

cDNA Cloning, Biological and Immunological Characterization of the Alkaline Serine Protease Major Allergen from *Penicillium chrysogenum*

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Key Words

Penicillium chrysogenum · Allergen · Alkaline serine protease · cDNA cloning · Purification · Enzymatic activity · Histamine release · IgE cross-reactivity

Abstract

Background: *Penicillium chrysogenum* (*Penicillium notatum*) is a prevalent airborne *Penicillium* species. A 34-kD major IgE-reacting component from *P. chrysogenum* has been identified as an alkaline serine protease (Pen ch 13, also known as Pen n 13 before) by immunoblot and N-terminal amino acid sequence analysis. **Methods:** In the present study, Pen ch 13 was further characterized in terms of cDNA cloning, protein purification, enzymatic activity, histamine release and IgE cross-reactivity with alkaline serine protease allergens from two other prevalent fungal species – *P. citrinum* (Pen c 13) and *Aspergillus flavus* (Asp fl 13). **Results:** A 1,478-bp cDNA (Pen ch 13) that encodes a 398-amino-acid alkaline serine protease from *P. chrysogenum* was isolated. This fungal protease has pre- and pro-enzyme sequences. The previously determined N-terminal amino acid sequence of the *P. chrysogenum* 34-kD major allergen is identical to that of residues 116–125 of the cDNA. Starting from Ala116, the deduced amino acid sequence

(283 residues) of the mature alkaline serine protease has a calculated molecular mass of 28.105 kD with two cysteines and two putative N-glycosylation sites. It has 83 and 49% sequence identity with the alkaline serine proteases from *P. citrinum* and *A. fumigatus*, respectively. The recombinant Pen ch 13 was recovered from inclusion bodies and isolated under denaturing condition. This recombinant protein reacted with IgE antibodies in serum from an asthmatic patient and with monoclonal antibodies (PCM8, PCM10, PCM39) that reacted with the 34-kD component from *P. chrysogenum*. The N-terminal amino acid sequence of the purified native Pen ch 13 is identical to that determined previously for the 34-kD major allergen in crude *P. chrysogenum* extracts. The purified native Pen ch 13 has proteolytic activity with casein as the substrate at pH 8.0. This enzymatic activity was inhibited by phenylmethylsulfonyl fluoride or diethylpyrocarbonate. Pen ch 13 was also able to degrade gelatin and collagen but not elastin. Basophils from 5 asthmatic patients released histamine (12–73%) when exposed to the purified Pen ch 13. In ELISA (enzyme-linked immunosorbent assay) experiments, IgE for Pen ch 13 was able to compete with purified Pen ch 13, Pen c 13 or Asp fl 13 in a dose-related manner. **Conclusions:** These results demonstrated that the 34-kD major allergen of *P. chrysogenum* is an alkaline serine protease.

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These results also indicated that atopic patients primarily sensitized by either of these prevalent fungal species may develop allergic symptoms by exposure to other environmental fungi due to cross-reacting IgE antibodies against this protease.

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Introduction

Fungal spores in numbers greater than those of pollens are found in our environment. Many airborne mold spores have been implicated in causing respiratory allergic symptoms [1, 2]. Indoor airborne allergens play a significant role in human allergic disorders. *Penicillium* and *Aspergillus* species are the most commonly found indoor fungal species [1, 3–5]. Positive immediate skin reactivity to *Penicillium* components was detected in about 22–68% of atopic subjects tested [6–8]. However, allergens in prevalent *Penicillium* species and human responses to major known allergens have not been well characterized.

We have demonstrated by immunoblotting and N-terminal amino acid sequence analysis that alkaline and vacuolar serine proteases are major allergens in eight prevalent *Penicillium* and *Aspergillus* species [9–13]. They have been designated as the group 13 (alkaline serine protease) and the group 18 (vacuolar serine protease) allergens, respectively, for both fungal genera by the Allergen Nomenclature Subcommittee [14]. Monoclonal antibodies (MoAbs) against different epitopes on these fungal allergens have also been generated [9, 15]. Results from molecular characterization confirmed that the group 18 allergens were vacuolar serine proteases [16]. We established that the vacuolar serine protease allergen from *P. oxalicum* (Pen o 18) and *P. citrinum* (Pen c 18) underwent N- and C-terminal processing upon maturation [16].

P. chrysogenum (also known as *P. notatum*) is the most frequently used *Penicillium* for extraction for clinical testing. In this study, we isolated a full-length cDNA clone of the 34-kD alkaline serine protease major allergen (Pen ch 13, also known as Pen n 13 before) from *P. chrysogenum* [10]. The deduced N-terminal amino acid sequence of the cDNA clone correlated with that reported previously for the native protease from *P. chrysogenum* [10]. In addition, we report here the isolation and characterization of the 34-kD major allergen from the culture medium of *P. chrysogenum*. The N-terminal and the internal amino acid sequences of the purified 34-kD allergen corresponded to those deduced from the isolated cDNA clone. Results from proteolytic activity assays have shown that

this protein is an alkaline serine protease. The allergenicity of this enzyme was corroborated by histamine release from basophils of asthmatic patients.

Patients with fungal allergy often react to a range of different fungi [1, 17]. In addition to Pen ch 13, we have observed that alkaline serine proteases are the major IgE-binding components in the most prevalent *Penicillium* (*P. citrinum*, Pen c 13) [9, 14] and *Aspergillus* (*A. flavus*, Asp fl 13) [12, 14] species found in the Taipei area. In this study, the presence and the degree of IgE cross-reactivity between the group 13 fungal allergens were analyzed by inhibition ELISA using purified Pen ch 13, Pen c 13 and Asp fl 13. We demonstrated that the group 13 fungal allergens share common IgE-binding epitopes. These results provide valuable information in clinical mold allergy.

Methods

Cloning and Molecular Techniques

P. chrysogenum Thom (also known as *P. notatum*) was used in previous [10] and present studies. The fungal strain CCRC 30568 was obtained from the Food Industry Research and Development Institute, Hsinchu, Taiwan. It was originally obtained from the American Type Culture Collection (ATCC 7813). Total RNA of this fungal strain 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 mortar and pestle and resuspended in Trizol reagent (Life Technologies, Frederick, Md., USA). Total RNA was extracted according to the manufacturer's instructions. The first-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, Calif., USA) and oligo-(dT) as the primer. The cDNA of the alkaline serine protease from *P. chrysogenum* was isolated with polymerase chain reaction (PCR) using primers 210 and AP (table 1). The degenerated primer 210 was constructed according to the sequence of amino acid residues 2–7 (NVVQRN) of the fungal protease [10]. 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 210 and AUAP. The product from the nest PCR reaction was gel purified, inserted into the pGem-Teasy vector (Promega, Madison, Wisc., USA) 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, Calif., USA).

The full-length cDNA of the *P. chrysogenum* alkaline serine protease was obtained by 5' rapid amplification of cDNA end (RACE). The template cDNA for the 5'-RACE reaction was synthesized using Moloney murine leukemia virus reverse transcriptase and primer 233 (table 1). Primer 233 has a sequence identical to nucleotide No. 591–609 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 EZppt™ DNA carrier (GeneTeks BioScience, Taipei, Taiwan, ROC). An oligo-(dC) was added to the end of the

Table 1. Primers for PCR

Name	Nucleotide sequence in 5' to 3'-end orientation
210	AACGT(C/T)GT(C/T)CAGCG(C/T)AA(C/T)GC
AP	GGCCACGCGTCGACTAGTACT-(dT)16
AUAP	GGCCACGCGTCGACTAGTAC
233	GTCATCATTGTCAGCAGTG
AAP	GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGGIIIG
234	TCACCAGCAGTAGAGTCATAC
5PN13- <i>Bam</i> HI	CGGGATCCGCCAACGTGGTTACAGCGTAATG
3PN13- <i>Nsi</i> I	CCAATGCATCATTGGGCGTTGATACC

purified cDNA with terminal deoxynucleotidyl transferase (Promega). Primers AAP and 234 were used in the 5'-RACE reaction. Primer AAP (table 1) contains the sequence of primer AUAP and a stretch of oligo-(dG). The sequence of primer 234 encompasses nucleotide No. 489–509 of the alkaline 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.

Expression and Purification of Recombinant Protein

The alkaline serine protease from *P. chrysogenum* was expressed as 6 × His-tagged protein according to the manufacturer's instructions (Qiagen, Valencia, Calif., USA). The cDNA of the protease was amplified through PCR. The forward primer used in the reaction was 5PN13-*Bam*HI (table 1) that contains the *Bam*HI restriction site in addition to the sequence encoding the N-terminal 7-amino-acid residues (ANVVQRN) of the protease. The reverse primer 3PN13-*Nsi*I (table 1) contains a *Nsi*I restriction site and the nucleotide sequence encompassing bases, 1,243–1,260 (including the TGA stop codon) of the *P. chrysogenum* alkaline serine protease cDNA clone. The PCR product was separated by agarose gel electrophoresis and purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). It was digested with *Bam*HI and *Nsi*I restriction enzymes then ligated into the *Bam*HI and *Pst*I restriction sites of the pQE-30 vector. The resulting vector was transformed into *E. coli* JM109 and M15. The recombinant polypeptide expressed in *E. coli* M15 was affinity purified, under denaturing condition, from a Ni-NTA resin column (Qiagen). The reactivity of the recombinant protein with IgE antibodies in sera from asthmatic patients and MoAbs against fungal serine proteases was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting.

Purification of the Pen ch 13 Allergen

P. chrysogenum strain CCRC 30568 was grown for 5 days at 25°C in a soybean liquid culture medium. The culture supernatant was collected and proteins were fractionated by ammonium sulfate (40–80%) precipitation and centrifugation. The pellet was dissolved in distilled water and dialyzed against 50 mM Tris-HCl, pH 7.5, before loading onto a DEAE cellulose column (DE SeptraSorb, Sepragen, Hayward, Calif., USA) that has been pre-equilibrated in the same buffer. The column was then eluted with a step gradient of 0.1, 0.2 and 0.3 M NaCl. The collected fractions were pooled according to their immunoblot reactivity to MoAb PCM10 against the alkaline serine protease allergens from *Penicillium* species [15]. The pooled fractions were then dialyzed against 10 mM sodium phosphate (pH 6.8) and further purified on a CM Sephadex C50 (Pharma-

cia Biotech, Uppsala, Sweden) cation exchanger that has been equilibrated with 10 mM sodium phosphate (pH 6.8). Pen ch 13 was eluted off the column with the same buffer and identified according to its positive immunoblot reactivity to MoAb PCM10.

Pen c 13 was isolated from a 4 day-old culture supernatant of *P. citrinum* strain CCRC 38014 (strain 52-2) [9]. The growth condition and purification methods are essentially the same as that described above for Pen ch 13 with minor modifications. For the purification of Pen c 13, the anion and the cation exchanger were run at pH 8.5 and 7.5, respectively. In addition, CM Sepharose Fast Flow cation exchanger was used instead of the CM Sephadex C50. Asp fl 13 was purified as described [12]. The purity of the allergen preparations was analyzed by SDS-PAGE. The concentrations of the protein preparations were determined by a dye-binding assay according to the manufacturer's instruction (Bio-Rad, Richmond, Calif., USA).

Amino Acid Sequence Analysis

To characterize the purified 34-kD allergen from *P. chrysogenum*, the amino acid sequence of the N-terminus and peptide fragments generated from limited proteolysis was determined. Protein digestion was carried out using bands excised from SDS gels. Gel slices containing the purified 34-kD component were loaded in the sample wells of a second gel and were then overlaid with protease-containing sample buffer. Proteases used were sequencing grade endoproteinase Lys-C and Glu-C (Promega). Electrophoresis was performed until the dye front reached the interface between the stacking and the separating gels. The power to the electrophoretic unit was interrupted for 40 min, then resumed until the dye front just migrated out of the gel. SDS-PAGE-separated protein components were then transferred electrophoretically [18] onto a polyvinylidene difluoride (PVDF) membrane (0.45 µm; Millipore, Bedford, Mass., USA) and visualized by Coomassie blue staining. Protein bands of interest were excised from the membrane and inserted into an ABI Procise model 491 sequencer (Applied Biosystems) for automated cycles of Edman degradation according to the manufacturer's instruction. In addition, the N-terminal amino acid sequences of the purified Pen c 13 and Asp fl 13 allergens were also determined by Edman degradation. The protein preparations were subjected to SDS-PAGE, then transferred electrophoretically onto PVDF membranes. Protein bands were visualized by Coomassie blue staining and excised from the membranes for sequence analysis.

Subjects

Serum samples used in the present study were collected from adult patients with bronchial asthma and attending the allergy clinics

at the Cheng Hsin Rehabilitation and Medical Center, 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]. After collection, serum samples were stored in aliquots at -70°C . The presence of IgE antibodies to the Pen ch 13 allergen was analyzed by immunoblotting. For the histamine-release assay, heparinized venous blood samples (5 ml) were collected from 5 adult asthmatic patients with IgE-immunoblot reactivity to Pen ch 13.

Gel Electrophoresis and Immunoblotting

The reactivity of fungal components in crude extracts of *P. chrysogenum* (prepared as described [10]) and purified recombinant/native allergens with human IgE or mouse MoAbs was analyzed by immunoblot analysis as described [10, 15, 16]. Briefly, the protein components were separated by SDS-PAGE and transferred electrophoretically onto PVDF membranes. The membranes were blocked with 1% skimmed milk, then incubated with diluted human serum samples (1:5 dilution in Tris-buffered saline, pH 7.5, containing 0.05% Tween 20 and 1% skim milk) for 16 h at 4°C or with culture supernatants from hybridoma cells at room temperature for 1 h. The human IgE antibodies were probed with alkaline-phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmingen, San Diego, Calif., USA) for 1 h at room temperature. The human IgE on the blot was developed with a substrate solution of carbonate buffer (0.1 M NaHCO_3 , pH 9.8, and 1.0 mM MgCl_2) containing 0.35 mM 5-bromo-4-chloro-3-indolyl-phosphate and 0.37 mM *p*-nitroblue tetrazolium chloride for about 30 min. Serum from a normal laboratory volunteer was used as a negative control.

Six MoAbs against serine protease allergens of *Penicillium* and *Aspergillus* species were used in the immunoblotting studies. They were MoAb 55A [9], PCM8, PCM10, PCM16, PCM39 and FUM20 [15]. MoAbs PCM8, PCM10 and PCM16 react with the alkaline serine proteases from *Penicillium* species. FUM20 reacts with the alkaline and the vascular serine proteases from *Aspergillus* but only the vacuolar serine proteases from *Penicillium* species. MoAb PCM39 reacts with the alkaline and the vacuolar serine proteases of both *Penicillium* and *Aspergillus* species [15]. After incubating and washing, MoAbs on the PVDF membrane were detected with horseradish-peroxidase-labeled goat anti-mouse IgG (H+L; Jackson ImmunoResearch Laboratories, West Grove, Pa., USA). MoAbs on the blot were visualized by developing in a substrate solution of acetate buffer (50 mM, pH 5.0) containing 2 mM 3-amino-9-ethyl-carbazole (Sigma) and 0.01% (v/v) hydrogen peroxide for about 5 min. A hybridoma culture supernatant containing MoAb against house dust mite allergen Der p 7 [20] was used as a negative control. Low-molecular-weight standards (Pharmacia) were used to estimate the molecular mass of the proteins.

Proteolytic Activity of Pen ch 13

The proteolytic activity of the purified Pen ch 13 allergen was analyzed with casein (Sigma, St. Louis, Mo, USA; L-5890) as the substrate [12]. Briefly, the purified allergen and casein (0.5%) were mixed in 50 mM Tris-HCl (pH 8.0) and incubated at 37°C for 30 min. The reaction was stopped by adding 20% trichloroacetic acid and the precipitates were removed by centrifugation. Soluble peptides remaining in the supernatant were measured by the dye-binding assay of Bio-Rad. As a negative control, the purified Pen ch 13 was replaced with bovine serum albumin (BSA, Pierce, Rockford, Ill., USA) in the proteolytic assay.

For the inhibition studies, purified Pen ch 13 was pre-incubated with various amounts of inhibitors for 20 min at room temperature. The residual proteolytic activity was then measured as described above. The enzymatic activity of Pen ch 13 without inhibitor treatment was assayed simultaneously as the control. The inhibitors used were phenylmethyl-sulfonyl fluoride (PMSF, Sigma), diethyl pyrocarbonate (DEPC, Sigma) and benzamidine (Sigma).

The elastinolytic activity of the purified Pen ch 13 was analyzed with elastin-Congo red (Sigma) as the substrate. The reaction was performed at 37°C for 30 min in 50 mM Tris-HCl, pH 8.5. The reaction mixture was centrifuged and the absorbance of the released dye in the supernatant was measured at 495 nm. Porcine pancreatic elastase (Boehringer Mannheim, Mannheim, Germany) was used as a positive control under the same assay conditions.

The collagenolytic activity of the purified Pen ch 13 against human placental type IV collagen (Sigma) was analyzed at 37°C for 1 h in 50 mM Tris-HCl, pH 8.0. The profile of the digests was analyzed by SDS-PAGE.

The proteolytic activity of the purified Pen ch 13 was also analyzed in 10% zymogram ready gels with gelatin (Bio-Rad). After electrophoresis, the gels were washed for 1 h in 2.5% Triton X-100 then incubated in 0.1 M glycine-NaOH (pH 8.3) at 37°C for 3 h. The gels were then fixed and stained for 1 h in 0.1% solution of amido black in methanol:acetic acid:water (30:10:60). The gels were destained in the same solution without the dye as described by Heussen and Dowdle [21].

Histamine-Release Activity of Pen ch 13

Heparinized venous blood samples (5 ml) were collected from 5 adult asthmatic patients with IgE-immunoblot reactivity to Pen ch 13 and attending the allergy clinics at the Cheng Hsin Rehabilitation and Medical Center. Samples were also obtained from an atopic volunteer sensitized to *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) and 4 healthy laboratory staff members as controls. Basophil histamine release activity was analyzed with a commercial enzyme immunoassay kit (histamine kit, Immunotech, Marseille, France) according to the manufacturer's instructions. Briefly, heparinized whole blood samples were diluted 7-fold with buffer then incubated at 37°C for 30 min with serially diluted preparations of purified native Pen ch 13, BSA, affinity-purified goat anti-human IgE (specific for ϵ chains; Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA). A buffer control was included for measuring spontaneous histamine release. After incubation, the mixtures were centrifuged at 4°C , the supernatants were collected and stored at -20°C . The histamines in the supernatants were quantified with an immunoassay kit. Total cellular histamines were determined after cell lyses with distilled water. Basophil histamine release was calculated as:

$$\frac{\text{antigen-stimulated histamine release} - \text{spontaneous release}}{\text{total histamine content} - \text{spontaneous release}} \times 100\%.$$

Inhibition ELISA

Purified native Pen ch 13 was dissolved in 10 mM sodium phosphate (pH 6.8) at a concentration of 1.6 $\mu\text{g}/\text{ml}$ and coated onto polyvinyl microtiter plates at 4°C for 16 h. The free sites on the microtiter plates were blocked with 0.05% Tween 20 and 1% skimmed milk in phosphate-buffered saline. Diluted serum samples from asthmatic patients were preincubated, individually, with various amounts of purified native Pen ch 13, Pen c 13 or Asp fl 13 at room temperature for 1 h. The reaction mixtures were then added to the Pen ch 13

Fig. 1. Nucleotide and deduced amino acid sequences of the alkaline serine protease major allergen from *P. chrysogenum* (accession No. AF 321100). Numbers to the right indicate the positions of the nucleotide and the deduced amino acid residues of the sequences, respectively. The amino acid triad (D157, H188 and S343) that is characteristic of serine proteases is depicted in bold letters. Potential N-glycosylation sites are in bold letters and underlined. The first 20-amino-acid residues (No. 1–20) of the deduced sequence with a hydrophobic stretch might represent the pre-sequence. Amino acid residues (No. 116–125) that are identical to those determined previously for the N-terminus of the 34-kD major IgE-binding component in the *P. chrysogenum* crude extracts are underlined. The stop codon TGA is denoted with an asterisk. The vertical arrows mark the putative cleavage site of the signal peptide and the amino terminus of the mature alkaline serine protease.

ATCAGCATCTTCCTGAGTTTCATCATCTCCATCTTCATCTTTATATCATTTCTAACCAACAAAC															63	
ATG	GGT	TTC	CTA	AAG	CTC	CTC	TCG	ACG	TCC	CTT	GCG	ACT	CTG	GCA	GTC	111
M	G	F	L	K	L	L	S	T	S	L	A	T	L	A	V	16
GTT	AAT	GCC	GGT	AAG	CTC	CTT	ACC	GCC	AAT	GAC	GAC	GAG	GTC	GAT	GTT	159
V	N	A	G	↑K	L	L	T	A	N	D	G	D	E	V	V	32
CCT	AGC	TCC	TAC	ATT	GTC	GTC	ATG	AAT	GAT	GGT	GTT	AGC	ACC	GCG	CAG	207
P	S	S	Y	I	V	V	M	N	D	G	V	S	T	A	Q	48
TTT	GAA	ACC	CAC	CGT	AAC	TGG	GCC	GCA	AAT	GTT	CAT	GCT	CGT	ACC	AGG	255
F	E	T	H	R	N	W	A	A	N	V	H	A	R	T	R	64
AGT	CTC	AAG	GGT	GGC	GAG	TCT	GGA	CCA	GGG	AAG	CAT	TTC	GAC	ATC	AAT	303
S	L	K	G	G	E	S	G	P	G	K	H	F	D	I	N	80
GGC	ATG	AAA	GGA	TAC	AGC	GCT	AGC	TTC	GAT	GAT	CGT	ACC	GTC	AAA	GAC	351
G	M	K	G	Y	S	A	S	F	D	D	R	T	V	K	D	96
ATT	GCT	AGT	GAC	CCG	ACG	GTC	AAG	TAC	GTT	GAA	CCA	GAC	ATG	GTT	GTG	399
I	A	S	D	P	T	V	K	Y	V	E	P	D	M	V	V	112
AAC	GCG	ACC	GCC	AAC	GTG	GTT	CAG	CGT	AAT	GCT	CCT	TCA	TGG	GGT	CTC	447
N	A	T	↑A	N	V	V	Q	R	N	A	P	S	W	G	L	128
TCT	CGC	ATC	TCT	AGC	AAG	AAG	TCC	GGT	GCT	ACC	GAC	TAT	GTG	TAT	GAC	495
S	R	I	S	S	K	K	S	G	A	T	D	Y	V	Y	D	144
TCT	ACT	GCT	GGT	GAG	GGC	ATC	GTG	ATC	TAT	GGC	GTC	GAC	ACC	GGC	ATT	543
S	T	A	G	E	G	I	V	I	Y	G	V	D	T	G	I	160
GAC	ATT	GGG	CAT	GCG	GAC	TTT	GGG	GGC	CGC	GCT	GAG	TGG	GGC	ACC	AAC	591
D	I	G	H	A	D	F	G	G	R	A	E	W	G	T	N	176
ACT	GCT	GAC	AAT	GAT	GAC	ACT	GAC	GGC	AAT	GGC	CAT	GGC	ACC	CAC	ACT	639
T	A	D	N	D	D	T	D	G	N	G	H	G	T	H	T	192
GCA	TCT	ACT	GCA	GCG	GGC	AGC	AAG	TTC	GGT	GTT	GCA	AAG	AAG	GCC	TCT	687
A	S	T	A	A	G	S	K	F	G	V	A	K	K	A	S	208
GTC	GTT	GCT	GTT	AAG	GTC	CTT	GGT	GCC	GAT	GGT	TCT	GGA	ACC	AAT	TCG	735
V	V	A	V	K	V	L	G	A	D	G	S	G	T	N	S	224
CAA	GTC	ATC	GCT	GGC	ATG	GAT	TGG	GCC	GTC	AAA	GAT	TCA	AAG	TCT	CGC	783
Q	V	I	A	G	M	D	W	A	V	K	D	S	K	S	R	240
GGC	GCC	ACT	GGC	AAG	TCT	GTT	ATG	AAC	ATG	TCT	CTG	GGT	GGT	GCC	TAT	831
G	A	T	G	K	S	V	M	N	M	S	L	G	G	A	Y	256
TCC	CGG	GCT	ATG	AAT	GAC	GCC	GCT	GCC	AAT	GTC	GTT	AGA	TCC	GGT	GTC	879
S	R	A	M	N	D	A	A	A	N	V	V	R	S	G	V	272
TTT	CTC	TCT	GTT	GCT	GCC	GGC	AAT	GAA	GCC	CAG	GAT	GCG	AGT	AAC	AGC	927
F	L	S	V	A	A	G	N	E	A	Q	D	A	S	N	S	288
TCC	CCT	GCT	TCT	GCT	CCG	AAT	GTA	TGC	ACT	ATT	GCC	GCT	TCT	ACC	AAC	975
S	P	A	S	A	P	N	V	C	T	I	A	A	S	T	N	304
TCA	GAC	GGA	AGT	GCT	TCC	TTC	ACC	AAC	TTC	GGT	TCC	GTT	GTC	GAC	CTC	1023
S	D	G	S	A	S	F	T	N	F	G	S	V	V	D	L	320
TAT	GCT	CCT	GGG	AAG	GAC	ATC	ACG	GCC	GCA	TAC	CCT	GGT	GGT	GGC	TCA	1071
Y	A	P	G	K	D	I	T	A	A	Y	P	G	G	G	S	336
AAG	ACC	CTG	TCC	GGA	ACC	TCT	ATG	GCT	GCC	CCG	CAT	GTT	GCT	GGT	GCT	1119
K	T	L	S	G	T	S	M	A	A	P	H	V	A	G	A	352
GCC	GCC	TAC	CTG	ATG	GCT	CTC	GAG	GGT	GTG	ACC	TCT	GAT	AAA	GCT	TGC	1167
A	A	Y	L	M	A	L	E	G	V	T	S	D	K	A	C	368
GCC	CGT	ATT	GTC	GAG	CTT	GCT	ATC	TCG	TCT	ATC	TCC	AGC	GCT	CCG	TCC	1215
A	R	I	V	E	L	A	I	S	S	I	S	S	A	P	S	384
GGC	ACT	ACC	AGC	AAG	CTT	CTG	TAT	AAC	GGT	ATC	AAC	GCC	CAA	TGA	1260	
G	T	T	S	K	L	L	Y	N	G	I	N	A	Q	*	398`	
ATGGCCTGATCGTGTGGCGGATGATTACGGTATGGCGCCGTGAGTAGCCTATGTGTATGTTCTT															1324	
GGGCGAGCTTTGGTAAAGCCAACCTAAGCCATGTTTATGCGTATTTAACGCTTGTTTGATTATC															1388	
TGGCTTCGCATACTCTCATTTTAAGTTTGCGGAGATAGTTTATAAGTACAAGTTCACGGTCATA															1452	
AAAAAAAAAAAAAAAAAAAAAAAAAAAA															1478	

coated microtiter plates and incubated for 16 h at 4°C. After washing, the microtiter plates were incubated with alkaline-phosphatase-labeled monoclonal anti-human IgE (Pharmingen) for 2 h at 37°C. Wells were washed again and a solution containing 1 mg/ml of *p*-nitrophenyl phosphate in 10% diethanolamine (pH 9.8) was added and incubated at 37°C for 30 min. The color change was detected by measuring the absorbance at 405 nm on a SPECTRAMax 190 (Molecular Devices, Sunnyvale, Calif., USA). Readings from three separate determinations were averaged for each datum. The ratio between the absorbance of the samples with and without inhibitor antigens in the fluid phase was expressed as percent inhibition.

Results

cDNA Cloning and Sequencing

In the present study, a 1,478-bp cDNA (Pen ch 13) encoding the *P. chrysogenum* alkaline serine protease was obtained through reverse transcriptase (RT)-PCR and the 5'-end RACE reaction. The nucleotide (GenBank accession No. AF 321100) and the deduced amino acid sequences of the isolated clone are presented in figure 1.

Pen ch 13	<u>ANVV--QRNA</u> PSWGLSRISS <u>KKSGATDYVY</u> DSTAGEGIVI <u>YGVDTGIDIG</u>	48
Pen n 13*	--S.V A.... .RT.T.S.T. V.F S	
Pen c 1	--S.V A.... .RT.T.S.T. V.F S	
Asp f 13	.LTT--KG. GS..H .GQAS...I. .TS..A.TYA ..V.S..NVN	
Pen ch 13	<u>HADFGGRAEW</u> GTNTADNDDT <u>DGNHGHTHTA</u> STAAGSKFGV <u>AKKASVVAVK</u>	98
Pen n 13*	.S.....K. ...VV...N. Y.. TL....	
Pen c 1	.S.....K. ...VV...N. Y.. TL....	
Asp f 13	.VE.ES..SL AY.A.GGSHV .SI.....V. G.IG.KTY.. ...TNLLS..	
Pen ch 13	VLGADGSGTN SQVIAGMDWA VKDSKSRG-- ---ATGKSVM <u>NMSLG</u> GAYSR	143
Pen n 13*	G.S..... ...A..... ---N.Y.. <u>.T...</u> EF.K	
Pen c 1	G.S..... ...A..... ---N.Y.. <u>.T...</u> EF.K	
Asp f 13	.FQGES.S.- .IILD.FN.. .N.IV.K.R- ----.K.AAI <u>.....G..Y</u>	
Pen ch 13	AMNDAAANVV RSGVFLSVAA <u>GNEAQDASNS</u> <u>SPASAPNVCT</u> IAASTNSDGS	193
Pen n 13*	.V..... K..I..... <u>...EN...</u> <u>...AEA..</u> ST...	
Pen c 1	.V..... K..I..... <u>...EN...</u> <u>...AE..</u> ST...	
Asp f 13	.F.N.VE.AF DE..LSV... ..NS... <u>T</u> AL. V.. <u>INK..</u> NAR	
Pen ch 13	ASFTNFGSVV DLYAPGKDIT AAYPGG--GS KTLSGTSMMA PHVAGAAAYL	241
Pen n 13*	QS.. V....	
Pen c 1	QS.. V....	
Asp f 13	...S.Y.... .IF...Q..L S.WI.STTAT N.I.....T ..IV.LSV..	
Pen ch 13	MALEGVTSDK A-CARIVELA ISSISSAPSG TTSKLLYNGI NAQ	283
Pen n 13*	SAGN -_-.....Q.. T....R.... V	
Pen c 1	SAGN -_-.....Q.. T....R.... V	
Asp f 13	.G.. <u>NLSGPA</u> .VT...K... TNGVVTNVK. SPN..A.... ..	

Fig. 2. Sequence alignment of the putative mature forms of the alkaline serine proteases from *P. chrysogenum* (Pen ch 13, accession No. AF 321100), *P. citrinum* (Pen c 1, accession No. AF084546), and *A. fumigatus* (Asp f 13, accession No. Z11580). Pen n 13* (accession No. AF193420) represents the amino acid sequence of the putative mature form of the alkaline serine protease from *P. chrysogenum* (*P. notatum*) reported by Chow et al. [22]. Amino acid residues that are identical to those of Pen ch 13 are presented with dots. Dashes are

introduced to optimize the alignment. The catalytic triad that is characteristic of the serine proteases is in hold type. Cys (C) residues in the sequences are underlined. The potential N-glycosylation sites are in boldface and underlined. The N-terminal and the partial internal amino-acid residues of the deduced Pen ch 13 sequence that were found to be identical to those determined for the native Pen ch 13 allergen are also underlined.

The cDNA encodes a 398-amino-acid polypeptide, assuming translation is initiated at the first AUG codon. The deduced N-terminus of the polypeptide has the characteristic of a hydrophobic leader sequence. Residues 116–125 of the encoded protein have a sequence identical to that of the N-terminus of the 34-kD major allergen from *P. chrysogenum* [10]. The mature alkaline serine protease has Ala116 at its N-terminus and a calculated molecular mass of 28.105 kD. Sequence analysis reveals the presence of two cysteines (Cys297, Cys368), two putative N-glycosylation sites and three active-site residues for

serine proteases (Asp157, His188 and Ser343) on the protein (fig. 1). The typical 'AATAAA' polyadenylation signal in the 3'-non-coding region for mammalian genes is absent upstream of the polyA tail.

On alignment, the deduced amino acid sequence of the mature alkaline serine protease from *P. chrysogenum* (283 residues) has 83 and 49% sequence identity with those from *P. citrinum* (Pen c 1, AF084546) and *A. fumigatus* (Asp f 13, Z11580), respectively (fig. 2). The protease active-site residues (aspartic acid, histidine, and serine) and both of the potential N-glycosylation sites are

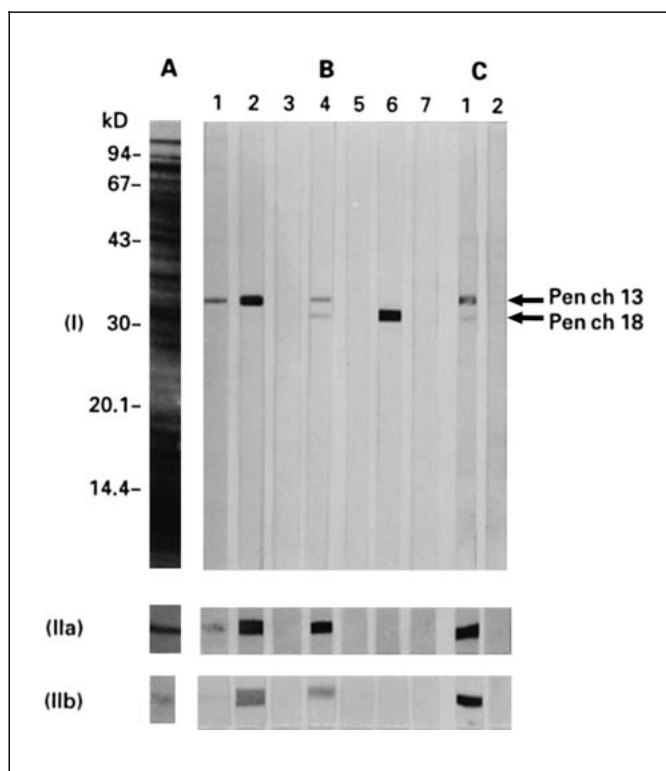


Fig. 3. Immunoblot reactivity of components from *P. chrysogenum* (part I), recombinant Pen ch 13 (part IIa) and native Pen ch 13 (part IIb) to 6 MoAbs against fungal serine proteases (**B**) and IgE antibodies in serum from an asthmatic patient (**C**). **A** The coomassie-blue-stained protein profiles of the *P. chrysogenum* crude extract, the purified recombinant and native Pen ch 13 proteins on PVDF blots were presented in parts I, IIa and IIb, respectively. **B** Strips 1–6 show immunoblot results generated with MoAbs PCM8, PCM10, PCM16, PAM39, 55A and FUM20, respectively. **C** Strip 1 shows IgE-immunoblot reactivity in a serum sample from an asthmatic patient. Strips 7 (**B**) and 2 (**C**) represent the negative controls using culture supernatant containing MoAb against the mite allergen Der p 7 [20] and serum from a normal laboratory volunteer, respectively. The molecular mass standards used were phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD) and α -lactalbumin (14.4 kD).

conserved among these fungal proteases. The two Cys residues are also conserved between *P. chrysogenum* and *P. citrinum*. In addition, the putative pro-enzyme region of the alkaline serine protease from *P. chrysogenum* has 72 and 30% sequence identity with those from *P. citrinum* and *A. fumigatus*, respectively (data not shown).

Recently, Chow et al. [22] have reported the cDNA sequence of a putative alkaline serine protease from *P. chrysogenum* (*P. notatum*; Pen n 13* in fig. 2). The deduced amino acid sequence of Pen ch 13 (398 residues)

in this study has 80% sequence identity with that of Pen n 13* (397 residues). However, Pen n 13* and Pen c 1, an alkaline serine protease from *P. citrinum* [23] share 99% (393/397) sequence identity and differ by only 4 amino acids. Two of these 4-amino-acid residues that can be found in the mature proteins were shown in figure 2. Moreover, these 4-amino-acid substitutions are results of single nucleotide replacements of the corresponding codons (data not shown).

Characterization of the Recombinant Polypeptide

We expressed the mature alkaline serine protease (283-amino-acid residues) as His-tagged protein (residues 116–398 in fig. 1), excluding the proposed N-terminal presequences. The resultant recombinant protein (rPen ch 13) was recovered from inclusion bodies and purified in denaturing condition. It has an apparent molecular mass of about 36 kD as estimated by SDS-PAGE (data not shown).

The reactivity of rPen ch 13 to MoAbs against fungal serine proteases and IgE antibodies in serum from an asthmatic patient (patient A) was analyzed by immunoblotting. As shown in our previous study [15], MoAbs PCM8, PCM10 and PCM39 reacted with the 34-kD alkaline serine protease allergen (Pen ch 13) in the crude extracts of *P. chrysogenum* (strips 1, 2 and 4, respectively, in section I-B of fig. 3). These three MoAbs also reacted with rPen ch 13 (strips 1, 2 and 4, respectively, in section IIa-B). MoAbs PCM16 and 55A did not react with components in *P. chrysogenum* (strips 3 and 5, respectively, in section I-B), while MoAb FUM20 (strip 6, section I-B) reacted with the 32-kD vacuolar serine protease (Pen ch 18) of *P. chrysogenum*. These three MoAbs showed negative immunoblot reactivity to rPen ch 13 (strips 3, 5 and 6, respectively, in section IIa-B). Serum sample from an asthmatic patient (patient A) with IgE reactivity to the 34-kD alkaline serine protease allergen (strip 1 of section I-C) showed IgE binding to the purified rPen ch 13 (strip 1 of section IIa-C). For negative controls, MoAb against house dust mite allergen Der p 7 and serum sample from a normal laboratory volunteer were used (strips 7 and 2 in sections B and C, respectively).

Purification of Pen ch 13 Allergen

A 34-kD component that reacts with MoAb PCM10 was purified from the culture medium of *P. chrysogenum* by ammonium sulfate precipitation, DEAE and CM Sephadex C50 ion exchange chromatography. This component was recovered from the loading flow through with a 30-kD protein from the DEAE column (data not shown).

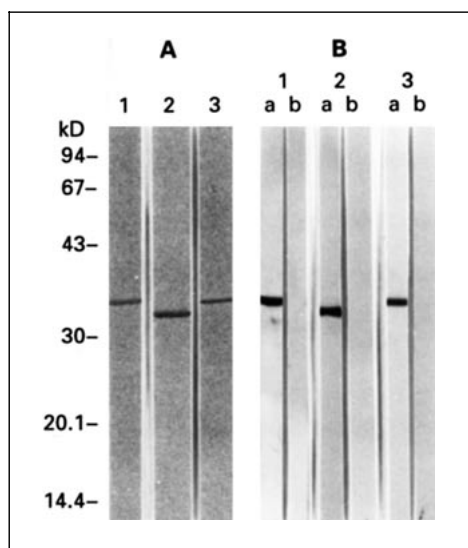


Fig. 4. SDS-PAGE and IgE-immunoblot profiles of purified Pen ch 13, Pen c 13 and Asp fl 13. **A** The Coomassie-blue-stained SDS-PAGE protein profiles of the purified Pen ch 13 (strip 1), Pen c 13 (strip 2) and Asp fl 13 (strip 3). Strips 1a, 2a and 3a (**B**) correspond to IgE-immunoblots of purified Pen ch 13, Pen c 13 and Asp fl 13, respectively, generated with serum from an asthmatic patient. Strips 1b, 2b and 3b (**B**) are negative controls using serum from a normal laboratory personnel.

Using a cation exchanger in the next step of the purification, the 34-kD immuno-positive component was separated from the 30-kD immuno-negative protein. The purity of the 34-kD protein preparation is shown in strip 1 of panel A (fig. 4). The N-terminal sequence of the purified protein (ANVVQRNAPS) is identical to that of the 34-kD major IgE-binding component in the crude extract of *P. chrysogenum* [10] and the sequence of residues 116–125 deduced from the cDNA clone Pen ch 13. The corresponding sequence from Pen n 13* was found to be ANVVQSNVPS (fig. 2).

The purified 34-kD component was further digested with endoproteases and the resulting fragments were subjected to amino acid sequence analysis. Peptides with sequences of KGATDYVYDSTAGEGIVY (Lys-C-digested), GIVYGVDTGIDIGHADFG and AQDASNS-SPA (Glu-C-digested) were identified. The N-terminal amino acid sequence of a 31-kD component that was found occasionally in the purified allergen preparation (data not shown) was determined to be GVDTGIDIGH-ADFGG. All these peptides show sequence identity with the corresponding peptides deduced from Pen ch 13 but not with those deduced from Pen n 13* (fig. 2). The only exception was the amino acid S that was the 21st residue

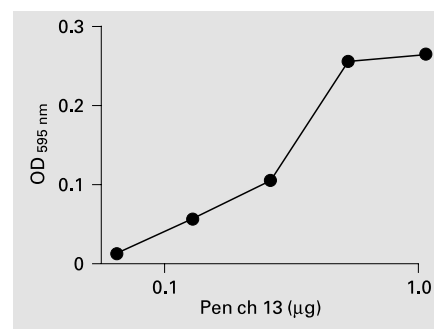


Fig. 5. Dose-response curve of the enzymatic activity of purified Pen ch 13 with casein (0.5%) as the substrate at pH 8.0. The OD₅₉₅ records the dye binding activity of peptides released by the protease.

in the deduced sequence of the mature Pen ch 13 protein, which was not detected in the Lys-C-digested peptide.

A serum sample from an asthmatic patient (patient A) with IgE reactivity to the 34-kD alkaline serine protease allergen (strip 1 of section I-C, fig. 3) also showed IgE reactivity to the purified native Pen ch 13 (nPen ch 13; strip 1 of section IIb-C). The purified nPen ch 13 reacted with PCM8, PCM10 and PCM39 (strips 1, 2 and 4, respectively, in section IIb-B of fig. 3) but not with PCM16, 55A and FUM20 (strips 3, 5 and 6, respectively, in section IIb-B of fig. 3). The immunoblot profile observed was found to be identical to that of the 34-kD major allergen in the crude extract of *P. chrysogenum* and the recombinant Pen ch 13 (section I-B and IIa-B, respectively, of fig. 3). For negative controls, the same MoAb against house dust mite allergen Der p 7 and serum sample from a normal laboratory volunteer as described above were used (strips 7 and 2 in sections IIb-B and IIb-C, respectively).

The SDS-PAGE protein profiles of the purified Pen c 13 and Asp fl 13 are shown in strips 2 and 3 of figure 4A, respectively. The N-termini of the purified proteins have amino acid sequences that are identical to those of the 33- and 34-kD major IgE-reactive components in the crude extracts of *P. citrinum* [9] and *A. flavus* [12], respectively (data not shown). The purified proteins also reacted with IgE antibodies in serum from an asthmatic patient (patient B; fig. 4B, strips 1a, 2a and 3a).

Proteolytic Activity of the Purified Pen ch 13 Allergen

The purified nPen ch 13 had a significant proteolytic activity with casein (0.5%) in neutral to alkaline pH (fig. 5, pH 8.0). It could also utilize gelatin and collagen

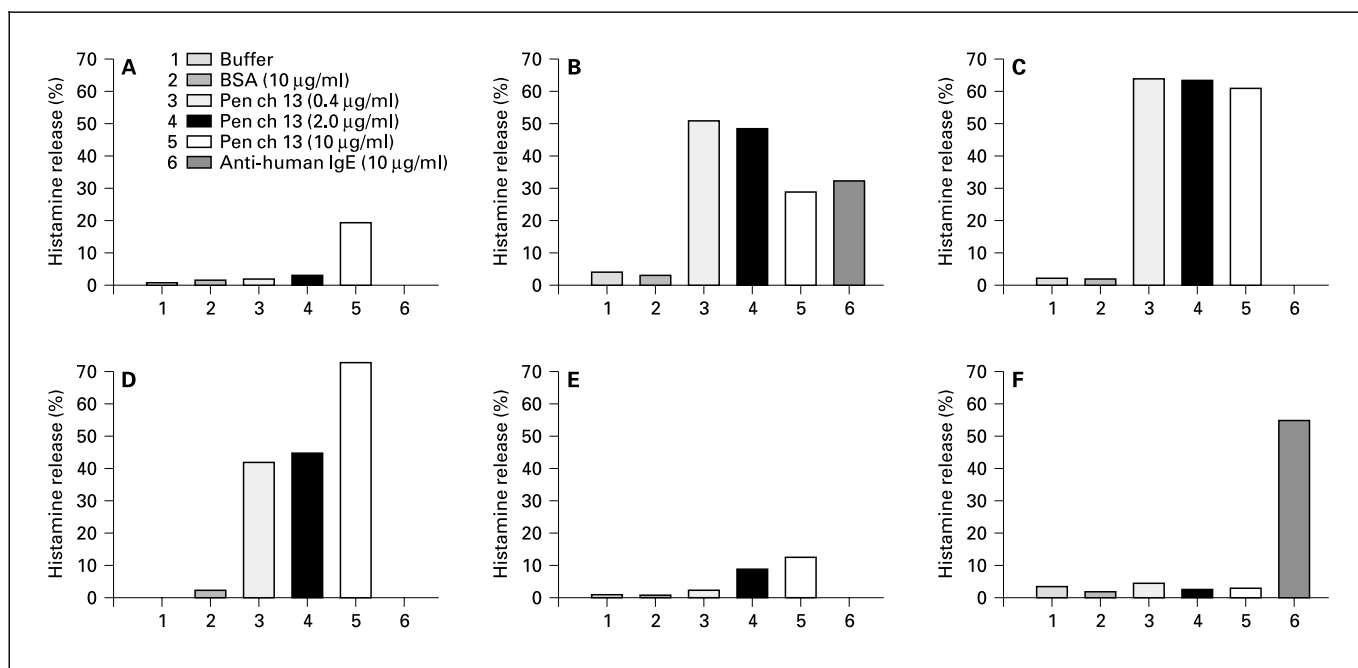


Fig. 6. Pen-ch-13-induced histamine release from basophils obtained from 5 asthmatic patients with IgE-immunoblot reactivity to Pen ch 13. **A–E** Results obtained with cells from these 5 asthmatic patients. **F** Results obtained from a patient allergic to house dust mite *D. pteronyssinus*. Histamine release is expressed as a percentage of total histamine. **A** The caption shows the allergen concentrations added to the basophil culture. Anti-IgE was added for positive controls (**B, F**).

but not elastin as the substrate (data not shown). The protease activity of 1.0 µg of nPen ch 13 at pH 8.0 was inhibited by 0.25 mM PMSF or DEPC (to 91 and 92%, respectively). Benzamidine (0.25 mM), a trypsin-type serine protease inhibitor, induced 0% inhibition.

Histamine-Release Induced by the Purified Pen ch 13 Allergen

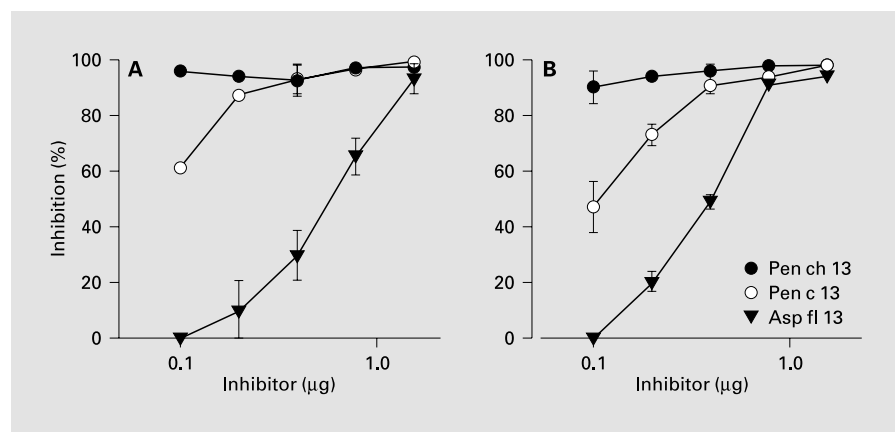
Besides IgE binding, Pen ch 13 can induce histamine release from basophils of asthmatic patients. Basophils from 5 patients (patients A–E) with positive IgE-immunoblot reactivity to Pen ch 13 released histamine in a dose-dependent fashion upon exposure to the purified native protein (fig. 6A–E). The maximum values of histamine release from patients A–E were determined to be 19 (fig. 6A), 51 (fig. 6B), 66 (fig. 6C), 73 (fig. 6D) and 12% (fig. 6E), respectively. The corresponding values from these patients stimulated with BSA were less than 3%. Alternatively, basophils from a patient allergic to *D. pteronyssinus* (fig. 6F) and 4 non-atopic laboratory personnel have only 4.5% histamine release upon exposure to nPen ch 13 (data not shown). As a positive control, basophils

from patient B and a mite-allergic patient have histamine release values of 33 (fig. 6B) and 55% (fig. 6F), respectively, upon exposure to a goat anti-human IgE.

IgE Cross-Reactivity

IgE cross-reactivity among the group 13 alkaline serine protease allergens was analyzed by inhibition ELISA. Sera from 2 asthmatic patients (patients B and C, fig. 6) that showed IgE binding to the group 13 fungal allergens were used in these studies. Results in figure 7 show that 0.1 µg of Pen ch 13 in fluid phase caused greater than 90% inhibition of IgE reactivity to Pen ch 13. About 50–60% inhibition of IgE binding to Pen ch 13 was detected in the presence of 0.1 µg of Pen c 13. This inhibitory effect can be increased to more than 90% by increasing Pen c 13 to 0.4 µg. Asp fl 13 at 0.1 µg did not inhibit the binding of IgE to Pen ch 13. There was 30–50% inhibition of IgE binding to Pen ch 13 at 0.4 µg of Asp fl 13. Almost complete inhibition was detected when 1.6 µg of Asp fl 13 was used as an inhibitor in both sera tested (fig. 7A, B).

Fig. 7. Dose-dependent inhibition of the IgE-ELISA reactivity in sera from 2 asthmatic patients to Pen ch 13 by purified Pen ch 13, Pen c 13 and Asp fl 13. The amount of inhibitors used were 0.1, 0.2, 0.4, 0.8 and 1.6 μ g of individually purified group 13 allergens. Percentage of inhibition was calculated by setting the absorbance obtained at 405 nm of a sample without inhibitor antigen in the fluid phase as 0% inhibition. **A, B** Results using sera from 2 asthmatic patients (patients B and C in fig. 6).



Discussion

The present study describes the cDNA sequence and biological activity of the polypeptide from the 34-kD major allergen (Pen ch 13) from *P. chrysogenum*. This allergen has previously been designated Pen n 13 because it was obtained from cultures of the strains *P. chrysogenum* with the obsolete name of *P. notatum* but has now been correctly renamed based on its genus and species [ftp://biobase.dk/pub/who-iuis/allergen.list]. The deduced amino acid sequence of the cDNA has confirmed the N-terminal sequence data showing that the allergen is an alkaline serine protease. It has significant sequence homology with other fungal alkaline serine proteases throughout the molecule and contained the active site for serine proteases (Asp-His-Ser). The amino acid sequence from residues 116–125 was identical to the N-terminal sequence of the 34-kD allergen in *P. chrysogenum* (*P. notatum*) extracts. Thus the mature protein is formed by the removal of the preprosequence leaving Ala116 as the N-terminus and creating a 28.105 kD polypeptide. The remaining mass of the 34-kD allergen is probably due to posttranslational modifications. The IgE binding of rPen ch 13 and the similarity of the MoAb binding profile to the natural 34-kD allergen indicates that much of the antigenicity is found in the recombinant polypeptide.

The amino acid sequence deduced from the cDNA was confirmed by N-terminal and partial amino acid sequences taken from the *P. chrysogenum* cultures and additionally confirming previous N-terminal studies. Furthermore, the combined results of the amino acid and cDNA analysis indicated that the 31-kD band found in some purified preparation of Pen ch 13 is a degradation product. Reichard et al. [24] have also encountered auto-

proteolysis in the purification of serine proteases from *A. fumigatus*.

Recently, Chow et al. [22] have reported the cloning of an alkaline serine protease from *P. chrysogenum* (*P. notatum*) (Pen n 13* in fig. 2). There was only an 80% amino acid sequence identity between the Pen ch 13 described here and the Pen n 13*, and the database analysis revealed only 4-amino-acid differences between Pen n 13* and Pen c 1, an alkaline serine protease of *P. citrinum* [23]. The close identity with *P. citrinum* is further emphasized by the fact that the 4-amino-acid changes were due to substitutions of single nucleotides and that the Pen n 13* reacts with the anti-*P. citrinum* monoclonal antibody 55A [22]. The nature of the cDNA described for Pen n 13* is thus unusual and different to the Pen ch 13 described here which shows the typical differences between the fungal species.

Functionally, the purified protein has proteolytic activity with casein as the substrate at neutral to alkaline pH. It is inhibited by PMSF and DEPC but not benzamide. In addition, gelatin and human collagen type IV can also be used as substrates for nPen ch 13. The significance of the proteolytic activity of Pen ch 13 in allergenicity and airway pathophysiology is currently unknown and needs to be clarified. Hanzi et al. [25] showed that invasive *Aspergillus* species (*A. fumigatus*, *A. flavus*, *A. terreus* and *A. nidulans*) secreted an alkaline protease that degraded collagen in vitro. The high activity of the protease at physiological pH against structural proteins such as collagen suggests a possible role for this enzyme as a virulence factor in invasive disease. It has also been suggested that the collagenolytic serine protease allergen Der p 9 was evolved to participate in the digestion of collagenous substrates in the form of human skin scales [26]. Further-

more, a chymotrypsin-like serine protease from *A. fumigatus* could induce human epithelial cell detachment [27]. We showed here that Pen ch 13 could degrade human type IV collagen, a component of the basement membrane. By virtue of its enzymatic activity, Pen ch 13 may have the potential to detach viable epithelial cells, increase the permeability of the bronchial mucosa, enhance tissue injury, activate inflammatory cells and possibly promotes its interaction with the immune system. In addition, changes in airway permeability induced by proteolytic activity might facilitate the access of additional allergens to mast cells in the airway submucosa. Thus, the enzymatic activity of Pen ch 13 may be associated with the processes of allergen presentation, inflammatory cell activation and chronic tissue injury.

In addition to IgE binding, the allergenicity of the purified Pen ch 13 was further confirmed by a histamine release analysis in the present study. Pen ch 13 induced histamine release (12–73%) from basophils of 5 asthmatic patients who have in their sera IgE antibodies against Pen ch 13. Inhibition of the allergen-induced histamine release was not detected when the protease inhibitor PMSF (1 mM) was included in the histamine release analysis (data not shown). Pen ch 13 can also induce immediate skin reactivity in 2 asthmatic patients with positive immediate skin response to a crude extract of *P. chrysogenum* obtained from the Greer Laboratories (Lenoir, N.C., USA; data not shown). Thus, Pen ch 13 is the first *Penicillium* allergen that conferred both immunological and biological activities.

Mold-allergic individuals often show sensitivity to several different fungal species. It may be that many fungi

show allergenic cross-reactivity or allergic subjects have independent parallel sensitivities to individual species. The knowledge of cross-reactivity of fungi is important in refining diagnosis, allergen avoidance, and in immunotherapy of mold allergies. IgE cross-reactivity between homologous allergens of different fungal species has been demonstrated [28, 29]. In the present study, the ELISA reactivity of serum IgE from 2 asthmatic patients to solid-phase-bound Pen ch 13 was inhibited by increasing amounts of Pen ch 13, Pen c 13 or Asp fl 13 in the fluid phase. It indicates that these proteins share common IgE-binding epitopes. Although all three proteins are able to fully inhibit the binding of IgE, Pen ch 13 is about 4 and 16 times more potent than Pen c 13 and Asp fl 13 with regard to ELISA inhibition. It suggests that serum IgE antibodies bind to these group 13 fungal allergens with different affinities.

In conclusion, results of the present study indicate that the 34-kD alkaline serine protease is a major allergen in *P. chrysogenum*. Present findings suggest that the presence of IgE cross-reactivity among major allergens from prevalent airborne *Penicillium* and *Aspergillus* species may play a significant role in clinical mold allergy.

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