

Original article

Molecular and immunological characterization of Pen ch 18, the vacuolar serine protease major allergen of *Penicillium chrysogenum*

Background: We have suggested previously that the 32 and 34 kDa major allergens of *Penicillium chrysogenum* (also known as *P. notatum*) are the vacuolar (Pen ch 18) and the alkaline (Pen ch 13) serine proteases, respectively, of *P. chrysogenum*. The purpose of this study is to characterize the 32 kDa allergen of *P. chrysogenum* and its immunoglobulin E (IgE) cross-reactivity with Pen ch 13 allergen.

Methods: The full-length cDNA of Pen ch 18 was isolated by reverse transcriptase-polymerase chain reaction and the 5'-rapid amplification cDNA end reaction. Recombinant Pen ch 18 was expressed as his-tagged proteins in *Escherichia coli*. Its reactivity with IgE and monoclonal antibodies against fungal serine protease allergens was analyzed by immunoblotting. The IgE cross-reactivity between Pen ch 18 and Pen ch 13 was analyzed by immunoblot inhibition. Overlapping recombinant fragments and synthetic peptides were used to map the B cell epitopes on Pen ch 18.

Results: In this study, we isolated a 1857 bp cDNA fragment containing an open reading frame of 494 amino acids that encodes the preproenzyme of Pen ch 18. Similar to other vacuolar serine proteases, this precursor appears to undergo N- and possibly C-terminal cleavage upon maturation. The his-tagged recombinant Pen ch 18 containing the putative sequence of the mature protein reacted with IgE antibodies in serum samples from asthmatic patients. In addition, IgE-binding to the 32 kDa major allergen of *P. chrysogenum* was inhibited when a positive serum sample was absorbed with recombinant Pen ch 18 before immunoblotting. Both inhibition and almost no inhibition of IgE-binding to the 32 kDa major allergen of Pen ch 18 were detected when eight positive serum samples were preabsorbed individually with purified Pen ch 13 before immunoblotting. The major IgE binding region was located in a fragment (PN1) encompassing the N-terminal 102 amino acid residues of the recombinant Pen ch 18. A dominant linear IgE epitope was further mapped within residues 73–95 (peptide PN1-e) of the N-terminally processed allergen. Monoclonal antibody FUM20 that reacts with Pen ch 18 but not with Pen ch 13 binds a synthetic peptide with sequence encompassing the N-terminal 23 residues of the recombinant Pen ch 18. Monoclonal antibody PCM39 that reacts with both Pen ch 13 and Pen ch 18 recognizes a peptide containing residues 132–154 of the allergen.

Conclusions: Our results confirm that the Pen ch 18 allergen is a vacuolar serine protease of *P. chrysogenum* that matures through N- and possibly C-terminal processing. The finding that there are cross-reactive and allergen-specific IgE epitopes for Pen ch 18 and Pen ch 13 suggests that both major allergens should be included in clinically diagnostic *P. chrysogenum* extracts.

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Key words: allergen; cDNA cloning; epitope mapping; *Penicillium chrysogenum*; vacuolar serine protease.

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Abbreviations: MBP, maltose-binding protein; MMLV-RT, Moloney murine leukemia virus reverse transcriptase; MoAbs, monoclonal antibodies; PVDF, polyvinylidene difluoride; RACE, rapid amplification cDNA end; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Penicillium species are prevalent indoor airborne fungi (1–7). Inhalation of *Penicillium* spores in quantities comparable with those encountered by natural exposure can induce atopic asthma in sensitive persons (8). Intracutaneous skin test was performed on 1000 asthmatic children in Taiwan and the results showed that the

positive rate for *Penicillium* species was 22.3% (9). In another study of 3550 asthmatic patients, 54.5% showed positive immediate skin reactivity to *Penicillium* (10). Furthermore, intradermal skin reactivity in 100 atopic subjects over a 2-year period with 30 different mold extracts demonstrated that *Penicillium chrysogenum* (also known as *P. notatum*) has the highest positive skin test reactivity (68%) (11).

Penicillium citrinum, *P. oxalicum*, *P. chrysogenum*, *P. brevecompactum* and *P. spinulosum* (12) are most regularly recovered among the more than 100 different species of *Penicillium* (13). *Penicillium chrysogenum* is now the most frequently used *Penicillium* species in clinical diagnosis of fungal allergy. However, our knowledge about the allergens of this prevalent *Penicillium* species is still quite limited. Consequently, there is a lack of standardized *Penicillium* extracts for clinical diagnostics.

Our results from studies of *Penicillium* allergens demonstrated that the alkaline and the vacuolar serine proteases with molecular mass of 32–34 kDa are the major allergens of *P. citrinum*, *P. chrysogenum*, *P. brevicompactum* and *P. oxalicum* (14, 15). They have been designated as the group 13 (alkaline serine protease) and the group 18 (vacuolar serine protease) allergens of *Penicillium* species by IUIS Allergen Nomenclature Subcommittee (16). Immunoblotting data showed that immunoglobulin E (IgE) antibodies against components of these prevalent *Penicillium* species could be detected in the sera of about 15–30% of asthmatic patients (16). Most of the positive serum samples tested showed IgE-binding to the 32/34 kDa serine protease(s) with a frequency >80% in different fungal species tested (16). A panel of six monoclonal antibodies (MoAbs) against different epitopes on these serine protease allergens has been generated (14, 17).

We have characterized the 34 kDa alkaline serine protease major allergen (Pen ch 13) from *P. chrysogenum* at molecular level (18). In the present study, we characterize the 32 kDa vacuolar serine protease allergen (Pen ch 18) of *P. chrysogenum* by molecular cloning and heterologous expression. The reactivities of the recombinant Pen ch 18 protein against IgE antibodies in sera from asthmatic patients and the MoAbs against serine protease allergens were analyzed by immunoblotting. The presence of IgE cross-reactivity between Pen ch 13 and Pen ch 18 allergens was analyzed by immunoblot inhibition using purified Pen ch 13 as inhibitor. Epitopes on Pen ch 18 recognized by IgE and MoAbs were analyzed using recombinant and synthetic peptides. Results obtained provide valuable information for clinical mould allergy.

Material and methods

Serum samples

Serum samples used in this study were collected from adult patients with bronchial asthma who attended the allergy clinics at the

Veterans General Hospital-Taipei. These patients had a clinical history consistent with intermittent and reversible airway obstruction and were diagnosed according to the statement provided by the American Thoracic Society (19).

Preparation of allergenic extracts

The *P. chrysogenum* strain (CCRC 30568) was obtained from the Food Industry Research and Development Institute, Hsinchu, Taiwan. The fungus was grown in a liquid medium (CYA) containing Czapek concentrate (13) (10 ml/l), K₂HPO₄ (1.0 g/l), yeast extract (Difco, Detroit, MI; 5 g/l) and sucrose (30 g/l) at 25°C for 6 days. The fungal mat containing both spores and mycelia was collected. The allergenic extracts of *P. chrysogenum* were prepared essentially as described previously (15). The protein content of the extract was determined by the dye-binding method provided by the manufacturer (Bio-Rad, Richmond, CA).

Cloning and molecular techniques

Total RNA of *P. chrysogenum* (CCRC 30568) was extracted from a 4-day-old fungal mat culture grown in a soybean (30 g/l) liquid culture medium at 25°C without shaking. The fungal mat was ground under liquid nitrogen with a mortar and pestle and was re-suspended in Trizol-reagent (Life Technologies, Inc., Frederick, MD). Total RNA was extracted according to the instructions provided by the manufacturer. The sequences of the primers and oligonucleotides used in this study are listed in Table 1. The first-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Stratagene, La Jolla, CA) and oligo-(dT) as primer. The cDNA of the vacuolar serine protease from *P. chrysogenum* was isolated with polymerase chain reaction (PCR) using primers 255 and AP. The degenerated primer 255 was constructed according to the amino acid sequence (residues 11–17, WGLARIS) of the mature fungal protease (15). The primer AP contains the sequence of primer AUAP plus a stretch of oligo-(dT). The PCR product obtained was used as a template in a subsequent PCR with primers 255 and AUAP. The product from the nest PCR reaction was gel purified, inserted into the pGem-Teasy vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5 α competent cells. The plasmid DNA was purified and the nucleotide sequence of the cDNA insert was determined with an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA).

Table 1. Primers for polymerase chain reactions

Name	Nucleotide sequence in 5' to 3'-end orientation
255	TGGGG(C/T)CTTGC(T/C)CG(C/T)ATCTC
AP	GGCCACGCGTCGACTAGTACT-(dT)16
AUAP	GGCCACGCGTCGACTAGTAC
129	GGAGGCGCCAACAGTGT
AAP	GGCCACGCGTCGACTAGTACGGGIIIGGGIIIG
127	AACAGCGTAGACGTTGGC
5NPNBamHI	GCGGATCCGATGACGATGACAAATCCGAAGGTAGTGTGAG
3CPNHindIII	CCCAAGCTTCTAGTAACCAACATCGGCAAG
PN1NE	GCGAATTCTCGAAGGTAGTGTGAG
PN1CH	CCCAAGCTTCTAAACAGCGTAGACGTTGGC
PN2NE	GCGAATTCAAGGCCAACGTCTACGCT
PN2CH	CCCAAGCTTCTAGGAGGCGCCAACAGTGT
PN3NE	GCGAATTCGCTGACGCATGCAACTAC
PN3CH	CCCAAGCTTCTAGTAACCAACATCGGCAAG

The full-length cDNA of the *P. chrysogenum* vacuolar serine protease was obtained by 5' rapid amplification of cDNA end (RACE) reaction. The template cDNA for the 5'-RACE reaction was synthesized by using MMLV-RT and primer 129. The sequence of primer 129 encompasses nucleotides 1077–1094 of the serine protease cDNA that was obtained in the previous PCR. After reaction, the RNA was removed by RNase H (Life Technologies) digestion and the remaining cDNA was purified by precipitation with EZpptTM DNA carrier (GeneTeks BioScience Inc., Taipei, Taiwan). An oligo-(dC) was added to the end of the purified cDNA with terminal deoxynucleotidyl transferase (Promega, Madison, WI). Primers AAP and 127 were used in the 5'-RACE reaction. Primer AAP contains the sequence of primer AUAP and a stretch of oligo-(dG). The sequence of primer 127 encompasses nucleotides 792–809 of the vacuolar serine protease cDNA that was obtained in the previous PCR. The product from the RACE reaction was purified, subcloned, transformed and sequenced as described above.

Preparation of recombinant Pen ch 18

It has been hypothesized that fungal vacuolar serine proteases are synthesized as a larger precursor that undergoes both N- and C-terminal cleavage upon maturation (20–22). The N-terminus of the recombinant allergen was constructed according to the experimentally determined sequence (SEGTSVE) of the mature protein (15). Hereafter in this manuscript, Pen ch 18 is synonymous with the N-terminally processed protein. We number the amino acids on the recombinant and natural allergen according to the protein sequencing results with serine as the first residue. Pen ch 18 was expressed as a 6 × His-tagged protein. The cDNA of Pen ch 18 was amplified through PCR. The forward primer used in the reaction contains the enterokinase and the *Bam*HI-recognizing sites in addition to the cDNA sequence (nucleotides 504–521) encoding the experimentally determined N-terminus of the mature Pen ch 18 (15). We hypothesize that the Pen ch 18 precursor is cleaved at or near the tyrosine residue encoded by nucleotides 1452–1454 of the isolated clone, analogous to the proposed C-termini of the vacuolar serine proteases of *Aspergillus niger* (PEPC) and *P. oxalicum* (20, 22). Thus, the reverse primer contains a *Hind*III restriction site, a newly added stop codon (TAG) and the sequence (nucleotides 1437–1454) encoding the hypothetical C-terminus (LADGGY) of Pen ch 18. The PCR product was ligated into the pQE-30 vector after digesting with *Bam*HI and *Hind*III restriction enzymes. The resulting vector was transformed into *E. coli* M15 for protein expression. The

recombinant protein was affinity-purified with a Ni-NTA resin column (Qiagen, Valencia, CA, USA) under denaturing condition according to the manufacturer's instructions.

Epitope mapping

Recombinant proteins covering three overlapping fragments of Pen ch 18 were prepared to map the epitope(s) recognized by human IgE and mouse MoAbs. Fragments encompassing amino acid residues 1–102 (PN1), 96–197 (PN2) and 178–317 (PN3) (Fig. 1) of Pen ch 18 were expressed as maltose-binding protein (MBP)-fusion proteins (New England Biolabs Inc., Beverly, MA) using the pMAL-c2 expression vector. The cDNA inserts were generated with PCR using three separate sets of primers. These primers are PN1NE and PN1CH, PN2NE and PN2CH, and PN3NE and PN3CH, for fragments PN1, PN2 and PN3, respectively. The PCR products were purified and restricted with *Eco*RI and *Hind*III. They were ligated into the pMAL-c2 vector and transformed into *E. coli* TB1 for protein expression. Epitopes within PN1 and PN2 were further evaluated by synthetic peptides. Peptides with amino acid residues 1–23, 19–41, 37–59, 55–77, 73–95, 91–102, 96–118, 114–136, 132–154, 150–172, 168–190, and 186–197 (Fig. 1) were custom synthesized and arrayed on membranes (ResGen, Invitrogen Corporation, Huntsville, AL). The membrane was then blocked with skim milk and incubated with serum samples from asthmatic patients or with culture supernatants from hybridoma cells. The IgE antibodies and/or MoAbs reacted with the peptides were detected with enzyme-conjugated anti-human IgE or anti-mouse IgG and developed with enzyme substrates as described below in immunoblotting.

Gel electrophoresis and immunoblotting

The crude fungal extract, the purified recombinant protein and the recombinant fusion proteins in *E. coli* lysates were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gels). After separation, protein components were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 µm, Millipore, Bedford, MS) (23). The membranes were blocked with 1% skim milk. Protein components on PVDF membranes reacted with IgE antibodies in serum samples from asthmatic patients and/or MoAbs against serine protease allergens were detected essentially as previously described (15, 17, 18, 22). Serum sample from a mite-sensitive asthmatic patient was used as negative control.

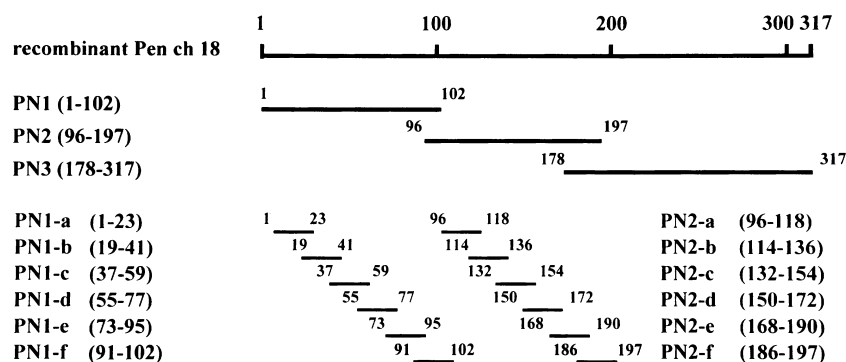


Figure 1. Schematic representation of recombinant fragments (PN1, PN2 and PN3) and synthetic peptides (PN1, a–f and PN2, a–f) covering the amino acid sequence of recombinant Pen ch 18 used in epitope mapping studies. Numbers in brackets stand for the amino acid residues of Pen ch 18.

Six MoAbs against serine protease allergens of *Penicillium* and *Aspergillus* species were used in the present study. They are MoAb 55A (14, 17), PCM8, PCM10, PCM16, PCM39 and Fum20 (17). A culture supernatant containing MoAb against house dust mite allergen Der p 7 (24) was used as a negative control.

Immunoblot inhibition

Immunoblot inhibition was performed to test the antigenic/allergenic relationship between the recombinant Pen ch 18 protein and the native 32 kDa allergen in the crude extracts of *P. chrysogenum*. The presence of allergenic cross-reactivity between Pen ch 13 and Pen ch 18 major allergens was also analyzed by immunoblot inhibition. A serum sample with IgE positivity against the 32 kDa Pen ch 18 allergen was preincubated at 4°C for 1 h with a PVDF membrane blotted with 5 µg of recombinant Pen ch 18 before immunoblotting. In addition, eight IgE-positive serum samples against both Pen ch 13 and Pen ch 18 allergens were mixed individually with 5 µg of the purified Pen ch 13 by end over end rotation for 1 h at room temperature. These samples were then incubated individually at 4°C for 16 h with PVDF blots containing *P. chrysogenum* extracts. As controls, the blots were incubated with the same positive serum samples that have been pre-mixed with 5 µg of bovine serum albumin (BSA; Pierce, Rockford, IL). The blots were washed and incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies and developed with enzyme substrates.

Results

cDNA cloning and sequencing

In the present study, a 1857 bp cDNA encoding Pen ch 18 was obtained through RT-PCR and 5'-end RACE reaction. The nucleotide sequence of this clone has been submitted to GenBank (accession no. AF263454). The

typical 'AATAAA' polyadenylation signal sequence for mammalian genes is missing from the upstream of the polyA tail of the insert (data not shown). This clone encodes a 494 amino acids polypeptide with the assumption that translation is initiated at the first AUG codon (data not shown). The N-terminal 16 amino acids (MKGFLSLTLLPLLVA) of the encoded polypeptide have the characteristic of a hydrophobic leader sequence. We have previously determined the N-terminal sequence of the 32 kDa major allergen of *P. chrysogenum* (15) and it is identical to residues 137–156 (SEG-SVEKNAPWGLARISHRE) of the isolated clone. We designate the Ser137 deduced from cDNA sequencing as Ser1 of Pen ch 18. Without further post-translational modifications upon maturation, Pen ch 18 has 358 residues and a calculated molecular mass of 37 112 Da. Three cysteines (Cys82, Cys181, Cys212) and four putative N-glycosylation sites are identified on the protein. The active-site residues for serine proteases (Asp46, His78 and Ser240) can also be easily located on Pen ch 18 (Fig. 2).

Amino acid sequence alignment revealed that the Pen ch 18 has 83, 78.5 and 63% sequence identity with those of the vacuolar serine proteases of *P. oxalicum* (Pen o 18, accession no. AF243425), *A. fumigatus* (Asp f 18, accession no. Y13338) and *S. cerevisiae* (PRB1, accession no. M18097), respectively. The sulphhydryl residues, the protease active-site amino acids (aspartic acid, histidine, and serine), and three of the four potential N-glycosylation sites are conserved among these fungal proteases (data not shown). In addition, the pro-enzyme region of Pen ch 18 has 71, 73.5 and 26% sequence identity with

Pen ch 18	SEGSVEKNAP	-WGLARTISHR	ESLSFGNFNK	LYAEEGGEGV	DAYVIDTGAN	50
Pen ch 13	AN-VVQRNAP	SHGLSRISSK	KS---GATD	VYDSTAGEGI	VIYGVDTGID	
Pen ch 18	VKHVDFEGRA	NWCKTIPOGD	AEEDGNGHGT	HCSGTIAGKK	FGVAKKANVY	100
Pen ch 13	IGHADFEGRA	EWGTNTADND	-ETDGNCHGT	HTASTAAGSK	FGVAKKASVV	
Pen ch 18	AVKVLRSNGS	GTMSDVVKGV	EWAAEAHIKK	SKKGDKKFKG	SVANMSLGGA	150
Pen ch 13	AVKVLGADGS	GTNSQVIAGM	DWA---VKD	SKSRGATGK-	SVNMMSLGGA	
Pen ch 18	SSRTLDLAVN	AAVDAGIHFA	VAAGNDNADA	CNYSFAAAEK	AITVGASTLA	200
Pen ch 13	YSRAMNDAAA	NVVRSGVFLS	VAAGNEAQA	SNSSPASAPN	VCTIAASTNS	
Pen ch 18	DERAYFSNYG	KCTDIFAPGL	NELSTWVGSD	HATNFIISGTS	MASPHIAGLL	250
Pen ch 13	DGSASTNFQ	SVVDLYAPGK	DITAAYPGG-	-GSKTISGTS	MAAPHVAGAA	
Pen ch 18	AYYVSLAPAK	DSAYAVADVT	PKQLKALIS	VATEGTLTDI	PSDTPNLLAW	300
Pen ch 13	AYLM-----	----ALEGVT	SDKACARIVE	LAI-SSISSA	PSGTTSKILY	
Pen ch 18	NGGGSANYTK	ILADGGYKAH	NAETTVEDRI	GGIIDSAEKA	PHKELGAIYS	350
Pen ch 13	NGINAQ					
Pen ch 18	EIKDAVSA					358

Figure 2. Composite alignment of the deduced amino acid sequences of Pen ch 18 and the mature alkaline serine protease major allergen (Pen ch 13) from *Penicillium chrysogenum*. Dashes denote spaces introduced to optimize the alignment. The active site aspartic acid, histidine, and serine residues are in bold type letters. Identical amino acid residues are shaded. Potential N-glycosylation (Asn-X-Ser or Asn-X-Thr) sites are underlined.

those of the vacuolar serine proteases of Pen o 18, Asp f 18 and PRB1, respectively (data not shown).

The 32 kDa vacuolar serine proteinase (Pen ch 18) and the 34 kDa alkaline serine proteinase (Pen ch 13) have been identified as two major allergens of *P. chrysogenum* (15). Amino acid sequence alignment shows 47% identity between Pen ch 18 and the mature Pen ch 13. The protease active-site residues (aspartic acid, histidine, and serine) are also conserved in both fungal proteases (Fig. 2).

Characterization of the recombinant Pen ch 18

Results from literature (20, 21) and also from our previous study (22) suggest that vacuolar serine protease precursors are processed at both the N- and C-termini. Thus, we expressed Pen ch 18 as His-tagged protein that contains only 317 amino acid residues (no. 1-317 in Fig. 2), excluding the proposed N- and C-terminal prosequences (see Material and methods). The resultant recombinant Pen ch 18 was recovered from inclusion bodies and purified in denaturing condition. It has an apparent molecular mass of about 35.5 kDa as estimated by SDS-PAGE (Fig. 3).

The reactivity of the recombinant Pen ch 18 to IgE antibodies in sera from asthmatic patients and MoAbs against fungal serine proteases was analyzed by immuno-

blotting. Serum samples used were obtained from 41 asthmatic patients. Thirteen of the 41 sera tested showed positive IgE-immunoblot reactivity to components in the *P. chrysogenum* extracts (Fig. 3, part I, panel B). The IgE-binding to a 32 kDa component (Pen ch 18) in *P. chrysogenum* was observed in 10 of the 13 positive serum samples. These 10 sera showed also IgE-binding to recombinant Pen ch 18 (Fig. 3, panel B of part II). The 32 kDa component was found to react with MoAbs PCM39 and FUM20 (Fig. 3, part I, strips 4 and 6 of panel C, respectively). In addition, immunoblot reactivity of MoAb PCM39 and FUM20 to recombinant Pen ch 18 was also detected (Fig. 3, part II, strips 4 and 6 of panel C, respectively). Monoclonal antibodies PCM8 and PCM10 against the 34 kDa Pen ch 13 alkaline serine protease allergen (Fig. 3, strips 1 and 2 of panel C, part I) showed negative immunoblot reactivity to recombinant Pen ch 18 as shown in Fig. 3, strips 1 and 2 of panel C, part II. For negative controls, immunoblot reactivity of a serum sample from a mite-sensitive patient, and a MoAb against Der p 7 were used (strips 14 and 7 in panels B and C, respectively).

The antigenic/allergenic relationship between recombinant Pen ch 18 and the 32 kDa component of *P. chrysogenum* was analyzed by immunoblot inhibition using serum no. 6 shown in Fig. 3. The serum sample was

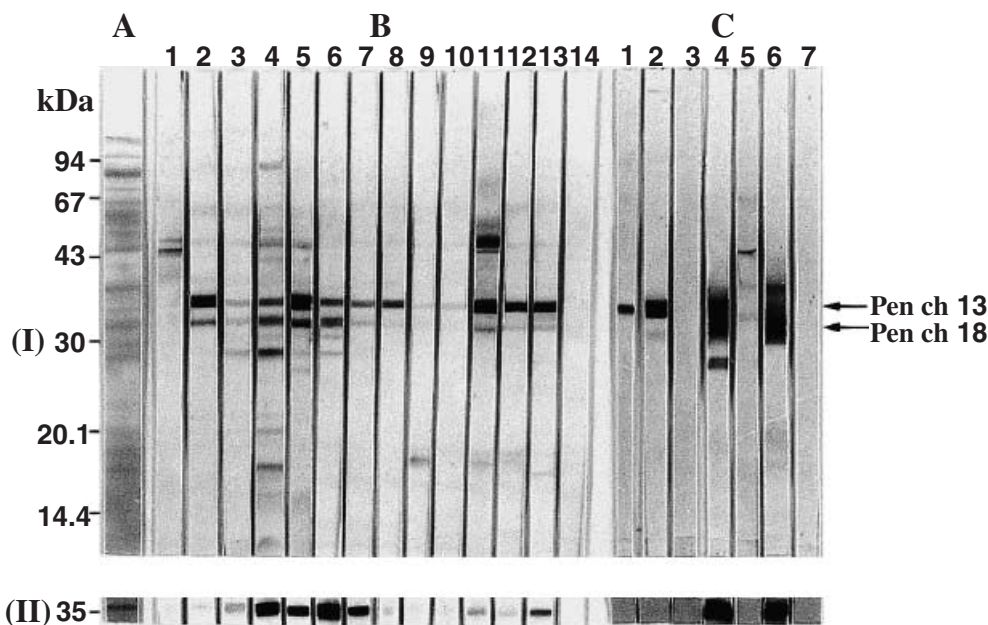


Figure 3. Immunoblot reactivity of immunoglobulin E antibodies in serum from asthmatic patients (panel B) and monoclonal antibodies (MoAbs) (panel C) to components of *Penicillium chrysogenum* (part I) and recombinant Pen ch 18 (part II). Panel A shows the Coomassie blue-stained protein profiles of a *P. chrysogenum* extract (part I) and the recombinant Pen ch 18 (part II) on polyvinylidene difluoride membranes. Strips in panel B show results of immunoblots generated from 13 serum samples of asthmatic patients. Serum 14 is a negative control obtained from a mite-sensitive asthmatic patient. Strips no. 1–6 in panel C are immunoblots generated with MoAbs PCM8, 10, 16, 39, 55A and FUM20, respectively. Strip no. 7 in panel C is a negative control obtained with a MoAb against mite allergen Der p 7.

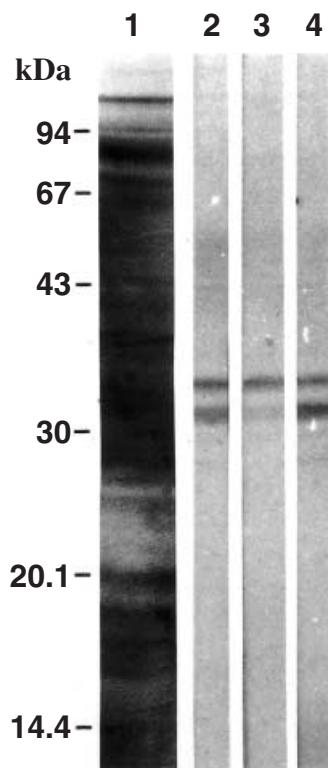


Figure 4. Inhibition of the immunoglobulin E-immunoblot reactivity of a serum sample (serum no. 6 in Fig. 3) that has shown positive reactivity to the 32 kDa component of *Penicillium chrysogenum* (strip 2). Strip 1 shows the Coomassie blue-stained protein profile of a *P. chrysogenum* extract on a polyvinylidene difluoride membrane. Strips 3 and 4 are the immunoblots generated with the serum sample that has been preincubated individually with 5 μ g of recombinant Pen ch 18 and bovine serum albumin, respectively.

preincubated with recombinant Pen ch 18 and inhibition of IgE-immunoblot reactivity to the 32 kDa component of *P. chrysogenum* was detected (Fig. 4, strip 3). This inhibition was not observed when bovine serum albumin was used as inhibitor (Fig. 4, strip 4).

IgE cross-reactivity between Pen ch 13 and Pen ch 18 allergens

The presence of IgE cross-reactivity between these two *P. chrysogenum* allergens was analyzed by immunoblot inhibition using eight asthmatic serum samples. Purified Pen ch 13 prepared as described previously (18) can inhibit the IgE immunoblot reactivity to the 34 kDa allergen in the crude *P. chrysogenum* extracts (Fig. 5, strip 2 in panels B–I). For certain serum samples (Fig. 5, strips 2 of panels G–I), purified Pen ch 13 can also inhibit the IgE immunoblot reactivity to the 32 kDa allergen in the crude extracts. However, this inhibition was not observed for samples presents in panels B–F of Fig. 5 (strips 2). Bovine serum albumin was used as

negative control in these experiments (Fig. 5, strip 3 in panels B–I).

Epitope mapping of Pen ch 18

Antigenic determinants of Pen ch 18 recognized by human IgE and mouse MoAbs PCM39 and FUM20 were analyzed by SDS-PAGE-immunoblot. Results in Fig. 6 demonstrate that 11 sera from asthmatic patients with IgE-binding to the 32 kDa component of *P. chrysogenum* (part I, strips no. 1–11 of panel B) showed IgE-binding to the PN1-MBP fusion protein (54 kDa) in the *E. coli*-lysate (part II, strips no. 1–11 of panel B). A close correlation in intensities of IgE-binding to the 32 kDa component and to the PN1-MBP fusion protein was observed for all the serum tested. Among the 11 serum samples tested, serum no. 6 and 11 of Fig. 6 also showed weak IgE immunoblot reactivity to the PN3-MBP and the PN2-MBP fusion proteins, respectively (data not shown). The fusion proteins (PN1-, PN2- and PN3-MBP) in this study can be recognized by immunoblotting with a rabbit anti-MBP antiserum (New England Biolabs, data not shown). Immunoblot reactivity of IgE-binding to MBP only was not detected for these 11 serum samples (data not shown). Serum from a mite-sensitive patient was used as a negative control (strips 12 in panels B). Monoclonal antibodies PCM39 and FUM20 showed immunoblot reactivity to PN2-MBP and PN1-MBP fusion proteins, respectively (data not shown).

Twelve overlapping peptides (Fig. 1) were constructed to map the linear epitopes within PN1 and PN2. These peptides were arrayed on membranes and results in Fig. 7 show that among the 6 PN1-positive asthmatic serum samples, four have IgE reactivity to peptide PN1-e covering amino acid residues 73–95 of Pen ch 18 (panel A, strips 3–6). One serum sample showed IgE binding to peptide PN1-a (residues 1–23) and another to PN1-d (residues 55–77) as shown in strips 1 and 2 of panel A. Among the six overlapping peptides that cover the sequence of PN2, PN2-b (residues 114–136) reacted with the IgE antibodies in one of the asthmatic serum tested (panel B, strip 1). Serum from a mite-sensitive patient was used as a negative control (data not shown). Monoclonal antibodies FUM20 and PCM39 reacted with peptides PN1-a (aa 1–23) (panel A, strip 7) and PN2-c (aa 132–154) (panel B, strip 2), respectively.

Discussion

Our previous results suggest that the 32 kDa major IgE-binding protein of *P. chrysogenum* is a vacuolar serine proteinase (15). This allergen has previously been designated Pen n 18 (16) of *P. chrysogenum* with the obsolete name of *P. notatum*. The Allergen Nomenclature Subcommittee has renamed this allergen as Pen ch 18. In the present study, the first 16 residues deduced from the

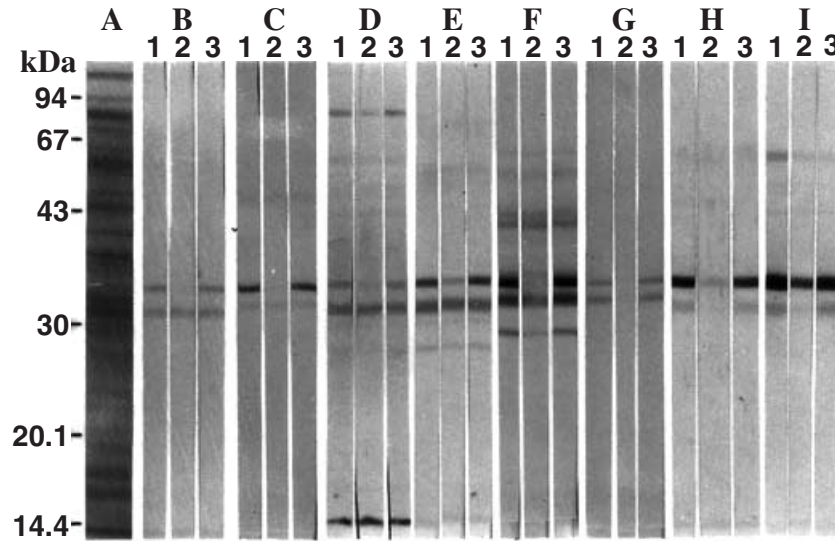


Figure 5. Inhibition of the immunoglobulin E (IgE)-immunoblot reactivity in sera from asthmatic patients to components in the crude extracts of *Penicillium chrysogenum* by purified Pen ch 13. Panel A shows the Coomassie blue-stained protein profile of a *P. chrysogenum* extract on polyvinylidene difluoride (PVDF) membrane. Strips 1 in panels B–I represent IgE immunoblots to components of *P. chrysogenum* generated by using eight asthmatic serum samples. Strips 2 in panels B–I represent immunoblots obtained by incubating the PVDF blots with individual serum sample that has been preincubated with 5 µg of Pen ch 13. Bovine serum albumin was used as a negative control in these experiments (5 µg, strips 3 in panels B–I).

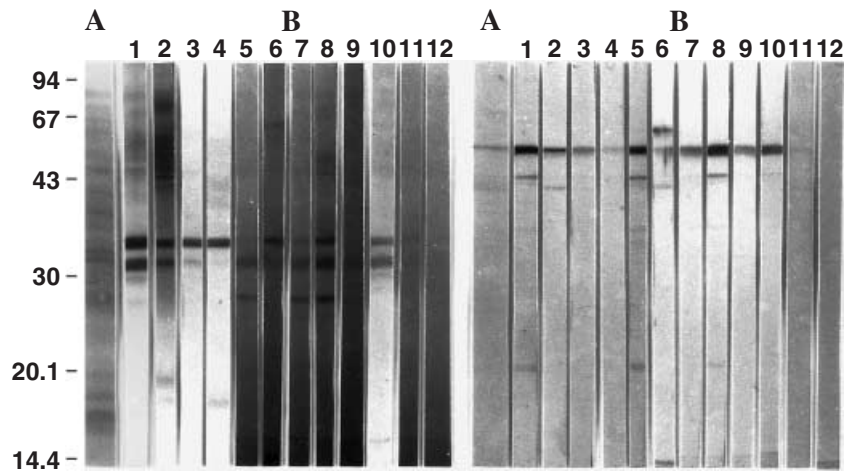


Figure 6. Immunoglobulin E-immunoblot reactivity in sera from asthmatic patients to components of *Penicillium chrysogenum* (part I) and the PN1-MBP fusion protein in *Escherichia coli* lysate (part II). Panels A show Coomassie blue-stained protein profiles of a *P. chrysogenum* extract (part I) and a PN1-MBP fusion protein in a crude *E. coli* lysate (part II) on polyvinylidene difluoride membranes. Strips 1–11 in panels B of both parts are immunoblots generated with sera from 11 asthmatic patients. Serum from a mite-sensitive patient was used as a negative control (strips 12 in panels B).

1857 bp cDNA insert (accession No. AF263454) are predominantly hydrophobic and characteristic of a leader sequence. Amino acid residues 137–156 deduced from the cDNA were found to be identical to those of the N-terminal 20 amino acids of the 32 kDa major allergen of *P. chrysogenum* (15). In addition, Pen ch 18 with the experimentally determined N-terminal sequence shows considerable (63–83%) homology to those of the vacuolar

serine proteases of *A. fumigatus*, *S. cerevisiae* and *P. oxalicum*. A difference in two amino acid residues was also observed between Pen ch 18 and Pen n 18 which was reported recently by Yu et al. (25). Thus, the Pen ch 18 cDNA is translated as a precursor with an N-terminal preproenzyme sequence, as proposed for the vacuolar serine proteases of *A. niger* (PEPC) (20), *S. cerevisiae* (protease B) (21), and *P. oxalicum* (Pen o 18) (22).

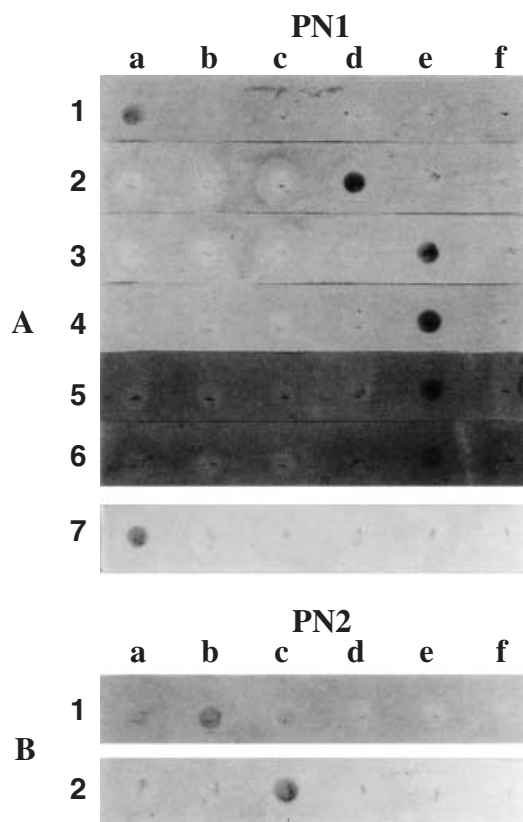


Figure 7. Mapping of epitopes on Pen ch 18 recognized by immunoglobulin E antibodies in sera from asthmatic patients and monoclonal antibodies (MoAbs) FUM20 and PCM39 using overlapping synthetic peptides (PN1, a–f and PN2, a–f) arrayed on membranes. Strips 1–6 of panel A and strip 1 of panel B represent the results obtained by using serum samples from seven asthmatic patients. Strip 7 of panel A and strip 2 of panel B represent results obtained using culture supernatants of hybridoma cells containing MoAb FUM 20 and PCM39, respectively.

Besides N-terminal cleavage, the mature vacuolar serine proteases also undergo C-terminal processing (20–22). The open reading frame for Pen ch 18 is 494 amino acids in length. After removing the N-terminal pro-sequence (136 residues), the remaining polypeptide (358 residues) has a molecular mass of 37 112 Da and is much larger than the 32–33 kDa observed for the mature Pen ch 18. There are several possible C-terminal cleavage sites on Pen ch 18. Cleavage at residue 317 results in a protein with 32 744 Da and a C-terminus homologous to those proposed for the vacuolar serine proteases of *A. niger* and *P. oxalicum* (20, 22). Alternatively, the protein can be cleaved at or near Ala306 or Pro291. The resulting polypeptide has a molecular mass of approximately 30–31 kDa and a C-terminus homologous to Pen ch 13 (Fig. 2), subtilisins (26, 27), proteinase K (28) or the mature protease B (21). Including possible glycosylations, the protein with a cleavage at residue 291 can

have a molecular mass close to that observed for the IgE- and MoAb-binding component in the crude extracts of *P. chrysogenum*. In the present study, our recombinant Pen ch 18 has 317 residues to ensure that it encompasses the complete sequence of the mature protein.

Thus, we postulate that the Pen ch 18 in this study also has a four-domain structure including a signal sequence, an N-terminal pro-sequence, the mature protein, and a C-terminal pro-sequence. The N-terminal pro-sequence seems to be involved in protein folding and processing of the precursor into an active conformation (29, 30). On the contrary, the C-terminal pro-sequence is required for translocating the proteases across the outer member (31–33).

The immunoblot reactivity of the recombinant Pen ch 18 to IgE antibodies in sera from asthmatic patients and to a panel of MoAbs to serine protease allergens was analyzed. Ten of the 13 sera that demonstrated IgE-binding to the 32 kDa component in the *P. chrysogenum* crude extracts also showed IgE-binding to the recombinant Pen ch 18. Among the six MoAbs tested, two (PCM39 and FUM20) showed immunoblot reactivity to the recombinant Pen ch 18. The antigenic profile obtained was found to be identical to that obtained with the 32 kDa *P. chrysogenum* component reported previously (15, 17) and also in the present study. In addition, the recombinant Pen ch 18 can inhibit IgE binding to the 32 kDa allergen. These results indicate that much of the antigenicity is found in the recombinant polypeptide.

Vacuolar serine proteinases have been identified as major allergens in *P. oxalicum* (15), *P. citrinum* (17), *A. fumigatus* (Y13338) and *A. niger* (PEPC) (34). Moreover, alkaline serine proteinases are also major allergens in prevalent airborne *Penicillium* and *Aspergillus* species (16) including *P. chrysogenum* (15). Our results showed that the mature Pen ch 13 (accession No. AF321100) (18) has 47% (132 of 283 amino acids) sequence identity with Pen ch 18. The antigenic cross-reactivity between both allergens was demonstrated with MoAb such as PCM39 (17). However, immunoblot inhibition results (Fig. 4) obtained with a single patient serum and purified recombinant Pen ch 18 as inhibitor indicate that only the IgE-binding to the 32 kDa vacuolar serine protease was inhibited. The IgE-binding to the 34 kDa alkaline serine protease was almost not affected. Results in Fig. 5 also indicate that Pen ch 13 may or may not inhibit IgE-binding to Pen ch 18. These data suggest that both serine protease major allergens of *P. chrysogenum* may have allergen cross-reactive and/or specific IgE epitope(s). Therefore, both allergens may induce individual sensitization in allergic patients and should be included in diagnostic fungal extracts. However, although both serine proteases were detected in extracts prepared in our laboratory, the vacuolar serine protease was absent from a commercially available diagnostic extract of *P. chrysogenum* (15). The MoAbs prepared may be useful in the standardization of crude diagnostic fungal extracts.

In the present study, antigenic determinants of Pen ch 18 recognized by human IgE antibodies and MoAbs PCM39 and FUM20 were analyzed. Our results showed that most of the IgE antibodies in sera from 11 asthmatic patients tested and MoAb FUM20 reacted with recombinant PN1 containing the N-terminal 100 amino acids of the Pen ch 18. However, the cross-reacting MoAb PCM39 reacted with recombinant PN2 covering the middle (residues 96–197) portion of the allergen.

We further narrowed down and found a major IgE epitope to a region within residues 73–95 of Pen ch 18, while FUM20 recognizes an epitope located within the N-terminal 23 amino acids of the recombinant protein. These observations are consistent with our previous findings. Protein spots of *P. chrysogenum* designated as “PN3” and “PN4” on a two-dimensional gel reacted with PCM39 but not with FUM20 (15, 17). The N-terminal amino acid residues of protein spots ‘PN3’ and ‘PN4’ were found to be identical (15) and matched exactly with residues 32–46 of Pen ch 18. It implies that the epitope recognized by FUM20 is located within the N-terminal 31 residues of the allergen.

The epitope recognized by the cross-reacting MoAb PCM39 is located at residues 132–154 of Pen ch 18. Antigenic sites are generally located on the protein surface, in regions of relatively high segmental flexibility and hydrophilicity. The major allergenic (aa 73–95) and antigenic (aa 132–154) peptides identified in this study

correspond to regions of high hydrophilicity as suggest by the GCG computing package (data not shown). However, there might also be discontinuous IgE epitopes that might not be detectable by strategies used in this study.

In conclusion, a full-length cDNA of Pen ch 18 was isolated. Based on sequence homology to other serine proteases and results obtained, the preproenzyme may undergo proteolytic processing at both termini to yield the mature active protease. Residues 73–95 and 132–154 of Pen ch 18 are a major allergenic and a conserved fungal serine protease antigenic epitopes, respectively. The finding that there are allergen-specific IgE epitopes for Pen ch 18 and Pen ch 13 suggests that both major allergens should be included in diagnostic *P. chrysogenum* extracts. The recombinant protein prepared, the MoAbs that have been characterized and information of allergenic/antigenic epitopes obtained in this study are useful in improving immunodiagnosis and developing therapeutic strategies.

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