 1-** ProDer p 3 (150 μM) was incubated at 37 °C in 20 mM Na citrate buffer (pH 6.5) with rDer p 1 (1 μM) previously activated in PBS pH 7.4 containing 1 mM DTT and 1 mM EDTA for several periods of time (0 to 120 min). Samples were analysed by SDS-PAGE and the rDer p 3 activity was measured as described below. To obtain mature rDer p 3, after the incubation of the glycosylated zymogen (150 μM) with rDer p 1 during 90 min, the reaction was stopped with 1mM E-64 and rDer p 3 was purified as described above.

**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 or Boc-Gln-Ala-Arg-MCA) 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 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 Procathepsin L, B and S (20,21). 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) (22-26) using the Grafit software Version 5.0.10 (Erithacus Software Ltd.) and the first pseudo-order rate constants (kobs) were calculated for each rDer p 1 concentration.

\[ P = y_{ss} + \frac{y_{ss} - y_0}{k_{obs}} e^\left((-k_{obs}t)-1\right) \]  
(Eq. 1)
where $P$, $v_0$, $v_s$ 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).

$N$-terminal sequencing- ProDer p 3 zymogen and mature rDer p 3 were sequenced in an Applied Biosystems Procise 492 sequencer (Applied Biosystems), based on Edman degradation. Samples were analysed by SDS-PAGE followed by electrophoretic transfer onto a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, USA).

Solid phase synthesis of NPILPASPNAT (WT) and NPILPASPNAR (TPR) peptides- Peptides were synthesized with the help of an Automated Solid Phase Peptide Synthesizer PS3 (Protein Technologies, Inc, Tucson, USA) using DMF as solvent, 20 % piperidine in DMF as deprotectant and 0.4 M N-methyl morpholine in DMF as activator. For one peptide synthesis, 0.1 mmol of resin (Fmoc-L-Arg(Pbf)-4-Methylbenzhydryl resin or Fmoc-L-Thr(tBu)-4-Methylbenzhydryl resin, CBL, Baltimore, Maryland, USA) was used with 0.4 mmol of each amino acid containing 0.4 mmol of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylyphenyl hexafluorophosphate (HBTU, Iris Biotech GmbH, Marktredwitz, Germany). The following side-chain-protecting groups were used: Trt for Asn, tBu for Ser and Thr and Pbf for Arg. The bound peptides were cleaved from the resin by treatment with a mixture of 5 ml trifluoroacetic acid, 500 µl anisole and 500 µl water during 3 hours at room temperature. The crude peptides 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 and 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 RP18column (150X4.6 mm, 3.5 µm, Waters, Milford, Massachusetts, USA).

Recombinant Der p 3 inhibition by its propeptide-

rDer p 3 activity was measured at 37°C with increasing concentrations (10-75 µM) of the IEGR-AMC substrate after the addition of its wild-type (NPILPASPNAT) or modified (NPILPASPNAR, TPR) propeptides (0, 1.25, 2.5, 3.5 and 5 mM) synthesized as described above. The measured reaction rates were analysed with the help of the Exploratory Enzyme Kinetics of Sigma Plot software Version 8.02 which permitted to fit the data to an inhibition model.

Results

Expression of recombinant proDer p 3-

Expression of Der p 3 zymogen in Escherichia coli yielded inclusion bodies at all tested temperatures (20, 28 and 37 °C), whereas a soluble form could be obtained in P. pastoris culture supernatant. This could be purified to homogeneity, yielding approximately 120 mg of proDer p 3 per liter of culture supernatant. N-terminal sequencing (YVNPILP[2]-) 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) indicated that proDer p 3 migrates as a wide 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 (−N3AT−). Staining of the electrophoresis gel with the Gel Code Glycoprotein Staining Kit® (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. 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 N-glycosylations are carried by the −N3AT- site in the propeptide, the asparagine residue at position P3 was replaced by a glutamine by mutagenesis. The N3Q mutant was also expressed in P. pastoris as a secreted soluble form. As shown by western-blotting (Fig. 2C), the mutant N3Q migrated as a single band of approximately 29 kDa, undistinguishable from that corresponding to the N-glycosylase F treated zymogen.
Activation mechanism of recombinant proDer p 3-
In analogy with the autocatalytic mechanism of trypsinogen maturation (12,13), both glycosylated and deglycosylated proDer p 3 were incubated at different pH (ranging from 2 to 12) and in the presence of increasing Ca²⁺ 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. In contrast, upon incubation at pH 8 in the presence of the mite cysteine protease recombinant Der p 1 (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 (12; VVGEKALAG). 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 tetrapeptidic substrate, LEGR-AMC, specific of trypsin-like proteases 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 inter-molecular activation of proDer p 3 occurs in one single step, corresponding to the loss of the entire propeptide.

In the course of the activation process, a minor band of approximately 19 kDa was also detected (Fig. 3A). It is characterized by the A113VGLP N-terminal sequence and corresponds to the hydrolysis of –NAK114, A113VGLP- in the mature recombinant Der p 3 (rDer p 3). Interestingly, a corresponding degradation site was previously reported for human trypsin (–NAR117, V118STIL-) (27). To determine the sensitivity of rDer p 3 to hydrolysis, pure rDer p 3 was incubated in 50 mM polybuffer at 37°C in presence or absence of rDer p 1 and its enzymatic activity was monitored at different times ranging from 0 to 240 hours. Figure 3C shows that the total inactivation of rDer p 3 occurs within 120 min. 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. SDS-PAGE analysis indicated that this rDer p 3 inactivation corresponded to its degradation in low molecular mass fragments (data not shown).

Effect of glycosylation on proDer p 3 maturation rate- Increase of the catalytic activity of rDer p 3 during its inter-molecular activation by rDer p 1 could be monitored in real time (see experimental procedures) (Fig. 4A). Under the experimental conditions described in Figure 4, proDer p 3 displayed a very weak activity, which remained constant over the time scale of the experiment (≤ 30 min). A similar phenomenon was reported with trypsinogen (28). In contrast, the rate of product formation (AMC) increased during the maturation of proDer p 3 and the activation process was faster in the presence of higher concentrations of rDer p 1 protease (Fig. 4A and 4B). For each proDer p 3 maturation curves, the constant velocities of rDer p 3 reached were different indicating that the real steady state 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 (kobs) values. These values were then plotted as a function of the rDer p 1 concentration (Fig. 4C). The observation that kobs increases linearly with the rDer p 1 concentration suggests an inter-molecular activation process. Moreover, in the absence of rDer p 1, extrapolation of the kobs values confirm that no intramolecular process occurs (i.e. kobs = 0). Data, in Fig. 4C, indicate that the non-glycosylated proDer p 3 is activated faster than its glycosylated counterpart. This can be explained by the location 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 Der p 1, recombinant Der p 3 was purified to homogeneity, using a high resolution Mono Q column. Western-blot analysis (Fig. 5) 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. Furthermore, N-terminal sequencing resulted in the expected Der p 3 sequence
(I,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 1EGR-AMC (10μM) as substrate. Figure 6 shows that Der p 3 retains activity between pH 5 and 11, with a maximum at pH ≈ 8.5 ; a similar behaviour has been reported for trypsin and natural Der p 3 (7,29). 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. 6). 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 I). According to the behaviour reported for trypsin (29), 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 protease rDer p 1 measured with the substrate QAR-AMC.

Specific inhibition of rDer p 3 by the wild-type (WT) and modified (TPrR) 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 (K_i > 5 mM). The single substitution of Thr by Arg at the C-terminal extremity of the propeptide (TPrR mutant) was sufficient, however, to dramatically enhance the inhibitory properties of the peptide. The results suggest that the TPrR behaves as a non competitive inhibitor, with dissociation constants K_i (for EI) and K'_i (for ESI) of about 1 and 2 mM respectively (data not shown). This indicates that a ternary rDer p 3-substrate-TPrR complex can be formed but is less stable than rDer p 3-TPrR (EI). The linearity of the Dixon plots (1/V vs I) showed that this ternary complex was not catalytically competent but 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 culture 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 (3,6). Consequently, production of recombinant Der p 3 is highly desirable. However, expression of the homologous mature Der f 3 in Escherichia coli was unsuccessful suggesting that the propeptide could play a critical role in the folding and thus in the production of the allergen (16).

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 N-glycosylation site present in the propeptide.

As expected, the zymogen was inactive indicating that its propeptide could act as an inhibitor of the protease domain. Indeed, many proteases are synthesized as inactive zymogens. It permits a spatially and temporally controlled activation of the proteases and avoids uncontrolled digestions (10). During the course of evolution of the trypsin’s sequence, Arg_{91} - or Ly_{91} 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 (14). Moreover, such acidic residues permit the control of the trypsinogen autoactivation in defined conditions like in presence of Ca^{2+} which can neutralize the carboxylate negative charges (14). 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 uncontrolled digestion of the pancreatic cells (12,13,30). Our results indicated that proDer p 3 could not be activated in presence of Ca^{2+} and/or by its incubation at different pH. The lack of a polyaspartyl motif in its prosequence and the replacement of the Arg_{91} or Ly_{91} 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 (14,29). 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 the zymogen activation (16).

The mite cysteine protease Der p 1 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 in vitro the putative maturation of proDer p 3 under the action of rDer p 1. 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 the cleavage of the propeptide of proDer p 3 as seen by N-terminal sequencing which revealed only the sequence corresponding to the mature form Der p 3 (I12VGGEKALAG-). 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). 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 – NAK114IA115VGLP-. A similar site is present in human trypsin (–NAR117V118STIL–) and could control the half-life of the active enzyme (27,31). Indeed, a natural R117H mutant of human trypsin seems to be involved in hereditary pancreatitis (31). Based on the available crystallographic structures of trypsin, a model of the Der p 3 protease was established by using the program CPHmodels 2.0 Server (32). Figure 7 indicates that the –NAR117– and –NAK114– 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 K114H mutation in Der p 3 could likely abolish this degradation.

The inter-molecular 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 potential proteolytic cascade, depicted in Figure 8, could imply first the maturation of the Der p 1 zymogen, under acidic conditions followed by proDer p 3 maturation, through Der p 1 action, in Der p 3 permitting both the mite digestive function and/or the proDer p 3 maturation. Preliminary results indicate that the maturation of the Der p 3 zymogen by rDer p 3 could be possible. However, the cleavage site would be different from that of the protease Der p 1 leading to another form of rDer p 3 (A16LAG-) previously identified for the natural enzyme (data not shown) (3). The role of Der p 3 in its own zymogen maturation as well as in the activation of different house dust mite precursors will be explored in details soon. 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. (33). 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, -LNAEP1 and -PNATPI respectively. From these results and in vitro 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 lysis of the cells (34-36).

The enzymatic test used to measure the inter-molecular activation of proDer p 3 by the protease rDer p 1 clearly highlighted 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 natural zymogen impossible. Our observations showed that the glycosylation of the house dust mite proDer p 3 zymogen contributed to the inhibition of the maturation process, as shown for the Der p 1 protease (18,37). It could thus indicate an additional protecting mechanism against attacks by other proteases or an earlier activation in the mite.

After the purification of the recombinant Der p 3 protease, we obtained a solution of pure rDer p 3 at 0.06 mg/ml. We determined the optimum pH which is approximately 8.5 in agreement with previous studies on trypsin and natural Der p 3 (7,29). The specificity of rDer p
3 was also explored for the P2 and P3 residues using four different synthetic substrates (IEGR-AMC, GPR-AMC, FSR-AMC and QAR-AMC). The latter is also a substrate used as a reference for measuring the Der p 1 activity. The catalytic efficiency of the natural Der p 1 previously determined by Schulz et al. was 4600 M⁻¹s⁻¹ (38). We measured a \(k_{cat}/K_M\) value of 5000 M⁻¹s⁻¹ for the recombinant Der p 1 protease in the same buffer at 37°C. The catalytic efficiency of rDer p 3 measured for this substrate was fifty-fold higher than that of rDer p 1. This observation could be related to a potential contaminant role of natural Der p 1, even in low quantities, in natural Der p 1 preparations. Indeed, previous studies of natural Der p 1 activity have shown that it was contaminated by a serine protease (39-41).

For the recombinant protease Der p 3, our data indicated that the variation of the residues in P2 and P3 position had little effect on its activity as demonstrated for trypsin (29). 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 (29,42).

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. showed that the trypsinogen fold was different from that of trypsin and that its maturation resulted in an irreversible conformation change in the protease domain (43). 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 T₃pR mutant was activated during the fermentation process (16). 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.

References


Footnotes

Aknowledgments ;

The abbreviations used are: WT, wild-type ; Boc, t-Butyloxy carbonyl ; CAPS, 3-(Cyclohexylamino)-1-propanesulfonic acid ; IPTG, Isopropyl-beta-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, Dimethyl formamide ; Fmoc, 9-Fluorenylmethoxycarbonyl ; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl ; tBu, tert-butyl ; Trt, Trityl ; rDer p 1, recombinant Der p 1 ; rDer p 3, recombinant Der p 3 ; Blo t 3, *Blomia tropicalis* protease, homologous to Der p 3.
Figure legends

Fig. 1. PreproDer p 3 sequence. The 18 residues 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).

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 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 Np3Q (lane 3, 25 µl culture supernatant) using anti-proDer p 3 polyclonal antibody. St, molecular mass standard.

Fig. 3. Inter-molecular activation of glycosylated proDer p 3 and stability of rDer p 3. (A) SDS-PAGE (15%) analysis; 150 µM of glycosylated proDer p 3 (lane A) was incubated at 37°C, in 20 mM Na-citrate buffer (pH 6.5) with 1 µM of rDer p 1 (lane B). St, molecular mass standard; *, rDer p 3 degradation band (A113,VGLP). (B) For each activation time (0 to 120 min), aliquots were diluted 2500-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 240 hours) in presence of rDer p 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. 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) Secondary data analysis of first pseudo order constants (kobs, s^-1) obtained from fits of the glycosylated (white points, standard deviations were between 0 and 20 %) and deglycosylated (black points, standard deviations < 2 %) proDer p 3 processing in function of the rDer p 1 concentration (nM).

Fig. 5. Analysis of purity of rDer p 3. Immunodetetection of proDer p 3 (1 µg, lanes 1), rDer p 3 (1 µg, lanes 2) and rDer p 1 (0.5 and 3 µg for lanes 3 and 4 respectively, molecular mass; 25kDa) by western-blotting using anti-proDer p 3 and anti-rDer p 1 polyclonal antibodies. St, molecular mass standard.

Fig. 6. pH activity profile of rDer p 3. Enzymatic activity was measured by using IEGR-AMC 10 µM as substrate. The relative activity (%) of rDer p 3 after incubation for 1 hour in 50 mM polybuffer, pH ranging from 2 to 12, at 37°C, is shown in black. The activity recovered after adjusting pH to 8 for 1 hour at 37°C is represented in white.

Fig. 7. Der p 3 model structure and human cationic trypsin RX structure. (A) Model of Der p 3 using the CPHmodels 2.0 Server program. The -NAK115- degradation site is in orange. (B) RX structure of human cationic trypsin (2R9P). The -NAR117- autolysis site is in green.

Fig. 8. Proposed proteolytic cascade occurring in the D. pteronyssinus house dust mite. The inter-molecular activation of the Der p 3 zymogen (Fig. 1) 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* are represented by solid arrows. The potential involvement of Der p 3 in its own activation and degradation is suggested by dotted arrows.
FIG 2.

A

B

C
FIG 3.  
proDer p 3 activation mechanism

A

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<th>kDa</th>
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<th>B</th>
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</table>

+ 1 μM Der p 1

B

[Graph showing activation time (min) vs. μM AMC/min]

C

[Graph showing relative activity (%) vs. time (min)]
FIG 4.

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
FIG 5.
FIG 6.
FIG 8.
TABLE I. Kinetic parameters for rDer p 3 and rDer p 1 in presence of 50 mM polybuffer at pH 8.5 or PBS pH 7.4 containing 1 mM EDTA and 1 mM DTT, respectively

<table>
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<tr>
<th>Substrate</th>
<th>Protease</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (10$^7$ M$^{-1}$s$^{-1}$)</th>
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<td>Boc-IEGR-AMC</td>
<td>rDer p 3</td>
<td>27 ± 1</td>
<td>17.3 ± 0.2</td>
<td>6.4 ± 0.3</td>
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<tr>
<td>Boc-GPR-AMC</td>
<td>rDer p 3</td>
<td>14 ± 1</td>
<td>12.4 ± 0.2</td>
<td>8.9 ± 0.7</td>
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<tr>
<td>Boc-FSR-AMC</td>
<td>rDer p 3</td>
<td>11 ± 1</td>
<td>9.9 ± 0.3</td>
<td>9 ± 0.9</td>
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<tr>
<td>Boc-QAR-AMC</td>
<td>rDer p 3</td>
<td>33 ± 3</td>
<td>8.6 ± 0.2</td>
<td>2.6 ± 0.2</td>
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<tr>
<td>Boc-QAR-AMC</td>
<td>rDer p 1</td>
<td>450 ± 80</td>
<td>2.3 ± 0.2</td>
<td>0.05 ± 0.01</td>
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