

# *Aspergillus* and *Penicillium* Allergens: Focus on Proteases

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*Penicillium* and *Aspergillus* species are prevalent airborne fungi. It is imperative to identify and characterize their major allergens. Alkaline and/or vacuolar serine proteases are major allergens of several prevalent *Penicillium* and *Aspergillus* species. They are also major immunoglobulin (Ig) E-reacting components of the most prevalent airborne yeast, *Rhodotorula mucilaginosa*, and the most prevalent *Cladosporium* species, *C. cladosporioides*. IgE cross-reactivity has been detected among these major pan-fungal serine protease allergens. In addition, the alkaline serine protease of *P. chrysogenum* (Pen ch 13) induces histamine release from basophils of asthmatic patients, degrades the tight junction protein occludin, and stimulates release of proinflammatory mediators from human bronchial epithelial cells. In addition to induction of IgE and inflammatory airway responses, the alkaline serine protease allergen of *A. fumigatus* (Asp f 13) has synergistic effects on Asp f 2–induced immune response in mice. Studies of these serine protease major allergens elucidate the diverse allergic disease mechanisms and facilitate the development of better therapeutic strategies.

## Introduction

The prevalence of respiratory atopic disorders is increasing worldwide. Among various causative agents, mold contributes to about 25% of problems [1,2]. Identification and characterization of fungal allergens will be beneficial in facilitating better diagnosis, treatment, and understanding of the pathogenic mechanisms of the diseases. *Penicillium*, *Aspergillus*, *Cladosporium*, and *Alternaria* are the most frequently found airborne fungi associated

with allergy [2–4]. *Penicillium* and *Alternaria* spores in relatively natural exposure states and numbers can be potent etiologic risk factors for asthma [5]. It has also been shown that *Penicillium* and *Aspergillus* allergens cause allergic sensitization and inflammation in mice [6,7,8•]. It is important to identify and comprehend the biologic significance of major allergens from these prevalent fungal genera.

## Identification of Prevalent Airborne *Penicillium* Species

The fungal genus *Penicillium* has more than 100 different species [9]. Various species have been selected as starting materials by different manufacturers for extract preparation. The components of the resulting extracts may differ from each other. It is important to identify the representative *Penicillium* species and characterize their major allergens before standardized crude extracts can be utilized for clinical purposes. A survey conducted in the Taipei area shows that among 5897 fungal isolates from 88 houses, 910 (15.4%) were *Penicillium* species [10]. About 41% of the 910 isolates (369 from 28 houses) were *P. citrinum* [10]. Moreover, *P. citrinum*, *P. spinulosum*, *P. brevicompactum*, *P. oxalicum*, and *P. chrysogenum* (*P. notatum*) were found to be the most frequently recovered *Penicillium* species in Topeka, KS [11]. Thus, major allergens of these prevalent *Penicillium* species should be characterized further.

## Identification of *Penicillium* Allergens

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) immunoblots were performed to identify major allergens of *Penicillium* species. Protein components with a molecular mass of about 33 kDa and an immunoglobulin (Ig) E-binding frequency of greater than 90% were identified as major allergens of *P. citrinum*, *P. chrysogenum*, and *P. brevicompactum* [12]. Characterization of these major IgE-reacting components by two-dimensional gel electrophoresis and N-terminal amino acid sequence analysis suggests that they are the alkaline and/or the vacuolar serine proteases

**Table 1. Serine protease major panallergens of prevalent airborne fungal species**

| Study                                 | Species                   | Allergen designation* | Properties                 | Frequency of IgE binding <sup>†</sup> |
|---------------------------------------|---------------------------|-----------------------|----------------------------|---------------------------------------|
| <i>Penicillium</i>                    |                           |                       |                            |                                       |
| Shen et al. [13],<br>Chou et al. [16] | <i>P. chrysogenum</i>     | Pen ch 13             | Alkaline serine proteinase | 88% (15/17)                           |
| Shen et al. [13],<br>Shen et al. [15] | <i>P. chrysogenum</i>     | Pen ch 18             | Vacuolar serine proteinase | 82% (14/17)                           |
| Shen et al. [12],<br>Su et al. [17]   | <i>P. citrinum</i>        | Pen c 13              | Alkaline serine proteinase | 100% (15/15)                          |
| Shen et al. [14],<br>Lin et al. [19]  | <i>P. citrinum</i>        | Pen c 18              | Vacuolar serine proteinase | 58% (19/33)                           |
| Shen et al. [13],<br>Shen et al. [14] | <i>P. oxalicum</i>        | Pen o 18              | Vacuolar serine proteinase | 94% (17/18)                           |
| Shen et al. [12]                      | <i>P. brevicompactum</i>  | Pen b 13              | Alkaline serine proteinase | 91% (10/11)                           |
| <i>Aspergillus</i>                    |                           |                       |                            |                                       |
| Shen et al. [20]                      | <i>A. fumigatus</i>       | Asp f 13              | Alkaline serine proteinase | 71% (10/14)                           |
| Shen et al. [20]                      | <i>A. fumigatus</i>       | Asp f 18              | Vacuolar serine proteinase | 79% (15/19)                           |
| Shen et al. [20]                      | <i>A. niger</i>           | Asp n 18              | Vacuolar serine proteinase | 100% (7/7)                            |
| Chou et al. [21]                      | <i>A. flavus</i>          | Asp fl 13             | Alkaline serine proteinase | 63% (19/30)                           |
| Shen et al. [22]                      | <i>A. oryzae</i>          | Asp o 13              | Alkaline serine proteinase | 71% (12/17)                           |
| <i>Cladosporium</i>                   |                           |                       |                            |                                       |
|                                       | <i>C. cladosporioides</i> | Cla c 9               | Vacuolar serine proteinase | 55% (41/74)*                          |
| <i>Curvularia</i>                     |                           |                       |                            |                                       |
| Gupta et al. [23]                     | <i>C. lunata</i>          | Cur l 1               | Alkaline serine proteinase | 80% (12/15)                           |
| <i>Rhodotorula</i>                    |                           |                       |                            |                                       |
| Chou et al. [24]                      | <i>R. mucilaginosa</i>    | Rho m 2               | Vacuolar serine proteinase | 57% (25/44)                           |

\*The allergens are designated as recommended by the Allergen Nomenclature Subcommittee.

<sup>†</sup>Frequency of IgE binding represents the percentage of the number of sera with IgE binding against the individual allergen divided by the number of sera with positive IgE reactivity against components of the fungal species tested.

\*Shen HD, Unpublished data.

of *Penicillium* species [13]. This conclusion was affirmed by cDNA cloning. They were designated as the group 13 (alkaline serine protease) and the group 18 (vacuolar serine protease) major allergens of *Penicillium* species [14–18] (Table 1).

### Identification of Serine Proteases as Major Pan-fungal Allergens

Similarly, the alkaline and/or the vacuolar serine proteases were identified as major allergens of *Aspergillus* species: *A. fumigatus* (Asp f 13 and Asp f 18) [20], *A. flavus* (Asp fl 13) [21], *A. niger* (Asp n 13) [20], and *A. oryzae* (Asp o 13) [22]. In addition, Cur l 1 (an alkaline serine protease) was characterized as a major allergen of *Curvularia lunata* [23]. About 80% of *C. lunata*–hypersensitive patients showed IgE reactivity against the major *Curvularia* allergen. The vacuolar serine protease was also identified as a major allergen of the most prevalent airborne yeast, *Rhodotorula*

*mucilaginosa* (Rho m 2) [24] and the most prevalent *Cladosporium* species, *C. cladosporioides* (Cla c 9; Shen HD et al., Unpublished results). Thus, the serine proteases can be considered as a major pan-fungal allergen group of prevalent airborne fungal species.

### IgE Cross-reactivity

In the analysis of IgE-reacting fungal components, all 10 *P. brevicompactum* 33-kDa component-positive serum samples showed IgE reactivity to the 33-kDa components of *P. citrinum* and *P. chrysogenum* [12]. Immunoblot inhibition studies demonstrated the presence of IgE cross-reactivity among the 33-kDa group major allergens of *P. citrinum*, *P. chrysogenum*, and *P. brevicompactum* [12]. Subsequent studies showed the presence of IgE cross-reactivity between and/or among serine protease allergens from *P. oxalicum*, *P. chrysogenum*, *P. citrinum*, *P. brevicompactum*, *A. fumigatus*, *A. flavus*, *A. oryzae*, *R. mucilaginosa*, and *C. cladosporioides* [12–14,16,20–22,24] (Shen HD

et al., Unpublished results). Results obtained suggest that atopic patients primarily sensitized by serine protease allergens from one of these prevalent fungal species may develop allergic symptoms by exposure to other environmental fungi due to cross-reacting IgE antibodies.

IgE cross-reactivity has been observed between Pen ch 13 and Pen ch 18. However, there are still allergen-specific IgE epitopes for Pen ch 18 and Pen ch 13 [14]. Both major allergens should thus be included in *P. chrysogenum* extracts for clinical diagnosis. Moreover, purified native Pen ch 13, Pen c 13, and Asp fl 13 in solution fully inhibit serum IgE from binding to solid-phase-bound Pen ch 13. But Pen ch 13 is about four to 16 times more potent than Pen c 13 and Asp fl 13 with regard to enzyme-linked immunosorbent assay (ELISA) inhibition [16]. These data indicate that these serine proteases share common IgE-binding epitopes. However, serum IgE antibodies bind to these group 13 allergens with different affinities.

### IgE Epitope Mapping

Interactions between an allergen and its specific IgE antibodies are fundamental to an allergic reaction. Characterization of IgE epitopes on allergens will enable us to better understand the molecular interactions between these macromolecules. We observed IgE antibodies in 35 serum samples reacted with at least one of the 11 peptides derived from the Pen ch 13 sequence [25]. The peptide encompassing residues 31 to 61 (peptide f-2n) showed an enhanced intensity and the highest frequency (77%) of IgE-binding. The frequencies of IgE binding to peptide f-4 (residues 93–133), f-1 (residues 1–37) and f-7 (residues 168–206) were 51%, 34%, and 31%, respectively. In addition, heterogeneity in IgE reactivity against Pen ch 13 in terms of antibody specificity and intensity in individual patients was also observed [25].

Substitution of His49 and/or Phe52 on Pen ch 13 with methionine by site-directed mutagenesis resulted in proteins with drastic loss of IgE binding in seven sera tested [25]. *P. cyclopium* subtilisin protease has greater than 90% sequence identity with Pen ch 13 and its structure has been elucidated. It was used as a template for constructing a Pen ch 13 3D model. The major epitope (<sup>48</sup>GHADFGGR<sup>55</sup>) on Pen ch 13 formed a loop-like structure and located on the surface of the allergen [25]. Results obtained have advanced our understanding of the molecular and structural features of the allergenicity of Pen ch 13. It is also of interest to find that His49 and Phe52 are highly conserved among the fungal serine protease allergens identified (Pen c 13, Pen ch 13, Asp f 13, Asp o 13, Pen c 18, Pen ch 18, Pen o 18, Asp f 18, Asp n 18, Rho m 2, and Cla c 9). Whether these two residues contribute to the allergenic cross-reactivity among these major fungal allergens needs further study.

### Monoclonal Antibodies against Serine Protease Major Allergens

Five monoclonal antibodies (MoAbs) against different epitopes of serine protease allergens have been generated [19]. Antibodies obtained may be useful tools in the standardization of serine protease allergens in commercially available crude fungal extracts. The lysine (K6), proline (P9), and tryptophan (W10) residues located on the N-terminal region of fungal serine proteases are critical core amino acid residues recognized by MoAb FUM20. This antibody can react with vacuolar serine proteases from *R. mucilaginosa* (Rho m 2), *P. chrysogenum* (Pen ch 18), *P. citrinum* (Pen c 18), *P. oxalicum* (Pen o 18), *A. fumigatus* (Asp f 18), and the alkaline serine proteases from *A. fumigatus* (Asp f 13) and *A. flavus* (Asp fl 13) [19,26]. It was found that a charged residue is needed at position 6 (K) for antigen-FUM20 interaction. The proline at position 9 bends the peptide backbone into a conformation for optimal protein-protein interaction. In addition, immunoblot reactivity of FUM20 was not detected when W10 of Rho m 2 was substituted with either alanine or phenylalanine. This suggests that an aromatic side chain is insufficient for FUM20 interaction at this position. These findings provide more information on structural elements required for the antigenicity of serine protease allergens.

### Characterization of Serine Protease Major Allergens

Pen ch 13 and Asp fl 13 have been purified from *P. chrysogenum* and *A. flavus*, respectively. They are proteolytically active with casein as a substrate at pH 8.0 [16,21]. The enzymatic activity is inhibited by phenylmethylsulphonyl fluoride (PMSF) or diethylpyrocarbonate [16,21]. In addition, purified native Asp f 13 was shown to induce IgE antibodies, inflammation, and airway hyperresponsiveness in mice [7]. Pen ch 13 also degrades gelatin and collagen but not elastin [16]. Basophils from five asthmatic patients released histamine (12%–73%) when exposed to purified Pen ch 13 [16]. The proteolytic activity of Pen ch 13 is needed for the induction of prostaglandin (PG) E<sub>2</sub>, interleukin (IL)-8, transforming growth factor (TGF)-β1, and cyclooxygenase (COX)-2 expression in A549 cells, 16HBE14o- cells, and primary cultures of human bronchial epithelial cells [27•]. Pen ch 13 also cleaves occludin, a tight junction protein from 16HBE14o- cells [27•]. Thus, Pen ch 13 may induce asthma by damaging the barrier formed by the airway epithelium and stimulating the release of mediators that orchestrate local immune responses as well as airway inflammatory and remodeling processes.

### Receptors of Fungal Serine Protease Allergens

Besides Pen ch 13, proteases present in fungal extracts of *A. fumigatus*, *A. alternata*, or *C. herbarum* also interact with epithelial cells and induce the production of proin-

flammatory cytokines [28–30]. It is important to identify potential receptor(s) responsible for the protease allergen-induced respiratory inflammatory manifestations. Protease-activated receptors (PARs) are G-protein-coupled receptors and four (PARs 1 to 4) have been reported [31]. Proteolytic cleavage of the PARs exposes an N-terminal cryptic receptor-activating sequence. The unmasked fragment then binds to the conserved extracellular domain of the cleaved receptor as a tethered ligand and activates PARs. Thus, PARs are able to detect proteolytic activities in the airways. PARs are found in epithelial cells, mast cells, eosinophils, endothelial cells, neutrophils, macrophages, lymphocytes, dendritic cells, and airway smooth muscle cells [31,32••]. All of these cells are involved in atopic asthma.

Among the four reported PARs, PAR2 has been implicated in the pathogenesis of airway inflammation. Asthmatic patients have increased PAR-2 expression in bronchial epithelium [33]. Fungal extracts and house dust mite serine proteases Der p 1, Der p 3, and Der p 9 can activate airway epithelial cells through PAR-2 receptors with subsequent release of proinflammatory cytokines [30,34–37]. Similarly, cockroach proteases activate PAR-2 on bronchial epithelial cells, resulting in extracellular signal-related kinase (ERK) stimulation and an increase in the tumor necrosis factor (TNF)- $\alpha$ -induced expression of IL-8 [38]. Furthermore, a PAR-2 agonist in the airways enhances ovalbumin-induced airway inflammation and airway hyperresponsiveness in mice [39•]. With an ovalbumin-sensitized mouse model, it has been shown that PAR-2 overexpression exacerbates eosinophil infiltration and bronchial hyperactivity [40]. Conversely, Adam et al. [41••] demonstrated that Der p 1-induced IL-8 production in human airway epithelial cells is independent of PAR-2 activation. Thus, more studies are needed to define the role(s) of PARs in protease allergen-induced respiratory inflammatory responses. Identification of a receptor(s) for fungal serine protease major allergens has not been firmly established and is the subject of current research.

### Role of Protease Allergens in Pathophysiology of Atopic Respiratory Diseases

Protease allergens may elicit both IgE-induced allergic responses and non-IgE-mediated airway reactions by provoking the innate immunity receptors, such as PARs, to activate epithelial cells, mast cells, and dendritic cells for further mediator release [42••]. They may also contribute to allergic manifestations by causing cell detachment, opening of tight junctions, modulating the respiratory mucosal permeability, and facilitating transepithelial allergen delivery [27•,43–46]. Der p 1 has shown to act on CD40, CD25, and CD23 to modulate the adaptive immune response towards T-helper type 2 (Th2) cells and enhance IgE immune responses [47–49]. Inactivation of a protease antagonist, such as elastase inhibitors by

Der p 1, may skew the mucosa balance towards a pro-inflammatory environment to facilitate the initiation and maintenance of allergic responses [50]. Whether serine protease pan-fungal allergens have similar effects on these immune-modulating molecules and contribute to asthmatic responses needs further study.

Asp f 2 (a major *A. fumigatus* allergen) alone failed to induce IgE and eosinophil responses in mice and showed less severe pulmonary inflammation compared to that in Asp f 13-challenged animals [7]. However, Asp f 13 in combination with Asp f 2 enhanced inflammatory responses in the lung and induced a marked elevation in eosinophils in the peripheral blood of mice [7]. Thus, allergens with protease activity may have a major role in contributing to a mature immune response and play a crucial role in the development of allergic reactions [7].

### Conclusions

The alkaline serine proteases and/or the vacuolar serine proteases have been identified as major pan-fungal allergens of prevalent airborne fungi including *Penicillium*, *Aspergillus*, *Rhodotorula*, *Curvularia*, and *Cladosporium* species. Except for the *Curvularia* alkaline serine protease, IgE cross-reactivity is detectable among these major pan-fungal allergens. Results from ongoing research suggest that the enzymatic activity of serine protease fungal allergens may contribute to asthma by degrading the airway epithelial barrier and stimulating the release of mediators that coordinate local immune responses and inflammatory processes.

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