

cDNA Cloning and Expression of Blo t 11, the *Blomia tropicalis* Allergen Homologous to Paramyosin

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Key Words

Blomia tropicalis · Paramyosin · Mite allergen · cDNA clone

Abstract

Blomia tropicalis is an important mite species in many parts of the world and the most predominant mite species in tropical countries. The prevalence of sensitization to this species has probably been underestimated because commercial extracts are largely unavailable. Identification and characterization of *B. tropicalis* allergens is an important step toward understanding the role of this species in allergic sensitization and could provide appropriate reagents for diagnostic and therapeutic procedures. This paper describes the isolation, sequence analysis, expression and allergenicity of a cDNA gene coding for a *B. tropicalis* allergen with homology to paramyosin, a high-molecular-weight allergen previously identified in *Dermatophagoides farinae*. The full-length Blo t 11 cDNA gene was isolated by cDNA library screening, 5'-rapid amplification of cDNA ends and long-distance PCR. Sequence analysis was performed with a combination of CLUSTAL W, CGC and BLAST program packages. The cDNA gene was expressed as a GST fusion protein in *Escherichia coli* and purified by affinity chromatography using the glutathione Sepharose column. Allergenicity

of the rBlo t 11 was tested by human IgE dot blot immunoassay. Blo t 11 is a 3,111-bp cDNA gene with a 2,625-bp open reading frame coding for an 875-amino acid protein, exhibiting significant homology with different invertebrate paramyosins. The human IgE dot blot immunoassay showed that the rBlo t 11 reacted positively to 52% (33/63) of sera from asthmatic patients. Blo t 11 is the homolog of Der f 11 exhibiting potentially important allergenic activity.

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Introduction

The role of house dust mites in the pathogenesis of asthma and atopic diseases has been well recognized [1]. Approximately 15% of the world population are afflicted and the most frequently implicated allergen source has been the house dust mites [2]. Dust mites are distributed worldwide but their prevalence varies from one region to the next [3]. *Blomia tropicalis* (Bt) is the most predominant mite species in tropical countries [4, 5].

Currently, crude mite extract preparation is the only available diagnostic and therapeutic reagent for mite allergy. However, due to the poor extraction method and the problem of protein degradation during extraction, the current extract for diagnosis fails to contain a complete

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profile of allergens, resulting in poor diagnosis and immunotherapy. Therefore, there is a great need for alternative diagnostic and therapeutic reagents that are of a high purity and specificity.

Over the past decade, the technique of molecular cloning had provided an avenue to develop pure and chemically well-defined allergens for both diagnosis and immunotherapy. However, despite the fact that more than 10 allergens had been cloned and characterized in *Dermatophagoides* spp. [6, 7], only 3 Bt allergens have been cloned, and their allergenicity established [8–11]. These Bt allergens include Blo t 5 [8, 9], Blo t 13 (previously known as Bt 6) [10] and Blo t 12 (previously known as Bt 11a) [11]. Thus far, the reported Bt allergens are low-molecular-weight proteins. Recent studies in other species of mites demonstrated the presence of high-molecular-weight allergens with significant IgE-binding properties from mite-sensitive patients [7, 12]. Der f 11 isolated from *Dermatophagoides farinae* is a 98-kD paramyosin allergen with a human IgE reactivity of 82% [13, 14] while Mag 3 (M-177) isolated from *Dermatophagoides pteronyssinus* is a 177-kD allergen with a human IgE reactivity of 70% [15].

We report in this paper the cDNA cloning, sequence analysis, expression and allergenicity of a high-molecular-weight Bt allergen, designated as Blo t 11, with a significant nucleotide and amino acid sequence homology to Der f 11 and other paramyosins from different invertebrates. The identification and characterization of Bt allergens is an important step toward understanding the role of this species in allergic sensitization. Likewise, recombinant allergens could provide appropriate reagents for diagnostic and therapeutic procedures in mite allergy.

Materials and Methods

Materials

The human sera (n = 63) used were obtained from asthmatic subjects with positive skin prick test (SPT) to Bt extract. The sera were stored at –80°C until use. The rDer f 11 was expressed as a GST fusion protein in *Escherichia coli* and purified as previously described [16]. The monoclonal antibody Df642 [13] was kindly provided by Dr. L.C. Tsai (Veterans General Hospital, Taipei, Taiwan). rBlo t 5-GST was purified from *E. coli* as previously described [8].

Bt mites were cultured in our laboratory following the methods described by Yi et al. [17]. In brief, Bt mites were cultured in powdered TetraMin fish flakes (Tetra Werke, Germany) for 3–4 weeks under natural environmental conditions with a mean annual temperature of 30°C and a mean relative humidity of 80%. The mites were harvested using a modified Tullgren apparatus. The Bt mite extract was prepared as previously described [17].

Bt cDNA Library Construction

Total RNA was isolated from live Bt cultured mites using commercial Trizol reagent (Gibco BRL, New York, USA). The Bt cDNA library was constructed according to standard protocol by inserting cDNA into the *Eco*RI and *Xho*I site of Uni-ZAP® expression vector (Stratagene, La Jolla, Calif., USA). The Bt cDNA library was titrated and amplified following the procedures in the Instruction Manual for Uni-ZAP®XR Library, Custom and Premaid Libraries (Stratagene).

Cloning of Full-Length Blo t 11 cDNA Gene

Screening of the Bt cDNA Library. Digoxigenin (DIG)-labeled Der f 11 gene fragments [14] were used as molecular probes in the Bt cDNA library screening process. Phages plated at 5–10,000 plaque-forming units were transferred onto Hybond-N+ Nylon transfer membranes (Amersham Pharmacia Biotech, UK) by the plaque lift method as described in the DIG System User's Guide for Filter Hybridization (Boehringer Mannheim, Germany) and detected by autoradiography. Positive plaque-forming units were further purified by a secondary and tertiary screening process prior to transformation into *E. coli* SOLR strain using the single-clone excision protocol described in the Uni-ZAP XR Library Instruction Manual (Stratagene).

5'-Rapid Amplification of cDNA Ends. The 5' end of Blo t 11 cDNA clone was isolated using the 5'-rapid amplification of cDNA ends (5'-RACE) following the protocol described in the SMART™ RACE cDNA Amplification Kit User Manual, protocol number PT3269-1 version PR93377 (Clontech Laboratories, Palo Alto, Calif., USA) with slight modifications. Briefly, first-strand cDNA synthesis was performed using 1 µg of total RNA isolated from Bt culture using the High Pure RNA Tissue Kit (Roche Diagnostics, Mannheim, Germany). A 50-µl PCR reaction mixture composed of 2.5 µl 5'-RACE-Ready cDNA, 5 µl of 10 × Universal Primer Mix, 1 µl of 10 µM Gene Specific Primer (5'-CTTCATCCTCCAATCGGTGT-3') and 41.5 µl of Master Mix (34.5 µl PCR grade water, 5 µl 10 × Advantage 2 PCR buffer, 1 µl 10 mM dNTP mix, and 1 µl 50 × Advantage 2 Polymerase mix). Amplification was performed by 25 cycles involving 94°C for 5 s, 65°C for 10 s and 72°C for 3 min using the PTC-10™ Programmable Thermal Controller (MJ Research, USA). Positive 5'-RACE products were resolved using agarose gel electrophoresis followed by purification using the GenElute Spin Columns (Supelco, USA). DNA samples were then precipitated by phenol/chloroform extraction and were analyzed by DNA sequencing using the ABI 377 DNA Sequencer (Applied Biosystems, USA).

Long-Distance PCR. The full-length Blo t 11 cDNA gene was isolated by long-distance PCR using gene-specific primers: 5'-GAC ATA TTC GAA TCA AAC TCT CGT CTA-3' and 5'-GAG GTG GTG ATA GTG GTG GTA AAC G-3'. Amplification was performed by 30 cycles using the PTC-100 Programmable Thermal Controller (MJ Research, USA). Each cycle involves 94°C for 30 s, 55°C for 30 s and 72°C for 3 min. The PCR products were purified as described above and were finally analyzed by DNA sequencing. The full-length Blo t 11 cDNA gene was ligated into pCR2.1-TOPO cloning vector and was subsequently transformed into TOP10 One Shot™ competent cells using the TOPO™ TA Cloning version E (Invitrogen, Calif., USA). Plasmid DNA purified by Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, Wisc., USA) was analyzed by DNA sequencing using the ABI 377 DNA Sequencer (Applied Biosystems, USA).

DNA Sequence Analysis

The Blo t 11 nucleotide sequence was compared and aligned with the Der f 11 nucleotide sequence using the CLUSTAL W version 1.6 multiple sequence alignment software (<http://www.clustalw.genome.ad.jp>). The reverse and complement sequences were performed using the GCG sequence analysis package. The deduced amino acid sequence and amino acid composition of the Blo t 11 gene was determined using the Translate Tool software and the ProtParam program found in the ExPASy Molecular Biology Server (<http://www.expasy.ch>). The deduced nucleotide sequence was submitted to the databases of the National Center for Biotechnology Information (NCBI), using the BLAST network server for nucleotide and amino acid homology searches. The secondary structure of Blo t 11 was analyzed using the PredictProtein Server (<http://cubic.bioc.columbia.edu/predictprotein>).

Protein Expression and Purification of Blo t 11 in *E. coli*

The full-length Blo t 11 cDNA gene was amplified by PCR using primers 5'-CGG GAT CCA TGG CGG CTC GAT CAG CA-3' and 3'-CGC TCG AGT TAA AAG TTC GAG CTC TCT TG-5', creating *Bam*HI and *Xho*I restriction sites, respectively. Amplification was performed for 30 cycles involving 94°C for 15 s, 55°C for 30 s and 72°C for 1 min using the PTC-100 Programmable Thermal controller (MJ Research). The gel-purified PCR product was digested with *Bam*HI and *Xho*I and was ligated into pGEX-4T-1 vector (Pharmacia Biotech, UK). The rBlo t 11 was expressed as a glutathione S-transferase (GST) fusion protein in *E. coli* (BL21 strain) by isopropyl- β -D-thiogalactopyranoside induction as described previously [16]. Purification of rBlo t 11 was performed by affinity chromatography using the Glutathione Sepharose Column (Sigma, USA) according to the protocol described in the GST Purification Module (Pharmacia Biotech, UK). Purified protein samples were analyzed by SDS-PAGE according to the method of Laemmli [18] and by Western blot analysis as previously described [17]. The purified protein was quantified by DC Protein Assay (BioRad, USA).

Human IgE Immunoassay

The allergenicity of the recombinant Blo t 11 was determined in vitro by dot blot immunoassay using a panel of sera from mite-sensitive subjects following the protocol previously described [14]. In brief, 2 μ g of rBlo t 11 was applied onto Hybond C nitrocellulose membrane (Amersham Life Sciences, UK) in duplicates using a Bio-Dot apparatus (BioRad, USA). Five micrograms of crude Bt extract and 2 μ g of rBlo t 5 were used as positive controls while 2 μ g of purified GST was used as negative control. Dot blots were then blocked with 5% nonfat milk powder in BPST (PBS with 0.05% Tween 20). Allergic sera were diluted 5 $\times$ in PBST and incubated with the dot blots for 12–16 h at 4°C prior to incubation with biotinylated anti-human IgE, 2 μ g/ml (Pharmingen, San Diego, Calif., USA) for 1 h. The membranes were then incubated with 5,000 $\times$ dilution of peroxidase and alkaline phosphatase-conjugated ExtrAvidin (Sigma, USA) for 1 h. The signal was detected using the ECL + PLUS detection system (Amersham Pharmacia Biotech, UK) followed by autoradiography. The color reaction was likewise performed by incubation of membranes for 20–30 min in 10 ml of 1 $\times$ AP Buffer containing 100 μ l each of AP color reagent A and reagent B (BioRad, USA).

Results

Full-Length Blo t 11 cDNA Gene

Screening of approximately 6×10^4 plaque-forming units from the Bt cDNA library resulted in 36 positive plaques using DIG-labeled Der f 11 cDNA fragments as probes. Four of these plaques were purified to homogeneity by a secondary and tertiary screening process. Nucleotide sequence analysis revealed that the 4 clones are related with a different degree of truncations on the 5' end. The nucleotide sequence of the 4 clones showed polyadenylation sequences. Sequence alignment revealed that the 4 clones exhibited significant homology to Der f 11 nucleotide sequence. Out of the 4 clones, clone 33 exhibited the longest size comprising of 2,598 bp with a polyadenylation sequence from position 2581 to 2598 (data not shown). Several rounds of the cDNA library screening process failed to isolate the full-length Blo t 11 gene. The 5'-RACE was then used to isolate the 5' end of the Blo t 11 cDNA gene using a gene-specific primer based on the nucleotide sequence of the truncated clone 33. The 5'-RACE product and the 5'-truncated clone 33 exhibit a 330-bp overlap.

The full-length Blo t 11 cDNA gene was isolated by a long-distance PCR using gene-specific primers designed based on the sequence of the truncated clone 33 and 5'-RACE product. The full-length Blo t 11 is composed of 3,111 nucleotide bp with a 2,625-bp open reading frame from position 326 to 2950. An ATG (M) initiation codon is found at position 326–328 while a TAA (stop) codon is found at position 2951–2953. The full-length sequence shows untranslated regions of 325 bp on the 5' end and a 158-bp on the 3' end (fig. 1). The gene codes for the 875-amino acid protein with a predicted molecular weight of 102,027.9 daltons.

Blo t 11 Codes for Bt Paramyosin

The amino acid sequence alignment shows a significant homology of the Blo t 11 to Der f 11 and other invertebrate paramyosins. The Blo t 11 amino acid sequence shows 90% sequence homology with the *D. farinae* paramyosin, Der f 11 [14] as shown in figure 2; however, they only share 78% nucleotide sequence homology. Moreover, the Blo t 11 amino acid sequence exhibited 58% nucleotide sequence homology. Moreover, the Blo t 11 amino acid sequence exhibited 58% homology with the *Drosophila melanogaster* paramyosin [19], 50% homology with *Onchocerca volvulus* [20] and *Dirofilaria immitis* [21] paramyosins, 49% homology with *Caenorhabditis elegans* paramyosin [22], 32% homology with *Taenia*

0001	g	aca	tat	tgc	aat	caa	act	ctc	gtc	tag	tga	gtt	gtg	cac	tgc	tgc	tgc	agt	att	cga	ccg	aac	aac	aag	gtg	tcc	caa	tac	ata	cac	aca	ctc	acc	aca	cat	att					
0113	cga	cct	gtc	cca	agt	ggc	gta	cac	gtt	att	cgc	aag	tct	ctc	aca	tca	tct	tca	cca	gaa	gca	gag	tag	tag	acg	atc	caa	gaa	atc	agt	ctc	atc	cta	ctt	cta	aag	cat				
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0557	gaa	ggg	tgc	agc	gaa	agc	gtt	acc	gag	atg	aac	aag	aaa	cga	gac	tcg	gaa	ttg	gct	agt	ctt	cga	aag	ttg	ctc	gaa	gat	gtc	cat	atg	gaa	agc	gag	act	gct	cac					
0668	e	g	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s					
0779	h	l	r	q	k	h	q	a	a	i	q	e	m	q	d	q	d	q	l	d	q	v	q	k	a	k	n	k	s	d	*	k	e	*	k	q	k	f	q	a	e
0890	gaa	atc	aat	cga	act	gtt	gtt	gag	gtt	acc	gct	cac	cga	caa	cga	ttg	tcg	caa	gag	aat	tcg	gaa	ttg	atc	aag	gaa	ggt	cat	gaa	tac	aag	att	tca	ttg	gac	aat	gct				
1001	aac	cac	ttg	aag	gga	caa	att	gca	caa	aaa	ttg	gag	cac	acc	cga	cac	cga	ttg	gag	gat	gaa	gaa	cga	aaa	cga	tca	agc	ttg	gag	aac	cac	gcc	cac	aca	ttg	gaa	gtt				
1112	gaa	ttg	gaa	tca	ttg	aag	gta	caa	ttg	gaa	gag	gaa	tct	gag	gcc	cga	tta	gaa	ttg	gaa	cga	cga	caa	ttg	atc	aaa	gct	aat	ggc	gat	gct	ctc	tga	ttg	aaa	tcc	aaa	tac			
1223	e	a	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e	e					
1334	gca	ttg	gaa	aag	caa	aag	gct	cga	ctc	caa	tcg	gag	gta	gaa	gtt	ttg	atc	atg	gac	ttg	gaa	aag	gct	acc	gca	cat	gcc	cag	gca	ttg	gaa	aaa	cgc	gtt	tcc	caa	ttg				
1445	gag	aag	atc	aac	ctc	gac	ctc	aag	agc	aag	ttg	gag	gag	gtc	agc	atg	ttg	ttg	gaa	caa	cat	caa	aat	gaa	gat	ctc	cgt	gtc	aag	att	gca	gac	ttg	caa	ttg	caa	cat				
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1667	cat	gaa	caa	gag	atc	gat	atc	aaa	cga	ttg	gag	aac	gag	cga	gaa	gaa	ttg	gct	gcc	cca	tac	aag	gaa	gct	gaa	acc	ttg	cga	aaa	caa	gaa	gag	gca	aag	aat	caa	cga				
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1889	aac	atg	cga	ttg	gct	gag	gct	gaa	gct	aaa	ttg	aag	acc	gag	gtt	gca	cga	ctc	aag	aag	aag	tac	caa	cga	caa	atc	act	gaa	ttg	gaa	ttg	tcg	ttg	gat	gcc	gct	aat				
2000	aag	gct	aac	atc	gac	ttg	cag	aag	acg	atc	aag	aag	caa	gct	ctc	caa	atc	act	ggg	ctt	caa	gcc	cat	tat	gat	gat	gta	cat	cgt	caa	ttg	caa	gaa	gct	gtt	gat	caa				
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2222	gag	gca	gtt	gct	cga	gtc	aac	gaa	ctt	aca	acc	aat	gtc	aac	ctc	gct	tcg	gcc	aag	agc	aaa	ttg	gaa	act	gaa	tac	ttc	tct	gct	ctt	caa	aac	gat	tac	gat	gaa	gta				
2333	cac	aag	gaa	ctc	cga	atc	tct	gaa	cga	gtg	caa	aaa	ctt	acc	att	gag	gtc	aag	tcg	aaa	gag	acc	ttg	ctc	gaa	tca	caa	acg	gaa	cgt	gtc	aca	aat	ttg	gaa	aca	aca				
2444	atc	aag	aaa	tca	ttg	gaa	acc	gaa	gta	cga	aat	ctc	caa	atc	cga	att	gaa	gaa	gct	gag	gct	aat	gca	ttg	gct	ggt	ggt	ggt	ggt	ggt	ggt	ggt	ggt	ggt	ggt	ggt	ggt				
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2777	ttg	acc	cgt	gtc	cga	ctc	caa	cga	gag	ttg	gaa	gcc	gaa	cga	gct	gat	caa	gct	gat	caa	gct	gat	caa	gct	gat	caa	gct	gat	caa	gct	gat	caa	gct	gat	caa	gct	gat	caa			
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2999	aat	ttg	aaa	ttc	aaa	cca	aac	tat	gat	ata	aat	cta	ata	aaa	aag	ttt	taa	aaa	aat	cac	aac	ctc	tta	aac	aca	cct	cga	tta	gag	ttt	acc	acc	act	atc	acc	acc					
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Blot 11	MAARSAKYMYQSSRAGHGGDISIEYGTDLGALTRLEDKIRLLSLEDSELERLRQRVEREKSDITVQLMNLTERLEETEGSSSESVTEMNKKRDSLEAKLRKLLLEDVHMESEETAHHLRQKH	120
Derf 11	MNKKRDSLEAKLRKLLLEDVHIESEETAHHLRQKH *****	(034)
Blot 11	QAAIQEMQDQLDQVOKAKNKSDEKEKQFOAEVFELLAQVETANKDKLVAQKTVEKLEYTHVHNLKIEINRTVVEVTAHRQLRSQENSELIEKVEHYKISLDNANHKGQIAQOQLEDTR	240
Derf 11	QAAIQEMQDQLDQVOKAKNKSDEKEKQFOAEVFELLAQVETANKDKLVAQKTVEKLEYTHVHNLKIEINRTVVEVTAHRQLRSQENSELIEKVEHYKISLDNANHKGQIAQOQLEDTR *****	(154)
Blot 11	HRLEDEERKRSSLENHAHTLEVELESLKVLQEESEARLELERQLTKANGDAASWKSXYEALQAHVDEVEELRRKMAQKISEYGEQLEALLNKCSALEKQKARLQSEVEVLIMDEKAT	360
Derf 11	HRLEDEERKRSSLENHAHTLEVELESLKVLQEESEARLELERQLTKANGDAASWKSXYEALQAHVDEVEELRRKMAQKISEYGEQLEALLNKCSALEKQKARLQSEVEVLIMDEKAT *****	(274)
Blot 11	AHAQALEKRVSQLEKINLDLKSLEEVSMLEQTKDLRVKADLQKLQHEYEKLDRQKEALARENKKLADDLAEAKSOLNDAHRRHIEQEIEIKRLNENRELEAAAYKEAETLRKQEEA	480
Derf 11	RHAQOLEKRVSQLEKINLDLKNKLEEVSMLEQTKDLRVKADLQKLQHEYEKLDRQKEALARENKKLADDLAEAKSOLNDAHRRHIEQEIEIKRLNENRELEAAAYKEAETLRKQEEA *****	(394)
Blot 11	KNQRLTAELAQTRHDYKRLAQKEEIEALRKQYQIEIEQLNMRLAEAEAKLKEVEARLKKKYQAQITELSLDAANKANIDLQKTIKKQALQITGLQAHYDEVHRQLQVADQLGVTO	600
Derf 11	KNQRLTAELAQTRHDYKRLAQKEEIEALRKQYQIEIEQLNMRLAEAEAKLKEVEARLKKKYQAQITELSLDAANKANIDLQKTIKKQALQITGLQAHYDEVHRQLQVADQLGVTO *****	(514)
Blot 11	RRCQALTAELAEEMRVNLEQALRAKRAAEQMHEEAVVRVNLTTINVNLSAKSKLETFESALQNDYDEVHKLRIISDERVQKLTIEVKSTDKLSETERVTKLETIKKSLETEVRNLQI	720
Derf 11	RRCQALTAELAEEMRVNLEQALRAKRAAEQMHEEAVVRVNLTTINVNLSAKSKLETFESALQNDYDEVHKLRIISDERVQKLTIEVKSTDKLSETERVTKLETIKKSLETEVRNLQI *****	(634)
Blot 11	RIEEVEANALAGGKRVIKLESIRIDVEIEVEEERRRHAETEMKLRKKDHRVKELLQNEEDHKQIQLQEMSDKLNKVKVYKRMQEQEGMSQQNLTRVRRFQRELAEDRADQAES	840
Derf 11	RIEEVEANALAGGKRVIKLESIRIDVEIEVEEERRRHAETEMKLRKKDHRVKELLQNEEDHKQIQLQEMSDKLNKVKVYKRMQEQEGMSQQNLTRVRRFQRELAEDRADQAES *****	(692)
Blot 11	NLSFIRAKHRSSWVTSQVPGGTQRQVFVTEQESSNF	875

2

Fig. 1. Nucleotide and deduced amino acid sequence of the full-length Blo t 11. The numbers label the positions of the nucleotides (left margin) and amino acids (right margin). The predicted N-linked glycosylation sites are underlined while the predicted leucine zipper motifs are shown in bold italicized letters. The predicted phosphorylation sites (*), N-meristoylation sites (') and amidation site (+) are indicated.

Fig. 2. Amino acid sequence alignment of Blo t 11 and Der f 11. The numbers indicate the amino acid positions of Blo t 11 and Der f 11 (in parentheses). Identical (*), conserved (-) and semiconserved (-) residues are indicated. The full-length Blo t 11 is longer by 86 and 97 amino acids on the N- and C-terminus, respectively, as compared to the truncated Der f 11.

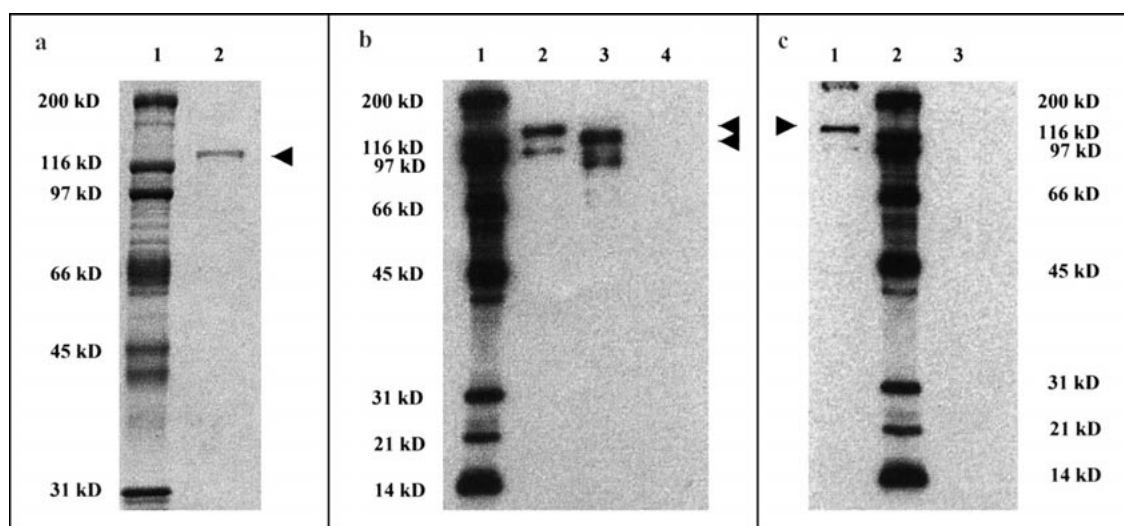


Fig. 3. SDS-PAGE (**a**) and Western blot analysis (**b**, **c**) of rBlo t 11. The rBlo t 11-GST (1 μ g) fusion protein was estimated as about 128 kD in a 10% Tris-glycine gel (**a**, lane 2). Western blot analysis with mAb Df642 (**b**) shows reactivity with rBlo t 11-GST (0.4 μ g, lane 2) and the positive control, rDer f 11-GST (0.4 μ g, lane 3), but not the negative control, GST (0.4 μ g, lane 4). **c** Western blot analysis of rBlo t 11-GST (lane 1) reacted with human IgE from an individual with positive SPT to Bt crude extract. Negative reaction was observed with GST (**c**, lane 3). Lane 1 (**a**, **b**) and lane 2 (**c**) are broad-range molecular weight markers (BioRad, USA). Protein samples used in the Western blot analysis were resolved in 12% Tris-glycine gel.

soleum paramyosin [23], and 31% homology with *Schistosoma mansoni* paramyosin [24].

Comparison of the amino acid composition along the region of homology between Blo t 11 (positions 87–778) and Der f 11 reveals a nearly identical percentage of amino acid composition (fig. 2). Slight differences were observed in the percentage of two uncharged amino acids glutamine (8.2 vs. 9.0%) and threonine (4.3 vs. 3.9%), and three apolar amino acids alanine (10.0 vs. 9.5%), leucine (12.9 vs. 13.7%) and valine (5.6 vs. 4.9%) (data not shown). Blo t 11 protein exhibits a single proline residue at position 859, 16 amino acids away from the C terminus (fig. 1, 2). A proline residue is reported to destabilize the coiled coil alpha helix structure of paramyosin [22]. Blo t 11 and Der f 11, likewise, exhibited the same percentage of total number of negatively charged residues ($\approx 20\%$) and the total number of positively charged residues ($\approx 17\%$).

Secondary structure prediction using the PHD program at the PredictProtein Server revealed that the Blo t 11 protein exhibits an all-alpha helix secondary structure with 95.2% in a helix form, 0.2% extended sheets and 4.6% loops. There are five predicted N-glycosylation sites present at positions 69–71, 139–141, 191–194, 817–819 and 841–843 (fig. 1). The sequence also exhibits four predicted leucine zipper pattern motifs at positions 331–352,

384–405, 408–429 and 641–662 (fig. 1). These motifs may play an important role in the folding of the protein rather than as binding sites for DNA-binding proteins. Interestingly, the sequence contains putative motifs for cAMP- and cGMP-dependent protein kinase phosphorylation, protein kinase C phosphorylation, casein kinase II phosphorylation, tyrosine kinase phosphorylation, N-myristoylation, and amidation as predicted using the PHD secondary structure prediction program (fig. 1).

In line with the predicted all-alpha helix secondary structure of Blo t 11, the amino acid sequence can be optimally arranged into the 7/28 repeat pattern characteristic of paramyosins and other alpha-helical coiled coil proteins [19–24]. As observed in other paramyosin structures, the Blo t 11 amino acid sequence exhibits a nonhelical amino and carboxyl terminus. There are 20 and 31 residues on the N-terminus and C-terminus, respectively, that do not follow the heptad structure. These nonhelical regions are potential sites of phosphorylation as observed in *C. elegans* paramyosin [25].

rBlo t 11 in E. coli

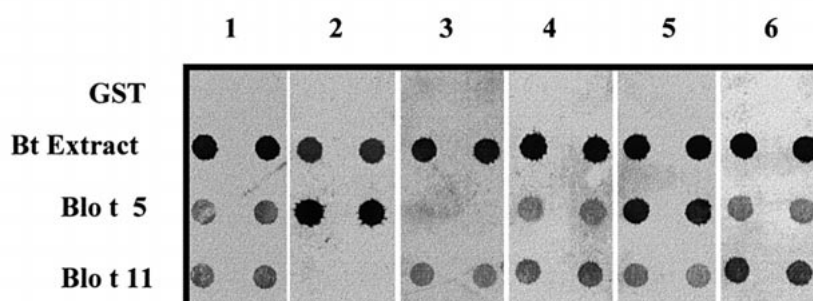
The rBlo t 11 was expressed as a GST fusion protein with an estimated molecular weight of about 128 kD on an SDS-PAGE gel (fig. 3a, lane 2). Affinity-purified rBlo t

a

Serum Number	Antigen		Serum Number	Antigen		Serum Number	Antigen	
	Bt Extract	rBlo t 11		Bt Extract	rBlo t 11		Bt Extract	rBlo t 11
1	+	-	22	+	-	43	+	-
2	+	+	23	+	-	44	+	-
3	+	+	24	+	+	45	+	-
4	+	-	25	+	-	46	+	-
5	+	-	26	+	+	47	+	-
6	+	+	27	+	+	48	+	-
7	+	+	28	+	+	49	+	-
8	+	+	29	+	-	50	+	-
9	+	+	30	+	+	51	+	-
10	+	+	31	+	+	52	+	-
11	+	+	32	+	-	53	+	-
12	+	+	33	+	+	54	+	-
13	+	+	34	+	+	55	+	-
14	+	+	35	+	+	56	+	+
15	+	+	36	+	-	57	+	+
16	+	+	37	+	-	58	+	-
17	+	-	38	+	+	59	+	-
18	+	+	39	+	-	60	+	+
19	+	+	40	+	-	61	+	+
20	+	+	41	+	-	62	+	+
21	+	+	42	+	-	63	+	+
Total number of Bt extract positive sera: 63								
Total number of rBlo t 11 positive sera: 33								

b

Fig. 4. Allergenicity of rBlo t 11 by dot blot immunoassay. **a** Human IgE reactivity of the rBlo t 11 protein with 63 Bt extract-positive sera is summarized. Positive (+) and negative (-) reactions are shown. **b** Six representative panels of the IgE dot blot immunoassay are shown. Purified rBlo t 11, rBlo t 5 and GST were used at 2 µg/dot and Bt extract was used at 5 µg/dot.



11-GST protein reacted strongly with the monoclonal antibody against Der f 11, Df642, as shown in the results of the Western blot analysis (fig. 3b, lane 2). The rBlo t 11 likewise reacted with human IgE from an individual with positive SPT to Bt crude extract (fig. 3c, lane 1).

IgE Reactivity of rBlo t 11

A panel of 63 sera with positive IgE reactivity with the crude Bt mite extract was used to assess the allergenicity of the rBlo t 11. Results of dot blot immunoassay showed 52% (33/63) positive reaction with human IgE from the panel of sera tested. Figure 4a summarizes the results of the IgE dot blot immunoassay for 63 Bt extract-positive sera. The GST protein was used as a negative control, while the Bt mite crude extract and rBlo t 5 were included as positive controls (data not shown). All sera presented registered a negative reactivity with GST. Figure 4b shows six representative samples of the dot blot immunoassay showing different intensities of reactivity to the different antigens used.

Discussion

Der f 11, a 98-kD protein with significant homology with invertebrate paramyosins, was the first high-molecular-weight allergen reported in house dust mites [13]. The reported Der f 11, however, is a truncated clone on both the amino and carboxyl ends [16]. In this study, we report for the first time a full-length cDNA clone, designated as Blo t 11, with 78% nucleotide and 90% amino acid sequence homology with the truncated Der f 11 sequence. Such observation indicates that there is a significant difference in the codon usage between these two mite species. The high sequence homology between Blo t 11 and Der f 11 is supported by the analysis of amino acid composition of the two allergens. Blo t 11 and Der f 11 exhibit a nearly identical percentage of amino acid composition. These data indicate that the isolated Blo t 11 cDNA gene is the homolog of Der f 11 gene.

The present study reports for the first time the cDNA sequence coding for a high-molecular-weight allergen in the mite species *Blomia tropicalis*. Thus far, the three reported Bt allergens are low-molecular-weight proteins with sizes ranging from 14 to 15 kD [8–11]. Blo t 5, the first Bt allergen to be cloned with 40% amino acid homology to Der p 5, is a major allergen isolated from Bt with 70% IgE reactivity to mite-allergic sera [8, 9]. Blo t 13 is a 14.8-kD allergen with an IgE reactivity to mite allergic sera of only 11% [10]. On the other hand, Blo t 12 is a 14.206-kD allergen with 50% specific IgE reactivity [11]. The presence of high-molecular-weight allergens in Bt was first observed in the results of crossed radioimmuno-electrophoresis studies [26] and immunoblotting experiments [27] using the whole Bt extract, which revealed the presence of multiple species-specific IgE-binding antigens with molecular weights ranging from 11 to 85 kD.

Blo t 11 amino acid sequence aligns significantly with the known invertebrate paramyosins [19–24] supporting the previously proposed idea that paramyosin is a highly conserved protein among the invertebrates. Its evolution is constrained by its function as a rod-like structure found on the core of thick filaments of invertebrate muscles and interacts with myosin [21]. The alpha helical structure of paramyosin with mostly apolar amino acids at positions d and a of the heptad is the main factor involved in the formation of paramyosin dimers [21]. Interestingly, no helix-destabilizing prolines are observed in the alpha-helical region of Blo t 11 indicating that the isolated Blo t 11 cDNA conforms with an uninterrupted alpha helical structure as observed in all paramyosin structures reported to date [19–24]. The only proline residue in Blo t 11 sequence is found on the nonhelical C terminus. Like other paramyosins, Blo t 11 contains putative motifs with regulatory roles such as leucine zipper patterns and phosphorylation sites. The presence of these motifs suggested that the mite paramyosin may exhibit significant regulatory functions relating to physiological or immunological processes. It has been reported that invertebrate paramyosins have the ability to inhibit the function of complement C1 in human serum [28]. Likewise, paramyosin has been reported to be localized in a nonfilamentous membrane-bound form in schistosomal tegument [29].

The conserved structure of paramyosin across invertebrate species is evidenced by its cross-reactive antigenicity [30]. It has been reported that people infected with *S. mansoni* and *O. volvulus* produced cross-reactive IgE antibodies to paramyosin [31]. In this study, the monoclonal antibody Df642 was able to recognize the recombinant Blo t 11 (fig. 3) indicating a potential cross-reactivity

between the two allergens. This observation, however, needs further validation by performing cross-inhibition studies between the two dust mite paramyosin allergens.

Initial characterization of the recombinant Blo t 11 by dot-blot immunoassay (fig. 4) shows that the allergen has a high frequency of IgE reactivity with sera from asthmatic patients. In most of the dot blots observed, there is an apparent weak signal among the Blo t 11-positive sera as compared to the Blo t 5. This could be explained by a potential masking effect on the IgE epitope as a result of the improper folding of the protein expressed in *E. coli*. As noted by Tsai et al. [14], several smaller fragments of the Der f 11 are more allergenic than the large Der f 11 fragment, suggesting that some internal IgE epitopes may have been masked by improper folding of the larger recombinant peptides. The observed weak signal exhibited by the rBlo t 11 could also be explained as a result of a dilution effect of the specific IgE present in the serum with the use of 5 × dilution of the sample sera tested. The titer of IgE specific to a particular antigen varies from one sample to the next and the intensity of reactivity could be increased by lower dilution (unpubl. data). The isolation of the native Blo t 11 and IgE epitope mapping using recombinant overlapping fragments of Blo t 11 continues in our laboratory, and it is hoped that this contributes to establishing the actual IgE reactivity of the allergen.

Interestingly, several sera tested show a positive reactivity to Blo t 11 but not to the major allergen Blo t 5. Likewise, some sera showed reactivity to Blo t 5 but not to Blo t 11. This observation suggests that the apparent high reactivity of Blo t 11 to the sera tested represents a high incidence of sensitization to the mite paramyosin comparable to that of Blo t 5 although a larger panel of sera is needed to confirm the observed results. Further studies with a larger panel of sera and comparative studies with rBlo t 11 and native Blo t 11 protein will help to further elucidate this observation. The identification of a high-molecular-weight Blo t 11 exhibiting potentially important allergenic activity confirms the previous results of studies showing the presence of high-molecular-weight antigens in the whole Bt mite extract.

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