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Development of recombinant stable house dust mite allergen Der p 3 molecules for component-resolved diagnosis and specific immunotherapy

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Summary

Background The allergen Der p 3 is underrepresented in house dust mite (HDM) extracts probably due to autolysis. Recombinant stable molecule of the allergen is thus needed to improve the diagnosis of allergy and the safety and efficacy of immunotherapy.

Objective The current study reports the immunological characterization of two recombinant molecules of the HDM allergen Der p 3 as useful tools for diagnosis and immunotherapy.

Methods Recombinant mature (rDer p 3) and immature (proDer p 3) Der p 3 and their corresponding S196A mutants were produced in *Pichia pastoris* and purified. The stability, IgE-binding capacity and allergenicity of the different proteins were analysed and compared with those of the major mite allergen Der p 1 used as a reference. Additionally, the immunogenicity of the different allergens was evaluated in a murine model of Der p 3 sensitization.

Results Compared to the IgE reactivity to recombinant and natural Der p 3 (nDer p 3), the mean IgE binding of patient's sera to rDer p 3-S196A (50%) was higher. The poorly binding to nDer p 3 or rDer p 3 was due to autolysis of the allergen. Contrary to Der p 3, proDer p 3 displayed very weak IgE reactivity, as measured by sandwich ELISA and competitive inhibition, rat basophil leukaemia degranulation and human basophil activation assays. Moreover, proDer p 3 induced a T_H1-biased immune response that prevented allergic response in mice but retained Der p 3-specific T-cell reactivity.

Conclusion rDer p 3-S196A should be used for the diagnosis of HDM allergy elicited by Der p 3, and proDer p 3 may represent a hypoallergen of Der p 3.

Keywords Der p 3, diagnosis, hypoallergen, inactive enzyme, recombinant mite allergen, specific immunotherapy

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Introduction

House dust mite (HDM) allergens are one of the major sensitization factors in the development of allergy and asthma worldwide [1, 2]. In the USA and Europe, allergens from *Dermatophagoides* (D.) species are prevalent [3]. Currently, complex *Dermatophagoides* natural extracts are largely employed for subcutaneous immunotherapy (SCIT) or sublingual immunotherapy (SLIT)

as well as for the diagnosis of allergy [4, 5]. However, despite the development of standardized methods to measure allergen content [6–8], these extracts are highly variable in allergen composition and content, which can jeopardize the outcome of diagnostic tests [9] but also the safety and efficacy of immunotherapy [5, 10]. For these reasons, recombinant allergens and hypoallergens are increasingly used for component-resolved diagnosis and therapy, respectively [11–16].

Between 70% and 100% of allergic patients exhibit high levels of IgE against Der p 1 and Der p 2, which are therefore considered as the two major allergens from *Dermatophagoides pteronyssinus* [17, 18]. However, other allergens, such as the serine proteases Der p 3, Der p 6 and Der p 9, may also play an important role in allergy [18]. Indeed, several studies have reported the enzymatic activity of mite proteases as a potential adjuvant factor in the development and chronicity of allergy [19–21]. There is evidence for the interaction of the protease Der p 3 with the respiratory epithelium, although the protease's allergenicity remains poorly investigated [22–25]. Furthermore, the prevalence of anti-Der p 3 IgE is controversial and varies between 10% and 100%. This finding could be due to the heterogeneity of the preparations of the natural allergen but could also be the result of the high susceptibility of Der p 3 to autolysis, hampering diagnosis performed with the purified natural allergen [17, 26, 27]. Therefore, a more stable and homogeneous Der p 3 allergen is highly desirable to ascertain the real prevalence of sensitization to this allergen.

In a previous study [28], we expressed the precursor form of Der p 3 (proDer p 3) as a soluble recombinant protein in *Pichia pastoris*. Fully mature, active Der p 3 was obtained from the cleavage of the prosequence by the major mite allergen Der p 1. We obtained a homogeneous preparation of recombinant Der p 3 with the biochemical and structural characteristics similar to those of the natural Der p 3 enzyme. We showed that, as for its natural counterpart, the half-life of rDer p 3 is tightly controlled by the autodegradation of the protein. Although proDer p 3 was shown to be enzymatically less active than Der p 3 and thus less sensitive to autolysis, the proteases' secondary structures appeared to be similar [28].

To explore the involvement of the proteolytic activity of Der p 3 in the protease's autolysis, we generated an S196A mutant in which the active serine was replaced by an alanine. The immunogenicity and allergenicity of rDer p 3, its more stable precursor form proDer p 3, and the corresponding inactive counterparts, rDer p 3-S196A and proDer p 3-S196A, respectively, were then investigated. Considering that the propeptide of the recombinant proform of Der p 1 (proDer p 1) was shown to block certain IgE epitopes on Der p 1 and that proDer p 1 produced in *Escherichia coli* could represent a hypoallergenic variant of Der p 1 and was a candidate for mite allergy vaccination [29, 30]; the hypoallergenic characteristics of proDer p 3 were also analysed.

Methods

Human sera and blood samples and allergens

Sera were obtained from patients with an HDM allergy and from non-allergic, non-asthmatic subjects from the

unit of Immunology and Allergology of the Centre Hospitalier de Luxembourg, Luxembourg, and the Asthma clinic, Centre Hospitalier Universitaire (CHU), Liège, Belgium. EDTA-anticoagulated peripheral blood samples were obtained from patients with HDM allergy and non-allergic, non-asthmatic subjects from the Asthma clinic, CHU, Liège, Belgium. The diagnosis of HDM allergy was based on a positive case history, positive skin prick test results and serum IgE antibodies against HDM extracts [ImmunoCAP, Phadia, Uppsala, Sweden, IgE (kU/L) $\geq$ 1.4–100]. The samples were collected after informed consent was provided and were analysed in an anonymized manner with the approval of the ethics committee of the CHU of Liège (B707201214995).

Unglycosylated proDer p 1 and proDer p 3 and the corresponding catalytically inactive molecules proDer p 1C34A and proDer p 3-S196A, respectively, were expressed in *P. pastoris*, purified and activated into mature rDer p 1 and rDer p 3 and their inactive counterparts, rDer p 1-C34A and rDer p 3-S196A, respectively, as previously described [28, 31–33]. Natural Der p 3 (nDer p 3) was purified to homogeneity from HDM extracts (see below). Endotoxin levels of purified allergens were determined using the limulus amoebocyte lysate assay (Hycult Biotech, Uden, the Netherlands) according to the manufacturer's instructions. The endotoxin levels of the allergens used for the immunization were < 20 U/mg protein.

Purification of nDer p 3

For purification, 1 g of whole *D. pteronyssinus* culture was stirred overnight at 4°C in a phosphate buffer saline pH 7.5 (PBS). Insoluble material was removed by ultracentrifugation at 130 000 g for 1 h. Supernatants containing solubilized proteins were dialysed at 4°C against 20 mM Tris-HCl buffer, pH 7.5 (buffer A). The solution was loaded onto a Q-HP-Sepharose column (60 mL, 2.6×10 cm; GE Healthcare, Uppsala, Sweden) previously equilibrated with buffer A. The elution of bound proteins was performed with a linear gradient of buffer A added to 1 M NaCl. The resultant fractions were analysed for Der p 1, Der p 3, Der p 6 and Der p 9 activities using specific chromogenic substrates, as previously described [28]. The fractions containing Der p 3 activity (and also Der p 6 and Der p 9 activities) were applied onto a benzamidine Sepharose affinity column equilibrated with buffer A. nDer p 3 and nDer p 6 were eluted from the column with 100 mM acetate buffer, pH 4. Finally, to separate Der p 6 and Der p 3, fractions containing nDer p 3 were dialysed against buffer A and applied onto a heparin affinity column (GE Healthcare) equilibrated in buffer A. Adsorbed material was eluted with a linear gradient of buffer A with 1 M NaCl. Purified nDer p 3 was concentrated by ultrafiltration (cut-off: 10 kDa) and stored at -20°C .

Circular dichroism measurements

Far UV circular dichroism (CD) spectra were recorded at 25°C in a 1-mm-pathlength cell using a Jasco J-810 CD spectropolarimeter (Jasco Inc., Easton, MD USA). Experiments were performed in 20 mM Tris pH 8.5 at a final protein concentration of 0.13 mg/mL. Five spectra were acquired at a scan speed of 3 nm/min, and were averaged and corrected for baseline contribution of the buffer.

Fluorescence measurements

The intrinsic fluorescence spectra of proteins (4 µM) were recorded at 25°C in a 1-cm-pathlength cell on a Perkin Elmer LS 50 B spectrofluorimeter (Perkin Elmer, Wellesley, MA, USA) using a scan rate of 350 nm/min. The excitation wavelength was 280 nm, and emission spectra were recorded from 300 to 420 nm. Five spectra were recorded, averaged and corrected as explained above.

Enzymatic activity measurements

The rDer p 3, proDer p 3, rDer p 1 and proDer p 1 allergens were incubated at 37°C for various lengths of time in PBS, pH 7.4. Allergens were analysed by SDS-PAGE, and Der p 3 or Der p 1 activities were measured in 10-fold diluted aliquots using 150 µM QAR-AMC as substrate on a Perkin Elmer LS 50 B spectrofluorimeter as previously described [28, 33].

IgE-binding activity assay

nDer p 3, rDer p 3, rDer p 3-S196A and recombinant Der p 1 (rDer p 1) were coated overnight (500 ng/well) at 4°C on immunoplates. Plates were washed with PBS containing 0.01% Tween-20 and then saturated for 1 h at 37°C with PBS-Tween-20 supplemented with 1% BSA (Sigma, Bornem, Belgium). Sera from 22 patients with an HDM allergy and from 8 healthy donors were used at a 1 : 5 dilution and were then incubated for 1 h at 37°C. After washing with PBS-Tween-20, the allergen-IgE complexes were detected with a mouse anti-human IgE antibody (ImTec Diagnostics) coupled to alkaline phosphatase (dilution 1 : 500). The enzymatic activity was detected using the 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) dissolved in TRIS buffer (pH 9.4). OD 405 nm was measured in Bio-Rad Novapath ELISA reader (Nazareth Eke, Belgium). ODs ≥ 3 times the control values were considered to be ELISA-positive results.

For IgE inhibition assays, plates were coated with rDer p 3-S196A (500 ng/well) and 6 sera were previously incubated with increasing concentrations of nDer

p 3, rDer p 3, rDer p 3-S196A, proDer p 3 or proDer p 3-S196A (0, 0.25, 0.5, 2.5, 5, 10, 25 and 50 µg/mL). The sera were then incubated on ELISA plates, and IgE-binding activity was measured as described above.

Sandwich ELISA for IgE-binding activity

Plates were coated with a mouse anti-human IgE monoclonal antibody (BD Pharmingen San Jose, CA, USA) (250 ng/well) for 16 h at 4°C, washed with Tris-buffered saline-0.01% Tween-80 and then saturated with the same buffer supplemented with 1% BSA. Sera (dilution 1 : 5) were incubated for 1 h at 37°C. After washing, rDer p 1, proDer p 3 or rDer p 3S196A (500 ng/well) was added to saturation buffer and incubated for 1 h at 37°C. The plates were then washed and incubated with rabbit anti-proDer p 3 or anti-proDer p 1 antibodies for 1 h at 37°C. After washing, a donkey anti-rabbit biotinylated Ig (Amersham, Roosendaal, the Netherlands) (dilution 1 : 5000) was added for 1 h at 37°C. The assay was developed using a streptavidin-HRP conjugate (Amersham) (dilution 1 : 1000) and 3,3',5,5'-tetramethylbenzidine (Sigma) as the substrate. The reaction was stopped by adding 0.4 M H₂SO₄, and the absorbance was measured at 450 nm. ODs ≥ 3 times the control values were considered to be ELISA-positive results.

Long-term stability assay

The rDer p 3 and rDer p 1 allergens and their corresponding precursor forms (5 µM) were incubated at 37°C. Samples were taken over time and analysed by 15% SDS-PAGE and for Der p 1 and Der p 3 activity using the fluorogenic substrate Boc-QAR-7-Amino-4-methylcoumarin (AMC) (10 µM), as previously described [28, 31].

Rat basophil leukaemia (RBL) cell mediator release assay

The five sera used, coming from patients with HDM allergy (see above), were selected by ELISA to be positive to Der p 1 and Der p 3. RBL-SX-38 cells (a kind gift of Professor Kinet, Beth Israel Deaconess Medical Center, Boston, MA, USA), expressing human FcεRI, were sensitized with sera (dilution 1 : 5) for 17 h. Triggering of RBL cells was induced by adding various concentrations (0.0001–1 µg/mL) of nDer p 3, rDer p 3, rDer p 3-S196A, proDer p 3, proDer p 3-S196A and rDer p 1 for 30 min at 37°C. As a control, cells were also incubated in the absence of allergen to measure spontaneous release. Total release was obtained by adding 0.5% Triton X-100 to the medium. The release of β-hexosaminidase was analysed by incubating

supernatants (50 μ L) with 50 μ L p-nitrophenyl-N-acetyl- β -D-glucosaminide (2 mM in 0.2 M citrate buffer, pH 4.5; Sigma) for 3 h at 37°C. The reaction was stopped by adding 150 μ L 1 M TRIS-HCl, pH 9, and the absorbance at 405 nm was measured. Results were expressed as percentage of the total release minus the spontaneous release.

Human basophil activation assay

This assay was performed on EDTA-anticoagulated peripheral blood samples obtained from patients with HDM allergy and non-allergic, non-asthmatic subjects from the Asthma clinic, CHU, Liège, Belgium (see above) using an Allergen Kit (Beckman Coulter ZA Paris, France) according to the manufacturer's instructions. The nDer p 3, rDer p 3, rDer p 3-S196A, proDer p 3, proDer p 3-S196A or rDer p 1 allergens were used at different concentrations (0.0001–10 μ g/mL). An anti-IgE antibody and PBS were used as positive and negative controls, respectively. Leucocytes were analysed using a Navios flow cytometer (Beckman Coulter). Basophils (700 target events) were gated as the CRTH₂-positive/CD3-negative population. Responses were quantified as percentages of CD203c-expressing basophils in the Q2 region. This region was adjusted to contain 5% of basophils (i.e. activated basophils) in the unstimulated sample (negative control). A fivefold increase in the number of activated basophils (> 25%) compared with the negative control (5%) was considered to be a positive response [34].

Animals

Female Balb/c mice (6 weeks old) were obtained from Harlan (Horst, the Netherlands). Animal care and experimental procedures were performed in accordance with local institutional guidelines (laboratory licence no. LA1500474).

Immunization protocol

Groups of five Balb/c mice were immunized three times at 2-week intervals with 10 μ g rDer p 3, rDer p 3-S196A, proDer p 3 and rDer p 1 adjuvanted with alum (ratio of allergen/adjuvant of 1/200; intraperitoneal injection). As a control, two groups of mice were immunized with adjuvant alone (Naïve and IN). The mice were bled on days 0, 28, 42 and 47, and sera were collected. Except for the naïve group that was sensitized with adjuvant alone, airway inflammation was induced by challenging the mice 14 days after the last sensitization (day 42) by the intranasal instillation of 100 μ g of crude *D. pteronyssinus* extract (Greer, Lenoir, NC, USA)

for 3 consecutive days. For each mouse, serum, bronchoalveolar lavage fluid (BAL) and the spleen were collected on day 47.

Proliferation and cytokines assays

The rDer p 3- and rDer p 1-specific T-cell proliferative responses were measured. Spleen cells (4×10^5 /well/ in triplicate) from immunized mice were stimulated with rDer p 1, rDer p 3, proDer p 3 or with rDer p 3-S196A (1.25 μ g/mL). As control, cells were incubated with RPMI medium only. After 72 h, cells were pulsed with [³H]-thymidine (Amersham) for 16 h. Cells were harvested, and [³H]-thymidine uptake was measured by scintillation counting. Proliferate responses were expressed as stimulation index (SI), which corresponds to the ratio of counts per min (CPM) measured in the culture stimulated with the allergen to the CPM measured in the culture medium (RPMI). A stimulation index value > 2 was considered positive.

Production of IFN γ and IL-5 in supernatant from restimulated splenocytes was measured using ELISA kits supplied by BD Pharmingen.

Bronchoalveolar lavage

One day after the final challenge, mice were bled. The lungs were immediately washed via the trachea cannula with 1 mL PBS, which was instilled and gently recovered by aspiration three times. The lavage fluid was centrifuged and the cells were counted in a Thoma hemocytometer (Sigma). Cytospin preparations from 50 μ L aliquots were stained with May–Grünwald–Giemsa stain for differential cell counts.

Measurement of Der p 3-specific IgG1 and IgG2a

Immunoplates were coated overnight with rDer p 3-S196A (500 ng/well) at 4°C. Plates were then washed 3 times with Tris-buffered saline–0.01% Tween-80 (TBS-T) and saturated for 1 h at 37°C of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 h at 37°C. Plates were washed five times with TBST-T buffer. Antigen-bound antibodies (IgG1, IgG2a) were detected with anti-mouse subclass IgG1 or IgG2a monoclonal antibodies (dilution 1 : 7000, Biosource). Phosphatase alkaline conjugated to streptavidin (dilution 1 : 1000, Amersham) was added to each well and the enzymatic activity was measured as described above.

ODs ≥ 3 times the control values were considered to be ELISA-positive results.

Statistical analysis

Group data were compared using a Student's *t*-test. Differences were considered significant for $P < 0.05$.

Results

Characteristics of allergens

The purity of the preparations of allergens was assessed by silver-stained SDS-PAGE analysis (Fig. 1a). As previously shown, no Der p 1 could be detected in Der p 3 preparations [28]. The intrinsic fluorescence and far UV-CD analysis showed that rDer p 3 and its inactive counterpart rDer p 3-S196A exhibit a similar tertiary and secondary structure content, respectively (Figs 1b and c). In contrast, proDer p 3 and proDer p 3-S196A exhibited a blue shift of their maximum emission wavelength (333 nm) compared to those of rDer p 3 and rDer p 3-S196A (350 nm) (Fig. 1b) [33].

The proteolytic activity of Der p 3 reduces its stability

To investigate the effect of the enzymatic activity of Der p 3 on its stability, the active protease, rDer p 3, and its less active precursor form, proDer p 3, were incubated at 37°C, and their catalytic activities were quantified at multiple time points. After 6 h of incubation, rDer p 3 lost approximately 70% of its activity

(Fig. 2a), whereas more than 90% of the weak activity exhibited by proDer p 3 was maintained after 24 h. In comparison, rDer p 1 and proDer p 1 conserved more than 75% of their initial activity after 24 h of incubation (Fig. 2b). The higher stability displayed by proDer p 3, rDer p 1 and proDer p 1 was confirmed by SDS-PAGE analysis (data not shown). In contrast, after incubation of 4 h at 37°C, the band corresponding to rDer p 3 (25 kDa) continuously decreased in intensity with time, leading to the appearance of low molecular weight degradation fragments (Fig. 2c). Importantly, the inactive mutant rDer p 3-S196A did not undergo autolysis, even after 24 h of incubation (Fig. 2d). These results clearly show that due to its enzymatic activity, rDer p 3 is far less stable than rDer p 1 and that the propeptide plays an important role in the protection of Der p 3 against spontaneous hydrolysis. Moreover, mutation of the active serine in the enzyme's catalytic site completely abolishes the phenomenon of autolysis.

Underestimation of the IgE-binding capacity of Der p 3 due to autolysis

To determine the impact of the autolysis of Der p 3 during the indirect ELISA ($N = 22$), the IgE-binding capacities of rDer p 3 and nDer p 3 were compared with the capacity of the catalytic mutant rDer p 3-S196A, using rDer p 1 as a reference. Interestingly, both natural

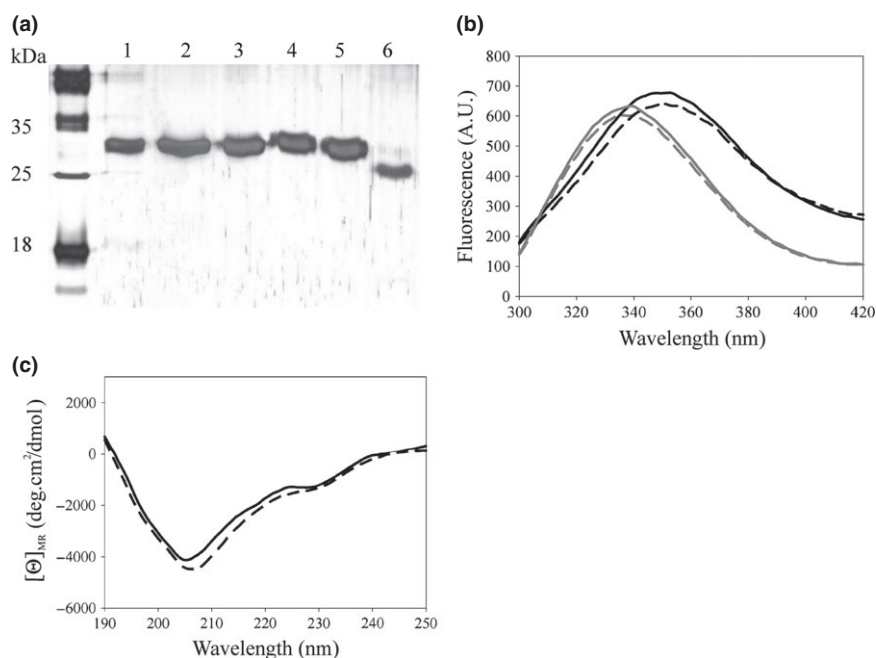


Fig. 1. Characteristics of purified allergens. (a) SDS-PAGE (15%) silver-stained analysis of 4 μ m purified 1: nDer p 3, 2: rDer p 3, 3: rDer p 3-S196A, 4: proDer p 3, 5: proDer p 3-S196A, 6: rDer p 1; (b) Intrinsic fluorescence spectra of rDer p 3 (solid black), rDer p 3-S196A (dashed black), proDer p 3 (solid grey) and proDer p 3-S196A (dashed grey); (c) Far UV-CD spectra of rDer p 3 (solid black) and rDer p 3-S196A (dashed black).

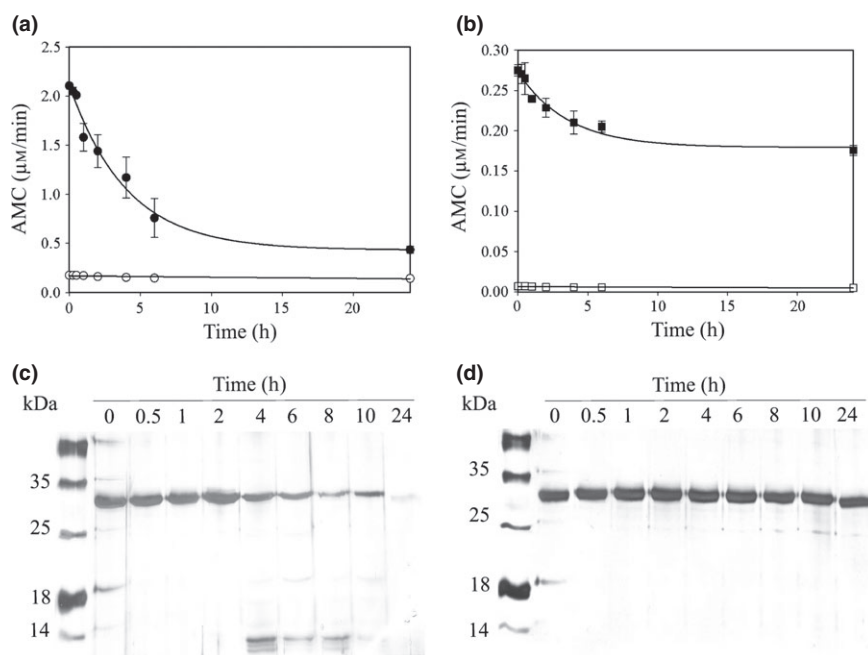


Fig. 2. The proteolytic activity of Der p 3 reduces the protease's stability. (a) For each incubation at 37°C, the residual activity of (a) rDer p 3 (●) and proDer p 3 (○) and (b) rDer p 1 (■) and proDer p 1 (□) was measured in 10-fold diluted aliquots. Standard deviations are < 10%. SDS-PAGE (15%) silver-stained analysis of 4 μM (c) rDer p 3 and (d) rDer p 3-S196A incubated at 37°C during increasing times.

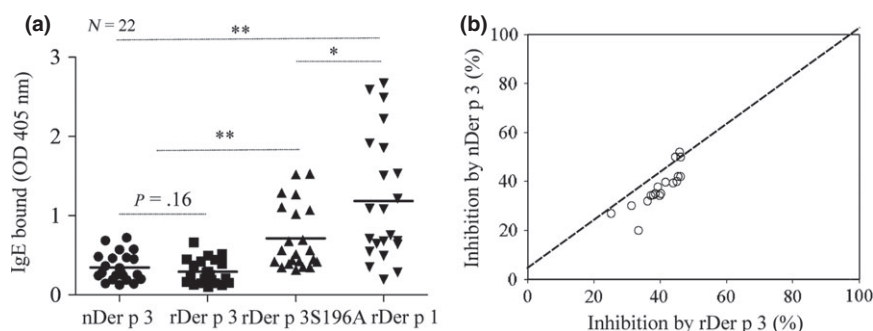


Fig. 3. The autolysis of Der p 3 reduces the protease's IgE reactivity. (a) The IgE reactivity of nDer p 3, rDer p 3, rDer p 3-S196A and rDer p 1 was evaluated by indirect ELISA using positive sera against *Dermatophagoides pteronyssinus* ($N = 22$). * $P < 0.01$ and ** $P < 0.001$; (b) Inhibition ($N = 20$) ELISAs were performed using ImmunoCAP-positive sera against *D. pteronyssinus*. The binding of IgE to rDer p 3-S196A was inhibited by nDer p 3 and rDer p 3. The percentage inhibition is shown as $100 - (\% \text{ IgE binding in the presence of inhibitor} / \% \text{ IgE binding in the absence of inhibitor})$. The dashed line represents the diagonal.

and recombinant forms of Der p 3 displayed similar IgE-binding reactivity with all of the tested sera (Fig. 3a).

As expected, the IgE reactivity to the stable allergen rDer p 3-S196A (ranging from 0.3 to 1.5; median value of 0.7) was two times higher than the rate measured for rDer p 3 or nDer p 3 (ranging from 0.2 to 0.7 and from 0.1 to 0.7, respectively; median values of 0.3) (Fig. 3a). The negative controls obtained from experiments realized with sera coming from non-allergic patients exhibited values ranging from 0.02 to 0.04.

If we consider approximately 10% and 25% of the tested sera to be Der p 3 positive when considering the

active nDer p 3 and rDer p 3 IgE-binding activities, respectively, which is in agreement with the findings of Thomas's group [17], 50% of sera should be Der p 3 positive when considering the IgE response to rDer p 3-S196A.

To evaluate the possibility that Der p 3 may digest IgE during test, sera previously incubated with active Der p 3 during increasing times were analysed by Western blot using an anti-IgE antibody. Noteworthy, no IgE degradation was observed (data not shown) confirming that the discrepancy of IgE-binding rate observed between the active and inactive Der p 3 forms is due to allergen autolysis.

The IgE reactivity of nDer p 3 and rDer p 3 were next analysed by competitive inhibition assays using the same sera pre-incubated with nDer p 3 or rDer p 3 before incubation with solid phase-bound rDer p 3-S196A. The two allergens inhibited the binding of patients' IgE to rDer p 3-S196A in a comparable manner (Fig. 3b; $R = 0.89$). However, strikingly, no more than 60% inhibition was observed. These results indicate again that rDer p 3 retains the relevant IgE epitopes of nDer p 3 and that the IgE-binding capacity of the catalytic mutant rDer p 3-S196A is higher than the capacity of the two active forms.

The recombinant catalytic mutant of Der p 3, rDer p 3-S196A, could thus represent a valuable tool for addressing the underestimation of the prevalence and titre of IgE against Der p 3 in HDM allergy diagnosis.

proDer p 3 has weak IgE reactivity and allergenicity in vitro

To test the hypoallergenic potential of the precursor form of Der p 3, the IgE reactivity of proDer p 3 was evaluated by sandwich ELISA and inhibition assay. The IgE-binding capacity of proDer p 3, estimated by sandwich ELISA, was drastically reduced compared with the capacity of rDer p 3-S196A (Fig. 4a, $P < 0.01$). In the inhibition assay, the IgE reactivity of rDer p 3-S196A was analysed using six patient sera pre-incubated with

various concentrations of proDer p 3 or rDer p 3-S196A. The inhibition capacity of proDer p 3 was also drastically reduced in this assay compared with the capacity of rDer p 3-S196A (Fig. 4b). Those results were confirmed using 20 other sera incubated with a fixed proDer p 3 or rDer p 3-S196A concentration (50 $\mu\text{g/mL}$) (Fig. 4c).

Mediator release assays were performed with RBL cells expressing the human Fc ϵ RI receptor passively sensitized with five sera from patients with a *D. pteronyssinus* allergy and stimulated with the different forms of Der p 3 or with rDer p 1, used as a positive control. Overall, the induced degranulation levels were similar for nDer p 3, rDer p 3, rDer p 3-S196A and rDer p 1 and were much higher than those levels induced by proDer p 3 (Fig. 5a). Note that no autolysis of Der p 3 occurred during this rapid test which requires a short incubation of 30 min at 37°C. This finding indicates that the reduced IgE-binding capacity of proDer p 3 is correlated with lower allergenic activity.

To confirm this result in human cells, blood basophils from allergic donors were also stimulated with different concentrations of the different forms of Der p 3 or with rDer p 1. Importantly, CD203c was significantly upregulated (Fig. 5b) in the same manner in basophils stimulated with nDer p 3, rDer p 3, rDer p 3-S196A or rDer p 1, whereas the precursor forms of Der p 3, up to a concentration of 10 $\mu\text{g/mL}$, did not activate IgE-sensitized

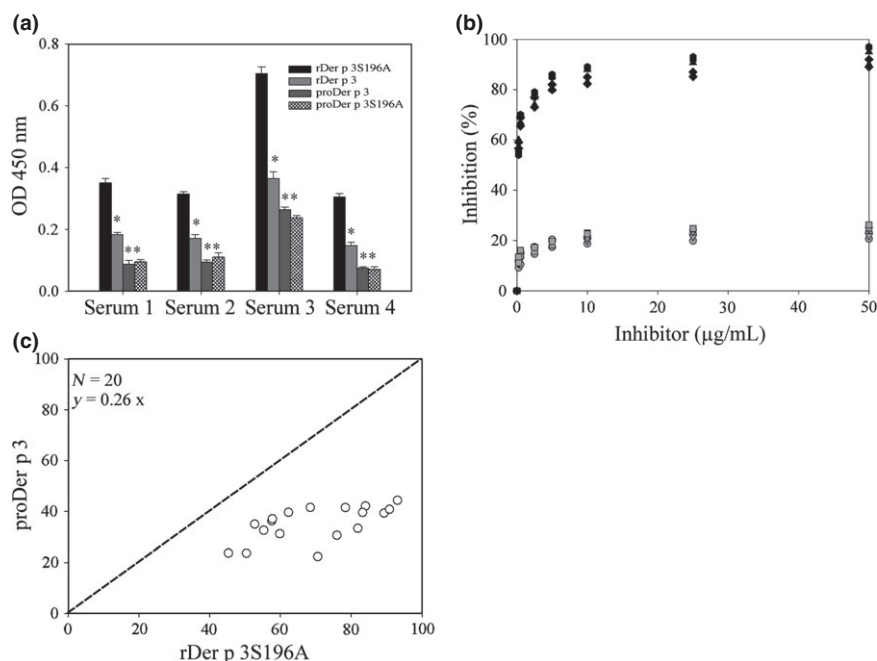


Fig. 4. proDer p 3 exhibits weak IgE reactivity. (a) IgE reactivity measured with a sandwich ELISA using positive sera against *Dermatophagoides pteronyssinus* ($N = 4$). * $P < 0.05$ and ** $P < 0.01$. The binding of IgE to rDer p 3-S196A was inhibited by incubating (b) positive sera ($N = 6$, the average curve is shown) with increasing concentrations of rDer p 3-S196A (black panel) or proDer p 3 (grey panel) or by incubating (c) positive sera ($N = 20$) with a fixed concentration (50 $\mu\text{g/mL}$) of rDer p 3-S196A or proDer p 3.

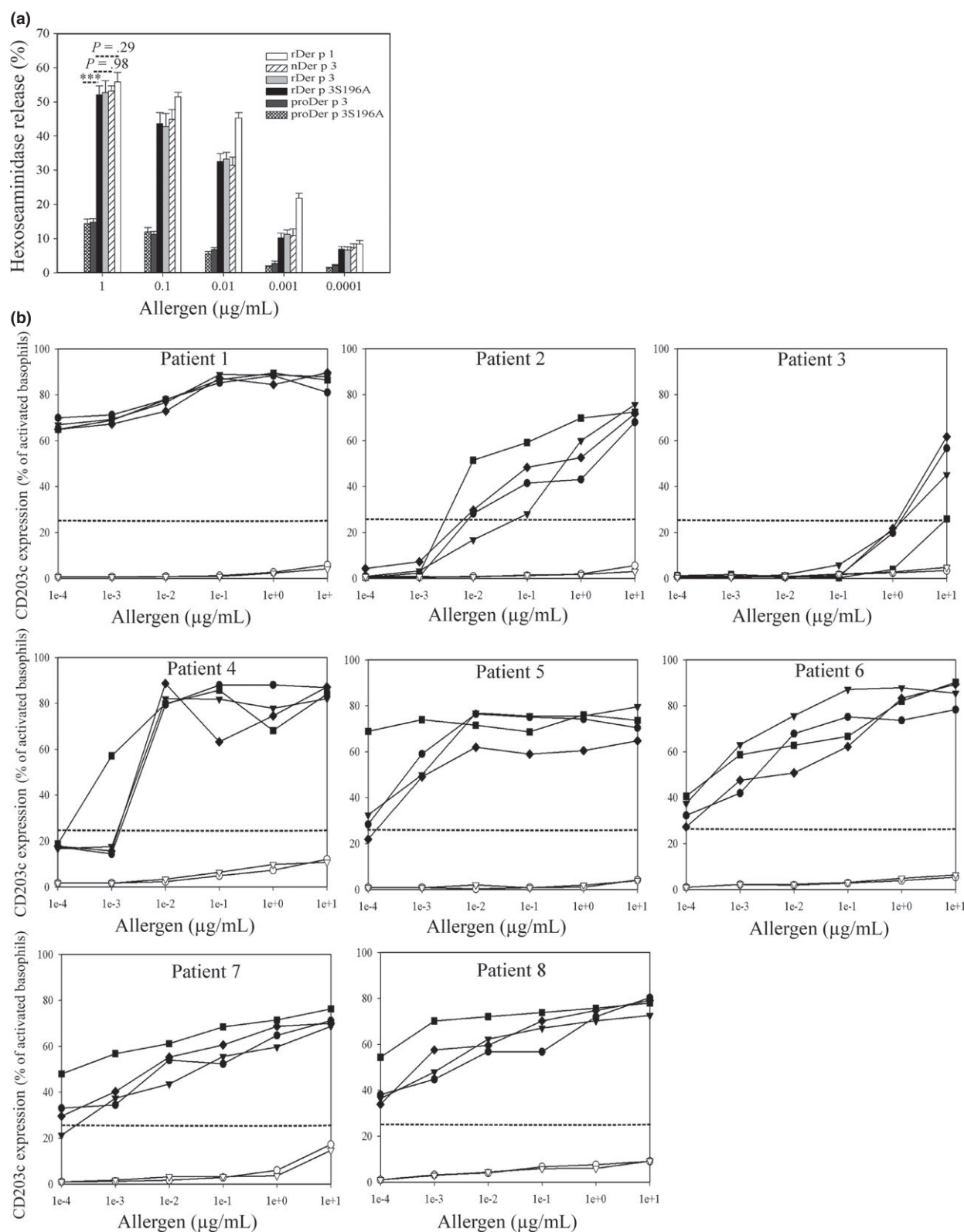


Fig. 5. proDer p 3 displays very weak allergenicity *in vitro*. (a) Mediator release of humanized rat basophil leukaemia (RBL) stimulated with different concentrations of allergens. The results are expressed as the percentage of the total release of β -hexosaminidase. $***P < 0.001$. (b) CD203c expression at the surface of human blood basophils stimulated with different concentrations of rDer p 1 (■), nDer p 3 (◆), rDer p 3 (●), rDer p 3-S196A (▼), proDer p 3 (○) and proDer p 3-S196A (▽). The percentage of activated basophils is defined as CRTH2/CD203c positive cells.

basophils. Similarly, basophils from a non-allergic individual did not express CD203c when stimulated with the different allergens (data not shown).

In vivo evaluation of Der p 3 and proDer p 3 allergenicity

Balb/c mice were sensitized with rDer p 3, rDer p 3-S196A, proDer p 3 or rDer p 1 adjuvanted with alum. As a control, two groups of mice were sensitized with adjuvant alone (Naïve and IN). The pretreated mice (except for the Naïve group) subsequently received HDM extracts by intranasal instillation to provoke airway inflammation (Fig. 6a).

Mice sensitized with rDer p 3 or rDer p 3-S196A exhibited high titres of specific IgE (Fig. 6b) and anti-Der p 3 IgG₁ antibodies (Fig. 6c), whereas the titres of anti-Der p 3 IgG_{2a} antibodies were very low (Fig. 6d). These results demonstrate that rDer p 3 and rDer p 3-S196A induce a typical allergic T_H2-type response, similar to rDer p 1. In contrast, the specific IgE and IgG₁ responses induced by proDer p 3 were significantly lower than those responses induced by rDer p 3 (Fig. 6c, $P < 0.001$). Moreover, proDer p 3 induced the significant production of anti-Der p 3 IgG_{2a} (Fig. 6d, $P < 0.001$). These results clearly suggest that proDer p 3 induces a T_H1-biased immune response, even in the presence of a pro-T_H2 adjuvant such as alum.

Splenocytes isolated from mice sensitized with proDer p 3, rDer p 3 or rDer p 3-S196A were stimulated *ex vivo* with rDer p 3. The cells's proliferation levels were similar for the different groups of mice (Fig. 6e). Moreover, after their stimulation with rDer p 3, proDer p 3 or rDer p 3-S196A, the stimulus index was similar (data not shown), thus suggesting that proDer p 3 retains relevant Der p 3-specific T-cell epitopes. By contrast, splenocytes isolated from mice that were sensitized with different forms of Der p 3 and stimulated *ex vivo* with rDer p 1 and vice versa did not proliferate, thereby serving as a negative control.

Concomitantly, supernatants from cultured stimulated lymphocytes were harvested and analysed for IFN- γ and IL-5. A typical T_H2 cytokine profile, characterized by the high production of IL-5 and low or no secretion of IFN- γ , was elicited in rDer p 3- and rDer p 3-S196A-sensitized mice (Fig. 6f). Again, the T_H1 bias of the immune response induced by proDer p 3 was confirmed because animals sensitized with proDer p 3 secreted high and low amounts of IFN- γ and IL-5, respectively, upon restimulation (Fig. 6f, $P < 0.001$).

Finally, animals sensitized with rDer p 3 and challenged with HDM extracts developed pronounced airway eosinophilia in the BAL fluids (Fig. 6g, $P < 0.01$). This hypereosinophilia was similar to that observed in mice sensitized with rDer p 1. In contrast, eosinophils

were not observed in BAL from mice sensitized with proDer p 3.

Discussion

Der p 1 and Der p 2 are described as the two major allergens of *D. pteronyssinus* because these allergens correspond to 70–100% of the total IgE-binding activity of HDM extracts [17, 18, 35]. However, the IgE-binding activity of these extracts could be biased due to the lack or to the poor stability of several allergens [9]. This bias is observed for mite serine proteases that are highly sensitive to degradation, which can compromise diagnosis [26, 27]. In particular, our group and others have shown that nDer p 3 and rDer p 3 undergo to autolysis after 4 h of incubation at 37°C [26, 28]. Here, we investigated the allergenicity of both forms and compared the allergenicity to that of the catalytically inactive mutant rDer p 3-S196A. Thus, for the first time, we evaluated the role of the degradation of an allergenic protease in the context of HDM allergy diagnosis.

Indirect ELISAs and inhibition assays showed that the IgE reactivities of nDer p 3 and rDer p 3 are highly similar, suggesting that the main IgE-binding epitopes of nDer p 3 are present in the protease's recombinant form. Accordingly, the two forms induced a similar release of β -hexosaminidase by humanized basophils in RBL assays. More interestingly, the forms also induced a similar upregulation of CD203c expression on the surface of blood basophils from allergic donors. These results, together with those findings obtained in our previous study, indicate that rDer p 3 has the same structural, biochemical and allergenic properties as its natural counterpart [28].

However, ELISAs and competitive inhibition assays performed with rDer p 3 and rDer p 3-S196A revealed that the rDer p 3's instability during tests due to its proteolytic activity drastically decreases its IgE-binding capacity. It is thus likely that the IgE-binding activity of Der p 3 was underestimated in the past due to the autolysis of nDer p 3 during testing. Using rDer p 3-S196A in ELISAs, we estimated a new prevalence of Der p 3 of approximately 50%. Thus, this catalytic mutant, rDer p 3-S196A could be considered as a suitable alternative for the diagnosis of HDM allergy in the future.

Moreover, due to its proteolysis, the concentration of nDer p 3 in the HDM extracts must decrease with time. Therefore, in the context of immunotherapy with HDM extracts, it would be of interest to stabilize nDer p 3 protein using specific serine protease inhibitors treatment. Additionally, rDer p 3-S196A variants could be used to develop a robust and accurate ELISA allowing the quantification of Der p 3 in the HDM extracts. Such

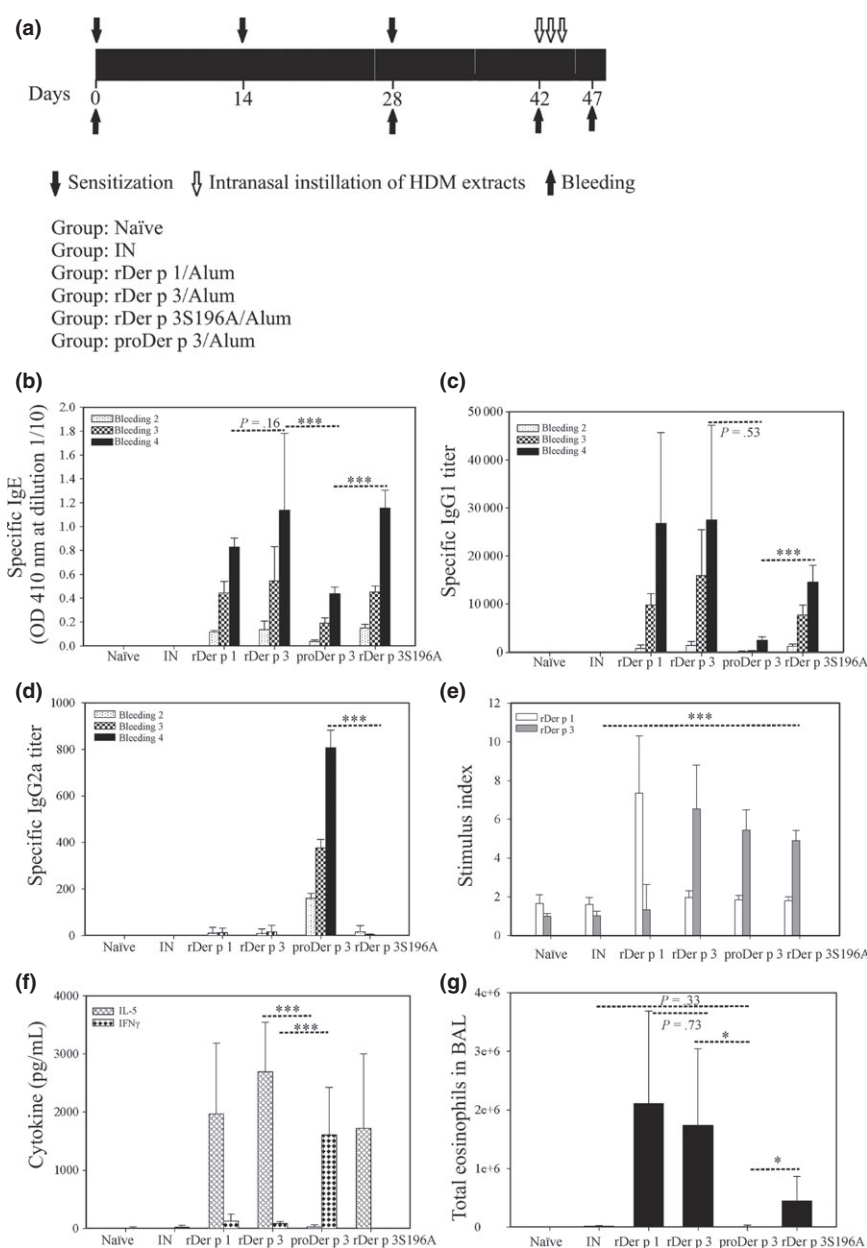


Fig. 6. Evaluation of experimental house dust mite (HDM) allergy by sensitization with proDer p 3. (a) Experimental protocol for the evaluation of immune responses in naïve and sensitized mice. Antibody titres [(b) IgE, (c) IgG₁ and (d) IgG_{2a}], (e) the lymphoproliferative response and (f) the splenocyte cytokine production of *in vitro*-stimulated splenocytes. (g) Airway eosinophilia. The results are expressed as the means of individual titres $\pm$ SD from five mice.

an assay could also be applied to assess the stability of the extracts over time and contribute to the standardization of HDM materials.

If the broader use of rDer p 3-S196A reveals that the allergenic role of Der p 3 has been underestimated in allergic subjects, the development of Der p 3-based hypoallergens would be interesting. Here, we describe the immunological characterization of recombinant proDer p 3 as a hypoallergen of nDer p 3. Although the secondary structures of nDer p 3 (24.9 kDa) and proDer

p 3 (26.4 kDa) were previously shown to be similar [28], the intrinsic fluorescence spectra of these proteins, which are characteristic of the proteins' tertiary structures, are different [33]. Indeed, when compared with the precursor proDer p 3, the mature forms, nDer p 3 and rDer p 3, exhibit a red shift of their maximum emission wavelength, suggesting the rearrangement of the catalytic domain of the protease after maturation. This phenomenon, affecting the loops but not the secondary structures, has been reported during the activa-

tion of trypsin [33]. The role of the Der p 3 propeptide in allergenicity was thus evaluated. For all tested sera, proDer p 3 showed less allergenicity than each form of Der p 3 (n, r or S196A) in sandwich ELISAs and in competitive inhibition, RBL and human basophil activation assays, as shown for the proDer p 1-Der p 1 couple [29]. The mechanisms behind the weak IgE-binding activity of proDer p 3 are not clear. This mechanism could be related to the blocking of conformational IgE-binding sites on the surface of the protease domain by the N-terminally located propeptide, as was demonstrated for proDer p 1 [29], or to the increase of epitopes accessibility after protease activation.

We thus compared the immunogenicity of proDer p 3 and the mature forms of Der p 3 in mice. Mice sensitized with rDer p 3 exhibited a typical T_H2 -biased immune response, as described for Der p 1 [30], characterized by high titres of Der p 3-specific IgG₁ and IgE and the absence of IgG_{2a} production. In contrast, proDer p 3 induced a T_H1 -biased immune response characterized by the production of specific IgG_{2a} and the weak production of specific IgE antibodies over time. The cytokine pattern of T-cell proliferation confirmed that proDer p 3 is a T_H1 inducer because the drastic reduction of IL-5 production was accompanied by enhanced IFN- γ secretion. proDer p 3 immunization also drastically reduced the infiltration of eosinophils in the BAL fluids, which is most likely correlated with low IL-5 secretion and reduced IgE-dependent mast cell activation in proDer p 3-sensitized mice after intranasal instillation with HDM extracts [36]. Together with the maintenance of the Der p 3-specific T-cell reactivity, these data clearly increase the interest for the use of hypoallergenic proDer p 3, as a safe recombinant vaccine in allergen-specific immunotherapy. It is noteworthy that the rDer p 3 and rDer p 3-S196A allergens induced both a similar humoral and cellular immune

response *in vivo*; which confirms that the IgE epitopes of Der p 3 are maintained in the catalytically deficient mutant. However, the infiltration of the eosinophils in the BAL fluids was reduced for rDer p 3-S196A, which indicates that the proteolytic activity of Der p 3 is important to trigger this inflammation mechanism. Indeed, it was previously shown that Der p 3 induces the activation of the PAR-2 receptor and that PAR-2 mediates eosinophil infiltration [25, 37]. Finally, the treatment of asthmatic mice with serine protease inhibitors decreased the eosinophilia [38].

In conclusion, we report the first example of the underestimation of the IgE-binding capacity of an allergen due to its instability. We suggest that the variant rDer p 3-S196A should be used to correctly evaluate Der p 3-specific IgE binding in the context of HDM allergy diagnosis. Moreover, based not only on a murine model but also on human blood basophil experiments, our data clearly show that proDer p 3 could be used as a hypoallergenic variant of Der p 3 suitable for allergen-specific, systemic immunotherapy.

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Conflict of interests

The authors declare no conflict of interest.

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