

Isolation and Characterization of a cDNA Clone Encoding One IgE-Binding Fragment of *Penicillium brevicompactum*

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Key Words

Allergen · Fungi · *Penicillium brevicompactum* · cDNA cloning · IgE immunoblot · Ribosomal P1 phosphoprotein

Abstract

Background: The abundance of allergenic *Penicillium* species has been associated with an increased incidence of childhood asthma and pulmonary bleeding. *Penicillium brevicompactum* has been identified as the most prevalent indoor species of this genus. However, detailed studies on the allergens of the ubiquitous *Penicillium* species are still lacking. For the characterization of allergens of prevalent *Penicillium* species, molecular cloning of the allergen genes of *P. brevicompactum* was performed in the present study. **Methods:** A phage cDNA library of *P. brevicompactum* was constructed in Uni-ZAP[®] XR vector using mRNA isolated from the organism. The cDNA library of *P. brevicompactum* was screened using pooled atopic sera. **Results:** Screening of *P. brevicompactum* cDNA library resulted in one positive clone encoding an estimated molecular weight of 11 kDa polypeptide, rich in acidic residues (>20%), with a pI of 3.87. This clone was designated as *Pen b 26* and found to be reactive only against the atopic sera obtained from indi-

viduals sensitive to *P. brevicompactum*. The amino acid sequence analysis of *Pen b 26* revealed that it had strong homology to the 60S acidic ribosomal protein P1 family from different eukaryotic sources, predominantly fungal aero-allergens. Other features of *Pen b 26* including having high alpha-helical content (>50%), alanine-rich residues (>20%), and a well-conserved C-terminal epitope region fits well into the common properties of 60S acidic ribosomal proteins. **Conclusions:** The results obtained suggest that the allergenic clone, *Pen b 26* is a 60S acidic ribosomal protein P1 of *P. brevicompactum* and shows strong similarity to other P1 family proteins.

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Introduction

In the modern western world, the majority of people spend more than 90% of their life in indoor environments. These air-controlled environments often allow growth of indoor mold species in the presence of excess humidity. Excessive growth of indoor mold species creates numerous health problems in sensitive individuals, including rhinitis, conjunctivitis, pulmonary bleeding and extrinsic bronchial asthma. Major indoor mold allergens include species of *Penicillium*, *Alternaria*, *Clado-*

sporium and *Aspergillus* [1, 2]. *Penicillium* is one of the most frequently encountered and diverse mold genera found in indoor air. The abundance of allergenic *Penicillium* species also correlates with the increased incidence of childhood asthma and mold allergies. In our laboratory, 14 *Penicillium* species were investigated for their allergenic components [3]. Among them, *P. brevicompactum*, *P. viridicatum*, *P. janthinellum*, *P. citrinum* and *P. oxalicum* were found to be highly allergenic. *P. brevicompactum* has been reported to be one of the major indoor aero-allergens [4].

Molecular characterization of these allergenic proteins is essential for a better understanding of the pathogenesis of atopic diseases and also for developing immunoassays to quantify indoor and outdoor mold and other aero-allergens. Allergens purified using conventional procedures are often obtained in low yields and tend to be of inconsistent quality. In particular, fungal allergens are subject to considerable variation between isolates of the same species, due to the fact that molds rapidly adapt to new environments. Stable and consistently pure allergenic proteins can be prepared using recombinant DNA techniques. Expression of these proteins in expression systems, such as *Pichia pastoris* would yield allergens in large quantities. In our laboratory, major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum* have been cloned [5, 6]. Hence, in the present study, a similar strategy was used to clone and express allergenic components of *P. brevicompactum*. A phage cDNA library of *P. brevicompactum* was screened using human atopic serum IgE. The clones were expressed and their protein products were characterized by immunological techniques. One of the isolated clones designated as *Pen b* 26 according to the recommendations of the International Union of Immunological Societies [7] will be studied in this paper.

Materials and Methods

Molecular biological techniques were performed according to the standard protocols unless otherwise stated [8, 9].

RNA Isolation and Construction of the cDNA Library of P. brevicompactum

An isolate of *P. brevicompactum* was grown in liquid malt extract broth (Difco, Detroit, Michigan, USA) at 22°C. Mycelia were harvested on days 3, 5, 7 and 12 by washing (4×) with 0.6 M MgSO₄ and stored at -85°C. For RNA extraction, the mycelial mass was ground using a mortar and pestle under liquid nitrogen. Total RNA was extracted from the mycelial powder with TRIZOL® Reagent (Invitrogen Life Technologies, Carlsbad, Calif., USA) according to the manufacturer's instructions. Briefly, the mycelial powder was

resuspended in TRIZOL® Reagent with the addition of 10 vol of this reagent. The mixture was homogenized using a Polytron® PT 10-35 (Kinematica, Littau/Lucerne, Switzerland) 8× with short pulses and intervals. Chloroform was added to 10% of the total volume. The mixture was centrifuged at 12,000 g for 10 min. The supernatant was collected and RNA was precipitated with the addition of equal volumes of isopropanol and high salt solution (0.8 M sodium citrate and 1.2 M sodium chloride) to 50% of the final volume. The RNA pellets were washed once with 80% ethanol, dried briefly and resuspended in RNase-free water. The cDNA library of *P. brevicompactum* was constructed in Uni-ZAP® XR vector using the total RNA and oligo(dT) linkers by Stratagene (La Jolla, Calif., USA). Amplification and screening of the cDNA were performed in *Escherichia coli* XL1-blue host strain.

Antisera and Immunoscreening of P. brevicompactum cDNA Library

Immuno plaque hybridization assay was carried out using STRATAGENE picoBlue™ immunoassay kit. Sera from individuals recognized as *P. brevicompactum* sensitive by positive skin test were collected and assayed by a radioallergosorbent test (RAST) using *P. brevicompactum* cultures prepared in our laboratory [3]. Sera scoring 20% or greater by RAST were utilized in pools for immunoassay and individually when assessing reactivity of recombinant fusion proteins. For the initial high density screening, the library was plated at 50,000 plaque-forming units per 150-mm Petri plate. Plaques were lifted by placing duplicate isopropylthio-β-D-galactoside (IPTG)-absorbed nitrocellulose membranes (150 mm diameter) onto the growing plaques after 2 and 5 h, respectively. The membranes were first blocked in 1% BSA in TBST (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h at RT, followed by incubation overnight at 4°C, in an atopic serum pool (pooled from three sensitive individuals). Normal human serum was used as negative control. To eliminate background nonspecific cross-reactivity, both atopic and control sera were pre-absorbed with *Escherichia coli* lysate, diluted 5-fold in the above blocking solution. The membranes were washed 4× for 5 min each with TBST, and incubated with 1/500 dilution of a mouse monoclonal anti-human IgE-alkaline phosphatase conjugate (BD Biosciences, Franklin Lakes, N.J., USA). The membranes were washed as described earlier and incubated with 0.3 mg/ml NBT (nitroblue tetrazolium) and 0.15 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate) in color development buffer (100 mM Tris-HCL, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) until positive plaques were clearly visible (5–10 min). Further color development was stopped by washing the membranes with distilled water once and incubating in 20 mM Tris-HCL pH 2.9 and 1 mM EDTA. Positive clones were identified by comparing the orientation of the plaques on the primary- and secondary-lifted membranes. The positive clones were further purified by serial dilution until all the plaques showed positive reaction in the same plate.

Allergen Expression in Purified Phage Clone

The IgE-positive phage clone was inoculated with 100 ml of mid-log growing *E. coli* XL1-Blue host cells in a 500-ml flask. After 30 min incubation, IPTG was added to induce the expression of cloned protein. The culture was centrifuged and the supernatant containing the phage was collected. The phages were pelleted by the addition of 20% PEG-1.5 M NaCl solution to 30% of the original culture volume, followed by centrifugation. The resulting pellet was

briefly sonicated in a microcentrifuge tube with a micro-tip probe at medium intensity ($3 \times$ for 10 s). About 10 μ g sonicated sample in loading buffer containing β -mercaptoethanol was heated to 95°C for 7 min. The sample was electrophoresed in 12% polyacrylamide using a vertical slab mini gel SDS-PAGE [10] system (BIO-Rad, Mississauga, Ont., Canada). Broad range molecular weight markers (BIO-Rad) were used for size determination of protein bands. The gel was electro-transferred onto PVDF membrane [11]. Subsequently, standard Western blot hybridization was performed using primary atopic antibodies and secondary monoclonal anti IgE-AP antibodies. In a separate but identical gel, proteins were staining with Coomassie Brilliant Blue R-250 to ensure that sufficient amount of protein was present (data not shown).

Isolation of cDNA Clones

The excision of the cloned fragments from the phage vector was performed according to the manufacturer's manual (Stratagene). Briefly, *E. coli* XL1-Blue cells were co-infected with the phage clones and helper phage ExAssist for 15 min. The co-infected host cells were heated to 70°C for 20 min to kill the host cells and release the phagemids. The heated cell-phage mixture was centrifuged, and the supernatant containing phagemids was incubated with *E. coli* SOLR cells to produce plasmid clones.

DNA Sequencing

For DNA sequencing, plasmid DNA was purified using Qiagen miniprep kit. Asymmetric polymerase chain reaction (PCR) was carried out using fluorescein-labelled ddNTPs and M13 forward and reverse primers with Big Dye Terminator version 3.1 Cycle sequencing kit (Applied Biosystems, Foster City, Calif., USA) and ABI 310/3100 Genetic Analyser instrumentation. The results were analysed using the software of Sequencher (Gene Codes, Ann Arbor, Mich., USA). The open reading frame was detected using GENSCAN software [12].

Amplification of cDNA Clones by PCR

The cDNA sequence from the pBluescript clones were amplified using flanking M13 forward and reverse primers with hot-start Taq polymerase (Invitrogen). PCR was performed by initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s denaturation at 94°C, 15 s annealing at 55°C and 30 s extension at 72°C. A 7-min extension in the end of the 30 cycles was also incorporated.

Northern Blot Analysis of Clone Pen b 26

About 20 μ g total RNA from *P. brevicompactum* together with RNA molecular weight markers (0.24–9.5 kb RNA Ladder, Invitrogen) were fractioned in 1.3% formaldehyde-agarose gel and transferred to a positively charged nylon membrane in $20 \times$ SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) with overnight capillary action. RNA was UV-cross-linked to the membrane. DIG Northern blot hybridization was performed according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland) with slight modifications [13] as follows. Briefly, the membrane was incubated for 1 h in pre-hybridization solution (20% SDS, 0.25 M Na₂HPO₄, 1 mM EDTA and 1% blocking agent). Alkali-labile, DIG-11-dUTP-labelled *Pen b 26* cDNA probe was prepared by using above PCR. The probe was added to the freshly prepared pre-hybridization solution at 5 pmol/ml and incubated overnight at 65°C. The probe solution was removed and washed with $2 \times$ SSC and 0.4% SDS for 4×5 min intervals at 65°C, followed by a higher stringency wash

with $0.5 \times$ SSC and 0.1% SDS, 4×5 min intervals at 60°C. The membrane was blocked with 1% blocking solution in wash buffer (3 M NaCl, 0.1 M maleic acid, 0.3% Tween 20, pH 7.5) for 1 h. Anti-DIG-AP conjugate was added into the same freshly made blocking solution at 1/10,000 dilution and incubation continued for another 30 min, followed by $4 \times$ for 10 min washes with the wash buffer, and incubated for 2 min in 0.1 M Tris, pH 9.5. Finally, the membrane was incubated briefly with CDP-Star between transparency pages. The excess liquid was removed and exposed to an X-ray film until the bands became clearly visible.

Sequence Analyses of Clone Pen b 26

Amino acid sequence *Pen b 26* was analyzed using various internet software. The secondary structure analyses of *Pen b 26* protein was carried out using PSIPRED software from University College London, UK web site [14]. The hydrophobicity [15] and hydrophilicity [16] plots were drawn using the software of Colorado State University, USA web site (<http://arbl.cvmbs.colostate.edu/molkit/hydropathy/index.html>). The BLAST algorithm was used for homology searches in GenBank, National Centre for Biotechnology Information, USA web site [17]. MultAlin (from Institut National de la Recherche Agronomique, France), multiple alignment software was used to align the most homologous sequences [18]. Peptide analyses was performed by EMBOSS Pepstats using European Bioinformatics Institute web site [19]. Phosphorylation sites were predicted using NetPhos 2.0 server of Technical University of Denmark [20].

Database Submissions

The cDNA and predicted amino acid sequence of *Pen b 26* was submitted to GenBank under the accession number AY786077. Data obtained from this study was also submitted to the Allergen Nomenclature Sub-Committee for approval of the new allergen, designated as *Pen b 26*.

Results

Isolation of Clone Pen b 26 by Plaque Immunohybridization Assay

A phage cDNA library of *P. brevicompactum* was constructed in Uni-ZAP[®] XR vector using total RNA isolated from the organism. The phage plaques of the cDNA library of *P. brevicompactum* were screened using pooled atopic sera from individuals sensitive to *P. brevicompactum*. Among several allergenic clones, one clone, designated as *Pen b 26*, was found to be strongly allergenic. This clone was further purified by phage dilution method. The purified clone was found to be only reactive with the atopic sera obtained from individuals sensitive to this mold (fig. 1a). The *Pen b 26* phage clone was amplified in *E. coli* host and a crude extract was made by sonicating the phage particles. The crude extract was analyzed by Western blot as shown in figure 1b. The allergenic clone *Pen b 26* had an approximate MW weight of 10 kDa.

Fig. 1. Isolation and purification of clone *Pen b* 26 from a bacteriophage cDNA library of *P. brevicompactum*. Immuno-plaque hybridization (a) and Western blot analysis (b) of the purified phage extracts of the allergenic clone *Pen b* 26. Normal (N) and atopic (A) sera.

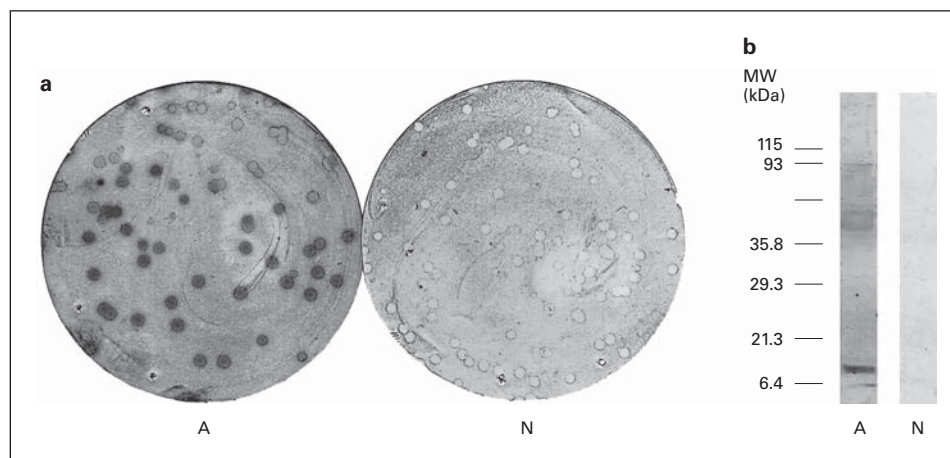


Fig. 2. Transcript of the *Pen b* 26 clone and the predicted amino acid sequence (*italicised*). Denotations: start (ATG), stop (TAA) codons and polyadenylation signals (AAA AAA) are underlined. Potentially phosphorylated residues are T-29 and S-97.

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1  GGCACGAGGC TTCAAGACAC CCAGTCTACC CGTCGACATG TCTACCGCTG
51  AGCTCGCTGT CTCTTACGCC GCCCTCATCC TGGCCGATGA CGGTATTGAG
101 GTCTCCGCCG ACAAGATCCA GACCATCCTC GGTGCCGCCA AGGTCCAGGA
151 GGTGAGCCC ATCTGGGCCA CCATCTTCGC CAAGGCCCTC GAGGGTAAGG
201 ACATCAAGGA GATCCTGACC AACGTCGGCT CCGCTGGTCC CGCCACCGCC
251 GGTGCCCCCG CCGCTGCTGG TGCCGCCGCT CCCGCTGAGG AGAAGAAGGA
301 GGAGAAGGAA GAGGAGAAGG AGGAGTCCGA TGAGGACATG GGCTTCGGTC
351 TCTTCGACTA AGCGTCTTAA AAACCTCCGTA TCGAATTTGA ACTCGTTTCC
401 TTTTTCAAA AAAAAAAAA AAAAAAAAA AAAAAAAAA AAAAAAAAA
451 AAAAAA

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DNA Sequencing, PCR and Northern Blot Hybridization

Performing single colony excision protocol, phage clone *Pen b* 26 was converted to phagemid catalyzed by the helper phage. Phagemid was purified as plasmid in pBluescript vector and further analyzed for DNA sequence and insert size. The DNA sequence of *Pen b* 26 in pBluescript is shown in figure 2. The cDNA insert had a nucleotide sequence of 455 bp. An open reading frame deduced from the cDNA sequence data using the largest predicted sequence encoded a polypeptide of 107 residues with a predicted molecular size of 11 kDa (table 1) which was in close agreement with the Western blot analysis as shown earlier (fig. 1b). Furthermore, a PCR was performed using flanking primers from pBluescript vector (fig. 3a). A PCR product of about 0.5 kb, including the external primer sequences was obtained, which confirmed the size of the cDNA sequence obtained from the sequence data. The

size of the transcript was further confirmed by Northern blot hybridization using the cDNA of clone *Pen b* 26 as a probe (fig. 3b). This probe was hybridized strongly with a band of about 0.5 kb. However, there were two more unrelated bands, one is around 0.3 kb and the other one is a little higher than 0.5 kb, cross-reacted with the same probe despite higher stringency washes. Hence, the size of the cDNA fragment was further confirmed.

Protein Sequence Analysis

Pen b 26 protein appears to have strongly acidic and negatively charged residues, 21.5% with an isoelectric point of 3.87 (table 1). This protein was also found to be unusually rich in alanine (20.5%) and glutamic acid (15%), and together they comprised more than one-third of all the residues. Furthermore, the amino acid sequence of *Pen b* 26 was analyzed using NetPhos 2.0 Server [20] for the prediction of phosphorylated residues. It was

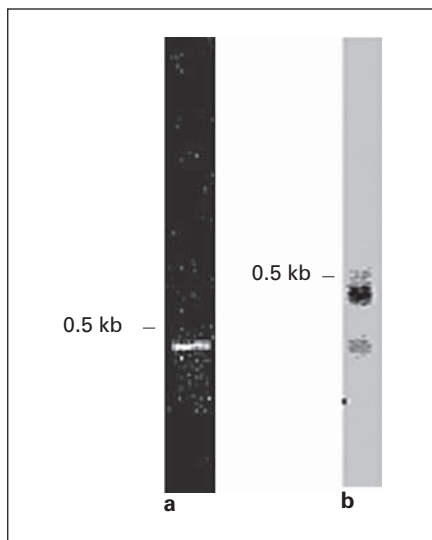


Fig. 3. Analysis of clone *Pen b 26* by PCR using flanking M13 forward and reverse primers (**a**) and Northern blot hybridization (**b**) probed with *Pen b 26* cDNA.

found that two potential residues, Ser-97 and Thr-29 had high scores of 0.997 and 0.764, respectively.

The secondary structure analyses of *Pen b 26* protein carried out using PSIPRED software (fig. 4) which predicted extensive alpha-helical regions from the N-terminus to the middle of the gene intercepted by short coil regions. However, towards the C-terminus larger segment of coil regions appeared. There was little indication of a strand structure closer to the N-terminus.

Furthermore, the hydrophobicity and hydrophilicity plots were analyzed (fig. 5). *Pen b 26* protein had hydrophobic regions closer to its N-terminus, indicating that N-terminus could be buried while strong hydrophilic regions were found towards its C-terminus region. Hydrophilic regions on the protein are exposed on the surface and most likely play a key role in antigenicity and allergenicity.

Amino acid sequence homology analysis of *Pen b 26* protein was performed first using BLAST program and about 100 sequences appeared to have homology to *Pen b 26*. Later, Multalin program was used to align the most prominent examples of the homologous sequences (fig. 6). Further sequence analysis indicated that *Pen b 12* protein had greater than 50% amino acid sequence homology to 60S ribosomal P1 proteins from a variety of species, primarily from fungal sources, including *Aspergillus fumigatus*, *Cladosporium herbarum*, *Neurospora crassa*, *Alternaria alternata*, and *Schizosaccharomyces pombe* (fig. 6).

Table 1. EMBOSS Pepstats analysis results for *Pen b 26* protein

Residue	n	Mol%	DayhoffStat*
A = Ala	22	20.561	2.391
B = Asx	0	0.000	0.000
C = Cys	0	0.000	0.000
D = Asp	7	6.542	1.189
E = Glu	16	14.953	2.492
F = Phe	3	2.804	0.779
G = Gly	9	8.411	1.001
H = His	0	0.000	0.000
I = Ile	8	7.477	1.661
K = Lys	9	8.411	1.274
L = Leu	7	6.542	0.884
M = Met	2	1.869	1.100
N = Asn	1	0.935	0.217
P = Pro	4	3.738	0.719
Q = Gln	2	1.869	0.479
R = Arg	0	0.000	0.000
S = Ser	5	4.673	0.668
T = Thr	5	4.673	0.766
V = Val	5	4.673	0.708
W = Trp	1	0.935	0.719
X = Xaa	0	0.000	0.000
Y = Tyr	1	0.935	0.275
Z = Glx	0	0.000	0.000

Property	Residues	n	Mol%
Tiny	(A+C+G+S+T)	41	38.318
Small	(A+B+C+D+G+N+P+S+T+V)	58	54.206
Aliphatic	(I+L+V)	20	18.692
Aromatic	(F+H+W+Y)	5	4.673
Non-polar	(A+C+F+G+I+L+M+P+V+W+Y)	62	57.944
Polar	(D+E+H+K+N+Q+R+S+T+Z)	45	42.056
Charged	(B+D+E+H+K+R+Z)	32	29.907
Basic	(H+K+R)	9	8.411
Acidic	(B+D+E+Z)	23	21.495

Molecular weight = 11,066.32; residues = 107; average residue weight = 103.424; charge = -14.0; isoelectric point = 3.8730; probability of expression in inclusion bodies = 0.978.

* DayhoffStat is the amino acid's Dayhoff statistic divided by the molar percent. The Dayhoff statistic is the amino acid's relative occurrence per 1,000 aa normalized to 100 by ris@ebi.ac.uk (original work from 1993).

Discussion

Penicillium species have long been associated with indoor mold allergies. A number of allergens have been isolated from various *Penicillium* species. These includes *Pen c 1* [21], *Pen c 2* [22] and *Pen c 3* [23] of *P. citrinum*, *Pen o 18* of *P. oxalicum* [24], *Pen ch 13* (a.k.a. *Pen n 13*)

% Identity	1						60
1- (100%)	MSTAEELAVSY	AALILADDGI	EVSADKIQTI	LGAAKVQVEE	PIWATIFAKA	LEGKDIKEIL	
2- (76%)	MSTAEELACSY	AALILADDGV	EITADKIQTL	LGAAKVADVE	PIWTSIFAKA	LEGKDIKDLL	
3- (73%)	MSTAEELATSY	AALILADDGV	EITADKIQTI	IKAAKIEDVE	PIWASLFAKA	LEGKDVKDLL	
4- (71%)	MSHAELASSY	AALILADDGI	EITADKLQTL	IKAAANVEEVE	PIWTSIFAKA	LEGKDVKDLL	
5- (67%)	MSTSELATSY	AALILADDGV	DITADKLQSL	IKAAKIEEVE	PIWTTLFAKA	LEGKDVKDLL	
6- (67%)	MSASELATSY	TALILADEGI	EITSDKLSSL	TKAGNV.EVE	PIWATIFAKA	LEGKDLKELL	
Consensus	MStaELAtSY	aALILAD#G!	#!taDKlqt1	.kAak!.#VE	PIWatIFAKA	LEGKD.K#1L	

	51						112
1- (100%)	TNVGSAG...	.PATAGAPAA	AGAAAPAEK	KEE.KEEKE	ESDEDMGFGL	FD	
2- (76%)	TNVGSGGAAA	.PAAVGGAAA	GAAAPAEAAA	AEEKKEEKE	ESDEDMGFGL	FD	
3- (73%)	SNVGSGGAAA	APAAAGAAAA	GGAA..EEAK	AEE.KVEEKE	ESDEDMGFGL	FD	
4- (71%)	VNVGSGGGAA	.PAAGGAAAA	GGA...EEAA	PEEAKKEEKE	ESDEDMGFGL	FD	
5- (67%)	LNVGSGGGAA	.PLPEALLLR	WRAAD.AAPA	AEEKKEEKE	ESDEDMGFGL	FD	
6- (67%)	LNIGSAGAAS	APTAAGAGTA	APAEAAEEK	KEEAKEEE..	ESDEDMGFGL	FD	
Consensus	.N!GSgGaaa	.Paaaga.aa	..Aa...ee.k	.EE.KeEEke	ESDEDMGFGL	FD	

Fig. 6. Amino acid sequence homology among 60S ribosomal proteins from different fungal sources and *Pen b* 26 protein of *Penicillium brevicompactum*. Sequences: 1 = *Penicillium brevicompactum* *Pen b* 26 (GeneBank Accession No. AY786077); 2 = *Aspergillus fumigatus* (AF268869.1); 3 = *Neurospora crassa* (XM_331351.1); 4 = *Cladosporium herbarum* (*Cla h* 12) (AACM01000433.1); 5 = *Alternaria alternata* (*Alt a* 12) (X84216.1); 6 = *Schizosaccharomyces pombe* (P1-alpha 1) (NC_003423.1). Consensus symbols: ! = anyone of I, V; # = anyone of N, D, Q, E, B, Z.

[25, 26], *Pen ch* 18 [27] of *P. chrysogenum*. However, not all *Penicillium* species are well-studied and a number of allergens from *Penicillium* species are awaiting discovery. In this study, we attempted to isolate and clone the allergenic proteins from *P. brevicompactum*. Isolation of cloned allergens or allergenic clones by the use of atopic sera has been a method of choice. In this study, a phage cDNA library of *P. brevicompactum* has been screened with sera obtained from individuals allergic to this mold. A clone designated as *Pen b* 26 was successfully screened using atopic sera.

The *Pen b* 26 protein appeared to have a low molecular weight of only 11 kDa which was confirmed by Western blotting, DNA sequence analysis, PCR and Northern blot hybridization. BLAST analysis of its amino acid sequence indicated that this protein had strong sequence homology to a well-known 60S ribosomal P1 protein from several species of fungi and also several other eukaryotic organisms (data not shown).

Ribosomal P proteins are phospho-proteins and characterized from a variety of species [28–34]. In humans, P1 protein, together with two other acidic P proteins, namely P2 and P0, form a distinct lateral protuberance on the 60S ribosomal subunit [30]. They interact with elongation factors, Ef1 and Ef2, and their level of phosphorylation is a regulatory mechanism for the overall rate

of translation [35]. In the yeast *Saccharomyces cerevisiae*, the P proteins, unlike most riboproteins, are found in multi copies and form a pentameric complex with two isomeric copies of P1 (α/β) and P2 (α/β) joined with one copy of P0 [34]. Yeast strains with all four disrupted P1 and P2 genes are viable, indicating that these genes are not absolutely essential, although these cells exhibit slower growth rate, cold-sensitivity and inability to sporulate [31]. In contrast, P0 protein is absolutely essential for cell viability and ribosome activity [32]. Actively growing yeast cells synthesize higher numbers of P proteins than the stationary cells [36]. Although most proteins in the 60S ribosome are basic, P proteins are among the few acidic proteins. P1 and P2 proteins, after synthesis, shuttle between cytoplasmic pool and the ribosome whereas the P0 protein remains exclusively in the ribosomes [37].

The presence of autoantibodies against the ribosomal P proteins in autoimmune diseases [38] and their association in IgE-mediated allergic diseases [39] confirm the allergenicity of *Pen b* 26 protein of *P. brevicompactum*. All P proteins seem to contain an important epitope structure in their C-terminus that is recognized by the antibodies in the sera of individuals with autoimmune diseases, such as systemic lupus erythematosus [40], protozoan infections like leishmaniasis [41], and Chagas' heart disease

[42]. In addition to the common C-terminus epitope, P proteins contain epitope regions in their N-terminal regions [38]. Based on hydrophilicity plots (fig. 5) and comparison of the sequence homologies from different 60S ribosomal P1 proteins (fig. 6), the C-terminus of *Pen b* 26 also contains this common epitope region. The cellular location of P proteins has recently been re-investigated and in addition to cytoplasmic location, fully phosphorylated P proteins were also found on the cell wall [43]. The new data may explain their allergenicity. Since P1 proteins are on the fungal cell wall, they can easily induce allergies by direct contact to the skin or nasal membranes of the sensitive individuals without lysis of the cells.

The secondary structure analysis of *Pen b* 26 protein conforms to the secondary structure of other P proteins. It has high alpha-helical content (>50%) in the N-termi-

nus and a flexible hinge region. The central region and its high alanine content (~20%) and acidic nature (high in acidic residues and low pI), in conjunction with the predicted phosphorylation site in the conserved C-terminal epitope region, like other known P1 proteins support the hypothesis that the isolated clone *Pen b* 26 is indeed an acidic ribosomal P1 protein. This clone does react with atopic sera from allergenic individuals and has structure similar to other acidic ribosomal P1 proteins.

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