

Production and characterization of monoclonal antibodies to serine proteinase allergens in *Penicillium* and *Aspergillus* species

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Summary

Background Alkaline and/or vacuolar serine proteinases are major allergens in prevalent airborne *Penicillium* and *Aspergillus* species.

Objective The object of this study is to generate and characterize monoclonal antibodies against these serine proteinase allergens.

Methods BALB/c mice were immunized individually with the *Penicillium citrinum* culture medium or the crude extract and culture medium preparations of *Aspergillus fumigatus*. Hybridoma cells that secrete monoclonal antibodies against serine proteinase allergens were selected by immunoblotting. Antigens in three different *Penicillium* (*P. citrinum*, *P. notatum* and *P. oxalicum*) and two different *Aspergillus* species (*A. fumigatus*, and *A. flavus*) recognized by these monoclonal antibodies were analysed by sodium dodecyl sulphate and two-dimensional polyacrylamide gel electrophoresis immunoblotting and N-terminal amino acid sequence analysis.

Results Four (PCM8, PCM10, PCM16 and PCM39) and one (FUM20) monoclonal antibodies against serine proteinase allergens were generated after fusion of NS-1 cells with spleen cells obtained from BALB/c mice immunized with antigens from *P. citrinum* and *A. fumigatus*, respectively. Immunoblotting results showed that PCM8 reacted with an alkaline serine proteinase allergen in *P. citrinum* and *P. notatum*. PCM10 and PCM39 reacted with the alkaline serine proteinase in two *Penicillium* (*P. citrinum*, *P. notatum*) and two *Aspergillus* species (*A. fumigatus*, and *A. flavus*) tested. PCM16 reacted with the alkaline serine proteinase allergen in *P. citrinum*, *A. fumigatus* and *A. flavus* but not with that in *P. notatum*. MoAb FUM20 reacted with the alkaline serine proteinase allergen in two *Aspergillus* species (*A. fumigatus* and *A. flavus*) but not with that in two different *Penicillium* species (*P. citrinum*, *P. notatum*) tested. Among these five monoclonal antibodies generated, only PCM39 and FUM20 can react with the vacuolar serine proteinase allergen in *P. notatum*, *P. oxalicum* and in *A. fumigatus*. The 35 kDa *P. citrinum* component that reacted with FUM20 has an N-terminal amino acid sequence of DSPSVEKNAP.

Conclusion Five monoclonal antibodies against different epitopes of the serine proteinase major allergens in prevalent *Penicillium* and *Aspergillus* species were generated in the present study. Antibodies obtained may be useful in the characterization and standardization of serine proteinase allergens in crude fungal extracts.

Keywords: monoclonal antibodies, serine proteinase allergens, *Penicillium* and *Aspergillus* species

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Introduction

About 10% of the general population suffer from allergic disorders and the number of allergic patients is still on the rise. Among various causative allergens such as house dust mites, pollens and fungal spores, mould allergens are the least characterized.

A major difficulty associated with studies on mould allergens is the multiplicity of factors that contribute to the results obtained [1]. For instance, more than 100 different species have been identified in *Penicillium* and *Aspergillus*, two taxonomically closely related fungal genera [2,3]. It is important to understand the allergenic profiles of the prevalent airborne *Penicillium* and *Aspergillus* species, to detect the presence of antigenic/allergenic cross-reactivity among major allergens of these ubiquitous fungi, and to identify the representative species for clinical diagnosis. In addition, the lack of standardized allergens and/or allergenic extracts has become a major drawback in conventional diagnosis and immunotherapy of allergic disorders [4].

Recently, we have identified that alkaline and/or vacuolar serine proteinases are major allergens of the prevalent airborne *Penicillium* and *Aspergillus* species including *P. citrinum* [5], *P. brevicompactum* [5], *P. notatum* [6], *P. oxalicum* [6], *A. fumigatus* (manuscript accepted), *A. oryzae* [7], *A. flavus* [8] and *A. niger* (manuscript accepted). Our immunoblotting data showed that IgE antibodies against components in prevalent airborne *Penicillium* and *Aspergillus* species could be detected in about 15–30% of serum samples from asthmatic patients [9]. Most of the positive serum samples showed IgE-binding to the 32/34 kDa serine proteinase(s) with a frequency $\geq 60\%$ in different fungal species tested. In addition, IgE cross-reactivity among these major serine proteinase allergens has also been detected [6–9]. However, the mould strain used, the composition of the culture media, the culturing condition and the extraction method might all contribute to the variations in the crude allergenic extracts prepared from different sources [1,4].

Monoclonal antibodies (MoAbs) have been shown to be useful tools in the quantification of major allergens [10–12] and in the standardization of crude allergenic extracts [13,14]. They may also be useful in the characterization [15], purification [10,16] and cDNA cloning [17] of allergens. The purpose of the present study is to prepare MoAbs against serine proteinase allergens in prevalent *Penicillium* and *Aspergillus* species. Fungal components reacted with MoAbs generated were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional (2D) immunoblotting. Antigenic cross-reactivity among serine proteinase allergens in different fungal species was also analysed by immunoblotting using MoAbs obtained. Our results showed that MoAbs generated

against different epitopes of serine proteinase allergens may be useful in the characterization and standardization of these major allergens in crude allergenic fungal extracts.

Materials and methods

Fungal antigen preparations

Crude fungal extracts of *P. citrinum* [5], *P. notatum* [6], *P. oxalicum* [6] and *A. flavus* [8] were prepared essentially as described previously. The *A. fumigatus* strain used in the present study was CCRC33476 that was kindly provided by Professor Shean-Shong Tzean (Department of Plant Pathology and Entomology, National Taiwan University). It was isolated from the air of Tainan, Taiwan and verified by the Food Industry Research & Development Institute, Hsinchu, Taiwan. An *A. fumigatus* extract was prepared as described [5,6,8]. Briefly, *A. fumigatus* CCRC33476 was grown in a liquid medium containing Czapek concentrate [2] (10 mL/L), K_2HPO_4 (1.0 g/L), yeast extract (Difco, Detroit, MI, USA; 5 g/L) and sucrose (30 g/L) for 6 days at 25 °C. The fungal matt containing both spores and mycelia was ground under liquid nitrogen with mortar and pestle and was then extracted (1:5, v/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) for 4 h at 4 °C, with constant shaking. After centrifugation, the supernatant was dialysed against 50 mmol/L of NH_4HCO_3 , pH 8.0 for 48 h at 4 °C. The dialysate was sterilized by filtration (0.2 μ m; Sartorius, Hayward, CA, USA), lyophilized in aliquots and stored at –20 °C. The proteins in the extract were quantified with the protein assay dye concentrate (catalogue number 500–0006) of Bio-Rad (Richmond, CA, USA). In addition, the culture media from different fungal species grown were also collected individually. They were filtered, lyophilized and stored at –20 °C as described above.

Development of MoAbs

Initially, MoAbs against serine proteinase allergens were prepared essentially as described previously [5,15]. *P. citrinum* culture medium in complete Freund adjuvant (CFA, Difco Laboratories, Detroit, Michigan, USA) and crude extract and culture medium preparations of *A. fumigatus* in the same adjuvant were used in the intraperitoneal immunization of BALB/c mice individually. Two weeks later, a booster immunization with the same antigen preparations in incomplete Freund adjuvant (IFA, Difco) was performed. Since antibody immune response against serine proteinase antigens was not detected one week later, crude fungal extract and/or the fungal culture medium that has been denatured in 0.5% SDS at 80 °C for 10 min and mixed with the IFA was injected intraperitoneally into BALB/c mice. The

same denatured and heat-treated antigen preparations in IFA were used for booster immunization after a week interval. Three days before fusion, mice were received a tail vein injection of the denatured and heat-treated antigen preparations in phosphate buffer (10 mM, pH 7.2). Spleen cells from immunized animals were fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol (MW 1500 Da; Boehringer Mannheim, Mannheim, Germany). Hybrid cells secreting antibodies were screened by enzyme-linked immunosorbent assay (ELISA) and immunoblot using the crude fungal extracts and/or the culture medium preparations. Fungal proteins reacted with the antibodies in the hybridoma cell culture supernatants were characterized by SDS-PAGE and/or 2D-immunoblot. Hybridomas producing antibodies against the 34/32 kDa serine proteinases were subcloned at least twice by limiting dilutions and then further expanded. The isotype of the MoAbs was determined by ELISA with class-specific antisera (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

ELISA

The ELISA was performed essentially as described [15]. The untreated antigens or the SDS (0.5%) and heat-treated antigen preparations (80 °C, 10 min) in carbonate buffer, pH 9.6 were coated separately onto wells of polyvinyl microtitre plates. After blocking with 1% skimmed milk, culture supernatant solutions of hybridoma cells were added to each well and incubated for 1 h at 37 °C. After washing with phosphate-buffered saline (150 mM, pH 7.4) containing 0.05% Tween 20 (PBST), an affinity-purified, horseradish peroxidase labelled goat antimouse IgG (H + L) preparation (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was added to each well and incubated for another hour at 37 °C. Wells were washed again with PBST and a substrate solution (Bio-Rad, peroxidase substrate kit 172–1064, containing 2,2'-di-[3-ethylbenzthiazoline-6-sulphonic acid] and hydrogen peroxide) was added to each well for 30 min at 37 °C. A colour change was detected by measurement of the absorbance at 415 nm on SPECTRAMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Culture supernatant solutions of hybridoma cells containing MoAbs against the house dust mite allergen Der p 7 generated by us [15] were used as negative control. An average of two readings from separate determinations was calculated. Positive ELISA activity in the screening was attributed to supernatant which showed absorbance greater than 2.0 times the absorbance obtained with the negative control supernatant.

Immunoblot analysis

Components in the crude fungal extracts or culture media

that could be recognized by IgE antibodies in sera from asthmatic patients and/or MoAbs generated in our laboratory were analysed by immunoblotting after SDS-PAGE or 2D gel electrophoresis essentially as described [5–8]. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (0.45 µm; Millipore, Bedford, MS, USA). The membrane was blocked with 1% skimmed milk, then incubated with diluted human serum samples (1 : 5 dilution in Tris-buffered saline, pH 7.5 containing 0.05% Tween 20 and 1% skimmed milk) for 16 h at 4 °C or with culture supernatant of individual hybridoma cells at room temperature for 1 h. After washing, the blots were incubated for 1 h with alkaline phosphatase-conjugated monoclonal antihuman IgE antibodies (Pharmingen, San Diego, CA, USA) or with affinity-purified, horseradish peroxidase-labelled goat antimouse IgG (H + L) (Jackson ImmunoResearch Laboratories) at room temperature. The blots were washed and developed either for 1 h in a substrate solution of carbonate buffer (0.1 M NaHCO₃, 1.0 mM MgCl₂, pH 9.8) containing 0.35 mM BCIP (5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt) and 0.37 mM NBT (p-nitro blue tetrazolium chloride), or for about 5 min in a substrate solution of acetate buffer (50 mM, pH 5.0) containing 1 mM 3-amino-9-ethyl-carbazole (Sigma) and 0.015% (v/v) hydrogen peroxide. The deposition of the purple or red stain indicated antigen-antibody binding was recorded by photography. Serum separated from cord blood and serum from a mite-allergic patient, and MoAb WH22 against Der p 7 [15] were used as negative control. Low molecular weight standards (Pharmacia, Uppsala, Sweden) were used to estimate the molecular mass of the protein components. Carbamylated creatine kinase (CPK) obtained from BDH (Poole, UK) was used as charge standards for calculating the isoelectric point of the resolved proteins.

N-terminal amino acid sequence analysis

Components in crude fungal extract were separated by 2D gel electrophoresis and transferred electrophoretically onto PVDF membranes. The proteins were visualized by Coomassie blue staining. Protein spots that reacted with IgE and MoAbs in immunoblotting were excised from the membrane and inserted into an ABI Procise Model 491 sequencer (Applied Biosystems, Inc., Foster City, CA, USA) for automated cycles of Edman degradation according to the manufacturer's instruction.

Results

Selection and characterization of MoAbs to serine proteinase allergens

Hybridomas were obtained by fusion of immunized murine spleen cells with plasmacytoma NS-1 cells. Hybrid cells

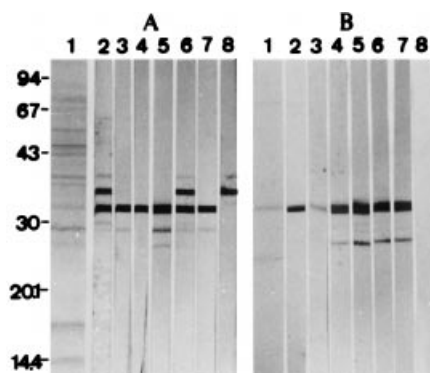


Fig. 1. Immunoblot reactivity of MoAbs generated to components in the crude extract (panel A) and the culture medium (panel B) of *P. citrinum*. Strips 1 in panels A and B show the Coomassie blue-stained protein profiles of the crude extract and the culture medium of *P. citrinum* on PVDF membranes, respectively. Strips 2 in both panels show the reactivity of IgE antibodies in serum from an asthmatic patient to components of *P. citrinum*. Strips 3–8 in both panels correspond to immunoblots generated by using MoAbs PCM8, PCM10, PCM16, PCM39, 55A and FUM20, respectively. The molecular mass standards (Pharmacia, Uppsala, Sweden) used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

secreting antibodies against fungal antigens were screened by ELISA. Since most of the culture supernatants of hybridoma cells gave negative and/or weak ELISA activity to nondenatured antigen preparations, SDS-PAGE immunoblot was used in the screening of hybridomas secreting antibodies against serine proteinases. The 33 kDa alkaline serine proteinase has been identified as a major allergen in *P. citrinum* [5]. In the present study, our results showed that 4 hybridomas (PCM8, PCM10, PCM16 and PCM39) originated from *P. citrinum*-immunized spleen cells produce MoAbs that are reactive against a 33-kDa component in a crude extract of *P. citrinum* (Fig. 1, panel A, strips 3–6). The protein profiles of the extract and the culture medium of *P. citrinum* on PVDF blots were shown in strips 1 of panels A and B of Fig. 1, respectively. This same set of MoAbs obtained also showed immunoblot reactivity to the 33 kDa component in the culture medium of *P. citrinum* (Fig. 1, panel B, strips 3–6). The MoAb-reacting 33 kDa component in fungal extract and culture medium also showed immunoblot reactivity with IgE antibodies in serum from an asthmatic patient (strips 2 in panels A and B). MoAb 55 A against the 33 kDa alkaline serine proteinase allergen of *P. citrinum* [5] reacted with the same 33 kDa component in both crude extract and culture medium (strips 7 of panels A and B, respectively). In addition to the 33 kDa component, IgE antibodies in serum from the same asthmatic patient and MoAb PCM39 also reacted with a 35-kDa component in the

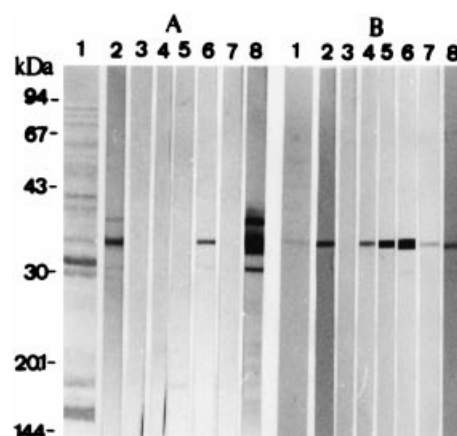


Fig. 2. Immunoblot reactivity of MoAbs generated to components in the crude extract (panel A) and the culture medium (panel B) of *A. fumigatus*. Strips 1 in panels A and B show the Coomassie blue-stained protein profiles of the crude extract and the culture medium of *A. fumigatus* on PVDF membranes, respectively. Strips 2 in both panels show the reactivity of IgE antibodies in serum from the same asthmatic patient used in Fig. 1 to components of *A. fumigatus*. Strips 3–8 in both panels correspond to immunoblots generated by using MoAbs PCM8, PCM10, PCM16, PCM39, 55A and FUM20, respectively. The molecular mass standards (Pharmacia, Uppsala, Sweden) used were the same as shown in Fig. 1.

crude extract of *P. citrinum*, as shown in strips 2 and 6 of panel A, respectively.

MoAb FUM20 was originated from mouse spleen cells immunized with antigens from *A. fumigatus*. The immunoblot reactivity of this MoAb against components of *P. citrinum* was shown in strips 8 in both panels A and B of Fig. 1. FUM20 reacted with a 35-kDa IgE-binding component in the extract of *P. citrinum* (strip 8, panel A). However, immunoblot reactivity of FUM20 to component(s) in the culture medium of *P. citrinum* was not detected (strip 8 of panel B).

Immunoblot reactivity of the six MoAbs mentioned above against components in the crude extract and the culture medium of *A. fumigatus* was shown in panels A and B of Fig. 2, respectively. IgE antibodies in serum from the same asthmatic patient as shown in Fig. 1 reacted with a 34-kDa component in the crude extract (strip 2, panel A) and in the culture medium (strip 2, panel B) of *A. fumigatus*, respectively. MoAbs PCM39 and FUM20 reacted with the 34 kDa component in the crude extract of *A. fumigatus* (strips A6 and A8). MoAbs PCM8, PCM10, PCM16 and 55 A showed negative immunoblotting results as shown in strips A3, A4, A5 and A7, respectively. However, although PCM8 showed negative immunoblot to component(s) in the culture medium of *A. fumigatus* (strip B3), MoAbs PCM10, PCM16, PCM39, 55 A and FUM20 reacted with a 34-kDa component in the culture medium of *A. fumigatus* as shown in Fig. 2, strips 4–8, respectively.

Antigenic cross-reactivity among serine proteinase allergens in different fungal species detected by immunoblot using MoAbs generated

Antigenic cross-reactivity among serine proteinase allergens in prevalent airborne *Penicillium* and *Aspergillus* species was detected with the MoAbs generated. Results from our previous studies showed that the 34 kDa alkaline and the 32 kDa vacuolar serine proteinases are major allergens in *P. notatum* [6]. It was also found that the 34 kDa vacuolar and alkaline serine proteinases are major allergens in *P. oxalicum* [6] and in *A. flavus* [8], respectively. In the present study, immunoblot reactivity of MoAbs PCM8, 10, 16, 39, 55 A and FUM20 to components in the crude extracts of *P. notatum* (panel A), *P. oxalicum* (panel B) and *A. flavus* (panel C) was analysed, respectively. The protein profile and the reactivity of these three fungal species against IgE antibodies in serum from the same asthmatic patient as shown in Figs 1 and 2 were demonstrated in strip 1 and 2 of Fig. 3, respectively. MoAbs PCM8 and PCM10 reacted with the 34 kDa component of *P. notatum* (strips A3 and A4). PCM39 reacted with the 34, 32 and 28 kDa components (strip A6), and FUM20 reacted with the 37 and the 32 kDa components in the extract of *P. notatum* (strip A8). The immunoblot reactivity of MoAbs PCM16 and 55 A to *P. notatum* was not detectable as shown in strips A5 and A7, respectively.

Results in strips 6 and 8 of panel B show that PCM39 reacted with the 37, 34 and 29 kDa components while

FUM20 reacted with the 37 and 34 kDa components of *P. oxalicum*, respectively. Immunoblot reactivity of MoAbs PCM8, 10, 16 and 55 A to *P. oxalicum* was not detected (strips B3, B4, B5 and B7, respectively).

MoAbs PCM10 and 55 A gave relatively weak immunoblot reactivity to the 34 kDa component of *A. flavus* (strips C4 and C7, respectively). MoAb PCM16 reacted with the 34 kDa component (strip C5), while PCM39 (strip C6) and FUM20 (strip C8) reacted with the 34, 30 and 24 kDa components of *A. flavus*. PCM8 does not react with *A. flavus* (strip C3, Fig. 3).

To summarize the results obtained, the immunoblot profiles of MoAbs obtained to serine proteinase allergens in different fungal species tested were presented in Table 1. In addition, the isotypes of MoAbs PCM8, 10, 16, 39 and FUM20 are IgG1, κ as analysed by ELISA using class-specific antisera (data not shown). In this experiment, SDS (0.5%) and heat (80 °C, 10 min)-treated culture medium preparations of *P. citrinum* and *A. flavus* were used as antigens in the coating of the ELISA microtitre plates.

Characterization of fungal antigens reacted with MoAbs by 2D-immunoblot

2D immunoblotting further characterized the antigens recognized by the MoAbs. The 2D protein profile of the *P. citrinum* extract on PVDF membrane was presented in panel A of Fig. 4. IgE-immunoblot reactivity against *P. citrinum* was determined with a mixture of serum samples from five asthmatic patients and presented in panel B of Fig. 4. MoAbs PCM8, 10 and 16 reacted with the 33 kDa allergen (panels C, D and E). In addition to the 33 kDa components, PCM39 also showed a relatively weak immunoblot reactivity against a 35-kDa component of *P. citrinum* (panel F). 2D-immunoblot reactivity of MoAb FUM20 to the 35 kDa component was detected as shown in panel G of Fig. 4. The N-terminal amino acid sequence of the 35 kDa component was determined to be DSPSVEKNAP.

In 2D immunoblot analysis, IgE antibodies in sera from asthmatic patients can react with the 34, 30.5 and 30 kDa components in a crude extract of *A. fumigatus* (manuscript accepted). MoAbs PCM39 and FUM20 reacted with the same 34 kDa IgE-binding component in the crude extract of *A. fumigatus* (data not shown). Results from N-terminal amino acid sequence analysis suggest that the 30.5 and 30 kDa components probably represent the degraded products of the 34 kDa vacuolar serine proteinase allergen of *A. fumigatus* (manuscript accepted).

The 2D protein profile of an extract of *P. notatum* and the immunoblot reactivity of IgE antibodies and MoAb PCM39 to components of *P. notatum* that have been

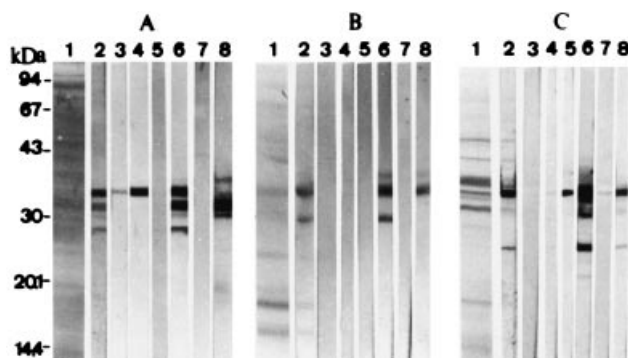


Fig. 3. Immunoblot reactivity of MoAbs generated to components in crude extracts of *P. notatum* (panel A), *P. oxalicum* (panel B) and *A. flavus* (panel C). Strips 1 in all 3 panels show the Coomassie blue-stained protein profiles of individual fungal extract on PVDF membrane. Strips 2 show the reactivity of IgE antibodies in serum from the same asthmatic patient used in Figs 1 and 2 to components of individual fungal species tested. Strips 3–8 in 3 panels correspond to immunoblots generated by using MoAbs PCM8, PCM10, PCM16, PCM39, 55A and FUM20, respectively, to components of individual fungal species tested. The molecular mass standards (Pharmacia, Uppsala, Sweden) used were the same as shown in Fig. 1.

Table 1. Reactivity of MoAbs generated to the alkaline and/or vacuolar serine proteinase allergens in *Penicillium* and *Aspergillus* species detected by SDS-PAGE immunoblot

Fungal allergens	MoAbs					
	PCM8	PCM10	PCM16	PCM39	55A*	FUM20
Alkaline serine proteinase						
<i>P. citrinum</i> (E)†	+	+	+	+	+	–
<i>P. citrinum</i> (M)‡	+	+	+	+	+	–
<i>P. notatum</i> (E)	+	+	–	+	–	–
<i>A. fumigatus</i> (M)	–	+	+	+	+	+
<i>A. flavus</i> (E)	–	+	+	+	+	+
<i>A. flavus</i> (M)§	–	+	+	+	+	+
Vacuolar serine proteinase						
<i>P. citrinum</i> (E)	–	–	–	+	–	+
<i>P. notatum</i> (E)	–	–	–	+	–	+
<i>P. oxalicum</i> (E)	–	–	–	+	–	+
<i>A. fumigatus</i> (E)	–	–	–	+	–	+

*55A: MoAb against the 33 kDa alkaline serine proteinase allergen of *P. citrinum* generated by us previously [5]. †E: crude extract of the fungal species. ‡M: culture medium of the fungal species. §Data not shown in the present study.

shown previously by us [6] were demonstrated again in panels A, B and E in Fig. 5, respectively. PCM 39 reacted with the 34, 32, 28.5 and 28 kDa allergens of *P. notatum*. MoAbs PCM8 and 10 reacted with the 34 kDa allergen (panels C and D) whereas FUM 20 reacted with the 32 kDa allergen (panel F) in *P. notatum*. Results from Edman degradation suggest that the 28.5 and 28 kDa components may be breakdown products of the 32 kDa vacuolar serine proteinase allergen of *P. notatum* [6].

We have shown previously by 2D immunoblot analysis that MoAb PCM39 reacted with 34 kDa IgE-binding components in an extract of *P. oxalicum* [6]. In the present study, a 2D immunoblot profile similar to those for IgE and PCM39 to *P. oxalicum* was observed for MoAb FUM20 (data not shown). Results from N-terminal amino acid sequence analysis suggest that the 34 kDa component is possibly the vacuolar serine proteinase of *P. oxalicum* [6].

We have reported the 2D protein profile of an *A. flavus* extract [8]. IgE antibodies in sera from asthmatic patients reacted with the 34 and 24 kDa components of *A. flavus* [8]. The first nine N-terminal amino acid residues of the 24 kDa component were found to be identical to those of the 34 kDa major allergen [8]. It was suggested that the 24 kDa component may be a degraded product of the 34 kDa alkaline serine proteinase allergen [8]. In the present study, analysed by 2D immunoblotting, MoAbs PCM10 and 16 reacted with the 34 kDa IgE-binding component in *A. flavus* (data not shown). In addition to the 34 kDa component, an immunoblot reactivity to a 24-kDa IgE-binding component of

A. flavus was also detected for MoAbs PCM39 and FUM 20 (data not shown).

Discussion

Allergenic extracts used in clinical applications contain miscellaneous components. Fungal extracts are especially notorious for their complexity and variability. Many factors contribute to the marked batch-to-batch variability in fungal extracts [1]. The lack of readily available high quality extracts is a major problem in studying clinical mould allergy. The preparation of these reagents can be improved by carefully identifying, characterizing and standardizing the major allergens in the extracts. Alkaline and/or vacuolar serine proteinases have been identified as major allergens in several prevalent airborne *Penicillium* and *Aspergillus* species [5–9]. In addition, we have identified as major allergens a 34-kDa vacuolar serine proteinase in the crude extract and an alkaline serine proteinase with approximately the same molecular mass in the culture medium of *A. fumigatus* (manuscript accepted). MoAbs against these major allergens were generated in the present study to provide reagents for characterization and standardization purposes.

In the course of this study, initial attempts to produce specific antibody response with native proteins as the immunogen were not successful. In literature, denaturing the proteins in sodium dodecyl sulphate and heating can increase the immunogenicity of the protein antigens [18]. The elastase from *Aspergillus fumigatus* was only weakly

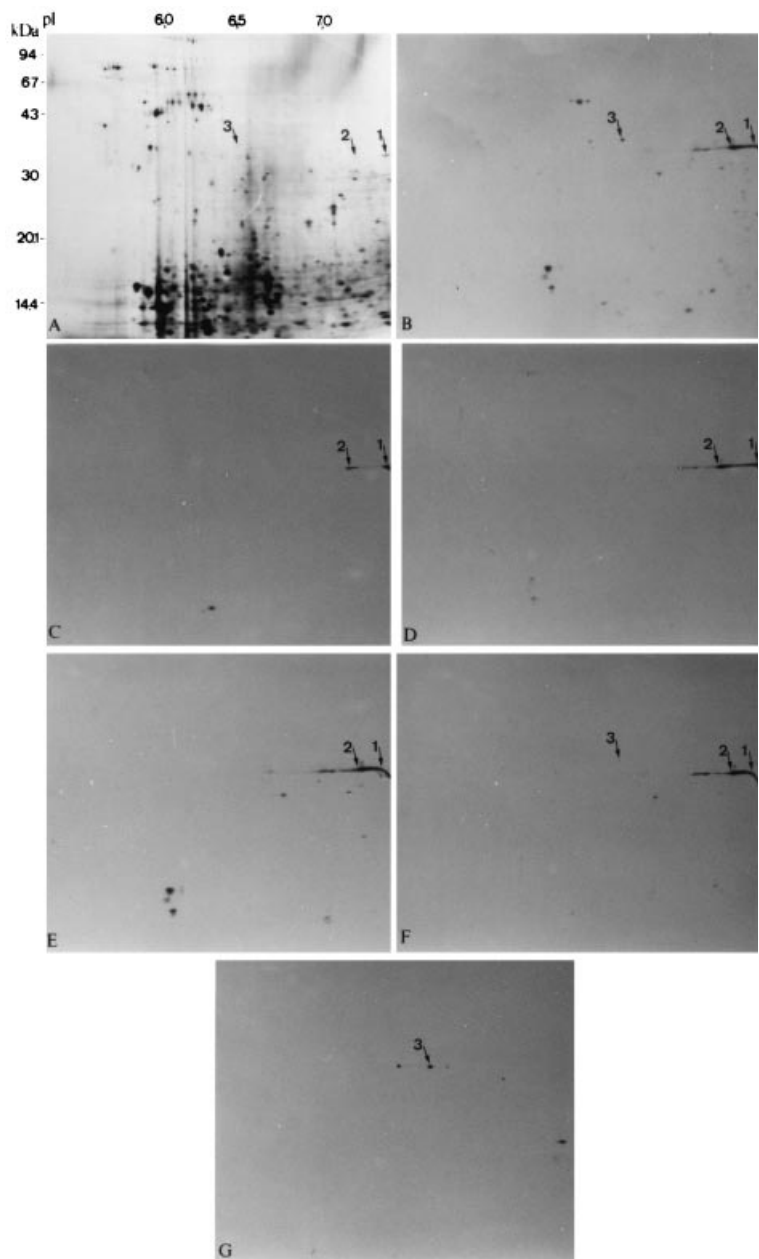


Fig. 4. 2D immunoblot analysis of components in the extract of *P. citrinum* reacted with human IgE antibodies and MoAbs generated. Panel A shows the Coomassie blue-stained two-dimensional protein profile of the crude extract on a PVDF blot. Panel B shows the immunoblot reactivity of IgE antibodies in a mixture of serum samples from five asthmatic patients to components in the crude *Penicillium* extract. Panels C-G show the reactivity of MoAbs PCM8, PCM10, PCM16, PCM39 and FUM20 to components in *P. citrinum*, respectively. Protein spots indicated in panel A (1, 2 and 3) correspond to those that showed enzymatic colour reaction in immunoblot as shown in panels B-G. The same molecular mass standards as shown in Fig. 1 were used. Carbamylated creatine kinase (CPK) obtained from BDH (Poole, UK) was used as charge standards for calculating the isoelectric point of the resolved proteins.

immunogenic in BALB/c mice [19]. However, the titres of antiserum can be increased by denaturing the elastase in 0.5% sodium dodecyl sulphate at 80 °C for 10 min prior to injection. Of the eight hybridomas selected, seven produced IgG reactive with the enzyme and one produced nonspecific IgM. The nondenatured immunogen yielded mainly IgM producers. Immunoblots and ELISA showed that the IgG MoAbs were reactive against both the denatured and nondenatured fungal elastase [19]. According to Frosco *et al.* the elastase from *Aspergillus fumigatus* is also a serine proteinase [19]. We utilized a similar approach and our

results here show that the denatured fungal proteins elicited antibody titres and resulted in production of specific IgG MoAbs. However, the MoAbs obtained in this study as well as MoAb 55 A showed strong immunoblot reactivity with the denatured (in immunoblots) but weak/or negative ELISA reactivity with the native (nondenatured in ELISA) antigen preparations. MoAb 55 A is reactive against the alkaline serine proteinase allergen of *P. citrinum* and was prepared by immunizing the mouse with nondenatured crude fungal extract [5].

Results obtained indicate that the fungal serine proteinases may be poorly immunogenic in their native forms.

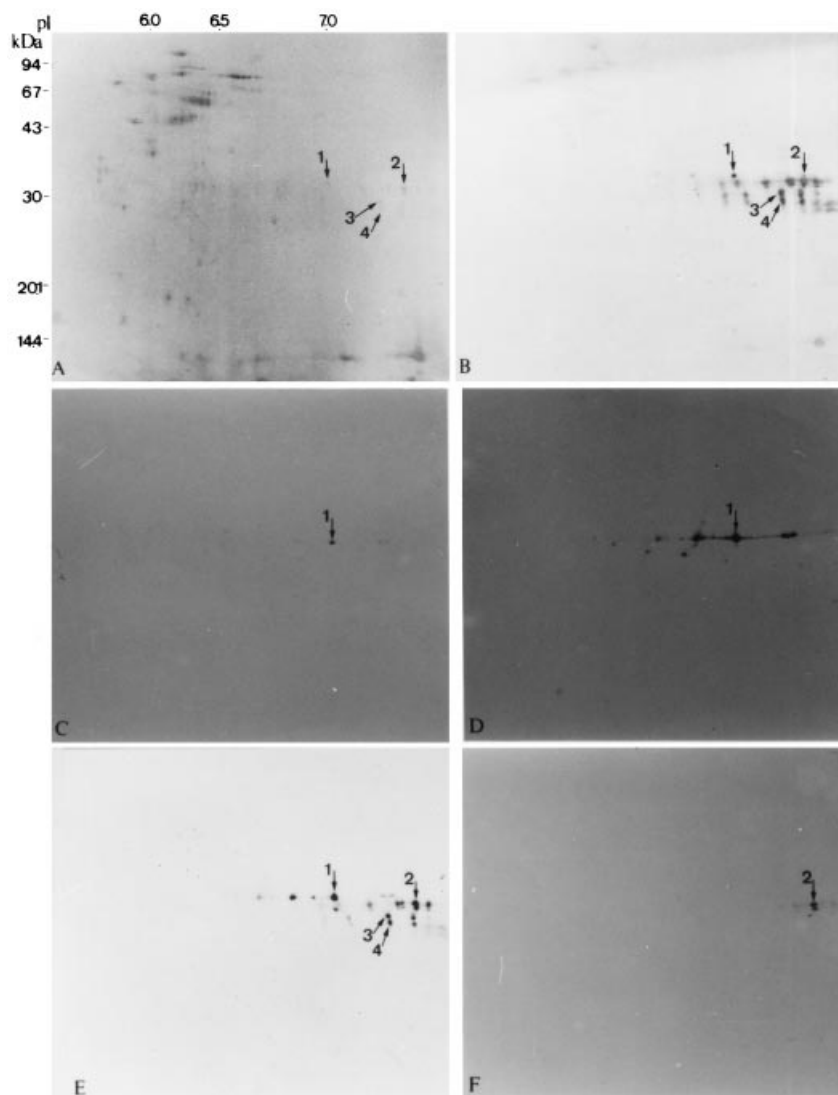


Fig. 5. 2D immunoblot analysis of components in the extract of *P. notatum* reacted with human IgE antibodies and MoAbs generated. Panel A shows the Coomassie blue-stained two-dimensional protein profile of the crude extract on a PVDF blot. Panel B shows the immunoblot reactivity of IgE antibodies in serum from an asthmatic patient to components in the crude *Penicillium* extract. Panels C-F show the reactivity of MoAbs PCM8, PCM10, PCM39 and FUM20 to components in *P. notatum*, respectively. Protein spots indicated in panel A (1, 2, 3 and 4) correspond to those that showed enzymatic colour reaction in immunoblot as shown in panels B-F. The same molecular mass and charge standards as used in Fig. 1 and Fig. 4, respectively, were used.

Denaturation in SDS may actually expose previously hidden antigenic determinants. The MoAbs obtained reacted with the denatured rather than the native proteins. Thus, these MoAbs probably recognize linear epitopes that are stable in the presence of detergent and heating instead of conformational epitopes on the fungal serine proteinases.

MoAbs PCM10, PCM16, PCM39 and 55 A react with the alkaline serine proteinase in *Penicillium* and *Aspergillus* species (Table 1). MoAb PCM10 and PCM39 reacted with the alkaline serine proteinase in two *Penicillium* and two *Aspergillus* species tested. However, PCM16 and 55 A showed immunoblot reactivity to the alkaline serine proteinase in *P. citrinum*, *A. fumigatus* and *A. flavus* but not to that in *P. notatum*. It was also found that MoAb PCM8 reacted with the alkaline serine proteinase in two *Penicillium* species tested, and MoAb FUM20 reacted with the same

proteinase in two *Aspergillus* species tested (Table 1). In addition to the reactivity with the alkaline serine proteinase, MoAb PCM39 and FUM20 also showed immunoblot reactivity to the vacuolar serine proteinase allergen in *P. notatum*, *P. oxalicum* and *A. fumigatus*. Results obtained suggest that different antigenic epitopes may have been recognized by five MoAbs generated in the present study.

Since MoAb FUM20 has been found to be reacted with the vacuolar serine proteinase in *P. notatum* and *P. oxalicum*, the 35 kDa FUM20-reacting component in *P. citrinum* may be the corresponding vacuolar serine proteinase allergen in *P. citrinum*. The N-terminal amino acid sequence of this 35 kDa component was determined to be DSPSVEKNAP that has high similarity to that of the vacuolar serine proteinase allergen in *P. notatum* (SEGSVEKNAP) [6] and in *A. fumigatus* [EDPTVEKSAP] (manuscript accepted).

Thus, the FUM20-reacting 35 kDa IgE-binding component may be the vacuolar serine proteinase of *P. citrinum*. It was also found that the 35 kDa component reacted with IgE antibodies in 19 (58%) of 33 *P. citrinum* IgE-positive asthmatic serum samples (data not shown). Therefore, the 35 kDa vacuolar serine proteinase may also be a major allergen of *P. citrinum*.

In the present study, fungal components with molecular mass less than 32/34 kDa have been found to be reactive with the MoAbs generated. They are possibly degrade products of the 32/34 kDa serine proteinase allergens as mentioned in the Results section. An easily autolytic 32 kDa extracellular serine proteinase from *A. fumigatus* has also been reported by Reichard *et al.* [20]. In ELISA, the proteinase inhibitor PMSF (phenylmethylsulphonyl fluoride) was added to the purified serine proteinase solution in coating the microtitre plates [20]. Antibodies to the 32 kDa alkaline protease of *Aspergillus fumigatus* were detected with high titres in the sera of two of six patients with aspergilloma when PMSF was used in coating the antigen onto plate [20].

In Fig. 4, the MoAb PCM16 also recognizes low molecular weight components with a pI of about 6.0 (panel E). These components are also recognized by IgE antibodies in sera from asthmatic patients (panel B). These low molecular weight components may be degraded products of the 32/34 kDa serine proteinase allergens. They may also be components of *P. citrinum* that have antigenic cross-reactivity with serine proteinase major allergens.

MoAbs against different epitopes of an allergen component have been used successfully in the standardization of allergenic extracts by a sandwich ELISA approach [10–12]. Although MoAbs generated in this study gave weak ELISA activity to serine proteinase allergens, they may be useful in immunoblot as an approach in the standardization of allergenic fungal extracts. In the analysis of IgE-binding components in crude fungal extracts, it was found that the 32 kDa vacuolar serine proteinase was not detectable in a commercial extract of *P. notatum* obtained from Greer Laboratories, Inc. (Lenoir, North Carolina, USA) [6]. Both the alkaline and the vacuolar serine proteinase allergens were detected in an extract of *P. notatum* prepared in our laboratory [6]. MoAbs obtained may thus be useful in the identification of individual serine proteinase allergen in crude fungal extracts.

The significance of serine proteinases being major allergens of prevalent *Penicillium* and *Aspergillus* species has not been established. The proteinase might trigger the allergic response by detaching areas of epithelium, causing an increase in penetration of antigens (allergens) into the submucosa [21]. It might enhance tissue injury, activate inflammatory cells and possibly promote its interaction with the immune system. The proteinase might also have effect

on IgE synthesis by exerting its proteolytic effect on regulatory molecules as observed for the major house dust mite allergen Der p 1 [22]. In addition, extracellular proteases have been considered to play a role in the pathogenesis of aspergillosis [20,21,23,24]. MoAbs against serine proteinases obtained in the present study may also be useful in the study of proteases as major allergens and as virulence factors of pathogenic *Aspergilli*.

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