Molecular and immunological characterization and IgE epitope mapping of Pen n 18, a major allergen of *Penicillium notatum*

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The mould genus, *Penicillium*, is a significant source of environmental aero-allergens. A major allergen from *Penicillium notatum*, Pen n 18, was identified by two-dimensional immunoblotting using monoclonal antibody G11A10, raised against the vacuolar serine protease of *Penicillium citrinum*, followed by matrix-assisted laser-desorption ionization–time-of-flight MS analysis of the peptide digest. Pen n 18 was then cloned and the amino acid sequence deduced from the cDNA sequence. The cDNA encoded a 494 amino acid protein, considerably larger than mature Pen n 18, the differences being due to the N- and C-terminal prosequences. The deduced amino acid sequence showed extensive similarity with those of vacuolar serine proteases from various fungi. The Pen n 18 coding sequence was expressed in *Escherichia coli* as a His-tagged fusion protein and purified by Ni^{2+}-chelate affinity chromatography. On immunoblots, the purified recombinant protein specifically bound IgE from mould-allergic patients, and cross-inhibition assays demonstrated the presence of common IgE-binding epitopes on Pen n 18 and a major allergen of *P. citrinum*, Pen c 18. When mapping of the allergenic epitopes was performed, at least nine different linear IgE-binding epitopes, located throughout the Pen n 18 protein, were identified. Of these, peptide C12, located in the N-terminal region of the molecule, was recognized by serum from 75% of the patients tested and therefore appears to be an immunodominant IgE-binding epitope.

Key words: IgE-binding epitope, proteomics, vacuolar serine protease.

INTRODUCTION

Atopic disorders, such as asthma, are becoming increasingly prevalent in developed countries. It has long been recognized that inhalation of fungal spores can produce allergic symptoms in susceptible individuals. Indeed, fungi are regarded as one of the main sources of allergens [1,2]. More than 80 genera of the major fungal groups are known to be allergenic [3], of which respiratory symptom disorders related to *Candida*, *Cladosporium*, *Alternaria*, *Penicillium* and *Aspergillus* species are among the most frequently reported [4,5]. *Penicillium notatum* is a well-known indoor airborne mould and is a major source of fungal inhalant allergens. Sensitization to this mould species is demonstrated using skin-prick test panels for allergic diagnosis [6].

Using molecular cloning techniques, some of the major allergens of *Penicillium* spp. have been characterized. A major *Penicillium citrinum* allergen, Pen c 13 (formerly known as Pen c 1), binds IgE from at least 95% of patients with mould allergy and shares high homology with a group of alkaline serine proteases [7]; the cDNA coding for this allergen has been isolated and characterized [8]. In addition, Pen c 18 (formerly known as Pen c 2) has been characterized as a novel *P. citrinum* allergen with a high degree of sequence identity to the vacuolar serine protease family and also binds human IgE [9]. A 68 kDa major glycoprotein allergen with IgE-binding capacity from *P. notatum* has been cloned and shown to share sequence homology with β-*N*-acetylglucosaminidase [10]. Additionally, allergenic cross-reactivity between a major allergen of *P. notatum*, Pen n 13, and the 33 kDa major allergens of *P. citrinum*, *P. notatum* and *Penicillium brevicompactum* has been demonstrated [11]. In addition, Pen n 13 has been cloned, expressed and shown to display IgE-binding capacity [12]. Data from inhibition studies indicated the presence of IgE cross-reactivity between the 33 kDa major allergens of *Aspergillus flavus* and *P. citrinum* [13], and a similar phenomenon is seen with the 34 kDa major allergens of *Aspergillus oryzae* and *P. citrinum* [14]. These studies have thus revealed the presence of shared allergenic activity in different mould species; shared epitopes also occur within several fungal groups, but only a few species have been examined.

A 32 kDa protein from *P. notatum* was found to bind IgE from 14 of 17 sera (i.e. 82%) from patients with mould allergy, and the N-terminal region of the native protein shows significant sequence similarity with that of vacuolar serine protease from *Aspergillus fumigatus* [15]. We were interested in this protein because it is a major allergen of *P. notatum* in patients allergic to this organism and because of its IgE-binding ability. The allergen has been designated Pen n 18 according to the recommendations of the International Union of Immunological Societies [16]. In the present study, we analysed Pen n 18 allergenic proteins by two-dimensional Western blotting, followed by matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF MS) analysis. In addition, Pen n 18 was cloned and expressed in *Escherichia coli* and its immunological reactivity characterized in terms of the binding of patients' sera, cross-reactivity with Pen c 18 and reactivity of patients' sera with characterized purified Pen n 18-derived peptides. This study increases our understanding

Abbreviations used: MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; PSD, post-source decay; RACE, rapid amplification of cDNA ends; IPTG, isopropyl β-D-thiogalactoside; r, recombinant; mAb, monoclonal antibody.

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The sequence of the Pen n 18 cDNA has been deposited in the GenBank Nucleotide Sequence Database under accession number AF264027.

of Pen n 18 epitopes and provides important information on immunotherapeutic approaches to mould-allergic disease.

MATERIALS AND METHODS

Materials

Oligo(deoxythymidine)-cellulose and Pharmalyte 3-10 were purchased from Amersham Bioscience (Uppsala, Sweden). PVDF membranes were from Millipore (Bedford, MA, U.S.A.). The MALDI matrix α-cyano-4-hydroxy-cinnamic acid was from Fluka Chemical Corp. (Buchs, Switzerland). Restriction enzymes were obtained from New England BioLabs (Beverly, MA, U.S.A.). *Taq* DNA polymerase and the pGEM-T vector were purchased from Promega (Madison, WI, U.S.A.). Other chemicals for molecular-biological techniques were purchased from Gibco BRL (Eggenstein, Germany). The Marathon™ cDNA amplification kit was from Clontech (Palo Alto, CA, U.S.A.). Alkaline phosphatase-labelled horse anti-human IgE antibodies and horseradish peroxidase-conjugated goat anti-mouse IgG antibodies were purchased from Pharmingen (San Diego, CA, U.S.A.). The bicinchoninic acid (BCA) protein assay reagent kit was from Pierce (Rockford, IL, U.S.A.). All other chemicals were purchased from Sigma and were of analytical grade.

Patients' sera and monoclonal antibodies

Serum samples, collected from eight allergic patients with a clinical history of bronchial asthma, in whom IgE reactivity to *P. notatum* antigen had been demonstrated using the Pharmacia CAP system, were used in immunoblotting studies. Sera from normal donors were used as negative controls. To characterize Pen n 18 allergen by immunoblotting, monoclonal antibody (mAb) G11A10, raised against the vacuolar serine protease of *P. citrinum* (EC 3.4.21.–), was used.

Preparation of *P. notatum* allergen extract

P. notatum crude extract was prepared as described previously [15]. Briefly, *P. notatum* CCRC 30568 was grown for 6 days at 25 °C in CzapekDox medium. Then the fungal mats were harvested, extracted with PBS, pH 7.0, for 4 h at 4 °C with constant shaking, and centrifuged at 12000 ***g*** for 15 min at 4 °C. The supernatant was dialysed for 48 h at 4 °C against 50 mM NH_4HCO_3, pH 8.0, then lyophilized to yield the crude extract used for two-dimensional immunoblotting. Protein assays were performed colorimetrically using the BCA protein assay reagent kit.

Two-dimensional immunoblotting and N-terminal sequencing

Crude extracts were analysed by two-dimensional immunoblotting essentially as described previously [9]. Briefly, for the first separation, 1 mg of *P. notatum* extract was applied to an immobilized pH-gradient gel strip containing ampholytes in the pH range 3–10, and isoelectric focusing performed on a Multiphor II electrophoresis system (Amersham Bioscience) according to the manufacturer's instructions. After isoelectric focusing the strip was applied to SDS/PAGE (12.5% gel) [17], blotted on to a PVDF membrane and stained with Coomassie Brilliant Blue R-250 or used for immunoblotting using mAb G11A10. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as a secondary antibody. Following addition of the substrate, 3-amino-9-ethyl-carbazole, and hydrogen peroxide, in acetate buffer [8], protein spots recognized by mAb G11A10 were excised from the membrane and subjected to N-terminal sequence analysis in a Procise ABI 494 protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Peptide mass fingerprinting and post-source decay (PSD) fragment-ion spectra

Protein spots reacting with mAb G11A10 were excised and subjected to in-gel tryptic digestion as described previously [18]. The resulting digests were mixed with saturated α-cyano-4-hydroxycinnamic acid solution in acetonitrile/water and spotted on to a MALDI sample plate. MALDI mass-spectroscopic analysis was performed on a Voyager DE-STR workstation (PerSeptive Biosystems, Framingham, MA, U.S.A.). Peptide spectra, acquired in reflectron mode at an accelerating voltage of 20 kV, were the sum of 50 laser shots. Mass spectra were calibrated externally using low-mass peptide standards. This procedure typically results in mass accuracies of 50–100 p.p.m. The peptide mass fingerprint data were compared with those in the NCBInr protein database using the MS-Fit search tool.

Ions of interest for PSD analysis were obtained after isolation of the appropriate derivative precursor ions using timed ion selection. Fragment ions were refocused on to the final detector by stepping the voltage applied to the reflectron in the following mirror ratios: 1.0 (precursor ion segment), 0.8, 0.75, 0.6, 0.5, 0.4, 0.3, 0.2, 0.15, 0.1, 0.07 and 0.05 (fragment-ion segments). The individual segments were stitched together using software developed by PerSeptive Biosystems. The PSD mass spectra were searched using the MS-Tag program.

Isolation of mRNA

Total RNA was extracted from the mycelium of *P. notatum* using the acid guanidinium thiocyanate/phenol/chloroform method described by Chomczynski and Sacchi [19]. Poly(A)$^+$ RNA was purified using an oligo(dT) cellulose kit.

cDNA cloning and sequencing

The rapid amplification of cDNA ends (RACE) method was employed to produce cDNA fragments coding for Pen n 18 using the Marathon™ cDNA amplification kit [20]. For PCR, a degenerate sense primer, 5′-AACGCYCCYTGGGGYCTTGC-BCG-3′, synthesized according to the N-terminal amino acid sequence (NAPWGLAR), and an antisense primer, 5′-TGAG-GAGAGGCCATGGAAGTGCC-3′, encoding the highly conserved region (GTSMASPH) of the vacuolar serine protease, were used. PCR amplification was performed as described previously [8].

The 5′ end was amplified by 5′-RACE using Marathon-ready cDNAs as templates with the anchor primer AP1, 5′-CCAT-CCTAATACGACTCACTATAGGGC-3′, and a gene-specific primer, 5′-TCCTCGGCATACAAATACTTGTT-3′, corresponding to bp 554–576 of Pen n 18 cDNA. The 3′-end was amplified by 3′-RACE using a method essentially similar to that used for the 5′-end, using the same anchor primer, AP1, and a gene-specific primer, 5′-TGGGGTCTTGCCCGTATCTCT-3′, encoding bp 483–523 of Pen n 18 cDNA. The nested PCR was carried out using anchor primer AP2, 5′-ACTCACTATA-GGGCTCGAGCGGC-3′, and a gene-specific primer, 5′-AAC-ATCCTCTCTACCTGGGTTG-3′, corresponding to bp 1133–1154. The amplified PCR fragments were separated by agarose gel electrophoresis and subcloned into the pGEM-T vector, then transformed into *E. coli* strain JM109. After transformation, plasmids from positive clones were subjected to sequence analysis

using an ABI 377 DNA sequencer (Applied Biosystems) and the dye terminator cycle-sequencing FS Ready reaction.

Purification of recombinant (r) Pen n 18

To produce His-tagged rPen n 18 protein, the cDNA encoding mature Pen n 18 allergen was obtained from *P. notatum* cDNA by PCR amplification using a sense primer, 5′-GCGGATCC-GATGACGATGACAAATCCGAAGGTAGTGTTGAG-3′, and an antisense primer, 5′-CCCAAGCTTCTAGTAACCAC-CATCGGCAAG-3′, designed to introduce *Bam*HI and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively. The amplified fragments were subcloned into the pGEM-T vector and the plasmid DNA isolated, digested with *Bam*HI/*Hin*dIII, and ligated into the similarly digested expression vector, pQE 30 (Qiagen, Chatsworth, CA, U.S.A.). The ligation product was transformed into expressing cells, *E. coli* M15 (Qiagen). Cultures of transformed cells were grown to a D_{600} value of 0.6, induced with 1 mM isopropyl β-D-thiogalactoside (IPTG), and harvested after 2 h. Under these conditions, recombinant $(His)_6$-Pen n 18 fusion protein, isolated as inclusion bodies, was purified under denaturing conditions using a Ni^{2+}-chelate affinity column as described previously [8].

One-dimensional immunoblotting and cross-inhibition assays

Immunoblotting was performed using membrane-bound rPen n 18 and either pooled sera from *Penicillium*-sensitized patients or mAb G11A10. Bound human IgE was detected using alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies and the 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) phosphatase substrate system [8]. Bound mouse mAb G11A10 was detected as described above.

For blotting inhibition analysis, patients' sera were pre-incubated for 2 h at 4 °C with purified rPen c 18 (10 μg/ml), BSA (10 μg/ml) or buffer, then tested for binding to rPen n 18 blotted on to a PVDF membrane and the bound IgE visualized as described above.

Peptide purification and identification

rPen n 18 was digested overnight at 37 °C with lysyl endopeptidase (EC 3.4.21.50), chymotrypsin (EC 3.4.21.1) or Glu-C endopeptidase (EC 3.4.21.19) at an enzyme/substrate ratio of 1:50. The lysyl endopeptidase and chymotrypsin digestions were carried out in 0.1 M pyridine/acetate/collidine, pH 8.2, and the Glu-C endopeptidase digestion in 0.1 M pyridine/acetate, pH 6.5. The resulting peptides were fractionated by reversed-phase HPLC on a Beckman ODS column (Beckman, Fullerton, CA, U.S.A.) using a linear gradient of 5–60 % acetonitrile in 0.06 % trifluoroacetic acid and a flow rate of 1 ml/min. Peptide elution was monitored at 220 nm, and all fractions were collected and analysed by N-terminal sequencing (Procise ABI 494 sequencer) and MS (Perseptive Biosystems Voyager DE-STR mass spectrometer) for peptide mass, sequence and purity.

Peptide dot-blot immunoassays

A total of 19 peptides, covering most of the protein sequence, were immobilized on PVDF membrane as described previously [21], and screened for their ability to bind human IgE in individual serum samples from allergic subjects. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-human IgE and the CDP-Star™ detection system (Amersham Bioscience) according to the manufacturer's instructions.

Molecular modelling of Pen n 18

All computations were performed on an Indigo2 workstation (Silicon Graphics). Two closest homologues of Pen n 18, proteinase K (Protein Data Bank code 2PRK) [22] and subtilase (Protein Data Bank code 1SUP), were selected from available structures in the Brookhaven Protein Data Bank using the 3D-PSSM program [23]. The initial three-dimensional model was built using the X-ray structure of proteinase K as a template, which shared the highest sequence identity (48 %) with Pen n 18. Energy minimization and molecular dynamics were carried out using the molecular modelling package QUANTA (Accelrys, San Diego, CA, U.S.A.). Sequence-conserved regions were located under the QUANTA environment, and the main-chain co-ordinates of Pen n 18 were copied from the proteinase K sequence (code 2PRK). However, most of the gap regions were adjusted manually using XtalView [24], and the resulting model was subsequently energy-minimized. The new model was structurally closer to subtilase than proteinase K, even though the former shared approx. 30 % sequence identity with Pen n 18. The 1SUP sequence was thus used to generate the final (present) model for Pen n 18, after performing another round of energy minimization and molecular dynamics using QUANTA.

RESULTS

Detection of the Pen n 18 allergen

In order to identify the *P. notatum* antigens recognized by mAb G11A10, the proteins in a crude extract were subjected to two-dimensional gel electrophoresis followed by immunoblotting. As shown in Figure 1(A) , protein staining demonstrated the presence of a large number of spots, which were heterogeneous in terms of molecular mass and isoelectric point (pI), the majority having molecular masses of 20–66 kDa and pI values of 4.0–7.0. Immunoblotting showed that mAb G11A10 reacted with components with molecular masses of 37, 34 and 29 kDa (Figure 1B). The 34 and 29 kDa spots (labelled as spots 1 and 2, respectively, on Figure 1B) had identical N-terminal amino acid sequences (SEGSVEK), which showed sequence identity with the known partial sequence of the vacuolar serine protease from *P. notatum* [15]. The 37 kDa spot was not obtained in sufficient amounts for analysis.

Spot 1, the 34 kDa protein with an experimental pI value of 6.5, was then excised and examined by *in situ* trypsin digestion and MALDI-TOF MS analysis. The MS profile showed multiple peaks ranging from 500 to 1800 Da (Figure 2A). Six peaks, with molecular masses of 859.4, 884.4, 949.4, 1142.5, 1210.6 and 1303.7 Da, were selected for comparison with established databases, and the protein with the highest correlation with spot 1 was Pen c 18 allergen (accession number AF098517) [9], corresponding to 50 % sequence coverage. To characterize further the internal sequence of Pen n 18, the signal at 884.4 Da was selected for a MALDI-PSD experiment, and the generated fragment-ion spectrum identified this peptide as having the sequence NAPWGLAR (Figure 2B). Spot 2, the 29 kDa protein with an experimental pI value of 6.7, also showed a high correlation with Pen c 18, its mass spectrum being almost identical with that of spot 1 (results not shown). The mass signals for the 37 kDa spot were too low to be read with confidence.

Sequence of the Pen n 18 cDNA and comparison with vacuolar serine proteases

The Pen n 18 cDNA contains 1750 nucleotides with an open reading frame of 1482 nucleotides (excluding the stop codon), coding for a protein of 494 amino acids. Based on signal-peptide-

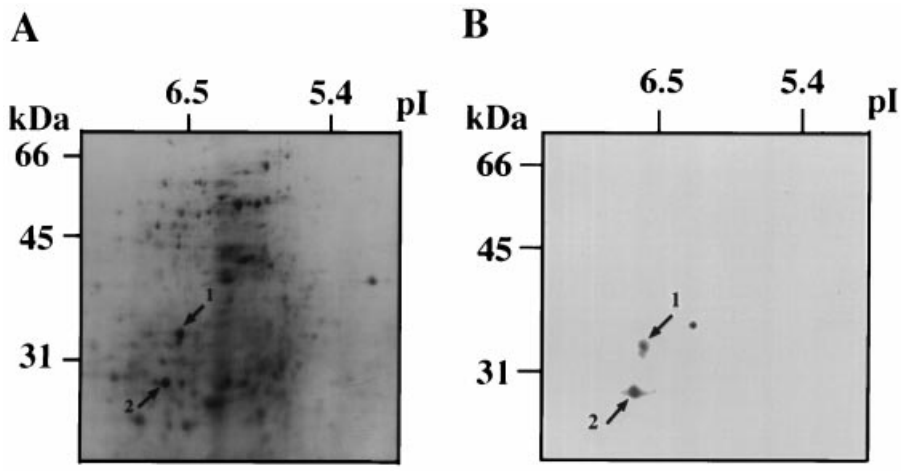

Figure 1 ***P. notatum*** **crude extract separated by two-dimensional electrophoresis**

(**A**) Coomassie Brilliant Blue-stained blot. (**B**) Immunoblots probed with mAb G11A10. The N-terminal sequences of immunoreactive spots 1 and 2 were determined and are given in the text.

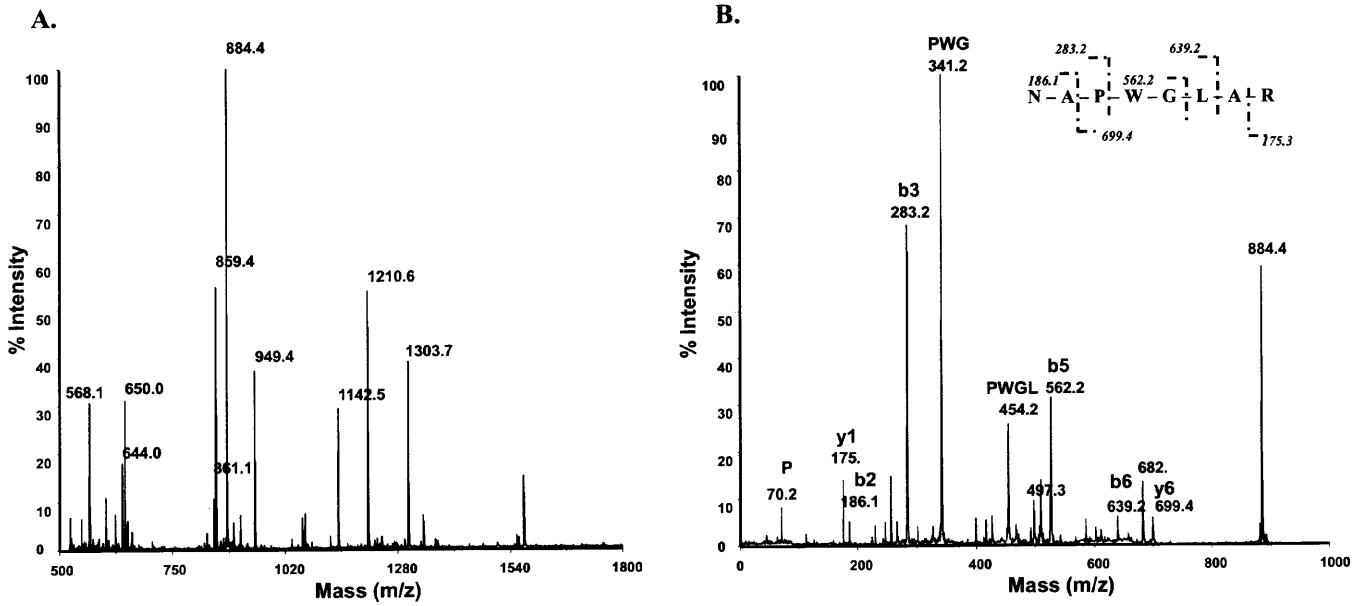

Figure 2 MALDI-TOF MS profile of tryptic digests of spot 1

(**A**) The spot 1 protein was digested *in situ* with trypsin and the six prominent mass peaks chosen for database searches (see the text for details). (**B**) PSD spectrum of the peptide with a mass of 884.4 Da (see **A**); peptide sequence ions from the N-terminus (*b* series) and C-terminus (*y* series) are indicated.

sequence cleavage prediction [25], the precursor of Pen n 18 contains a 16 amino acid hydrophobic leader peptide. This is followed by a 120 amino acid prosequence, a typical feature of serine proteases, in which proteolytic removal of the prosequence is an important step in the generation of active protease from the inactive zymogen. If no additional processing occurs, the mature antigen would be composed of 358 amino acids with a calculated molecular mass of 37.1 kDa and a theoretical pI value of 5.8. The size estimated is similar to the 37.1 kDa determined from the electrophoretic mobility of the protein on the two-dimensional gel.

Database searches showed significant sequence similarity between the deduced amino acid sequence of Pen n 18 and those of vacuolar serine proteases from other fungal species (Figure 3). Conserved amino acid motifs were identified surrounding aspartic acid, histidine and serine residues (Figure 3, asterisks) that form the catalytic triad characteristic of this enzyme family. Pen n 18 shared the highest degree of amino acid sequence identity with Pen c 18 (87.1 %) [9], although other enzymes also showed high similarity, including the vacuolar serine protease PEPC from the pathogenic fungus *Aspergillus niger* (74.5 % identity) [26] and vacuolar serine protease B from *Saccharomyces cerevisiae* (PRTB; 64.1 % identity) [27].

The 87.1 % sequence identity between Pen n 18 and Pen c 18 suggests that they have similar structures. The sequence alignment (Figure 3) reveals that Pen n 18 has three deletions with respect

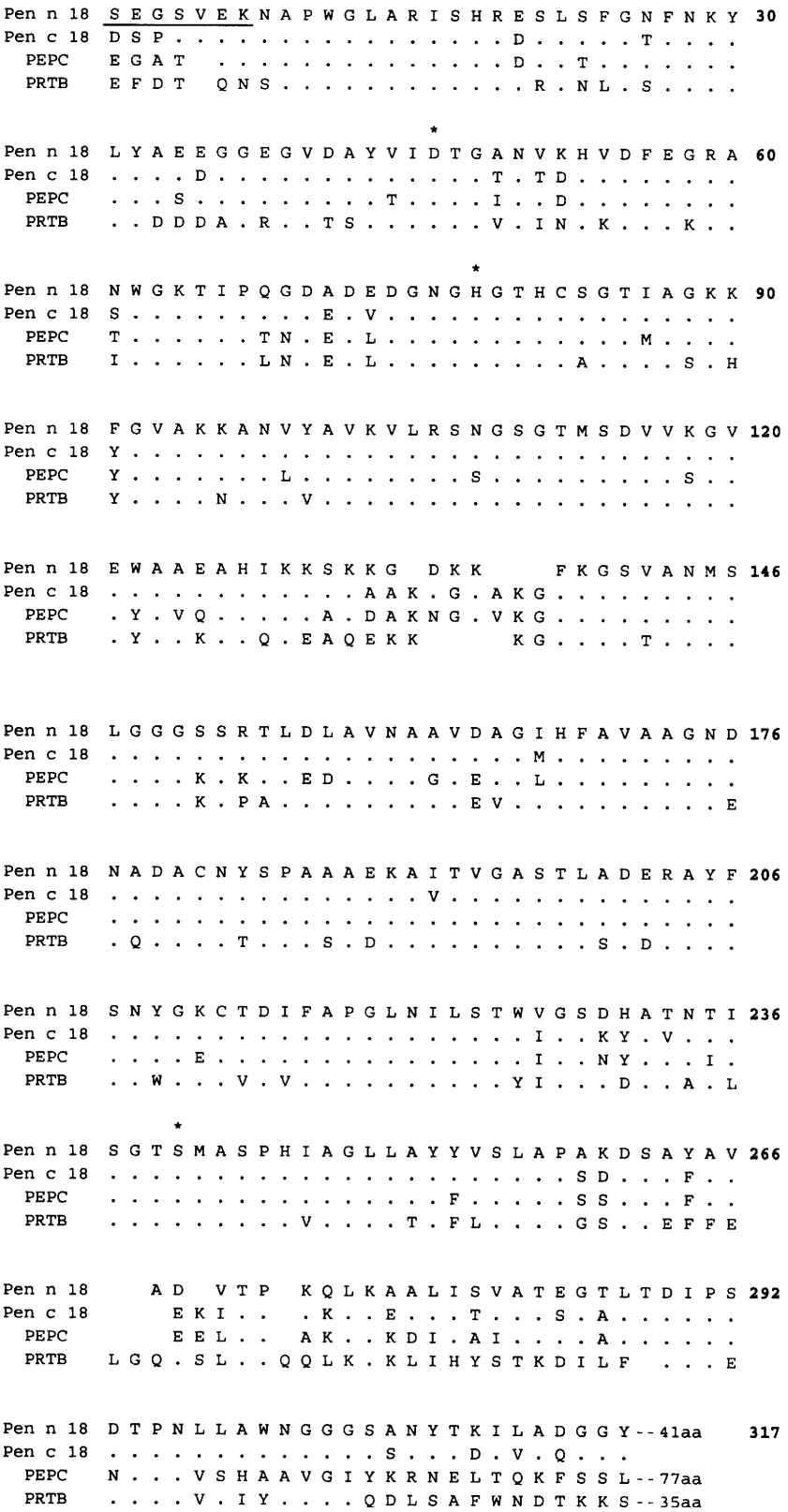

Figure 3 Sequence alignment of Pen n 18 with Pen c 18, PEPC and PRTB protease

The sequences other than Pen n 18 are those for Pen c 18 from *P. citrinum* [9], PEPC, the vacuolar protease from *A. niger* [26], and PRTB, a vacuolar protease B from *S. cerevisiae* [27]. The numbering system is based on the Pen n 18 sequence. Gaps were introduced for optimal alignment and to give maximal homology between all compared sequences. Identical amino acids are shown as dots. The highly conserved and consensus amino acid residues involved in the active site are indicated by asterisks. The underlined amino acids are those found at the N-terminus of Pen n 18.

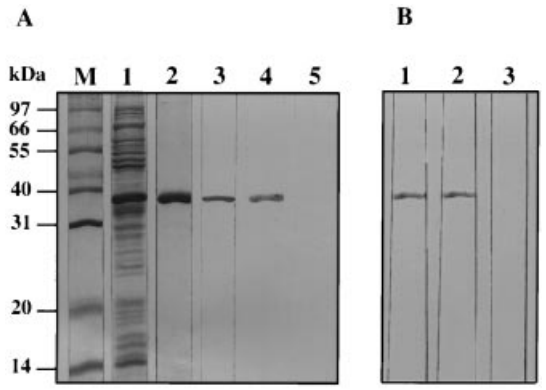

Figure 4 Immunoblot analysis of recombinant Pen n 18 protein

(**A**) Specific antibody recognition. The samples were subjected to SDS/PAGE (12.5% gel) under reducing conditions and the gel transferred to PVDF membranes. Lane M, molecular-mass markers. Lanes 1 and 2, IPTG-induced *E. coli* cell lysate producing rPen n 18 (lane 1) or purified rPen n 18 (lane 2) stained with Coomassie Brilliant Blue. Lanes 3–5, purified rPen n 18 reacted with mAb G11A10 (lane 3), pooled sera from allergic patients (lane 4) or normal control human serum (lane 5). (**B**) Inhibition analysis. A pool of sera from allergic patients was preincubated with rPen c 18 or control samples, as described in the Materials and methods section, then tested for binding to purified rPen n18 blots. Lane 1, preincubation with PBS; lane 2, preincubation with BSA; lane 3, preincubation with rPen c 18.

to Pen c 18, which are as follows: a one-residue deletion after Gly-134, a three-residue deletion after Lys-137 and a one-residue deletion after Asp-268. The most striking difference between the Pen n 18 and Pen c 18 sequences is the presence of an extra 41 residues at the extreme C-terminal part of Pen n 18. The C-terminus of Pen n 18 is perhaps more similar to that of yeast protease B and PEPC (Figure 3), in which the mature protein is thought to be produced by post-translational proteolytic cleavage of the C-terminal extension [26,28].

Expression and purification of rPen n 18

Pen n 18-encoding cDNA was expressed as a $(His)_6$-tagged fusion protein in *E. coli* M15. Heterologous expression of the rPen n 18 allergen resulted in a prominent target protein band in the bacterial lysates (Figure 4A, lane 1), and purification by Ni^{2+}-chelate chromatography resulted in a homogeneous protein solution (Figure 4A, lane 2). On immunoblots, purified rPen n 18 bound mAb G11A10 (Figure 4A, lane 3) and serum IgE from mould-allergic patients (Figure 4A, lane 4), but not IgE from non-allergic donors (Figure 4A, lane 5).

Inhibition of IgE-binding

Immunoblot inhibition was performed to detect the presence of IgE cross-reactivity among allergens from *P. citrinum* and *P. notatum*. Preincubation of mould-allergic patients' sera with rPen c 18 completely abolished IgE binding to recombinant Pen n 18 (Figure 4B, lane 3), whereas preincubation with buffer or BSA (Figure 4B, lanes 1 and 2) had no effect. These inhibition experiments suggest that Pen c 18 and Pen n 18 share identical IgE-binding epitopes.

Identification of linear IgE-binding epitopes of rPen n 18

Reversed-phase HPLC profiles of peptides from the chymotrypsin-, lysyl endopeptidase- or Glu-C endopeptidase-digested

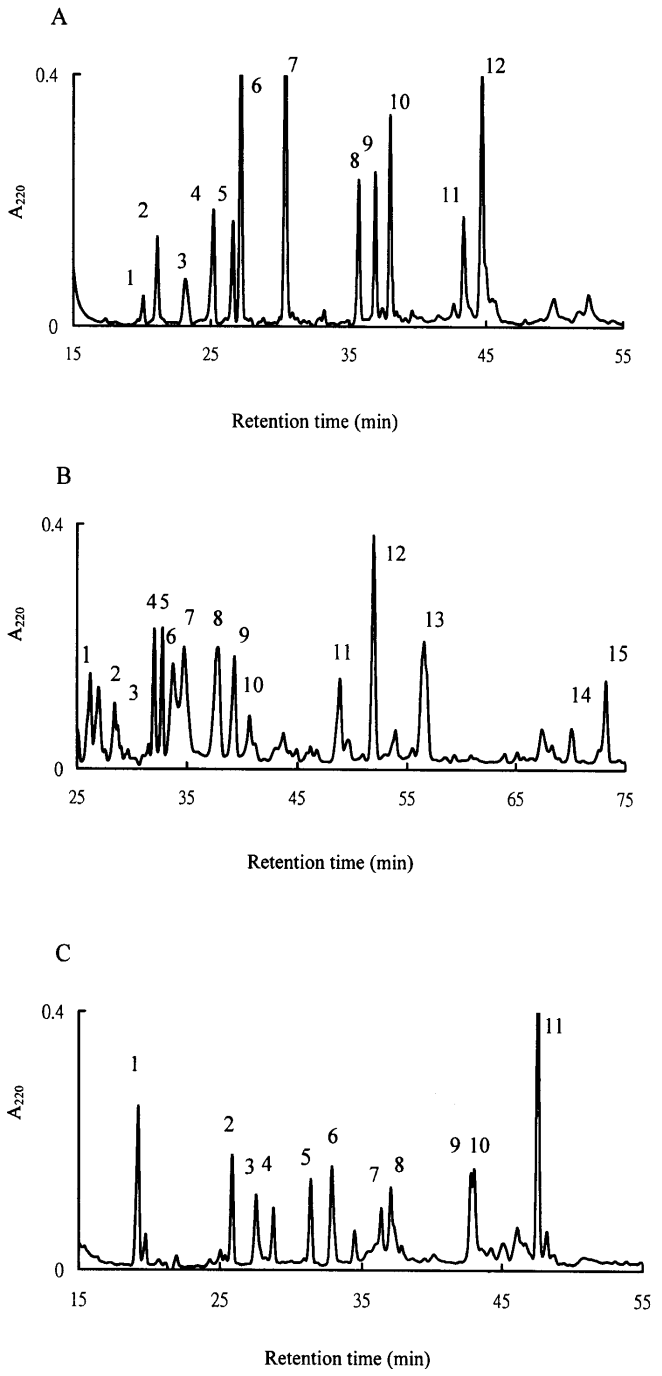

Figure 5 HPLC elution profile of rPen n 18 digests

(**A**) Lysyl endopeptidase digest; (**B**) chymotrypsin digest; (**C**) Glu-C endopeptidase digest. The isolated fragments were then sequenced and subjected to MS analysis. See the text for details.

protein are shown in Figure 5 . The isolated fragments were sequenced and their mass spectra analysed. To test the binding of specific IgE to these peptides, 19 peptides, covering almost the entire amino acid sequence of rPen n 18, were screened on dot-blots using sera from eight *Penicillium*-allergic individuals (Figure 6). The intensity of IgE binding and the epitopes recognized by serum IgE from different individuals varied markedly. At least nine different IgE-binding determinants were found. Antibody-binding regions were identified in peptides C10, C11, C12, C13,

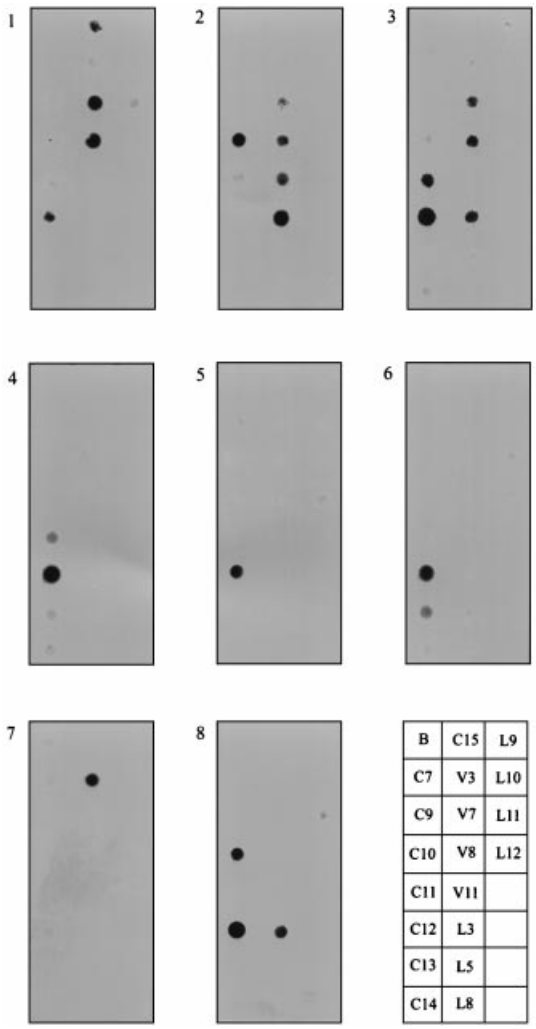

Figure 6 Dot-blot immunoassay using peptides and sera from allergic patients

The selected peptides were probed with serum from eight *Penicillium*-allergic patients (panels 1–8). The box shows the peptides used; B indicates the buffer control.

C15, V3, V7, V8, V11 and L3, corresponding to residues 265–274, 222–242, 44–62, 106–122, 275–298, 126–156, 39–57, 36–57, 285–317 and 65–89, respectively. Since peptides V7 and V8 are virtually the same and showed identical reactivity in all tests, they are considered as a single antibody-binding region. Table 1 summarizes the IgE reactivity of these nine IgE-binding epitopes and their respective positions in the Pen n 18 molecule. Epitope C12 was recognized by serum IgE from 75 % (six in eight) of the mould-allergic patients, classifying it as a dominant epitope in the sampled population. Other epitopes were recognized by serum IgE from 12.5 % (one in eight) to 37.5 % (three in eight) of the patients tested. The control serum sample showed no IgE reactivity (results not shown).

Location of the IgE-binding epitopes on the molecular model of Pen n 18

Based on 48 % sequence identity between Pen n 18 and proteinase K, the crystal structure of proteinase K was used initially for modelling Pen n 18. However, subtilase, sharing approx. 30 % sequence identity with Pen n 18, was proven to be a very good template for building a reasonable model [29]. As seen in Figure 7(A), the molecule consists of an α/β core flanked by several amphipathic helices and anti-parallel β-sheets. Epitope V8 is situated on the N-terminal β-sheet, whereas epitopes C11, C10 and V11 are located on β-sheets close to the C-terminus. Epitope V3 forms a β-sheet in the middle part of the protein. Epitopes L3 and C13 are α-helices adjacent to the N-terminal region, whereas the α-helical epitope C15 covers the extreme C-terminal domain. The immunodominant IgE-binding epitope, C12 (Figure 7B), comprises solvent-exposed residues present in structurally conserved β-sheets and turns.

DISCUSSION

P. notatum is a well-known indoor mould and is frequently included in skin-test panels for allergic diagnosis. One of its proteins, Pen n 18, has been identified as a major allergen in 82 % of patients with atopic dermatitis with mould allergy [15]. In this study, the antigenic profile of *P. notatum* was analysed by two-dimensional immunoblotting using mAb G11A10 raised against the vacuolar serine protease of *P. citrinum*, and three components of the crude extract were found to bind the mAb. These antigens were studied by proteomic analysis, and the largest was cloned and expressed in *E. coli* and its immunological properties tested. Epitope mapping of the recombinant protein was then performed by dot-blotting using purified peptides, covering most of the protein sequence, and IgE from allergic patients.

Fungal extracts used for *in vivo* and *in vitro* diagnosis of sensitization have thus far been prepared from fungal spores and mycelia [30] and the composition of allergenic components may

Table 1 Human IgE-binding epitopes of Pen n 18

N-terminal residues were determined by Edman degradation. Mass was determined by MALDI-TOF MS.

Peptide no.	IgE reactivity (% of patients' sera)	N-terminal sequence	Mass (Da) $[M+H]^+$	Position in Pen n 18
C10	25	AVADV ...	1041.86	Ala^{265}–Leu^{274}
C11	25	ILSTW ...	2149.12	Ile^{222}–Ala^{242}
C12	75	VIDTG ...	2127.91	Val^{44}–Trp^{62}
C13	12.5	RSNGS ...	1809.39	Arg^{106}–Trp^{122}
C15	12.5	KAALI ...	2440.81	Lys^{275}–Leu^{298}
V3	12.5	AHIKK ...	3259.74	Ala^{126}–Asp^{156}
V7	37.5	GVDAY ...	2049.43	Gly^{39}–Glu^{57}
V8	37.5	Not determined	2292.32	Gly^{36}–Glu^{57}
V11	12.5	GTLTD ...	3352.94	Gly^{285}–Tyr^{317}
L3	37.5	TIPQG ...	2438.22	Thr^{65}–Lys^{89}

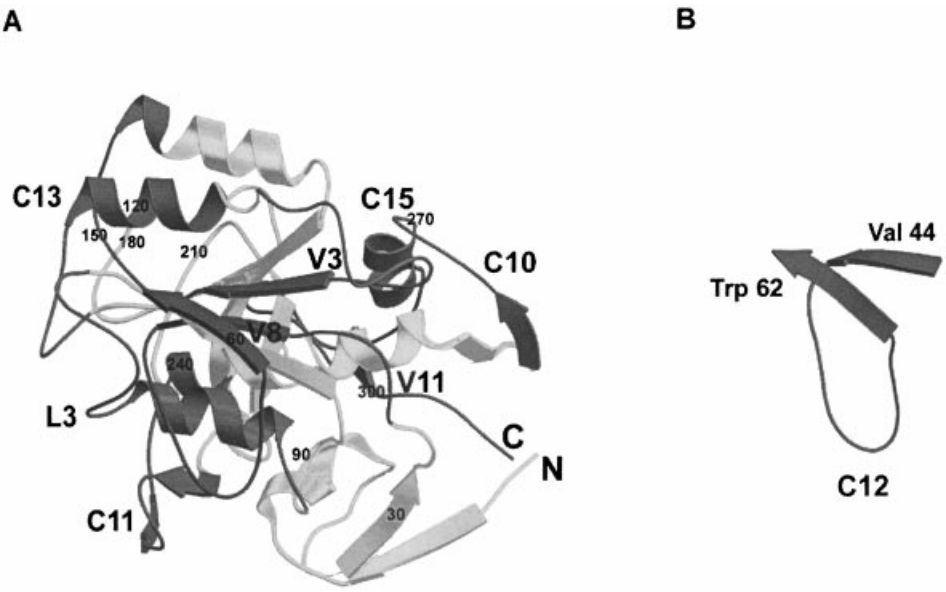

Figure 7 Molecular models and immunodominant IgE-binding epitope of Pen n 18

The ribbon diagrams were produced using Molscript [39] and Raster3D [40]. (**A**) A ribbon diagram representing the Pen n 18 model. The immunodominant and other IgE-binding epitopes are displayed in dark grey. (**B**) A ribbon diagram showing the immunodominant IgE-binding epitope, C 12, of Pen n 18.

vary depending on culture conditions and extraction methods [31]. We have now extracted allergenic components from *P. notatum* and found that the native Pen n 18 allergens have molecular masses of approx. 37, 34 and 29 kDa, somewhat greater than the molecular masses of 32, 28.5 and 28 kDa reported previously [15]; this difference may be due to differences in the preparations.

To the best of our knowledge, this is the first time that the method of peptide mass fingerprinting by MALDI-TOF MS has been used to identify immunoreactive proteins in a crude extract. We also showed that PSD on selected peptides leads to spectra that can be used for peptide sequence analysis. This strategy provides a powerful tool for the identification of allergenic proteins.

Examination of the gene sequence shows that the Pen n 18 precursor comprises four domains, the signal peptide (16 amino acid residues), the N-terminal prosequence (120 residues), mature Pen n 18 (317 residues) and the C-terminal prosequence (41 residues). The N-terminal prosequence seems to be involved in protein folding and precursor processing [32]. The sequence similarity with other vacuolar serine proteases suggests that Pen n 18 may undergo proteolytic processing at its C-terminus [28].

In terms of the expression of a specific rPen n 18 allergen, the fusion protein showed immunological reactivity, indicating that IgE-reactive epitopes were present on the recombinant protein. In cross-inhibition experiments, IgE binding to rPen n 18 was inhibited completely by rPen c 18, implying that Pen n 18 shares epitopes with Pen c 18. The recombinant protein thus bound specific IgE and showed cross-reactivity with allergens in other moulds, suggesting that recombinant allergens can be used for clinical testing and should greatly improve the accuracy of the diagnosis of mould allergy.

At present, only a few recombinant molecules are available for the diagnosis of pollen, mite and mould allergies, but a small number of recombinant proteins is sufficient to diagnose pollen [33] and mould [34] allergy with more than 90% accuracy. The relevant allergens for the precise diagnosis of mould allergy still need to be defined. The accuracy of tests with recombinant allergens will improve significantly in the near future.

Knowledge of the IgE-binding epitope is important in designing effective peptide immunotherapy strategies [35]. To obtain more detailed information on the binding of IgE to the Pen n 18 molecule, we mapped IgE-binding epitopes using enzymic cleavage fragments. Immunoblotting using sera from eight allergic patients showed that patients' IgE bound to different fragments of Pen n 18; their localization is summarized in Figure 7. At least nine distinct IgE-binding epitopes were found, distributed throughout the entire molecule. In molecular modelling studies, these regions were predicted to be on the protein surface and contain mostly solvent-exposed residues, including several conserved amino acids [29]. Moreover, since IgE-binding epitopes are classically defined as being present on the surface of the native antigen, most of the exposed surface of a protein may be antigenic [36]. Consistent with this prediction, the current model of Pen n 18 reveals that the antigenic regions are located on the protein surface.

The results presented provide a detailed description of the IgE-reactive epitopes of the allergen. The identification of an immunodominant IgE-binding hapten could result in safer immunotherapy with a minimum of anaphylactic side effects by local saturation of IgE-producing cells, preventing subsequent activation by contact with native allergen [37]. Alternatively, a cocktail of IgE-binding haptens might be used to saturate IgE bound to effector cells, thus preventing undesirable reactions, such as mediator release or degranulation [38]. Immunotherapeutic approaches utilizing peptides representing IgG epitopes can also shift the balance of antigen-specific antibody production from IgE to IgG [36]. Hapten therapy represents a novel approach to the specific treatment of allergic diseases.

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