Identification and expression of Pen c 2, a novel allergen from *Penicillium citrinum*

Lu-Ping CHOW*[1], Ning-Yuan SU*, Chia-Jung YU*, Bor-Luen CHIANG† and Horng-Der SHEN‡

*Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, Taipei, Taiwan 100, †Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan, and ‡Department of Medical Research, Veterans General Hospital, Taipei, Taiwan

The mould genus, *Penicillium*, is known to be a significant source of environmental aero-allergens. One important allergen from *Penicillium citrinum*, Pen c 2, has been identified by means of two-dimensional immunoblotting using IgE-containing patients' sera. This novel allergen was cloned, sequenced and expressed in *Escherichia coli*. The cloned cDNA encodes a large 457-amino acid protein precursor containing a 16-amino acid signal peptide, a 120-amino acid propeptide and the 321-amino acid mature protein. Comparison of the Pen c 2 sequence with known protein sequences revealed shared high sequence similarities with two vacuolar serine proteases from *Aspergillus niger* and *Saccharomyces cerevisiae*. Asp-46, His-78 and Ser-244 were found to constitute the catalytic triad of the 39-kDa Pen c 2. The DNA coding for Pen c 2 was cloned into vector PQE-30 and expressed in *E. coli* as a His-tag fusion protein that bound serum IgE from *Penicillium*-allergic patients on immunoblots. Recombinant Pen c 2 could therefore be used effectively for diagnosis and also potentially for the treatment of mould-derived allergic disorders.

Key words: allergy, cDNA cloning, IgE-binding activity, mould allergens, vacuolar serine protease.

INTRODUCTION

The Fungi Imperfecti include a number of species that can cause IgE-mediated allergic diseases in atopic individuals. Many fungi, such as *Cladosporium* spp., *Alternaria* spp., *Penicillium* spp. and *Aspergillus* spp., are major sources of allergens [1]. *Penicillium* and *Aspergillus*, less common outdoors, are usually considered the major indoor fungi [2]. Bronchial challenge with *Penicillium* spp. spores induces immediate- and delayed-type asthma in sensitized subjects [3], and the genus *Penicillium* is thought to be an important inducer of allergic asthma and allergic rhinitis.

In a previous study on *Penicillium citrinum*, several proteins with molecular masses ranging from 26 to 67 kDa that bound serum IgE from atopic allergic patients were identified immunochemically [4]. Recently, we reported the isolation, cloning and expression of one of these allergenic proteins with a molecular mass of approximately 33 kDa, referred to as Pen c 1 [5], and showed it to be a major *P. citrinum* allergen, since IgE from more than 70 % of mould-allergic patients recognized this protein [4]. In addition to Pen c 1, other allergenic proteins have been detected in *P. citrinum* extracts; of these, components with molecular masses of around 39 kDa, which bind IgE from 53 % of *P. citrinum*-positive sera, can also be considered as important *P. citrinum* allergens [4].

Antigenic cross-reactivity between components of different fungal species has been reported previously. Anti-*Penicillium* IgG can also react with several *Aspergillus fumigatus* antigens with molecular masses ranging from 28 to 130 kDa [6]. A 33-kDa component in extracts of seven different *Penicillium* species reacted with monoclonal antibody (mAb) 55A, raised against Pen c 1 from *P. citrinum* [7]. Culture-filtrate antigens from two *Fusarium* spp. were all able to inhibit the binding of IgE to the specific allergen bands on immunoblots of extracts of *Fusarium solani* [8]. Cross-reactivity between *P. citrinum* and *Aspergillus oryzae* has also been detected, and a major 34-kDa allergen has been found in both species [9]. The major allergen of *Penicillium* has been shown to share common epitopes with related proteins of similar sizes in *Aspergillus* spp. [9]. These findings suggest that both shared and unique epitopes exist in different fungal species. Shared allergenic and antigenic epitopes appear to be very common among ascomycete anamorphs and, consequently, it may be possible to prepare a common antigen with representative antigenic/allergenic properties from a highly purified extract of a single isolate of fungal species for use in allergic diagnosis.

In this paper, we describe the identification of a novel allergen from the common house mould *P. citrinum*, and the characterization of its immunological reactivity with patients' IgE. In addition, the molecule was cloned and expressed in *Escherichia coli*. The cloned allergen was named Pen c 2 according to the recommendations of the WHO/IUIS Allergen Nomenclature Subcommittee [10].

EXPERIMENTAL

Materials

A Trizol kit for RNA extraction was obtained from Life Sciences (Petersburg, FL, U.S.A.). Oligo(dT)–cellulose was purchased from Pharmacia (Uppsala, Sweden). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA, U.S.A.). *Taq* DNA polymerase and pGEM-T vector were purchased from Promega (Madison, WI, U.S.A.). The Marathon™ cDNA amplification kit was from Clontech (Palo Alto, CA, U.S.A.). Alkaline phosphate-labelled horse anti-human IgE antibodies were purchased from Pharmingen (San Diego, CA, U.S.A.). The digoxigenin (DIG) luminescence-detection kit was from Boehringer Mannheim (Mannheim, Germany). Chemicals

Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; mAb, monoclonal antibody; DIG, digoxigenin.

[1] To whom correspondence should be addressed (e-mail lupin@ha.mc.ntu.edu.tw).

for sequence analysis were obtained from Applied Biosystems (Foster City, CA, U.S.A.). All other chemicals were of analytical grade.

Patients' sera and mAb production

Sera from patients with mould allergies were collected in the National Taiwan University Hospital, Taipei, Taiwan. The atopic phenotype was confirmed by clinical history and diagnosis and characterized by carrier-polymer-system measurements for IgE reactivity (Pharmacia). Control sera were collected from non-atopic donors. The anti-Pen c 2 mAb, mAb 39, was produced by immunization of mice with the crude protein from *P. citrinum* [7].

Strain and culture

Strain 52-5 of *P. citrinum* was used in the present study [11]. After cultivation on potato/dextrose/sugar plates for 7 days at 25 °C, 1×10^5 conidia were inoculated into 100 ml of Czapek-Dox broth medium (Difco, Detroit, MI, U.S.A.) in a 500-ml Erlenmeyer flask and incubated for 4 days at 25 °C. The mycelia were harvested by filtration through six layers of gauze, washed with PBS and lyophilized.

Protein extraction

Approx. 1 g of lyophilized mycelia were ground in a mortar filled with liquid nitrogen. The ground mycelia and spores were then mixed with 4 ml of extraction buffer [50 mM Tris/HCl (pH 6.8)/10 mM $MgCl_2$/0.1 % (v/v) β-mercaptoethanol/2 mM PMSF/0.2 mM dithiothreitol]. After centrifugation, the supernatant was mixed with a final concentration of 5 % trichloroacetic acid. The resultant precipitate was washed three times with cold diethyl ether, dried and stored at −80 °C. The protein concentration was measured with the bicinchoninic acid protein-assay reagent kit (Pierce, Rockford, IL, U.S.A.) using BSA as standard.

SDS/PAGE and immunoblotting

SDS/PAGE was carried out as described by Laemmli [12] using a separation gel of 12.5 % polyacrylamide under reducing conditions (5 % β-mercaptoethanol). After electrophoresis, the separated proteins were transferred on to PVDF membranes (Millipore, Bedford, MA, U.S.A.) by semi-dry blotting as described previously [13]. The blotted membranes were then either stained directly with Coomassie Brilliant Blue R-250 or blocked with 1 % skimmed milk in Tris-buffered saline [20 mM Tris/500 mM NaCl (pH 7.5)] at room temperature for 1 h then incubated overnight at 4 °C with patients' sera diluted 1:10 in Tris-buffered saline containing 1 % Tween-20 (TBST) and 1 % skimmed milk. After three washes with TBST, the blots were incubated with anti-human-IgE/alkaline phosphatase antibody (1:2000) for 1 h at room temperature and development was carried out using a mixture of Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate toluidinium as substrate [5]. When the monoclonal anti-Pen c 2 antibody was used, the bound mAb 39 was detected using horseradish peroxidase-labelled goat anti-mouse IgG as secondary antibody, and development was carried out using a substrate solution of acetate buffer containing 3-amino-9-ethyl-carbazole and hydrogen peroxide [5].

Two-dimensional PAGE

The final powder (2 mg) was suspended in 100 μl of sample solution (9.0 M urea/2 % carrier ampholytes/60 mM dithiothreitol/0.5 % Triton X-100/0.003 % Bromophenol Blue), incubated at 37 °C for 30 min with continuous stirring, and centrifuged at 19000 ***g*** for 15 min. Two-dimensional PAGE was carried out using a procedure described previously [13]. For the first dimension, an immobilized pH gradient of 3–10 on a horizontal electrophoresis system (Multiphor II, Pharmacia) was used, whereas, for the second dimension, SDS/PAGE was used. Immunoblotting was performed as described above.

Table 1 Oligonucleotide primer sequences

Primer	Nucleotide sequence (in 5′–3′ orientation)
G1	GT(C/T) GA(A/G) AA(A/G) AA(C/T) GC(T/C/G) CCC TGG
G2	GGC CAT GGA AGT (A/G)CC AGA
G3	GGC GTA GAG GTA CTT GTT AAA GG
G4	TGG GGT CTT GCT CGC ATC TC
G5	AAC ATT CTG TCC ACC TGG ATT GG
AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC
AP2	ACT CAC TAT AGG GCT CGA GCG GC
T7	TAA TAC GAC TCA CTA TAG G
SP6	GAT TTA GGT GAC ACT ATA GA
N	GCG GAT CCG ATG ACG ATG ACA AAG ATT CCC CCT CCG TT
C	CCC AAG CTT CTA GTA GCC ACC CTG GGC GAC

N-terminal microsequencing

The blotted proteins were visualized using Coomassie Brilliant Blue R-250. After destaining, the blots were rinsed in deionized water and air dried. The protein spots of interest were excised and placed directly into a blot cartridge fitted to an ABI model 494 Procise sequencer (Applied Biosystems) with an online phenylthiohydantoin amino acid analyser. Routinely, 12 Edman degradation cycles were performed on each spot. The partial sequences were compared for similarity with known proteins using the Basic Local Alignment Search Tool (BLAST) algorithm.

Cloning and sequencing of Pen c 2

Total RNA was extracted from the mycelia of *P. citrinum* using the Trizol reagent kit [14]. Poly(A)$^+$ RNA was isolated from total RNA by oligo(dT)–cellulose chromatography. mRNA (1 μg) was reverse transcribed to cDNA using the Marathon™ cDNA amplification kit (Clontech), and the double-stranded cDNAs ligated to Marathon™ cDNA adaptors [15].

Two degenerate primers, based either on the N-terminal sequences or on highly conserved positions, were synthesized. The sense primer, G1, encoding the N-terminal amino acids (VEKNAPW), and the antisense primer, G2, encoding the highly conserved amino acid sequences (SGTSMA), were used (Table 1). PCR amplification was performed at 94 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min for 30 cycles, followed by a final extension at 72 °C for 10 min.

For the generation of the 5′-end cDNA fragment, Marathon-ready cDNAs as templates were amplified using the anchor primer, AP1, and the gene-specific primer, G3, corresponding to bp 629–651 of the Pen c 2 cDNA (Table 1). The 3′ end was amplified by rapid amplification of 3′-cDNA ends using a method essentially similar to that used for the 5′ end, using the anchor primer, AP1, and the gene-specific primer, G4, encoding bp 582–602 of the Pen c 2 cDNA (Table 1). The nested PCR was carried out using anchor primer AP2 and a gene-specific primer,

G5, corresponding to bp 1225–1248. All primers are listed in Table 1.

The amplified products were analysed by electrophoresis and subcloned into the pGEM-T vector, then transformed into *E. coli* strain JM109. After transformation, positive clones were selected by blue/white screening and PCR screening with the primers T7 and SP6. Sequencing was performed on an ABI 373A automatic fluorescent sequencer (Applied Biosystems). A homology search of the GenBank and EMBL databases was performed using BLAST and FASTA software algorithms.

Northern-blot analysis

mRNA (3.6 μg) from *P. citrinum* was denatured and electrophoresed on 1.0% agarose/formaldehyde gels. The RNA was then transferred on to positively charged nylon membranes and hybridized with a Pen c 2 cDNA that was DIG-labelled by random priming (Boehringer Mannheim). Hybridization was carried out at 42 °C, as described previously [5]. The DIG-labelled hybridization signal was detected using a DIG luminescence detection kit according to the manufacturer's specifications.

Expression and purification of recombinant Pen c 2

To express the mature allergen Pen c 2 in *E. coli*, the cDNA was modified using the PCR method to remove the signal and prepropeptide sequences. The N and C primers were designed such that they contained the *Bam*HI and *Hin*dIII restriction sites, respectively. After PCR amplification and digestion with *Bam*HI and *Hin*dIII, the fragment was ligated into the similarly digested expression vector, PQE-30 (Qiagen, Chatsworth, CA, U.S.A.), for expression as a His-tag fusion protein. *E. coli* M15, transformed with PQE-30 harbouring Pen c 2, was grown at 37 °C to an absorbance of 0.8 at 600 nm. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 1 mM, and incubation continued for another 2 h. The cells were then harvested by centrifugation, and the pellet resuspended in Tris/HCl (pH 8.0), containing 1 mg/ml lysozyme, and incubated for 30 min on ice. The nucleic acids were broken down by mild sonication. Since the expression product accumulated as an insoluble inclusion body, the lysate was centrifuged and the precipitate was solubilized by incubation with 8 M urea in column buffer [5 mM imidazole/0.5 M NaCl/20 mM Tris/HCl (pH 7.9)]. The recombinant protein was purified under denaturing conditions on a packed column of Ni^{2+}-chelate affinity resin [16]. To obtain soluble proteins in a physiological buffer, the samples were dialysed against phosphate buffer (pH 7.4). Immunoblots was performed using either sera from sensitized patients and non-atopic donors or mAb 39.

RESULTS

Allergen identification

Single-dimension SDS/PAGE

When 55 serum samples were tested on SDS/PAGE immunoblots for IgE binding to a *P. citrinum* extract, 13 gave positive results (shown in Figure 1). The apparent molecular masses of the IgE-binding components ranged from 27 to 97 kDa. Of the prominent IgE-binding bands, allergens of approx. 33 and 39 kDa were detected by all 13 sera. A 33-kDa component with the highest IgE-binding activity and -binding frequency has previously been noted and identified as a major allergen [5]. Other IgE-binding components with various molecular masses were detected at frequencies of less than 30%, e.g. sera 2, 4, 8 and 9 gave a positive reaction with a 64-kDa component in the crude extract. No IgE binding was seen when sera from healthy donors were used (lane 15).

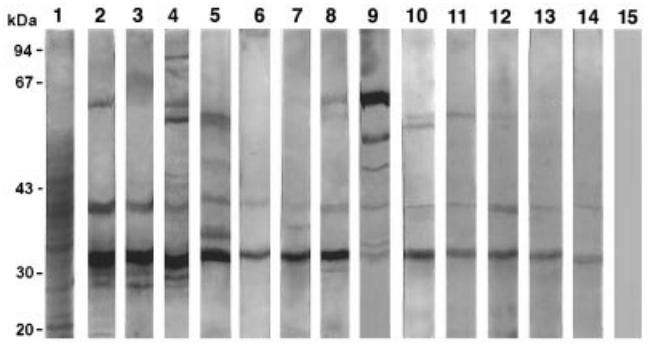

Figure 1 Binding of patients' sera IgE to a *P. citrinum* extract on immunoblots

Lane 1 shows Coomassie Brilliant Blue-staining of the *P. citrinum* extract on PVDF blots. Lanes 2–14 show the result of immunoblotting using the 13 different serum samples from allergic patients that gave a positive reaction on immunoblots. Lane 15 shows non-allergic patient who gave a negative reaction on immunoblots.

Two-dimensional immunoblotting

To characterize further the antigens recognized by the serum antibodies, two-dimensional gel electrophoresis and immunoblotting were performed. Figure 2(A) shows the Coomassie Brilliant Blue-stained two-dimensional protein profile of the *P. citrinum* crude extract; more than 200 protein spots were detected. To identify spots corresponding to allergens, IgE-binding spots on a two-dimensional gel were visualized by immunoblotting using sera from asthmatic patients with a positive immediate skin test to an extract of *Penicillium* spp. After immunoblotting with a mixture of sera (patients 2, 3, 8 and 12), six spots, two with a molecular mass of 33 kDa, three of 39 kDa and one of 64 kDa, and pI values ranging from about 5.0 to greater than 7.0 were shown to be reactive with IgE antibodies (Figure 2B). Similar staining was seen using individual sera from allergic patients (results not shown). The arrows in Figure 2(A) indicate the corresponding protein spots. No positive spots were detected when the negative serum was used (results not shown).

N-terminal amino acid sequencing

The immunoreactive protein spots were excised from the Coomassie Brilliant Blue-stained blot (Figure 2A) and subjected to N-terminal amino acid microsequencing. The N-terminal sequence information of two protein spots is detailed here. Spot 1, a 33-kDa protein with a pI of 7.1 (N-terminal sequence, ANVVQSNVPSWGL), corresponded to the recently described Pen c 1 allergen [5]. A weaker neighbouring spot was also detectable at this molecular-mass position, but with a different pI (pH 6.6); this protein was not sequenced, but, like spot 1, bound mAb 55A (results not shown). The N-terminal sequence of spot 2, a 39-kDa protein with a pI of 5.4, was DSPSVEKNAPWGL. When compared with the GenBank database, this was found to be highly similar to several vacuolar serine proteases [17,18]. Two other weaker neighbouring spots had the same molecular mass, but different pI values (pH 5.25–5.35). The N-terminus of the 64-kDa spot was blocked.

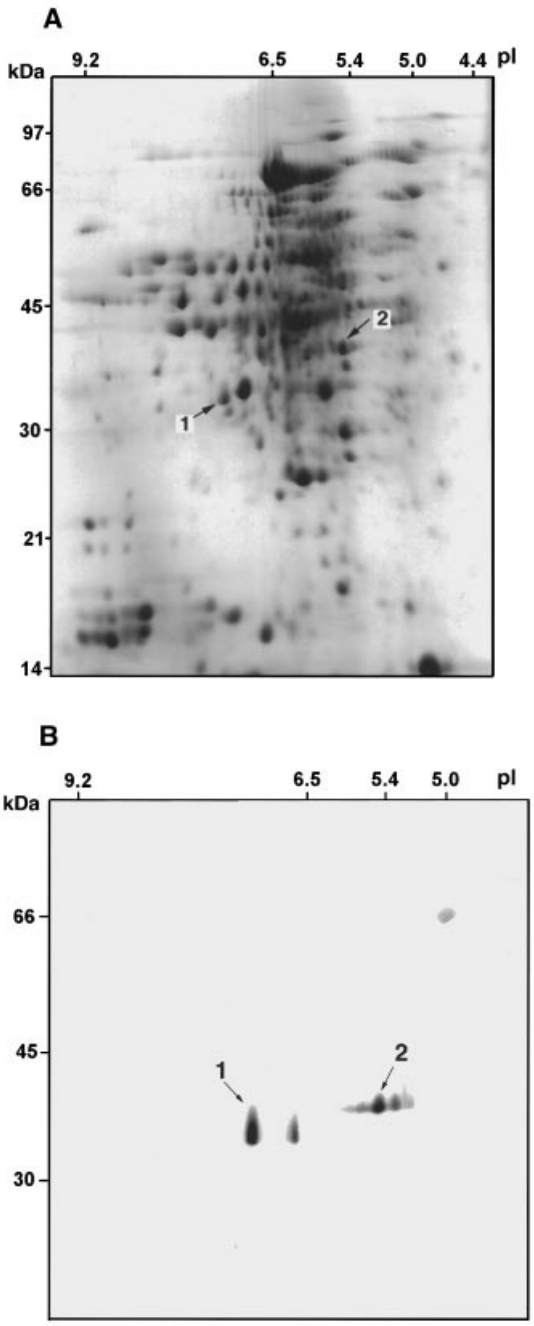

Figure 2 Two-dimensional immunoblot analysis of IgE binding to a crude *P. citrinum* extract

After two-dimensional PAGE and semi-dry electroblotting on to PVDF membranes, the blots were: (**A**) stained with Coomassie Brilliant Blue; and (**B**) tested for binding of IgE from pooled sera from patients 2, 3, 8 and 12. The arrows indicate the binding of IgE antibodies to components corresponding to protein spots 1 and 2, respectively. The N-terminal sequences of these protein spots were determined and are given in the text.

Analysis of the DNA and derived amino acid sequences of the *Pen c 2* gene

The sequence of the cDNA clone encoding Pen c 2 is shown in Figure 3. This Pen c 2 cDNA was 1902 bp in length, had an open reading frame of 1371 nucleotides and contained a 5′-non-coding region of 144 bp and a 3′-non-coding region of 387 bp upstream of the poly(A)$^+$ tail. The open reading frame started with an ATG codon at position 145 and ended at the TAG stop codon at position 1518 of the cDNA. The encoded protein had a predicted molecular mass of 48 kDa and contained 457 amino acids.

In the N-terminal region the protein contained a series of uncharged amino acid residues with a high content of hydrophobic amino acids. Based on signal-peptide-sequence cleavage prediction [19], it may generate a 16-amino acid signal peptide. The cleavage site of the signal peptide is presumably located before Ser-17 [20]. The 16-amino acid leader segment is a signal sequence with a higher content of hydrophobic amino acids, which is expected to direct the transport of nascent polypeptide chains across the endoplasmic-reticulum membrane into the endoplasmic-reticulum lumen [21]. The N-terminal sequence of the mature protein is predicted to begin at Asp-137, located 137 residues from the N-terminus of the primary translation product. A 120-amino acid polypeptide located between the leader and the N terminus is presumably removed during processing of the pre-proenzyme. The mature enzyme contains 321 amino acids with a calculated molecular mass of 35043 Da. Examination of the organization of the Pen c 2 sequence revealed domains characteristic of this enzyme, i.e. a hydrophobic signal pre-sequence, a pro-domain that may be released by autoproteolysis and a subtilisin-like catalytic domain. Thus the Pen c 2 precursor itself may be a secreted protein and probably passes through the endoplasmic reticulum and Golgi apparatus in transit to the vacuole [22]. In the case of subtilisin E, it has been pointed out that the propeptide seems to be involved in the proteolytic processing of the precursor, leading to the correct folding of the active enzyme [23,24].

Sequence similarities and comparisons between vacuolar serine proteases

As shown in Figure 4, the deduced protein sequence showed similarity with known vacuolar serine proteases, i.e. it showed 77.6 and 66.3 % identity of residues with *Aspergillus niger* PEPC (a vacuolar serine protease) [17] and *Saccharomyces cerevisiae* protease B [18], respectively. The residues of the catalytic triad of Pen c 2 were immediately recognized by sequence comparison to be Asp-46, His-78 and Ser-244. Pen c 2 contained four consensus glycosylation sequences at Asn^{108}-Gly-Ser, Asn^{148}-Gly-Ser, Asn^{186}-Tyr-Ser and Asn^{311}-Tyr-Thr. It also had three conserved Cys residues, one of which was situated near the active site and has been suggested to play a role in catalysis [20]. This Cys corresponds to the known free Cys in PEPC and is also found in protease B, and it is therefore probable that it is also free in Pen c 2. The remaining two Cys residues may form a disulphide bond.

Northern-blot hybridization

When the RNA from the organism was hybridized with the DIG-labelled Pen c 2 cDNA probe to determine the actual size of the gene transcript, the probe hybridized to a single band of about 1.9 kb (Figure 5). Since the size of the mRNA coding for the allergen was close to that of the cDNA, this suggests that the cloned cDNA represented almost the full length of the transcript.

Expression and purification of recombinant Pen c 2

To investigate whether IgE antibodies were elicited in response to Pen c 2 in patients, we expressed Pen c 2 in *E. coli* M15 as a

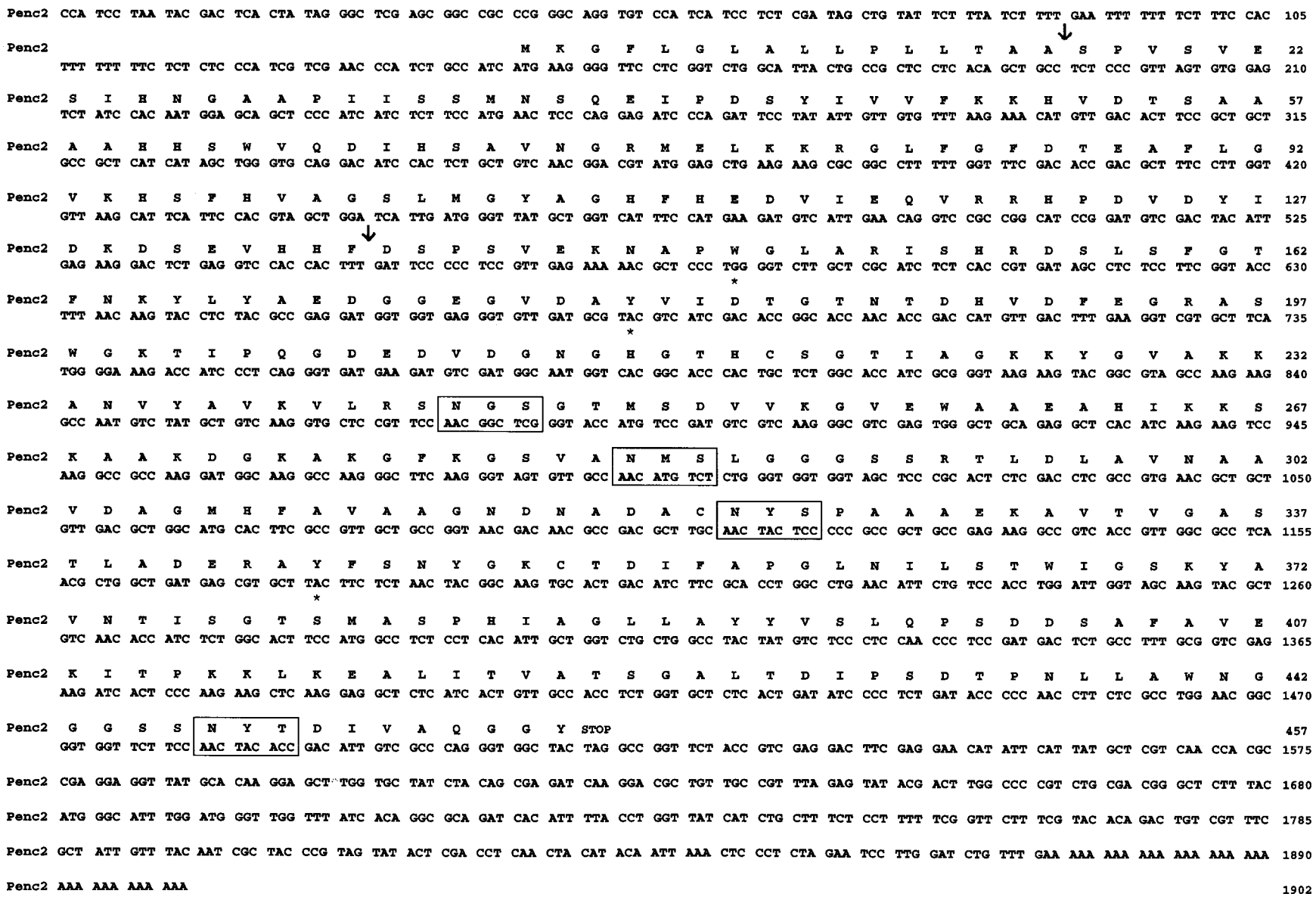

Figure 3 Complete nucleotide and deduced amino acid sequences of Pen c 2

The numbers on the right indicate amino acid residues (top line) and nucleotide positions (bottom line). The putative signal peptide is amino acids 1–16; the propeptide domain is from 17 to 136. The mature protein sequence is numbered from 137 to 457. The arrows indicate cleavage sites for the signal and pro-sequences. The catalytically important residues, Asp, His and Ser, are marked by asterisks. The potential consensus sequences for N-glycosylation are boxed. The GenBank accession number for the cDNA is AF098517.

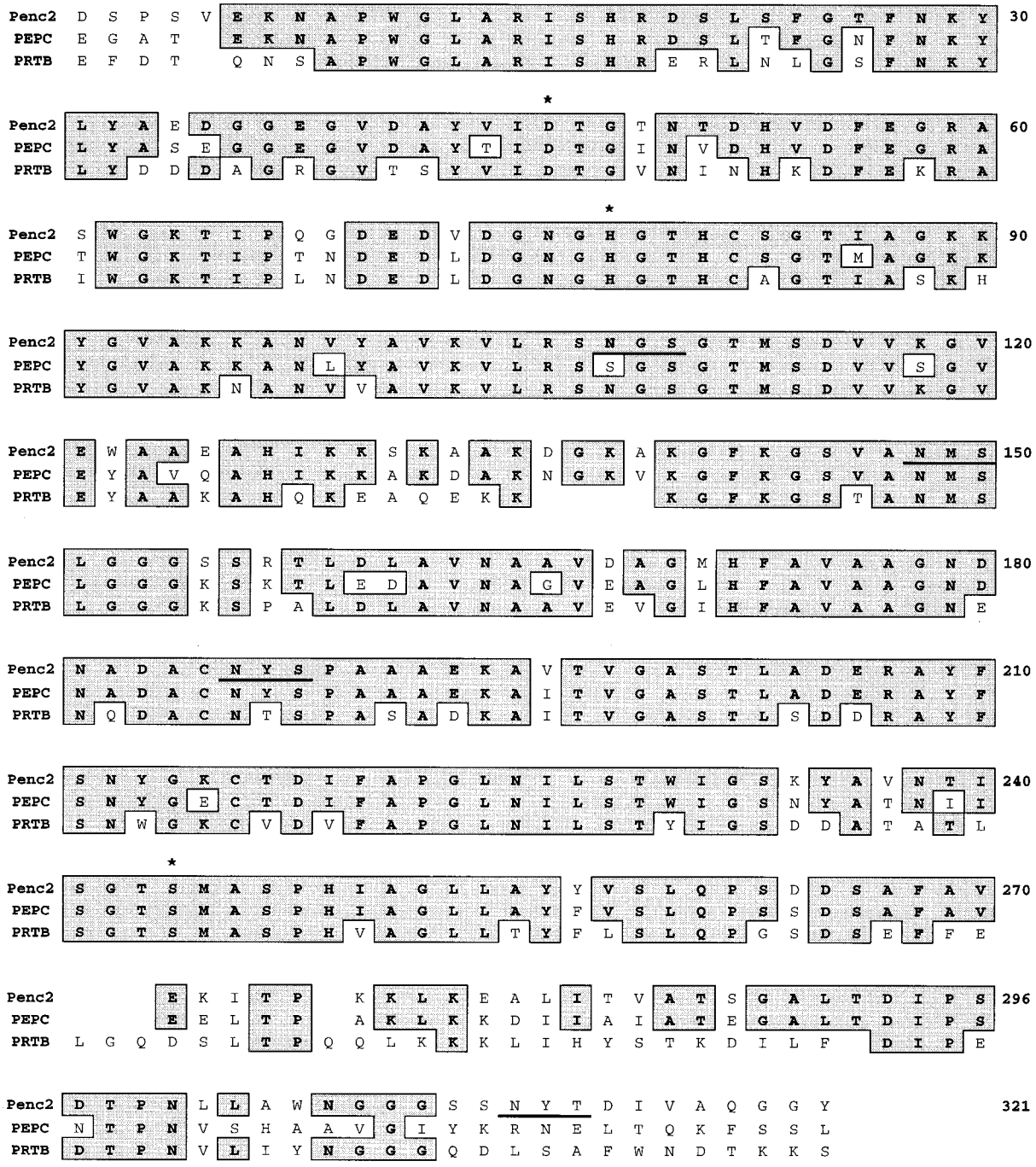

Figure 4 Comparison of Pen c 2 with different vacuolar serine proteases

PEPC is the vacuolar serine protease of *A. niger* [17]; PRTB is the vacuolar protease B of *Saccharomyces cerevisiae* [18]. The numbering system is based on the Pen c 2 sequence. The gaps were introduced for optimal alignment and maximum similarity 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.

His-tag fusion protein. Figure 6(A) shows Coomassie Brilliant Blue-stained crude extracts from transformed *E. coli* M15 before and after induction by IPTG; the induced protein with an apparent molecular mass of 43 kDa can be seen clearly. The Pen c 2 fusion protein was purified by metal-chelate affinity chromatography. Recombinant Pen c 2 was extracted from inclusion bodies under denaturing conditions, applied to the affinity column and eluted by increasing imidazole concentrations. Purified recombinant Pen c 2 consisted of a single band with a molecular mass of approx. 43 kDa (Figure 6A), which bound both the Pen c 2-specific mAb 39 and serum IgE from mould-allergic patients (Figure 6B).

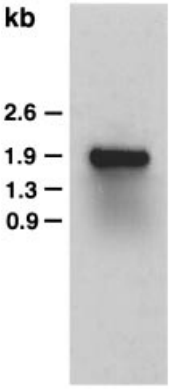

Figure 5 Northern-blot analysis of the Pen c 2 transcript

Pen c 2 cDNA was hybridized to *P. citrinum* mRNA extracted after 1 day of culture. Hybridization was performed using labelled Pen c 2 cDNA as the probe.

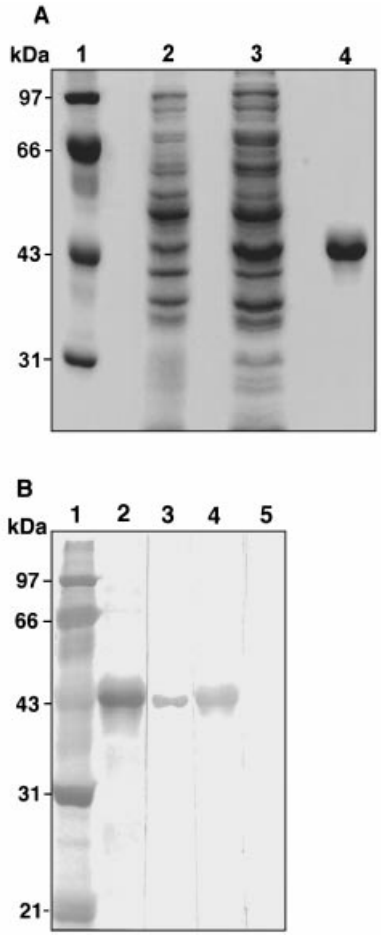

Figure 6 Expression of recombinant Pen c 2 protein in *E. coli*

(**A**) Coomassie Brilliant Blue-stained SDS/PAGE of the recombinant allergen. Lane 1, molecular-mass markers; lane 2, non-IPTG-induced *E. coli* cell lysate; lane 3, IPTG-induced *E. coli* cell lysate producing the recombinant Pen c 2; lane 4, recombinant Pen c 2 purified to homogeneity by affinity chromatography. (**B**) Immunoblot of recombinant Pen c2. Lane 1, molecular-mass markers; lane 2, Coomassie Brilliant Blue-stained recombinant Pen c 2; lanes 3–5, reaction with antibodies (lane 3, mAb 39A; lane 4, serum from an allergic patient; lane 5, serum from a non-allergic patient).

Antigenic structure and epitopes of Pen c 2

Predictive algorithms were used to investigate the antigenic structure of Pen c 2. Antigenic determinants are presumed to be composed of amino acid residues on the protein surface; therefore predictive algorithms were used to probe potential surface residues on Pen c 2. The degree of hydrophilicity, surface probability and flexibility of the primary sequences were the parameters used for antigenic identification. As shown in Figure 7, these features were highly correlated. Several peptide segments were predicted to be major specific epitopes of Pen c 2. These analyses suggest that the IgE-binding epitopes are probably correlated with the extent of protrusion of the polypeptide molecule, and the sequences of the corresponding regions may provide information on the binding between allergen and IgE. These antigenic regions are important for evaluating the mapping of B-cell and T-cell epitopes, and for clarifying the mechanism of the IgE immune response. More detailed immunological analysis and crystallographic work will be required to clarify these points.

DISCUSSION

Moulds are well-known triggers of bronchial hyper-responsiveness or the exaggeration of the response to inflammation and rhinitis seen in allergic asthma. *Penicillium*, one of the most allergenic moulds, has been studied [11]. To date, only one allergen, Pen c 1, has been studied in detail and has been shown to be a caseinolytic enzyme [5]. In the present study, the allergenic profiles of *P. citrinum* were analysed by SDS/PAGE immunoblots using sera from mould-sensitive patients. The antigenic make-up of *P. citrinum* consists of a very heterogeneous group of components. Maximal IgE binding was seen with a 33-kDa protein, shown by sequencing to be Pen c 1. In addition to the 33-kDa allergen, an IgE-binding component with a molecular mass of about 39 kDa was also noted. We propose that this 39-kDa protein is a novel allergen. On two-dimensional analysis, multiple spots were seen in the 33- and 39-kDa regions that could be isomers of the corresponding proteins. The significance of the existence of multiple isoforms identified by serum from allergic patients has yet to be clarified.

A number of clinically significant allergens have been suggested to have protease activity and one of the common allergenic proteases belongs to the serine protease group. Bacterial subtilisins are widely used in the detergent industry [25] and their role in allergic disease has been investigated extensively [26]. The serine protease from *A. oryzae*, which plays a key role in producing the taste of soy sauce and in the brewing industry [27], may also be an important allergen responsible for allergic disorders [9]. The house dust mite allergens Der p 3 and Der f 3 are reported to possess tryptic activity [28,29]. In addition to trypsin, at least two more proteins with chymotryptic and elastase activity, respectively, have been found in extracts from *Dermatophagoides* spp. [30]. Dust mite serine protease allergens have been shown to have a direct effect on epithelial integrity and permeability [31], to cause mast-cell degranulation in a non-IgE-dependent manner [30] and to induce cytokine release from the respiratory epithelium [32]. We suggest that vacuolar serine proteases induce a persistent allergic response, perhaps by facilitating the breaching of epithelial barriers or by participation in the inflammatory processes.

Immunotherapy with mould-allergen extracts is an accepted treatment for allergic disorders. One of the most notable properties of fungal antigens is their complexity, which causes great problems for diagnosis. Difficulties in extracting, purifying and standardizing natural allergens make them less suited for diag-

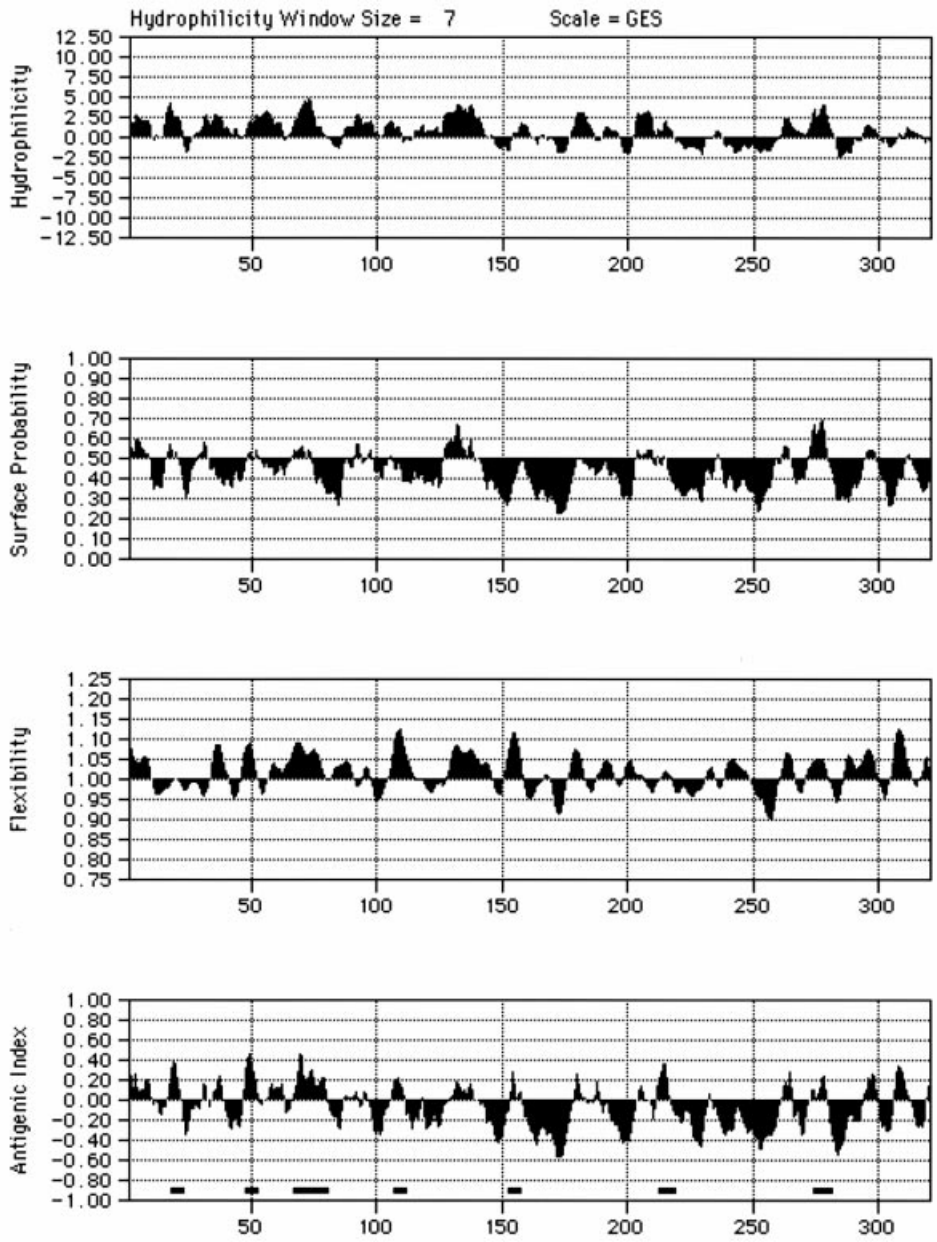

Figure 7 Hydrophilicity, surface probability, flexibility and antigenic index analyses of the protein sequence deduced from the Pen c 2 cDNA

The deduced amino acid sequence was analysed by the Mac Vector computer program. The amino acid numbers are shown below the profiles. The predicted epitopes are indicated by bars at the bottom of the Figure.

nostic and therapeutic purposes than their recombinant counterparts. The characterization and standardization of many species of fungal allergens will be necessary prior to clinical trials, and standardized recombinant allergens may therefore be instrumental in studying the molecular basis of the IgE response and the development of new diagnostic and therapeutic strategies. We have therefore undertaken this pilot study of a mould allergen using recombinant techniques.

We here present our successful attempt to further characterize the allergens of *P. citrinum*; one of these, Pen c 2, has been characterized by cDNA cloning, sequencing and expression in *E. coli*, and the product of the *Pen c 2* gene has been shown to react well with IgE from patients' sera. This recombinant approach seems to be an effective way of obtaining a well-characterized pure allergen for diagnosis and therapy. The availability of the recombinant allergen would facilitate the analysis of its epitopes as well as of the antigenicity and allergenicity of the allergen.

This work was supported by grant NSC-88-2314-B-002-210 from the National Science Council, Taipei, Taiwan. We thank Professor S.-H. Chiou of the Institute of Biochemical Sciences, National Taiwan University, for critical reading of, and comments on, the manuscript.

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Received 25 January 1999/25 March 1999; accepted 21 April 1999