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# Major House Dust Mite Allergens *Dermatophagoides pteronyssinus* 1 and *Dermatophagoides farinae* 1 Degrade and Inactivate Lung Surfactant Proteins A and D*

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**Lung surfactant proteins (SP) A and D are calcium-dependent carbohydrate-binding proteins. In addition to playing multiple roles in innate immune defense such as bacterial aggregation and modulation of leukocyte function, SP-A and SP-D have also been implicated in the allergic response. They interact with a wide range of inhaled allergens, competing with their binding to cell-sequestered IgE resulting in inhibition of mast cell degranulation, and exogenous administration of SP-A and SP-D diminishes allergic hypersensitivity *in vivo*. House dust mite allergens are a major cause of allergic asthma in the western world, and here we confirm the interaction of SP-A and SP-D with two major mite allergens, *Dermatophagoides pteronyssinus* 1 and *Dermatophagoides farinae* 1, and show that the cysteine protease activity of these allergens results in the degradation of SP-A and SP-D under physiological conditions, with multiple sites of cleavage. A recombinant fragment of SP-D that is effective in diminishing allergic hypersensitivity in mouse models of dust mite allergy was more susceptible to degradation than the native full-length protein. Degradation was enhanced in the absence of calcium, with different sites of cleavage, indicating that the calcium associated with SP-A and SP-D influences accessibility to the allergens. Degradation of SP-A and SP-D was associated with diminished binding to carbohydrates and to *D. pteronyssinus* 1 itself and diminished capacity to agglutinate bacteria. Thus, the degradation and consequent inactivation of SP-A and SP-D may be a novel mechanism to account for the potent allergenicity of these common dust mite allergens.**

Lung surfactant proteins A (SP-A)[2] and D (SP-D) are two of four surfactant proteins (SP-A, SP-B, SP-C, and SP-D) predominantly synthesized and secreted in the lung by alveolar type II cells and Clara cells. SP-A and SP-D belong to the collectin family of carbohydrate-binding proteins, other members of which include mannose binding lectin in humans and conglutinin, CL-43 and CL-46, in Bovidae (1). Recent genome analyses have identified two related human collectins, CL-L1 and CL-P1, that are expressed by liver and vascular endothelial cells, respectively (2, 60).

Collectins are multimeric proteins, and each polypeptide chain is composed of four domains; a cysteine-rich N-terminal region, which is involved in the formation of interchain disulfide bonds, a collagen-like triple helical region, an $\alpha$-helical coiled-coil neck region, and a C-terminal carbohydrate recognition domain (CRD) (4, 5). Each polypeptide associates with two others via their N-terminal, collagen, and $\alpha$-helical neck region, to form a trimeric subunit, and six of these trimeric subunits make up the overall "bouquet"-like structure of SP-A, whereas SP-D is composed of four trimeric subunits arranged in a cruciform-like structure (6–8).

Both SP-A and SP-D play important roles in innate host defense against inhaled pathogens. The immunomodulatory properties of SP-A and SP-D are diverse and include bacterial agglutination, opsonization, modulation of phagocyte function, and direct inhibition of microbial growth (9, 10). In most cases the CRDs mediate $Ca^{2+}$-dependent interactions of SP-A and SP-D with their ligands, and whereas CRD monomers can recognize these ligands, trimerization is necessary for high affinity binding.

There is mounting evidence to suggest that SP-A and SP-D are also important regulators of allergy. SP-A and SP-D levels increase severalfold in allergic asthma (11–13), and furthermore, the kinetics of the increase in SP-D levels after allergen challenge have been reported to coincide with the resolution of inflammation (14). Mouse models of house dust mite (*Dermatophagoides pteronyssinus* (Der p)) allergy have clearly demonstrated a protective role for SP-D during secondary allergen exposure. Intranasal treatment of allergen-sensitized mice with native full-length human (Nh) SP-D or a 60-kDa trimeric recombinant fragment of human SP-D comprising just the $\alpha$-helical neck region and CRDs (rfhSP-D) after allergen challenge was effective in reducing eosinophilic inflammation and allergen-specific IgE production, with a concomitant reduction in bronchial hyperresponsiveness and a shift in cytokine profile from the typical Th2 response toward a Th1 response (15, 16).

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[2] The abbreviations used are: SP-A, surfactant protein A; Der p 1, *D. pteronyssinus* allergen 1; Der f 1, *D. farinae* allergen 1; NhSP-A/D, native human (*Nh*) lung surfactant protein-A/D; rfhSP-D, recombinant fragment of human lung surfactant protein-D; CRD, carbohydrate recognition domain; HDM, house dust mite; BALF, broncheolar lavage fluid; IAA, iodoacetamide.

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Similar results were observed with both SP-A and SP-D in mouse models of allergy to *Aspergillus fumigatus* (17). The mechanisms by which SP-A and SP-D bring about these protective effects are not clearly defined but *in vitro* studies have shown that SP-A and SP-D bind to glycosylated Der p and *A. fumigatus* allergens through their CRDs in a calcium-dependent manner (18, 19) with consequent inhibitory effects on allergen-induced histamine release (19, 20), and furthermore, SP-A and SP-D have also been shown to inhibit allergen-induced T lymphocyte proliferation (20). These *in vitro* properties of SP-A and SP-D may account for some of their protective effects seen *in vivo*. SP-D has also been shown to enhance the removal of apoptotic cells *in vivo* (21), and because clearance of apoptotic cells is important in the resolution of an inflammatory response (22–23) SP-D may minimize the inflammation that occurs during and after allergen challenge.

Allergens of the house dust mite *D. pteronyssinus* are a major cause of allergic disease in the Western World. Der p 1, one of several such mite allergens, elicits IgE antibody responses in more than 80% of patients who are sensitive to *D. pteronyssinus* and is considered to be the most immunodominant allergen involved in the expression of IgE-mediated dust mite sensitivity (24). The reason for this potent IgE-eliciting property of Der p 1 is likely to be due to its cysteine protease activity, which has been shown to cleave CD40 on dendritic cells, resulting in decreased interleukin-12 production (25), CD23 (IgE receptor) on B cells, which may disrupt a negative feedback mechanism resulting in excessive IgE production (26), and CD25 (the $\alpha$ subunit of the interleukin-2 receptor) on T cells, diminishing the proliferation and interferon $\gamma$ production of T cells (27, 28). The cleavage of these molecules would collectively favor a Th2-mediated response.

The proteolytic activity of Der p 1 also facilitates its entry into subepithelial tissues. Occludin, a protein component of intercellular tight junctions, is another substrate of Der p 1 (29), thus allowing the destruction of the integrity of tight junctions between epithelial cells. The importance of the enzyme activity of Der p 1 in inducing allergy has also been demonstrated *in vivo*, where intranasal administration of proteolytically active Der p 1 to sensitized mice leads to enhanced inflammatory cellular infiltration of the lungs and systemic production of IgE, in comparison to inactive Der p 1, which has no effect (30).

The less common house dust mite *Dermatophagoides farinae* (Der f) is found predominantly in North America, Japan, and the Far East (31). Der f 1, one of the major allergens, is also a cysteine protease and shows 82% homology to Der p 1. Similar to Der p 1, a recombinant form of Der f 1 has been shown to cleave human CD23 and CD25 *in vitro* (32) and reduce barrier function of the skin through its proteolytic activity (33).

The present study was undertaken to determine whether SP-A and SP-D, important modulators of allergy, were susceptible to degradation by the major house dust mite allergens Der p 1 and Der f 1, with resultant effects on their function. Here we show that Der p 1 and Der f 1 cleave NhSP-A, NhSP-D, and rfhSP-D in a time- and concentration- dependent manner at multiple sites within the CRD, $\alpha$-helical neck region, and collagen-like-region, and cleavage of these collectins abrogates their lectin activity and lectin-associated functions such as bacterial agglutination and allergen binding. The cleavage and consequent inactivation of SP-A and SP-D may be a novel mechanism to account for the potent allergenicity of Der p 1 and Der f 1.

## EXPERIMENTAL PROCEDURES

*Protein Purification*—Native human SP-D (NhSP-D) and native human SP-A (NhSP-A) were purified from bronchiolar lavage fluid (BALF) obtained from alveolar proteinosis patients as described previously (34, 35).

The cDNA for the $\alpha$-helical neck/CRD of human SP-D (rfhSP-D) including a short region of the collagen stalk, representing residues 179–345 of the mature protein sequence, was inserted into a pET24d vector (Altana Pharma AG, Konstanz, Germany). The plasmid was transformed into BL21 (λDE3) pLysS, and a single colony was selected and diluted into 25 ml of Luria-Bertani broth (LB) supplemented with 25 $\mu$g/ml ampicillin (LB+), and grown overnight with shaking at 37 °C. An aliquot of 5 ml of this was used to inoculate 2-liter flasks containing 500 ml of LB+. The optical density at 600 nm was measured at regular intervals, and at $A_{600} = 0.6$ expression was induced by adding a final concentration of 1 mM isopropyl 1-thio-$\beta$-D-galactopyranoside. After 3 h, cells were spun at 5000 × *g* for 5 min. Pellets were weighed and stored at −20 °C before use.

Pellets were suspended in Bugbuster™ (Novagen) (6 ml/g pellet) containing 1 mM PEFA Block and left mixing at room temperature for 20 min. Samples were then centrifuged at 15,000 × *g* for 20 min, and the pellet was resuspended in 10% (v/v) Bugbuster™ (36 ml/g pellet). After thorough mixing, the samples were centrifuged at 14,000 × *g* for 20 min to pellet the inclusion body which contained rfhSP-D. The inclusion body pellet was weighed and solubilized in 20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 5% (v/v) glycerol (solubilization buffer), 8 M urea at a concentration of 20 mg/ml and left to mix at 4 °C for 1 h. The protein was then refolded through the following series of dialysis steps; solubilization buffer with 2 M urea for 3 h, solubilization buffer with 1 M urea overnight, solubilization buffer with 0.5 M urea for 3 h, solubilization buffer for 24 h, and finally 20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ for 24 h to remove glycerol. Correctly folded rfhSP-D was separated from misfolded rfhSP-D by maltose affinity chromatography and purified further by gel filtration as described previously (36).

Der p 1 was isolated from house dust mite fecal pellets (Allergon, Angelholm, Sweden) using a multistep procedure involving immunoaffinity chromatography on immobilized monoclonal anti-Der p 1 antibody (clone 4C1, Indoor Biotechnologies, Manchester, UK), removal of contaminating serine proteases on immobilized soybean trypsin inhibitor (Sigma), and finally fast protein liquid chromatography to remove low molecular weight contaminants (37). Natural Der f 1 was purchased from Indoor Biotechnologies.

The purity of all proteins was verified through SDS-PAGE and N-terminal sequencing, and the purity of the Der p 1 preparation was further confirmed by demonstrating that enzymatic activity is completely dependent on pre-activation with cysteine and is totally blocked by cysteine protease inhibitor iodoacetamide (IAA).

*Enzyme-linked Immunosorbent Assays*—96-Well maxisorp microtiter plates (Nunc) were coated with varying concentra-

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tions of Der p 1 and Der f 1 in 35 mM $Na_2C0_3$, 15 mM $NaHCO_3$, pH 9.6, and left overnight at room temperature. Wells were then washed with 20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4, (TSC) 0.05% (v/v) Tween and blocked with 300 μl of TSC, 0.05% (v/v) Tween, 3% (w/v) bovine serum albumin for 1 h at 37 °C to eliminate nonspecific binding. After washing again, 2 μg of NhSP-A, 2 μg of NhSP-D, or 3 μg of rfhSP-D were added to each well in 200 μl of BALF with 5 mM $CaCl_2$ (BALF/Ca). The addition of proteins to uncoated wells served as background controls. After incubating for 2 h at 37 °C and washing, bound NhSP-A was detected using biotinylated polyclonal anti-SP-A antibody (1:1000 dilution; Antibody Shop, Gentofte, Denmark), and bound NhSP-D/rfhSP-D was detected using biotinylated rabbit polyclonal anti-rfhSP-D antibody (1:4000 dilution) by incubating at 37 °C for 1 h. After washing, 100 μl of streptavidin-horseradish peroxidase (1:10,000; Sigma) was added to each well, and plates were incubated at 37 °C for 30 min before washing. Finally, tetramethylbenzene substrate (Bio-Rad) was added to each well, and after sufficient color development, the reaction was stopped with 100 μl of 0.5 M $H_2SO_4$. Absorbance values were measured at 450 nm, and background control values were subtracted from binding to protein-coated wells. In some cases BALF/Ca was replaced with BALF with 5 mM EDTA (BALF/E) or BALF/Ca with 100 mM mannose (for NhSP-A) or 100 mM maltose (for NhSP-D and rfhSP-D).

*Degradation Assays*—Der p 1 and Der f 1 were made enzymatically active by incubating a final concentration of 62.5 μg/ml with 5 mM cysteine at 37 °C for 10 min. To investigate time-dependent cleavage of the collectins by Der p1 and Der f 1, a final concentration of 1.25 μg/ml allergen was incubated with 100 μg/ml NhSP-A, NhSP-D, or rfhSP-D in a total volume of 30 μl in 20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4, (TSCa) for 0, 1, 3, 6, 12, 24, and 48 h. Reactions were stopped by adding a final concentration of 10 mM IAA. To verify Der p 1/Der f 1-specific activity, these assays were also conducted in the presence of 10 mM IAA. Proteins were then resolved by 12% (w/v) SDS-PAGE under reducing conditions. In some cases, the assays were performed in 20 mM Tris-HCl, 150 mM NaCl (without calcium).

To examine dose-dependent cleavage of collectins, varying amounts of activated Der p 1 and Der f 1 (final concentrations ranging from 40 ng/ml to 5 μg/ml) were incubated with collectins as described above for 20 h. Proteins were then resolved by 12% (w/v) SDS-PAGE under reducing conditions. Degradation of endogenous NhSP-D and SP-A in BALF was also investigated by incubating BALF with 2.5 μg/ml Der p 1 and Der f 1 for various times ranging from 0 to 48 h and then detecting endogenous NhSP-D and NhSP-A by immunoblotting.

*N-terminal Sequencing of Degradation Products*—150 μg/ml NhSP-D or rfhSP-D was incubated with 1.25 μg/ml Der p 1 and Der f 1 in 20 mM Tris-HCl, 150 mM NaCl with or without 2 mM $CaCl_2$ for 20 or 6 h, respectively. The N-terminal sequences of cleaved products were determined by automated Edman degradation (Sequentor 472; Applied Biosystems, Foster City, CA).

*Der p 1 and Der f 1 Enzyme Activity Assay*—Continuous rate assays were performed in 20 mM Tris-HCl, 150 mM NaCl, 5 mM cysteine, pH 7.4, at 25 °C in a total volume of 200 μl in the presence of varying concentrations of calcium as described previously (38). Each reaction contained 280 μM *N-tert*-butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin, which acted as the substrate for the enzymes. The assays were started by adding cysteine-activated Der p 1 or Der f 1 to a final concentration of 10 nM. Hydrolysis of the 7-amino-4-methylcoumarin (AMC) substrate to AMC was monitored using a fluorescent spectrophotometer ($\lambda_{ex}$ = 355 nm, $\lambda_{em}$ = 460 nm). Results were related to a 7-amino-4-methylcoumarin standard curve and are expressed as the number of mol of substrate converted/min.

*Surface Plasmon Resonance*—Biotinylated Der p 1, Der f 1, and mannan were immobilized on streptavidin-coated BIAcore chips as described previously (39). 60 μg/ml NhSP-D or 120 μg/ml rfhSP-D had been incubated with 1.25 μg/ml enzymatically active Der p 1 and Der f 1 for 20 h in 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$ (HSC) in the presence or absence of 10 mM IAA were flowed over the cells in HSC, 0.05% (v/v) surfactant P20. Data were analyzed using BIAevaluation 2000 software, and responses for binding to the empty flow cell were subtracted from values for binding to flow cells immobilized with mannan or the allergens.

*Bacterial Agglutination*—Bacterial agglutination assays were performed with *Escherichia coli* K12. *E. coli* K12 was grown in 5 ml of LB overnight at 37 °C. The cells were then pelleted and washed 3 times with 20 mM Tris-HCl, 150 mM NaCl. Cells were then diluted to an $A_{600}$ of 1.0 with TSCa. Then 2 μg of NhSP-A, 2 μg of NhSP-D, or 5 μg of rfhSP-D that had previously been digested for 24 h in TSCa with Der p 1 or Der f 1 in the presence or absence of IAA were added to the cells in a total volume of 1 ml. Absorbance was measured at 660 nm at regular intervals for 180 min. Aggregation was observed as a decrease in absorbance as bacterial cells precipitated out of solution. Additional controls were performed using bovine serum albumin and an equivalent amount of protease that would have been associated with the collectin degradation assays.

## RESULTS

*Interaction between Collectins and House Dust Mite Allergens in BALF*—The ability of purified NhSP-D, NhSP-A, and rfhSP-D to bind to the major HDM allergens Der p 1 and Der f 1 were tested in the solid phase by using allergen-coated microtiter wells. Previous studies have demonstrated interactions between lung collectins and Der p allergens (18), but these assays were carried out in BALF to determine whether the interactions could take place in the presence of competitor proteins and lipids that would normally be present in the lung environment. NhSP-D, NhSP-A, and rfhSP-D all bound to Der p 1 and Der f 1 in a dose-dependent manner. The binding to these allergens was inhibited in the presence of maltose for NhSP-D and rfhSP-D and mannose for NhSP-A and with EDTA, confirming a carbohydrate-mediated interaction through the CRD (Fig. 1).

*Potential Cleavage Sites of Der p in SP-A and SP-D*—Based on cleavage sites of known protein substrates and using a panel of synthetic peptide substrates, it appears that Der p 1 has a preference for small aliphatic residues (Gly/Ala/Val) in the P2 position, charged residues (Asp/Glu/Arg) in the P1 position, and small hydrophobic/hydrophilic residues (Thr/Ser/Ala/Gly) in

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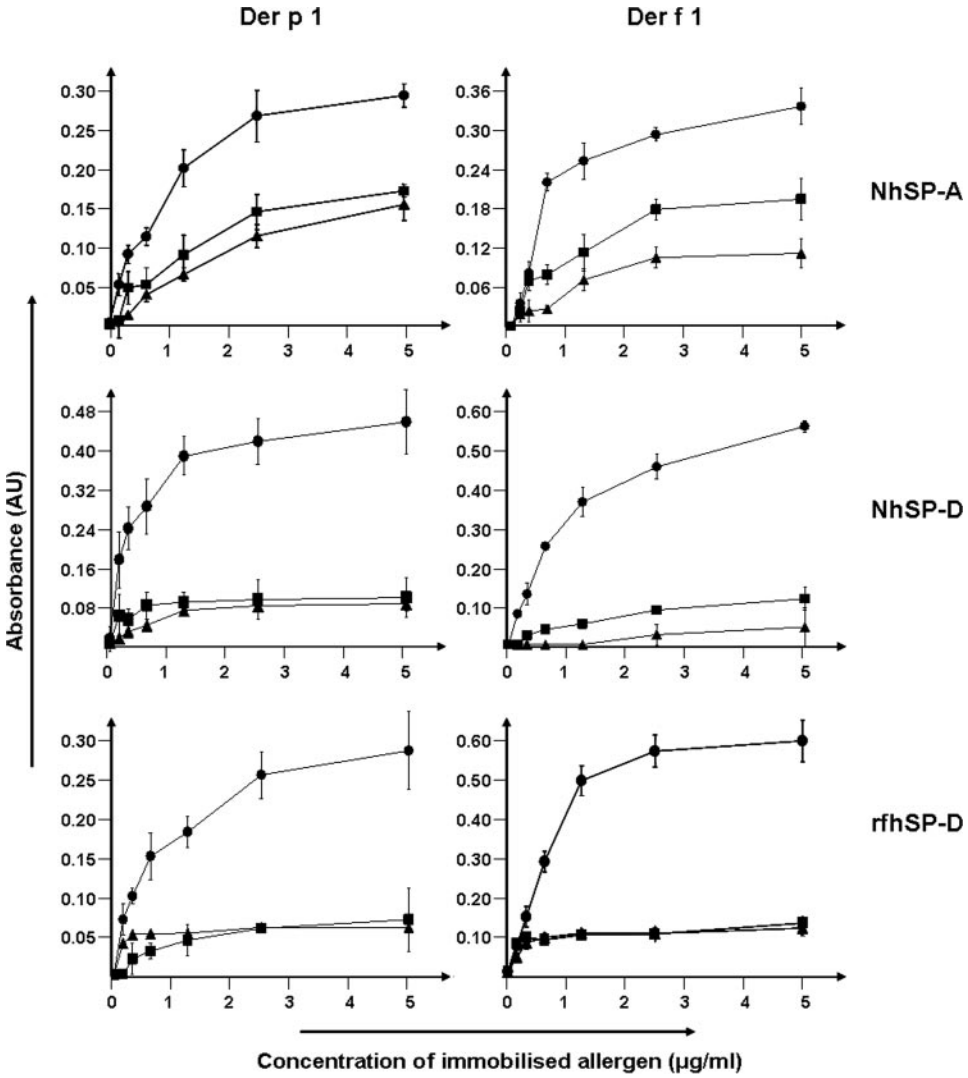


FIGURE 1. **Binding of NhSP-A, NhSP-D, and rfhSP-D to immobilized Der p 1 and Der f 1 in BALF.** Varying concentrations of Der p 1 and Der f 1 were immobilized onto microtiter plates. 1 $\mu$g of NhSP-A and NhSP-D or 2.5 $\mu$g of rfhSP-D was then added in BALF with 5 mM $CaCl_2$ (●), BALF with $CaCl_2$ plus 100 mM mannose (for NhSP-A) or 100 mM maltose (for NhSP-D and rfhSP-D) (■), or BALF with 5 mM EDTA (▲). Bound collectin was detected with the corresponding biotinylated antibody followed by the addition of horseradish peroxidase-conjugated streptavidin and the addition of TMB substrate, which resulted in color development at 450 nm. Binding of all three collectins to the allergens was diminished in the presence of sugars and EDTA, confirming a CRD-mediated interaction. Results are expressed as the mean of three independent experiments ±S.E. AU, absorbance units.

the P1′ position (where the sequence surrounding the cleavage site is represented by P3, P2, P1, P1′, P2′, and P3′, and the site of cleavage is between P1 and P1′) (38, 40). NhSP-D appeared to have several such potential cleavage sites throughout its sequence, whereas NhSP-A had only one within the CRD (Fig. 2).

*Der p 1 and Der f 1 Degrade SP-A and SP-D*—Having determined that there were potential cleavage sites in NhSP-D and NhSP-A, NhSP-A, NhSP-D, and rfhSP-D were incubated with the cysteine proteases Der p 1 and Der f 1 under physiological conditions, and the proteins were subsequently resolved by SDS-PAGE to determine whether degradation had taken place. Using molar enzyme:substrate ratios of 1:19, 1:15, and 1:33 for assays with NhSP-A, NhSP-D, and rfhSP-D respectively (where the number of moles of substrate are based on the number of trimeric units present), NhSP-A, NhSP-D, and rfhSP-D were all susceptible to cleavage by Der p 1 and Der f 1 (Fig. 3). Degradation of NhSP-A by Der p 1 and Der f 1 was visible after 3 h, as indicated by the decrease in intensity of the 35-kDa and 70-kDa bands representing the monomer and dimer of NhSP-A, respectively. No distinct cleavage products were visible, suggesting that there were multiple cleavage sites in NhSP-A and that it was degraded into small fragments that were not visible on a gel.

Degradation of NhSP-D by Der p 1 and Der f 1 was also observed after 6 h, but in this case, two distinct cleavage products of ~24 and 18kDa were visible (a third minor cleavage product of 11 kDa could be detected when using very high concentrations (>150 $\mu$g/ml) of NhSP-D; data not shown). By comparing the intensities of the 45-kDa band representing the intact monomer, after 24 h of incubation there was less than ~30% of the original uncleaved protein.

rfhSP-D was most susceptible to degradation by Der p 1 and Der f 1, with ~50% of the protein having been degraded by 1 h. The major cleavage product for both Der p 1 and Der f 1 digestion was 18 kDa. By 12 h none of the original uncleaved monomer was visible. No cleavage of NhSP-A, NhSP-D, or rfhSP-D was observed in the presence of IAA, suggesting that the degradation was specific to the action of Der p 1 or Der f 1.

Varying enzyme concentrations may be encountered *in vivo*, and to determine the minimum concentration at which degradation could be observed, a wide range of enzyme:substrate ratios were tested (Fig. 4). NhSP-A, NhSP-D, and rfhSP-D were all degraded in a concentration-dependent manner by both Der p 1 and Der f 1. The lowest enzyme:substrate ratio at which degradation of NhSP-A was visible was 1:38, as indicated by the decrease in intensity of the 35- and 70-kDa bands representing the monomer and dimer, respectively. NhSP-D showed a similar response to dose-dependent Der p 1 and Der f 1 digestion, and the minimal enzyme:substrate ratio at which cleavage was observed was 1:28. Consistent with the time-dependent cleavage assays rhfSP-D was most susceptible to degradation by Der p 1 and Der f 1. Even at the lowest concentration of enzyme tested (molar enzyme:substrate ratio of 1:500), cleavage of rfhSP-D was apparent with a distinct cleavage product of 14 kDa and some minor cleavage products at 10 kDa and less. As the concentration of the enzymes was increased, these frag-

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**NhSP-A**

[1]mwlcplalnl ilmaasgavc evkdvcvgsp gipgtpgshg lpgrdgrdgl
kgdpgppgpm gppgempcpp gndglpgapg ipgecgekge pgergppglp
ahldeelqat lhdfrhqilq trgalslqgs imtvgekvfs sngqsitfda iqeacaragg
riavprnpee neaiasfvkk yntyayvglt egpspgdfry sdgtpvnytn
wyrgepagrg keqcvemytd gqwndrncly srlticef[248]

**NhSP-D**

[1]aemktyshrt mpsactlvmc ssvesglpgr dgrdgregpr gekgdpglpg
aagqagmpgq agpvgpkgdn gsvgepgpkg dtgpsgppgp pgvpgpagre
gplgkqgnig pqgkpgpkge agpkgevgap gmqgsagarg lagpkgergv
pgergvpgnt gaagsagamg pqgspgargp pglkgdkgip gdkgakgesg
lpdvaslrqq vealqgqvqh lqaafsqykk velfpngqsv gekifktagf
vkpfteaqll ctqaggqlas prsaaenaal qqlvvaknea aflsmtdskt
egkftyptge slvysnwapg epnddggsed cveiftngkw ndracgekrl vvcef[355]

FIGURE 2. **Potential Der p 1 cleavage sites in the primary sequences of SP-D and SP-A based on known substrates of Der p 1.** The protein sequences of SP-A and SP-D were retrieved from PubMed (SP-D ID: CAA46152 and SP-A ID: NM005402) and were searched for potential cleavage sites based on the known specificity of Der p 1. Der p 1 has a preference for small and hydrophobic residues (Gly/Ala/Val) in the P2 position, charged residues (Asp/Glu/Arg) in the P1 position, and small hydrophobic/hydrophilic residues (Thr/Ser/Ala) in the P1′ position (cleavage would occur between P1 and P1′). Amino acids are represented by their standard one-letter code. P2, P1, and P1′ positions are *underlined*; the *arrow* indicates the start of rfhSP-D. SP-D appeared to have several potential cleavage sites, whereas SP-A appeared to have only one.

ments appeared to be degraded further and were no longer visible on the gel, and another major cleavage product of 18 kDa became visible that was also seen in the time-dependent cleavage assays.

*Degradation of SP-D, but Not SP-A, Is Enhanced in the Absence of Calcium*—Calcium ions are integral to the structure of the CRDs of both SP-A and SP-D (41–44). Therefore, to determine whether the absence of calcium and any consequent changes in structure of the collectins could influence the allergen-induced degradation of collectins, NhSP-A, NhSP-D, and rfhSP-D were incubated with Der p 1 and Der f 1 in the absence of calcium, and cleavage products were resolved by SDS-PAGE (Fig. 5). At 3 h, NhSP-A did not appear to have been degraded by Der p 1 and perhaps only slightly by Der f 1, as judged by the change in intensity of the 35-kDa band corresponding to the size of the monomer. This corresponds to the assays performed in the presence of calcium whereby degradation was not observed within 3 h (Fig. 3). However, the cleavage of NhSP-D and rfhSP-D were enhanced in the absence of calcium. By 1 h, with both Der p 1 and Der f 1 incubation with NhSP-D, most of the uncleaved monomer (45-kDa band) had disappeared, and a major cleavage product of 37 kDa was visible. In the presence of calcium, only a small amount of NhSP-D had been degraded at 6 h. Furthermore, the cleavage pattern was also different from that observed in the absence of calcium; in the presence of calcium minor cleavage products corresponding to bands of 20 and 17 kDa were detected after 12 h, whereas in the absence of calcium a single major cleavage product corresponding to a band of 37 kDa was detected within 1 h with both Der p 1 and Der f 1 digestion. These results suggested that the site of cleavage was altered in the absence of calcium. rfhSP-D was also more susceptible to degradation in the absence of calcium. After 1 h of digestion with Der p 1 and Der f 1, all the rfhSP-D had been degraded with no visible degradation products. This is in contrast to the degradation observed in the presence of calcium whereby a single cleavage product (17 kDa) was visible after 3 h of digestion and remained even after 48 h of digestion.

Because the cleavage of NhSP-D and rfhSP-D by Der p 1 and Der f 1 was modulated in the absence of calcium both in terms of their susceptibility to degradation and the actual site of cleavage, it was necessary to determine whether this was due to a calcium-induced modulation of enzyme activity or whether this could be attributed to a calcium-induced modulation of tertiary structure and consequent modulation of the accessibility of the enzymes to their sites of cleavage.

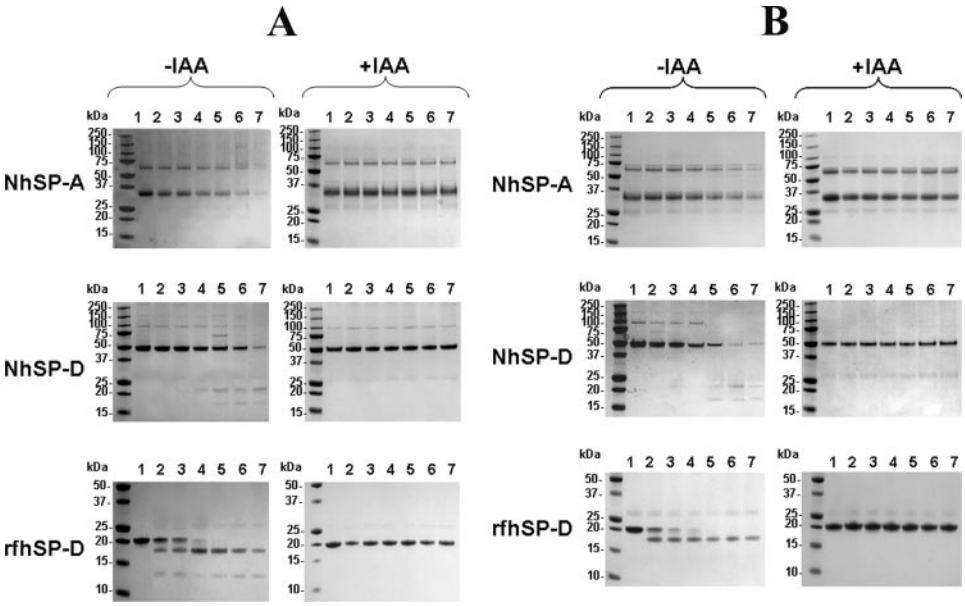


FIGURE 3. **Time-dependent cleavage of NhSP-A, NhSP-D, and rfhSP-D by Der p 1 (*A*) and Der f 1 (*B*) under physiological conditions.** 100 $\mu$g/ml NhSP-A, NhSP-D, and rfhSP-D was incubated with 1.25 $\mu$g/ml Der p 1 or Der f 1 in the presence or absence of IAA in TSCa for various times. Proteins were resolved be SDS-PAGE under reducing conditions. *Lane 1*, 0 min; *lane 2*, 1 h; *lane 3*, 3 h; *lane 4*, 6 h; *lane 5*, 12 h; *lane 6*, 24 h; *lane 7*, 48 h. NhSP-A, NhSP-D, and rfhSP-D were all susceptible to degradation under these conditions. After 48 h of incubation with Der p 1 and Der f 1, most of the NhSP-A had become degraded with no visible cleavage products. Minor cleavage products of 24 and 18 kDa were detected when NhSP-D was incubated for 12 h or more with Der p 1 and Der f 1. These fragments were still visible after 48 h of incubation. rfhSP-D was most susceptible to degradation, with a major cleavage product of 18 kDa visible after 1 h of incubation with Der p 1 and Der f 1. A minor cleavage product of 11 kDa was also visible with Der f 1 digestion. No degradation was observed in the presence of IAA, confirming Der p 1/Der f 1-specific activity. Data are representative of at least three independent experiments.

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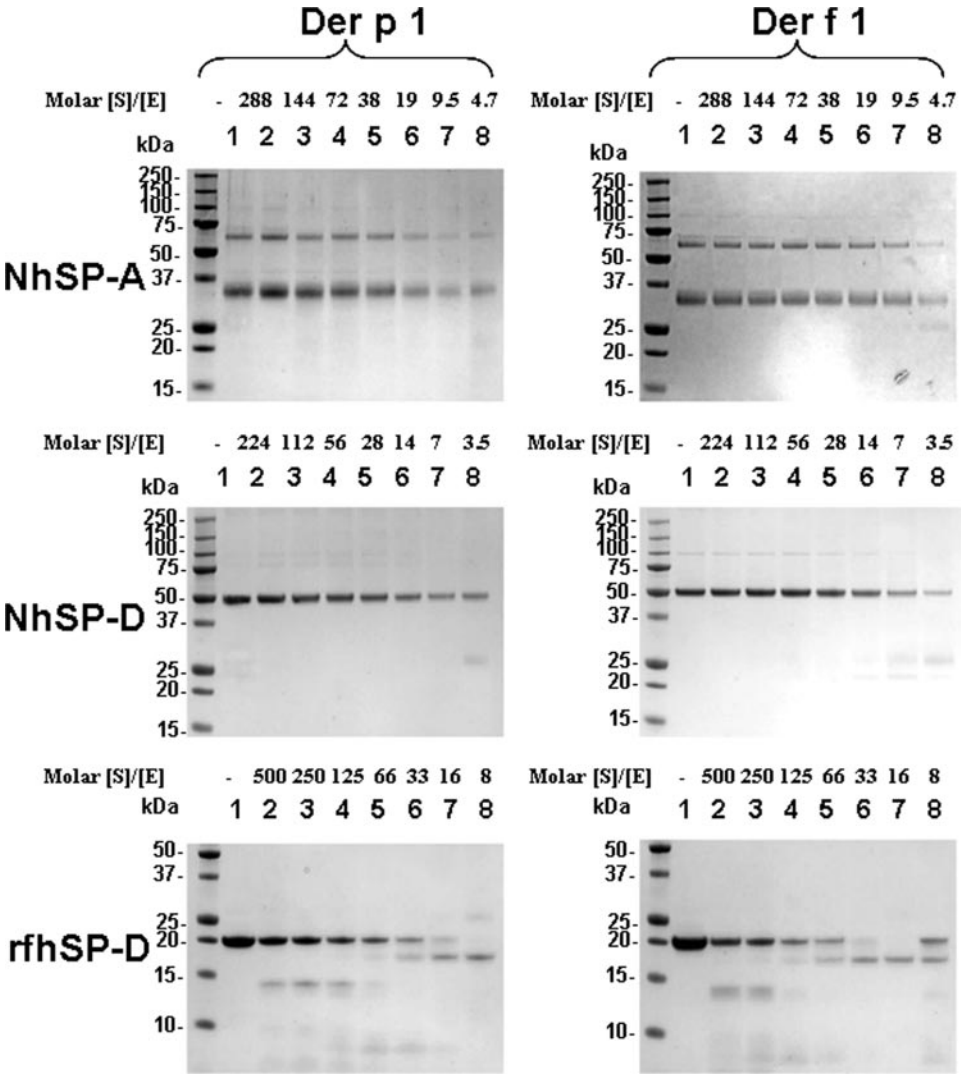


FIGURE 4. **Dose-dependent cleavage of NhSP-A, NhSP-D, and rfhSP-D by Der p 1 and Der f 1.** 100 μg/ml NhSP-A, NhSP-D, or rfhSP-D were incubated with 2-fold serial dilutions of Der p 1 and Der f 1 for 20 h in TSCa. Proteins were resolved by SDS-PAGE under reducing conditions. *Lane 1*, no enzyme; *lane 2*, 78 ng/ml; *lane 3*, 156 ng/ml; *lane 4*, 312 ng/ml; *lane 5*, 625 ng/ml; *lane 6*, 1.25 μg/ml; *lane 7*, 2.5 μg/ml; *lane 8*, 5 μg/ml. *Numbers above the gels* indicate molar substrate (*[S]*):enzyme (*[E]*) ratios where the number of moles of substrate represents the number of trimeric units present. The extent of degradation was inversely proportional to molar [S]:[E]. rfhSP-D was the most susceptible to degradation, with degradation observed at [S]:[E] ratios of as high as 500:1, whereas degradation of NhSP-A and NhSP-D could only be observed at ~10-fold higher enzyme concentrations. Data are representative of three independent experiments.

Der p 1 and Der f 1 activity was, therefore, measured by examining cleavage of a synthetic peptide substrate (*N-tert*-butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin) in the presence of varying concentrations of calcium. There was no significant decrease in Der f 1 and Der p 1 activity with calcium, suggesting that the enhancement of degradation observed in the absence of calcium was likely to be attributable to structural changes of the collectins (data not shown).

*N-terminal Sequencing of Cleavage Products*—The N-terminal sequences of the cleavage products arising from exposure of NhSP-D and rfhSP-D to Der p 1 and Der f 1 in the presence and absence of calcium were determined by Edman degradation to locate the sites of cleavage and determine sequence specificity of the enzymes (Fig. 6; there were no visible cleavage products from NhSP-A degradation, and hence, it was not possible to determine the sites of cleavage in this way). In the presence of calcium Der p 1 and Der f 1 both cleaved NhSP-D at the same three sites located within the end of the collagen region, start of the α-helical neck region, and CRD, respectively. Cleavage within the collagen region and α-helical region is likely to have resulted in unfolding of the oligomeric structure of NhSP-D, resulting in increased susceptibility to extensive degradation, and this could explain why after 48 h of cleavage, most of the NhSP-D was not visible on the gel (Fig. 3). The site of cleavage by Der p 1 and Der f 1 within NhSP-D was altered in the absence of calcium. A single major cleavage product was visible that had the same N terminus as the uncleaved molecule. However, from the size of the cleavage product (40 kDa) we can estimate the cleavage site to be within the CRD.

Exposure of rfhSP-D to Der p 1 in the presence of calcium resulted in a major and minor cleavage product of 18 and 12 kDa respectively. The minor cleavage product was not visible with Der f 1 exposure, possibly due to further degradation by this enzyme. The major site of cleavage was common to both NhSP-D and rfhSP-D $^{196}$KGE↓SGL located within the α-helical neck region (Fig. 6). The minor Der p 1 cleavage site was within the CRD. When rfhSP-D was degraded by Der p 1 and Der f 1 in the absence of calcium, there were no visible degradation products.

As described above, it appears that Der p 1 has a preference for small and hydrophobic residues (Gly/Ala/Val) in the P2 position, charged residues (Asp/Glu/Arg) in the P1 position, and small hydrophobic/hydrophilic residues (Thr/Ser/Ala) in the P1′ position (where the sequence surrounding the cleavage site is represented by P3, P2, P1, P1′, P2′, and P3′, and the site of cleavage is between P1 and P1′). Consistent with this, all three cleavage sites within NhSP-D (in the presence of calcium) and the major cleavage site within rfhSP-D, which is also common to NhSP-D, all have a small, aliphatic residue (Gly or Ala) at the P2 position, a charged or polar amino acid (Glu, Arg, or Gln) at the P1 position, and a small amino acid (Ser or Gly) at the P1′ position. The minor cleavage site of rfhSP-D digestion with Der p 1 has a charged residue (Lys) at the P1 position, but the amino acids at the P2 and P1′ positions (Tyr and Lys, respectively) do not conform to the usual specificity of Der p 1.

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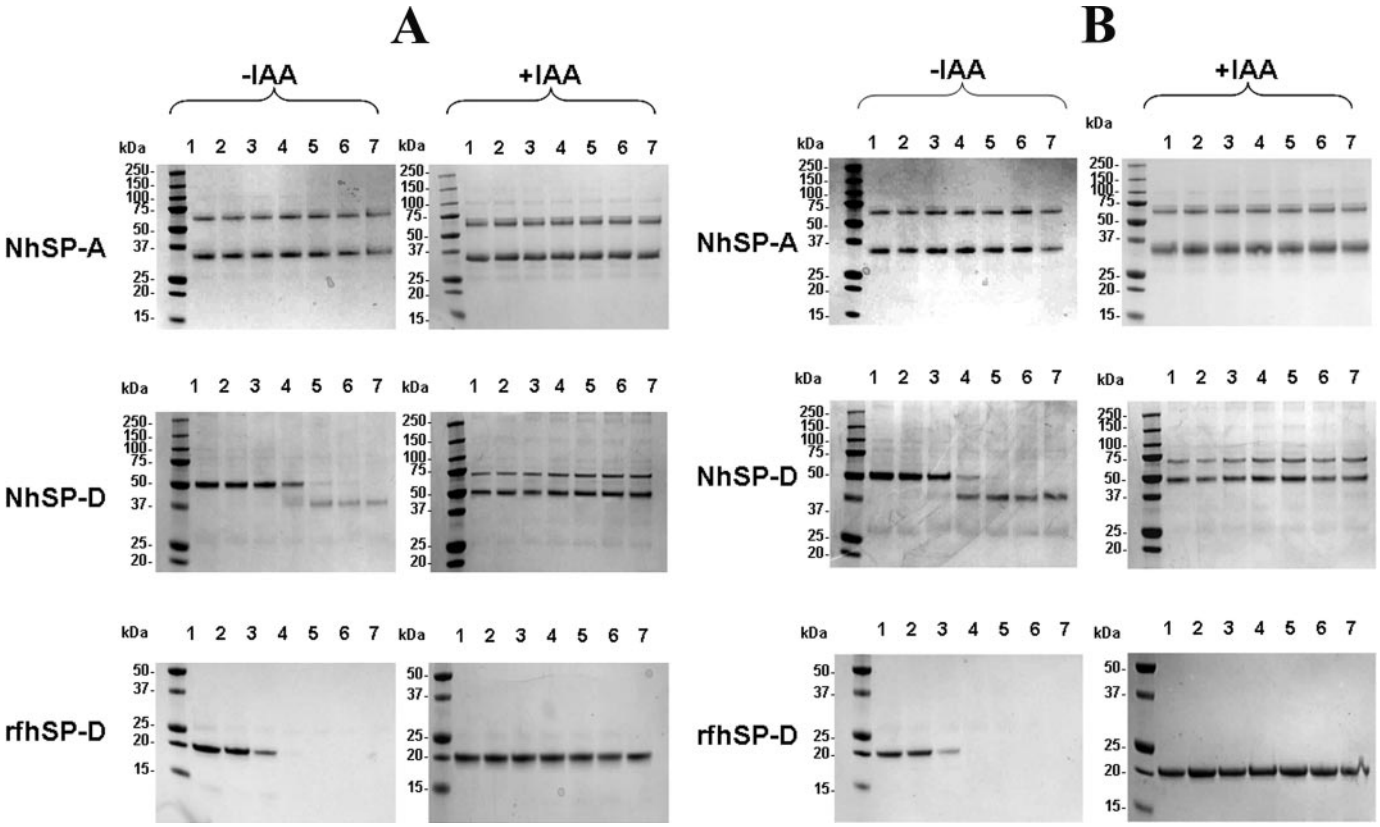


FIGURE 5. **Cleavage of NhSP-A, NhSP-D, and rfhSP-D by Der p 1 in the absence of calcium.** 100 μg/ml NhSP-A, NhSP-D, or rfhSP-D was incubated with 1.25 μg/ml Der p 1 (*A*) and Der f 1 (*B*) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 for various times in the presence or absence of IAA. Proteins were subsequently resolved by reducing SDS-PAGE. *Lane 1*, 0 min; *lane 2*, 10 min; *lane 3*, 30 min; *lane 4*, 1 h; *lane 5*, 2 h; *lane 6*, 3 h; *lane 7*, 6 h. NhSP-A, NhSP-D, and rfhSP-D were all susceptible to degradation under these conditions. Degradation of NhSP-D and rfhSP-D, but not NhSP-A, was more extensive in the absence of calcium. After 3 h of incubation with Der p 1 and Der f 1, a significant amount of uncleaved NhSP-A was still visible, as was observed in the assays with calcium (Fig. 3). However, after 2 h of incubation with Der p 1 and Der f 1, all the NhSP-D becomes degraded into a stable 37-kDa fragment. rfhSP-D was the most susceptible to degradation, and it was completely degraded by Der p 1 and Der f 1, with no visible cleavage products by 1 h. No degradation was observed in the presence of IAA, confirming Der p 1/Der f 1 specific activity. Data are representative of at least three independent experiments.

*Der p 1 and Der f 1 Cleave Endogenous SP-D, but Not SP-A, in BALF*—To see whether NhSP-A and NhSP-D were susceptible to degradation in their normal lung environment where they would be associated with lipids and perhaps other proteins, BALF was isolated from alveolar proteinosis patients, cells were removed through centrifugation, and the cell-free supernatant was incubated with Der p 1 and Der f 1 for various times at 37 °C. NhSP-A and NhSP-D were then detected by immunoblotting (Fig. 7). Endogenous NhSP-A was not degraded by Der p 1 or Der f 1 even after 48 h of incubation. However, NhSP-D was degraded by both Der p 1 and Der f 1 in a time-dependent manner, as indicated by the decrease in ~45-kDa band representing the monomer, and after 3 h, almost all the NhSP-D had become degraded. This degradation was not apparent in the presence of excess IAA, suggesting allergen-specific activity (data not shown). A small proportion of NhSP-D appeared to be resistant to degradation even after 48 h of incubation with the allergens. This may be a very large, aggregated, multimeric form of NhSP-D.

*Effects of Cleavage on the Biological Functions of SP-A and SP-D*—To determine whether exposure to Der p 1 and Der f 1 could alter the biological functions of collectins, many of which are mediated through carbohydrate binding, the mannan binding capacity of NhSP-D and rfhSP-D that had been incubated with Der p 1 and Der f 1 in the presence or absence of IAA was investigated through surface plasmon resonance. NhSP-D that had been exposed to Der p 1 and Der f 1 in the absence of IAA shows approximately an 85% reduction in mannan binding compared with that incubated with the allergens in the presence of IAA (Fig. 8*A*). Similarly, rfhSP-D that had been exposed to Der p 1 and Der f 1 in the absence of IAA showed approximately an 80 and 90% decrease in binding to mannan, respectively, compared with that incubated in the presence of IAA. Thus, the cleavage of these collectins by Der p 1 and Der f 1 significantly abolishes their carbohydrate binding capacity. Consistent with these results, exposure of NhSP-D and rfhSP-D to Der p 1 in the absence of IAA also significantly diminished their binding to immobilized Der p 1 compared with the iodoacetamide controls (Fig. 8*B*). Binding of NhSP-A to mannan and Der p 1 was not observed by surface plasmon resonance in these conditions perhaps due to calcium-induced aggregation, as has been reported previously (45).

Aggregation of bacteria is an important function of SP-A and SP-D that is mediated by the lectin domain. As a consequence of the diminished carbohydrate binding capacity, we hypothesized that NhSP-A, NhSP-D, and rfhSP-D that had been exposed to Der p 1 and Der f 1 would fail to aggregate bacteria. These collectins were incubated with the allergens before add-

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| Enzyme | Cleavage condition | N-terminal sequence * | Size of fragment (kDa) | Position within structure | Sequence surrounding cleavage site (P3,P2,P1,P1',P2',P3') |
|---|---|---|---|---|---|
| Der p 1 | NhSP-D with calcium | $G^{179}$PPGLK | 24 | end of collagen region | GARGPP |
| | | $S^{199}$GLPDV | 18 | start of α-helical neck region | KGESGL |
| | | $S^{239}$VGEKL | 11 | Start ofCRD | NGQSVG |
| | NhSP-D without calcium | $A^{1}$EMTKY | 40 | Start of NhSP-D | N/A |
| Der f 1 | NhSP-D with calcium | $G^{179}$PPGLK | 24 | end of collagen region | GARGPP |
| | | $S^{199}$GLPDV | 18 | start of α-helical neck region | KGESGL |
| | | $S^{239}$VGEKL | 11 | Start of CRD | NGQSVG |
| | NhSP-D without calcium | $A^{1}$EMTKY | 40 | Start of NhSP-D | N/A |
| Der p 1 | rfhSP-D with calcium | $S^{199}$GLPDV | 18 | Start of α-helical neck region | KGESGL |
| | | $K^{230}$VELFP | 12 | Start of CRD | QYKKVE |
| Der f 1 | rfhSP-D with calcium | $S^{199}$GLPDV | 18 | Start of α-helical neck region | KGESGL |

FIGURE 6. **N-terminal sequences of cleaved products.** 150 μg/ml NhSP-D or rfhSP-D were incubated with 1. 25 μg/ml Der p 1 or Der f 1 in the presence or absence of calcium for 20 h or 6 h, respectively. N-terminal sequences of degradation products were determined by Edman degradation to locate the sites of cleavage (*, number refers to position of amino acid within NhSP-D sequence). In the presence of calcium three different Der p 1 and Der f 1 cleavage sites were determined in NhSP-D; that is, at the end of the collagen region, start of α-helical neck region, and start of the CRD. Two of these sites were common to both NhSP-D and rfhSP-D. Der p 1 and Der f 1 did not cleave at these sites in the absence of calcium.

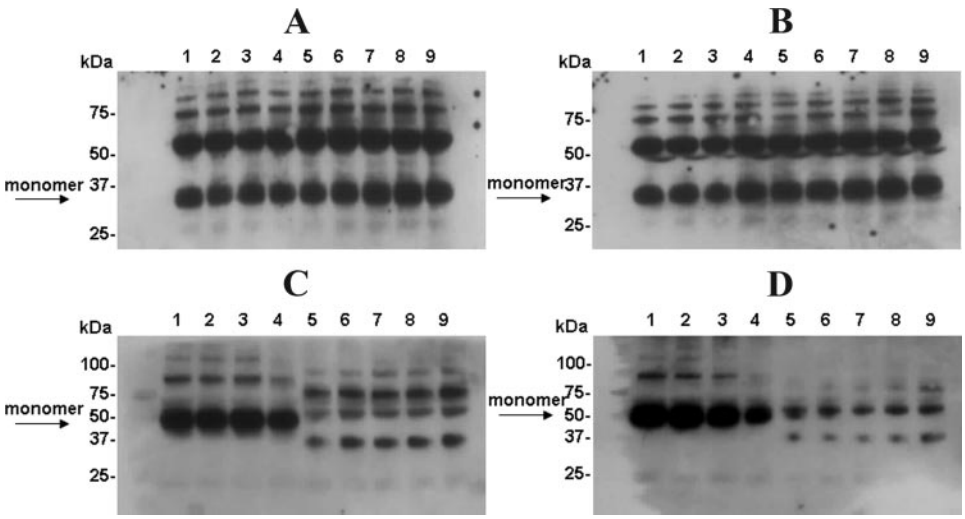


FIGURE 7. **Der p 1 and Der f 1 cleave NhSP-D but not NhSP-A in BALF.** 2. 5 μg/ml Der p 1 (*A* and *C*) and Der f 1 (*B* and *D*) were added to 30 μl of BALF and incubated at 37 °C for various times (*lane 1*, 0 min; *lane 2*, 10 min; *lane 3*, 30 min; *lane 4*, 1 h; *lane 5*, 3 h; *lane 6*, 6 h; *lane 7*, 12 h; *lane 8*, 24 h; *lane 9*, 48 h). Endogenous SP-A (*A* and *B*) and SP-D (*C* and *D*) were subsequently detected by immunoblotting. NhSP-D, but not SP-A, was susceptible to degradation by Der p 1 and Der f 1, as indicated by the decrease in intensity of the ~45-kDa band representing the monomer and increasing intensity of the ~37-kDa band representing a cleaved product. Results are representative of three independent experiments.

ing to an *E. coli* K12 cell suspension, and agglutination was measured by a decreased in absorbance at 660 nm. The ability of NhSP-A, NhSP-D, and rfhSP-D to agglutinate *E. coli* was lost after exposure to Der p 1 and Der f 1, confirming a Der p/f 1-dependent loss of CRD-dependent activity (Fig. 9). Collectins preincubated with Der p 1 and Der f 1 in the presence of IAA still retained their ability to agglutinate bacteria (data not shown).

## DISCUSSION

IgE-mediated hypersensitivity is the underlying mechanism of atopic conditions, such as asthma, that affects ~20% of the population (46). Given its high prevalence, it is necessary to understand the mechanisms involved in triggering and perpetuating the allergic response. Recent work has suggested that the non-immunological properties, such as enzyme activity, of some allergens may contribute to their allergenicity (40). This is best exemplified by the major HDM allergen Der p 1, which elicits IgE responses in more than 80% of patients who are sensitive to *D. pteronyssinus* and is the most immunodominant allergen involved in the expression of IgE-mediated dust mite hypersensitivity (24). In addition to acting as an immunogen, Der p 1 may bias the immune response toward a Th2-mediated response through its cysteine protease activity (25–28). Der f 1 is the major allergen from the house dust mite *D. farinae* and, with 82% homology to Der p 1, also exhibits cysteine protease activity, although its biological activity has not been well characterized.

The data presented in this study confirm a CRD-mediated interaction between the lung collectins NhSP-A, NhSP-D, and rfhSP-D and Der p 1/Der f 1. We examined the functional consequences of this interaction and show that Der p 1 and Der f 1 cleave NhSP-A, NhSP-D, and rfhSP-D in a time- and concentration-dependent manner *in vitro*, with a consequent abrogation of their capacity to bind carbo-

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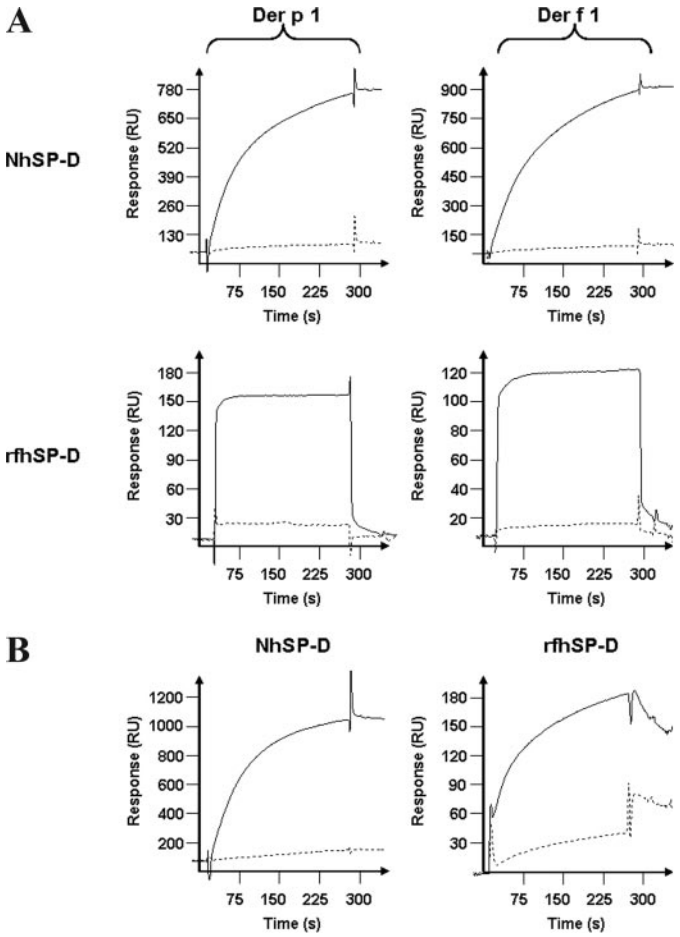


FIGURE 8. **Surface plasmon resonance response analyses of cleaved and uncleaved NhSP-D and rfhSP-D binding to mannan (*A*) and Der p 1 (*B*).** 60 $\mu$g/ml NhSP-D or 120 $\mu$g/ml rfhSP-D were incubated with 1.25 $\mu$g/ml Der p 1and Der f 1 (mannan binding only) in the presence (*solid line*) or absence (*dotted line*) of IAA in HSC before flowing over immobilized mannan (*A*) or Der p 1 (*B*). Exposure to these allergens in the absence of IAA significantly diminished mannan and Der p 1 binding. Results are representative of three independent experiments. *RU*, response units.

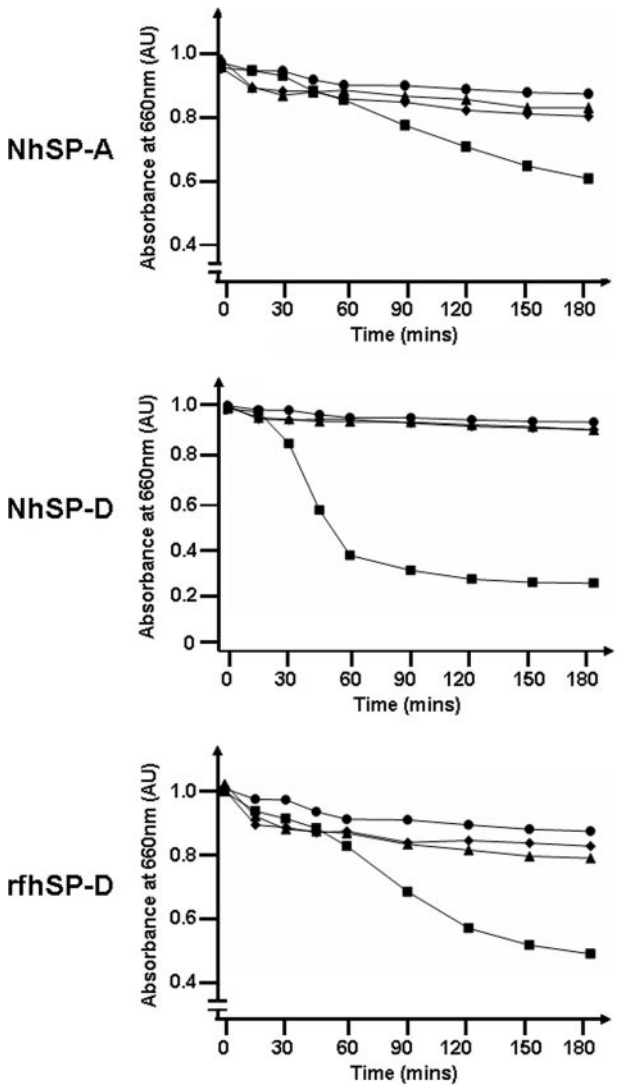


FIGURE 9. **Der p 1- and Der f 1- cleaved NhSP-A, NhSP-D, and rfhSP-D do not aggregate *E. coli*.** 2 $\mu$g of NhSP-A, 2 $\mu$g of NhSP-D, or 5 $\mu$g of rfhSP-D were digested with Der p 1 or Der f 1 in TSCa or left undigested before adding to 1 ml of *E. coli* suspension in TSCa. Aggregation of bacteria was assessed by regularly measuring the absorbance at 660 nm for 180 min. (●, bacteria alone; ■, bacteria with collectin; ♦, bacteria with collectin pre-incubated with Der p 1; ▲, bacteria with collectin preincubated with Der f 1. A decrease in absorbance indicated the precipitation of bacterial aggregates over time. Exposure to Der p 1 and Der f 1 diminished the ability of all collectins to aggregate bacteria. Results are representative of three independent experiments. *AU*, absorbance units.

hydrates and agglutinate bacteria. The cleavage and inactivation of lung collectins, which are important regulators of allergy, may potentially enhance the allergenicity of Der p 1 and Der f 1 *in vivo*.

It is plausible that many of the protective, anti-allergic effects of collectins seen *in vitro* and *in vivo* are mediated through interactions with the allergens themselves (15–19). To determine whether the collectins could bind to the allergens within the lung environment, solid phase binding assays were performed in BALF, which contains lipids and competitor proteins that would normally be present in the lung environment. NhSP-A, NhSP-D, and rfhSP-D all bound to Der p 1 and Der f 1 in BALF in a calcium- and carbohydrate-dependent manner, indicating that the interaction may well take place *in vivo*. It is known that NhSP-A and NhSP-D bind to ordered arrays of carbohydrates found on the surface of bacterial and viral pathogens but not to the carbohydrate structures found on eukaryotic proteins, as multivalent binding does not take place (4). It has been recently shown that Der p 1 has multiple glycosylation sites (47), and the arrangement of carbohydrate structures on Der p 1 and Der f 1 must mimic the ordered arrays seen on the surface of bacteria and viruses, enabling them to be recognized and bound by SP-A and SP-D.

We also demonstrate that NhSP-A, NhSP-D, and rfhSP-D are all susceptible to degradation by the cysteine proteases Der p 1 and Der f 1 under physiological conditions (Fig. 3). In the dose-dependent assays, the lowest molar enzyme:substrate ratios at which degradation was observed were 1:38, 1:28, and 1:500 for NhSP-A, NhSP-D, and rfhSP-D, respectively (Fig. 4). Der p 1 is found at concentrations as high as 10 mg/ml within fecal pellets (24). Moreover, nanogram quantities of Der p 1 have been detected in BALF from HDM-sensitive individuals, and thus, before dilution Der p 1 may be found in microgram quantities in the lung (48). The amount of NhSP-A and NhSP-D found within a healthy lung falls in the ranges of 5–10 and 0.5–2

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μg/ml, respectively, and thus, the enzyme:substrate ratios found *in vivo* could favor the cleavage of NhSP-A and NhSP-D. Furthermore, the high levels of glutathione found within the airways (~430 μM) (49) are likely to maintain Der p/f 1 in an active state, although this has not been tested directly.

NhSP-A and NhSP-D were cleaved by Der p 1 and Der f 1 at multiple sites. Cleavage within the collagen and/or α-helical neck region in NhSP-D may disrupt the oligomerization with consequent unfolding of the protein structure, exposing other potential cleavage sites and making the rest of the molecule susceptible to degradation. This could explain why most of the cleaved NhSP-A and NhSP-D could not be visualized by SDS-PAGE.

The sequences surrounding the cleavage sites in NhSP-D and the major cleavage site in rfhSP-D were consistent with the known specificity of Der p 1 enzyme activity and, therefore, coincided with some of the predicted sites of cleavage. However, despite only one predicted cleavage site for NhSP-A, NhSP-A appeared to be degraded completely by Der p 1 with no distinct cleavage products. Furthermore, the region of the NhSP-D N terminus to the mapped cleavage sites was not visible through SDS-PAGE analysis, suggesting it had also been degraded completely despite the absence of multiple predicted cleavage sites in this region. This indicates that the primary sequence specificity of Der p 1 enzyme activity determined from known protein and synthetic peptide substrates is not comprehensive.

Cleavage of NhSP-D and rfhSP-D, but not NhSP-A, was enhanced in the absence of calcium. This was not due to direct effects on enzyme activity, as determined by the effects of calcium on cleavage of a synthetic substrate (data not shown). Calcium also modulated the sites of cleavage (Fig. 6). A coordinated $Ca^{2+}$ ion is integral to the primary carbohydrate binding site of collectins and most other C-type lectins (41), and three additional $Ca^{2+}$ binding sites have been identified within the CRDs based on the crystal structure of rfhSP-D (42, 43). The $Ca^{2+}$ ions that are not directly involved in coordinating sugars are likely to influence protein structure and, thus, could potentially alter accessibility to Der p 1 and Der f 1, accounting for the decreased cleavage of SP-D and rfhSP-D in the presence of calcium. Calcium-dependent alterations in the CD spectrum of SP-D have also been described, providing further evidence for calcium-dependent conformational changes (50). Because the calcium binding sites differ in affinity (43), their differential occupancy could also influence susceptibility to degradation.

The regulation of calcium concentration in the airspaces of the normal or injured lung is not well understood; however, hypocalcemia or local decreases in free calcium could enhance SP-D degradation by HDM allergens *in vivo*. Normal levels of ionized calcium in the blood are ~1.4 mM, which is similar to the concentration used in these degradation assays, but calcium levels have been known to decrease in intensive care patients and in sepsis conditions (51, 52). Despite reports that the binding of calcium to SP-A causes a conformational change of the octadecamer and aggregation *in vitro* (53, 54), the degradation of NhSP-A by Der p 1 and Der f 1 was not significantly altered in the absence of calcium, suggesting that the cleavage sites may be far away from the calcium-binding sites.

Non-reducing SDS-PAGE was used to verify that the small amount of cysteine present in the reaction buffers from the activation step of Der p 1 and Der f 1 did not reduce the disulfide bonds in the collectins and, thus, alter susceptibility to degradation (data not shown). The minimum concentration of cysteine required to see any reduction of NhSP-A, NhSP-D, or rfhSP-D with calcium far exceeded the maximum concentration of cysteine present in the degradation assays with calcium, and thus, the cleavage observed was not due to disulfide bond reduction and subsequent changes in structure. However, in the absence of calcium, rfhSP-D was very susceptible to reduction by very low concentrations of cysteine, which may partially account for the significant enhancement in its degradation that was observed in the absence of calcium.

The vast majority of SP-A recovered from broncheolar lavage is associated with surfactant phospholipids, primarily dipalmitoylphosphatidylcholine, particularly in the form of tubular myelin (55). Although there is no evidence to suggest that SP-D is constrained within tubular myelin *in vivo*, SP-D normally exists in the alveolar hypophase in the presence of surfactant lipids, and it has been reported that lipids co-isolate with SP-D from the lavage of rats (56), and furthermore, SP-D has been shown to bind to phosphatidylinositol, a component of surfactant lipids (57). In our study endogenous NhSP-D, but not NhSP-A, was degraded by Der p 1 and Der f 1 in BALF. The association of NhSP-A with lipids in BALF (which are likely to be lost during the purification process of NhSP-A) and/or association with other proteins may account for the lack of susceptibility to cleavage in these conditions even after 48 h of incubation. NhSP-A is also susceptible to aggregation, which may have occurred in these conditions and would limit accessibility of the enzymes. In addition, because the concentration of NhSP-A in the healthy lung is significantly higher (5–10 μg/ml) than that of NhSP-D (0.5–2 μg/ml), a higher substrate:enzyme ratio would exist for NhSP-A than NhSP-D *in vivo*. It is, thus, likely that NhSP-A would not be degraded by these allergens *in vivo*. Degradation of NhSP-D was observed after 1 h of incubation with physiological concentrations of the enzymes despite any lipid association, and by 3 h almost all of it had become degraded. Thus, it appears that SP-D function may be critically compromised in the very acute phase (within the first few hours) of allergen exposure if the rate of degradation surpasses any increase in expression that may arise during this time.

Exposure of NhSP-D and rfhSP-D to the allergens significantly abolished their carbohydrate binding capacity, as assessed by their binding to mannan and Der p 1 and their ability to agglutinate bacteria. This is to be expected with NhSP-D, as exposure to the allergens results in extensive degradation with a few minor cleavage products. rfhSP-D is cleaved at the start of the α-helical neck region to generate a stable 18-kDa fragment, and it is possible that cleavage at this part of the molecule disrupts the trimerization of the monomers, and hence, avidity of binding to carbohydrates is reduced. The binding of NhSP-D to allergens may be one way in which the anti-allergic properties of NhSP-D are mediated, such as the inhibition of allergen-induced histamine release (19, 20), and thus, allergen-induced cleavage may abrogate some of the anti-allergic effects of NhSP-D.

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Despite the increased susceptibility of rfhSP-D to cleavage by HDM allergens reported here, rfhSP-D is still effective in down-regulating allergic hypersensitivity to HDM allergens *in vivo* (15, 16). It is likely that the high concentrations of exogenously administered SP-D would simply "mop-up" (neutralize) the allergen and in doing so protect endogenous SP-D from degradation. However, the increased susceptibility of rfhSP-D to degradation should be taken into account when considering its potential as a therapeutic agent in the treatment of asthma. A modified form of rfhSP-D with a mutation in the Der p/f 1-cleavage site(s), thus rendering it less resistant to cleavage, may be even more efficient in down-regulating allergic hypersensitivity *in vivo*.

In conclusion, the cleavage and consequent inactivation of lung collectins by HDM allergens may represent a novel mechanism by which the allergenicity of the HDM is enhanced. Interestingly, CD23, the low affinity IgE receptor, and DC-SIGN and DC-SIGNR, pattern recognition molecules, which are all also calcium-dependent lectins, are susceptible to cleavage by Der p 1 (3, 26). Thus Der p 1 and Der f 1 may influence the immune response through cleavage of a diverse range of immune molecules.

It remains to be seen whether SP-A and SP-D are susceptible to degradation by these allergens *in vivo*. Clearly, any extrapolation to the *in vivo* situation would also have to consider the expected concentrations of allergens in lung tissues. There is no doubt that chronic exposure, which is so characteristic of HDM sensitivity, would result in the cumulative build up and continuous replenishment of very high concentrations of allergens in lung tissues. Despite some reported increases in SP-A and SP-D levels 24 h after allergen challenge, which may well represent an innate immune defense mechanism (11, 12, 15, 59), given our findings, we believe that as well as very short-term exposure, which has been discussed above, long-term exposure to HDM allergens would disrupt the physiological levels and functions of SP-D in the lungs, and this could have profound consequences. Consistent with this, decreased levels of SP-A and SP-D in BALF are seen in the chronic phase of allergen exposure in murine models of allergic hypersensitivity to HDM allergens (58).

Enzymatic activity is not just restricted to HDM allergens, but the plant pollen allergen *Phleum pratense* 1 and the cat allergen *Felis domesticus* 1 have cysteine and serine protease activity, respectively. In a similar fashion to Der p 1, which cleaves molecules that bias the immune response toward a Th2-mediated response, it may be that the enzymatic activity of these allergens is essential for eliciting powerful IgE responses. Thus, the possibility that SP-A and SP-D, which have protective roles in allergy, are susceptible to degradation by all these allergens merits further investigation.

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