

Recombinant Der p 1 and Der f 1 with in vitro Enzymatic Activity to Cleave Human CD23, CD25 and α_1 -Antitrypsin, and in vivo IgE-Eliciting Activity in Mice

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Key Words

Recombinant allergen · House dust mite · Dermatophagoides · Der p 1 · Der f 1 · Cysteine protease · CD23 · CD25 · α_1 -Antitrypsin · IgE-eliciting activity

Abstract

Background: The major house dust mite group 1 allergens Der p 1 and Der f 1 are the most potent indoor allergens. Der p 1 cleaves human cell surface molecules, the low-affinity IgE receptor (CD23/FcεRII), the α -subunit of the IL-2 receptor (CD25), and a protease inhibitor α_1 -antitrypsin, and in vitro and in vivo studies suggested the importance of its proteolytic activity in the pathogenesis of allergy. Recently, we established an efficient system to prepare correctly folded active recombinant Der p 1 and Der f 1 (Der p 1-N52Q and Der f 1-N53Q) with similar molecular sizes, secondary structures and allergenicities as their natural types. To evaluate whether Der p 1-N52Q and Der f 1-N53Q are suitable for use in future in vitro and in vivo studies as alternatives to the natural types, we investigate their proteolytic activity to cleave

the human proteins and IgE-eliciting activity in mice. **Methods:** Proteolytic activities of Der p 1-N52Q and Der f 1-N53Q against a short peptide substrate, a collagen substrate Azocoll, human CD23 and CD25 expressed on the cells and human α_1 -antitrypsin were analyzed by kinetic assays for proteolysis of the fluorogenic or colorimetric substrates, flow cytometry and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, respectively. Mice were intraperitoneally immunized with Der p 1-N52Q and Der f 1-N53Q adsorbed on Alum, and the serum IgE levels were measured by sandwich ELISA. **Results:** Der p 1-N52Q and Der f 1-N53Q showed proteolytic specificities against the short peptide substrate, Azocoll, human cell surface CD23 and CD25 and human α_1 -antitrypsin, and elicited significant serum IgE levels in immunized mice. **Conclusion:** The recombinant forms, Der p 1-N52Q and Der f 1-N53Q, will be useful tools as alternatives to the natural Der p 1 and Der f 1 for various in vitro and in vivo analyses.

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Introduction

House dust mites of two species of *Dermatophagoides* (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are a major source of allergens associated with allergic diseases such as bronchial asthma, rhinitis and atopic dermatitis [1–3]. Group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) allergens are major allergens derived from house dust mites based on the frequency of patients sensitized, the amount of specific IgE and the content in mite extract [4]. Group 1 allergens are the most clinically relevant allergen proteins, because they exist in abundance in mite fecal pellets and account for over 50% of IgE antibodies against total mite extract [5–10].

Der p 1 and Der f 1 have cysteine protease activity [11–15]. The cysteine protease activity of Der p 1 has been suggested to be involved in the pathogenesis of allergy [16] by facilitating a passage of their own and other allergens across the epithelium [17, 18], cleaving and/or interacting with intrinsic protease inhibitors [19–21] and modulating the function of various cells by cleaving cell surface molecules [19, 22–25]. Two research groups reported on the enhancement of IgE synthesis [26–28] and induction of Th2 responses [29] by the proteolytic activity of Der p 1 in vivo. Herbert et al. [17] speculated that cysteine protease activity of natural Der p 1 could be activated in vivo by glutathione in the airway on the basis of their in vitro observations. However, which molecule(s) and which cell type(s) are the most critical targets of the cysteine protease activity of Der p 1 and Der f 1 for the pathogenesis of allergy and where Der p 1 and Der f 1 become active and work effectively are not well investigated.

Recently, we and others reported on efficient systems that can be used to produce recombinant mature Der f 1 and Der p 1 with IgE-binding and proteolytic activities by acid treatment of the proforms expressed in yeast *Pichia pastoris* [30–35] or *Escherichia coli* [36]. Furthermore, we prepared recombinant Der p 1 and Der f 1 without yeast-derived hyperglycosylation [30–32] and demonstrated that they exhibited similar molecular sizes, secondary structures and allergenicities as their natural types [30]. To evaluate whether these recombinant forms are suitable for use in future in vitro and in vivo studies, we investigated their proteolytic activity to cleave human cell surface molecules, CD23 and CD25, and a protease inhibitor α_1 -antitrypsin, which were reported to be cleaved by natural Der p 1 [16, 19, 20, 22, 23], and IgE-eliciting activity in mice.

Materials and Methods

Preparation of Recombinant Der p 1 and Der f 1

A previously described method was followed with some modifications [30–32]. Briefly, proforms of recombinant house dust mite group 1 allergens Der p 1-N52Q and Der f 1-N53Q, in which an N-glycosylation motif in the mature portion was disrupted by site-directed mutagenesis, were secreted into the culture supernatant of yeast *P. pastoris* transfectant cells and converted into the prosequence-removed mature forms. The mature forms were purified by anion exchange and size exclusion column chromatography. The purities were checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentrations were determined by the Bradford procedure using a protein assay kit (Bio Rad, Richmond, Calif., USA) with bovine IgG (Bio Rad) as the standard.

Natural Der p 1 and Der f 1

Natural Der p 1 and Der f 1 used for proteolysis assays were purified from whole culture extracts of house dust mites, *D. pteronyssinus* and *D. farinae*, respectively, as previously described by Yasueda et al. [37]. For immunization of the mice, commercially available natural Der p 1 and Der f 1 (Indoor Biotechnologies, Charlottesville, Va., USA) were used, because the amounts of our preparations for the purified natural allergens were limited.

Measurement of Proteolytic Activity Using a Short Synthetic Substrate

Cysteine protease activity was measured as previously described with modifications [31]. Enzymes were activated with 1 mM dithiothreitol (DTT) by incubation for 5 min at 37°C. After further incubation with or without the cysteine protease-specific inhibitor E-64 (Peptide Institute, Osaka, Japan) [38] for 15 min, assays were conducted in reaction buffer (50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA) with 1 mM DTT at 37°C. The final concentrations of natural and recombinant Der p 1 and Der f 1 were 100 nM. The substrate butyloxycarbonyl-Gln-Ala-Arg-MCA (Peptide Institute) [39] was used at 0.1 mM, and the fluorescence of aminomethylcoumarin released from the substrate was measured.

Measurement of Proteolytic Activity Using the Collagen Substrate Azocoll

The insoluble diazotized collagen substrate Azocoll (>100 Mesh; Calbiochem, San Diego, Calif., USA) was used as a protein substrate [40]. Enzymes were activated with 2.5 mM L-cysteine by incubation for 10 min at 37°C. Then, a suspension of Azocoll (10 mg/ml) was added to each serially diluted enzyme solution and incubated in the reaction buffer under gentle shaking for 30 min at 37°C. The reaction was stopped on ice, and the suspension was centrifuged at 2,000 g for 5 min at 4°C. The absorbance of the supernatant at 490 nm was then measured.

Cleavage of Human CD23 and CD25

The methods used were those described by Schulz et al. [19, 23]. Briefly, human B cell lymphoma RPMI8866 cells or human peripheral blood mononuclear cells stimulated with phytohemagglutinin (2×10^6 cells/ml) were incubated with activated enzymes for 30, 60 or 120 min. The cells were then washed and stained with fluorescein isothiocyanate (FITC)-labeled anti-human CD23 monoclo-

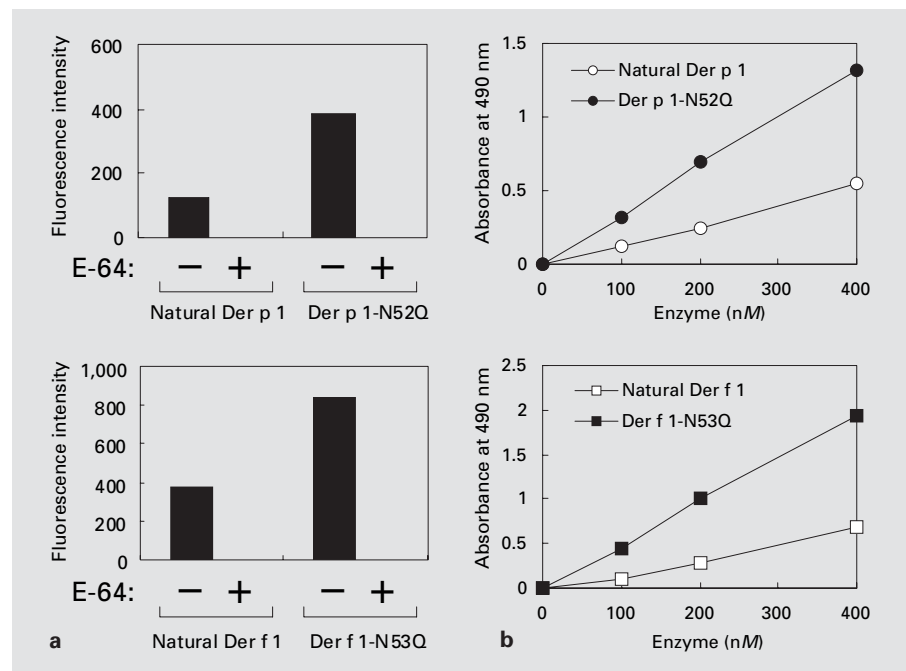


Fig. 1. Proteolytic activity of recombinant and natural Der p 1 and Der f 1. **a** Blockade of proteolytic activity with the cysteine protease-specific inhibitor E-64. Butyloxycarbonyl-Gln-Ala-Arg-MCA was used as the substrate, and the values of the fluorescence intensities at 10 min were recorded. E-64: the allergens were incubated with (+) or without E-64 (–) prior to reaction with the substrate. **b** Proteolysis of collagen substrate Azocoll. Means of the duplicate data are shown.

nal antibody or FITC-labeled anti-human CD25 monoclonal antibody, respectively. They were washed again and analyzed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, Calif., USA).

Cleavage of Human α_1 -Antitrypsin

Recombinant Der p 1 and Der f 1 (400 nM) activated with L-cysteine were added to purified human α_1 -antitrypsin (100 nM; Sigma, St. Louis, Mo., USA) in reaction buffer (50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA). After incubation for 2 h at 37°C, the samples were subjected to SDS-PAGE.

Immunizations of Mice with Activated Recombinant and Natural Der p 1 and Der f 1

Six- to 8-week-old female CBA/J mice were purchased from Charles River Japan, Inc., Yokohama, Japan. The mice were maintained in the specific pathogen-free animal facility at Juntendo University throughout the study. Before use, recombinant and natural Der p 1 and Der f 1 were preincubated with 5 mM L-cysteine (Sigma) at a neutral pH for 10 min at 37°C. Then, the antigens were diluted with PBS, incubated with Alum and further diluted to appropriate volumes with saline. The mice were given three weekly intraperitoneal injections with 10 μ g of antigens adsorbed on Alum (ImjectAlum; Pierce, Rockford, Ill., USA). Peripheral blood was collected from the retroorbital plexus at 1 week after the last injection. All sera were stored at –20°C before analysis for IgE levels.

Detection of Serum IgE by ELISA

Total IgE was detected by a sandwich ELISA using rat anti-mouse IgE monoclonal antibody (clone R35–72; BD Biosciences, San Jose, Calif., USA) and horseradish peroxidase-conjugated rat anti-mouse IgE monoclonal antibody (clone LO-ME-2; Serotec,

Oxford, UK) with purified mouse IgE (BD Biosciences) as the standard. Binding of the conjugates was visualized with tetramethylbenzidine (Sigma) in citrate buffer (pH 5.0) containing H₂O₂. The color reaction was stopped by adding sulfuric acid, and optical density at 450 nm was measured.

Results and Discussion

Der p 1 and Der f 1 have an N-glycosylation motif in their mature amino acid sequences, and therefore, recombinant forms designated Der p 1-WT and Der f 1-WT with wild-type sequences are modified by yeast-derived hyperglycosylation [30–32]. To obtain recombinant proteins without hyperglycosylation, we generated mutants designated Der p 1-N52Q and Der f 1-N53Q, in which the N-glycosylation motif within the mature sequence was disrupted. Recently, we have demonstrated that Der p 1-N52Q and Der f 1-N53Q retained identity to their natural types in terms of structure and allergenicity [30]. In the present study, we investigated proteolytic activities of Der p 1-N52Q and Der f 1-N53Q to cleave the human low-affinity IgE receptor (CD23/Fc ϵ R2), the α -subunit of the human IL-2 receptor (CD25) and human α_1 -antitrypsin, and IgE-eliciting activity in mice to evaluate whether the recombinant types are suitable for use in future studies as alternatives to the natural types. Natural

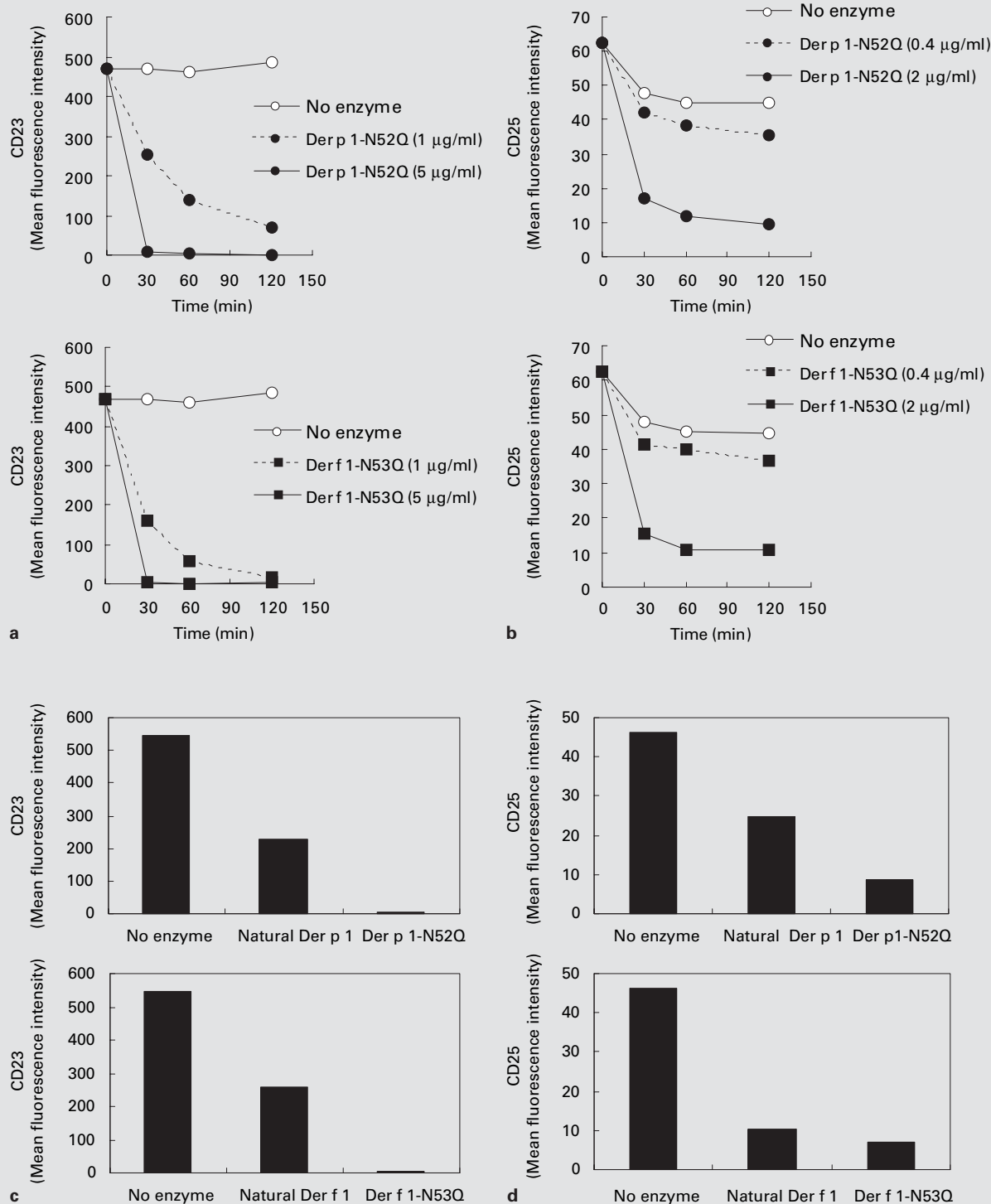


Fig. 2. Cleavage of human CD23 and CD25 on the cell surface by the enzymatic activities of recombinant and natural Der p 1 and Der f 1. Cells were incubated with enzymes activated by *L*-cysteine and stained with FITC-labeled monoclonal antibodies. The values shown are mean fluorescence intensities calculated by subtracting the background of the unstained cells. **a, b** Time- and dose-depen-

dent cleavage of CD23 on human B cells (**a**) and CD25 on human T cells (**b**) by recombinant Der p 1 and Der f 1. **c, d** Cleavage of CD23 (**c**) and CD25 (**d**) by recombinant and natural Der p 1 and Der f 1 (10 µg/ml) after incubation for 60 min. No enzyme: cells were incubated with the buffer alone. The results shown are representative of two or three independent experiments.

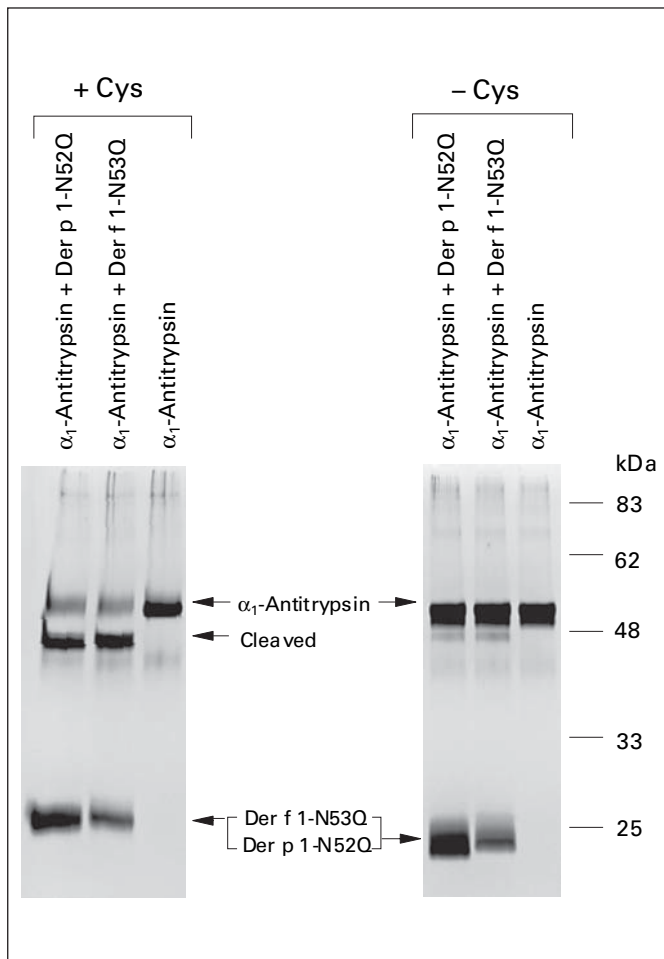


Fig. 3. Cleavage of human α_1 -antitrypsin by cysteine protease activities of recombinant Der p 1 and Der f 1. The allergens were incubated in a buffer with (+ Cys) or without *L*-cysteine (– Cys) prior to reaction with the human α_1 -antitrypsin. Bands for the intact α_1 -antitrypsin, α_1 -antitrypsin truncated by cleavage with the recombinant Der p 1 and Der f 1 (Cleaved), recombinant Der p 1 and recombinant Der f 1 were indicated by arrows. The gels after SDS-PAGE (15–25%) under nonreducing conditions were silver stained.

Der p 1 purified from mite culture cleaves human cell surface molecules CD23 [19, 22], CD25 [23], and a protease inhibitor α_1 -antitrypsin [19, 20], and in vitro and in vivo studies suggested the importance of its proteolytic activity in the pathogenesis of allergy [16–18, 24–29, 41–45].

Der p 1-N52Q and Der f 1-N53Q showed cysteine protease activity which was completely inhibited by the addition of E-64, a cysteine protease-specific inhibitor, as well as natural types purified from the mite culture ex-

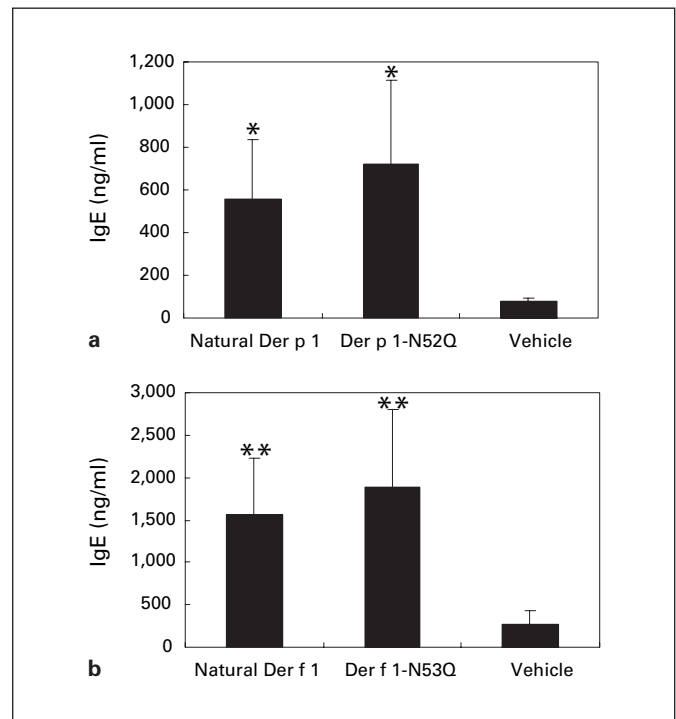


Fig. 4. Induction of serum IgE in mice immunized with recombinant and natural Der p 1 and Der f 1. Vehicle: the buffer alone was mixed with Alum and injected to mice. Data shown represent the means $\pm$ SD of four (a) or five (b) mice. * $p < 0.05$ and ** $p < 0.01$ compared with the vehicle control by unpaired *t* test (two-tailed).

tracts (fig. 1a). Before assays, we activated them with reducing reagents, DTT or *L*-cysteine, to regenerate the thiol group at the catalytic center that had become oxidized during purification; some other research groups also activated natural Der p 1 prior to use [19, 20, 23–28, 39, 41, 43–45]. Der p 1-N52Q and Der f 1-N53Q showed proteolytic activity against the collagen substrate Azocoll (fig. 1b). We found that Der p 1-N52Q, Der f 1-N53Q and natural Der f 1 also cleave human CD23 and CD25 on the cell surface in a time- and dose-dependent manner, as well as natural Der p 1 (fig. 2) [19, 23]. Freshly prepared recombinant mature forms showed a higher activity than the natural types (fig. 1, 2), suggesting that irreversible inactivation of natural allergens might have occurred during the cultivation of mites, purification and/or storage.

The human α_1 -antitrypsin was also cleaved by activated Der p 1-N52Q and Der f 1-N53Q (fig. 3, + Cys) but not without activation (fig. 3, – Cys). Kalsheker et al. [20] reported similar activity of activated natural Der p 1 to cleave the human α_1 -antitrypsin, which was blocked with E-64.

Immunization of mice with activated Der p 1-N52Q and Der f 1-N53Q elicited significant serum IgE levels as well as activated natural Der p 1 and Der f 1 (fig. 4). Gough et al. [26] reported that immunization of mice with activated natural Der p 1 resulted in an enhancement in IgE synthesis compared with mice immunized with Der p 1 inactivated with E-64. The recombinant Der p 1-N52Q and Der f 1-N53Q and their mutants would be useful for further analysis of the role of the enzymatic activity of Der p 1 and Der f 1 in eliciting in vivo IgE/Th2 responses.

Thus, Der p 1-N52Q and Der f 1-N53Q showed proteolytic specificities against the short peptide substrate butyloxycarbonyl-Gln-Ala-Arg-MCA, collagen substrate Azocoll, human cell surface CD23 and CD25 and human α_1 -antitrypsin, and IgE-eliciting activity in mice. Now, we can consider Der p 1-N52Q and Der f 1-N53Q to retain overall identity to their natural types in terms of structure, allergenicity [30, 31], proteolytic specificity against human protein substrates and IgE-eliciting activ-

ity in mice. The recombinant forms will be useful tools as alternatives to the natural Der p 1 and Der f 1 for various in vitro and in vivo analyses for the relation between the enzymatic activity and pathogenesis of allergy, and will be the basis for allergen standardization and the design of safer and more effective allergen vaccines for allergen-specific immunotherapy [46, 47].

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