

Characterization of the 33-kilodalton major allergen of *Penicillium citrinum* by using MoAbs and N-terminal amino acid sequencing

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Summary

Background The 33 kD component has been identified as a major allergen of *Penicillium citrinum*, the most prevalent *Penicillium* species in the Taipei area of Taiwan.

Objective This study analyses the isoforms, antigenic cross-reactivity and the N-terminal amino acid sequence of the 33 kD allergen of *P. citrinum*.

Methods The composition of isoforms and antigenic cross-reactivity was analysed by SDS-PAGE and 2D-immunoblotting using MoAbs generated. The N-terminal sequence was analysed by using an automatic gas/liquid phase sequencer.

Results Two MoAbs (55A and 34H) against the 33 kD allergen were generated in the present study. In addition to the 33 kD component, MoAb 34H also showed immunoblot reactivity to other components in the crude extract of *P. citrinum*. Analysed by 2D-immunoblotting, at least six different isoforms of the 33 kD component with pI values ranging from 6.75 to greater than 7.0 were shown to be reactive to both MoAbs and IgE antibodies in serum of an asthmatic patient. Different immunoblot patterns were observed when both MoAbs were reacted with four different strains of *P. citrinum* used in the present study. Among another six different *Penicillium* and four different *Aspergillus* species tested, only an immunoblot reactivity of MoAb 55A to the 33 kD component of *P. brevicompactum* was observed. In 2D-immunoblotting, components of *P. brevicompactum* with an MW of about 33 kD and pI values similar to those of the 33 kD component of *P. citrinum* reacted with MoAb 55A and IgE antibodies in serum of the asthmatic patient. The N-terminal amino acid sequence of the 33 kD component of *P. citrinum* was determined to be ANVVQSNVP which was identical to the first 9 N-terminal amino acids of a heat-labile alkaline serine proteinase from *P. citrinum*.

Conclusion Results obtained in the present study suggest that the 33 kD major allergen of *P. citrinum* may be an alkaline serine proteinase.

Keywords: *P. citrinum*, 33 kD allergen, MoAbs, N-terminal amino acid sequence

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Introduction

Airborne fungal spores are potential respiratory allergens. Over 60 species of fungi are known to be allergenic [1].

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Although *Cladosporium* is far more prevalent in the outdoor environment [2], *Penicillium* and *Aspergillus* are the most common indoor fungal species [3,4]. Since more than 150 different species have been discovered in the genus *Penicillium* [5], a study was conducted by our laboratories to isolate and identify indoor *Penicillium* species in the Taipei area of Taiwan. The results have shown that among 910

colonies of *Penicillium* isolated, 369 (40.5%) were *P. citrinum* which could be found in 28 (32%) of the 88 houses studied [6]. Although *P. chrysogenum* (*P. notatum*) was used most frequently in skin prick tests clinically, *P. citrinum* has also been identified as one of the five most frequently recovered species of *Penicillium* in and out of doors in Topeka KS [7].

A 33 kD component has been identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as a major allergen of *P. citrinum* [8,9]. It was found that among the 67 asthmatic serum samples tested, 15 showed IgE reactivity to 12 different components of *P. citrinum* [9]. Furthermore, all 15 *P. citrinum*-positive serum samples showed IgE-binding to the 33 kD extract component of this species [9]. For characterization of the 33 kD major allergen of *P. citrinum*, MoAbs against this particular component were generated. The N-terminal amino acid sequence of the protein blotted onto PVDF (polyvinylidene difluoride) membrane and identified with these MoAbs was also determined. The result showed that the sequence obtained was the same as that of the first nine N-terminal amino acids of a heat-labile serine proteinase of *P. citrinum* reported by Yamamoto *et al.* [10].

Materials and methods

Allergenic extracts

For preparation of the allergenic extract, *P. citrinum* strain 52–5 isolated from the air in the Taipei area [6] was used. The species was identified by us and verified by the American Type Culture Collection. Fungal mats of *P. citrinum* 52–5 containing both spores and mycelia were collected after its growth in a liquid medium containing Czapek concentrate [5] (10 ml/l), K_2HPO_4 (1.0 g/l) and sucrose (30 g/l) for 3 weeks at 25°C. The crude extract of *P. citrinum* was prepared by extracting the fungal mats (1:10, 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 36 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. After sterile filtration (0.2 µm; Sartorius, Hayward, CA, USA), the extract was lyophilized in aliquots and stored at 4°C. Furthermore, extracts of different strains of *P. citrinum* prepared by the same procedure mentioned above were also included in the present study. In addition to 52–5, the strains used were a strain of *P. citrinum* (30143) obtained from NRRL (NRRL 24977; ARS Culture Collection, Northern Regional Research Center, Peoria, IL, USA) and another two strains of *P. citrinum* (strain no. 62–1 and 23–98) isolated from the

air of the Taipei area which have also been verified by the American Type Culture Collection.

For cross-reactivity studies, the ground and freeze-dried preparations of different fungal species were obtained from Allergon AB (Engelholm, Sweden). They were six species of *Penicillium* and four species of *Aspergillus*. The *Penicillium* species obtained were *P. notatum*, *P. brevicompactum*, *P. oxalicum*, *P. spinulosum*, *P. roseopurpureum* and *P. frequentans*. The *Aspergillus* species obtained were *A. fumigatus*, *A. niger*, *A. flavus* and *A. nidulans*. Allergenic extracts of all these fungal species were prepared individually as mentioned above. Furthermore, the *P. notatum* extract obtained from Greer Laboratories (Lenoir, North Carolina, USA) used for skin testing has also been included in the cross-reactivity study using MoAbs generated. The protein content of the extracts was determined by the dye-binding method of Bio-Rad (Richmond, CA, USA).

Development of MoAbs

MoAbs against the 33 kD allergen were prepared essentially as described previously [11]. Spleen cells obtained from BALB/c mice immunized with the crude extract of *P. citrinum* strain 52–5 were fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol (MW 1500 daltons; Merck, Hohenbrunn, Germany). Antibody-producing hybrid cells were screened by the enzyme-linked immunosorbent assay (ELISA) [11] using the same crude extract of *P. citrinum*. Antigens of *P. citrinum* recognized by MoAbs produced by hybridomata were analysed by SDS-PAGE and immunoblotting. Hybridomata producing MoAbs against the 33 kD component of *P. citrinum* 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). Reactivity of MoAbs to components of different fungal species was analysed by SDS-PAGE-immunoblotting.

Gel electrophoresis and immunoblotting

The components of crude fungal extracts recognized by MoAbs produced or by IgE antibodies in serum of asthmatic patient were analysed by gel electrophoresis and immunoblotting essentially as described [11,12]. Briefly, components of the crude allergenic extracts were separated by SDS-PAGE or by the two-dimensional (2D) gel electrophoresis [12] and transferred electrophoretically onto PVDF membrane (0.45 µm, Millipore, Bedford, MS, USA). After blocking with 1% skimmed milk, the blots were incubated with culture supernatant solutions of hybridoma cells at

room temperature for 1 h or 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. After washing, the blots were incubated with affinity-purified, horseradish peroxidase-labelled goat anti-mouse IgG (H+L) (BioRad) or with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, CA, USA) for 1 h at room temperature.

After washing, the blots were developed 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 for about 5 min or in a substrate solution of carbonate buffer (0.1 M NaHCO₃, 1.0 mM MgCl₂, pH 9.8) containing 0.35 M BCIP (5-bromo-4-chloro-3-indoyl-phosphate p-toluidine salt) and 0.37 M NBT (p-nitro blue tetrazolium chloride) for about 30 min. After washing with water, the deposition of the red or the purple stain indicating antigen-antibody binding was recorded by photography. The culture supernatant solution of hybridoma cells containing MoAb WH9 against the house dust mite allergen Der p 7 [12] and the cord serum were used as negative controls. Low MW standards (Pharmacia, Uppsala, Sweden) were used to determine the MW of the protein components. Carbamylated creatine kinase obtained from BDH (Poole, UK) was used as the charge standard for calculation of isoelectric point of the resolved proteins.

Periodate oxidation

Periodate oxidation of antigens was performed as described by Woodward *et al.* [13]. The antigens of *P. citrinum* coated onto microtitre plates were exposed to varying concentrations of sodium m-periodate (0, 0.1, 1.0, 10 and 20 mM; May & Baker, Dagenham, England). The activity of MoAbs to antigens treated by periodate oxidation was then determined by ELISA.

N-terminal amino acid sequence analysis

Components of *P. citrinum* strain 52-2 separated by 2D gel electrophoresis were transferred electrophoretically onto a PVDF membrane. The membrane-bound proteins were visualized by Coomassie blue staining. The protein spot of interest was then excised from the membrane and inserted into a sequencer for direct sequencing. Automated cycles of Edman degradation were performed with an ABI gas/liquid phase Model 470A sequencer (Applied Biosystems Inc., Foster City, CA, USA) with an on-line Model 120A (ABI) phenylthiohydantoin (PTH)-amino acid analyser as described [14].

Results

Preparation of MoAbs

Hybridomata were obtained by fusion of immunized murine spleen cells with plasmacytoma NS-1 cells. Hybridomata secreting MoAbs against *P. citrinum* were first screened by ELISA. In order to identify antigens recognized by antibodies, protein components in the crude extract of *P. citrinum* were separated by SDS-PAGE. Figure 1A shows that there were at least 25 different components with molecular weights (MWs) ranging from more than 100 to about 10 kD in the crude extract of *P. citrinum*. A hybridoma (55A) producing MoAbs which showed

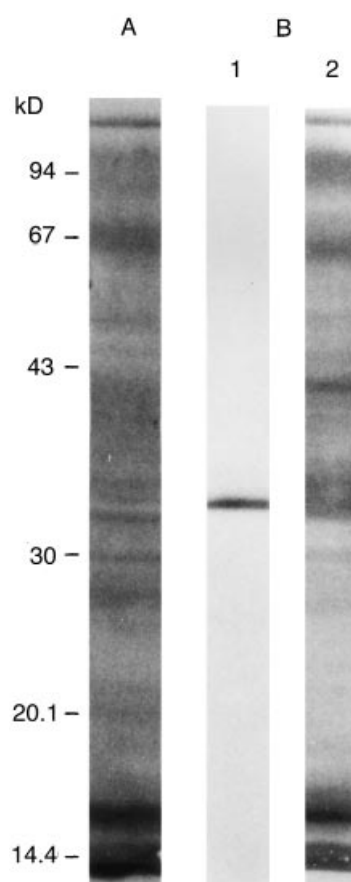


Fig. 1. SDS-PAGE-immunoblot analysis of antibody reactivity to components of *P. citrinum*. (A) Coomassie blue-stained protein profile of the crude extract of *P. citrinum* strain 52-5 separated by SDS-polyacrylamide gel electrophoresis. (B) Lanes 1 and 2 show the immunoblotting reactivity of MoAbs 55A and 34H, respectively to the crude fungal extract. The MW standards (Pharmacia, Uppsala, Sweden) 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).

immunoblotting reactivity to the 33 kD component (Fig. 1B-1) and another hybridoma (34H) producing MoAbs which showed immunoblotting activity to multiple components (including the 33 kD protein) (Fig. 1B-2) were finally chosen and expanded. The isotype of both MoAbs was IgG1, κ as analysed by ELISA using class-specific antisera.

2D-immunoblotting

In order to characterize further antigens recognized by MoAbs, 2D gel electrophoresis and immunoblotting were performed. Figure 2A shows the silver-stained two-dimensional protein profile of the crude extract of *P. citrinum*. More than 150 protein spots were detected in the

silver-stained 2D gel. To identify components corresponding to the 33 kD allergen, IgE-binding spots in 2D gel were visualized by immunoblotting using serum from an asthmatic patient with positive immediate skin test to an extract of *Penicillium* species supplied by the Taiwan Allergy Center, Taipei. After immunoblotting, six different components with MW of 33 kD and pI values ranging from about 6.75 to greater than 7.0 were shown to be reactive to IgE antibodies (Fig. 2B). The arrows in Fig. 2A indicate protein spots corresponding to those which showed enzymatic colour reaction in immunoblotting as shown in Fig. 2B. Components with similar pI values and MW were also shown to be reactive to MoAb 55A (Fig. 2C). In addition to these IgE-binding 33-kD components, MoAb 34H also showed reactivity to other components in the extract of

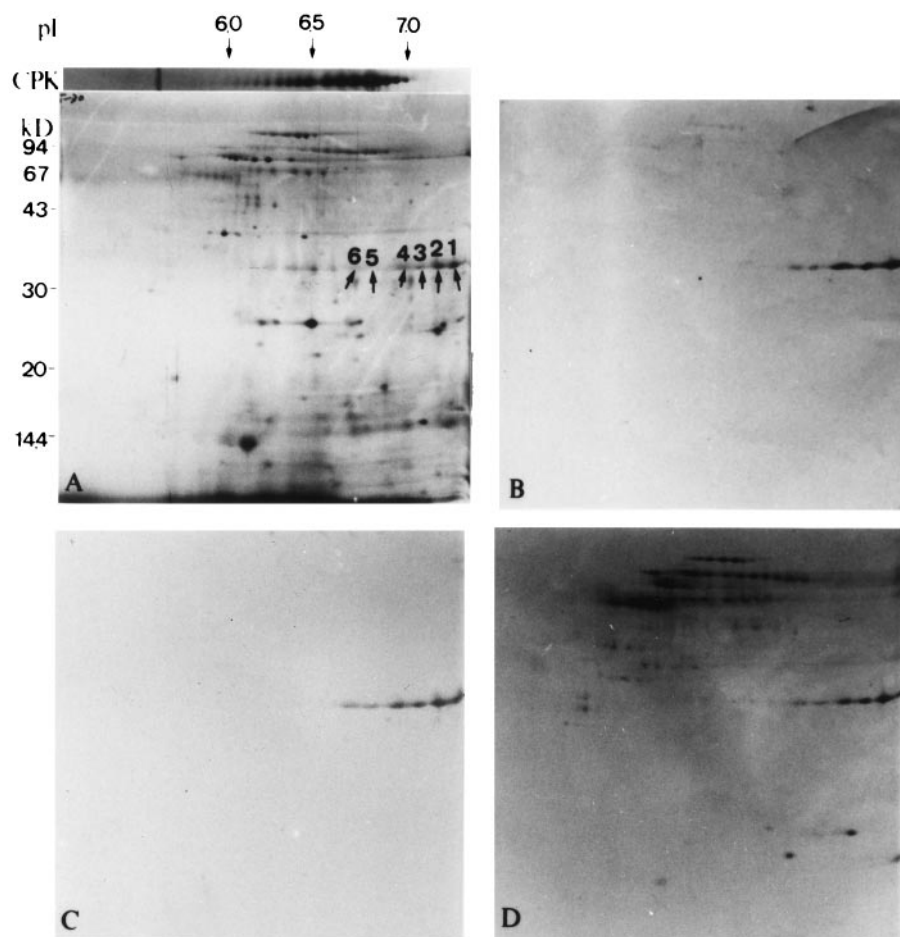


Fig. 2. 2D-immunoblot analysis of reactivity of IgE and monoclonal antibodies to components in the crude extract of *P. citrinum*. Panel A shows the silver-stained two-dimensional protein profile of the crude extract of *P. citrinum* strain 52-5. Panels B-D show the immunoblotting reactivity of IgE antibodies (B) and MoAbs 55A (C) and 34H (D), respectively, to components in the crude fungal extract. The arrows in panel A indicate protein spots corresponding to those which showed enzymatic colour reaction with an MW of about 33 kD in immunoblotting as shown in panels B-D. The same MW standards as shown in Fig. 1 were used to determine the MW of the protein components. Carbamylated creatine kinase (CPK) obtained from BDH (Poole, UK) was used as the charge standard for calculation of isoelectric point of the resolved proteins.

P. citrinum (Fig. 2D). For control, enzyme colour reaction was not detected when MoAb WH9 against Der p 7 or when the cord serum was used (data not shown).

Cross-reactivity

To test the presence of cross-reactivity among antigens of different fungal strains and species, SDS-PAGE-immunoblotting was performed using MoAbs obtained. Among four different strains of *P. citrinum* tested, MoAb 55A showed immunoblotting reactivity to the 33 kD components of strains 52–5, 23–98 and 30143 (Table 1). However, MoAb 34H showed immunoblotting reactivity to components of strains 52–5 and 30143 only (Table 1). A multiple-component reactivity similar to that observed between MoAb 34H and *P. citrinum* 52–5 was also detected when MoAb 34H was reacted with the crude extract of *P. citrinum* strain 30143 (data not shown). When both MoAbs were reacted with fungal extracts of another six different species of *Penicillium* and four different species of *Aspergillus* as mentioned in the materials and methods section, only immunoblotting reactivity of MoAb 55A to the 33 kD component of *P. brevicompactum* was observed (data not shown).

In 2D-immunoblotting, components of *P. brevicompactum* with an MW of about 33 kD and pI values ranging from about 6.8 to greater than 7.0 were shown to be reactive to MoAb 55A (Fig. 3B). The arrows in Fig. 3A indicate protein spots of *P. brevicompactum* in 2D gels corresponding to those reacted with MoAb 55A as shown in Fig. 3B. The 55A-binding 33 kD components of *P. brevicompactum* also showed 2D-immunoblotting reactivity to IgE antibodies in the same asthmatic serum sample used in Fig. 2B (data not shown).

Periodate oxidation

The epitopes on the 33 kD component of *P. citrinum* 52–5 recognized by both MoAbs 55A and 34H were relatively

resistant (less than 20% reduction of ELISA activity) to treatment of periodate oxidation with concentration of NaIO₄ up to 20 mM (data not shown).

N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the 33 kD component of *P. citrinum* strain 52–5 corresponding to the protein spot no. 2 in Fig. 2A analysed by using the automatic amino acid sequencer was determined to be ANVVQSNVP (Fig. 4). When it was compared with the protein data bank of IntelliGenetics (IntelliGenetics Inc, Mountain View, California, USA), seven of the nine amino acids were identical to those of a serine proteinase inactivating m-subunit of lactate dehydrogenase from *P. citrinum* KE-1 (KE) (Fig. 4) reported by Watazu *et al.* [15] (accession no. Pc2084). Furthermore, it was also found that the sequence obtained was the same as the sequence of the first nine N-terminal amino acids of a heat-labile serine proteinase of *P. citrinum* (PC in Fig. 4) reported by Yamamoto *et al.* [10].

Discussion

House dust mites, airborne pollens and fungal spores have long been considered as causative agents of extrinsic bronchial asthma. At least seven different groups of mite allergens have been characterized based on nucleotide sequences of known cDNAs, N-terminal sequences of the allergenic components or enzymatic functions [16]. Major allergens of weed pollens such as Amb a 1 and Amb a 2, of grass pollens such as Lol p 1, Lol p 2 and Lol p 3, and of tree pollens such as Bet v 1 and Bet v 2 have all been characterized through molecular biological approaches [17]. However, among numerous airborne fungi, only the sequence data of the 65, 90 and 55 kD allergens of *Aspergillus fumigatus*, the 40 kD allergen of *Candida albicans*, the 28 kD allergen of *Alternaria alternata* and the 30 kD allergen of *Trichophyton tonsurans* are available [17]. Recently, genes of several allergens of *Alternaria alternata* and *Cladosporium herbarum* have been cloned and sequenced [18,19]. Concerning allergens of *Penicillium*, only the 68 kD allergen of *P. notatum* has been identified by us as a beta-N-acetylglucosaminidase [20]. The difficulties associated with studies on mould allergens such as fungal identification, strain and species variability, differences in culture conditions and methods used for the preparation of allergenic extracts may all contribute to variations concerning the results obtained.

The 33 kD component has been identified as an important allergen of *P. citrinum*, the most prevalent *Penicillium* species in the Taipei area of Taiwan [8,9]. In the present study, two MoAbs against the 33 kD allergen of *P. citrinum* were generated. Analysed by 2D-immunoblotting using IgE antibodies and MoAbs generated, the 33 kD allergen has

Table 1. SDS-PAGE-immunoblotting reactivity of MoAbs 55A and 34H to different strains of *P. citrinum*.

<i>P. citrinum</i> strain	Immunoblotting reactivity	
	MoAb 55A	MoAb 34H
52–5	+	+
62–1	–	–
23–98	+	–
30143	+	+

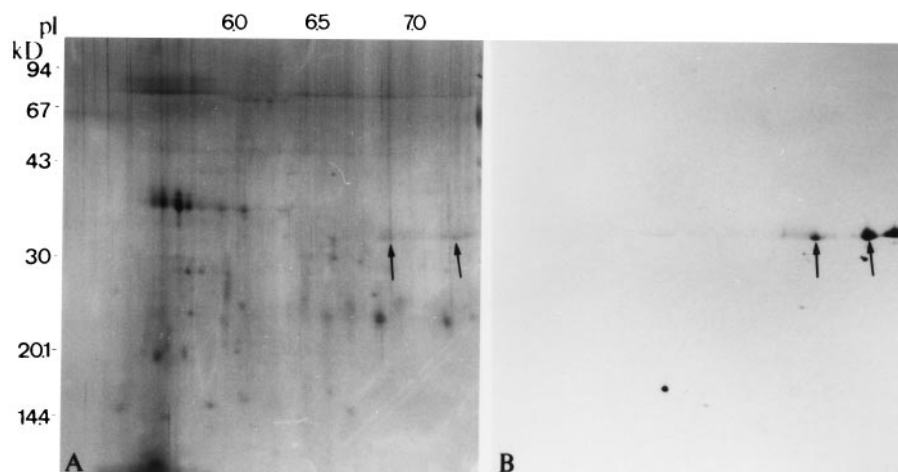


Fig. 3. 2D-immunoblot analysis of reactivity of MoAb 55A to components in the crude extract of *P. brevicompactum*. Panel A shows the silver-stained two-dimensional protein profile of the crude extract of *P. brevicompactum*. Panel B shows the immunoblotting reactivity of MoAb 55A to components in the fungal extract. The arrows in panel A indicate protein spots corresponding to those which showed enzyme colour reaction in immunoblotting as shown in panel B. The charge and MW standards used were the same as those described in Fig. 2.

been shown to contain at least six different isoforms with pI values ranging from about 6.75 to greater than 7.0 in the crude extract of *P. citrinum*. However, the significance of these different isoforms is currently unknown and needs to be clarified further. MoAbs obtained also demonstrated antigenic variability among components of different strains of *P. citrinum* tested. Significant strain variation among different strains of *A. fumigatus*, *C. herbarum*, *A. alternata* and *C. albicans* has also been reported before [21–25]. It was suggested that antigen from more than one single isolate of a certain fungal species should be used to prepare a high quality extract with representative antigenic/allergenic composition for diagnosis and treatment [21].

In the present study, antigenic cross-reactivity among components of different fungal species has also been analysed using MoAbs prepared. Among extracts of seven different *Penicillium* and four different *Aspergillus* species tested, only immunoblotting reactivity of MoAb 55A to the

33 kD components of *P. citrinum* and *P. brevicompactum* was observed. Results obtained from a separate study conducted by us showed that the 33 kD component is a major allergen of *P. brevicompactum* [9]. Furthermore, the 33 kD component in the extract of *P. notatum* obtained from Greer Laboratories (Lenoir, North Carolina, USA) used for skin testing has also been identified as an important IgE-binding component of this *Penicillium* species [9]. Thirteen (93%) of the 14 *P. notatum*-positive serum samples and 10 (91%) of the 11 *P. brevicompactum*-positive sera showed IgE reactivity to the 33 kD component in individual *Penicillium* extract [9]. In addition, IgE cross-reactivity among the 33 kD group major allergens of *P. citrinum*, *P. notatum* and *P. brevicompactum* was also detected [9]. All these results suggest that it is worthwhile to investigate further the antigenicity/allergenicity of components with an MW of about 33 kD of the other prevalent airborne *Penicillium* species.

The N-terminal amino acid sequence of the 33 kD allergen of *P. citrinum* was determined to be ANVVQSNVP which showed a high degree of sequence homology to that of a serine proteinase from *P. citrinum* KE-1 (KE) [15]. Besides, this sequence was also found to be identical to the first nine amino acids of an alkaline serine proteinase of *P. citrinum* (PC) reported by Yamamoto *et al.* [10]. The MWs of purified KE and PC were 32 000 and 29 000 Daltons, respectively, analysed by SDS-poly-acrylamide gel electrophoresis [10,15]. The isoelectric point of both purified enzymes was shown to be 9.5 [10,15].

According to Watazu, KE proteinase was unrelated to PC based on characteristics such as thermostability, molecular weight and amino acid composition [15]. However, the most

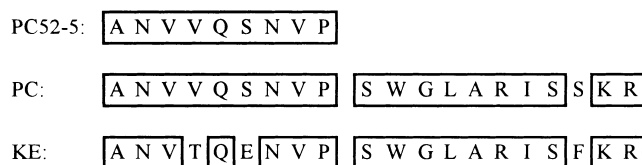


Fig. 4. The N-terminal amino acid sequence of the 33 kD allergen of *P. citrinum* strain 52–5 (PC 52–5) compared with the sequences of a heat-labile serine proteinase from *P. citrinum* (PC) (see ref. 10) and a serine proteinase inactivating m-subunit of lactate dehydrogenase from *P. citrinum* KE-1 (KE) (ref. 15).

susceptible bond of the initial site of cleavage by both enzymes KE and PC towards the oxidized B-chain of insulin was between Leu15 and Tyr16 where amino acids with large hydrophobic side chains are clustered [10, 15]. Thus, based on similarities at the N-terminal amino acid sequence, MW and alkaline pI values, it was suggested that the 33 kD major allergen of *P. citrinum* may be a serine proteinase of this prevalent *Penicillium* species. Supportive evidence for this suggestion comes from our recent finding that the nine N-terminal amino acid sequence (ANVVQSNVP) of the IgE-binding 33 kD component of *P. brevicompactum* is identical to that of *P. citrinum* strain 52-5 (data not shown).

The serine proteinase is widely distributed in various organisms [26]. The group 3 and group 6 allergens of house dust mites have been shown to belong to the trypsin and chymotrypsin-like serine proteinase family, respectively [17]. We are going to purify the 33 kD component of *P. citrinum* to characterize more exactly properties such as the enzymatic activity and the isoelectric point of this important *Penicillium* allergen. Furthermore, molecular cloning of the cDNA using MoAbs generated and oligonucleotides designed according to the N-terminal amino acid sequence obtained will also be undertaken to understand more about this 33 kD *Penicillium* allergen.

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