

Cloning and expression of Blo t 1, a novel allergen from the dust mite *Blomia tropicalis*, homologous to cysteine proteases

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Summary

Background House dust mite allergens have been shown to be a very important stimulus in the causation of asthma and triggers for the exacerbation of symptoms. Therefore, characterization of mite-derived allergens at the molecular level is an important step for the development of effective diagnostic and therapeutic approaches, as well as for epidemiological studies.

Objective To clone, express and characterize at the molecular level the cysteine protease from *Blomia tropicalis* (Bt).

Methods A full-length cDNA encoding Blo t 1 was cloned from a Bt cDNA library using a PCR and RACE-based strategy. The cDNA was PCR-amplified, sequenced and subcloned into a prokaryotic expression vector. The allergenicity of the recombinant Blo t 1 was evaluated for IgE reactivity by Western blot.

Results Blo t 1 cDNA encodes a 221 amino acids polypeptide with an estimated molecular weight of 25 kDa. The recombinant protein is 35% identical to other mite cysteine proteases. Recombinant Blo t 1 (rBlo t 1) bound IgE from 62% of Bt skin test-positive serum. *Dermatophagoides pteronyssinus* (Dp) skin test-positive sera did not react with rBlo t 1, indicating the possible presence of unique IgE epitopes on the rBlo t 1 molecule. A three-dimensional image of Blo t 1, constructed based on predicted analysis, showed conserved secondary and tertiary structure with other cysteine proteases.

Conclusion We report the cloning, expression and IgE reactivity of Blo t 1, a novel allergen from the domestic mite *Blomia tropicalis* (Bt), highly homologous to cysteine proteases. This putative cysteine protease, designated Blo t 1, may play a major role as an immunodominant allergen involved in dust mite-specific IgE-mediated hypersensitivity.

Keywords allergen, Blo t 1, *Blomia tropicalis*, cysteine protease, house dust mite

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Introduction

Since the establishment of *Dermatophagoides pteronyssinus* (Dp) and *D. farinae* (Df) as a main source of house dust allergens, immunochemical, physicochemical and molecular studies have identified multiple allergens in these species [1–5]. Of these allergens, cysteine proteases or group 1 allergens have long been recognized as immunodominant allergens involved in dust mite-specific IgE-mediated hypersensitivity [6]. It has been proposed that proteolytic activity is the major contributor of the hypersensitivity associated with group 1 dust mite allergens such as Der p 1 [7]. Der p 1 causes disruption of intercellular tight junctions (TJs), which are the principal components of the epithelial paracellular permeability barrier [8]. This suggests that opening of TJs by environmental proteinases may be the initial step in the development of allergic asthma. Other studies have established that Der p 1 selectively cleaves human CD25,

the alpha subunit of the T cell IL-2 receptor [9]. As a result of this cleavage, Th1 cells show diminished proliferation and IFN- γ secretion, leading to an augmentation of the Th2 subset and, presumably, the development of allergy.

It is expected that the design of better diagnostic and therapeutic strategies for allergic asthma will be advanced by efforts to clone and characterize the major dust mite allergens, including those of *Blomia tropicalis* (Bt). The important role of Bt as a trigger of allergic asthma and allergic rhinitis has been previously documented [10–13]. Although many studies have been performed to identify and characterize the allergens from Df and Dp, few studies have been applied to defining the spectrum and relative clinical importance of Bt allergens. It has been reported that Bt has 21 different IgE binding antigens detected by crossed-radioimmunoelectrophoresis. Most of these allergens are species-specific [14] or have relatively low cross-reactivity with *Dermatophagoides* species allergens [15]. Other studies have demonstrated that Bt has one major allergen (11–13 kDa), and three important allergens (64 kDa, 36 kDa, 33 kDa) with about 51% IgE reactivity [16].

Defining the relevance of Bt in allergic respiratory diseases has important clinical and epidemiological implications, especially

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due to their high prevalence in tropical and subtropical areas of the world. As an important first step in the characterization of the allergenic components of Bt and their individual roles in the IgE-mediated immune response to this mite, we report the cloning and expression of Blo t 1, a new allergen from the house dust mite Bt, highly homologous to cysteine proteases.

Materials and methods

Blomia tropicalis culture

Bt mites were grown in 8-inch diameter culture dishes on Tetra-Fin fish food (TetraWerke, Blacksburg, VA, USA). After 28 days, live cultures containing mites, fecal particles and culture media were placed on a funnel with 150 size mesh and exposed to a 40-watt light bulb for 4 h. The live mites, which dropped through the mesh, were collected in 50 mL conical centrifuge tubes, immediately placed on a 1-inch deep and 12-inch diameter brass sieve pan (Dual Manufacturing Co., Chicago, IL, USA) and evenly distributed to form a uniform layer. Mites were again exposed to the 40-watt light for 15 min and after this period the brass sieve pan was turned face down and gently tapped so that food particles and dead mites were eliminated. The live mites, which had adhered to the brass sieve pan, were then used for poly A⁺ mRNA isolation.

Serum samples

Sera from 27 patients with positive skin reactions by the skin prick test (> 3 mm weal-erythema) to Bt and Dp extracts were used to establish the IgE-binding frequency of the recombinant protein. Negative control sera ($n=12$) were obtained from subjects from Michigan, USA, who are not likely to be exposed to Bt (kindly provided by Dr Robert Bull, Laboratory of Human Genetics, Michigan State University). Low level (< 2 IU/mL) IgE reactive sera characterized by fluorescent allergosorbent test (FAST) were also used as controls ($n=7$; available through Dr F. Montealegre, Laboratory of Immunochimistry, Ponce School of Medicine)

Construction of the Bt cDNA library

Bt poly A⁺ mRNA (21.5 µg/mL) was isolated from 300 mg of live mites using the QIAGEN Oligotex Direct mRNA Midi/Maxi Kit according to the manufacturer's specifications (QIAGEN Inc, Valencia, CA, USA). The integrity and size distribution of purified poly A⁺ mRNA was verified by formaldehyde-agarose gel electrophoresis (1.2%) and ethidium bromide staining. cDNA was synthesized from 1 µg of mRNA using the Clontech Marathon cDNA Amplification Kit according to the manufacturer's protocol (Clontech Laboratory Inc., Palo Alto, CA, USA).

Polymerase chain reaction (PCR)-based Blo t 1 cloning strategy

Gene-specific primers derived from highly conserved amino acids regions from the cysteine protease active site sequences of Dp, Df and *Euroglyphus maynei* (Em) were designed. The 5' primer corresponded to N-terminal amino acid region QGGCGSCW (5'-CAAGGAGGCTGTGGTTCATGTTGG-3') and the 3' primer was specific for the C-terminal amino acid region WIVRNSWD (5'-ATCCCAACTGGTTCGTACGATCCA-3'). 'Hot Start' PCR amplification using the Bt

cDNA library as template DNA was performed with the Taq PCR Core Kit and HotStart Taq DNA polymerase (QIAGEN Inc.). Final concentrations per reaction (50 µL final volume) were as follows: 10 pmol of each primer, 200 µM of each dNTP, 1 × PCR buffer including 1.5 mM MgCl₂, and 2.5 units of Taq DNA Polymerase. PCR was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) using an initial incubation of 15 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C. After a final incubation of 10 min at 72 °C, amplicons were analysed by agarose electrophoresis, cloned and sequenced.

Cloning and DNA sequencing

PCR-amplified DNA was purified using the QIAquick PCR Purification Kit (QIAGEN Inc.) according to the manufacturer's specifications and cloned into TOPO TA pCR 2.1 plasmid (Invitrogen, Carlsbad, CA, USA). Recombinant plasmids were transformed into *E. coli* XL1 Blue cells (Stratagene, La Jolla, CA, USA) and identified by blue/white screening on Luria-Bertani (LB) plates containing 100 µg/mL ampicillin, 2% 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal) and 10 mM isopropylthio-beta-D-galactoside (IPTG). Plasmids were purified using the Plasmid Mini-Prep Kit following the manufacturer's protocol (QIAGEN Inc.). The presence of insert DNA was confirmed by *Eco*RI restriction analysis and sequencing. Automated cycle sequencing was performed in both directions using the Cy5 AutoCycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) with the M13-40 and M13 reverse primer on an ALFExpress sequencer (Amersham Pharmacia Biotech Inc.).

5' and 3' rapid amplification of cDNA ends (RACE)

5' and 3' RACE were performed using the Bt cDNA library as template DNA. Gene-specific primers for the 5' and 3' RACE reactions were designed according to the DNA sequence obtained from the first round PCR product. 5' RACE was performed using the 5' primer (5'-TGCCTCGGGAGACA-TACCCGATTG-3'; nucleotide residues 211–234) and the adaptor primer 1 (AP1), which binds to the Marathon cDNA adaptor. For the 3' RACE reaction, the gene-specific primer (5'-GGTGATCAAGAGATTCAAGCAGCAATCATG-3'; nucleotide residues 370–399) and the AP-1 primer were used for amplification. 'Hot-Start-Touchdown' PCR amplification from Bt cDNA library was performed as described above. Cycle parameters included an initial incubation time of 15 min at 94 °C, five cycles of 30 s at 94 °C, 2 min at 70 °C, and five additional cycles of 30 s at 94 °C, 2 min at 68 °C. After 25 cycles of 30 s at 94 °C, 2 min at 66 °C and a final incubation at 72 °C for 5 min, amplicons were subcloned and analysed as described above.

Full-length cDNA generation

A 'Hot Start' standard PCR reaction using primers based on the 5' and 3' ends of the characterized RACE products was used to amplify the full-length Blo t 1 cDNA from the Bt cDNA library. The oligonucleotides used were: 5'-ATTCCAGCCAATTTT-GATTGG-3' (nucleotide residues 1–21) in the forward direction and 5'-AAGGGATGCATAGATTGG-3' (nucleotide residues 646–663) in the reverse direction. 'Hot Start' PCR amplification was performed as described above. Cycling parameters were as follows: 15 min at 94 °C; 30 cycles of 30 s

at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, and a final incubation at 72 °C for 5 min.

Nucleotide sequence and inferred amino acid sequence analysis

Sequence analysis was performed using the Mac VECTOR 5.0 software (IBI, New Haven, CT, USA). Hydrophilicity plots were computed with the software according to the Kyte-Doolittle algorithm using a seven-residue window [17]. Identity and similarity searches were conducted by comparing the sequence with those present in GeneBank, National Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST) heuristic search algorithm.

Preparation of expression construct

The full-length Blo t 1 cDNA was cloned into pDS56/RBSII-E/6His-*Eco* (pDS56) vector [18] by directional cloning of the PCR-amplified cDNA using primers engineered to include *Bam*HI (forward) and *Hind*III (reverse) sites. The PCR product was first cloned into the TOPO TA vector. Insert DNA was obtained by *Bam*HI and *Hind*III digestion followed by gel purification using the QIAGEN Gel Extraction Kit (QIAGEN Inc.). Full-length Blo t 1 cDNA was subcloned into pDS56 cleaved with *Bam*HI and *Hind*III. After transformation into *E. coli* XL1 Blue cells (Stratagene), coding frame was checked by restriction analysis and sequencing of transformants.

Expression of rBlo t 1 in E. coli

Expression of Blo t 1 in *E. coli* XL1 Blue cells was induced with 1 mM IPTG for 2 h. After centrifugation, cell pellet was lysed with buffer B (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-Cl, 20 mM imidazole, pH 8.0) under denaturing conditions. Purification of the recombinant histidine-tagged protein was done using a Ni-NTA Superflow resin (Qiagen Inc.) followed by elution with buffer D (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-Cl, pH 5.9) following the manufacturer's protocol.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Non-denaturing SDS-PAGE and immunoblotting were performed according to standard methods [19, 20]. Fifty µg of rBlo t 1 were loaded per track in a 15% polyacrylamide gel. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA, USA). Proteins were visualized by silver staining. For immunoblotting, proteins were transferred to a PVDF membrane (Immobilon-P Millipore, Bedford, MA, USA). Each PVDF strip was incubated overnight with 150 µL of patient's serum. IgE binding was visualized by incubating the strips with horseradish peroxidase anti-human IgE monoclonal antibody (1/100 000 dilution in 0.5% BSA) (The Binding Site, San Diego, CA, USA) and the Supersignal West Femto Maximum Sensitivity Substrate (PIERCE, Rockford, IL, USA). Chemiluminescence detection was performed by autoradiography.

Secondary and three-dimensional structure analysis

Secondary structure analysis was performed using the Mac VECTOR 5.0 software (IBI, New Haven, CT, USA) based in the Chou-Fasman and Robson-Garnier methods [21–23]. The structural model of rBlo t 1 was compared with the crystal

structure of human cathepsin L (NCBI MMDB PDB files 1CS8A) and porcine cathepsin H (NCBI MMDB PDB files 8PCHA) [24, 25]. Three-dimensional modelling was performed using the San Diego Protein Structure Modelling Server (San Diego Supercomputing Center, San Diego, CA, USA) [26] and the RasMol software [27].

Results

Cloning of a cysteine protease cDNA from Blomia tropicalis

In order to clone the Bt cysteine protease cDNA, the primary structures of published mite cysteine proteases (Der p 1, Accession number AAA28296 [28]; Der f 1, Accession number P16311 [29]; and Eur m 1, Accession number P25780 [30]) were aligned using the Clustal W algorithm and compared. Oligonucleotide primers were designed from the conserved active sites QGGCGSCW and WIVRNSWD. These primers amplified a PCR product of approximately 518 bp from the Bt cDNA library, which was subcloned and sequenced. Homology searches using BLAST revealed an open reading frame lacking the 5' and 3' termini and homologous to dust mite cysteine proteases. Gene-specific primers based on the partial sequence were designed to be used in 5' and 3' RACE reactions. The 5' RACE oligonucleotide was complementary to nucleotide residues 211–234 and the 3' RACE primer to nucleotide residues 370–399. PCR reactions with each RACE primer plus the adaptor-specific AP-1 primer amplified two single products of approximately 350 bp (5' RACE) and 500 bp (3' RACE). Cloning, sequencing and analysis of these products showed high amino acid sequence similarities to 5' and 3' termini of cysteine proteases from various organisms. A full-length cDNA clone was generated by PCR amplification using oligonucleotides complementary to the 5' (nucleotide residues 1–21) and 3' (nucleotide residues 646–663) sequences.

Sequence analysis of a cysteine protease cDNA from Blomia tropicalis

Nucleotide sequence analysis of the full-length cDNA included a 663 base pairs coding region of the mature protein terminating at the TAA stop codon (Fig. 1). Homology searches in the NCBI protein database revealed significant similarities to other cysteine proteases from dust mites and other organisms. BLAST analysis of the inferred amino acid sequence of the mature protein showed 35% identity to Der p 1, Der f 1 and Eur m 1. The 40 amino acid sequence of the Blo t 1 5' RACE product was also analyzed using the BLAST algorithm and was found to be homologous to the pro-peptide sequences of a number of cysteine proteases from insects, parasites, and plants, but not to the group 1 dust mite allergens. Isoleucine was predicted to be the initial amino acid of the mature protein of Blo t 1 based on sequence homology to cysteine proteases from organisms such as *Aphis gossypii* (plant, ACCESSION# CAD33266), *Fasciola hepatica* (trematode, ACCESSION# AAA29137), and *Rhodnius prolixus* (insect, ACCESSION# AAL34984). The residues making up the cysteine protease active sites are conserved (Glx19, Gly23, Ser24, Cys25, His169, Ser190 and Trp191) except for Thr189, which was substituted for Asn. Blo t 1 maintains five of the seven cysteine residues present in Der p 1, Der f 1 and Eur m 1, while Cys67 is unique to Blo t 1. In addition, aspartic acid

10 20 30 40 50 60 70
ATTCCAGCCAATTTTGATTGGAGACAAAAGACACATGTAAACCCGATCCGAAACCAAGGAGGCTGTGGTT
I P A N F D W R Q K T H V N P I R N Q G G C G G

80 90 100 110 120 130 140
CATGTTGGGCATTTCGCTGCCTCTTCGGTTGCCGAAACATTGTATGCAATCCATCGTCAACAAAATATAAT
S C W A F A A S S V A E T L Y A I H R H Q N I I>

150 160 170 180 190 200 210
TCTATCGGAACAAGAGTTGCTCGATTGCACATACCACCTTATATGACCCAACATATAAGTGTTCATGGATGT
L S E Q E L L D C T Y H L Y D P T Y K C H G C>

220 230 240 250 260 270 280
CAATCGGGTATGTCTCCGAGGCATTTAAATACATGAAACAAAAGGGATTGTTGGAAGAAAGCCATTATC
Q S G M S P E A F K Y M K Q K G L L E E S H Y>

290 300 310 320 330 340 350
CATATAAAATGAAACTTAACCAATGTCAAGCCAATGCCAGAGGAACAAGATACCATGTAAGCAGCTATAA
P Y K M K L N Q C Q A N A R G T R Y H V S S Y N>

360 370 380 390 400 410 420
TTCACCTCGATACCGAGCAGGTGATCAAGAGATTCAAGCAGCAATCATGAACCATGGTCCAGTTGTGATC
S L R Y R A G D Q E I Q A A I M N H G P V V I>

430 440 450 460 470 480 490
TACATTACGGTACCGAAGCTCATTTCCGTAATTTGCGTAAGGGAATCCTTCGTGGAGCTGGTTATAACG
Y I H G T E A H F R N L R K G I L R G A G Y N>

500 510 520 530 540 550 560
ATGCTCAAATCGATCATGCCGTCGTTTTGGTTGGTTGGGGAACCTCAAATGGAATCGATTACTGGATCGT
D A Q I D H A V V L V G W G T Q N G I D Y W I V>

570 580 590 600 610 620 630
ACGAACCAGTTGGGGAACTCAATGGGGTGATGCTGGTTATGGTTTTGTTGAACGACACCACAATTCGTTG
R T S W G T Q W G D A G Y G F V E R H H N S L>

640 650 660
GGTATCAACAACTATCCAATCTATGCATCCCTTTAA
G I N N Y P I Y A S L>

Fig. 1. Nucleotide and deduced amino acid sequence of Blo t 1 cDNA. Numbers are nucleotide positions. The stop (TAA) signal is underlined. Conserved cysteine active site residues are double-underlined.

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Fig. 2. Comparison of the derived amino acid sequences of Der f 1, Eur m 1, Der p 1 and Blo t 1. Conserved cysteine protease active site residues are in bold. Arrows indicate conserved cysteine residues.

residue 168 (D168), which in papain and other cysteine proteases influences the enzyme catalytic activity, is conserved in Blo t 1 but not in the other mite-derived cysteine proteases (Fig. 2).

Expression and purification of rBlo t 1 in *E. coli*

The inducible pDS56 expression vector containing the full-length Blo t 1 cDNA was transformed into *E. coli* XL1 Blue cells. Upon induction with IPTG, cells were collected by centrifugation and lysed. The recombinant protein was affinity-purified on a Ni-NTA superflow resin by batch purification under denaturing conditions. Forty-three milligrams per litre of culture of the recombinant protein were obtained. SDS-PAGE showed a single band with a molecular weight of about 25 kDa (data not shown). The recombinant protein expression and molecular weight were also confirmed by Western blotting using an anti-histidine antibody (data not shown).

Reactivity of IgE antibodies against rBlo t 1

The reactivity of IgE from serum of non-immunotherapy adult patients with positive skin test (> 3 mm weal-erythema) to Bt ($n = 21$) and Dp ($n = 6$) extracts was tested. Sera from individuals who live in Michigan, USA, where *Blomia* is not present ($n = 12$), and sera shown to have low IgE levels (< 2 IU/mL; $n = 7$) by FAST were used as controls. Thirteen out of 21 patients (62%) skin test-positive to Bt extracts demonstrated strong IgE binding to recombinant Blo t 1. All six sera from patients reactive to Dp (but not Bt) did not reveal any IgE binding to rBlo t 1. Control sera from Michigan individuals and sera with low IgE levels also failed to react with rBlo t 1.

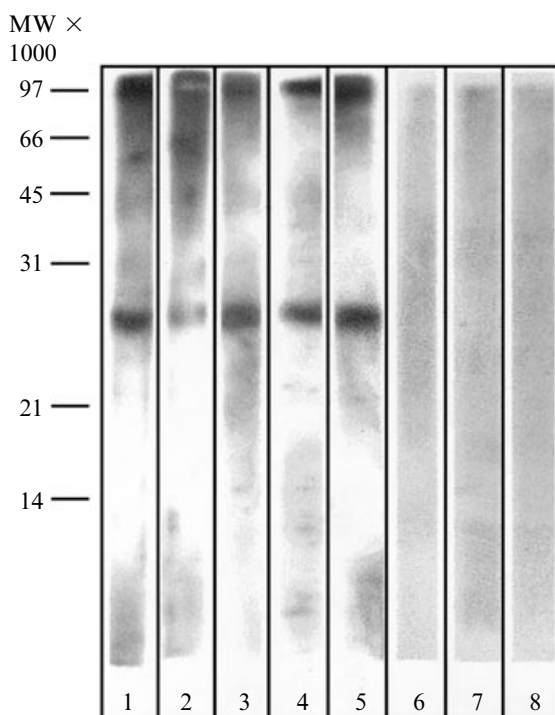


Fig. 3. Immunoblotting (15% SDS-PAGE) of rBlo t 1 with test serum samples. The IgE reactivity to rBlo t 1 was tested against non-immunotherapy Bt-positive skin test patient sera (representative samples 1–5) and sera from individuals from Michigan, USA, where *Blomia tropicalis* is not present (representative samples 6–8).

Figure 3 shows the immunoblotting of rBlo t 1 with representative serum samples from Bt-positive skin test patients and control individuals.

Secondary structure and three-dimensional prediction analysis

Results from the secondary structure analysis show that rBlo t 1 amino acid sequence has a similar distribution of alpha helix and beta sheet motifs as human cathepsin L and porcine cathepsin H, with the exceptions of amino acid regions 4–7, 114–119 and 215–221. These are predicted to be 'turns' instead of beta sheets. Analogous to human cathepsin, rBlo t 1 has an alpha helix (AA 50–56) and a beta sheet (AA 151–156) structure not present in the porcine cathepsin. Three-dimensional structure of rBlo t 1 suggests that this molecule consists of two domains with a deep cleft between them. A large portion of the divergent amino acids in rBlo t 1 are hydrophilic, as predicted by the Kyte-Doolittle algorithm (data not shown).

Discussion

The importance of Bt in triggering allergic responses has been extensively described [10–13, 15, 31–32]. Many studies have characterized and identified allergens from Dp, Df and *Euroglyphus maynei* (Em); in contrast, Bt allergens have been less studied. To overcome this limitation, we report the identification of a cDNA cysteine protease allergen denominated Blo t 1. Blo t 1 was cloned, sequenced and expressed as a recombinant protein. rBlo t 1 consists of 221 amino acids with an estimated molecular mass of 25 kDa, which is consistent with those described for other cysteine proteases, including those of mite origin. Gel analysis (data not shown) and Western blotting (Fig. 3) confirm the estimated molecular weight.

rBlo t 1 shares 35% identity with three other mite cysteine proteases previously characterized (Der p 1, Der f 1 and Eur m 1). There is about 50% degree of divergence in the primary amino acid sequence of Blo t 1 when compared with its dust mite counterparts, while Der p 1, Der f 1 and Eur m 1 cysteine proteases maintain 85–86% similarity to each other [29]. Cysteine protease active sites QGGCGSCW and WIVRNSWD were conserved except for one amino acid change at position 189 (Thr for Asn). Other critical amino acid residues were all conserved in rBlo t 1: Glx19, Gly23, Ser24, Cys25, His169, Ser190 and Trp191 (Fig. 2). Another important difference is the presence of aspartic acid 168 (D168), which is critical for enzymatic activity, while other mite-derived cysteine proteases have a tyrosine at this position. Also, five out of seven cysteine residues present in the mature protein of Der p 1, Der f 1 and Eur m 1 were conserved in Blo t 1. The sixth cysteine of Blo t 1 is residue #67, which can be found as part of an 8-residue stretch that is unique to the Bt cysteine protease. Cysteine residue pairs (Cys22–Cys70, and Cys56–Cys102) are likely to be involved in disulphide bond formation as described for other cysteine proteases [33, 34]. Because most of the cysteine proteases have an odd number of these residues present, the contribution of Cys67 in the overall protein structure or functionality is currently unclear. Taken together, sequence analyses indicate that regions involved in secondary structure and enzymatic activity are highly conserved among dust mite cysteine proteases,

including Blo t 1, as would be expected for this important physiological enzyme. The effects of the observed amino acid substitutions (Thr189Asn and Asn117Cys) on the enzymatic activity and allergenicity of Blo t 1 require further investigation.

Despite being purified under denaturing conditions, rBlo t 1 was recognized by 13/21 (62%) of Bt skin test-positive patient sera (Fig. 3), which suggests a possible role of this protein as a major allergen and re-enforces the clinical importance of this protein. Interestingly, Dp skin test-positive sera (Bt negative) did not react with rBlo t 1, which suggests the presence of unique IgE-binding epitopes on the rBlo t 1 molecule. A possible explanation for this observation is the fact that *Blomia* is taxonomically distant (*Echymyopodidae*) from *Dermatophagoides* and *Euroglyphus* species (*Pyroglyphidae*). This is supported by studies reporting a low level of cross-reactivity between *Blomia* and *Dermatophagoides* [15, 35, 36]. Arruda et al. [11] found no cross-reactivity between group 1 and group 2 allergens of Dp and allergens from Bt and showed that only 30 to 36% of Bt allergens may be shared with Dp. To date, only three Bt allergens have been cloned: Blo t 5, a 310-bp partial clone, and Bt 6 (probably a fatty acid-binding protein) [28, 32, 37, 38]. rBlo t 5 is 43% similar to the Der p 5 allergen, but despite this high degree of similarity, they only exhibit moderate allergenic cross-reactivity [15]. Studies of atopic subjects in temperate regions, who are likely to be exposed to Dp and not Bt, showed that the reactivity to rBlo t 5 was much lower than to rDer p 5 [31, 35, 36]. The small percentage sequence homology between Blo t 1 and the other three mite cysteine proteases also supports the allergenic differences observed among mite species. Taken together, these data demonstrates that Bt contains unique epitopes with relatively low cross-reactivity with those of the *Pyroglyphidae* family and that *Echymyopodidae* and *Pyroglyphidae* families are antigenically and allergenically different.

Cysteine proteases share similar sequences and three-dimensional structures [39]. Analysis of the human cathepsin L and porcine cathepsin H three-dimensional structures indicate that despite a 50% divergence at the amino acid level it is highly likely that Blo t 1 shares common secondary and tertiary structure with other cysteine proteases, including those of mite origin. With regard to specific IgE epitopes, it is interesting to note that all IgE cross-reactions described so far have been found to result from shared features at both primary and tertiary protein structures [40]. While all cross-reactive proteins have a similar fold, the reverse is not true: proteins with a similar fold are not necessarily cross-reactive. More importantly, protein folding is liberal with respect to single amino acid substitution for many positions in the sequence. Such substitutions may markedly affect the outer protein surface and thus reduce antibody reactivity. Similar protein folds are found with as little as 25% amino acid identity, whereas cross-reactivity is rare below 50% identity [40]. In most situations cross-reactivity requires more than 70% identity. Because Blo t 1 has only 35% identity to Der p 1, the absence of cross-reactivity from Dp positive serum to Blo t 1 as shown by immunoblotting is not surprising based on this partial identity.

Residents of tropical regions are continuously exposed to Bt, Dp and other mite allergens. This explains the high level of sensitization to these allergens among Puerto Ricans and the high prevalence of asthma in this population. Our studies resulted in the characterization of a novel cDNA that, based on amino acid sequence homology, codes for a cysteine protease

from Bt, denominated Blo t 1. rBlo t 1 expressed in *E. coli* retains its allergenicity, despite being purified under denaturing conditions. This recombinant product therefore constitutes an important tool to study the mechanisms of allergy and for diagnosing Bt exposures. As dust mite-derived cysteine proteases have been shown to be important triggers of asthma and other allergic disorders, cloning their genes is of utmost importance for the generation of novel therapeutic strategies, including modified recombinant vaccines and generation of hypoallergenic variants by site-directed mutagenesis. These studies will improve *in vitro* diagnostic immunoassays and will help elucidate the mechanisms involved in mite-associated allergic disorders.

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GenBank Accession Numbers: Blo t 1 (AAK58415), Der p 1 (AAA28296), Der f 1 (P16311), Eur m 1 (P25780).

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