

The Importance of Serine Proteinases as Aeroallergens Associated with Asthma

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Key Words

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Abstract

Penicillium and *Aspergillus* species have been identified as prevalent indoor airborne fungi that are associated with extrinsic bronchial asthma. We have recently analyzed the IgE-binding components in 8 prevalent *Penicillium* and *Aspergillus* species (*P. citrinum*, *P. notatum*, *P. oxalicum*, *P. brevicompactum*, *A. fumigatus*, *A. flavus*, *A. oryzae* and *A. niger*) by immunoblotting and N-terminal amino acid sequence analysis. Our results show that the alkaline and/or vacuolar serine proteinases are the major allergens in these prevalent fungal species. IgE cross-reactivity among these major allergens was also detected. Results obtained provide an important basis for clinical allergy. In addition, monoclonal antibodies against alkaline and/or vacuolar serine proteinase allergens have been generated. These antibodies can be applied for the standardization of allergenic extracts.

Introduction

It has long been established that inhaling airborne mold spores can cause respiratory allergic symptoms [1]. More than 60 genera of fungi have been associated with symptoms of respiratory tract allergy [2]. Most studies on fungi as aeroallergens have been limited to the fungi imperfecti. Besides *Alternaria alternata*, *Aspergillus fumigatus* and *Cladosporium herbarum* of the fungi imperfecti [3–6], information on allergens from the vast number of other fungal species are scarce.

Aldehyde dehydrogenase [7], ribosomal protein P2 [7], the YCP4 protein [7], and the 28-kD Alt a 1 [8] have been identified as allergens in *A. alternata*. In addition to the aldehyde dehydrogenase [7], the ribosomal protein P2 [7, 9], and the YCP4 protein [7], other allergens that have been identified in *C. herbarum* are the 48-kD enolase [7], the heat shock protein 70 [10], the 13-kD Ag-32 and the 25-kD Ag-54 components [11]. Allergens that have been recognized in *A. fumigatus* include metallo (Asp f 5) and aspartic (Asp f 10) proteinases [6], the ribotoxin (Asp f 1) [12, 13], the peroxisomal protein (Asp f 3) [14], the Mn superoxide dismutase (Asp f 6) [15], the peptidyl-prolyl isomerase (Asp f 11) [5], and a heat shock protein (Asp f 12) [5, 16]. Four other components with molecular mass of 37 kD (Asp f 2) [17, 18], 30 kD (Asp f 4) [6], 11.6 kD (Asp f 7) [6] and 33.7 kD (Asp f 9) [6] are also comprised in this list. Our results have also shown that the heat shock protein 70 [19],

Table 1. Serine proteinase allergens identified in various *Penicillium* and *Aspergillus* species

Allergen designation ¹	Originating species	Apparent MW, kD	Properties	References
Pen c 13	<i>P. citrinum</i>	33	ASP ² , N-AA ³ , AF084546 ⁴	29, 31
Pen b 13	<i>P. brevicompactum</i>	33	ASP, N-AA	29
Pen n 13	<i>P. notatum</i>	34	ASP, N-AA	28
Pen n 18	<i>P. notatum</i>	32	VSP ⁵ , N-AA	28
Pen o 18	<i>P. oxalicum</i>	34	VSP, N-AA	28
Asp f 13	<i>A. fumigatus</i>	34	ASP, N-AA, P28296 ⁴	ms ⁶ , 37
Asp f 18	<i>A. fumigatus</i>	34	VSP, N-AA, Y13338 ⁴	ms
Asp n 18	<i>A. niger</i>	34	VSP, N-AA, m96758 ⁴	ms, 38
Asp fl 13	<i>A. flavus</i>	34	ASP, N-AA	35
Asp o 13	<i>A. oryzae</i>	34	ASP, N-AA, P12547 ⁴	34, 36

¹ The allergens are designated as recommended by the Allergen Nomenclature Subcommittee.
² ASP, alkaline serine proteinase.
³ N-AA denotes the N-terminal amino acid sequence of the native allergen has been determined.
⁴ The GenBank accession number of a cDNA clone that has a deduced N-terminal amino acid sequence identical to that of the allergen determined by Edman degradation.
⁵ VSP, vacuolar serine proteinase.
⁶ ms, manuscript submitted.

the N-acetyl glucosaminidase [20] and the peroxisomal membrane protein [manuscript submitted] are allergens in *Penicillium* species.

A major difficulty associated with studies on mould allergens is the multiplicity of factors contributing to the results obtained. For example, more than 100 different species have been identified in each of the taxonomically closely related fungal genera *Penicillium* and *Aspergillus* [21, 22]. It is important to understand the allergenic profiles of the prevalent airborne *Penicillium* and *Aspergillus* species, the presence of IgE cross-reactivity among major allergens of these ubiquitous fungi, and identify the representative species for clinical diagnosis. Recently, we have identified the major allergens in several prevalent airborne *Penicillium* and *Aspergillus* species by immunoblotting using sera from adult asthmatic patients. Major IgE-binding components were characterized by N-terminal amino acid sequence analysis (table 1). The presence of IgE cross-reactivity among major allergens of these fungal species was analyzed by immunoblot inhibition.

Characterization of Allergens of *Penicillium* Species

Penicillium are among the prevalent indoor airborne fungi [1, 3, 23]. Their spores have been identified as potential respiratory allergens [1–3, 24]. However, little is known

about the allergens of *Penicillium* species. We surveyed 88 homes in the Taipei area in order to isolate and identify the indoor *Penicillium* species. Our results show that *Penicillium citrinum* is the most prevalent *Penicillium* species [25]. Among the 910 isolated *Penicillium* colonies, 369 (40.5%) were *P. citrinum* that were found in 28 (32%) of the 88 houses studied [25]. Muilenberg et al. [26] have reported that *P. citrinum*, together with *P. spinulosum*, *P. brevicompactum*, *P. oxalicum*, and *P. chrysogenum* (*P. notatum*) were the five most frequently recovered species of *Penicillium* in Topeka, Kans., USA.

*IgE-Binding Components in Prevalent *Penicillium* Species*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-immunoblot was performed to identify the IgE-binding components of the prevalent *Penicillium* species. Among the 67 asthmatic serum samples tested, 15, 14 and 11 samples have IgE reactivity against components of *P. citrinum*, *P. notatum* and *P. brevicompactum*, respectively [27]. The crude extracts of *P. citrinum* and *P. brevicompactum* were prepared in our laboratory while the *P. notatum* extract used was obtained commercially from Greer Laboratories (Lenoir, N.C., USA). All 15 *P. citrinum*-positive samples showed IgE binding to a 33-kD component in the extract. Thirteen (93%) and 10 (91%) of the *P. notatum*- and *P. brevicompactum*-positive samples, respectively, also showed IgE reactivity to a 33-kD protein in the respective

Penicillium species. Moreover, all 10 *P. brevicompactum* 33-kD-component-positive samples demonstrated IgE reactivity to the 33-kD components of the other two *Penicillium* species tested. Dose-dependent inhibition of IgE-binding to these major allergens was observed when the positive serum sample was preabsorbed with extracts prepared from any one of these three *Penicillium* species [27]. The results obtained suggest that there is IgE cross-reactivity among the 33-kD major allergens of *P. citrinum*, *P. notatum* and *P. brevicompactum* [27].

The IgE-binding components in the crude extracts of *P. oxalicum* and *P. notatum* that were prepared in our laboratory were also analyzed by immunoblot using another batch of 70 adult asthmatic serum samples. Among the 70 sera tested, 18 (26%) and 17 (24%) have IgE immunoblot reactivity towards components of *P. oxalicum* and *P. notatum*, respectively [28]. Major allergens (>80% frequency of IgE binding) from both species are the 34- and 30-kD proteins of *P. oxalicum* and the 34- and 32-kD proteins of *P. notatum* [28]. IgE cross-reactivity among these major allergens and the 33-kD major allergen of *P. citrinum* can also be detected by immunoblot inhibition studies [28].

Characterization of the Major IgE-Binding Components in *Penicillium* Species

The major IgE-binding components in different *Penicillium* species were characterized further by two-dimensional immunoblot and N-terminal amino acid sequence analysis. Results demonstrated that at least six different isoforms in *P. citrinum* with pI values ranging from 6.75 to greater than 7.0 were reactive to monoclonal antibody (MoAb) 55A and IgE antibodies in the serum of an asthmatic patient [29]. The 33-kD IgE-binding components of *P. citrinum* and *P. brevicompactum* have an N-terminal protein sequence of ANVVQSNVP [29] that is identical to that of the N-terminus of a heat-labile alkaline serine proteinase from *P. citrinum* [30]. The 34-kD allergen from *P. notatum* has an N-terminal sequence of ANVVQRNAPS [28]. The similarity among these protein sequences is rather obvious. These 33- to 34-kD major allergens of *P. citrinum*, *P. brevicompactum* and *P. notatum* are probably alkaline serine proteinases. The cDNA of the 33-kD alkaline serine proteinase allergen has recently been isolated from *P. citrinum*. Upon refolding, the purified recombinant protein has full caseinolytic activity as the native protein [31].

The N-terminal amino acid sequences of the *P. oxalicum* 34-kD allergen (EDPAIEKNAPWGLARISHKD), the *P. notatum* 32-kD (SEGSVEKNAPWGLARISHRE) and the 28-kD (YAEEGGEGVDAYVID) allergens share homology with that of the vacuolar serine proteinase (accession No.

Y13338) from *A. fumigatus* [28]. In addition, immunoblotting results showed that MoAb PCM39 reacted with the 34-, 30- and 16-kD IgE-binding components of *P. oxalicum*, and with the 34-, 32- and 28-kD IgE-binding components of *P. notatum* [28]. These results imply that the 34-kD major allergen of *P. oxalicum* and the 32-kD major allergen of *P. notatum* are probably vacuolar serine proteinase. The 30- and 16-kD IgE-binding components of *P. oxalicum* and the 28-kD IgE-binding component of *P. notatum* are possibly the breakdown products of the postulated major vacuolar serine proteinase allergens.

Characterization of Allergens of *Aspergillus* Species

Fungi of the genus *Aspergillus* are associated with extrinsic bronchial asthma [1–3, 32]. Studies through molecular approaches have identified at least 12 different *A. fumigatus* allergens [5, 6]. However, *A. flavus* and *A. niger* (>50% occurrence frequency) are the predominant airborne fungi in the residences of atopic children in the Taipei area. *A. fumigatus* and *A. clavatus* have only a 4% occurrence rate [33]. *A. flavus* and *A. niger* are, therefore, recommended to be included on a skin test panel. In addition, a 34-kD component in *A. oryzae* can react with MoAb 55A, an antibody that recognizes the 33-kD major allergen of *P. citrinum* [34]. The IgE-binding components in *A. oryzae*, *A. flavus*, *A. fumigatus* and *A. niger* were analyzed with a similar approach as that for the allergens in *Penicillium* species.

IgE-Binding Components in *Aspergillus* Species

IgE-binding components in *A. oryzae* were firstly identified by immunoblot using sera from 70 adult asthmatic patients. Thirteen components of *A. oryzae*, ranging from 16 to 42 kD react with IgE antibodies in 17 (24.3%) of the 70 sera tested [34]. A 34-kD component that reacted intensely and most frequently (with the highest IgE-binding frequency of 71%) was considered as a major allergen of *A. oryzae* [34]. Similarly, a 34-kD component with 63% IgE-binding frequency was identified as a major allergen from *A. flavus* [35]. In addition, among two batches (70 and 41 sera) of adult asthmatic sera tested, 19 (27%) and 14 (34%) samples, respectively, have IgE immunoblot reactivity towards components of *A. fumigatus*. A 34-kDa protein that reacts with IgE antibodies in 15 (79%) and 11 (79%) of the 19 and 14 positive samples, respectively, may be considered a major allergen of *A. fumigatus* [manuscript submitted].

Characterization of the Major IgE-Binding Components in *Aspergillus* Species

Results from two-dimensional immunoblot analysis demonstrated that the 34-kD major IgE-binding component in *A. oryzae* could react with MoAb 55A, an antibody used in our previous studies on *Penicillium* species [34]. Data from immunoblot inhibition studies indicated the presence of IgE cross-reactivity between the 34-kD major allergens of *A. oryzae* and *P. citrinum* [34]. A similar phenomenon was observed among the 34-kD major allergens of *A. flavus*, *A. fumigatus* and *P. citrinum* [35].

The N-terminal amino acid sequence of the *A. oryzae* 34-kD (GLTTQKSAPWGLGSI) and the *A. flavus* 34-kD (GLTTQKSAP) IgE-binding components were found to be identical to that of the alkaline serine proteinase from *A. oryzae* [34–36]. In addition, the *A. flavus* 34-kD major allergen (Asp fl 13) can be purified from the culture medium by ammonium sulfate precipitation and DEAE-ion exchange chromatography. The purified Asp fl 13 has proteolytic activity with casein as substrate at pH 8.0 and can be inhibited by either phenylmethylsulfonyl fluoride or diethylpyrocarbonate [35]. Our results suggest that these 34-kD major allergens from *A. flavus* and *A. oryzae* are alkaline serine proteinases.

The N-terminal amino acid sequences of the 34-kD (EDPTVEKGA) major allergen, the 30.5 (YASEG)- and 30 (YASEGGEGV)-kD IgE-binding components of *A. fumigatus* have sequence identity to that of the vacuolar serine proteinase (accession No. Y13338) from *A. fumigatus* [manuscript submitted]. The 34-kD major allergen of *A. fumigatus* is probably also a vacuolar serine proteinase. The 30.5- and 30-kD IgE-binding components possibly represent the degraded products of the 34-kD vacuolar serine proteinase allergen of *A. fumigatus*. In addition, immunoblot inhibition studies show IgE cross-reactivity among major allergens of *A. fumigatus*, *P. notatum* and *P. oxalicum* [manuscript submitted].

We have identified a 34-kD IgE-binding component in the culture medium of *A. fumigatus*. IgE antibodies in 10 of the 11 sera that have IgE-binding activity against vacuolar serine proteinase reacted with this 34-kD component [manuscript submitted]. The N-terminus of this protein has a sequence of ALTTQKGAPWGLG that is identical to that of the *A. fumigatus* alkaline serine proteinase [37]. Thus, the alkaline serine proteinase may also be a major allergen of *A. fumigatus*. It should also be noted that the alkaline [37] and the vacuolar (accession No. Y13338) serine proteinases of *A. fumigatus* share 48% sequence identity.

We have also performed immunoblotting for the identification of allergens from another prevalent airborne *As-*

pergillus species, *A. niger* [manuscript submitted]. Among the 55 atopic (patients with bronchial asthma and/or allergic rhinitis) sera tested, 7 showed IgE binding to an *A. niger* protein extract. In particular, these 7 positive serum samples reacted with a 34-kD component of *A. niger*. This 34-kD protein has an N-terminal sequence (EGATEKNAPW?LARI) that is almost identical to that of residues 137–151 (EGATEKNAPWGLARI) of an intracellular serine proteinase (PEPC) from *A. niger* [38]. It has been proposed that PEPC is synthesized as a preproprotein and the mature protein has its N-terminus started close to residue 142 [38]. According to our results, the mature PEPC protein begins at Glu 137. PEPC shares about 75% amino acid sequence identity with the vacuolar serine proteinase of *A. fumigatus* (accession No. Y13338). Therefore, the intracellular (vacuolar) serine proteinase of the subtilisin family might also be a major allergen of a prevalent airborne *Aspergillus* species, *A. niger*.

Conclusions and Perspectives

In conclusion, our immunoblotting data showed that IgE antibodies against components in prevalent airborne *Penicillium* and *Aspergillus* species could be detected in about 15–30% of adult asthmatic serum samples. Different batches of serum samples have been used in the identification of allergens. Most of the positive serum samples showed IgE binding to the 32/34-kD serine proteinase(s) with a frequency >60% in different fungal species tested. In addition, IgE cross-reactivity was observed among major serine proteinase allergens from different fungal species. Moser et al. [39] have not been able to detect the IgE binding to *A. fumigatus* alkaline serine proteinase. However, a 1:100 dilution of 19 allergic bronchopulmonary aspergillosis (ABPA) sera was used in their study. Cramer et al. [6] analyzed a panel of 35 sera from allergic asthmatics with *A. fumigatus* sensitization but without ABPA [6]. The frequencies of IgE binding to recombinant Asp f 1, Asp f 3, Asp f 5, Asp f 7, Asp f 9 and Asp f 10 are 46, 49, 74, 29, 31 and 3%, respectively [6], while specific IgE reactivity against Asp f 4 and Asp f 6 was not discernible in these 35 sera. However, among the 54 ABPA sera studied, 42 (78%) and 30 (56%) showed IgE binding to recombinant Asp f 4 and Asp f 6, respectively. The frequencies of IgE binding to Asp f 1, Asp f 3, Asp f 5, Asp f 7, Asp f 9 and Asp f 10 of the 54 ABPA sera analyzed are 83, 94, 93, 46, 89 and 28%, respectively [6]. These values are much higher than those obtained with sera from patients without ABPA.

The composition of allergenic components may vary in crude fungal extracts from different sources [40]. In *P. no-*

tatum, IgE-binding components of 34 kD and higher can be detected in extracts prepared in our laboratory or from Greer Lab. However, IgE-binding components of less than 34 kD (for example, the vacuolar serine proteinase) were detected only in self-prepared extracts [28]. Thus, the sources of the mould allergens need to be carefully categorized for test reagent preparations.

In the course of these studies, we generated MoAb 55A that reacts with the alkaline serine proteinase in *P. citrinum* [29], *P. brevicompactum* [29] and *A. oryzae* [34]. Another MoAb, PCM39, reacts with both the alkaline and the vac-

uolar serine proteinase allergens [28]. In addition, four more MoAbs against serine proteinase allergens have also been generated [manuscript in preparation]. These MoAbs may provide the basis for the standardization of serine proteinase major allergens in crude fungal extracts.

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