

cDNA Cloning and Immunological Characterization of a Newly Identified Enolase Allergen from *Penicillium citrinum* and *Aspergillus fumigatus*

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Key Words

Penicillium citrinum · *Aspergillus fumigatus* · Allergen ·
Enolase · cDNA cloning · IgE cross-reactivity ·

Abstract

Background: *Penicillium citrinum* and *Aspergillus fumigatus* are prevalent indoor airborne fungal species that have been implicated in human respiratory allergic disorders. It is important to understand the allergenic profile of these fungal species. The purpose of the present study is to characterize a newly identified enolase allergen from *P. citrinum* and *A. fumigatus*. **Methods:** Fungal proteins were separated by two-dimensional (2D) gel electrophoresis and blotted onto polyvinylidene difluoride membranes. Protein spots that reacted with IgE antibodies in serum samples from asthmatic patients were identified and the N-terminal amino acid sequences were determined by Edman degradation. The peptide sequences obtained were utilized in cloning the cDNA of the allergen genes by reverse transcriptase-polymerase chain reaction and the 5'- and 3'-rapid amplification cDNA end reactions. **Results:** Our results from 2D immunoblotting identified a 47-kD IgE-reactive component in the extracts of *P. citrinum* and *A. fumigatus*. The N-termi-

nal amino acid sequences of the 47-kD proteins are homologous to those of fungal enolases. The corresponding enolase cDNA from *P. citrinum* contains 1,552 bp and encodes a protein of 438 residues. In *A. fumigatus*, the isolated enolase cDNA has 1,649 bp and contains a 438-amino acid open reading frame. The deduced amino acid sequences of these two enolases have 94% identity. These enolases from *P. citrinum* and *A. fumigatus* were expressed in *Escherichia coli* as a His-tagged protein and designated as rPen c 22 and rAsp f 22, respectively. Sera from 7 (30%) of the 23 *Penicillium*-sensitized asthmatic patients showed IgE binding to the 47-kD *P. citrinum* component (Pen c 22) and rPen c 22. In addition, six of seven Pen c 22-positive serum samples have IgE immunoblot reactivity to the 47-kD *A. fumigatus* component (Asp f 22) and rAsp f 22. A polyclonal rabbit antiserum generated against the N-terminal peptide of Pen c 22 can react with Pen c 22, rPen c 22, Asp f 22 and rAsp f 22. In addition, the presence of IgE cross-reactivity between rPen c 22 and rAsp f 22 and between enolases from *A. fumigatus* and *Alternaria alternata* was also detected by immunoblot inhibition. **Conclusions:** These results demonstrated that a novel enolase allergen from *P. citrinum* (Pen c 22) and *A. fumigatus* (Asp f 22) was identified. In addition, IgE cross-reactivity between eno-

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lase allergens from *A. fumigatus* and *P. citrinum* and between enolases from *A. fumigatus* and *A. alternata* was also detected. Results obtained provide more information on fungal enolase allergens.

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Introduction

Fungal spores are ubiquitous in our environment. They have been implicated in human respiratory allergic disorders [1, 2]. More than 60 genera of fungi have been associated with symptoms of respiratory tract allergy [3, 4]. Among fungi of allergologic interest, *Penicillium* and *Aspergillus* are the two most frequently encountered genera of indoor fungi [1, 5–9]. Extracts of both fungal genera have long been used in clinical diagnosis of mould allergy. More than 100 different species have been identified in the fungal genera *Penicillium* and *Aspergillus* [10, 11]. However, information on allergens and allergenic relationship between allergens from these fungal species is still quite limited. Consequently, clinical diagnosis of mould allergy is far from satisfactory [4, 8].

We have shown previously that the alkaline/vacuolar serine proteases are major allergens in eight prevalent *Penicillium* and *Aspergillus* species (*Penicillium citrinum*, *Penicillium brevicompactum*, *Penicillium notatum*, *Penicillium oxalicum*, *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*) [12–17]. IgE cross-reactivity among these serine protease allergens was also detected [13–16]. In addition, we have documented that a heat shock protein in the hsp70 family [18] and an 18-kD peroxisomal membrane protein [19] are also important allergens of *P. citrinum*. In addition to the serine protease and peroxisomal membrane protein [20] allergens, more than 10 different *A. fumigatus* allergens have also been identified through molecular approaches [21, 22].

In order to elucidate the full-spectrum allergenic profile of prevalent fungal species, protein components of fungal extracts from *P. citrinum* and *A. fumigatus* were separated by using two-dimensional (2D) gel electrophoresis and immunoblotted with serum samples from asthmatic patients in this study. A 47-kD IgE-binding component was detected in both extracts. The N-terminal amino acid sequences of these 47-kD components were determined and the corresponding complementary DNA (cDNA) clones were isolated. These components were identified as enolases and were designated as Pen c 22 and Asp f 22 allergens of *P. citrinum* and *A. fumigatus*, respec-

tively, by the Allergen Nomenclature Sub-Committee. IgE cross-reactivity between the enolase allergens from both fungal species was also detected. The enolase has been identified a conserved allergen in many different fungal species [23]. In addition to Pen c 22 and Asp f 22, IgE reactivity in serum samples from asthmatic patients to enolase allergens from *Cladosporium herbarum*, *Alternaria alternata*, *Saccharomyces cerevisiae* and *Candida albicans* was also determined in the present study. Our results showed partial IgE cross-reactivity between enolase allergens from *A. fumigatus* and *A. alternata*.

Methods

Serum Samples

Serum samples were collected from patients with bronchial asthma and attending the allergy clinics at the Veterans General Hospital-Taipei or the Cathay General Hospital-Taipei. These 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 Study [24]. After collection, serum samples were stored in aliquots at -70°C .

Allergenic Extracts of *P. citrinum* and *A. fumigatus*

Crude allergenic extracts of *P. citrinum* and *A. fumigatus* were prepared essentially as previously described [12, 16]. The *P. citrinum* strain 52-5 [12] was isolated from the air in the Taipei area and verified by the American Type Culture Collection. The *A. fumigatus* strain used was CCRC33476 [16]. It was isolated from the air of the Tainan area in Taiwan and verified by the Food Industry Research and Development Institute, Hsinchu, Taiwan. The fungal strains were grown individually in a liquid medium containing Czapek concentrate [10] (10 ml/l), K_2HPO_4 (1.0 g/l), yeast extract (Difco, Detroit, Mich., USA; 5 g/l) and sucrose (30 g/l). Incubation was carried out without shaking at 25°C for 4 days for *P. citrinum* and with shaking at 30°C for 3 days for *A. fumigatus*. The fungal mat containing both spores and mycelia was collected and ground under liquid nitrogen with mortar and pestle. It was then resuspended in a phosphate-buffered saline (85 mmol/l of NaCl, 2.6 mmol/l of KH_2PO_4 , and 49 mmol/l of Na_2HPO_4 , pH 8.0) and extracted for 4 h at 4°C with constant shaking. After centrifugation, the supernatant was dialyzed 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 μm ; Sartorius, Hayward, Calif., USA), lyophilized in aliquots and stored at -20°C . The protein content of the extracts was determined by the dye-binding method of Bio-Rad (Richmond, Calif., USA).

Gel Electrophoresis and Immunoblotting

Protein components in the crude fungal extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or by 2D gel electrophoresis and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membrane (0.45 μm , Millipore, Bedford, Mass., USA) as described [12–16]. The membrane was blocked with 1% skim milk, then incubated with diluted human serum samples (1:5 dilution in Tris-buffered saline, pH 7.5, 0.05% Tween 20 and 1% skim milk) for 16 h at 4°C . After washing,

Table 1. Primers used in PCR

Name	Nucleotide sequence in 5'- to 3'-end orientation
Eno2	GTGAGAGACCATGACACCCCA
Eno3	GTTTACGACTCCCGCGGTAAC
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
AUAP	GGCCACGCGTCGACTAGTAC
183	TACTTCTTCTTGGCCAGAG
AP	GGCCACGCGTCGACTAGTACT-(dT)16
Eno5	TGCTCAAGGTCAACCAGATC
192	TTGTCAACGTCACGGAGCTC
5BamHI-Eno-Pc	GCGGATCCATGCCTATCGCCAAGGTTC
5BamHI-Eno-Af	GCGGATCCATGCCTATCTCCAAGATC
3PstI-Eno	AACTGCAGCTGCATTTACAAGTTGACG
5BamHI-Eno-Ca	CGGGATCCTCTTACGCCACTAAAATC
3PstI-Eno-Ca	AACTGCAGTTACAATTGAGAAGCCTTTTG

the human IgE antibodies bound to the allergens on the blot were detected with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, Calif., USA) for 1 h at room temperature. The human IgE on the blot was then developed with a substrate solution of carbonate buffer (0.1 mol/l NaHCO₃, pH 9.8, and 1.0 mmol/l MgCl₂), containing 0.35 mmol/l 5-bromo-4-chloro-3-indolyl-phosphate and 0.37 mmol/l *p*-nitroblue tetrazolium chloride for about 30 min. Serum samples from healthy laboratory personnel were used as negative controls. Low molecular weight standards (Pharmacia, Uppsala, Sweden) were used to estimate the molecular mass 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.

A peptide with sequence identical to that of the N-terminal 21-amino acid residues of the 47-kD IgE-reacting component from *P. citrinum* was custom-made by Genemed Biotechnologies (South San Francisco, Calif., USA). This peptide was used to raise rabbit polyclonal antibodies (Genemed Biotechnologies) for immunoblotting experiments. The rabbit antibodies on the blot were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA). For the visualization of rabbit antibodies, the blot was developed in a substrate solution of acetate buffer (50 mmol/l, pH 5.0) containing 1 mmol/l 3-amino-9-ethyl-carbazole (Sigma, St. Louis, Mo., USA) and 0.015% (v/v) hydrogen peroxide for about 5 min. A normal rabbit serum was used as negative control.

N-Terminal Amino Acid Sequence Analysis

Components of *P. citrinum* and *A. fumigatus* were separated by 2D gel electrophoresis and transferred electrophoretically onto PVDF membranes. The 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 instruction.

Cloning and Molecular Techniques

Total RNA was extracted from a 1-day-old fungal mat culture grown at 30°C with constant shaking. The fungal mat was ground

under liquid nitrogen with mortar and pestle, then resuspended in a Trizol reagent (Life Technologies, Grand Island, N.Y., USA). Total RNA was extracted according to the instructions provided by the manufacturer. The first-strand cDNA was synthesized by using reverse transcriptase (MMLV-RT, Moloney murine leukemia virus reverse transcriptase, Stratagene, La Jolla, Calif., USA) and oligo-(dT) as primer. The cDNA of the *P. citrinum* enolase was cloned with polymerase chain reaction (PCR). The primers (table 1) used were designed according to the conserved amino acid sequences among fungal enolases. The sense (Eno3) and the antisense (Eno2) primers encode the N-terminal (VYDSRGN) and the C-terminal (WGVMVSH) conserved amino acids, respectively. The PCR product was gel purified and subcloned into the pGem-Teasy vector (Promega, Madison, Wisc., USA) and transformed into *Escherichia coli* JM109 competent cells. The plasmid DNA was purified and the nucleotide sequence of the cDNA insert was determined with an ABI 377 automatic sequencer (Applied Biosystems).

The full-length cDNA of the *P. citrinum* enolase was obtained through the 5'- and the 3'-end RACE (rapid amplification of cDNA ends) reactions. The template cDNA used for the 5'-RACE was synthesized by MMLV reverse transcriptase using primer Eno2 (table 1). After reaction, the RNA was removed by RNase H (Life Technologies) digestion and the remaining cDNA was purified by precipitation with EzpptTM DNA carrier (GeneTeks BioScience, Taipei, Taiwan). An oligo-(dC) was added to the end of the purified cDNA with terminal deoxynucleotidyl transferase (Promega, Madison, Wisc., USA). The 5'-RACE experiment was carried out with primers AAP and 183 (table 1). Primer AAP contains the sequence AUAP and oligo-(dG) while primer 183 was synthesized according to the internal sequence (nucleotide No. 697–715) of the *P. citrinum* enolase cDNA obtained in the previous PCR reaction.

The template cDNA used for the 3'-RACE was synthesized by MMLV reverse transcriptase using primer AP (table 1) that contains the sequence of primer AUAP and oligo-(dT). The primers used for the 3'-RACE were primers AUAP and Eno 5 (table 1). Primer Eno 5 corresponds to nucleotide No. 1120–1139 of the *A. fumigatus* enolase cDNA. It also comports to nucleotide No. 1147–1166 of the *P. citrinum* enolase cDNA with only one nucleotide difference (C to G substitution at No. 1151). Primer Eno 5 was thus used in the 3'-RACE reactions for both enolase allergens. Products from the RACE

reactions were purified individually, subcloned, transformed and sequenced as described above.

The cDNA of the *A. fumigatus* enolase was isolated essentially as described for the *P. citrinum* enolase. Primer 192 (table 1) synthesized according to the internal sequence (nucleotide No. 228–247) of the *A. fumigatus* enolase was used in the 5'-RACE reaction.

Preparation of Recombinant Proteins

The enolases of both fungal species were expressed as 6 × His-tagged proteins according to the manufacturer's instructions (Qiagen, Valencia, Calif., USA). The cDNAs of the enolases were amplified through PCR. The forward primers used in the reactions are 5BamHI-Eno-Pc and 5BamHI-Eno-Af (table 1) that contain the *Bam*HI restriction site in addition to the sequences encoding the N-terminal 6-amino acid residues of the enolases from *P. citrinum* and *A. fumigatus*, respectively. The same reverse primer, 3PstI-Eno (table 1) was used in the PCR amplification of cDNAs for both enolases. It contains a *Pst*I restriction site and encompasses nucleotide sequence 1418–1436 of the 3'-end of the *P. citrinum* enolase cDNA. It also covers nucleotide sequence 1391–1409 of *A. fumigatus* enolase with a single nucleotide substitute (T to C) at position 1398 but results in no amino acid change. The PCR products were separated by agarose gel electrophoresis and purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). It was digested with *Bam*-HI and *Pst*I restriction enzymes and ligated into the pQE-30 vector after repurification. The resulting vector was transformed into *E. coli* JM109 and M15. The recombinant polypeptides expressed in *E. coli* M15 was affinity-purified, under denaturing conditions from a Ni-NTA resin column (Qiagen). The reactivity of the recombinant proteins with IgE antibodies in serum samples from asthmatic patients and a rabbit anti-N-terminal peptide antiserum was analyzed by SDS-PAGE immunoblot as described above.

In this study, the enolase of *C. albicans* was also expressed as 6 × His-tagged protein prepared essentially as described above. The *C. albicans* strain used (CCRC21538) was obtained from the Food Industry Research and Development Institute, Hsinchu, Taiwan. The primers used in the PCR amplification of cDNA for the *C. albicans* enolase were 5BamHI-Eno-Ca and 3PstI-Eno-Ca (table 1). The forward primer 5BamHI-Eno-Ca contains the *Bam*HI restriction site in addition to the sequences encoding the N-terminal 6-amino acid residues (SYATKI) of the enolase from *C. albicans* (P30575). The reverse primer 3PstI-Eno-Ca has a *Pst*I restriction site and sequences encoding a stop codon and the C-terminal 6-amino acid residues (QKASQL) of the *C. albicans* enolase. The recombinant polypeptide expressed in *E. coli* M15 was affinity-purified from a Ni-NTA resin column (Qiagen). In addition, recombinant enolase of *C. herbarum* used in this study was a kind gift from Dr. Birgit Simon-Nobbe of the Department of Genetics and General Biology, University of Salzburg, Austria. The recombinant enolase of *A. alternata* and the purified enolase of *S. cerevisiae* were obtained from Biomay Productions (Vienna, Austria) and from Sigma, respectively. Reactivity of the enolase proteins with IgE antibodies in serum samples from asthmatic patients was analyzed by SDS-PAGE immunoblot as described previously.

Results

2D Immunoblot and N-Terminal Amino Acid Sequence Analysis

The allergens of *P. citrinum* and *A. fumigatus* were characterized with 2D immunoblotting using serum samples from asthmatic patients. Preliminary studies have shown that serum samples used have IgE reactivity to components in both fungal species (data not shown). The Coomassie blue-stained 2D protein profiles of the *P. citrinum* and the *A. fumigatus* extracts on PVDF membranes are shown in figure 1a and 2a, respectively. Fungal components that reacted with IgE antibodies in serum samples tested were shown in figures 1b and 2b. Components as indicated by arrows in figure 1 (PC1) and figure 2 (AF1) with an apparent molecular mass of about 47 kD were selected for N-terminal amino acid sequence analysis. The sequences determined are PIAKVHARSVY-DSRGNPTVEV and PISKIHARSVYDSRGNPTVEV, respectively, for spots PC1 and AF1. They have high sequence homology to those of the enolases from *A. oryzae* (accession No. Q12560), *C. albicans* (P30575) and *S. cerevisiae* (ENO1, P00924; ENO2, P00925). The 47-kD allergens of *P. citrinum* and *A. fumigatus* identified have been designated as Pen c 22 and Asp f 22, respectively, by the Allergen Nomenclature Sub-Committee.

cDNA Cloning and Sequencing

The cDNAs of Pen c 22 and Asp f 22 were constructed by reverse transcriptase-polymerase chain reaction (RT-PCR) coupled with the 5'- and the 3'-end RACE reactions. The nucleotide sequence of the 1,552-bp Pen c 22 cDNA was submitted to GenBank with the accession No. AF254643. The putative polyadenylation signal 'AA-TAAG' is located 12–17 nucleotides upstream of the poly A tail (data not shown). The deduced amino acid sequence of the open reading frame encodes a polypeptide of 438 residues including the initiator methionine. Except for the 12th residue, the peptide sequence determined experimentally for the N-terminal 21 amino acids of Pen c 22 is identical to that of residues 2–22 deduced from the cDNA. Therefore, the mature Pen c 22 starts from Pro2 and has a calculated molecular mass of 47,144 daltons, excluding further posttranslational modifications. It was also found that Pen c 22 has one cysteine (Cys341) and one putative N-glycosylation site (Asn70Glu71Thr72).

The nucleotide sequence of the 1,649-bp Asp f 22 cDNA was also submitted to GenBank with the accession No. AF284645. The putative polyadenylation signal (AATGAA) is located 16–21 nucleotides upstream of the

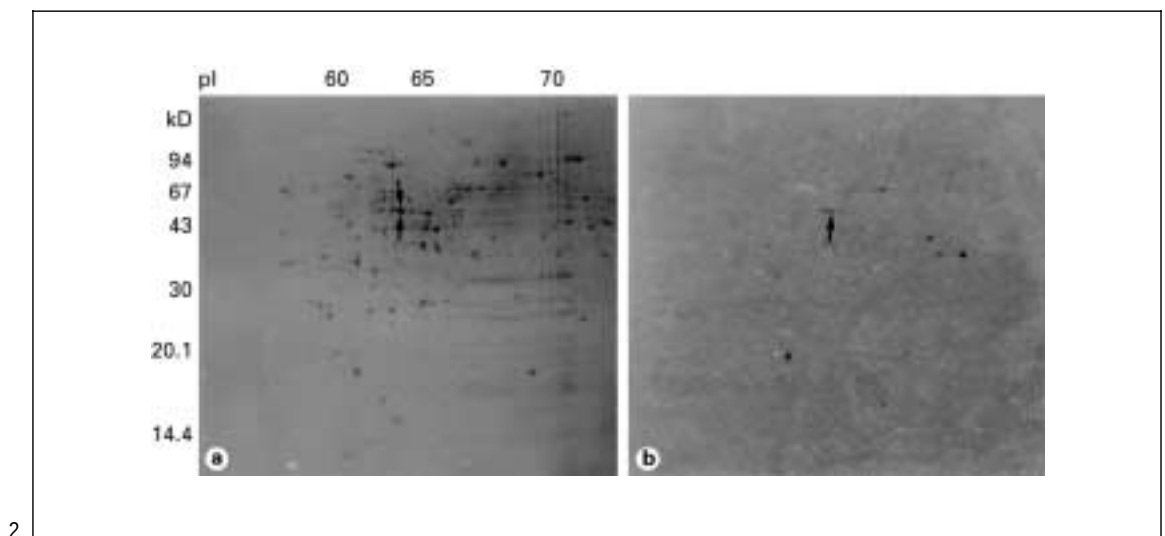
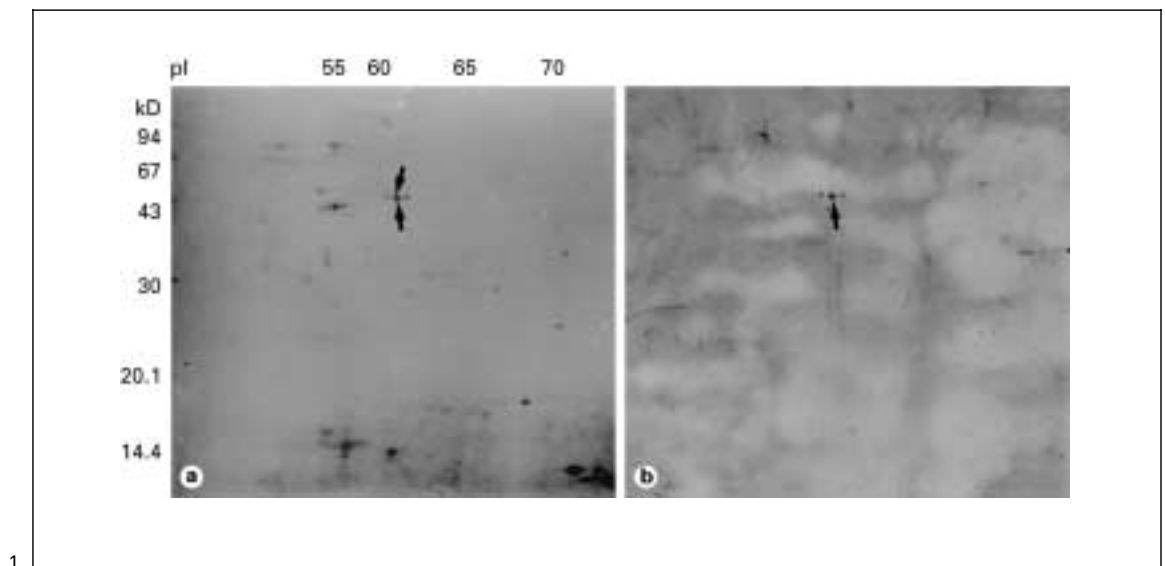


Fig. 1. 2D immunoblot analysis of components in *P. citrinum* 52-5 extracts reacted with human IgE antibodies. a Coomassie blue-stained 2D protein profile of the crude extracts on a PVDF blot. b Immunoblot reactivity of IgE antibodies in serum samples from asthmatic patients to components in the crude *P. citrinum* 52-5 extracts. The arrows in a indicate the protein spot (PC1) which corresponds to that identified in the immunoblot of b by enzymatic color reaction.

Fig. 2. 2D immunoblot analysis of components in *A. fumigatus* CCRC33476 extracts reacted with human IgE antibodies. a Coomassie blue-stained 2D protein profile of the crude extracts on a PVDF blot. b Immunoblot reactivity of IgE antibodies in serum samples from asthmatic patients to components in the crude *A. fumigatus* CCRC33476 extracts. The arrows in a indicate the protein spot (AF1) which corresponds to that identified in the immunoblot of b by enzymatic color reaction.

poly-A tail (data not shown). The deduced amino acid sequence of the open reading frame also encodes a 438-amino acids polypeptide. The N-terminal 21 amino acids (No. 2–22) of the encoded polypeptide, excluding the initiator methionine, are identical to those determined for

Asp f 22 by Edman degradation. Therefore, the mature Asp f 22 starts from Pro2 and has a calculated molecular mass of 47,204 daltons, excluding posttranslational modifications.

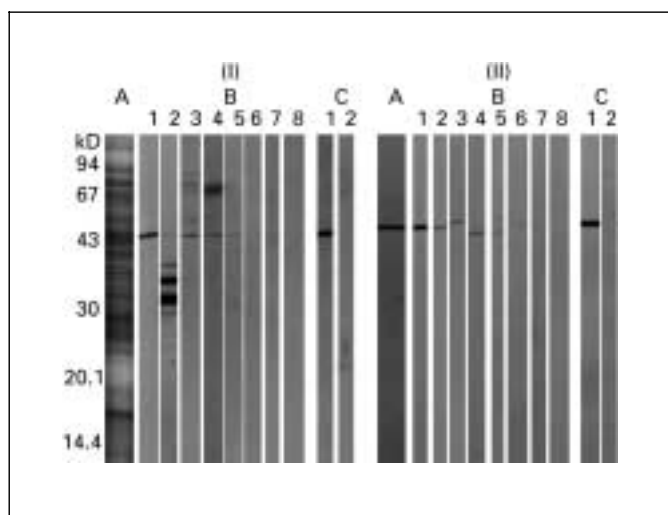


Fig. 3. Immunoblot reactivity of IgE antibodies in serum samples from asthmatic patients and a rabbit antiserum against the N-terminal peptide (21 residues) of Pen c 22 to components of *P. citrinum* and rPen c 22. Panels I-A and II-A show the Coomassie blue-stained protein profiles of the *P. citrinum* extracts and the rPen c 22 on PVDF membranes, respectively. Immunoblots generated with serum samples from 7 asthmatic patients are presented in strips 1–7 of both panels I-B and II-B. Immunoblots generated with the rabbit antiserum are presented in strip 1 of panels I-C and II-C. Strip 8 in panels I-B and II-B represents a negative control using serum obtained from a healthy laboratory volunteer. Strip 2 in panels I-C and II-C represents a negative control obtained with a normal rabbit serum.

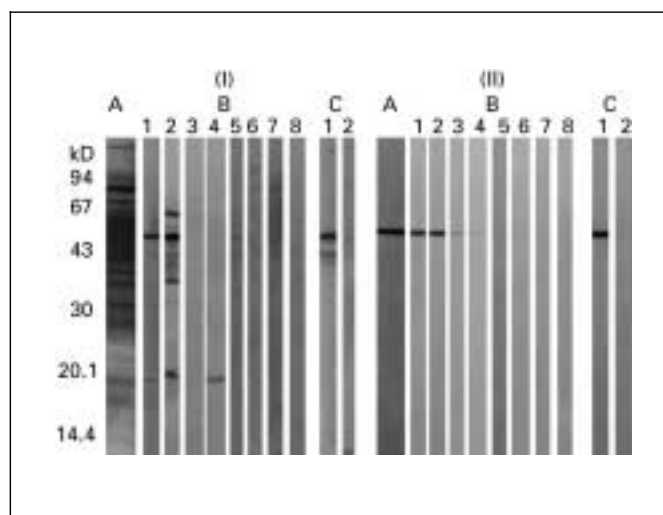


Fig. 4. Immunoblot reactivity of IgE antibodies in serum samples from asthmatic patients and a rabbit antiserum against the N-terminal peptide (21 residues) of Pen c 22 to components of *A. fumigatus* and rAsp f 22. Panels I-A and II-A show the Coomassie blue-stained protein profiles of the *A. fumigatus* extracts and the rAsp f 22 on PVDF membranes, respectively. Immunoblots generated with the same 7 serum samples from asthmatic patients as shown in figure 3 are presented in strips 1–7 of panels I-B and II-B. Immunoblots generated with the rabbit antiserum are presented in strip 1 of panels I-C and II-C. Strip 8 in panels I-B and II-B represents a negative control using serum obtained from the same healthy laboratory volunteer as shown in figure 3. Strip 2 in panels I-C and II-C represents a negative control obtained with the same normal rabbit serum used in figure 3.

Amino acid sequence alignment revealed that Asp f 22 and Pen c 22 share 94% sequence identity (412 out of 438 amino acids). Asp f 22 has two cysteines (Cys341, Cys403) and two putative N-glycosylation sites (Asn70Glu71Thr72, Asn102Lys103Ser104). Cys341 and one of the two potential N-glycosylation sites are conserved in both Pen c 22 and Asp f 22. Protein database search unveiled that 382 (87%), 374 (85%), 313 (72%) and 328 (75%) of the 438-amino acid residues in Asp f 22 are identical to those of the enolase allergens from *A. alternata* (accession No. U82437) [23], *C. herbarum* (accession No. X78226) [25], *S. cerevisiae* (accession No. P00925) [26] and *C. albicans* (accession No. P30575) [27], respectively. It has also been shown that the 3 potential N-glycosylation sites of *C. albicans* enolase were not glycosylated [28].

Characterization of Recombinant Pen c 22 and Asp f 22

Pen c 22 and Asp f 22 were expressed as His-tagged proteins in *E. coli*. The recombinant proteins (rPen c 22 and rAsp f 22) were produced as inclusion bodies and purified in denaturing conditions. The purified proteins have an estimated molecular mass of 49 kD upon SDS-PAGE (fig. 3, 4, strips II-A).

A panel of 97 serum samples from asthmatic patients was used in the analysis of IgE reactivity to components of *P. citrinum*. Seven (30%) of the 23 *P. citrinum*-positive serum samples showed IgE reactivity to a 47-kD component of *P. citrinum* (fig. 3, strips 1–7 of panel I-B). A peptide that has the sequence of the N-terminal 21 residues of Pen c 22 was synthesized (Genemed Biotechnologies). A rabbit antiserum raised against this peptide can react with the 47-kD component (fig. 3, strip 1 of panel I-C) and rPen c 22 (fig. 3, strip 1 of panel II-C). In addition, the seven serum samples with IgE reactivity to the 47-kD

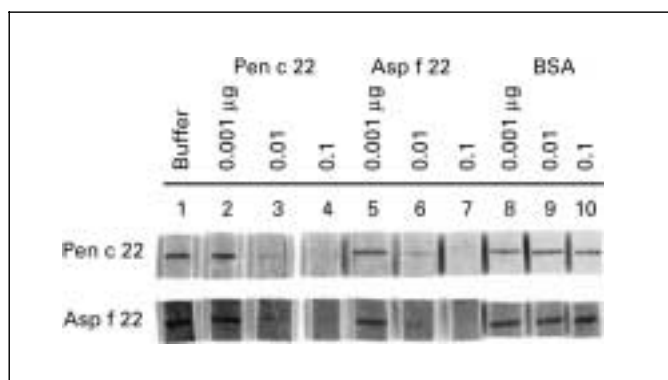


Fig. 5. Inhibition of IgE immunoblot reactivity to rPen c 22 and rAsp f 22 using recombinant proteins. Strips 1 in both panels show the IgE immunoblot reactivity of a serum sample (serum No. 1 in fig. 3 and 4) against individual recombinant allergen. Strips 2–10 represent results of immunoblot obtained by incubating the PVDF blots with the same serum sample that has been mixed and preincubated with 0.001, 0.01 and 0.1 µg, respectively, of rPen c 22 (strips 2–4), rAsp f 22 (strips 5–7) and bovine serum albumin (BSA, strips 8–10).

component also have IgE immunoblot reactivity against rPen c 22 (fig. 3, strips 1–7 of panel II-B).

The reactivity of IgE antibodies in serum samples from the 7 asthmatic patients to components of *A. fumigatus* and rAsp f 22 was also analyzed by immunoblotting. Six (sera No. 1–6) of the seven positive serum samples showed IgE binding to a 47-kD component of *A. fumigatus* (fig. 4, strips 1–6 of panel I-B) and the rAsp f 22 (fig. 4, strips 1–6 of panel II-B). In addition, an immunoblot reactivity of the 47-kD protein (fig. 4, strip 1 of panel I-C) and the rAsp f 22 (fig. 4, strip 1 of panel II-C) to the rabbit antiserum against the N-terminal peptide of Pen c 22 was also detected. For negative controls, immunoblot reactivity of a serum sample from a normal laboratory volunteer and a normal rabbit serum were used (fig. 3 and 4; strips 8 and 2 in panels B and C, respectively).

IgE Cross-Reactivity

The presence of IgE cross-reactivity between Pen c 22 and Asp f 22 was analyzed by immunoblot inhibition using serum No. 1 in figures 3 and 4. Since the recombinant enolases were produced in inclusion bodies, they were solubilized in 8 M urea and affinity purified under denaturing conditions. The purified recombinant proteins were refolded by dialysis against phosphate-buffered saline solutions with decreasing concentrations of urea (6, 4, 2, 1 and 0 M). The renatured rPen c 22 and rAsp f 22 proteins were used as inhibitors in the immunoblot inhi-

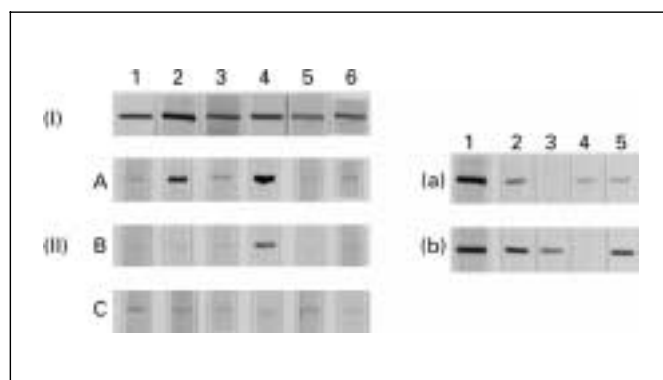


Fig. 6. Immunoblot reactivity of IgE antibodies in three serum samples (A, B and C) from asthmatic patients to six fungal enolase proteins and inhibition of IgE immunoblot reactivity of serum sample A to enolases of *A. fumigatus* and *A. alternata* using recombinant proteins. Strips 1–6 of part I show the Coomassie blue-stained protein profiles of enolases of *P. citrinum*, *A. fumigatus*, *C. herbarum*, *A. alternata*, *S. cerevisiae* and *C. albicans* on PVDF membranes, respectively. Immunoblots generated with serum samples from 3 asthmatic patients are presented in strips 1–6 of parts II-A, II-B and II-C. Strips 1 of parts a and b show the Coomassie blue-stained protein profiles of enolases of *A. fumigatus* and *A. alternata* on PVDF membranes, respectively. Strips 2 in both parts a and b show the IgE immunoblot reactivity of serum sample A against individual recombinant allergen. Strips 3–5 represent results of immunoblot obtained by incubating the PVDF blots with the same serum sample that has been mixed and preincubated with 10 µg of *A. fumigatus* enolase (strips 3), *A. alternata* enolase (strips 4) and bovine serum albumin (strips 5), respectively.

bition study. The serum aliquots were preincubated at 4°C for 2 h with 0.001, 0.01 and 0.1 µg of inhibitor proteins, and tested for IgE binding by SDS-PAGE immunoblot with membranes blotted with rPen c 22 or rAsp f 22. Dose-dependent inhibition of the IgE reactivity to rPen c 22 (fig. 5) and rAsp f 22 proteins was detected with serum that has been preincubated with allergen polypeptides. No inhibition was detected when 0.001, 0.01 and 0.1 µg of bovine serum albumin was used as an inhibitor (fig. 5).

Detection of IgE reactivity to fungal enolases was also performed by SDS-PAGE immunoblot by screening another batch of serum samples from asthmatic patients. Three serum samples (serum A, B and C) showed IgE binding to enolase proteins from *P. citrinum* and *A. fumigatus* (fig. 6, strips 1 and 2 of part II). Serum C also showed IgE reactivity to enolases of *C. herbarum*, *A. alternata*, *S. cerevisiae* and *C. albicans* (fig. 6, strips 3–6 of part II-C). IgE antibodies in serum B displayed positive, relatively strong, negative, and borderline IgE-binding capacities to enolase proteins from *C. herbarum*, *A. alter-*

nata, *S. cerevisiae* and *C. albicans*, respectively (fig. 6, strips 3–6 of part II-B). Serum A showed IgE binding to enolases of *C. herbarum*, *A. alternata* and *C. albicans*, and borderline IgE reactivity to *S. cerevisiae* enolase (fig. 6, strips 3–6 of part II-A).

The presence of IgE cross-reactivity between enolases from *A. fumigatus* and *A. alternata* was also analyzed by immunoblot inhibition. The aliquots of serum sample A were mixed and preincubated at 4°C for 6 h with 10 µg of inhibitor proteins, and tested for IgE binding by SDS-PAGE immunoblot with membranes blotted with enolases from *A. fumigatus* and *A. alternata*. Inhibition of IgE binding to *A. fumigatus* enolase by about 92 and 39% was detected with serum aliquots that have been preincubated with 10 µg of enolases from *A. fumigatus* (strip a-3) and *A. alternata* (strip a-4), respectively (fig. 6). Inhibition of IgE binding to *A. alternata* enolase by 30 and 96% was also detected by preincubation of the same serum with 10 µg of the enolase proteins of *A. fumigatus* (strip b-3) and *A. alternata* (strip b-4), respectively. Inhibition of IgE binding to enolases of *A. fumigatus* (strip a-5) and *A. alternata* (strip b-5) by 15 and 9%, respectively, was detected when 10 µg of bovine serum albumin was used as a control inhibitor. The intensities of the bands were analyzed in ImageMaster VDS equipped with TotalLab software version 1.0 (Amersham Pharmacia Biotech, Piscataway, N.J., USA).

Discussion

In the present study, results from 2D immunoblot and N-terminal amino acid sequence analyses suggest that the 47-kD IgE-reacting components of *P. citrinum* and *A. fumigatus* are enolases. Based on the amino acid obtained, the full-length cDNAs of the enolases were cloned through RT-PCR and RACE reactions. The N-terminal peptide sequences of the 47-kD IgE-reacting components are almost identical to those deduced from the cDNA clones. The only deviation observed is the valine at residue 13 of the Pen c 22 cDNA clone. The corresponding amino acid is an aspartic acid on the 47-kD protein from *P. citrinum*. However, the PCR product obtained in the preparation of the Pen c 22 recombinant protein was sequenced. The corresponding amino acid residue deduced from the nucleotide sequence determined was found to be an aspartic acid. It was found that, instead of a T, an A at nucleotide position 151 was determined. Thus, the original GTC codon that encodes for a valine was replaced by GAC that encodes for an aspartic acid. In addition, the PCR prod-

uct for the preparation of rAsp f 22 was also sequenced. A C to T transition at nucleotide position 160 that results in a change of the deduced amino acid residue from A to V was also detected (data not shown). The discrepancy observed may represent the presence of isoforms or due to experimental variations. Our results from 2D immunoblot showed that at least three spots in the *P. citrinum* and *A. fumigatus* extracts are IgE-reacting (fig. 1, 2). These spots have a molecular mass of 47 kD and pIs ranging from 6.0 to 6.5. It suggests the possible presence of enolase isoforms.

A peptide was constructed according to the N-terminal sequence of the 47-kD IgE-reacting component from *P. citrinum*. Polyclonal rabbit antibodies were raised against this peptide. This rabbit antiserum as well as IgE antibodies in sera from asthmatic patients all showed immunoblot reactivity to the 47-kD fungal components and the corresponding recombinant enolases. Results obtained confirm that the 47-kD fungal allergens identified are enolases from *P. citrinum* and *A. fumigatus*, respectively, and have been designated as the group 22 allergen for *P. citrinum* (Pen c 22) and *A. fumigatus* (Asp f 22) by the Allergen Nomenclature Sub-Committee.

Enolase is a well-known cytoplasmic household enzyme active in glycolysis. It has also been found in the cell wall fraction and in the culture supernatant of *C. albicans* [29, 30]. It has been identified as a highly conserved allergens [23] in many fungal species including *S. cerevisiae* [31, 32], *C. albicans* [32–34], *C. herbarum* [25] and *A. alternata* [23, 25]. It appears to be the most important protein allergen of *S. cerevisiae* and *C. albicans* [31–34]. It was also recognized as an important allergen of *C. herbarum* and *A. alternata* [23, 35, 36]. The recombinant *Alternaria* enolase was recognized by serum antibodies in 22% of patients allergic to *A. alternata* [35, 36]. The *Cladosporium* enolase was recognized by IgE antibodies in 20% of patients with a positive *Cladosporium* immunoblot [25]. It was also found that in IgE immunoblots, more than 50% of patients sensitized to *Cladosporium* react positively to the purified recombinant *Cladosporium* enolase [23]. In the present study, 7 (30%) of the 23 *P. citrinum*-positive sera showed IgE reactivity to the native and the recombinant enolases of *P. citrinum*, with relatively weak IgE reactivity detected in 2 of these 7 patients.

Our results from 2D immunoblot showed that, in addition to the 47-kD enolase allergen, a 33-kD component with an alkaline pI value also reacted with IgE antibodies (fig. 1). This 33 kD has been identified as an alkaline serine protease allergen (Pen c 13) of *P. citrinum* [12, 17] and it can react with monoclonal antibody 55A [12] (data not

shown). Besides the 47-kD enolase and the 33-kD alkaline serine protease allergens, we detected by SDS-PAGE immunoblot an additional 35-kD protein that reacted strongly with IgE antibodies in serum No. 2 (fig. 3). This protein has been identified as the vacuolar serine protease allergen of this *Penicillium* species by using serine protease-specific monoclonal antibodies [37] (data not shown).

The 34-kD and the 18-kD IgE-reacting components in figure 2 reacted with monoclonal antibodies FUM20 [37] and PCE2-11 [19] (data not shown), respectively. They have been identified as the 34-kD vacuolar serine protease (Asp f 18) [16, 17] and the 18-kD peroxisomal membrane protein (Asp f 3) [20] allergens of *A. fumigatus*.

Except for serum No. 2, IgE antibodies in serum samples from other patients reacted only with enolase but not the serine protease major allergens of *P. citrinum* and *A. fumigatus* (fig. 3, 4). However, IgE antibodies in serum No. 1 showed relatively strong immunoblot reactivity to both Pen c 22 and Asp f 22 (fig. 3, 4). Apparently, the group 22 enolase allergens play a more significant role at least in this asthmatic patient.

In this study, a sequence identity of 94% was observed between the deduced amino acid sequences of the enolase allergens from *P. citrinum* and *A. fumigatus*. Six of the seven Pen c 22-positive serum samples have IgE immunoblot reactivity to Asp f 22 and rAsp f 22. However, strong IgE reactivity to Asp f 22 was detected only in two of these six serum samples tested. In addition, IgE cross-reactivity between both group 22 fungal allergens was detected by immunoblot inhibition using one serum sample that showed strong IgE binding to both Pen c 22 and Asp f 22.

An amino acid sequence identity of about 72–75% was found among the enolases from *A. fumigatus*, *S. cerevisiae* and *C. albicans*. However, two serum samples with relatively strong IgE immunoblot reactivity to Pen c 22 and Asp f 22 (samples 1 and 2 in fig. 3 and 4) were shown to have negative IgE immunoblot reactivity to purified *S. cerevisiae* enolase that was obtained from Sigma (data not shown). In addition, sera 1 and 2 of figures 3 and 4 showed negative and positive IgE immunoblot reactivity to the recombinant enolase of *C. albicans*, respectively (data not shown).

The enolases of *C. herbarum* and *A. alternata* have been identified as important allergens of both fungal species [23]. In the present study, IgE immunoblot reactivity to six fungal enolases including those from *P. citrinum*, *A. fumigatus*, *C. herbarum*, *A. alternata*, *S. cerevisiae* and *C. albicans* was also analyzed. The finding that IgE antibodies in serum C (fig. 6) reacted with all 6 fungal eno-

lases tested confirms that enolases are highly conserved fungal allergens even in a single allergic patient.

An amino acid sequence identity of 87% was found between enolases from *A. fumigatus* and *A. alternata*. However, our results also showed that enolases from *A. fumigatus* and *A. alternata* were only able to diminish IgE binding to *A. alternata* enolase and *A. fumigatus* enolase in the range of 30 and 39%, respectively, in 1 patient's serum (fig. 6, serum A). It indicates that both fungal enolases contain common and unique IgE-binding epitopes. In addition, results obtained also suggest that this patient may have independent parallel sensitivities to enolases of *A. fumigatus* and *A. alternata*.

The enolases from *S. cerevisiae* and *C. albicans* share 75% amino acid sequence identity. IgE cross-reactivity between both yeast enolases was also detected [31, 34]. However, results from Ito et al. [34] showed that detection of *S. cerevisiae* enolase-specific IgE antibodies was found in only 4 (20%) out of 20 patients positive for *C. albicans* enolase-specific IgE antibodies. Their results also suggest that *C. albicans* enolase contains several IgE-binding determinants some of which cross-react with *S. cerevisiae* enolase [34]. In the analysis of IgE cross-reactivity, results from Simon-Nobbe et al. [36] showed that *C. herbarum* enolase contains additional IgE-binding epitopes compared with *A. alternata* enolase. Thus, although enolases are highly conserved fungal allergens, results obtained previously and from the present study suggest that there may be cross-reactive and species-specific IgE epitopes of these fungal enolase allergens.

In conclusion, a novel enolase allergen from *P. citrinum* (Pen c 22) and *A. fumigatus* (Asp f 22) was identified and characterized in the present study. In addition, IgE cross-reactivity between enolase allergens from *A. fumigatus* and *P. citrinum*, and between enolases from *A. fumigatus* and *A. alternata* was also detected. Results obtained provide more information in clinical mould allergy.

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