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Alkaline Serine Proteinase: A Major Allergen of *Aspergillus oryzae* and Its Cross-Reactivity with *Penicillium citrinum*

Abstract

Background: *Aspergillus* species are common indoor airborne fungi and have been considered as causative agents of human allergic disorders. However, allergens of different *Aspergillus* species have not been effectively characterized. The object of this study was to identify and characterize IgE-binding components of *Aspergillus oryzae*. **Methods.** Allergens of *A. oryzae* were identified by immunoblot analysis using sera from asthmatic patients. The N-terminal amino acid sequences of allergens thus identified were determined by Edman degradation. The antigenic and the allergenic cross-reactivities between allergens of different fungi were analyzed by immunoblotting and immunoblot inhibition analysis, respectively, using a monoclonal antibody (MoAb) 55A against the 33-kD major allergen of *Penicillium citrinum* and a mixture of IgE-containing asthmatic serum samples. **Results:** Thirteen components of *A. oryzae* ranging in apparent molecular weight from 16 to 42 kD react with IgE antibodies. A 34-kD component that showed intense IgE-binding reactivity and was detectable in the highest frequency in our asthmatic serum samples tested was considered a major allergen of *A. oryzae*. The 34-kD component also reacted with MoAb 55A. Results from immunoblot inhibition studies also demonstrated the IgE cross-reactivity between the 34-kD major allergens of *A. oryzae* and *P. citrinum*. In addition, the sequence of the N-terminal 18 amino acid residues of the 34-kD major allergen of *A. oryzae* was found to be identical to that of the alkaline serine proteinase from the same *Aspergillus* species. **Conclusion:** The 34-kD major allergen of *A. oryzae* is an alkaline serine proteinase. There is IgE cross-reactivity between the major serine proteinase allergens of *A. oryzae* and *P. citrinum*.

Key Words

Aspergillus oryzae

Penicillium citrinum

Allergens

Alkaline serine proteinase

Immunoblot

Cross-reactivity

Introduction

Aspergillus and *Penicillium* species are common indoor airborne fungi and have been identified to be associated with human allergic disorders [1, 2]. In studies of *Penicillium* allergens, allergenic cross-reactivity among the 33-kD

major allergens of *P. citrinum*, *Penicillium notatum* and *Penicillium brevicompactum* has been reported [3]. Results from N-terminal amino acid sequence analysis suggest that the 33-kD major allergen of *P. citrinum*, the most prevalent airborne *Penicillium* species in the Taipei area [4] is an alkaline serine proteinase [5]. In addition, monoclonal antibody

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(MoAb) 55A against the 33-kD serine proteinase major allergen of *P. citrinum* cross-reacts with the 33-kD major allergen of *P. brevicompactum* but not with that of *P. notatum* [5].

In studies of *Aspergillus* allergens, it was found that the 65-kD IgE-binding protein of *Aspergillus fumigatus* shows homology with heat shock protein 90 [6]. It was also found that Asp f 1, a major 18-kD *A. fumigatus* allergen, is a member of the mitogillin family of cytotoxins [7]. Northern analysis confirmed the presence of Asp f 1 mRNA in *A. fumigatus* and *Aspergillus restrictus*, but not in other species such as *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus nidulans* [8]. In a recent review [9] on the biochemistry of common aeroallergens, enzymes such as pectinase I, cellulase, flaviastase and glucoamylase were considered as allergens of *A. niger*, while α -amylase and lactase were recognized as allergens of *A. oryzae*. The enzyme alkaline serine proteinase was identified as a major allergen of *P. citrinum* [5]. The same enzyme from *Bacillus* species was also identified as an occupational allergen in detergent industry [10, 11].

To explore the possibility whether the serine proteinase enzyme may also be a significant allergen of *Aspergillus* species, we studied the IgE-binding components of *A. oryzae* by immunoblotting and N-terminal amino acid sequence analysis. The presence of antigenic and allergenic cross-reactivities between allergens of *A. oryzae* and *P. citrinum* was analyzed by immunoblot using MoAb 55A and by immunoblot inhibition using IgE-containing sera, respectively. Results from these examinations show that the enzyme alkaline serine proteinase is a major allergen of *A. oryzae*. The antigenic and the allergenic cross-reactivities between the major serine proteinase allergens of *A. oryzae* and *P. citrinum* were also observed. Information obtained should be useful in clinical allergy.

Materials and Methods

Serum Samples

Seventy serum samples from patients with bronchial asthma attending the allergy clinics at the Veterans General Hospital and the Cathay General Hospital, Taipei, were collected. 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. oryzae* was analyzed by immunoblotting as described previously [3].

Allergen Extracts

A. oryzae used in the preparation of allergenic extracts was obtained from the Food Industry Research & Development Institute, Hsinchu, Taiwan (CCRC 30196). *P. citrinum* strain 52-5 isolated from the air of Taipei area [4] was used for cross-reactivity studies. Fungal matts of *A. oryzae* and *P. citrinum* containing both spores and mycelia

were collected individually from liquid medium containing Czapek concentrate [12] (10 ml/l), K_2HPO_4 (1.0 g/l), yeast extract (Difco, Detroit, Mich., USA; 5 g/l) and sucrose (30 g/l) after growing at 25°C for 6 days. The allergenic extracts of both fungal species were prepared by extracting the fungal matts (1:5, w/v) with phosphate-buffered saline (containing 85 mmol/l of NaCl, 2.6 mmol/l of KH_2PO_4 and 49 mmol/l of Na_2HPO_4 , pH 8.0) at 4°C for 4 h with constant shaking. After centrifugation, the supernatant was dialysed against 50 mmol/l of ammonium bicarbonate at 4°C for 48 h. After sterile filtration (0.2 μm ; Sartorius, Hayward, Calif., USA), the extract was lyophilized in aliquots and stored at 4°C . The protein content of the extracts was determined by using the dye binding assay of Bio-Rad (Richmond, Calif., USA) according to the manufacturer's instructions.

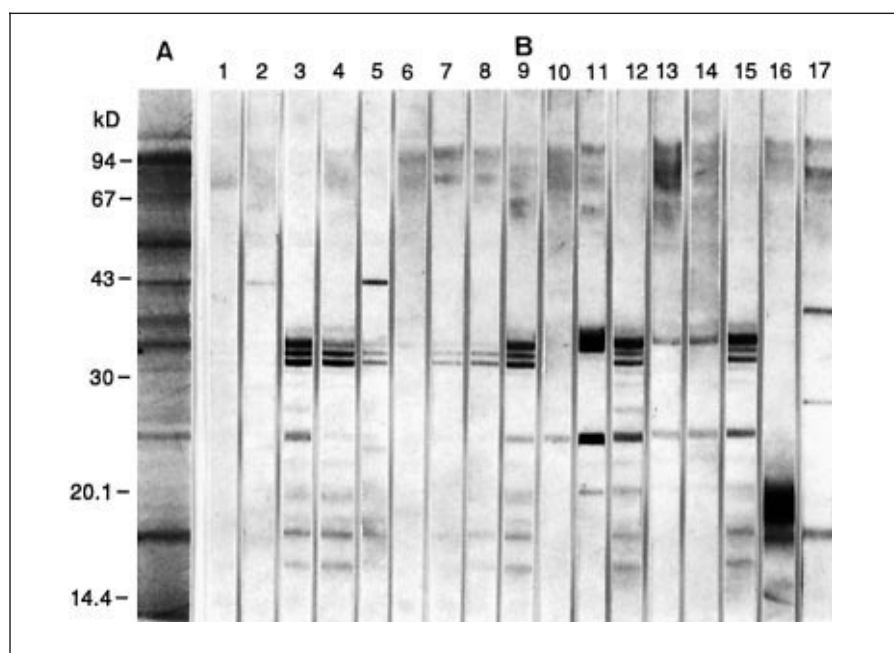
Gel Electrophoresis and Immunoblotting

Immunoblot analysis of *A. oryzae* components with human IgE and mouse MoAb was performed essentially as described [3, 13]. Briefly, components of the fungal extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or by two-dimensional (2D) gel electrophoresis [13] and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 μm ; Millipore, Bedford, Mass., USA). After blocking with 1% skimmed milk, the blots were incubated individually with diluted human serum samples (1:5 dilution in Tris-buffered saline, pH 7.5 containing 0.05% Tween 20 and 1% skimmed milk) at 4°C for 16 h or with a culture supernatant solution of hybridoma cells containing MoAb 55A at room temperature for 1 h. After washing, the blots were incubated with alkaline-phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, Calif., USA) or with affinity-purified, horseradish-peroxidase-labeled goat antimouse IgG (H+L) (Bio-Rad) at room temperature for 1 h. The blots were washed and developed in a substrate solution of carbonate buffer (0.1 M NaHCO_3 , 1.0 mM MgCl_2 , pH 9.8) containing 0.35 mM BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and 0.37 mM NBT (p-nitro blue tetrazolium chloride) for about 30 min or in a substrate solution of acetate buffer (50 mM, pH 5.0) containing 2 mM AEC (3-amino-9-ethyl-carbazole, Sigma) and 0.01% (v/v) hydrogen peroxide for about 5 min. The blots were then washed with water and the deposition of the purple or red stain indicating antigen-antibody binding was recorded by photography. The cord serum and the culture supernatant solution of hybridoma cells containing MoAb WH9 against the house dust mite allergen Der p 7 [13] were used as negative controls. Low-molecular-weight standards (Pharmacia, Uppsala, Sweden) 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 calculation of isoelectric points of the resolved proteins.

Immunoblot Inhibition

Immunoblot inhibition was performed to test the presence of IgE cross-reactivity between allergens of *A. oryzae* and *P. citrinum*. A mixture of 6 IgE-positive serum samples was mixed individually with 100 or 300 μg of *A. oryzae* or of *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 has been mixed with 100 or 300 μg of an extract of *Candida albicans* prepared as reported previously [14]. The blots were washed and incubated with alkaline-phosphate-conjugated monoclonal anti-human IgE antibodies and developed with the enzyme substrate as described above.

Fig. 1. IgE-binding components of *A. oryzae* analysed by SDS-PAGE immunoblotting. **A** Coomassie-blue-stained protein profile of an extract of *A. oryzae* on PVDF blot. **B** Strips 1–17 show the positive immunoblotting results of the *A. oryzae* extract using 17 different serum samples from asthmatic patients.



N-Terminal Amino Acid Sequence Analysis

Components of *A. oryzae* separated by 2D gel electrophoresis 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 Inc., Foster City, Calif., USA) for automated cycles of Edman degradation according to the manufacturer's instructions.

Results

IgE-Binding Components of A. oryzae

Protein components in a crude extract of *A. oryzae* 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 ranging in apparent molecular weight from about 10 to 100 kD were detected. IgE antibodies in 17 (24.3%) of the 70 different asthmatic serum samples tested reacted with the protein components of *A. oryzae* and the results were presented in figure 1B. Thirteen IgE-binding components ranging in apparent molecular weight from 16 to 42 kD were observed. A 34-kD component that reacted intensely and most frequently (with the highest binding frequency of 71%, table 1) may be considered as a major allergen of *A. oryzae*. Components with apparent molecular weights of about 33, 32 and 24 kD that reacted with 53% of the 17 asthmatic sera (table 1) may also be considered as important al-

lergens of *A. oryzae*. The 20-, 18- and 16-kD components reacted with IgE antibodies in 35–41% of the 17 serum samples (table 1). Other IgE-binding components reacted with IgE antibodies in less than 15% of the 17 sera. As a control, the cord serum was used with negative findings (data not shown).

IgE Cross-Reactivity

The presence of IgE cross-reactivity between allergens of *A. oryzae* and *P. citrinum* was analyzed by immunoblot inhibition. Figure 2 A α , B α show the Coomassie blue-stained protein profiles of electrophoretically separated extracts of *A. oryzae* and *P. citrinum* on PVDF blots, respectively. Strips a in figure 2A, B show the IgE immunoblot reactivity to components of *A. oryzae* and *P. citrinum*, respectively, using a mixture of positive serum samples (serum No. 3, 4, 9, 11, 12 and 15 in fig. 1). Inhibition of the IgE immunoblot reactivity was observed when 100 μ g (strips b and d) and 300 μ g (strips c and e) of extracts from *A. oryzae* (strips b and c) and *P. citrinum* (strips d and e), respectively, were used as inhibitors. Inhibition of IgE immunoblotting was not detected when 100 μ g (strips f) and 300 μ g (strips g) of extracts from *Candida albicans* were used (fig. 2).

2D Immunoblot Analysis

Protein components in the *A. oryzae* extract were further analyzed by 2D gel electrophoresis. At least 150 different spots were visualized in the silver-stained 2D electrophore-

Fig. 2. Competitive inhibition of IgE immunoblot reactivity to components of *A. oryzae* (**A**) and *P. citrinum* (**B**). Strips α of both panels show the Coomassie-blue-stained protein profiles of individual fungal species on PVDF blots. Strips a of both panels demonstrate the IgE immunoblot reactivity to components of *A. oryzae* (**A**) and *P. citrinum* (**B**), respectively using a mixture of positive serum samples (serum No. 3, 4, 9, 11, 12 and 15 in fig. 1). Inhibition of the IgE immunoblot reactivity was observed when 100 μ g (strips b and d) and 300 μ g (strips c and e) of extracts from *A. oryzae* (strips b and c) and *P. citrinum* (strips d and e), respectively, were preincubated with the positive serum mixture for 1 h at 4°C before immunoblot analysis. Inhibition of the IgE immunoblot reactivity was not observed when 100 μ g (strips f) and 300 μ g (strips g) of a *C. albicans* extract (strips f and g) were used as inhibitors.

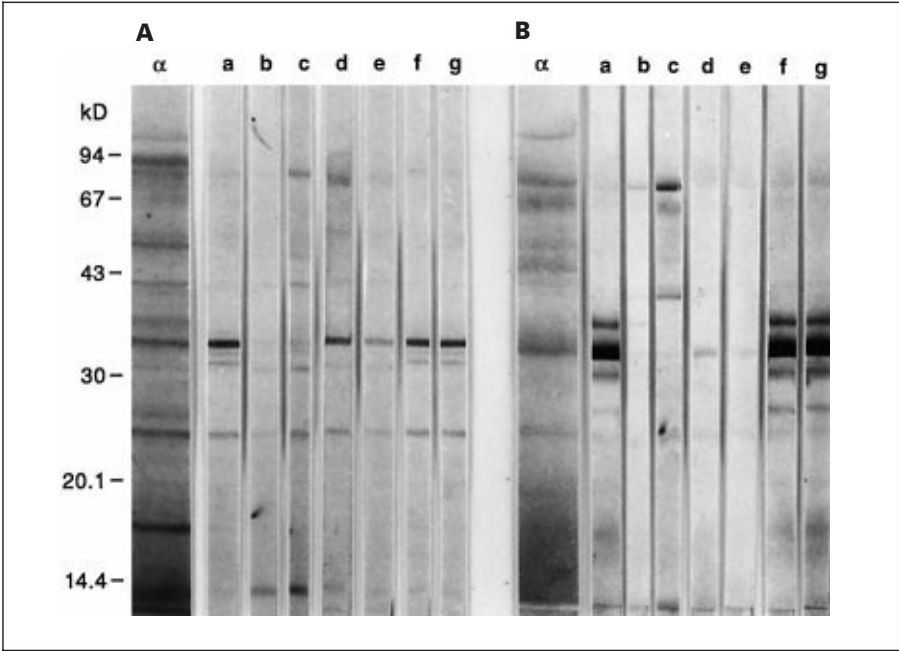
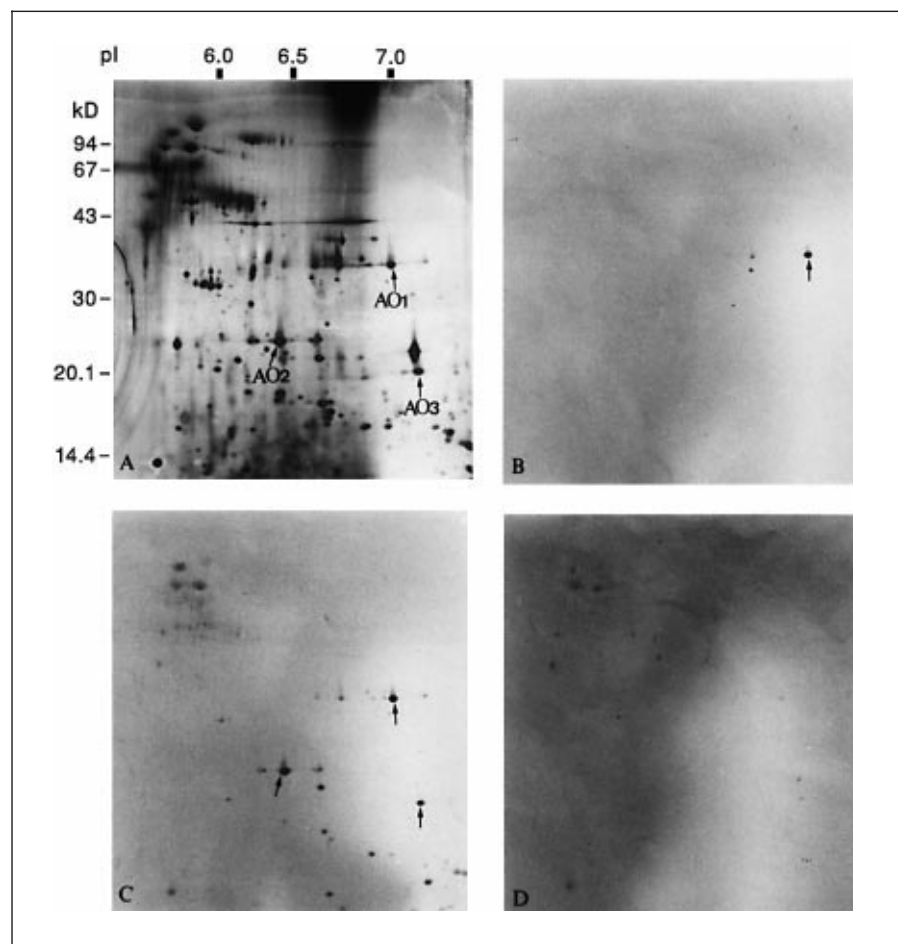


Table 1. Components of *A. oryzae* recognized by IgE antibodies in sera of 17 asthmatic patients analyzed by immunoblotting

Serum No.	MW ¹ IgE-binding ²	16 35	17.5 12	18 41	19 6	20 35	24 53	27 12	28 6	32 53	33 53	34 71	37 6	42 12
1										+	+			
2														+
3		+		+		+	+	+		+	+	+		
4		+		+		+	+			+	+	+		
5				+						+	+	+		+
6												+		
7										+	+	+		
8		+		+						+	+	+		
9		+		+		+	+			+	+	+		
10							+							
11						+	+					+		
12		+		+		+	+	+		+	+	+		
13							+					+		
14							+					+		
15		+		+		+	+			+	+	+		
16			+		+									
17			+						+				+	

¹ Apparent molecular weight (kD).
² Frequency of IgE-binding (%), which was calculated by dividing the individual number of positive serum samples by the total positive number of 17.

Fig. 3. 2D immunoblot analysis of reactivity of IgE and monoclonal antibodies against components of *A. oryzae*. **A** Shows the silver-stained two-dimensional protein profile of an *A. oryzae* extract. **B, C** show the reactivity of MoAb 55A and IgE antibodies in a mixture of serum samples (serum No. 3, 4, 9, 11, 12 and 15 in fig. 1), respectively, against components of *A. oryzae*. **D** shows the result of a negative control for IgE immunoblotting by using a cord serum sample. The arrow in **B** indicates the immunoblotting reactivity of MoAb 55A to the component corresponding to protein spot AO1 in **A**. Arrows in **C** indicate the reactivity of IgE antibodies to components which correspond to protein spots AO1, AO2 and AO3, respectively as shown in **A**.



togram (fig. 3A). Results in figure 3B show that components with an apparent molecular weight of about 34 kD and pI values of 6.6, 6.7, 7.0 and greater than 7.0 reacted with MoAb 55A. The same components were also reactive against IgE antibodies in the mixture of serum samples as shown in figure 3C. In addition, IgE-binding to components with apparent molecular weight of about 24, 20, 18 kD and less than 18 kD was also observed (fig. 3C). Figure 3D shows the negative control for IgE-immunoblotting by using a cord serum sample against the *A. oryzae* extract. Enzyme color reactivity was not detected when a control MoAb, WH9, was used in the 2D immunoblotting (data not shown).

N-Terminal Amino Acid Sequence Analysis

Components AO1, AO2 and AO3 in figure 3A that showed relatively strong IgE immunoblot reactivity (fig. 3C) were selected for N-terminal amino acid sequence analysis. The results are summarized in figure 4. AO1, AO2 and AO3 have been sequenced to the 18, 23 and 21 residues,

AO1	GLTTQKSAPWGLGSI*HK
AO2	GLTTQKSAPWGLGSISHKGQQ*T
AO3	GLTTQKSAPWGLGSISHK(K/G)QQ
Alp	GLTTQKSAPWGLGSISHKGQQST

Fig. 4. The N-terminal amino acid sequence of AO1, AO2, AO3 and Alp. AO1, AO2 and AO3 were isolated by 2D gel electrophoresis as indicated in figure 3A and subjected to Edman degradation. Cycles from protein sequencer with signals that are too low for positive amino acid identification are denoted with *. Alp represents the sequence of alkaline serine proteinase from *A. oryzae* as determined by Tatsumi et al. [15].

respectively, and they are identical to that of the alkaline serine proteinase of *A. oryzae* (fig. 4 [15]). We detected both lysine and glycine for residue 19 of AO3. Whether they represent a polymorphism in the allele remains to be investigated.

Discussion

In the present study, the 34-kD component that showed the highest frequency of IgE-binding (71%) was identified as a major allergen of *A. oryzae*. Results from N-terminal sequencing and protein data bank search suggest that the 34-kD major allergen is an alkaline serine proteinase that reported previously by Tatsumi et al. [15]. The conclusion was further affirmed by the positive immunoblot reactivity of the 34-kD major allergen with MoAb 55A, a MoAb that was raised against the 33-kD major serine proteinase allergen of *P. citrinum* [5].

From a taxonomical point of view, the genus *Aspergillus* is closely related to the genus *Penicillium* [16]. Cross-reactivity between *Aspergillus* and *Penicillium* species has been reported. For instance, anti-*Penicillium* IgG can react with several *A. fumigatus* antigens with molecular mass between 28 and 130 kD [17]. In addition, IgE antibodies in sera from patients with allergic bronchopulmonary aspergillosis can cross-react with *Penicillium* allergens [17]. Results in this study show that both antigenic and IgE cross-reactivities exist between the 34-kD major serine proteinase allergens of *A. oryzae* and *P. citrinum*. IgE cross-reactivity among the 33-kD major allergens of three different prevalent airborne *Penicillium* species, *P. citrinum*, *P. notatum* and *P. brevicompactum* has previously been reported [3]. Our results further suggest that there may be allergenic cross-reactivity among major allergens of different *Penicillium* and *Aspergillus* species.

The finding that components AO1, AO2 and AO3 have similar N-terminal amino acid sequence suggests that AO2 and AO3 with apparent molecular weight of about 24 and 18 kD, respectively, are possibly degraded products of AO1. IgE-binding components with different apparent molecular weight identified by SDS-PAGE immunoblot might thus represent different forms of a certain allergenic component. A similar observation was reported for the group 3 allergen of house dust mites. Stewart et al. [18] reported the isolation of allergenic, small-molecular-weight degradation products resulting from the autolysis of the group 3 trypsin allergen isolated from *Dermatophagoides pteronyssinus*. However, the contingency that AO1, AO2 and AO3 are members of an isozyme system could not be excluded.

Interestingly, MoAb 55A reacts with AO1 but not AO2 or AO3, suggesting that the antigenic determinant site of 55A may not be located at the N-terminal portion of the 34-kD major allergen. However, the N-terminal part of the 34-kD allergen should contain IgE epitope(s) which was present on components AO1, AO2 and AO3.

A. oryzae has been used in the manufacturing of soy sauce and the enzyme alkaline serine protease is considered to play a key role in producing the taste of soy sauce by hydrolyzing raw materials [15]. Bacillus serine proteinases (subtilisins) have their best-known application in detergent powders [10, 11]. Occupational allergy due to this enzyme has been found [10, 11]. Results obtained in the present study show that the serine proteinase of *A. oryzae* may also be an important allergen responsible for the allergic disorders in asthmatic patients. Although structurally unrelated to subtilisins, serine proteinases such as trypsin and chymotrypsin have been identified as the group 3 [18, 19] and the group 6 [20] allergens, respectively, of the house dust mite *D. pteronyssinus*. A chymotrypsin-like serine proteinase from *A. fumigatus* can induce human epithelial cell detachment [21]. Robinson et al. [21] suggest that the proteinase might trigger the allergic response by detaching areas of epithelium, causing an increase in penetration of *A. fumigatus* antigens into the submucosa. Alternatively, the proteinases might act as one of these antigens.

In the analysis of allergens of *A. oryzae*, the presence of a corresponding Asp f 1 allergen needs further study. Conversely, the presence of an alkaline serine proteinase allergen in *A. fumigatus* and other prevalent airborne *Aspergillus* species, and cross-reactivity among proteinase allergens of different *Aspergillus* and *Penicillium* species need further investigation.

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