



Expression and characterization of Pen b 26 allergen of *Penicillium brevicompactum* in *Escherichia coli*

M. Serdal Sevinc*, Veena Kumar, Makonnen Abebe, Susantha Mohottalage, Premkumari Kumarathasan, Renaud Vincent, Hari M. Vijay

Healthy Environments and Consumer Safety Branch, Health Canada, 251 F.G. Banting Driveway, Rm C229, Tunney's Pasture, Postal Locator 2202D2, Ottawa, Ont., Canada K1A 0K9

ARTICLE INFO

Article history:

Received 9 July 2008
and in revised form 23 December 2008
Available online 14 January 2009

Keywords:

His tag
Expression
Allergen
Ribosomal 60S P1 phosphoprotein
Penicillium brevicompactum
Protein characterization

ABSTRACT

Pen b 26 is one of the allergens produced by *Penicillium brevicompactum* which is one of the most prevalent indoor airborne fungi and a major source of respiratory problems, including asthma. Pen b 26 was cloned and expressed as an N-terminal as well as a C-terminal His₆ tagged fusion protein in *Escherichia coli*. This allergen was purified by immobilized Ni²⁺-affinity chromatography. The purified Pen b 26 was characterized by immunological, biochemical and biophysical methods. C-His₆ tagged Pen b 26 produced several fold greater yield than N-His₆ tagged Pen b 26. The affinity of C-His₆ tagged Pen b 26 for the specific antibody was also 2.75 times higher than N-His₆ tagged Pen b 26.

Crown Copyright © 2009 Published by Elsevier Inc. All rights reserved.

The prevalence of airborne allergic diseases has been on the rise, especially in the developed countries where the vast majority of the people live in artificially created and controlled indoor air environments [1]. This tightly regulated air is often prone to the overgrowth of ever-present mold species living indoor environments especially if the excess humidity is not controlled appropriately. The mold-overgrowth is often associated with a number of allergic symptoms involving respiratory system including asthma. *Penicillium* species have been identified as a major source of indoor mold allergies and respiratory problems including asthma and Farmer's Lung syndrome [2–6]. *Penicillium brevicompactum* is one of the most frequently isolated indoor *Penicillium* species. In addition, *P. brevicompactum* has also been reported to be one of the major food spoilage organisms and a source of mycotoxins [7–10].

Molecular characterization of allergens is of significant value to understand the pathogenesis of allergic diseases and the development of specific diagnostic and efficacious therapies. Classical methods of allergen protein purification often produce low yield of poor quality allergens. Allergens cloned using recombinant DNA technologies can be produced in large quantities with exceptional purity. Purified allergens are useful for developing diagnostic tests to screen susceptible populations allergic to mold as well as for the hypersensitization of these patients to induce the production of protective IgG antibodies. Affinity tags, which are incorporated into one end of a protein, have been frequently used for

protein detection and purification. The hexa-histidine (His₆)¹ tag is one of the most popular tags. His₆ tag provides a better advantage over other tags as it binds strongly to the immobilized metal ion affinity chromatography (IMAC) resin and its small size often eliminates the need for proteolytic removal. Although His₆ tag can be incorporated into either end of the protein, N-terminal His₆ tag has been known to produce better solubility and higher yield than the C-terminal His₆ tag [11].

We have recently analyzed the extracts of 14 *Penicillium* species, including *P. brevicompactum* [12]. The extracts of *P. brevicompactum* showed at least six allergenic bands resolved by immunoblot analysis. To date, very few allergens have been isolated and cloned from *Penicillium* species. Allergen Pen b 26 has been recently isolated from a cDNA library of *P. brevicompactum* in our laboratory [13]. In this study, Pen b 26 was over-expressed using His₆ tag expression system. In order to determine the best possible expression, we employed both the N- and the C-terminal His₆ tag containing expression vectors. This paper describes the expression of Pen b 26 in *Escherichia coli* and its characterization.

Materials and methods

Penicillium brevicompactum strain DAOM 231273 (Pen-01-0001) was obtained from Canadian Collection of Fungal Cultures, Agricul-

* Corresponding author. Fax: +1 613 941 6959.
E-mail address: serdal_sevinc@hc-sc.gc.ca (M.S. Sevinc).

¹ Abbreviations used: His₆, hexa-histidine; IMAC, immobilized metal ion affinity chromatography; PCR, polymerase chain reaction.

ture, and Agri-Food Canada and used to isolate total RNA and to construct a cDNA library as described earlier [13].

Construction of the expression vectors

The cDNA encoding Pen b 26 was amplified by polymerase chain reaction (PCR) using two internal forward and reverse primer pairs and cloned into two different His₆-tag containing expression vectors (Invitrogen Life Technologies, Carlsbad, California, USA): pTrcHisTOPO vector, which had a His₆ tag at the N-terminus (Fig. 1a) and pTrcHis2TOPO, which had a His₆ tag at the C-terminus (Fig. 1b). The forward primer (5'-ATGTCTACCGCTGAGCTCGTG-3', the first 22 nts of the protein coding sequence of the native Pen b 26) and the reverse primer (5'-GCTTAGTCGAAGAGACCGAAGC-3', the reverse complementary sequence of the last 18 nts in the 3' end of the coding sequence, stop codon and the flanking two nucleotides of the native Pen b 26) were used for the construction of pTrcHisTOPO-Pen b 26 (N-His₆ tagged) vector. The same forward primer and a His₆ tag containing reverse primer (5'-TTAATGATGATGATGATGATGGTCGAAGAGACCGAAGCCC-3', the reverse complementary sequence of the last 19 nts in the 3' end of the coding sequence followed by the coding sequence of 6 His residues and the stop codon) were used for the construction of pTrcHis2TOPO-Pen b 26 (C-His₆ tagged) vector. 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 at the end of the 30 cycles was also incor-

porated. Both constructs were transformed into *E. coli* TOP10 strain (Invitrogen).

The selected PCR clones were verified by DNA sequencing as described earlier [13]. In pTrcHisTOPO-Pen b 26 construct, total of 35 residues were added to the N-terminus of the native protein which contained the HisG epitope for detection and purification of the recombinant and the original stop codon was retained. The N-His₆ tag fused peptide added about 3.8 kDa to the size of the native Pen b 26, thereby increasing its estimated size from about 11 to 14.8 kDa. In pTrcHis2TOPO-Pen b 26 construct, three external residues from the vector in the N-terminus and the six histidine residues in the C-terminus added about 1 kDa to the size of the native Pen b 26, to give a protein of 12 kDa.

Expression of Pen b 26 fusion proteins in *E. coli*

The cultures of *E. coli* with the appropriate expression constructs were grown in LB (Luria–Bertani) medium with 50 µg/mL ampicillin at 37 °C, 225 rpm. The expression of Pen b 26 was induced by the addition of IPTG into the culture medium at mid-log phase ($A_{600nm} = 0.6$) to 1 mM final concentration. For small-scale, time-course expression, the cultures of 200 mL in 1 L flasks were grown and samples of 10 mL were taken with various time intervals. The samples were centrifuged for 5 min at 10,000g, 4 °C. The pellets were stored at –84 °C until needed. Large scale expression was performed in 4 × 500 mL LB medium with ampicillin (50 µg/mL) in 2 L flasks for the large-scale purification

a

Pen b26 with N-terminal His ₆ -tag														
ATG	GGG	GGT	TCT	CAT	CAT	CAT	CAT	CAT	CAT	GGT	ATG	GCT	AGC	ATG
M	G	G	S	H	H	H	H	H	H	G	M	A	S	M
ACT	GGT	GGA	CAG	CAA	ATG	GGT	CGG	GAT	CTG	TAC	GAC	GAT	GAC	GAT
T	G	G	Q	Q	M	G	R	D	L	Y	D	D	D	D
AAG	GAT	CCA	ACC	CTT	ATG	TCT	ACC	GCT	GAG	CTC	GCT	GTC	TCT	TAC
K	D	P	T	L	M	S	T	A	E	L	A	V	S	Y
GCC	GCC	CTC	ATC	CTG	GCC	GAT	GAC	GGT	ATT	GAG	GTC	TCC	GCC	GAC
A	A	L	I	L	A	D	D	G	I	E	V	S	A	D
AAG	ATC	CAG	ACC	ATC	CTC	GGT	GCC	AAG	GAC	CAG	GAG	GTT	GAG	
K	I	Q	T	I	L	G	A	A	K	V	Q	E	V	E
CCC	ATC	TGG	GCC	ACC	ATC	TTC	GCC	AAG	GCC	CTC	GAG	GGT	AAG	GAC
P	I	W	A	T	I	F	A	K	A	L	E	G	K	D
ATC	AAG	GAG	ATC	CTG	ACC	AAC	GTC	GGC	TCC	GCT	GGT	CCC	GCC	ACC
I	K	E	I	L	T	N	V	G	S	A	G	P	A	T
GCC	GGT	GCC	CCC	GCC	GCT	GCT	GGT	GCC	CGC	GCT	CCC	GCT	GAG	GAG
A	G	A	P	A	A	A	G	A	R	A	P	A	E	E
AAG	AAG	GAG	GAG	AAG	GAA	GAG	GAG	AAG	GAG	GAG	TCC	GAT	GAG	GAG
K	K	E	E	K	E	E	E	K	E	E	S	D	E	D
ATG	GGC	TTC	GGT	CTC	TTC	GAC	TAA	GC						
M	G	F	G	L	F	D	*							

b

Pen b26 with C-terminal His ₆ -tag														
ATG	GCC	CTT	ATG	TCT	ACC	GCT	GAG	CTC	GCT	GTC	TCT	TAC	GCC	GCC
M	A	L	M	S	T	A	E	L	A	V	S	Y	A	A
CTC	ATC	CTG	GCC	GAT	GAC	GGT	ATT	GAG	GTC	TCC	GCC	GAC	AAG	ATC
L	I	L	A	D	D	G	I	E	V	S	A	D	K	I
CAG	ACC	ATC	CTC	GGT	GCC	GCC	AAG	GTC	CAG	GAG	GTT	GAG	CCC	ATC
Q	T	I	L	G	A	A	K	V	Q	E	V	E	P	I
TGG	GCC	ACC	ATC	TTC	GCC	AAG	GCC	CTC	GAG	GGT	AAG	GAC	ATC	AAG
W	A	T	I	F	A	K	A	L	E	G	K	D	I	K
GAG	ATC	CTG	ACC	AAC	GTC	GGC	TCC	GCT	GGT	CCC	GCC	ACC	GCC	GGT
E	I	L	T	N	V	G	S	A	G	P	A	T	A	G
GCC	CCC	GCC	GCT	GCT	GGT	GCC	CGC	GCT	CCC	GCT	GAG	GAG	AAG	AAG
A	P	A	A	A	G	A	R	A	P	A	E	E	K	K
GAG	GAG	AAG	GAA	GAG	GAG	AAG	GAG	GAG	TCC	GAT	GAG	GAC	ATG	GGC
E	E	K	E	E	E	K	E	E	S	D	E	D	M	G
TTC	GGT	CTC	TTC	GAC	CAT	CAT	CAT	CAT	CAT	CAT	TAA	GC		
F	G	L	F	D	H	H	H	H	H	H	*			

Fig. 1. Nucleotide and amino acid sequence of (a) N- and (b) C-His₆ tagged (bold) Pen b 26. The additional residues to the native Pen b 26 are underlined.

of the recombinant protein. The induced culture was allowed to grow for about 4 h and harvested by centrifugation at 3000g for 15 min at 4 °C. The pellets were stored at –84 °C until needed. All pellets were thawed and re-suspended in appropriate amount of 10 mM phosphate buffer, pH 7.2, and sonicated until the lysate was clear (3–5 times in 10–15 s intervals) using a Branson Sonifier (Branson Ultrasonics, Danbury, CT, USA). The mixtures were centrifuged, and their supernatants were collected. Protein concentrations were determined by BCA™ protein assay (Pierce, Rockford, IL, USA) before being analyzed by SDS–PAGE [14] and immunoblotting [15].

SDS–PAGE and immunoblotting

Crude extracts containing about 30 µg protein per well in loading buffer with β-mercaptoethanol were heated to 85 °C for 10 min. Samples were electrophoresed in 15% polyacrylamide using a vertical slab mini gel SDS–PAGE system (Bio-Rad, Mississauga, ON, Canada). The gel was electro-transferred onto PVDF membrane. Subsequently, standard immunoblotting was performed. Immunoblots were routinely probed with primary mouse anti-His antibodies (anti-HisG antibody for N-His₆ tagged, anti-His (C-term) antibody for C-His₆ tagged protein, Invitrogen), followed by secondary goat anti-mouse HRP conjugate (Bio-Rad) for detection of the fusion protein. Immunoblots with rabbit primary antibody against Pen b 26 was followed by secondary goat anti-rabbit IgG HRP conjugate (Bio-Rad). For specific applications, human IgG antibody against ribosomal P antigens (P1α, P1β, P2α, P2β, and P0) (Immunovision, Springfield, AR, USA) was used as primary antibody. Individual atopic sera were used as primary antibodies for immuno-screening, followed by secondary goat anti-human IgE (epsilon)-HRP conjugate (KPL, Gaithersburg, Maryland, USA). In a separate but similar gel, proteins were stained with Coomassie brilliant blue R-250 to ensure that sufficient amount of protein was present. *Penicillium* allergic atopic sera used for immuno-screening were purchased from Plasma Lab International, Washington, USA.

Purification of Pen b 26 fusion proteins

Pen b 26 fusion proteins were purified using IMAC (ProBond™ purification system, Invitrogen). The fusion proteins were purified using native purification protocols, unless otherwise stated, according to the manufacturer's instructions (Invitrogen). After elution from the immobilized Ni²⁺-affinity column, the recombinant proteins were concentrated either through a 10,000 M_w cut-off centrifugal column (Millipore Corporation, Bedford, MA, USA) or Amicon filtration device under nitrogen-pressure. With regards to purity, no visible difference on SDS–PAGE gels was observed between the centrifugal and nitrogen-pressure concentration devices. The purified proteins were washed 5–7 times with 10 mM phosphate buffer, pH 7.2, and stored at –84 °C until needed.

Quantitation of the expression of Pen b 26

Analyses of the relative densitometric values for Pen b 26 bands on SDS–PAGE gels were determined after staining the gels with Coomassie brilliant blue R-250 using AlphaEaseFC version 6.0.0 software system (Alpha Innotech (San Leandro, CA, USA). Densitometric values for protein bands were reported in arbitrary units above the basal values. Values for Pen b 26 were normalized using the total protein values.

Generation of rabbit polyclonal antibody against Pen b 26

Two NZ albino rabbits were immunized against Pen b 26 using purified N-His₆ tagged Pen b 26 which was obtained using the

denaturing protocol for His₆ tagged proteins (Invitrogen). For primary immunization, 100 µg purified protein in 500 µL saline was mixed with an equal volume of complete Freund's adjuvant and injected at five different dorsal sites subcutaneously. Then, the rabbits were given three booster injections with 3-week intervals using incomplete Freund's adjuvant. The rabbits were bled and the sera were pooled and analyzed by immunodiffusion prior to use for immunoblots.

Analysis of Pen b 26 by enzyme linked immunosorbent assay (ELISA)

The affinity of N- and C-His₆ tagged Pen b 26 antigens for the rabbit polyclonal antibody raised against N-His₆ tagged Pen b 26 was compared by competitive ELISA. Immulon-2 HB 96-well microtiter plates were coated overnight at 4 °C with 100 µL of either N- or C-His₆ tagged Pen b 26 (500 ng/mL in PBS, 10 mM phosphate buffered saline, pH 7.4). The rest of the assay was performed at room temperature. After washing the wells with 3× with 200 µL of PBS-T (0.05% Tween-20 in PBS), the wells were incubated with 100 µL blocking solution (1% bovine serum albumen in PBS) for 1 h. A series concentrations of both antigens (0, 1, 2, 5, 10, 20, 50, 100, 200, and 500 ng/mL) were prepared in glass tubes and incubated with the rabbit polyclonal antibody (1/10,000 in blocking solution) for 1 h. The bound antigens, called inhibitor, caused the inhibition of the binding of the rabbit polyclonal antibody to the coated antigen. Antigen–antibody complexes (100 µL) were added to the wells after washing the wells 3× with PBS-T, and incubated for 1 h. The wells were incubated for 1 h with goat anti-rabbit HRP conjugate (1/20,000 dilution in blocking solution, Bio-Rad) after washing the wells 4× with PBS-T. TMB substrate (KPL, USA) was added at 100 µL/well after washing the wells 6× with PBS-T. After 10–12 min incubation, the reaction was stopped with the addition of 50 µL/well 1 M phosphoric acid. The absorbance was read at 450 nm. The ELISA was repeated 3 times in triplicates to confirm the data. The IC₅₀ was determined as the concentration of each antigen that incubated with the rabbit polyclonal antibody and caused 50% decrease at absorbance of the coated antigen.

Mass spectral analysis of Pen b 26 fusion proteins by MALDI-TOF

The IMAC purified Pen b 26 fusion proteins were analyzed by MALDI-TOF mass spectrometry in order to verify its estimated size. The samples for MALDI-TOF mass spectral analysis was prepared as reported [16].

Circular dichroism spectroscopy

CD spectroscopy experiments were performed using a Jasco J-810 spectrometer (JASCO). Proteins were adjusted to 1 mg/mL in 10 mM potassium phosphate buffer (pH 7.3). The data was collected over 175–260 nm at 50 nm/min in a 0.02 cm round cuvette. A total of five scans were acquired and the average was used for final analysis. The raw CD data were in converted into mean residue ellipticity and cropped to include only the region encompassing 178–260 nm. CD spectral data was then analyzed with the CDPro software package and using the SP29 basis set [17,18].

Structural analysis by softwares

The structure of Pen b 26 was further analyzed by using publicly available internet softwares. The secondary structure analyses were carried out using PSIPRED software from University College London, UK [19]. The hydrophobicity [20] and hydrophilicity [21] plots were drawn using the software of Colorado State University,

USA web site <http://arbl.cvmbs.colostate.edu/molkit/hydropathy/index.html>).

Results

Expression of Pen b 26 in *E. coli*

The expression construct for Pen b 26 was designed to allow large-scale production and ease of purification of the recombinant protein without affecting its antigenic determinants. Pen b 26 was successfully cloned and expressed with both N- and C-His₆ tag in *E. coli* after induction with IPTG. The time-course of the expression of Pen b 26 was monitored following induction with IPTG. The purified protein was visualized after SDS-PAGE with Coomassie brilliant blue stain (Fig. 2). The production of Pen b 26 was stabilized after 3 h of induction and no significant increase in the level of expression was observed thereafter (Fig. 3). However, the C-His₆ tagged protein was expressed at levels 3–4 times higher than that of the N-His₆ tagged Pen b 26. The purified preparations of recombinant Pen b 26 were made from the cultures of *E. coli* after 3–4 h induction. Pen b 26 was purified using affinity chromatography and the eluted fractions were analyzed by SDS-PAGE. At the end of the purification procedure, 15 and 75 mg purified proteins were obtained per litre culture for N- and C-His₆ tagged Pen b 26, respectively.

Mass spectral analysis of Pen b 26

The calculated molecular sizes of purified Pen b 26 fusion proteins were 14,790 Da for N-His₆ tagged protein and 12,073 Da for C-His₆ tagged protein, which were verified by MALDI-TOF mass spectral analysis, producing the values of 14,790 and 12,075 Da, respectively.

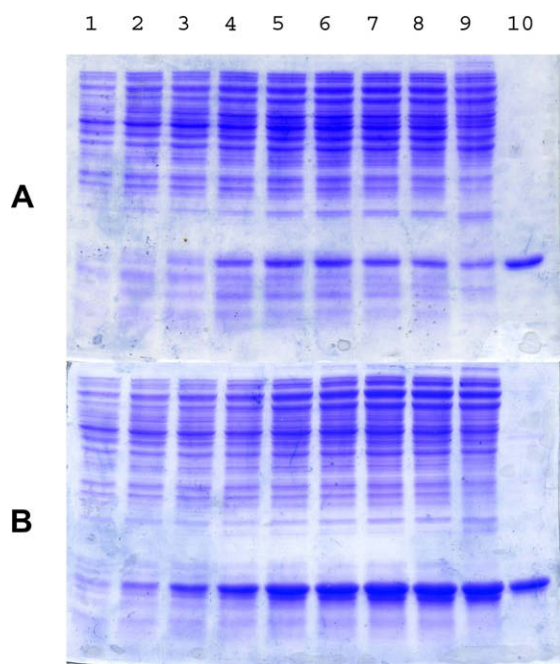


Fig. 2. The time-course expression of (A) N- and (B) C-His₆ tagged Pen b 26 in *Escherichia coli*. Time after induction with IPTG: lane 1, 0 min; lane 2, 30 min; lane 3, 1 h; lane 4, 2 h; lane 5, 3 h; lane 6, 4 h; lane 7, 5 h; lane 8, 6 h; lane 9, 16 h; lane 10, purified Pen b 26 (3 µg/lane). Approximately, total 30 g crude extracts per lane was loaded.

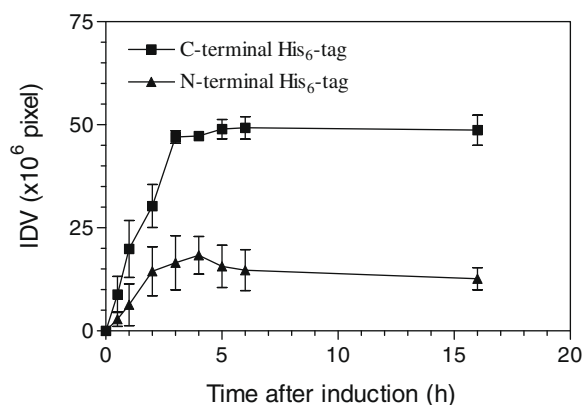


Fig. 3. Comparison of the expression of N- and C-His₆ tagged Pen b 26. Integrated density values (IDV) for Pen b 26 band in each lane in SDS-PAGE gels stained with Coomassie brilliant blue. Approximately, total 30 g crude extracts per lane was loaded. Values represent means $\pm$ SD of three separate experiments.

Analysis of Pen b 26 by ELISA

The affinity of the epitopes on N- and C-His₆ tagged Pen b 26 for the rabbit polyclonal antibody was compared by inhibition experiments using competitive ELISA (Fig. 4). When the microtiter plates were coated with C-His₆ tagged Pen b 26, IC₅₀ values for N- and C-His₆ tagged Pen b 26 were 6.33 ± 0.15 and 2.3 ± 0.15 ng/mL, respectively. Thus, C-His₆ tagged Pen b 26 had 2.75 times higher affinity for the rabbit polyclonal antibody than N-His₆ tagged Pen b 26. On the other hand, when the microtiter plates were coated with N-His₆ tagged Pen b 26, IC₅₀ values for both C- and N-His₆ tagged Pen b 26 were 6.03 ± 0.30 and 5.33 ± 0.45 ng/mL, respectively. Besides, N-His₆ tagged Pen b 26 produced about 10% higher absorbance

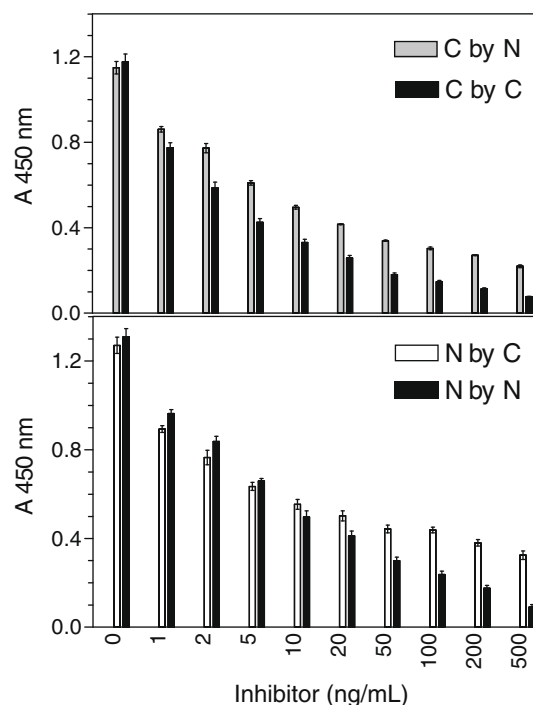


Fig. 4. Comparison of the affinity of N- and C-His₆-tagged Pen b 26 for the rabbit polyclonal antibody by ELISA. Top panel: microtiter plate coated by C-His₆-tagged Pen b 26, inhibited by either N- or C-His₆-tagged Pen b 26 (C by N or C by C) that was incubated with the rabbit polyclonal antibody. Bottom panel: microtiter plate coated by N-His₆-tagged Pen b 26, inhibited by either C- or N-His₆-tagged Pen b 26 (N by C or N by N) that was incubated with the rabbit polyclonal antibody. Values represent means $\pm$ SD of three separate experiments.

($A_{450\text{nm}}$) than C-His₆ tagged Pen b 26 in the absence of the inhibitor. At higher inhibitor concentrations (>10 ng/mL), always the same inhibitor antigen as the coated antigen produced higher absorbance than the non-coated antigen. The results were also reproducible with the different batches of Pen b 26 preparations.

Immunoblot analyses of Pen b 26

Purified N- and C-His₆ tagged Pen b 26 proteins were analyzed by immunoblotting with several different antibodies (Fig. 5). Both proteins reacted equally well with the rabbit polyclonal antibodies raised against N-His₆ tagged Pen b 26, human IgG antibody against ribosomal P antigens and *Penicillium*-sensitive atopic sera based on the intensity of the bands. Both proteins also specifically reacted with their N- and C-His₆ tag specific antibodies.

Secondary structure analyses of Pen b 26 by CD spectroscopy

Secondary structure analyses of purified N- and C-His₆ tagged Pen b 26 proteins by CD spectroscopy showed that both proteins were in their folded conformation (Fig. 6). No major differences were observed in the protein secondary structures despite the differences in their molecular sizes (Table 1). The differences observed were within the range of predictions.

Structural analysis of Pen b 26 by various softwares

Structure of Pen b 26 was further analyzed by processing the polypeptide sequences of the native and the two versions of recombinant Pen b 26 proteins with various publicly available internet softwares, including prediction of the secondary structure and hydrophobicity and hydrophilicity plots (data not shown). No significant differences were observed among the two versions of the recombinant Pen b 26 and the original cloned Pen b 26. The possible common epitope in the C-terminal region of Pen b 26 was preserved in both proteins while the location of the His₆ tag epitope varied.

Prevalence of allergies against Pen b 26

Purified C-His₆ tagged Pen b 26 was used as an antigen to screen the individual *Penicillium*-sensitive atopic sera to determine the prevalence of allergies against Pen b 26 by immunoblotting (Table 2). The results obtained from the screening of 41 individual sera indicated that 30 of the *Penicillium*-sensitive individuals also had IgE antibodies against Pen b 26 (73%). This data indicated that Pen b 26 was a major allergen of *P. brevicompactum*. However, the degree of allergenicity against Pen b 26 in the screened popu-

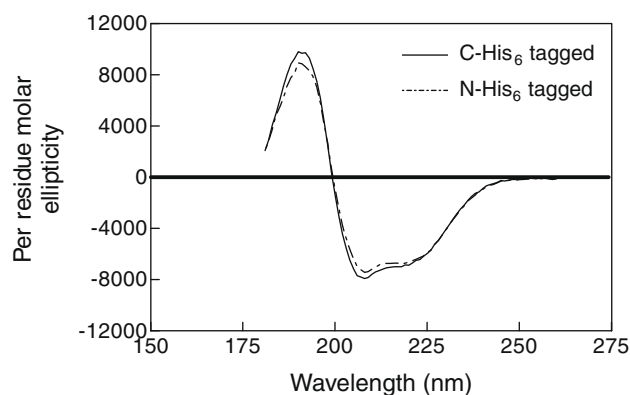


Fig. 6. Comparison of the CD spectra of Pen b 26 proteins with C- and N-His₆ tags.

lation varied considerably: from weak (22%) to medium (12%) to strong (39%) reactions as observed from the intensity of the bands on the immunoblot.

Discussion

A number of *Penicillium* species have been reported to cause indoor mold allergies [22–27]. However, very few of these allergens have been isolated or characterized. Most mold allergens are produced in small quantities by the mold species as integral part of the mycelia or spores. Classical method of purification of the native allergens is a tedious process as impurities are often encountered. Besides, the yield of the allergen is often not satisfactory for characterization studies. Therefore, it is essential to produce these allergens in large-scale and highest possible purity for further studies using recombinant DNA technology.

In this study, the allergen Pen b 26 was cloned and expressed successfully. The time-course expression of the cloned allergen was used to optimize the harvest time and thereby the production of Pen b 26 in abundant quantities. The expression of Pen b 26 with C-His₆ tag produced 3–5 times higher yield than its N-His₆ tag version although the proteins with the N-terminal leader sequence (35 residue including His₆ tag) is claimed to express better in *E. coli* by the manufacturer (Invitrogen). Perhaps, the production and folding of the smaller C-His₆ tagged protein was less burden for the cell than its larger N-His₆ tagged version. In the C-His₆ tagged Pen b 26, there were also three additional residues in the N-terminus in addition to the C-His₆ tag, which were essential for the expression of Pen b 26 in *E. coli*. When these three residues were deleted, no expression was observed (unpublished data).

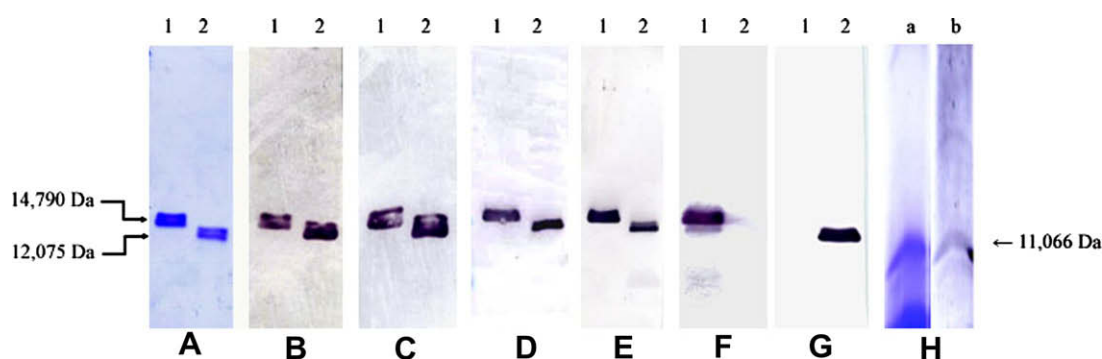


Fig. 5. Immunoblot analyses of purified Pen b 26 with various antibodies (B–G) and Coomassie brilliant blue stain (A), (B) human antibody against ribosomal P antigen, (C) rabbit polyclonal antibody against N-His₆ tagged Pen b 26, (D and E) *Penicillium*-sensitive atopic sera, (F) anti-hisG antibody specific for N-His₆ tag, (G) anti-his (C-term) antibody specific for C-His₆ tag. Lane 1, purified N-His₆ tagged Pen b 26; lane 2, purified C-His₆ tagged Pen b 26. Approximately, 3 μ g of protein per lane was loaded. (H) Partially purified native extracts of *P. brevicompactum* after staining with Coomassie brilliant blue (a) and its immunoblot with *Penicillium*-sensitive atopic serum (b).

Table 1Comparison of the calculated secondary structures of Pen b 26 proteins with C- and N-His₆ tags.

Method	Pen b 26	α_R	α_D	β_R	β_D	T	U
CONTINLL	With C-His ₆ tag	0.134	0.127	0.150	0.089	0.206	0.294
	With N-His ₆ tag	0.126	0.120	0.154	0.092	0.207	0.302
CDSSTR	With C-His ₆ tag	0.102	0.088	0.185	0.100	0.208	0.303
	With N-His ₆ tag	0.087	0.094	0.169	0.093	0.232	0.314

The secondary structure fractions are regular alpha-helix (α_R), distorted alpha-helix (α_D), regular beta-sheet (β_R), distorted beta-sheet (β_D), turns (T), and unordered (U).**Table 2**Prevalence of allergies against Pen b 26 among *Penicillium*-sensitive individuals.

Total no. of sera: 41	Positives: 30 (73%)			Negatives: 11
Degree of reaction	Strong (+++)	Medium (++)	Weak (+)	None (–)
Detailed analysis	16 (39%)	5 (12%)	9 (22%)	11 (27%)

The mass spectral analyses of both versions Pen b 26 by MALDI-TOF confirmed that the calculated sizes of both Pen b 26 proteins were correct and no modification had occurred as the method is accurate within ± 1 –2 Da.

The lower IC₅₀ value for C-His₆ tagged Pen b 26 indicated that it had 2.75 times higher affinity for the rabbit polyclonal antibody than the N-His₆ tagged antigen when the microtiter plates were coated with the C-His₆ tagged antigen. On the other hand, similar IC₅₀ values for both antigens on microtiter plates coated with the N-His₆ tagged antigen, and slightly higher absorbance (10%) for the N-His₆ tagged antigen without the inhibitor was probably due to better binding of N-His₆ tagged Pen b 26 to the microtiter plates than that of C-His₆ tagged Pen b 26. Furthermore, at higher inhibitor concentrations (>10 ng/mL), it was not possible to compare the affinity of both antigens for the rabbit polyclonal antibody because of the saturation of the epitopes, thus, similar results were obtained from both antigen-coated plates. As a result, C-His₆ tagged Pen b 26 may be a better choice for the large-scale expression and future studies than the N-His₆ tagged protein.

In *Saccharomyces cerevisiae*, it was shown that 60 S acidic ribosomal proteins form a pentamer of two isomeric copies of P1 (α/β) and P2 (α/β) joined with a copy of P0 [28]. All five subunits in the pentamer share one common epitope in their C-terminus. This epitope is also present in Pen b 26 and probably plays a significant role in the development of antibodies against Pen b 26. Immunoblot analysis of both versions Pen b 26 with different antibodies indicates that the common epitope is equally preserved in both proteins and reacted with the specific antibodies (Fig. 5). Earlier, it was shown that Pen b 26 had strong amino acid homology (>50%) to a number of 60 S acidic ribosomal P1 proteins from different fungal species using BLAST search [13]. In this study, our experimental evidence confirmed the interaction between the ribosomal P protein specific antibody and Pen b 26 (Fig. 5).

CD spectroscopy as well as theoretical computational analyses of the secondary structure and hydrophobicity and hydrophilicity of these two versions of the recombinant Pen b 26 did not show any major differences between them. However, it could be possible that the common epitope region may have been slightly secluded in the tertiary structure of the N-His₆ tagged protein due to the presence of the large number of additional residues in its N-terminus and thereby causing variation of affinity for the same polyclonal antibody.

Pen b 26 appears to be a major allergen of *P. brevicompactum* because of its high degree of prevalence (73%) among *Penicillium*-sensitive individuals. Screening of the *Penicillium* allergy population using recombinant Pen b 26 is of significant value. This recombinant protein could be used to screen large number of individuals not only allergic to *Penicillium* but also allergic to

60 S acidic ribosomal P proteins. The significance of the latter is that about half of the number of individuals carrying antibodies against 60 S acidic ribosomal P proteins have autoantibodies [29–30] and therefore prone to autoimmune diseases such as systemic lupus erythematosus [31], protozoan infections like leishmaniasis [32], trypanosomiasis (Chagas' heart disease) [33], toxoplasmosis [34]. It was shown that specific antibodies in the sera of systemic lupus erythematosus patients react with 60 S acidic ribosomal P proteins by forming an immunocomplex. Therefore, cloned and purified 60 S acidic ribosomal P proteins, like Pen b 26 with C-His₆ tag offers a valuable tool to screen and confirm the presence of autoantibodies as well as allergies to *Penicillium* in large number of individuals.

Acknowledgments

We thank Dr. W.L. Casley and Mrs. Michèle Lemieux (Health Products and Food Branch, Health Canada) for assistance with DNA sequencing and analyses and Dr. M.A. Hefford and John Mark (Biologics and Genetic Therapies Directorate, Health Canada) for assistance with CD spectroscopy.

References

- [1] P. Ellwood, M.I. Asher, R. Beasley, T.O. Clayton, A.W. Stewart, ISAAC Steering Committee. The international study of asthma and allergies in childhood (ISAAC): phase three rationale and methods, *Int. J. Tuberc. Lung Dis.* 1 (2005) 10–16.
- [2] V.P. Kurup, H.D. Shen, H. Vijay, Immunobiology of fungal allergens, *Int. Arch. Allergy Immunol.* 129 (2002) 181–188.
- [3] H.D. Shen, M.F. Tam, H. Chou, S.H. Han, The importance of serine proteinases as aeroallergens associated with asthma, *Int. Arch. Allergy Immunol.* 119 (1999) 259–264.
- [4] H.D. Shen, S.H. Han, Characterization of allergens of *Penicillium* and *Aspergillus* species, *J. Microbiol. Immunol. Infect.* 31 (1998) 141–145.
- [5] K. Nakagawa-Yoshida, M. Ando, R.I. Etches, J.A. Dosman, Fatal cases of farmer's lung in a Canadian family. Probable new antigens, *Penicillium brevicompactum* and *P. olivicolor*, *Chest* 111 (1997) 245–248.
- [6] R. Erkinjuntti-Pekkanen, M. Reiman, J.I. Kokkarinen, H.O. Tukiainen, E.O. Terho, IgG antibodies, chronic bronchitis, and pulmonary function values in farmer's lung patients and matched controls, *Allergy* 54 (1999) 1181–1187.
- [7] D.P. Overy, J.C. Frisvad, Mycotoxin production and postharvest storage rot of ginger (*Zingiber officinale*) by *Penicillium brevicompactum*, *J. Food Prot.* 68 (2005) 607–609.
- [8] J. Hubert, V. Stejskal, Z. Munzbergova, A. Kubatova, M. Vanova, E. Zd'arkova, Mites and fungi in heavily infested stores in the Czech Republic, *J. Econ. Entomol.* 97 (2004) 2144–2153.
- [9] C.F. Kure, I. Skaar, J. Brendehaug, Mould contamination in production of semi-hard cheese, *Int. J. Food Microbiol.* 93 (2004) 41–49.
- [10] T. Rundberget, I. Skaar, A. Flaoyen, The presence of *Penicillium* and *Penicillium* mycotoxins in food wastes, *Int. J. Food Microbiol.* 90 (2004) 181–188.
- [11] D. Busso, R. Kim, S.H. Kim, Expression of soluble recombinant proteins in a cell-free system using a 96-well format, *J. Biochem. Biophys. Methods* 55 (2003) 233–240.
- [12] H. Vijay, M. Abebe, V. Kumar, M. DeVouge, T. Schrader, A. Thaker, P. Comtois, P.B. Escamilla-Garcia, Allergenic and mutagenic characterization of 14 *Penicillium* species, *Aerobiologia* 21 (2005) 95–103.
- [13] M.S. Sevinc, V. Kumar, M. Abebe, W.L. Casley, H.M. Vijay, Isolation and characterization of a cDNA clone encoding one IgE-binding fragment of *Penicillium brevicompactum*, *Int. Arch. Allergy Immunol.* 138 (2005) 12–20.
- [14] U.K. Laemmli, Cleavage of structural proteins during assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [15] P. Matsudaira, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes, *J. Biol. Chem.* 262 (1987) 10035–10039.

- [16] P. Kumarathasan, S. Mohottalage, P. Goegan, R. Vincent, An optimized protein in-gel digest method for reliable proteome characterization by MALDI-TOF-MS analysis, *Anal. Biochem.* 346 (2005) 85–89.
- [17] N. Sreerama, R.W. Woody, On the analysis of membrane protein circular dichroism spectra, *Protein Sci.* 13 (2004) 100–112.
- [18] N.N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set, *Anal. Biochem.* 287 (2000) 252–260.
- [19] L.J. McGuffin, K. Bryson, D.T. Jones, The PSIPRED protein structure prediction server, *Bioinformatics* 16 (2002) 404–405 (<http://bioinf.cs.ucl.ac.uk>).
- [20] T.P. Hoop, K.R. Woods, Prediction of protein antigenic determinants from amino acid sequences, *Proc. Natl. Acad. Sci. USA* 78 (1981) 3824–3828.
- [21] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, *J. Mol. Biol.* 157 (1982) 105–132.
- [22] N.Y. Su, C.J. Yu, H.D. Shen, F.M. Pan, L.P. Chow, Pen c 1, a novel enzymic allergen protein from *Penicillium citrinum*. Purification, characterization, cloning and expression, *Eur. J. Biochem.* 261 (1999) 115–123. Erratum in: *Eur. J. Biochem.* 261 (1999) 821.
- [23] L.P. Chow, N.Y. Su, C.J. Yu, B.L. Chiang, H.D. Shen, Identification and expression of Pen c 2, a novel allergen from *Penicillium citrinum*, *Biochem. J.* 341 (1999) 51–59.
- [24] H.D. Shen, C.W. Wang, H. Chou, W.L. Lin, M.F. Tam, M.H. Huang, M.L. Kuo, S.R. Wang, S.H. Han, Complementary DNA cloning and immunologic characterization of a new *Penicillium citrinum* allergen (Pen c 3), *J. Allergy Clin. Immunol.* 105 (2000) 827–833.
- [25] H.D. Shen, C.W. Wang, W.L. Lin, H.Y. Lai, M.F. Tam, H. Chou, S.R. Wang, S.H. Han, CDNA cloning and immunologic characterization of Pen o 18, the vacuolar serine protease major allergen of *Penicillium oxalicum*, *J. Lab. Clin. Med.* 137 (2001) 115–124.
- [26] H. Chou, H.Y. Lai, M.F. Tam, M.Y. Chou, S.R. Wang, S.H. Han, H.D. Shen, CDNA cloning, biological and immunological characterization of the alkaline serine protease major allergen from *Penicillium chrysogenum*, *Int. Arch. Allergy Immunol.* 127 (2002) 15–26.
- [27] H.D. Shen, H. Chou, M.F. Tam, C.Y. Chang, H.Y. Lai, S.R. Wang, Molecular and immunological characterization of Pen ch 18, the vacuolar serine protease major allergen of *Penicillium chrysogenum*, *Allergy* 58 (2003) 993–1002.
- [28] M. Tchorzewski, D. Krokowski, A. Boguszczyńska, A. Liljas, N. Grankowski, Structural characterization of yeast acidic ribosomal P proteins forming the P1A–P2B heterocomplex, *Biochemistry* 42 (2003) 3399–3408.
- [29] C. Johanne, C. Andre, J. Sibilia, A. Baquey, F. Oksman, M. San Marco, R.L. Humbel, M.F. Taillie, P. Chretien, A. Escande, J. Cohen, A. Chevailler, J.C. Monier, J. Goetz, Clinical significance of antiribosomal antibodies. Study Group on Autoimmunity (GEAI), *Rev. Med. Interne* 21 (2000) 510–516.
- [30] U. Appenzeller, C. Meyer, G. Menz, K. Blaser, R. Cramer, IgE-mediated reactions to autoantigens in allergic diseases, *Int. Arch. Allergy Immunol.* 118 (1999) 193–196.
- [31] E. Bonfa, S.J. Golombek, L.D. Kaufman, S. Skelly, H. Weissbach, N. Brot, K.B. Elkon, Association between lupus psychosis and anti-ribosomal P protein antibodies, *N. Engl. J. Med.* 317 (1987) 265–271.
- [32] M. Soto, J.M. Requena, M. Garcia, L.C. Gomez, I. Navarrete, C. Alonso, Genomic organization and expression of two independent gene arrays coding for two antigenic acidic ribosomal proteins of *Leishmania*, *J. Biol. Chem.* 268 (1993) 21835–21843.
- [33] E.A. Mesri, G. Levitus, M. Hontebeyrie-Joskowicz, G. Dighiero, M.H. Van Regenmortel, M.J. Levin, Major *Trypanosoma cruzi* antigenic determinant in Chagas' heart disease shares homology with the systemic lupus erythematosus ribosomal P protein epitope, *J. Clin. Microbiol.* 28 (1990) 1219–1224.
- [34] H.J. Ahn, S. Kim, H.W. Nam, Molecular cloning of ribosomal P protein in *Toxoplasma gondii* and the availability to detect antibody against recombinant protein in toxoplasmosis patients, *Korean J. Parasitol.* 41 (2003) 89–96.