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Pen c 1, a novel enzymic allergen protein from *Penicillium citrinum*
Purification, characterization, cloning and expression

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A 33-kDa alkaline serine protease secreted by *Penicillium citrinum* strain 52-5 is shown to be an allergenic agent in this fungus. The protein, designated Pen c 1, was purified by sequential DEAE-Sepharose and carboxymethyl (CM)-Sepharose chromatographies. Pen c 1 has a molecular mass of 33 kDa and a pI of 7.1. The caseinolytic enzyme activity of this protein was studied. The protein binds to serum IgE from patients allergic to *Penicillium citrinum.* The cDNA encoding Pen c 1 is 1420 bp in length and contains an open reading frame for a 397-amino-acid polypeptide. Pen c 1 codes for a larger precursor containing a signal peptide, a propeptide and the 33-kDa mature protein. Sequence comparison revealed that Pen c 1 possesses several features in common with the alkaline serine proteases of the subtilisin family. The essential Asp, His, and Ser residues that make up the catalytic triad of serine proteases are well conserved. Northern blots demonstrated that mRNAs transcribed from this gene are present at early stages of culture. The allergen encoded by Pen c 1 gene was expressed in *Escherichia coli* as a fusion protein bearing an N-terminal histidine-affinity tag. The protein, purified by affinity chromatography with a yield of 130 mg of pure protein per liter of culture, was able to bind to both a monoclonal anti-Pen c 1 antibody and IgE from the serum of patients allergic to *Penicillium*. Recombinant Pen c 1 can therefore be expressed in *E. coli* in large quantities and should prove useful as a standardized specific allergen for immuno-diagnosis of atopic disorders. In addition, full caseinolytic enzyme activity could be generated in the purified recombinant protein by sulfonation and renaturation, followed by removal of the affinity tag, indicating that the refolded protein can assume the same conformation as the native protein.

Keywords: Pen c 1; mold allergens; IgE-binding activity; alkaline serine protease; *Penicillium citrinum*.

IgE-mediated allergy is a serious problem for patients with symptoms such as allergic rhinitis, conjunctivitis, dermatitis or asthma. It has long been recognized that inhalation of fungal spores can produce allergic symptoms in susceptible individuals, and fungi are regarded as one of the main sources of allergens. In Taipei, the prevalence of asthma in schoolchildren has become a serious medical problem. In addition, more than 45% of asthmatic children and 70% of asthmatic adults have positive allergic responses to molds [1]. Certain species of molds constitute a large part of this inhalant allergen reservoir that is a result of airborne spores [2,3]. Over 60 species of fungi are known to be allergenic [4], with *Cladosporium*, *Alternaria*, *Penicillium,* and *Aspergillus* being among the most frequently reported [5]. *Penicillium* and *Aspergillus* are commonly found indoors [5,6], while *Cladosporium* and *Alternaria* are most prevalent outdoors [7]. Licorish *et al*. have reported that *Alternaria* and *Penicillium* spores, in relatively natural states and numbers, are potent asthma immunopathogens [8]. More than 150 different species have been discovered in the genus *Penicillium* [9] and *Penicillium* species being common indoor fungi, are frequently included in skin test panels. About 40.5% of the *Penicillium* colonies isolated from the Taipei urban area are *Penicillium citrinum* [10] and, because of this high prevalence and their allergenic properties, the allergens from *P. citrinum* justify study. However, the allergenic relationships between different *Penicillium* species, and thus the species recommended for clinical use, have not yet been determined. Previous studies have suggested the presence of a 33-kDa major allergen in *P. citrinum* extracts, while N-terminal amino-acid sequence analysis results suggest that it might belong to a member of alkaline serine protease [11].

Several reports on the cross-reactivity of molds have been published. Binding of anti-(*Penicillium* IgG) to several *A. fumigatus* antigens with molecular masses between 28 and 130 kDa has been reported [12]. Similarly, an antigenic relationship between the different *Penicillium* species has been investigated by Shen *et al*. [13]. In a recent report, IgE cross-reactivity of *A. oryzae* and *P. citrinum* protease allergens has also been demonstrated [14]. Antigenic relationships between *Cladosporium* species have been demonstrated by Honbu *et al*. [15]. Taken together, these studies show the presence of shared unique antigenic and allergenic activity between the different mold species.

Although positive immediate and delayed skin reactivity can be produced using poor quality crude extracts of many fungal species, it would be advantageous to have well-characterized homogeneous fungal allergens in order to elucidate the

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Abbreviations: CM, carboxymethyl; PVDF, polyvinylidene difluoride.

Enzymes: Alkaline serine protease (EC 3.4.21-), *Staphylococcus aureus* V8 protease (EC 3.4.21.19).

Note: The GenBank accession number for the cDNA is AF084546.

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Table 1. Oligonucleotides used in PCR.

Primer	Nucleotide sequence in 5′-3′ orientation
p1	GC(A,C,T,G) AA(C,T) GT(A,C,T,G) GT(A,C,T,G) CA (A,G) TC(A,C,T,G) AA(C,T) GT
p2	TG(A,G) GGG GTG GCC ATG GAA GT(A,G) CC
p3	TGG GG(C,T) CTT GC(T,C,G) CGC ATC TC
p4	AA(T,G) (A,G)(A,G)A GC(A,G) (C,G)TG CCA TCT GT
p5	GGT GCC GAT GGT TCT GGC ACT AA
p6	CAT ACC AGA GAT GAC ACC
p7	GCA GTA GAA TCA TAT GTA TAG CT
AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC
AP2	ACT CAC TAT AGG GCT CGA GCG GC
p8	GCG GAT CCG ATG ACG ATG ACA AAG CGA ACG TGG TTC AAT CC
p9	CCC AAG CTT TTA GAC GTT GAT GCC ATT GTA

immunopathologic mechanisms leading to *P. citrinum* allergic diseases. In addition, the availability of pure immunoreactive antigens or allergens may assist the diagnosis, improve immunotherapy of allergic diseases, and lead to a better understanding of the allergic reactions underlying immunopathologic complications, such as IgE-mediated asthma. There is considerable interest in circumventing problems related to extract standardization by the isolation of specific allergens for use both in the standardization of allergen extracts and in studies of the mechanism involved in the allergic response in patients.

In this study, we focus our attention on a secreted allergen from *P. citrinum* reported to be a serine protease belonging to the subtilisin family [11] that we have now designated as Pen c 1, according to the new allergen nomenclature [16]. Herein, we purified this novel allergen from mold *P. citrinum* and characterized its immunologic reactivity with patients' IgE and with a monoclonal antibody raised against, and reacting with, Pen c 1. These studies were followed by the molecular cloning and expression of this allergen in *Escherichia coli* and its subsequent renaturation. The enzymatic activity and IgE-binding properties of the renatured recombinant protein were compared with those of the natural homolog.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. A Trizol RNA extraction kit was obtained from Life Sciences (Petersburg, FL, USA). Oligo dT-cellulose, HiTrap DEAE-Sepharose Fast Flow column and HiTrap CM-Sepharose Fast Flow columns were purchased from Pharmacia (Uppsala, Sweden). Hybond-N membrane was from Amersham (Oakville, Ontario, Canada). The Marathon™ cDNA amplification kit was from Clontech (Palo Alto, CA, USA). Deoxyribonucleotide primers were synthesized using the phosphoramite method in an Applied Biosystems automated DNA synthesizer. *Taq* DNA polymerase, the pGEM-T vector, and the AmpliTag FS Prism ready-reaction cycle sequencing kit were from Applied Biosystems. The (DIG) digoxigenin luminescent detection kit was from Boehringer Mannheim (Mannheim, Germany). Other chemicals were of analytical grade.

Allergenic extracts and patients' sera

To prepare the allergenic extract, *P. citrinum* strain 52-5, isolated from the air in the Taipei area was used [11]. Sera from patients allergic to *Penicillium* species were collected in the National Taiwan University Hospital, Taipei, and stored in aliquots at −70 °C. The atopic phenotype was characterized for IgE reactivity by Carrier Polymer system (Pharmacia) measurements, scores being determined according to the manufacturer's manual. Control sera were collected from non-atopic donors.

Pen c 1 purification

The fungal culture was grown in 1 L of production medium (0.5% glucose, 1.5% meat extract, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$, 0.0001% $CuSO_4{\cdot}7H_2O$, 0.0001% $ZnSO_4{\cdot}7H_2O$ and 0.0001% $MnSO_4{\cdot}4H_2O$, pH 4.5). After 4 days of culture, the broth was filtered through cheesecloth and the resulting clear filtrate used as starting material for allergen purification.

All purification procedures were carried out at 4 °C. Finely powdered ammonium sulfate was added to the culture filtrate to 80% saturation. After 1 h on ice, the precipitate formed was collected by centrifugation, redissolved in 10 mM sodium phosphate buffer, pH 7.8, and dialyzed against the same buffer. After dialysis, non-dissolved protein was removed by centrifugation and the clear supernatant applied to an anion-exchange column (HiTrap DEAE-Sepharose Fast Flow, 0.7 cm × 2.5 cm), equilibrated with 10 mM sodium phosphate buffer, pH 7.8, using a FPLC system (Pharmacia). All fractions were tested for IgE-binding activity by SDS/PAGE and immunoblotted using sera from patients with allergies to *Penicillium* species. The selected fractions were pooled, concentrated by ultrafiltration, dialyzed against 10 mM sodium phosphate buffer, pH 6.0, and chromatographed on a cation-exchange column (HiTrap CM-Sepharose Fast Flow, 0.7 cm × 2.5 cm) using an FPLC system (Pharmacia) equilibrated with 10 mM sodium phosphate buffer, pH 6.0. The column was washed with the same buffer until the 280 nm absorbance fell below 0.02, then the adsorbed proteins were eluted with a 6-mL linear gradient of 0–0.25 M sodium chloride in the same buffer, and the IgE-binding activity of the fractions determined. Allergen fractions were lyophilized and stored at −20 °C. Protein concentrations were determined using the bicinchonic acid protein assay reagent kit (Pierce), with BSA as the standard.

Polyacrylamide gel electrophoresis

SDS/PAGE analyses were performed as described by Laemmli [17] using 12% polyacrylamide gels. Protein bands were stained with Coomassie Brilliant Blue. The pI of Pen c 1 was estimated by isoelectric focusing on pH 3–10 Pharmalyte gradients (Pharmacia).

Gel filtration

Gel filtration of Pen c 1 was performed on a Pharmacia Superdex 200 column using 50 mM sodium phosphate buffer,

pH 6.6, containing 150 mM NaCl as buffer. About 0.5 mg of protein in 0.2 mL of buffer was loaded onto the column. The flow rate was 0.5 mL·min^{-1}.

Protease activity

For the azocasein assay [18], azocasein (Sigma) was dissolved in 0.1 M Tris/HCl buffer, pH 8.0; 400 μL of this substrate solution were mixed with 10–100 μL of the enzyme sample. After incubation at 37 °C for 1 h, 150 μL of 20% trichloroacetic acid was added. After 30 min, the precipitate was removed by centrifugation at 8000 ***g*** for 2 min, then the supernatant was mixed with an equal volume of 1 M NaOH, and the absorption of the dye released measured at 436 nm. Enzyme activity was expressed in arbitrary units; one azocasein unit was defined as an increase of 0.1 absorbance unit after incubation for 1 h at 37 °C.

Immunological assays

Immunoblot analysis using mouse mAb 55 A, an antibody raised against Pen c 1 was performed essentially as described [11], using horseradish peroxidase-conjugated goat anti-(mouse IgG) as secondary antibody a substrate solution of acetate buffer containing 3-amino-9-ethyl-carbazole and hydrogen peroxide [11]. In experiments in which the binding of IgE from patients' sera was tested, the monoclonal antibody was replaced by a 1 : 10 dilution of patient's serum in blocking buffer, the second antibody was alkaline phosphatase-conjugated monoclonal mouse anti-(human IgE) antibody (1 : 5000, Pharmingen), and the developing agent a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate toluidinium in the carbonate buffer [11]. Proteins on the membranes were detected by Coomassie Brilliant Blue.

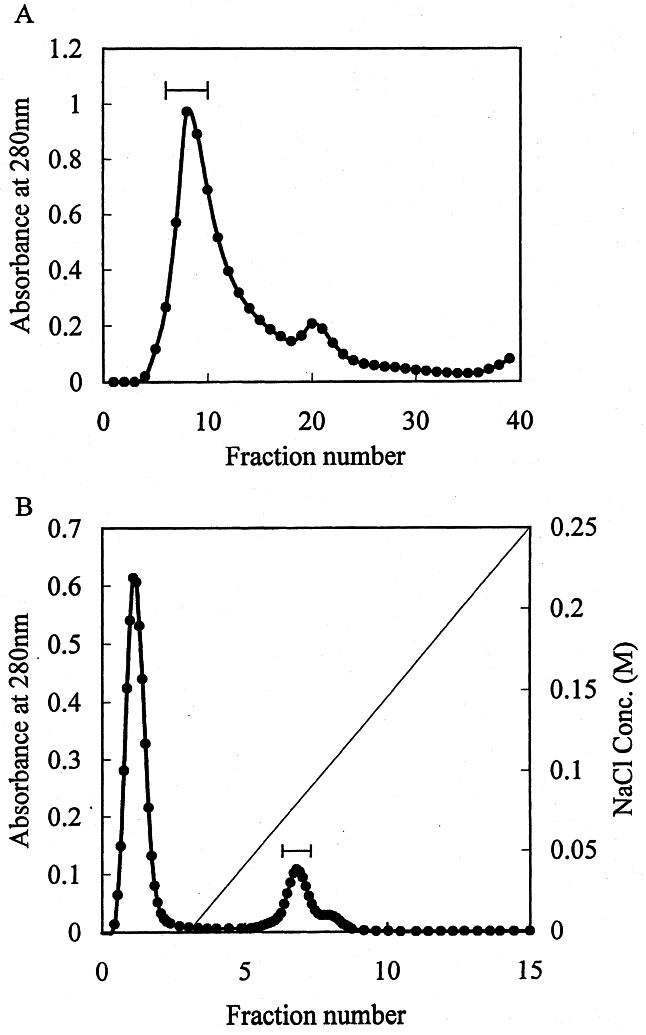

Fig. 1. Isolation of Pen c 1. Elution profiles of the chromatographic steps during the purification procedure. (A) Ion-exchange chromatography on HiTrap DEAE-Sepharose Fast Flow column. (B) Ion-exchange chromatography on HiTrap CM-Sepharose Fast Flow column. The pooled allergen-containing fractions are indicated by bars. The dotted line represents the NaCl gradient.

Sequence analysis

N-terminal Edman degradation of the purified proteins was performed on an Applied Biosystems model 494 Procise sequencer according to the manufacturer's instructions.

Digestion of 100 pmol of the alkylated protein with 0.1–1.0 μg of *Staphylococcus aureus* V8 protease was carried out for 6 h at 37 °C in 50 μL of 0.1 M pyridine acetate buffer, pH 6.5, containing 0.1 mM $CaCl_2$. The mixture was then lyophilized, dissolved in SDS/PAGE buffer, and applied to 15% SDS gels. After electrophoresis, the peptides were electroblotted onto PVDF membranes, stained with Coomassie Blue, and air dried. One internal peptide (about 6 kDa) was detected, the membrane segment cut out, and the peptide sequenced.

RNA isolation

Total RNA was extracted from the mycelium of *P. citrinum* by acid guanidinium thiocyanate/phenol/chloroform extraction according to the method of Chomczynski and Sacchi [19]. mRNAs were prepared by affinity chromatography on oligo-(dT)cellulose.

PCR amplification.

One microgram of mRNA was reverse transcribed using the Marathon™ cDNA amplification kit (Clontech), and the double-stranded cDNAs ligated to Marathon™ cDNA adapters [20]. The primers used were two degenerate synthetic oligonucleotides based on N-terminal amino acids 1–8 and the highly conserved positions 226–233 of Pen c 1 (Table 1, p1 and p2). The amplification protocol consisted of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 2 min. A final elongation was performed at 72 °C for 10 min. After 30 cycles of PCR amplification, 1 μL of PCR products was used as a template for a second PCR using sense primer p3, corresponding to the N-terminal amino acids 11–17, and the antisense primer p4, corresponding to positions 190–196 of the 6-kDa V8 peptide.

RACE on the 3′ end of the cDNA was performed using the Marathon™ cDNA amplification kit with the flanking primer AP1 and sense primer p3 (Table 1). These PCR products were then subjected to nested PCR using adapter primer AP2 and gene-specific primer p5. The 5′-end was amplified by 5′-RACE essentially as described for the 3′-end, but using the flanking primer AP1 and the gene-specific primer p6 (Table 1) then nested PCR was performed using AP2 and the gene-specific primer p7. All the primers used are listed in Table 1.

Cloning and sequencing

The amplified products were analyzed by electrophoresis and subcloned into pGEM-T vector, then used to transform *E. coli*

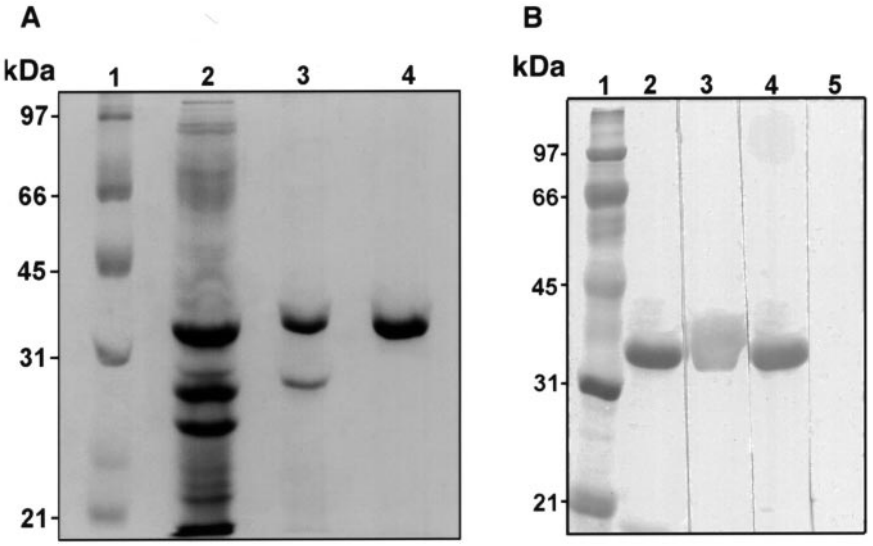

Fig. 2. SDS/PAGE analysis of the purification steps. (A) Coomassie blue staining of 12% SDS/PAGE gels. Lane 1, molecular mass markers; lane 2, crude preparation of secreted proteins from *P. citrinum*; lane 3, Pen c 1-containing fractions eluted from DEAE-Sepharose Fast Flow column; lane 4, Pen c 1 eluted from HiTrap CM-Sepharose Fast Flow column. (B) Binding of mAb 55A and IgE to natural Pen c 1 after transfer to PVDF membranes. Lane 1, molecular weight markers; lane 2, Coomassie blue staining; lane 3: treated with mAb 55A; lane 4, treated with 'positive' IgE serum; lane 5, treated with 'negative' IgE serum.

strain JM109. Positive clones were selected by blue/white screening and PCR screening using the primers T7 and SP6. DNA sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and a 373-A DNA sequencer (Applied Biosystems); the sequences were analyzed using the GCG program package (Genetics Computer Group Inc.).

Northern blots

Total RNA (20 μg) from *P. citrinum* at different stages was subjected to electrophoresis in the presence of formamide on 1% agarose/formaldehyde gels. The separated RNA molecules were transferred onto a Hybond-N^+ membrane using 20 × NaCl/Cit buffer, then cross-linked using a UV Stratalinker (Stratagene). The membrane was hybridized in 50% formamide, 5 × NaCl/Cit, 0.02% SDS, 0.1% *N*-lauroylsarcosine, 2% blocking solution (Boehringer Mannheim), and the digoxigenin-labeled hybridization signal detected using a digoxigenin luminescent detection kit (Boehringer Mannheim), following the manufacturer's protocol. In time-course studies, Pen c 1 cDNA, labeled with digoxigenin-11-dUTP using a random primed DNA labeling kit (Boehringer Mannheim), was used.

Expression of the allergen Pen c 1 in *E. coli*

To express the mature allergen Pen c 1 in *E. coli*, the cDNA was modified by PCR to remove the signal and other leading sequences of the mature Pen c 1. The sense primer was p8, and the antisense primer p9 (Table 1). These primers were designed to introduce *Bam*H1 and *Hind*III cleavage sites and an N-terminal extension consisting of the pentapeptide DDDDK [21]. The amplified cDNA was then cloned into the PQE-30 expression vector (Qiagen) allowing the N-terminal fusion a HHHHHH-tag sequence to generate the plasmid PQE-30/Pen c 1, which was then used to transform *E. coli* M15. Transformants were selected on Luria broth plates supplemented with 25 $\mu g \cdot mL^{-1}$ of kanamycin and 100 $\mu g \cdot mL^{-1}$ of ampicillin. To induce gene expression, the transformed *E. coli* M15 cells were grown at 37 °C in Luria broth containing 100 $\mu g \cdot mL^{-1}$ of ampicillin and 25 $\mu g \cdot mL^{-1}$ of kanamycin to a cell density of $D_{600} = 0.7$, then expression of the fusion protein was induced by the addition of isopropyl thio-β-D-galactoside to a final concentration of 1 mM. After incubation at 37 °C for 2 h, the cultures were harvested by centrifugation. Recombinant protein was designated rPen c 1.

Purification of histidine-tagged rPen c 1

The histidine-tagged fusion protein was affinity purified as described previously [22]. As the expression product accumulated in an insoluble inclusion body, the cell pellets were resuspended in 40 mL of the binding buffer (20 mM Tris/HCl, pH 7.9, containing 5 mM imidazole, 0.5 M NaCl) and sonicated on ice for 5 min, then the suspension was centrifuged and the supernatant loaded at a flow rate of 0.7 $mL \cdot min^{-1}$ onto a Ni^{2+}-immobilized resin column (Qiagen), pre-equilibrated in washing buffer (20 mM Tris/HCl, pH 7.9, containing 20 mM imidazole, 0.5 M NaCl and 8 M urea). After adsorption, the non-bound material was removed using washing buffer and the bound protein eluted using elution buffer (20 mM Tris/HCl, pH 7.9, containing 60 mM imidazole, 0.5 M NaCl and 8 M urea). Elution was monitored by UV absorbance at 280 nm and the fractions were collected and analyzed by 12.5% SDS/PAGE.

Sulfonation and refolding

For renaturation, the purified rPen c 1 was sulfonated using 2-nitro-5-sulfothio-benzoate in 8 M urea, 0.3 M Na_2SO_3, pH 8.0 to prevent disulfide formation and interchange during refolding [23], and the sulfonated protein precipitated by dialysis against 0.25% acetic acid, then solubilized in 50 mM sodium borate, pH 8.4, containing 5 mM EDTA, 8 M urea, and 4 mM reduced and 2 mM oxidized glutathione. Refolding was initiated by diluting the sample fourfold with the same buffer lacking urea, and the resulting solution left at room temperature for 24 h before being dialyzed overnight at 4 °C against H_2O. It was then centrifuged to remove insoluble material, and lyophilized.

Table 2. Summary of the Pen c 1 purifications. A unit defined as an increase of 0.1 absorption unit after incubation for 1 h at 37 °C.

Purification step	Total protein (mg)	Total activity (10^3 U)	Specific activity ($U \cdot \mu g^{-1}$)	Yield (%)	Purification (fold)
Crude extract	58.7	198.0	3.3	100	1.0
DEAE Sepharose Fast Flow	29.4	129.7	4.4	65.5	1.3
CM Sepharose Fast Flow	2.4	27.7	11.5	14.1	3.4

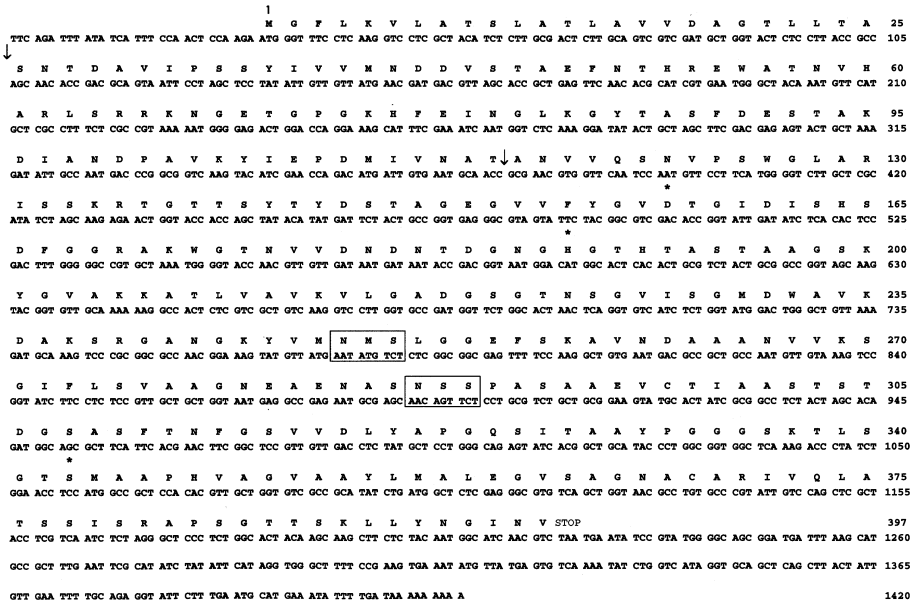

Fig. 3. Nucleotide and deduced amino-acid sequences of Pen c 1. The nucleotide and predicted amino-acid residues are numbered on the right. The mature protein sequence is numbered 115–397, the putative signal peptide 1–25 and the propeptide domain 26–115. The arrows indicate cleavage sites for the signal and pro-sequences. The catalytically important Asp, His and Ser residues are indicated by asterisks. The potential N-glycosylation consensus sequences are boxed. The Gene Bank accession number for the cDNA is AF084546.

Protein concentrations were determined using the bicinchonic acid protein assay kit with BSA as standard. The refolded rPen c 1 was tested by Western blotting, using sera from sensitized patients and monoclonal antibody mAb 55 A.

Non-fusion recombinant protein preparation

The DDDDK peptide and the histidine-affinity tag were removed from the refolded rPen c 1 by digestion with enterokinase for 16 h at room temperature at pH 7.5 [24], the digested protein was separated by SDS/PAGE, blotted onto PVDF membranes and the band excised for N-terminal sequencing. The enzymatic activity was assayed using azocasein as substrate [18].

RESULTS

Isolation of Pen c 1 from *P. citrinum*

Pen c 1 was purified to homogeneity from *P citrinum* culture medium by two simple steps. In the first step, using HiTrap DEAE-Sepharose Fast Flow chromatography, the majority of the protein eluted as a single peak which displayed immunoreactivity (Fig. 1A); this contained a major protein with a molecular mass (estimated by SDS/PAGE) of approximately 33 kDa, and a minor protein of around 27 kDa. The 27-kDa component was removed by the second step, HiTrap CM-Sepharose Fast Flow chromatography (Fig. 1B), while the 33-kDa protein corresponding to Pen c 1 was found in the second peak (Fig. 2A). Size-exclusion chromatography confirmed the molecular mass to be 33 kDa (data not shown). Using the Pharmacia Multipule II system, the pI of the protein was found to be 7.1, in agreement with that calculated from the amino-acid composition of Pen c 1 determined from the sequence. The results of the various purification steps are summarized in Table 2. About 2.4 mg of Pen c 1 was purified from 1 L of culture medium and the final purification was about 3.4-fold, with a 14.1% recovery.

Immunoreactivity against anti-(Pen c 1) antibodies

A Pen c 1-specific monoclonal antibody, mAb55 A, was used. The native allergen, purified from the culture filtrate, reacted strongly with mAb 55 A and an IgE-positive serum sample. The sera of non-allergic donors presented no specific IgE to the allergen Pen c 1 (Fig. 2B). Immunoblotting indicated that the protein was bound by IgE from patients known to be allergic to *Penicillium*.

Amino acid sequencing

The sequence of the first 16 N-terminal amino-acid residues of the native 33-kDa Pen c 1 was determined by Edman degradation to be ANVVQSNVPSWGLARI. The first 16 amino-acid residues from the 6-kDa V8 peptide were determined as VCTIAASTSTDGSASF.

Molecular cloning of the Pen c 1 coding sequence

The sequence of the cDNA coding for Pen c 1 is shown in Fig. 3. Sequence analysis of the cDNA clone showed it to be 1420 nucleotides in length. The open reading frame starts with an ATG codon at position 31 and ends at the TAA stop codon at position 1224. The encoded protein is predicted to have a molecular mass of 40 kDa and to contain 397 amino acids, which is larger that the purified 33-kDa protein. However, the hydropathy profile for the derived sequence and computer-based comparison of the N-terminal amino-acid sequence indicated the existence of a preprosequence, consisting of a hydrophobic signal peptide at the extreme N-terminus. Von Heijne's prediction method [25] indicated that this signal peptide would be cleaved after Ala25. This preprosequence is followed by a 90-amino-acid prosequence, a typical feature of proteases of the subtilisin family, in which proteolytic removal of the prosequence is an important step in the generation of the active protease from the inactive zymogen. The determined N-terminal sequence of the mature protein beginning at Ala1 was located 116 residues from the N-terminus of the amino-acid sequence of the primary translation product (Fig. 3). Thus, the mature enzyme contains 282 amino acids, giving a calculated molecular weight of 28 043.

Homology comparison

The predicted Pen c 1 protein sequence shows extensive similarity with members of the subtilisin family of serine proteases (Fig. 4) and shares 51.4%, 48.2%, 46.4%, 44.7% or

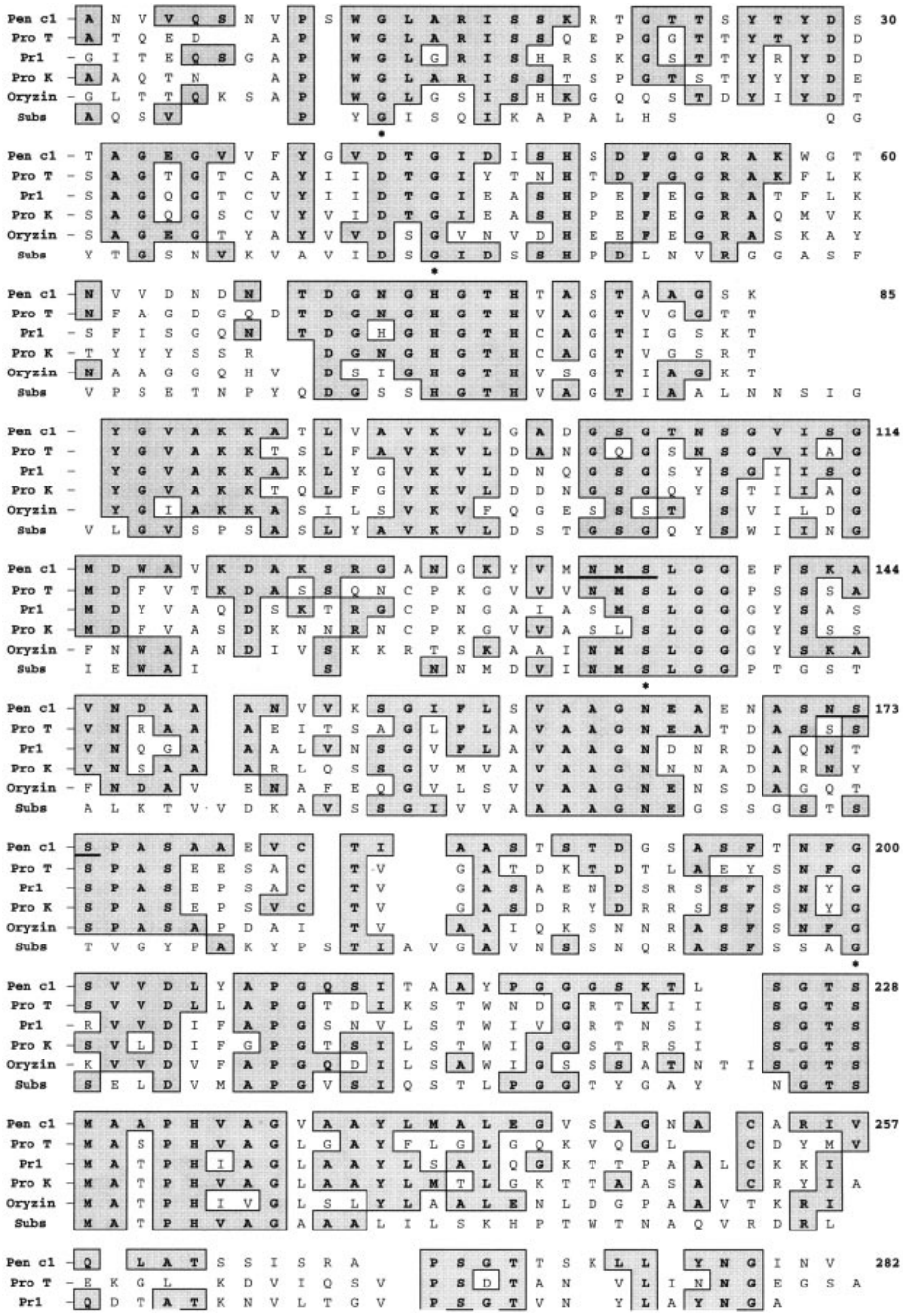

Fig. 4. Sequence comparison of Pen c 1 and other proteases. Homology between Pen c 1 and the subtilisin-family enzymes, *Tritirachium album* proteinase T (Pro T) (26), *Metarhizium anisopliae* cuticle-degrading proteinase (Pr1) (27), *Tritirachium album* proteinase K (Pro K) (28), *Aspergillus oryzae* alkaline serine proteinase (oryzin) (29) and *Bacillus subtilis* subtilisin (Sub) (30). The numbering system is based on the Pen c 1 sequence. Gaps were introduced for optimal alignment and to give the maximum homology between all compared sequences. Identical amino acids are shown in shaded boxes. The highly conserved and consensus amino-acid residues involved in the active site are indicated by asterisks. Potential N-glycosylation sites are underlined.

35.1% identity of residues with *Tritirachium album* proteinase T [26], *Metarhizium anisopliae* Pr1 [27], *Tritirachium album* proteinase K [28], *Aspergillus oryzae* oryzin [29] and *Bacillus subtilis* subtilisin [30], respectively.

The amino-acid residues Asp42, His73, and Ser228, common to all subtilisin-like proteases, were found in the mature Pen c 1 protein. The residues surrounding the catalytic site (catalytic triad) are similar to those essential for all of the subtilisin-like members to function as proteases [31]. The protein also contains an asparagine residue at position 165 that is highly conserved in these proteases and is believed to stabilize the tetrahedral transition state [32].

Northern hybridization

To determine the size of the gene transcript, RNA from the organism was hybridized with the digoxygenin-labeled Pen c 1 cDNA probe. The results showed that the probe hybridized to a single band at about 1.4 kb (Fig. 5). As this is similar to the size of the cDNA, this suggests that the cDNA contains almost the full length of the transcript. Comparison of RNA isolated from mycelia at different stages demonstrated that this transcript showed clear induction after 24 h of growth, reached a maximum at about 42 h, then showed a slight decay after 48 h.

Expression and purification of recombinant protease

In order to produce large amounts of pure allergen, we expressed Pen c 1 in *E. coli* M15 as a fusion protein. The Pen c 1-encoding cDNA was ligated into plasmid PQE-30 which codes for a histidine-tagged fusion protein with a predicted molecular mass of 35 kDa. Figure 6A shows expression of the Pen c 1 fusion protein in the transformed cells on induction with isopropyl thio-β-D-galactoside. The induced protein, with an

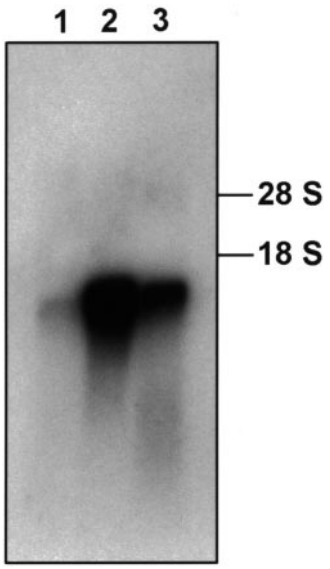

Fig. 5. Northern blot analysis of Pen c 1 transcripts. Pen c 1 cDNA was hybridized to total *P. citrinum* RNA extracted after 24, 42, or 48 h of culture. Hybridization was performed using the DIG-labeled Pen c 1 cDNA as probe.

apparent molecular mass on SDS gels of 35 kDa, represented at least 50% of the total protein, and was purified by metal-chelate affinity chromatography. Recombinant Pen c 1 was extracted from inclusion bodies under denaturing conditions and applied to the column and was then eluted using an imidazole gradient (20–100 mM). On SDS gels, the purified recombinant protease consisted of a single band with an apparent molecular mass of approxmately 35 kDa (Fig. 6A), slightly larger than the native protein because of the fusion tail. A total of 130 mg of rPen c 1 was purified from 1 L of *E. coli*.

The antibody-binding characteristics of the recombinant protein were similar to those of native Pen c 1, as it was recognized both by the Pen c 1-specific mAb55 A and by serum IgE from patients with allergic asthma. The sera of non-allergic donors presented no specific IgE to the rPen c 1 (Fig. .6B).

Refolding and caseinolytic activity

The non-refolded recombinant rPen c 1 was devoid of enzyme activity. After refolding the expressed protein was then subjected to enterokinase removal of the histidine-tag and DDDDK pentapeptide, the recombinant non-fusion rPen c 1 was concentrated by ultrafiltration to an final concentration of 1–2 mg·mL^{-1}. The sequence of amino acids 1–10 of the recombinant non-fusion protein rPen c 1, determined by Edman degradation, was identical to that of the natural Pen c 1. The specific activity of the purified non-fusion protein, determined using azocasein, was 10.5 U·μg^{-1} protein, similar to that of native Pen c 1 (11.5 U·μg^{-1}).

Although the non-refolded fusion protein bound to both the monoclonal anti-(Pen c 1) antibody and IgE from positive patients' sera, the correct three-dimensional structure is required for enzymatic activity. And the protease results therefore imply that the refolded Pen c 1 had a conformation similar to that of the native Pen c 1 from *P. citrinum*.

DISCUSSION

Mold is a well known trigger of bronchial hyperresponsiveness and can cause exaggeration of the response to inflammation and rhinitis, observed in allergic asthma. High levels of fungal allergens are present in the domestic environment in the Taipei area [33]. When *Penicillium* genus, one of the allergenic molds most prominent in eliciting an immunologic response, was studied, 22% of 67 asthmatic serum samples tested showed IgE reactivity to the 33 kDa component of *P. citrinum* [11].

The present study reports the purification of a major antigen, a 33-kDa serine protease from *P. citrinum*. Because of the extent of IgE-binding seen using a large panel of sera, it should be considered a dominant allergen from this mold. This allergen, named Pen c 1, was purified, characterized, cloned and expressed in *Escherichia coli*.

Pen c 1 purified from *P. citrinum* migrated as a 33-kDa protein on SDS/PAGE, while the calculated molecular mass of the mature protein was 28.0 kDa. A similar size difference between that seen for the purified protein and that deduced from the DNA sequence has been seen for the *A. oryzae* protease [29] and *B. subtilis* subtilisin [30], although the reason for this difference is not clear. One possibility is glycosylation. Two potential N-linked glycosylation sites are present in the Pen c 1 sequence, one at residue 134 (Asn-Met-Ser), and the other at residue 171 (Asn-Ser-Ser). However, the purified protein tested negative for carbohydrate using periodic acid Schiff staining (data not shown). In the three-dimensional structure of subtilisin [34], the Asn-Met-Ser site is hidden inside the molecule and

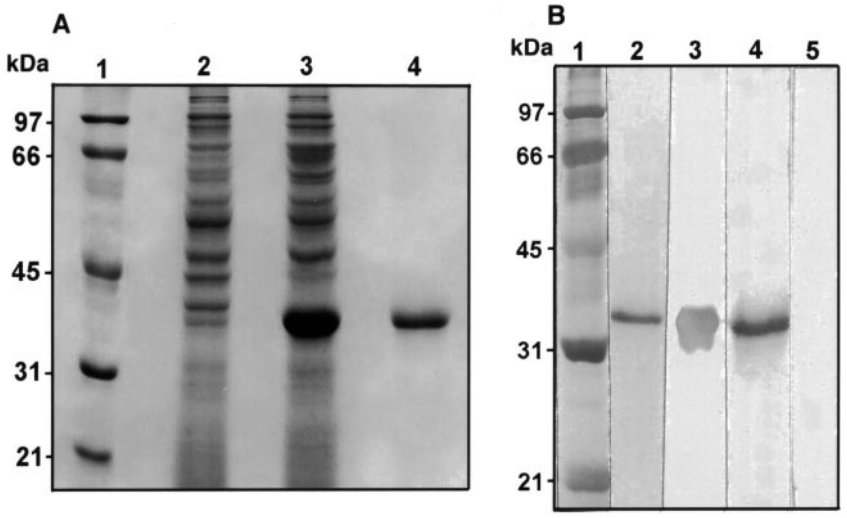

Fig. 6. Expression of His-tagged rPen c 1 protein in *E. coli*. (A) Coomassie blue-stained 12% SDS/PAGE to show amplification of the recombinant protease. Lane 1, molecular mass markers; lane 2, *E. coli* cell lysate; lane 3, isopropyl thio-β-D-galactoside-induced *E. coli* cell lysate producing rPen c 1; lane 4, rPen c 1 purified to homogeneity by affinity chromatography. (B) Binding of mAb 55A and IgE to recombinant rPen c 1. Lane 1, molecular weight markers; lane 2, Coomassie blue stained rPen c 1; lane 3, treated with mAb 55A, lane 4, treated with patient sera containing IgE antibodies., lane 5, treated with normal control serum.

therefore non-glycosylated and this might also be the case for Pen c 1. The other reason is that given by Gavel and von Heijne [35] who suggested that the proline next to Asn-Ser-Ser interferes with the glycosylation.

In the present study, the Pen c 1 precursor was found to contain three distinct domains, a 25-amino-acid putative signal peptide, a 90-amino-acid propolypeptide, and a 282-amino-acid mature polypeptide (the active enzyme). The signal peptide cleavage site is predicted to be C-terminal to Ala25 [25]. The 25-amino-acid leader segment is a secretory signal sequence with a high content of hydrophobic amino acids that is expected to direct transport of the nascent polypeptide chain across the ER membrane into the ER lumen [36].

The 90-residue propeptide may be involved in the processing of the precursor and the folding of the catalytic domain [37]. Within the mature polypeptide, the amino-acid sequence surrounding Asp42, His73, and Ser228 is the most salient feature of Pen c 1 and is closely related to those at the catalytic sites of the subtilisin-like serine proteinase [31,32].

X-ray diffraction analysis of single crystals has shown that the subtilisin-like protease K contains two disulfide bonds [38]. On the basis of its deduced amino-acid sequence, Pen c 1 may contain one disulfide bond, the cysteine comprising the other disulfide bond in protease K being replaced by Val and Ala. In a study on subtilisin, an additional disulfide bond was introduced into the molecule by means of site-directed mutagenesis, and the resulting enzyme was shown to have a higher melting temperature because of a decrease in entropy for the unfolded state [39]. The disulfide bonds in these enzymes may contribute to their thermal stability properties. As Pen c 1 may have, at most, a single S–S bridge, it may be heat-labile [40,41]. We therefore believe that Pen c 1 represents a new member of the subtilisin-like family of serine proteases.

Serine proteases are widely distributed in various organisms. Subtilisins are well-characterized occupational allergens in the detergent industry [42], and their role in allergic disease has been extensively investigated [43]. Although structurally unrelated to the subtilisins, the group 3 [44,45] and group 6 [46] allergens of house dust mites belong to the trypsin-like and chymotrypsin-like serine proteinase family, respectively. Recent data indicate that mite extracts also contain another allergenic serine protease with collagenolytic activity [47]. A chymotrypsin-like serine protease from *A. fumigatus* can directly induce human epithelial cell detachment [48], and might trigger the allergic response by causing detachment of areas of epithelium, resulting in increased penetration of *A. fumigatus* antigens into the submucosa. We suggest that, because of their serine protease activity, molds can also be involved in the inflammatory processes in allergic asthma by means of mechanisms other than those involving an immunologic component.

Many allergens are enzymes. They share a similar sequence at the active site and have a similar mode of action. However, they show differences in terms of amino-acid composition, isoelectric point, and molecular mass. For example, there is only 35.1% similarity in their amino-acid sequences when comparing subtilisin and Pen c 1 [30] Nevertheless, homology modeling based on protease K and subtilisin (data not shown), shows their structures are surprisingly similar with some main-chain/side-chain hydrogen bonds being highly conserved. Future X-ray analysis of Pen c 1 may provide definite answer to the modeled structure.

The suggestion that enzymatic activity may enhance, or be required for, the induction of a persistent allergic response by an allergen is supported by the finding that many environmental allergens possess such activity and also by the high degree of sensitization engendered by such enzymes in commercial, domestic and pharmaceutical use.

Here, we have reported the isolation and cloning of the major allergen from *P. citrinum* and its allergenic characterization. The Pen c 1 sequence data will facilitate the mapping of T and B cell epitopes on the allergen, and will help to clarify the mechanism of the human immune response to *P. citrinum*.

For ease of purification, we used the *E. coli* expression system to purify milligram quantities of recombinant Pen c 1. The purification procedure is suitable for the production of well-characterized pure recombinant allergens, which will allow more detailed immunologic characterization of these proteins and the development of much more accurate diagnostic measures and specific anti-allergic treatments. Although the non-renatured recombinant protein was able to bind IgE antibodies from patients' sera when tested on Western blots, following renaturation, the protein also regained caseinolytic activity essentially equivalent to that of the native protein, indicating that it was correctly folded, and this product may therefore be more suitable for studying conformationally dependent epitopes. The mapping of allergenic and immunogenic epitopes on pure Pen c 1 using the recombinant protein is currently in progress.

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