

cDNA cloning and immunologic characterization of Pen o 18, the vacuolar serine protease major allergen of *Penicillium oxalicum*

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Penicillium species are prevalent indoor airborne fungi that have been identified as causative agents of human extrinsic bronchial asthma. In the preparation of standardized diagnostic reagents, it is imperative to define the allergens of these ubiquitous fungi. Results from our previous study on *P. oxalicum* suggest that the 34-kd major immunoglobulin E-reacting component of this prevalent *Penicillium* species is probably a vacuolar serine protease. The purpose of the present study was to define this major *P. oxalicum* allergen (Pen o 18) through cDNA cloning and immunologic characterization. The cDNA of Pen o 18 was isolated through a combination of reverse transcriptase-polymerase chain reaction and 5'- and 3'-rapid amplification cDNA ends reactions. The primers used in these reactions were constructed according to the internal amino acid sequences of Pen o 18 and the conserved amino acid sequences of fungal serine proteases. Our results showed that a 1897-bp cDNA with an open reading frame of 503 residues was isolated for the proenzyme of Pen o 18. The encoded protein has a 16-residue signal peptide and a 119-residue prosequence. On maturation, the protein has an N-terminal glutamate that is the 136th residue encoded by the cDNA. Apparently the precursor also undergoes C-terminal processing with the cleavage of about 47 amino acids. The cDNA for Pen c 18 (the vacuolar serine protease allergen from *P. citrinum*) was also isolated for comparison. Contrary to a previous report, the C-terminal region of Pen c 18 is similar to that of Pen o 18. Recombinant proteins (rPen o 18 and rPen c 18) with the putative mature N-termini and a his-tag were obtained by expressing the corresponding cDNAs in *Escherichia coli*. Serum samples from 7 asthmatic patients with immunoglobulin E reactivity to the 34-kd component of *P. oxalicum* also react to his-tagged recombinant Pen o 18. The presence of immunoglobulin E cross-reactivity between rPen o 18 and rPen c 18 was detected by immunoblot inhibition. Two monoclonal antibodies (PCM39 and FUM20) against fungal serine proteases react with rPen o 18, rPen c 18, and the 35/34-kd components in the corresponding crude fungal extracts. These components also react with immunoglobulin E antibodies in serum samples from asthmatic patients. In conclusion, results obtained confirm that the 34-kd major allergen of *P. oxalicum* is a vacuolar serine protease. The cDNAs of Pen o 18 and Pen c 18 encode precursor molecules that appear to undergo both N-terminal and C-terminal processing. Constructs beginning with mature N-terminal can be expressed in *E. coli* to produce recombinant polypeptides that are reactive to monoclonal antibodies or immunoglobulin E antibodies in serum samples from asthmatic patients. Results obtained may provide useful information and materials for preparation of standardized diagnostic reagents in clinical mold allergy. (J Lab Clin Med 2001;137:115-24)

Abbreviations: AUG = ribonucleosides adenosine 5'-monophosphate, uridine 5'-monophosphate, and guanosine 5'-monophosphate; IgE = immunoglobulin E; mAb = monoclonal antibody; PVDF = polyvinylidene difluoride; RACE = rapid amplification cDNA ends; RT-PCR = reverse transcriptase-polymerase chain reaction; rPen o 18 = recombinant Pen o 18; rPen o 18s = recombinant Pen o 18 with a C-terminal 47-amino-acid deletion; rPen c 18 = recombinant Pen c 18; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Penicillium and *Aspergillus* are the most abundant genera of indoor fungi¹⁻⁴ that have been implicated in the development of extrinsic bronchial asthma.⁵⁻⁶ Surveys in Taipei, Taiwan, and Topeka, KS, have found that *P. citrinum*, *P. oxalicum*, *P. chrysogenum* (*P. notatum*), *P. brevicompactum*, and *P. spinulosum* are the most frequently isolated *Penicillium* species.^{7,8} Our results from immunoblotting and N-terminal amino acid sequence analyses suggest that the 34-kd major allergen (Pen o 18) of *P. oxalicum* is a vacuolar serine protease.^{9,10} Among 70 adult asthmatic patients, 18 (26%) of them had IgE antibodies in their serum against components from *P. oxalicum*.⁹ Seventeen (94%) of the positive serum samples had IgE reactivity to the 34-kd serine protease.

In this report we describe the cDNA cloning of the 34-kd IgE-reacting component of *P. oxalicum*. We present evidence that it is a vacuolar serine protease and is translated as a precursor polypeptide that undergoes both N-terminal and C-terminal processing. The cDNA encoding the vacuolar serine protease allergen from *P. citrinum* (Pen c 18)¹¹ was also isolated for comparison. In contrast to findings of a previous report,¹² the C-terminal region of Pen c 18 is similar to that of Pen o 18. We investigated the reactivity of his-tagged recombinant Pen o 18 and Pen c 18 proteins (rPen o 18 and rPen c 18) with IgE from serum samples of asthmatic patients and mAbs generated against serine protease allergens from *Penicillium* and *Aspergillus* species.¹¹ The IgE cross-reactivity between rPen o 18 and rPen c 18 was also determined. These vacuolar serine protease allergens cross-reacted at the IgE level and have antigenic cross-reactivity with the mAbs tested.

METHODS

Serum samples. Serum samples were collected from adult patients with bronchial asthma who were attending the allergy clinics at the Veterans General Hospital-Taipei. The samples were stored in aliquots at -70°C . The asthmatic subjects had a clinical history consistent with intermittent and reversible airway obstruction and were diagnosed according to the statement provided by the American Thoracic Society.¹³

Preparation of allergenic extracts. Extracts were prepared from *P. oxalicum* (CCRC33478)⁹ and *P. citrinum* (52-5).¹⁴ The identities of these fungi have been verified by the Food Industry Research and Development Institute of Taiwan (*P. oxalicum* CCRC33478) and the American Type Culture Collection (*P. citrinum* strain 52-5). They were grown in a liquid medium containing Czapek concentrate¹⁵ (10 mL/L), K_2HPO_4 (1.0 g/L), yeast extract (5g/L; Difco, Detroit, MI), and sucrose (30 g/L). Incubation was carried out at 25°C for 6 and 4 days for *P. oxalicum* and *P. citrinum*, respectively. Fungal mats containing both spores and mycelia were collected and ground under liquid nitrogen with mortar and pestle, then extracted with phosphate-buffered saline solution (85

mmol/L of NaCl, 2.6 mmol/L of KH_2PO_4 , and 49 mmol/L of Na_2HPO_4 , pH 8.0) for 4 hours at 4°C with constant shaking. After centrifugation, the supernatant was dialyzed against 50 mmol/L of NH_4HCO_3 , pH 8.0, for 48 hours at 4°C . The dialysate was sterilized by filtration (0.2 μm ; Sartorius, Hayward, CA), lyophilized in aliquots, and stored at -20°C . The protein content of the extracts was determined by the dye-binding method of Bio-Rad, Richmond, CA.

Cloning and molecular techniques. Total RNA was extracted from a 1- to 2-day-old fungal mat culture grown in tryptic soy broth (30 g/L) at 30°C with constant shaking. The fungal mat was ground under liquid nitrogen with mortar and pestle and then resuspended in a Trizol reagent (Life Technologies Inc, Frederick, MD) for total RNA extraction. The mRNA was isolated with an oligo-(dT)-cellulose column purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. The cDNA synthesis was performed with a cDNA amplification kit (Marathon; Clontech Laboratories Inc, Palo Alto, CA). The double-stranded cDNAs were ligated with the Marathon cDNA adaptors according to the manufacturer's instructions. The cDNA of Pen o 18 was isolated by PCR. The sequences of the primers used in this study are summarized in Table I. POF1 and POR2 were constructed according to the amino acid sequences that are conserved among fungal serine proteases. The sense (POF1) and the antisense (POR2) primers encode the N-terminal (EKNAPWGL) and C-terminal (SGTSMASP) conserved amino acid residues, respectively. The PCR product was purified electrophoretically on a gel, subcloned into the pGem-Teasy vector (Promega, Madison, WI), and transformed into JM109 competent cells. The plasmid DNA was purified and the nucleotide sequence of the cDNA insert was determined with an automatic sequencer (ABI 377; Applied Biosystems, Foster City, CA).

The full-length cDNA of Pen o 18 was obtained by 5'- and 3'-end RACE. The primers used were the anchor primer AP1 (Table I) and the gene-specific primers PO4N and PO4C (Table I) that were synthesized according to the internal sequences of Pen o 18 obtained in the previous PCR reaction. PO4N and PO4C cover nucleotide 621 to 643 and 1212 to 1233, respectively, of the Pen o 18 cDNA. Products from the RACE reactions were purified individually, subcloned, and transformed, and the complete nucleotide sequences were determined.

Preparation of recombinant proteins. Pen o 18 was expressed as a 6xHis-tagged protein according to the manufacturer's instructions (Qiagen Inc, Valencia, CA). The cDNA of Pen o 18 was amplified through PCR. The forward primer (5NPOBamHI) used in the reaction encodes the N-terminal amino acid residues (EDPAIE) of Pen o 18⁹ in addition to the enterokinase and the BamHI-recognition sites. The reverse primer (3CPOHindIII-1) contains a HindIII restriction site, a stop codon (TAG), and the sequence covering the 3'-end of the cDNA. The PCR product was isolated by agarose gel electrophoresis and further purified with an extraction kit (QIAquick gel; Qiagen). After BamHI and HindIII restriction enzyme digestion, ligation into the pQE-30 vector and transformation into *E. coli* JM 109 and M15 cells were performed. The recombinant polypeptides expressed in *E. coli* M15 cells

Table I. Primers for polymerase chain reactions

Name	Nucleotide sequence in 5'- to 3'-end orientation
POF1	GA(G/A)AAGAACGCCCTTGGGGTCT
POR2	AGGAGAGGCCATGGA(G/A)GTGCCAGA
AP1	CCATCCTAATACGACTCACTATAGGGC
PO4N	TCCTCGGCGTAAAGGTACTTGTT
PO4C	AACATTCTGTCCACCTGGATTG
5NPOBamHI	GCGGATCCGATGACGATGACAAAGAGGACCCTGCCATCGAG
3CPO-HindIII-1	CCCAAGCTTCTAAGCGCTAACAGC
3CPO-HindIII-2	CCCAAGCTTCTAGTAGCCACCCTTGAAAC
5NVSPBamHI	GCGGATCCGATGACGATGACAAAGATTCCCCCTCCGTT
3CVSPHindIII	CCCAAGCTTCTAAACGGCAACAGCGTCCTT
P1C1VSP5	GCTGATGAGCGTGCTTACTTC
P1C1VSP3	AAACCAACCCATCCAAATGCC

were affinity-purified with an Ni-NTA resin column under denaturing conditions (Qiagen).

The C-terminus of Pen o 18 is 47 amino acids longer than that of the published sequence for Pen c 18, a vacuolar serine protease from *P. citrinum*. Pen c 18 was previously designated as Pen c 2.¹² For comparison purposes, we constructed and expressed a Pen o 18 with a C-terminal 47-amino-acid deletion. This mutant was generated with PCR under identical procedures as outlined above. The primers used in the reaction were 5NPOBamHI and 3CPOHindIII-2 (Table I).

A full-length Pen c 18 was prepared by RT-PCR with mRNA from *P. citrinum* as template. The primers used were 5NVSPBamHI and 3CVSPHindIII (Table I). The forward primer (5NVSPBamHI) encodes the N-terminal five amino acid residues (DSPSV) of the *P. citrinum* sequence^{11,12} in addition to the enterokinase and the BamHI recognition sites. The reverse primer (3CVSPHindIII) contains a HindIII restriction site, a newly assigned TAG stop codon (nucleotide 1640-1642), and the sequence covering nucleotide 1622-1639 of the published Pen c 2 cDNA.¹² Recombinant Pen c 18 with a His-tag was expressed and purified essentially as described above.

SDS-PAGE and immunoblotting. Recombinant proteins and allergen extracts were separated by SDS-PAGE and transferred electrophoretically onto PVDF membrane (0.45 µm; Millipore, Bedford, MA).¹⁶ The membrane was blocked with 1% skim milk, then incubated either with diluted human serum samples (1:5 dilution in Tris-buffered saline solution, pH 7.5, with 0.05% Tween 20 and 1% skim milk) for 16 hours at 4°C or with culture supernatant from hybridoma cells at room temperature for 1 hour. The human IgE antibodies were probed with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmin-gen, San Diego, CA) for 1 hour at room temperature. The blot was then developed with a substrate solution of carbonate buffer (0.1 mol/L NaHCO₃, pH 9.8, and 1.0 mmol/L MgCl₂) containing 0.35 mmol/L 5-bromo-4-chloro-3-indolyl-phosphate and 0.37 mmol/L *p*-nitroblue tetrazolium chloride for about 30 minutes. Serum from a mite-sensitive patient was used as a negative control.

Six mAbs against serine protease allergens of *Penicillium* and *Aspergillus* species were used in the present study.

They are mAbs 55A,¹⁴ PCM8, PCM10, PCM16, PCM39, and FUM20.¹¹ PCM8, 10, 16, and 39 were obtained by immunizing BALB/c mice with culture medium of *P. citrinum* containing the alkaline serine protease. FUM20 was obtained by using the culture medium and the crude extract of *A. fumigatus* as an immunogen. After the blot was incubated with culture supernatant from hybridoma cells and was washed, mAbs on the PVDF membrane were detected with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (H+L; Jackson ImmunoResearch Laboratories Inc, West Grove, PA). The mAbs were visualized by developing the blot in a substrate solution of acetate buffer (50 mmol/L, pH 5.0) containing 2 mmol/L 3-amino-9-ethyl-carbazole (Sigma) and 0.01% (vol/vol) hydrogen peroxide for about 5 minutes. A culture supernatant containing mAb against house dust mite allergen Der p 7¹⁷ was used as a negative control.

RESULTS

cDNA cloning and sequencing. The cDNA for Pen o 18 was constructed by combining the product of RT-PCR with those from the 5'- and 3'-end RACE reactions. The nucleotide sequence of the resulting 1897 bp cDNA clone (GenBank accession number AF243425) is listed in Fig 1 together with the deduced amino acid sequence of the coding region. The AATAAA polyadenylation signal that typically locates within 10 to 30 nucleotides of the poly A tail is not present. Translation of this transcript initiates at the first AUG codon. The open reading frame encodes a 503-amino-acid polypeptide. The N-terminal 16 amino acids of the encoded polypeptide have the characteristic of a hydrophobic leader sequence. We have previously determined the N-terminal amino acid sequence of a 34-kd major allergen of *P. oxalicum* (PO2).⁹ The sequence thus determined is identical to that of residues 136 to 155 of Pen o 18. Therefore the mature Pen o 18 starts from Glu136 of the proenzyme and has a calculated molecular mass of 38,287 daltons, excluding post-translational modifications other than N-terminal cleavage. Pen o 18

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TCCTCTCTCTT CCCTTCCCAT CTCCTACCGC TCGTCGACAG CTTAGTCGAC ACCTATCCTA TCTGTGCTAT      70
CCCTTCCCTTG TGATATCTTT GAATCCCTGT CGTTTCATTGT CCATATCCTA TACCAATCGT CATC      134
  ATG AAG GGC CTA CTC AGC CTG ACA CTC CTT CCG CTG CTG GCA GCT GCA TCT CCC      188
  M K G L L S L T L L P L L A A A S P      18
  ACT TGG TCT GAA ACC ATC CAT AAC GGT GCA GCT CCC CTC ATC TCT TCC ACA AGT      242
  T W S E T I H N G A A P L I S S T S      36
  GCC AAG GAG ATC CCG GAT TCC TAT ATC GTC GTG TTT AAG AAG CAT GTC GGA GCT      296
  A K E I P D S Y I V V F K K H V G A      54
  TCC GCA GCG TCT GCT CAC CAC AGC TGG GTG CAG GAC ATC CAC TCG GAC AAC GTG      350
  S A A S A H H S W V Q D I H S D N V      72
  CGG ATG GAG CTG AAG AAG CGC TCC CTC TTC GGC TTT GAA AGC GAG CCT TAC CTC      404
  R M E L K K R S L F G F E S E P Y L      90
  GGC GTG AAG CAC ACC TTC CAC GTT GCG GGC TCG CTG ATG GGC TAC GCT GGT CAC      458
  G V K H T F H V A G S L M G Y A G H      108
  TTC CAT GAG GAC GTG ATC GAG CAG GTT CGC CGG CAT CCG GAT GTC GAG TAC ATC      512
  F H E D V I E Q V R R H P D V E Y I      126
  GAG AAG GAC TCG GAG GTC CAC CAC TTT GAG GAC CCT GCC ATC GAG AAG AAT GCC      566
  E K D S E V H H F E D P A I E K N A      144
  CCC TGG GGT CTG GCT CGT ATC TCT CAC CGT GAC AGT CTT TCC TTT GGT AGC TTC      620
  P W G L A R I S H R D S L S F G S F      162
  AAC AAG TAC CTT TAC GCC GAG GAT GGG GGT GAG GGC GTC GAC GCC TAT GTC ATC      674
  N K Y L Y A E D G G E G V D A Y V I      180
  GAT ACT GGT ACC AAC GTG GAC CAC GTT GAC TTC GAG GGC CGT GCT TCA TGG GGA      728
  D* T G T N V D H V D F E G R A S W G      198
  AAG ACC ATC CCT CAG GGT GAT CAG GAT GTC GAT GGC AAT GGT CAC GGC ACC CAC      782
  K T I P Q G D Q D V D G N G H* G T H      216
  TGC TCT GGC ACC ATC GCG GGT AAG AAG TAC GGC GTA GCC AAG AAG GCC AAT GTC      836
  C S G T I A G K K Y G V A K K A N V      234
  TAT GCT GTC AAG GTG CTC CGT TCC AAC GGC TCA GGT ACC ATG TCC GAT GTC GTC      890
  Y A V K V L R S N G S G T M S D V V      252
  AAG GGC GTC GAG TGG GCT GCA GAG GCT CAC ATC AAG AAG TCC AAG GCC GCC AAG      944
  K G V E W A A E A H I K K S K A A K      270
  GAT GGC AAG GCC AAG GGC TTC AAG GGT AGT GTT GCC AAC ATG TCT CTG GGT GGT      998
  D G K A K G F K G S V A N M S L G G      288
  GGT AGC TCC CGC ACT CTC GAC CTC GCC GTG AAC GCT GCT GTT GAC GCT GGC ATG      1052
  G S S R T L D L A V N A A V D A G M      306
  CAC TTC GCC GTT GCT GCC GGT AAC GAC AAC GCC GAC GCT TGC AAC TAC TCC CCC      1106
  H F A V A A G N D N A D A C N Y S P      324
  GCC GCT GCC GAG AAG GCC GTC ACC GTT GGC GCC TCA ACG CTG GCT GAT GAG CGT      1160
  A A A E K A V T V G A S T L A D E R      342
  GCT TAC TTC TCC AAC TAC GGC AAG TGC ACT GAC ATC TTC GCA CCT GGT CTG AAC      1214
  A Y F S N Y G K C T D I F A P G L N      360
  ATT CTG TCC ACC TGG ATT GGT AGC AAG TAC GCT GTC AAC ACC ATC TCC GGA ACT      1268
  I L S T W I G S K Y A V N T I S G T      378
  TCC ATG GCC TCC CCT CAT ATC GCC GGT CTC CTG GCC TAC TAT GTC TCT CTC CAG      1322
  S* M A S P H I A G L L A Y Y V S L Q      396
  CCT GCC TCC GAC TCC GCC TAC GCT GTC GAG GAG ATT ACC CCC AAG AAG CTC AAG      1376
  P A S D S A Y A V E E I T P K K L K      414
  GAT GCG CTG ATC ACC ATC GCT ACT TCC GGT GCT CTG TCT GAT ATT CCC TCC GAC      1430
  D A L I T I A T S G A L S D I P S D      432
  ACC CCT AAC CTC CTC GCC TGG AAC GGT GGT GGT TCC TCC AAC TAC ACC GAG ATC      1484
  T P N L L A W N G G G S S N Y T E I      450
  GTT TCC AAG GGT GGC TAC AAG GCT GGT GCT TCC GAG TCC ATG AAG AAG CAC CTC      1538
  V S K G G Y K A G A S E S M K K H L      468
  GAT GAG CTT GTT GGC AAG GTC GAG GAA GTG ATT GCC AAG GAG CAG AAG GTC CTG      1592
  D E L V G K V E E V I A K E Q K V L      486
  AGC CAC GAG CTC GGT GCT ATC TAC AGC GAG ATC AAG GAC GCT GTT AGC GCT TAG      1646
  S H E L G A I Y S E I K D A V S A      503
  AGAGACTTCG TGATCGAGAC CCGTCGTGTA CGGGCCGGTC TTTCTGGGC ATTTTGGAC GGAAGGATTC      1716
  ACATTGGCGC AGATCAGTTG CTTTGTTTTA TCCTGATTCA TGATTACTCT TTCCCCCTTT TTCTTATTGT      1786
  CATGGACTCT TCTATTATTT TTTTCGTCGG ATTAGTCGAT CCGGTGTGCT CAACCTATAG TATCTCACAG      1856
  ATTGTACTTT GAAAAAAAAA AAAAAAAAAA AAAAAAAAAA A      1897

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Fig 1. Nucleotide and deduced amino acid sequence of Pen o 18 cDNA. Numbers to the *right* are the nucleotide and amino acid positions. The first AUG of the transcript is assumed to be the site for translation initiation. A sequence identical to the N-terminal of the 34-kd IgE-reacting major allergen of *P. oxalicum* is underlined. The active site aspartic acid, histidine, and serine residues are marked with *asterisks*. The Asn-X-Ser or Asn-X-Thr potential N-glycosylation sites are also underlined.

Pen o 18	EDPAIEKNAP WGLARISHRD SLSFGSFNKY LYAEDGGEGV DAYVIDTGTN	50
" Pen c 2"	DS.SV.....T.....	
PRB1	.FDT-QNS..E R.NL...S..DD.A.R..TS.....V.	
Pen o 18	VDHVDPEGRA SWGKTIPQGD QDVDGNHGT HCSGTIAGKK YGVAKKANVY	100
" Pen c 2"	T.....E.....	
PRB1	IN.K...K..I.....LN.E.L.....A...S.H...N...V	
Pen o 18	AVKVLRSNGS GTMSDVVKGV EWAAEAHIKK SKAAKDGGKAK GFKGGSVANMS	150
" Pen c 2"		
PRB1	Y..K..Q.EAQEK-----T....	
Pen o 18	LGGGSSRTLD LAVNAAVDAG MHFAVAAGND NADACNYSPA AAEKAVTVGA	200
" Pen c 2"		
PRB1	...K.PA...EV.I.....E.Q...T...S.D..I....	
Pen o 18	STLADERAYF SNYGKCTDIF APGLNILSTW IGSKYAVNTI SGTSMASPHI	250
" Pen c 2"		
PRB1	...S.D....W...V.V.....Y...DD.TA.L.....V	
Pen o 18	AGLLAYYVSL QPASDSAY--AVEEITPKK LKDALITIAT SGALSDIPSD	297
" Pen c 2"	SD...F--...K.....E...V...T.....	
PRB1	T.FL...G...EFFE LGQDSL..QQ..KK..HYS.KDI.F...E.	
Pen o 18	TPNLLAWNGG GSSNYTEIVS KGGYKAGASE SMKKHLDELV GKVEEVIAKE	347
" Pen c 2"	D..A Q...	
PRB1	...V.IY...QDLSAFWND TKKSHSSGFK QELNM-..FI .SKTDL.FDQ	
Pen o 18	QKVLSELGA IYSEIKDAVS A	368
PRB1	VRDILDK.NI .	

Fig 2. Comparison of the deduced primary sequences of Pen o 18 and vacuolar serine proteinases from *P. citrinum* (Pen c 2) and *S. cerevisiae* (PRB1). Residues that are identical to that of Pen o 18 are indicated by periods. Dashes denote spaces introduced to optimize the alignment. A cDNA that encodes for a *P. citrinum* vacuolar serine protease allergen was previously termed Pen c 2 but has since been renumbered according to the IUIS allergen nomenclature subcommittee. It is now designated as a group 18 allergen.

has three cysteines (Cys217, Cys320, and Cys351) and four putative N-glycosylation sites. The active site residues for serine proteases (Asp181, His213, and Ser379) can also be readily identified (Fig 1).

High amino acid sequence identity can be found among Pen o 18 and other vacuolar serine proteases. Pen o 18 has 59% (218 amino acid residues) sequence identity with that of the vacuolar serine protease (protease B, PRB1) from *Saccharomyces cerevisiae*¹⁸ (Fig 2). The C-terminal of the yeast homologue PRB1 is 10 residues shorter than that of Pen o 18 (Fig 2). Sequence alignment also reveals that 304 (95%) of the N-termi-

nal 321 amino acid residues (number 1-321) of the mature Pen o 18 are identical to those of the *P. citrinum* vacuolar serine protease (previously designated Pen c 2 but now reclassified as Pen c 18)(Fig 2). The three cysteines; the four potential N-glycosylation sites; and the active-site aspartic, histidine, and serine residues of serine proteases are all found to be identical in both sequences (Fig 2). In addition, 108 (80%) of the N-terminal 135 amino acid residues in the proenzyme portion of Pen o 18 are identical to those of Pen c 2 (data not shown). However, sequence alignment reveals that the C-terminal of Pen o 18 is 47 amino acids longer

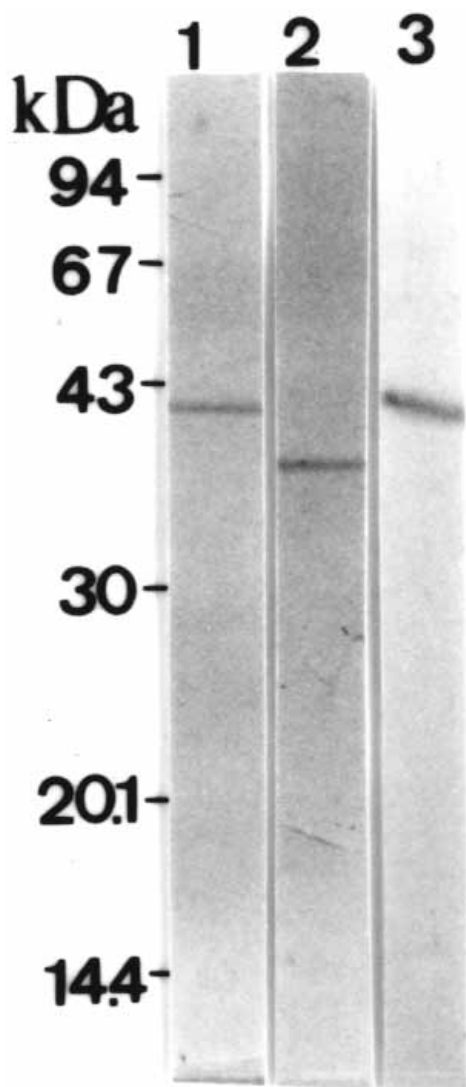


Fig 3. Coomassie blue-stained SDS-PAGE protein profiles of purified recombinant Pen o 18 (strip 1), Pen o 18-s (strip 2), and Pen c 18 (strip 3) on PVDF membranes.

than that of the published Pen c 2 sequence¹² (Fig 2). The nucleotide sequence homology between Pen o 18 and Pen c 2 after the TAG stop codon (number 1516-1518) of Pen c 2 is very high (data not shown). Careful examination of the Pen c 2 nucleotide sequence suggests that a mutation or a cloning error occurring at nucleotide 1516-1518 could possibly introduce a stop codon and result in the observed 47-amino-acid truncation.

We re-isolated the cDNA of Pen c 18 through RT-PCR with primers 3CVSPHindIII and 5NVSPBamHI. The RT-PCR product is 1.1 kbp in length (data not shown) and covers nucleotide 553-1642 of the published Pen c 2 sequence. The deduced amino acid

sequence of this fragment indeed encodes an additional 41 amino acid residues (KAGSTVEDFEEHHK-LVNHAAEEV MYKELGAIYSEIKDAVAV) at the C-terminus of the published Pen c 2 sequence.¹² Twenty-four (59%) of these 41 residues are identical to those on Pen o 18. Instead of a T, we found an A at nucleotide position 1516, thus replacing the TAG stop codon by AAG that encodes for a lysine. In addition, nucleotide 1558 (T) of the Pen c 2 sequence was not found in Pen c 18. Consequently, the TAG sequence at position 1640-1642 of the original Pen c 2 sequence is now a stop codon in Pen c 18. The sequence of the region encompassing nucleotides 1622-1642 was further confirmed by RT-PCR of the *P. citrinum* mRNA with P1C1VSP5 (nucleotide 1162-1182 of Pen c 2) and P1C1VSP3 (nucleotide 1684-1704 of Pen c 2) as the forward and the reverse primers, respectively (Table I).

The 1.1 kbp PCR product constructed here (accession number AF245168) encodes an N-terminally processed Pen c 18 of 358 amino acid residues with a calculated molecular mass of 37,400 daltons. Besides the C-terminal extension, other differences observed between Pen c 18 and Pen c 2 include a tetrapeptide deletion on Pen c 18 (DGKA, residue 272-275 of Pen c 2) and substitution of residues at positions 197, 204, 267, 270, 307, and 330. They were S, Q, S, A, M, and K in Pen c 2 and N, E, A, G, I, and N in Pen c 18, respectively.

Characterization of recombinant polypeptides. Pen o 18, Pen o 18 with a 47-amino-acid truncation in the C-terminus (Pen o 18-s), and Pen c 18 were expressed as His-tagged proteins in *E. coli*. These recombinant proteins were all produced as inclusion bodies and purified in denaturing conditions. The molecular masses of rPen o 18, rPen o 18-s, and rPen c 18 were estimated at 41, 36, and 41 kD, respectively, by SDS-PAGE analysis (Fig 3).

The reactivity of rPen o 18 and rPen o 18-s to IgE antibodies in serum samples from asthmatic patients was analyzed by immunoblotting. The serum samples used were obtained from 7 asthmatic patients and showed positive IgE immunostaining on Western blots against a 34-kD component from *P. oxalicum* (Fig 4, part I, section B). All 7 serum samples tested have IgE-immunoblot reactivity to Pen o 18 and Pen o 18-s, as demonstrated in Fig 4, section B, of part II-a and part II-b, respectively. The 34-kD component from *P. oxalicum* can react with mAbs PCM39 and FUM20, as shown in strips I-4 and I-6, respectively, of section C. In addition, immunoblot reactivity of mAb PCM39 and FUM20 to Pen o 18 and Pen o 18-s was also detected (Fig 4, section C, strips 4 and 6 of part II-a and part II-b, respectively). A serum sample from a mite-sensitive patient and a mAb against Der p 7 were used (strips

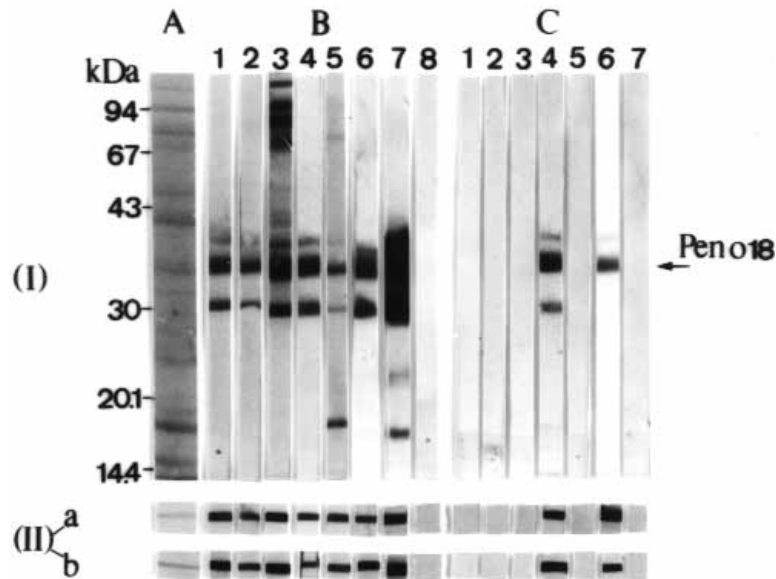


Fig 4. Immunoblot reactivity of IgE antibodies in serum from asthmatic patients (*section B*) and mAbs (*section C*) to components of *P. oxalicum* (*part I*), rPen o 18 (*part II-a*), and rPen o 18-s (*part II-b*). *Section A* shows the Coomassie blue-stained protein profiles of the *P. oxalicum* extract (*part I*), the rPen o 18 (*part II-a*), and the rPen o 18-s (*part II-b*) on PVDF membranes. Strips in *section B* show results of immunoblots generated from seven serum samples from asthmatic patients. Serum 8 is a negative control obtained from a mite-sensitive asthmatic patient. Strips 1 through 6 in *section C* show results of immunoblots generated with mAbs PCM8, 10, 16, 39, 55A, and FUM20, respectively. Strip 7 in *section C* is a negative control obtained with a mAb against mite allergen Der p 7.

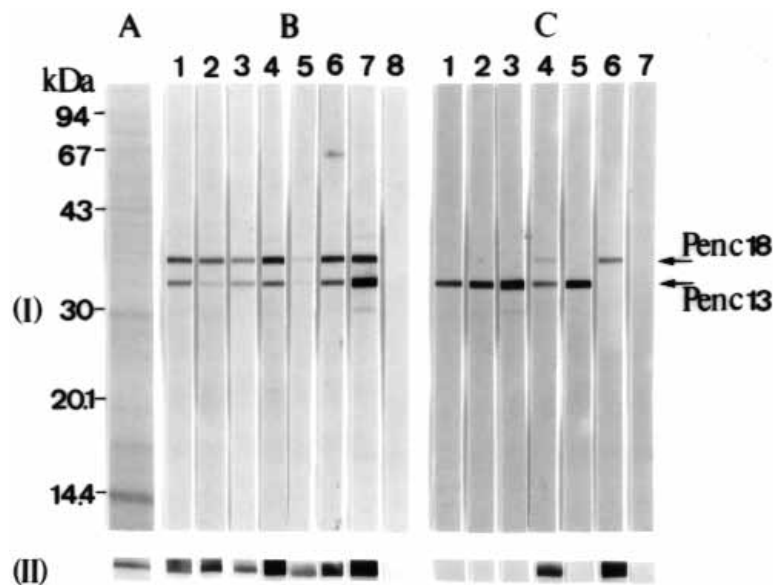


Fig 5. Immunoblot reactivity of IgE antibodies in serum from asthmatic patients (*section B*) and mAbs (*section C*) to components of *P. citrinum* (*part I*) and rPen c 18 (*part II*). *Section A* shows the Coomassie blue-stained protein profiles of the *P. citrinum* extract (*part I*) and the rPen c 18 (*part II*) on PVDF membranes. Strips in *section B* show results of immunoblots generated from seven serum samples (strips 1 through 7 in Fig 4) from asthmatic patients. Strip 8 is a negative control obtained from the same mite-sensitive patient as shown in Fig 4. Strips 1 through 6 in *section C* show results of immunoblots generated with mAbs PCM8, 10, 16, 39, 55A, and FUM20, respectively. Strip 7 in *section C* is a negative control generated with a mAb against mite allergen Der p 7.

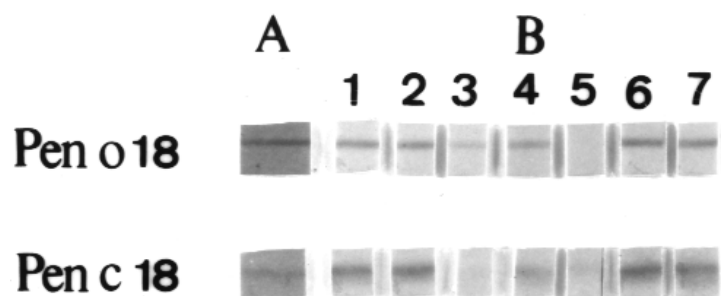


Fig 6. Inhibition of the IgE-immunoblot reactivity of a serum sample (strip 4 in Figs 4 and 5) that has shown positive reactivity to Pen o 18 (strip 1, upper panel) and Pen c 18 (strip 1, lower panel). Strips in section A show the Coomassie blue-stained protein profiles of rPen o 18 and rPen c 18 on PVDF membranes. Strips 2 through 7 show the immunoblots generated with the serum sample preincubated individually with 0.2 µg (strips 2, 4, 6) and 2.0 µg (strips 3, 5, 7) of rPen o 18 (strips 2, 3), rPen c 18 (strips 4, 5), and bovine serum albumin (strips 6, 7).

8 and 7 in sections B and C, respectively) as negative controls.

The same set of asthmatic serum samples was also used in the detection of IgE reactivity to components from *P. citrinum*. All 7 serum samples (Fig 5) tested have IgE reactivity to the 35- and 33-kd components of *P. citrinum* (part I, section B) and to rPen c 18 (part II, section B). mAbs PCM8, 10, 16, and 55A reacted with the 33-kd allergen (Pen c 13) of *P. citrinum* (strips 1, 2, 3, and 5 in part I, section C) as described previously.¹¹ All four mAbs showed negative immunoblot reactivity to Pen c 18 (strips 1, 2, 3, and 5 in part II, section C). mAb PCM39 reacted with the 33- and 35-kd components of *P. citrinum*, whereas mAb FUM20 reacted with the 35-kd component of *P. citrinum*, as shown in strips 4 and 6 of part I, section C. Both mAbs reacted with rPen c 18 (strips 4 and 6 of part II, section C).

The IgE cross-reactivity between Pen o 18 and Pen c 18 was analyzed by immunoblot inhibition with serum number 4 in Figs 4 and 5. Recombinant proteins were blotted individually onto PVDF membranes and were used as inhibitors. Serum aliquots were preincubated at 4°C for 6 hours with 0.2 and 2.0 µg of inhibitor before being used in immunoblotting experiments. Dose-dependent inhibition of the IgE reactivity to rPen o 18 (Fig 6, upper panel) and rPen c 18 (Fig 6, lower panel) was detected when serum aliquots were preincubated with allergen polypeptides. No inhibition was detected when 0.2 and 2.0 µg of bovine serum albumin was used as an inhibitor.

DISCUSSION

The vacuolar serine protease has been identified as a major allergen (Pen o 18) in a prevalent airborne *Penicillium* species, *P. oxalicum*.⁹ In the present study, a full-length 1.9 kbp cDNA of Pen o 18 was cloned

through RT-PCR and RACE reactions. As in most fungal genes,¹⁹ the first ATG codon of the cDNA is the translation initiation site. An adenosine is located at the -3 position (relative to the starting codon), which is in agreement with the consensus sequence observed for most (83%) fungal genes.¹⁹ The penultimate 16 amino acids have the characteristics of a typical hydrophobic leader sequence. The stop codon of the putative protein is located at nucleotide 1644-1646. Hence, Pen o 18 encodes a 503-residue polypeptide including the hydrophobic leader sequence.

The N-terminal amino acid sequences of four different variants of Pen o 18 have been determined.⁹ The first 20 amino acids of a protein designated PO2 are identical to residues 136 through 155 of the sequence deduced from the cDNA of Pen o 18. Apparently Pen o 18 is synthesized as a precursor with an N-terminal proenzyme sequence, as suggested for the vacuolar serine proteases of *A. niger*¹⁹ and *S. saccharomyces*.¹⁸ As found for other vacuolar hydrolases such as protease B and carboxypeptidase Y of the yeast *S. cerevisiae*,^{18,20} the precursors pass through the endoplasmic reticulum to the Golgi body, where they are sorted from secretory proteins and transported to the vacuole.

The mature Pen o 18 protein shows considerable amino acid sequence homology to vacuolar serine proteases of other fungal species. It shares 86%, 70%, 79%, and 59% sequence identity with Pen c 18 (*P. citrinum*), pepC (*A. niger*), Y13338 (*A. fumigatus*), and yeast protease B, respectively. This homology is most conspicuous at the central portion of the polypeptides, including the region containing the active site triad and the locations of the cysteines residues. Four potential N-glycosylation sites can be found in Pen o 18, and they are conserved in Pen c 18. Three of them can be found in the vacuolar serine proteases from *A. fumigatus*

and *S. cerevisiae*. Two of them are conserved in pepC. Whether any or all of them are glycosylated needs further study.

Apparently, vacuolar serine protease precursors are also processed at the C-terminus. Yeast protease B is synthesized as a precursor¹⁸ that undergoes both N- and C-terminal cleavages and glycosylation on maturation. This processing requires the action of protease A,^{21,22} which cleaves at or near residue 293 to yield protease B with a mature C terminus. The cleavage site on protease B was proposed to correspond to the C-termini of the homologous subtilisins, protease K and thermolysin.¹⁸ It has also been hypothesized that pepC is synthesized as a larger precursor. The mature form is formed after cleaving 130-140 and approximately 70 amino acids from its N- and C-terminal regions, respectively.¹⁹ This might also be the case for Pen o 18 and Pen c 18. The Pen o 18 cDNA encodes a 503-amino-acid polypeptide with a molecular mass of 53,297 daltons. On N-terminal cleavage, the resulting 368-residue polypeptide has a molecular mass of 38,287 daltons. Assuming Pen o 18 is cleaved as protease B and pepC at (or near) position 321 of the N-terminally processed protein, the resulting polypeptide would be 47 residues shorter with a molecular mass of 33,238 daltons. After glycosylation, the mature protein would have a size similar to that observed for the 34-kd (35-kd) component that reacts with the monoclonal and IgE antibodies in the *P. oxalicum* extracts.

The deduced amino acid sequence of Pen c 18 found in this study is also consistent with the notion of C-terminal processing. We have previously determined the N-terminal sequence (DSPSVEKNAP) of a 35-kd IgE-reacting component from *P. citrinum*.¹¹ This sequence is identical to that of the N-terminus of Pen c 18 deduced from the cDNA clone that encodes a 358-amino-acid polypeptide of 37,400 daltons. Assuming Pen c 18 is cleaved as in subtilisins at (or near) position 317, the resulting protein would be 41 residues shorter and has a molecular mass of 32,816 daltons. It can then be further glycosylated to 35,000 daltons.

Chow et al¹² have reported the cloning of a cDNA encoding a *P. citrinum* vacuolar serine protease allergen (Pen c 2). However, the C-terminus of the encoded protein is 41 amino acids shorter than that of Pen c 18 described here. The reported Pen c 2 can either be a vacuolar serine protease mutant or cloning/sequencing artifact. Other amino acid deletions and substitutions observed between Pen c 18 and Pen c 2 may reflect the polymorphism of the protease or may be due to experimental variations.

The serologic reactivity of rPen o 18, rPen o 18-s, and components of *P. oxalicum* were analyzed by immunoblotting with serum samples from 7 asthmatic

patients and a panel of mAbs produced against serine protease allergens. The antigenic profiles of the recombinant proteins are similar to that observed for the 34-kd component in the *P. oxalicum* extract. Thus the 34-kd protein in *P. oxalicum* corresponds antigenically to the mature protein encoded by the Pen o 18 cDNA isolated in the present study.

The N-terminally processed Pen o 18 and Pen c 18 have 86% sequence identity at the protein level. Therefore, cross-reactivity between these two proteins is expected. Indeed, serum samples from 7 asthmatic patients with IgE antibodies reactive against rPen o 18 and the 34-kd component of *P. oxalicum* demonstrated similar IgE reactivity to rPen c 18 and the 35-kd component of *P. citrinum*. These results again suggest an IgE cross-reactivity previously observed in SDS-PAGE blotting studies of the natural allergens.⁹ In the present study, the IgE cross-reactivity was confirmed by using immunoblot inhibition with rPen o 18 and rPen c 18. In addition, antigenic cross-reactivity between Pen o 18 and Pen c 18 was also demonstrated with mAbs PCM39 and FUM20.

The challenges facing studies of mold allergy include identifying the important allergens so that extracts can be standardized for the major specificities. We present in this report the cDNA sequences of major fungal allergens and immunologic characteristics of the recombinant proteins. The recombinant proteins and the mAbs utilized in this study might provide materials useful in the clinical study of mold allergy.

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