

Alkaline Serine Proteinase Is a Major Allergen of *Aspergillus flavus*, a Prevalent Airborne *Aspergillus* Species in the Taipei Area

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Key Words

Aspergillus flavus · Allergens · Alkaline serine proteinase

Abstract

Background: *Aspergillus* species are prevalent indoor airborne fungi and have been identified to be a causative agent of human allergic disorders. In the present study, we identified, purified and characterized the allergen(s) from *Aspergillus flavus*, a predominant airborne *Aspergillus* species in the Taipei area. **Methods:** The IgE-binding components of *A. flavus* were identified by SDS-PAGE immunoblotting with sera from asthmatic patients. The N-terminal amino acid sequences of the major allergens were determined by Edman degradation. The allergenic cross-reactivity among allergens from different fungi was analyzed by immunoblot inhibition using sera from asthmatic patients. The detected major allergen was purified from the culture medium. It was further characterized in terms of its N-terminal amino acid sequence, its IgE-binding activity and its enzymatic activity. **Results:** The results of the immunoblot analysis indicate that a 34-kD component that has high IgE-binding (63%) frequency is a major allergen of *A. flavus*. The N-terminus of this 34-kD major allergen (GLTTQKSAP) has high sequence identity with

that of the 34-kD alkaline serine proteinase major allergen of *A. oryzae*. Results from immunoblot inhibition studies indicate that IgE cross-reactivity occurs among the 34-kD major allergens of *A. flavus*, *A. fumigatus* and *Penicillium citrinum*. The 34-kD major allergen of *A. flavus* was purified from the culture medium by ammonium sulfate precipitation and DEAE ion exchange chromatography. The N-terminal amino acid sequence of the purified allergen (Asp fl 13) is identical to that determined previously for the 34-kD major allergen in the crude extract of *A. flavus*. The IgE immunoblot reactivity to the 34-kD major allergen in the crude extract can be dose-dependently inhibited by the purified Asp fl 13. The degree of IgE binding to the 34-kD major allergen in the crude extract correlates with that of the purified Asp fl 13 in sera of 8 asthmatic patients. The purified Asp fl 13 has proteolytic activity with casein as substrate at pH 8.0. This enzymatic activity can be inhibited by either phenylmethylsulfonyl fluoride or diethylpyrocarbonate. **Conclusion:** Our results suggest that the 34-kD alkaline serine proteinase is a major allergen of *A. flavus*. There was IgE cross-reactivity among allergens of *A. flavus*, *A. fumigatus* and *P. citrinum*.

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Introduction

It has long been established that respiratory allergic symptoms can be caused by the inhalation of airborne mold spores [1]. Although *Cladosporium* is far more prevalent in the outdoor environment [2], *Penicillium* and *Aspergillus* are the most common indoor fungal species associated with human allergic disorders [1, 3, 4]. The study by Licorish et al. [5] confirmed that *Alternaria* and *Penicillium* spores in relatively natural states and numbers were potent immunopathogens for asthma. *Aspergillus*-mediated allergic disorders include allergic asthma, allergic rhinitis, allergic sinusitis, allergic bronchopulmonary aspergillosis (ABPA), and hypersensitivity pneumonitis [6]. From skin tests with a variety of molds in 1,000 asthmatic children in Taiwan, the positive rates of *A. fumigatus*, *Cladosporium*, *Penicillium*, and *Alternaria* were observed to be 26.7, 22.8, 22.3 and 16.7%, respectively [7]. However, immunodiagnosis of mold-induced allergic diseases is greatly affected by nonavailability of reliable antigen preparations. *Penicillium* and *Aspergillus* belong to two taxonomically closely related genera, and more than 100 different species have been discovered [8, 9]. It is important to understand the allergenic profiles of the prevalent airborne *Penicillium* and *Aspergillus* species and the allergenic cross-reactivities among allergens of these ubiquitous fungi.

We have previously shown that *P. citrinum* is the most prevalent *Penicillium* species in the Taipei area of Taiwan [10]. In addition to *P. citrinum*, *P. chrysogenum* (*P. notatum*), *P. brevicompactum*, *P. oxalicum* and *P. spinulosum* have also been identified as the five most frequently recovered species of *Penicillium* indoors and outdoors in Topeka, Kans., USA [11]. Our results also showed that the 32 (34)-kD components of alkaline and/or vacuolar serine proteinase are major allergens of *P. citrinum*, *P. notatum*, *P. brevicompactum* and *P. oxalicum* [12, 13]. In addition, IgE cross-reactivity between major allergens of these prevalent *Penicillium* species has also been detected [12–14].

For the *Aspergillus* species, we have identified the 34-kD alkaline serine proteinase as a major allergen of *A. oryzae* [15]. IgE cross-reactivity between the major serine proteinase allergens of *A. oryzae* and *P. citrinum* has also been detected [15]. Studies using molecular approaches have identified at least 12 different *A. fumigatus* allergens [16, 17]. Among them, a major 18-kD *A. fumigatus* allergen (Asp f 1) has been correlated as a cytotoxin from the mitogillin family [18]. The complete nucleotide sequence of Asp f 1 has been determined [19], and recombinant Asp f 1 with IgE-binding activity has been produced [20]. However, Northern analysis has confirmed that Asp f 1 mRNA was

detected only in *A. fumigatus* and *A. restrictus*, but not in other *Aspergillus* species such as *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans* [19]. It was suggested that Asp f 1 could be a specific virulence factor for *A. fumigatus* [19].

Recently, results from a survey of atopic children in the Taipei area showed that *A. flavus* and *A. niger* were the predominant airborne fungi in their homes, with an occurrence frequency of >50%. However, the occurrence rates of *A. fumigatus* and *A. clavatus* were only 4% [21]. *A. flavus* and *A. niger* are, therefore, recommended to be included on a skin test panel. Glucoamylase, cellulase and hemicellulase preparations from *A. niger* were shown to contain allergens by skin prick tests and immunoblotting [22, 23]. Recently, β -xylosidase from *A. niger* was shown to be a new allergen in the baking industry [24]. However, little is known about the allergens of *A. flavus*. In order to clarify the allergenic profile of the prevalent airborne *Aspergillus* species, allergens of *A. flavus* were analyzed in the present study. Our results show that the alkaline serine proteinase from *A. flavus* is a major allergen. There is IgE cross-reactivity among allergens of *A. flavus*, *A. fumigatus* and *P. citrinum*.

Materials and Methods

Serum Samples

Two batches of serum samples containing 68 and 41 sera each were collected from adult patients (21–81 and 17–77 years old, respectively) with bronchial asthma attending the allergy clinics at the Veterans General Hospital, Taipei. Asthmatic subjects had a clinical history consistent with intermittent and reversible airway obstruction and were diagnosed according to the statement provided by the American Thoracic Society [25]. After collection, the serum samples were stored in aliquots at -70°C . The presence of IgE antibodies in these serum samples against components of *A. flavus* was analyzed by immunoblotting. The presence of IgE reactivity in the 41 serum samples against components of *Dermatophagoides pteronyssinus* and *A. fumigatus* was determined by the Pharmacia CAP system (RAST FEIA, Pharmacia, Uppsala, Sweden). The results were expressed as RAST class ranging from 0 to 6 according to the manufacturer's suggestions.

Allergenic Extracts

A. flavus strains used in the preparation of allergenic extracts were obtained from the Food Industry Research and Development Institute, Hsinchu, Taiwan. *A. flavus* strain CCRC32242 was originally isolated and identified by Dr. S.S. Tzean at National Taiwan University from stored paddy rice in Changhua, Taiwan. The species identification was reconfirmed by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. *A. flavus* strain CCRC30165 was originally obtained from The American Type Culture Collection (ATCC16883). *P. citrinum* strain 52-5 (CCRC38014) [10] and *A. fumigatus* (CCRC33476) used for cross-reactivity studies were isolated from the air of the Taipei and Tainan areas, respectively. Crude extracts of individual fungal species were prepared essentially as described pre-

viously [12–15]. The protein content of the extracts was determined by the dye-binding assay of Bio-Rad (Richmond, Calif., USA) according to the manufacturer's instructions.

Gel Electrophoresis and Immunoblotting

Immunoblot analysis of *A. flavus* components with human IgE was performed essentially as described previously [12–15]. Briefly, components of the fungal extracts or the purified allergen preparation were electrophoretically separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) or by the two-dimensional (2-D) gel [12, 13] and transferred onto polyvinylidene difluoride (PVDF) membranes (0.45 µm; Millipore, Bedford, Miss., USA). The blots were blocked with 1% skimmed milk and incubated individually with diluted human serum samples and then with alkaline-phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, Calif., USA). The blots were washed and developed in a substrate solution. The deposition of the purple stain indicating antigen-antibody binding was recorded by photography. A cord serum sample was used as a negative control. Low molecular weight standards (Pharmacia) were used to estimate the molecular weight of the protein components. Carbamylated creatine kinase obtained from BDH (Poole, UK) was used as the charge standard for calculating the isoelectric points of the resolved proteins.

Immunoblot Inhibition

Immunoblot inhibition was performed to test the presence of IgE cross-reactivity among allergens of *A. flavus*, *A. fumigatus* and *P. citrinum*. A mixture of IgE-positive serum samples was mixed individually with 200 or 600 µg of *A. flavus*, *A. fumigatus* or *P. citrinum* by end-over-end rotation for 1 h at 4°C. The PVDF blots were then incubated individually with the IgE-positive serum mixture or with serum extract mixtures for 16 h at 4°C. For control, the blots were incubated with the same positive serum mixture which had been mixed with 200 or 600 µg of a *Candida albicans* extract that was prepared as reported previously [26]. The blots were washed and incubated with alkaline-phosphate-conjugated monoclonal anti-human IgE antibodies and developed with the enzyme substrate as described above.

N-Terminal Amino Acid Sequence Analysis

Components of *A. flavus* separated by 2-D PAGE were transferred electrophoretically onto a PVDF membrane. The membrane-bound proteins were visualized by Coomassie blue staining. Protein spots of interest were excised from the membrane and inserted into an ABI Procise Model 491 sequencer (Applied Biosystems, Foster City, Calif., USA) for automated cycles of Edman degradation according to the manufacturer's instructions.

Purification of the 34-kD Major Allergen

The 34-kD major allergen identified was isolated from a 6-day-old culture supernatant of the *A. flavus* strain CCRC30165 grown at 25°C in the same liquid culture medium as described previously [12, 13]. The protein components in the culture medium were precipitated by adding powdered ammonium sulfate to a final concentration of 80% saturation. The precipitated proteins were dissolved in distilled water and dialyzed against 50 mM Tris-HCl, pH 8.7, before loading onto a DEAE SeptraSorb cellulose ion exchange column (DE SeptraSorb; Sepragen, Hayward, Calif., USA) preequilibrated with the same buffer. Fractions with positive immunoblot reactivity to a rabbit anti-serum (prepared by Genemed Biotechnologies, South San Francisco, Calif., USA) against a peptide containing the N-terminal 19 amino

acid residues (GLTTQKSAPWGLGSISHKG) [27] of the 34-kD alkaline serine proteinase major allergen of *A. oryzae* [15, 27] were collected and pooled. The purity of the allergen preparation was analyzed by SDS-PAGE, and the N-terminal amino acid sequence was determined as described above. The IgE-binding reactivity was analyzed by SDS-PAGE immunoblot and immunoblot inhibition using sera from asthmatic patients.

Proteolytic Activity of the Purified 34-kD Major Allergen

The proteolytic reactivity of the purified allergen was analyzed with casein (L-5890; Sigma Chemical Co., St. Louis, Mo., USA) as substrate. The purified allergen preparation and casein (0.5%) were mixed to a final volume of 1.2 ml at different pH in either citrate (50 mM, pH 4–6) or Tris-HCl buffer (50 mM, pH 7–10). Incubation was carried out at 37°C for 30 min. The reaction was stopped by adding 20% trichloroacetic acid, and precipitates were removed by centrifugation. Soluble peptides remaining in the supernatant were measured using the dye-binding assay from Bio-Rad. As a negative control, bovine serum albumin (Pierce, Rockford, Ill., USA) was used instead of the purified allergen preparation in the proteolytic assay.

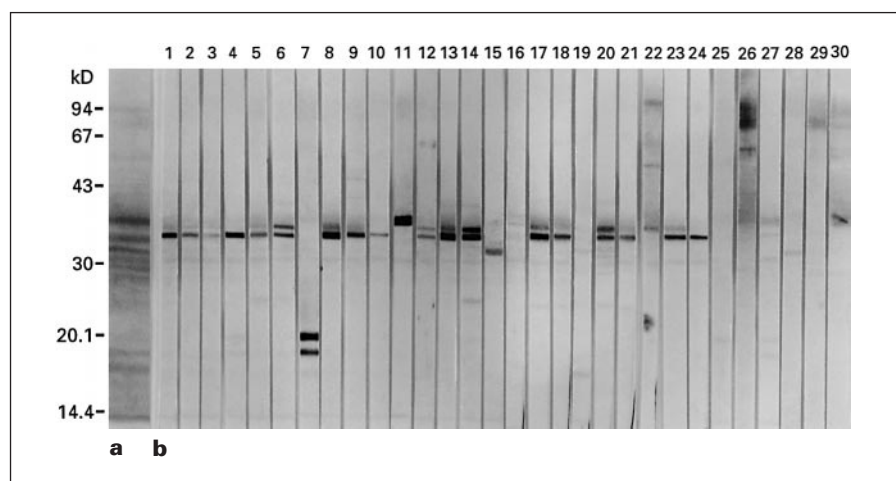
For the inhibition study, the purified allergen preparation was preincubated with different amounts of inhibitors for 20 min at room temperature. The residual proteolytic activity was measured by adding the substrate which was dissolved in a Tris-HCl buffer (50 mM, pH 8.0). A control containing the purified allergen preparation and substrate only was assayed simultaneously. The inhibitors used were phenylmethylsulfonyl fluoride (PMSF, Sigma), diethylpyrocarbonate (DEPC, Sigma) and ethylenediaminetetraacetic acid (EDTA, Merck, Germany).

Results

*IgE-Binding Components of *A. flavus**

Protein components in a crude extract of *A. flavus* (CCRC32242) were separated by SDS-PAGE. The protein profile of the extract on a PVDF blot visualized by Coomassie blue staining was presented in figure 1a. At least 20 different components with an apparent molecular mass ranging from 10 to 100 kD were detected. IgE antibodies in 30 (44%) of the 68 different asthmatic serum samples tested reacted with the protein components of *A. flavus*, and the results are presented in figure 1b. Fourteen IgE-binding components ranging in apparent molecular mass from 17 to >100 kD were observed. A 34-kD component that reacted intensely and most frequently (with the highest binding frequency of 63%, ^{19/30}) may be considered as a major allergen of *A. flavus*. Components with an apparent molecular mass of about 35 kD that reacted with 43% of the 30 asthmatic sera may be considered as an important allergen of *A. flavus*. Other IgE-binding components reacted with IgE antibodies in less than 25% of the 30 sera. Strong IgE binding to the 19- and 20-kD, the 36- and the 32-kD components of *A. flavus* was observed for sera No. 7, 11 and 15, respectively (fig. 1).

Fig. 1. IgE-binding components of *A. flavus* CCRC32242 analyzed by SDS-PAGE immunoblot. The apparent molecular weight standards (Pharmacia) used were phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD) and α -lactalbumin (14.4 kD). **a** Coomassie-blue-stained protein profile of the *A. flavus* extract on PVDF membrane. **b** Strips correspond to immunoblots generated from 30 different serum samples obtained from asthmatic patients.



The allergenic profile of *A. flavus* was also analyzed by preparing an extract from the *A. flavus* strain CCRC30165 and immunoblotted against another batch of 41 serum samples obtained from asthmatic patients (17–77 years old). Fourteen (34%) of the 41 sera showed positive IgE binding (fig. 2). The 34-kD component with an IgE-binding frequency of 64% was recognized as a major allergen (fig. 2). Twenty-five (61%) and 8 (19.5%) of the 41 sera showed positive results in the analysis of IgE reactivity to components of *D. pteronyssinus* and *A. fumigatus*, respectively, as analyzed by the Pharmacia CAP test. Seven of the 8 *A. fumigatus*-positive sera also showed positive results in IgE binding to *A. flavus* as shown in figure 2. Eleven of the 14 sera shown in figure 2 which showed positive IgE immunoblotting also gave a positive IgE response to *D. pteronyssinus* analyzed by the Pharmacia CAP test (data not shown). A cord serum sample was used as negative control in the immunoblot experiments (data not shown). In addition, sera from 5 normal laboratory personnel and from 2 house dust mite-allergic patients showed negative immunoblotting results (data not shown).

2-D Immunoblot Analysis

Protein components in the *A. flavus* extract (CCRC30165) were further analyzed by 2-D PAGE. At least 100 different spots were visualized in the Coomassie-blue-stained PVDF blot as shown in figure 3a. Components with an apparent molecular mass of about 34 and 24 kD and pI values ranging from 6.8 to >7.0 and 6.5, respectively, reacted with IgE antibodies (fig. 3b) in a mixture of serum samples (sera No. 1, 2, 3 and 5 in fig. 2). Enzyme color reactivity was not detected when a control cord serum sample was used (data not shown).

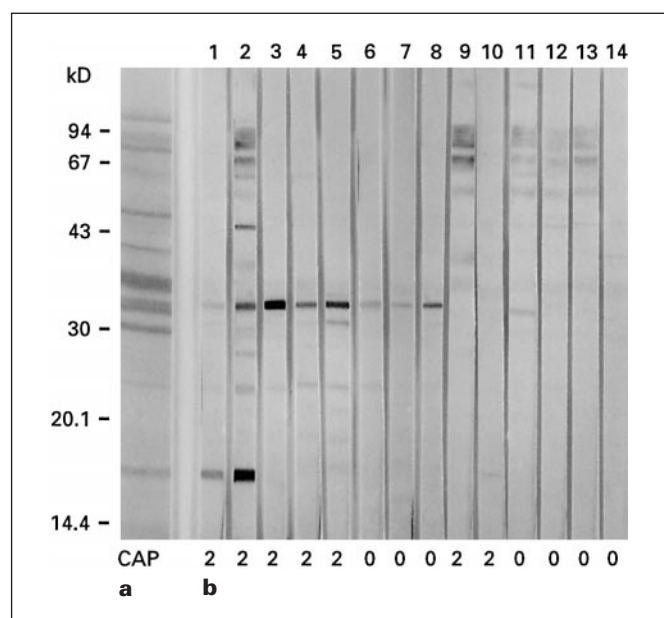
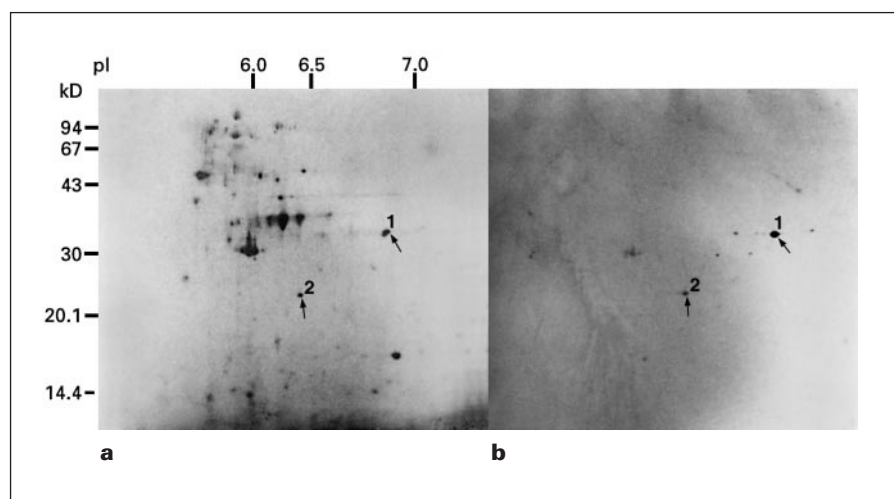


Fig. 2. IgE-binding components of *A. flavus* CCRC30165 analyzed by SDS-PAGE immunoblot. The apparent molecular weight standards used were the same as those described in figure 1. **a** Coomassie-blue-stained protein profile of the *A. flavus* extract on PVDF membrane. **b** Strips correspond to immunoblots generated from 14 asthmatic serum samples. The results of the commercial Pharmacia CAP test of the reactivity to *A. fumigatus* of individual serum samples tested are expressed as RAST classes.

N-Terminal Amino Acid Sequence Analysis

Spots 1 (AFLA1) and 2 (AFLA2) as indicated in figure 3a showed relatively strong IgE immunoblot reactivity (fig. 3b). They were selected for N-terminal amino acid sequence analysis. AFLA1 and AFLA2 have been sequenced

Fig. 3. 2-D immunoblot analysis of components in the crude extract of *A. flavus* CCRC30165 reacted with human IgE antibodies. The same apparent molecular weight standards as shown in figure 1 were used. Carbamylated creatine kinase obtained from BDH (Poole) was used as charge standards for calculating the isoelectric point of the resolved proteins. **a** Coomassie-blue-stained 2-D protein profile of the crude extract from *A. flavus* on a PVDF blot. Arrows (AFLA1 and AFLA2) indicate protein spots corresponding to those that showed enzymatic color reaction in immunoblotting as in **b**. **b** Immunoblot reactivity of IgE antibodies in a mixture of serum samples (serum no. 1, 2, 3 and 5 in figure 2) to components in the crude *Aspergillus* extract.



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AFLA1  GLTTQKSAP*GL
AFLA2  GLTTQKSAP*GL*I
AO      GLTTQKSAPWGLGSI
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Fig. 4. N-terminal amino acid sequences of AFLA1, AFLA2 and AO. AFLA1 and AFLA2 were isolated by 2-D gel electrophoresis as indicated in figure 3a and subjected to Edman degradation. Cycles from the protein sequencer with signals that are too low for positive amino acid identification are denoted with an asterisk. AO represents the sequence of alkaline serine proteinase from *A. oryzae* as determined by Tatsumi et al. [27].

to the twelfth and the fifteenth residue, respectively (fig. 4). Although residue 10 of AFLA1 and AFLA2 and residues 13 and 14 of AFLA2 have not been identified, the other amino acid residues determined are identical to the N-terminal sequence of the alkaline serine proteinase from *A. oryzae* (fig. 4) [27].

IgE Cross-Reactivity

The presence of IgE cross-reactivity among allergens of *A. flavus*, *A. fumigatus* and *P. citrinum* was analyzed by immunoblot inhibition. Strips 1 in figure 5a–c show the Coomassie-blue-stained protein profiles of the electrophoretically separated extracts of *A. flavus* (CCRC30165), *A. fumigatus* and *P. citrinum* on PVDF blots, respectively. Strips 2 in these three panels show the IgE immunoblot reactivity to components of *A. flavus*, *A. fumigatus* and *P. citrinum*, respectively, using a mixture of

serum samples (sera No. 1, 2, 3 and 5 in fig. 2). Inhibition of the IgE immunoblot reactivity to different fungal species was detected when 200 µg (strips 3, 5 and 7) and 600 µg (strips 4, 6 and 8) of extracts from *A. flavus* (strips 3 and 4), *A. fumigatus* (strips 5 and 6) and from *P. citrinum* (strips 7 and 8), respectively, were used as inhibitors. The 34-kD band in *A. fumigatus* inhibited was identified to be a vacuolar serine proteinase (manuscript submitted) and reacted with MoAb PCM39 [13] against serine proteinase allergens. The 33-kD band in *P. citrinum* inhibited was identified to be an alkaline serine proteinase [12] and reacted with PCM39 (data not shown). For all the immunoblots in figure 5b (strips 2–10), a band staining slightly below the 34-kD position (in line with the major protein band) was observed. It was considered a nonspecific binding in immunoblotting. A similar weak binding band was also detectable when a negative control cord serum sample was used (data not shown). Inhibition of IgE immunoblotting was not detected when 200 (strip 9) and 600 µg (strip 10), respectively, of an extract from *Candida albicans* was used as inhibitor (fig. 5).

Purification of the 34-kD Major Allergen

The 34-kD major allergen of *A. flavus* (CCRC30165) was purified from the culture medium by ammonium sulfate precipitation and DEAE ion exchange chromatography. The N-terminal amino acid sequence of the purified allergen was determined to be GLTTQKSAP*GL which was found to be identical to that of the 34-kD major allergen in the crude extract of *A. flavus* as shown in figure 4. The purified allergen preparation showed dose-dependent (0.2, 1.0 and 5.0 µg) inhibition of the IgE immunoblot reactivity to

Fig. 5. Inhibition of IgE immunoblot reactivity of a mixture of serum samples (serum no. 1, 2, 3 and 5 in figure 2) that has shown reactivity to components of *A. flavus* (**a**), *A. fumigatus* (**b**) and *P. citrinum* (**c**). Strips 1 in all three panels show the Coomassie-blue-stained protein profile of an individual fungal extract on PVDF membranes. Strips 2 in all three panels show the IgE immunoblot reactivity of the serum mixture against individual allergenic extracts tested. Strips 3–10 represent immunoblot results obtained by incubating the PVDF blots with the same serum sample that has been mixed and preincubated with 200 μ g (3, 5, 7, 9) and 600 μ g (4, 6, 8, 10) of *A. flavus* (3 and 4), *A. fumigatus* (5 and 6), *P. citrinum* (7 and 8), and *Candida albicans* (9 and 10) respectively.

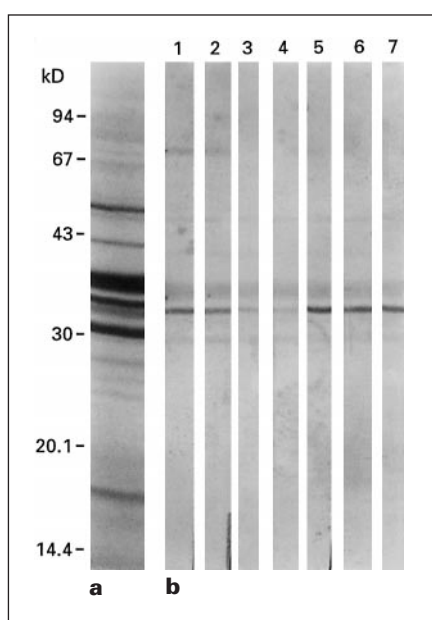
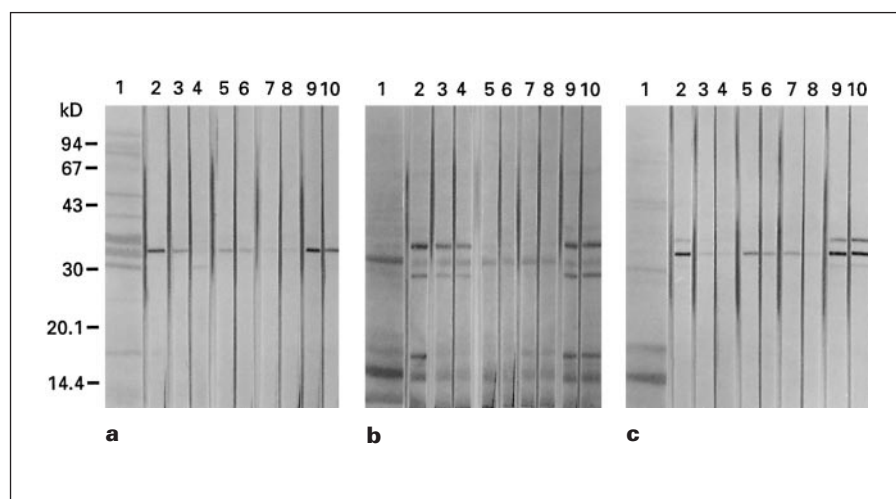


Fig. 6. Dose-dependent inhibition of IgE SDS-PAGE immunoblot reactivity (strip b1) to component(s) in the crude extract of *A. flavus* CCRC30165 (**a**) by 0.2 (strip b2), 1.0 (strip b3) and 5.0 μ g (strip b4) of the purified 34-kD major allergen of *A. flavus*, respectively. For control, 0.2 (strip b5), 1.0 (strip b6) and 5.0 μ g (strip b7) of bovine serum albumin were used as inhibitors, respectively.

the 34-kD allergen in the crude extract of *A. flavus* strain CCRC30165 (fig. 6, strips 2–4). A dose-dependent inhibition was not detected when the same amounts of bovine serum albumin were used as inhibitors (fig. 6, strips 5–7). The IgE-binding reactivity of the purified allergen was analyzed with the 14 positive sera shown in figure 2. Nine of

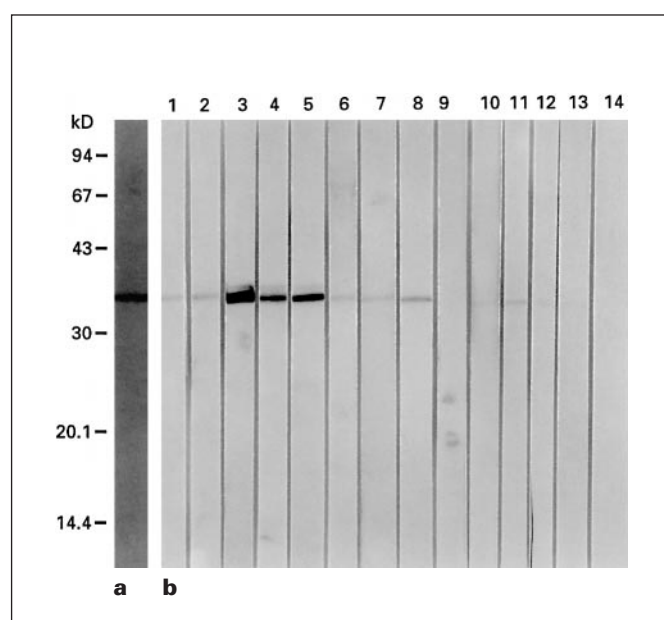


Fig. 7. IgE-binding reactivity of the purified 34-kD major allergen of *A. flavus* analyzed by SDS-PAGE immunoblot. The same apparent molecular weight standards as in figure 1 were used. **a** Coomassie-blue-stained protein profile of the purified allergen preparation on PVDF membrane. **b** Strips correspond to immunoblots generated from the same 14 asthmatic serum samples as in figure 2.

the 14 sera (sera No. 1–8 and 11) which showed positive IgE binding to the 34-kD allergen in the crude extract of *A. flavus* also reacted with the purified 34-kD allergen (fig. 7). A close correlation can be observed for the intensities of IgE-binding to the purified allergen and the 34-kD allergen in the crude extract with the asthmatic sera tested (fig. 2, 7).

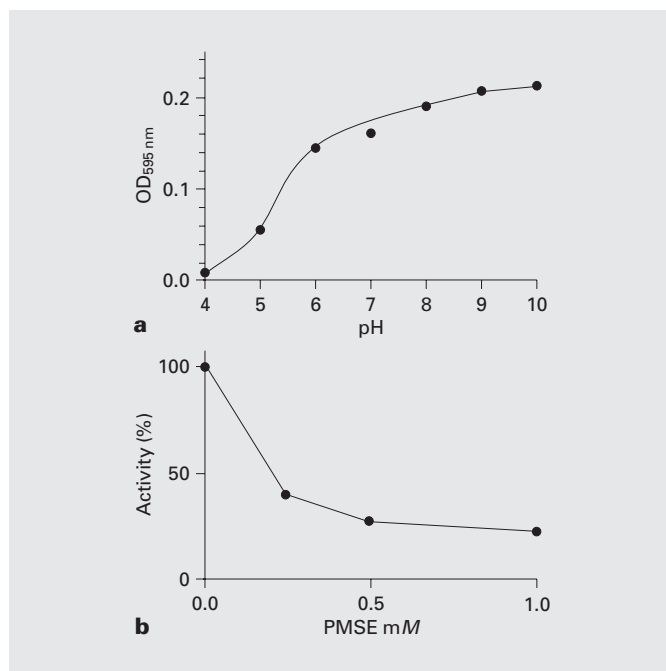


Fig. 8. **a** Proteolytic reactivity of the purified 34-kD major allergen of *A. flavus* to casein (0.5%) under different pH conditions. **b** Inhibition of the proteolytic reactivity of the purified 34-kD major allergen of *A. flavus* to casein (0.5%) at pH 8.0 by different concentrations of PMSF.

Proteolytic Activity of the Purified 34-kD Major Allergen

The purified allergen showed the highest proteolytic activity with casein (0.5%) as substrate in neutral to alkaline pH (fig. 8a). The protease reactivity was significantly inhibited in a dose-dependent manner (fig. 8b) by PMSF, a classical serine protease inhibitor. Serine proteases have a catalytic triad involving serine, histidine, and a carboxyl group. To further test for the involvement of such a catalytic triad, the effect of a histidine-modifying agent, DEPC, on proteolytic activity was also examined. Our results show that the proteolytic activity of the purified allergen can be completely abolished by treating the enzyme with 0.25 mM DEPC (data not shown). In addition, little to no inhibitory reactivity was observed when EDTA (0.5 and 1.0 mM), an inhibitor of the metalloproteinase, was used in the reaction (data not shown).

Discussion

The results obtained from the present study show that the 34-kD component is a major allergen from two different strains of a prevalent airborne *Aspergillus* species, *A. flavus*.

The N-terminal amino acid sequence of the 34-kD component was found to be identical to that of the 34-kD alkaline serine proteinase major allergen of *A. oryzae*, a typical subtilisin-type serine proteinase [27]. The 24-kD component in figure 3a shares the same N-terminal amino acid sequence as that of the 34-kD component and probably represents a C-terminally-truncated product of the 34-kD major allergen. A similar finding has also been observed for *A. oryzae* [15]. Components AO1, AO2 and AO3 have similar N-terminal amino acid sequences. This suggests that AO2 and AO3 with apparent molecular weights of about 24 and 18 kD, respectively, are possibly degraded products of AO1, a 34-kD major alkaline serine proteinase allergen of *A. oryzae* [15].

The 34-kD IgE-binding component is present in the crude extract of *A. flavus*, but can also be detected in the culture medium (data not shown). It was purified from the culture medium through ammonium sulfate precipitation and DEAE ion exchange chromatography. The purified 34-kD component showed IgE-binding activity and has the same N-terminal amino acid sequence as that for the major allergen in the crude extract. It also dose-dependently inhibited the IgE-binding activity of an asthmatic serum pool against the 34-kD major allergen in the fungal extract. The proteolytic activity of the purified protein is optimal at neutral to alkaline pH and can be inhibited by PMSF and DEPC but not EDTA. Therefore, it belongs to the family of alkaline serine proteinases that have already been identified as a major allergen for *P. citrinum* [12], *P. brevicompactum* [12], *P. notatum* [13], and *A. oryzae* [15].

The significance of alkaline serine proteinase being a major allergen of prevalent *Aspergillus* and *Penicillium* species has not been established. However, progress made in allergen characterization has shown that the majority of clinically important allergens have biochemical functions. Most allergens are enzymes or regulatory proteins [28]. Serine proteinases such as trypsin and chymotrypsin have been identified as the group 3 and the group 6 major allergens of the house dust mite *D. pteronyssinus* [29–31]. Bacillus serine proteinase (subtilisins) have previously been identified as occupational allergens in the detergent industry [32, 33]. In addition, extracellular proteases have been considered to play a role in the pathogenesis of aspergillosis [34–37]. The elastolytic activity of an *A. fumigatus* serine protease may enhance invasion of tissues [35]. A chymotrypsin-like serine proteinase from *A. fumigatus* can induce human epithelial cell detachment [36]. Robinson et al. [36] suggest that the proteinase might trigger the allergic response by detaching areas of the epithelium, causing an increase in penetration of *A. fumigatus* antigens into the

submucosa. Alternatively, the proteinases might act simultaneously as antigens. In addition, recent work suggests that some allergens, such as phospholipase A₂ from bee venom [38, 39] and Der p 1 from dust mite [39–42], have the intrinsic ability to subvert the regulatory process controlling IgE synthesis, thereby favoring an allergic outcome [43]. It was found that Der p 1 is a potent inducer of IgE synthesis probably by exerting its proteolytic effect on CD23 and CD25 [40–43]. The correlation of the allergenicity and the proteolytic activity of alkaline serine proteinase needs further studies.

Recognition of allergic cross-reactions is of major importance for the management of allergic patients [44]. Our results show that IgE cross-reactivity exists among the 32- to 34-kD major alkaline/vacuolar serine proteinase allergens of four prevalent *Penicillium* species (*P. citrinum*, *P. notatum*, *P. brevicompactum* and *P. oxalicum*) [13, 14]. The results obtained also show the presence of IgE cross-reactivity between alkaline serine proteinase allergens of *Aspergillus* (*A. oryzae*) [15], *A. flavus*) and *Penicillium* species (*P. citrinum*). In this study, IgE cross-reactivity between allergens of two different *Aspergillus* species (*A. flavus* and *A. fumigatus*) was also detected. Results from our preliminary study show that the 34-kD vacuolar serine proteinase is a major allergen of *A. fumigatus* [manuscript submitted]. The amino acid sequence identity was found to be about 48% between the alkaline [45] and the vacuolar (accession No. Y13338) serine proteinases *A. fumigatus*. The first 12 N-terminal amino acid residues of the alkaline serine proteinase in *A. fumigatus* are ALTTQKGAPWGL [45] and were found to be very similar to those of the alkaline serine proteinase in *A. flavus* and *A. oryzae* as shown in figure 4. An amino acid sequence identity of 84% was also found between the alkaline serine proteinases from *A. fumi-*

gatus [45] and from *A. oryzae* [27]. It was reported that the secreted alkaline protease of *A. fumigatus* did not elicit IgE antibodies in 19 patients with ABPA [46]. A 1:100 dilution of human sera was used in the detection of protease-specific IgE [46]. However, it is worth noting that there may be a difference between *Aspergillus* allergens recognized by patients with asthma and the allergens from *A. fumigatus* recognized by patients with other types of aspergillosis such as ABPA. The immunoblot pattern in patients with ABPA aspergillosis in different stages shows that a 32/34-kD band of *A. fumigatus* was recognized by several sera but was not dominant [47]. In this study, 7 of the 8 asthmatic sera with positive CAP IgE antibody response to *A. fumigatus* demonstrated IgE binding to components of *A. flavus* (fig. 2). This finding also suggests the presence of IgE cross-reactivity between allergens of *A. fumigatus* and *A. flavus*. Studies on the cross-reactive IgE epitopes of the major serine proteinase allergens of prevalent *Penicillium* and *Aspergillus* species are under way.

Fungal extracts contain a mixture of allergenic and non-allergenic components and are difficult to standardize. In addition to the characterization and purification of the allergens of *A. flavus*, monoclonal antibodies PCM39 and FUM20 that reacted with different epitopes of the 34-kD major alkaline serine proteinase allergen have also been generated [manuscript in preparation]. The results obtained should be useful in clinical fungal allergy analyses.

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