

ORIGINAL ARTICLE

Allergens

The *Blomia tropicalis* allergen Blo t 7 stimulates innate immune signalling pathways through TLR2

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Summary

Background: Although the house dust mite species *Blomia tropicalis* is a leading cause of allergic diseases in tropical and subtropical regions, the identification and characterization of the allergenic proteins remain incomplete.

Objective: We aimed to characterize a recombinant form of Blo t 7 (rBlo t 7) in terms of IgE reactivity, lipid-binding activity and ability to stimulate innate immunity.

Methods: The mature Blo t 7 cDNA was cloned by PCR methods for the expression of a secreted form of the allergen in *P. pastoris*. The IgE reactivity to purified rBlo t 7 as well as the potential cross-reactivity with Der p 7 was determined by ELISA. The lipid-binding capacity of rBlo t 7 was assayed using fluorescent lipid probes. The stimulation of TLR2 signalling pathway by rBlo t 7 was examined in cell activation and reporter assays.

Results: The amplified mature Blo t 7 cDNA revealed the presence of a 60 base pair insertion compared with the reference sequence registered in the GenBank database. Multiple protein sequence alignments of group 7 mite allergens confirmed that this longer deduced amino acid sequence was the authentic Blo t 7 polypeptide chain. Analysis of IgE reactivity can classify rBlo t 7 as an intermediate *B. tropicalis* allergen which displayed weak cross-reactivity with Der p 7. Purified rBlo t 7 was shown to bind selectively the naturally fluorescent lipid probe cis-parinaric (cPNA) with a dissociation constant of 2 µmol/L. The group 7 *Blomia* allergen stimulated the TLR2-, NF-κB- and MAPK-dependent production of IL-8 and GM-CSF in respiratory epithelial cells.

Conclusions & Clinical Relevance: Through its propensity to transport fatty acids/lipids and to stimulate TLR2 signalling pathways in airway epithelial cells, Blo t 7 can represent a key allergen for the initiation of the *B. tropicalis*-induced airway inflammation.

KEYWORDS

Blo t 7, *Blomia tropicalis*, IgE reactivity, lipid-binding protein, TLR2

1 | INTRODUCTION

House dust mites (HDM) represent one of the most important sources of allergens and trigger in world-wide sensitized patients allergic rhinitis and asthma.¹ Whereas the most studied allergenic proteins belong to *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* mite species, the in-depth characterization of the allergens from the storage mite *Blomia tropicalis* remains incomplete. *Blomia tropicalis* is a geographically-restricted allergenic source in tropical and subtropical environments² and sensitization to its allergens are commonly associated with the typical clinical manifestations of allergy.³

Although at least 25 IgE-binding protein bands/spots have been detected in *B. tropicalis* crude allergen extracts by IgE immunoblotting, around 20 of them were cloned but only 13 identified *B. tropicalis* allergens were produced as recombinant proteins and officially recognized as allergens by the WHO/IUIS Allergen Nomenclature Sub-Committee.⁴ Weak to intermediate levels of sequence identities (25%-66%) were shared with the corresponding allergenic proteins from *D. pteronyssinus*, which could likely explain that cross-reactivity with *D. pteronyssinus* is low to moderate.⁵ Exceptions are represented by tropomyosin Blo t 10 and paramyosin Blo t 11 which display 94% and 89% of identities with Der p 10 and Der p 11, respectively.^{6,7}

Whereas Der p 1, Der p 2 and the recently identified Der p 23 represent the most important major allergens in *D. pteronyssinus*,⁸ the major *B. tropicalis* allergenic proteins are represented, up to now, by Blo t 5 and probably Blo t 21.⁹ Surprisingly, the IgE-binding frequencies of Blo t 1 and Blo t 2 remained poorly characterized and such information gap is likely related to the difficulties to obtain properly folded recombinant allergens.¹⁰ Nevertheless, limited data from two studies suggested that both of them could be also considered as important *B. tropicalis* allergens as their IgE reactivity reached 65% and 55% in cohorts of *B. tropicalis*-sensitized patients.^{11,12} The recent elucidation of the Blo t 1 crystal structure shed light on the limited cross-reactivity between Der p 1 and Blo t 1 through the presence of unique IgE-binding epitopes which are not shared with Der p 1.¹³ Similar specific IgE-binding epitope repertoire could be also present in Blo t 2, as evidenced by the low cross-reactivity between rBlo t 2 and rDer p 2 measured in inhibition ELISA.¹²

The biological functions of *B. tropicalis* allergens were attributed, up to now, essentially by prediction through sequence and/or structural homologies with the corresponding allergens from *Dermatophagoides* mite species.⁴ To our knowledge, the biological activity of *B. tropicalis* allergens was confirmed experimentally only for Blo t 12 and Blo t 13, 2 proteins displaying chitin- and fatty acid-binding properties, respectively.^{14,15}

Highly purified and correctly folded *B. tropicalis* allergens remain essential not only to develop the component-resolved diagnosis of *Blomia* sensitizations but also to decipher their allergenic determinant(s). Particularly, their capacity to trigger key innate immune

signalling pathways which are critical to initiate the allergic response was totally unexplored as of today.

The group 7 *B. tropicalis* allergen, Blo t 7, is largely ignored by the community of molecular allergists. Indeed, with the exception of one paper describing the IgE reactivity to a recombinant form of Blo t 7 produced in *E. coli*, the characterization of this allergen is missing.¹⁶ The most characterized group 7 mite allergens are Der p 7 and Der f 7, which display intermediate IgE reactivities.^{17,18} However, group 7 mite allergens could contribute to the initiation of the allergic responses through induction of innate immunity as the elucidation of their crystal structures demonstrated that these allergenic proteins could be involved in the transport of hydrophobic ligands to trigger innate immunity. Notably, Der p 7 and Der f 7 were shown to display structural homologies with LPS-binding protein (LPB). However, it was reported that none of them binds lipopolysaccharide (LPS) but binds bacterial lipopeptide polymyxin B (PB).^{19,20}

In this study, we aimed to characterize a recombinant form of Blo t 7 not only for its ability to bind lipids/fatty acids but also to activate innate immunity in human airway epithelial cells.

2 | MATERIALS AND METHODS

2.1 | Cloning, expression and purification of recombinant Blo t 7 (rBlo t 7)

The cDNA encoding the presumed mature form of Blo t 7 (amino acids 18-192) was amplified by PCR from a total *B. tropicalis* cDNA preparation using the following primers based on the registered Pre-Blo t 7 mRNA sequence (GenBank accession number AY291326): forward primer: 5'-CCGCTCGAGAAAAGAGAGGCTGAAGCTGATGATGCCGCAACCAACT-3' (Xho I site underlined), reverse primer: 5'-CGGGCGGCCGCTTAAAAAGCTGGAGCGGGAGTTGTGTTTT-3' (Not I site, underlined). The amplicon was subcloned into the pGEM T easy vector for sequencing. Following a double digestion by Xho I and Not I, the Blo t 7 cDNA was subsequently cloned into the pPICZα A vector (Invitrogen) restricted with the same enzymes, directly downstream of sequence encoding the α-mating factor pre-peptide. The *P. pastoris* KM71H strain was transformed by electroporation with the pPICZα A -Blo t 7 vector previously linearized by Bgl II digestion and the recombinant clones selected on the YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 mol/L sorbitol, 2% agar) agar plate containing 100 µg/mL zeocin (Invitrogen). The presence of Blo t 7 cDNA in zeocin-resistant clones was confirmed by colony PCR.

For a typical expression assay, individual zeocin-resistant colonies were first grown at 30°C under shaking conditions in YPD starter culture medium (1% yeast extract, 2% peptone, 2% dextrose). These first cultures were subsequently used to initiate secondary cultures using the BMGY medium ([1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate, pH 6.0, 1.34% YNB, 4 × 10⁻⁵% biotin, 1% glycerol], Invitrogen) under the same culture conditions. When the cell density reached an OD_{600 nm} of 6-9, the cultures

were pelleted and the cells were resuspended at a final OD_{600 nm} of 5 in BMMY medium (BMGY medium with methanol replacing glycerol, Invitrogen) containing 1% methanol (w/v) to trigger Blo t 7 expression at 22°C. After 24 hours, methanol was added to maintain the inducer concentration. Culture supernatants were collected after 48 hours of induction by centrifugation at 13 000 g for 10 minutes stored at 4°C until used. The supernatants were diluted 10 times with MilliQ water and the pH was adjusted to pH 3.5. This material was applied onto a S Ceramic HyperD ion-exchange chromatography sorbents (PALL) equilibrated with 20 mmol/L sodium citrate pH 3.5. After washing the unbound proteins from the column with the equilibrating buffer, a first elution step was performed using 20 mmol/L sodium citrate pH 3.5 containing 1000 mmol/L NaCl to remove contaminants. The column was subsequently washed with the equilibrating buffer and rBlo t 7 was finally eluted with 20 mmol/L Tris-HCl pH 9.5. Such eluting fraction was concentrated by ultrafiltration using a Amicon Ultra-5K membrane (cut-off 5 kD). The allergen purification was achieved by a gel filtration step onto a Superdex 75 HR column 10/30 (GE Healthcare Lifesciences), equilibrated with PBS pH 7.3.

The fractions containing purified rBlo t 7 were pooled and stored at -20°C for further analysis. The protein concentration was estimated using the microBCA protein assay kit (Pierce).

2.2 | Spectrofluorometric lipid-binding assay

The ligand binding activity of rBlo t 7 was evaluated using the following fluorescent lipid probes as previously described²¹: the naturally fluorescent fatty acid cis-parinaric acid (cPNA, Invitrogen), the fluorophore-tagged fatty acids 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (Bodipy® FL C16, Invitrogen), 11-([5-dimethylaminonaphthalene-1-sulfonylamino]) undecanoic acid (DAUDA, Invitrogen) and dansyl-DL- α -aminocaproic acid (DACA, Sigma-Aldrich), the fluorescent hydrophobic probe 1-anilino-naphthalene-8-sulfonate (ANS, Sigma-Aldrich), 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS, Sigma-Aldrich), the naturally fluorescent sterol dihydroergosterol (DHE, Sigma-Aldrich). Fluorescence emission spectra were recorded using an Agilent Cary Eclipse fluorescence spectrophotometer (Agilent, USA). Binding assays were performed as well with the lipid-binding protein beta-lactoglobulin (positive control) and rDer p 23, a major HDM allergen which was proven not to have any sequence homologies with fatty acid-binding protein (negative control).²² Competition experiments were conducted in which oleic acid, was gradually added to the preformed cPNA-protein complexes.

The equilibrium dissociation constant (K_d) of the rBlo t 7 bound to cPNA was determined at pH 7.3 by adding increasing concentrations of rBlo t 7 (0.01–3 μ mol/L) directly into a solution of cPNA (30 μ mol/L). Fluorescence intensities were normalized to the peak fluorescence intensity and corrected for background fluorescence of the ligand alone at each increment of protein. Corrected data were analysed using the one-site non-competitive saturation model.

2.3 | Production of *Blomia tropicalis* mite bodies extract

One hundred and sixty mg of *Blomia tropicalis* mite bodies (Stallergenes Greer) were extracted with 5 mL of endotoxin-free PBS pH 7.3 for 16 hours on a rotator. The extract was subsequently isolated by ultracentrifugation at 200 000 g for 30 minutes and the protein concentration of the collected supernatant was estimated using the micro BCA protein assay kit to 2.9 mg/mL. The *B. tropicalis* extract was aliquoted and stored at -20°C until use.

2.4 | IgE reactivity to rBlo t 7

Sera from a total of 170 patients with HDM-associated allergic rhinitis or asthma were obtained from the King Chulalongkorn Memorial (n = 36), Children (n = 62), Ramathibodi (n = 61) and Phramongkutklao (n = 12) hospitals, respectively. HDM sensitizations were diagnosed by case history, skin prick test and Dpt ImmunoCap. Written informed consent was obtained for each case. As negative controls, sera from subjects without any history of allergy were also collected (n = 40). As previous results highlighted a low to moderate IgE cross-reactivity⁵ between *B. tropicalis* and *D. pteronyssinus*, sera were also collected at the King Chulalongkorn Memorial Hospital from 184 volunteers who gave their inform consent to investigate allergy testing. It was not possible to ask these blood donors any questions about their personal allergic histories or potential risk factors for HDM allergy. The study was approved by the Ethic Committees from the Faculty of Medicine, Chulalongkorn University (IRB 023/55), Children hospital (IRB 195/2556), Faculty of Medicine, Ramathibodi hospital, Mahidol University (IRB 03-56-34) and Phramongkutklao College of Medicine (IRB S039Q/57_EXP). The specific IgE titers to *D. pteronyssinus* allergens were determined using ImmunoCAP assay (Thermo Scientific, Uppsala, Sweden). For the screening of *B. tropicalis*-specific IgE antibodies in the sera, ELISA plates were coated with 10 μ g/well *B. tropicalis* allergen extract in 0.1 mol/L sodium carbonate, pH 9.6 at 4°C for overnight. The plates were then washed with PBS-Tween 20, 0.05% (PBS-T) and blocked with 150 μ L of 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS-T for 2 hours at 37°C. The plates were then washed again with PBS-T and incubated with goat biotinylated anti-human IgE (KPL, 1/5000 dilution) at 37°C for 1 hour. The plates were then washed again with PBS-T and incubated with Streptavidin-HRP (BD Biosciences, 1/2000 dilution). The allergen-IgE complexes were detected with the TMB substrate (BD Biosciences) and the reaction was stopped with 0.5 mol/L sulphuric acid. Optical density (OD) was measured at 450 nm using iMark microplate reader (Bio-Rad). The IgE cut-off value for the *B. tropicalis* allergen extract assays was defined as the mean ELISA OD values of negative control sera (sera from individuals without history of allergy) plus 2 standard deviations.

The identified positive sera (n = 33) to *B. tropicalis* were subsequently selected to determine the IgE reactivity to rBlo t 7, together with some negative sera (n = 25), used as negative controls. The sensitization profile to rBlo t 5 (expressed and purified following the

same cloning and purification strategies as used for rBlo t 7) was also evaluated using the same sera. In this assay, ELISA plates were coated with 500 ng/well rBlo t 7 or rBlo t 5 in 0.1 mol/L sodium carbonate, pH 9.6 at 4°C overnight. The plates were then washed with PBS-Tween 20, 0.05% (PBS-T) and blocked with 150 µL of 10% fetal bovine serum in PBS (FBS-PBS) for 2 hour at 37°C. Sera, diluted at 1/8 in 10% FBS-PBS were added to the plates and incubated at 37°C for 2 hour. After another washing step, the plates were then further incubated with goat anti-human IgE-HRP (KPL, 1/2000 dilution in PBS-FBS 10%) at 37°C for 1 hour. The allergen-IgE complexes were detected with TMB as substrate (BD Biosciences) and the reaction was stopped with 0.5 mol/L sulphuric acid. Optical density (OD) was determined at 450 nm using iMark microplate reader (Bio-Rad). Intensity higher than the mean OD value for the negative sera responses + 2 standard deviations was considered positive.

To evaluate cross-reactivities between rBlo t 7 and rDer p 7 (similarly produced in *P. pastoris*), 2 inhibition ELISA were performed. For these assays, 25 Dpt ImmunoCap and rDer p 7 positive sera as well as six sera positive for rBlo t 7 were pre-incubated (at 1/10 dilution) with rDer p 7 or rBlo t 7 at 4°C overnight. As control, sera were also mixed with rDer p 23 (10 µg/mL) or with PBS pH 7.2. The IgE reactivity to rDer p 7 and Blo t 7 was subsequently determined with the rDer p 7- and rBlo t 7-positive treated sera, respectively, and using the same protocol as described above. The percentage of inhibition was calculated as $(1 - A_i/A_o) \times 100$ where A_i and A_o correspond to OD_{450 nm} with and without inhibitor, respectively.

2.5 | Rat basophil degranulation assay

Rat basophil leukaemia cells expressing human FcεRI (RBL-SX38 cells) were plated in 96-well culture plate at a cell density of 1.5×10^5 cells/well in RPMI 1640 medium. Cells were subsequently primed for 16 hours with human sera (positive or negative for Blo t 7 or Blo t 5 specific IgE) at 1/10 dilution. Each serum was previously diluted 2 times with 4 mol/L glucose and incubated for 45 minutes at 56°C. Cells were then washed with PBS and incubated with phenol red-free RPMI 1640 containing 1 mg/mL BSA for 15 minutes before the addition of serial dilutions of rBlo t 7 or rBlo t 5 (0.0001–1 µg/mL) for 45 minutes. To evaluate the maximum degranulation, control cells were treated with 5% (v/v) Triton X-100. The cell degranulation was assayed by the detection of the β-hexosaminidase activity in the culture supernatants as previously described.²²

2.6 | Human bronchial epithelial cell activation

The BEAS-2B human bronchial epithelial cells (ATCC) were treated with rBlo t 7 or the TLR2 agonist Pam3Cys using the same experimental conditions previously described.²¹ The contribution of TLR2 was assessed by pre-incubation with anti-TLR2 blocking antibodies or an isotype control. In another set of experiments, cells were pre-treated for 30 minutes with the IκBα inhibitor BAY 11-7082 (10 µmol/L) or the MAPK inhibitors UO126 (10 µmol/L), SB203580

(10 µmol/L) and SP600125 (10 µmol/L). As control, cells were stimulated with DMSO (vehicle control) before addition of the agonists.

2.7 | HEK TLR2 reporter cell assays

HEK-Blue hTLR2 cells, expressing human CD14 and TLR2 as well as the control parental HEK-Blue Null1 (Invivogen) were cultured in DMEM Glutamax (Life Technologies) supplemented with 10% FBS. Both cells lines stably expressed the secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by NF-κB and activator protein 1 (AP-1). After the cells reached approximately 80% confluency, cells were incubated in serum-free DMEM for a further 24 hours. Cells were then treated with different concentrations of rBlo t 7 (5, 10 µg/mL), Pam₃CSK₄ (100 ng/mL, Invivogen) or TNF-α (100 ng/mL) for 16 hours at 37°C. As negative controls, stimulations were also performed with rDer p 23 or with a supernatant from wild-type KM71H cultured in BMMY and treated using the same purification protocol used for the isolation of rBlo t 7. Twenty µL of different cell supernatants were then collected and mixed with 180 µL of QUANTI-Blue™ detection medium (Invivogen) and incubated at 37°C for 1 hour to assess the SEAP activity. Optical density (OD) at 650 nm was measured using iMark microplate reader (Bio-Rad).

2.8 | Statistical analysis

Data were compared using Student's *t* test or Wilcoxon signed-rank test. *P*-value of less than .05 was considered to be significantly different.

3 | RESULTS

3.1 | Cloning of mature Blo t 7 cDNA reveals a new sequence

According to the sequence with the GenBank accession number AY291326, the full-length cDNA of Blo t 7 has 579 base pairs which codes for a polypeptide of 192 amino acids. As three nucleotides were not identified within the cDNA sequence, the nature of amino acids at position 157, 160 and 169 was unknown (full-length Blo t 7 numbering, Uniprot KB accession number A1KXI4). Protein analysis, through the use of the SignalP software for the prediction of leader peptide, suggested that this cDNA encodes Pre-Blo t 7 with a mature sequence spanning amino acids 18–192.

Consequently, 2 specific primers derived from the sequence AY291326 were designed to amplify the Blo t 7 mature cDNA from a *B. tropicalis* cDNA library. Surprisingly, the DNA sequence of the amplicon contained 585 base pairs, compared with the predicted size (525 bp) from the AY291326 sequence (Figure 1.A). The deduced 195 amino acid sequence clearly demonstrated the presence of a 20 amino acids long insertion between Ala60 and Gly61 residues (mature Blo t 7 numbering). Together with this insertion, 4 substitutions were evidenced in the cloned cDNA compared with the AY291326

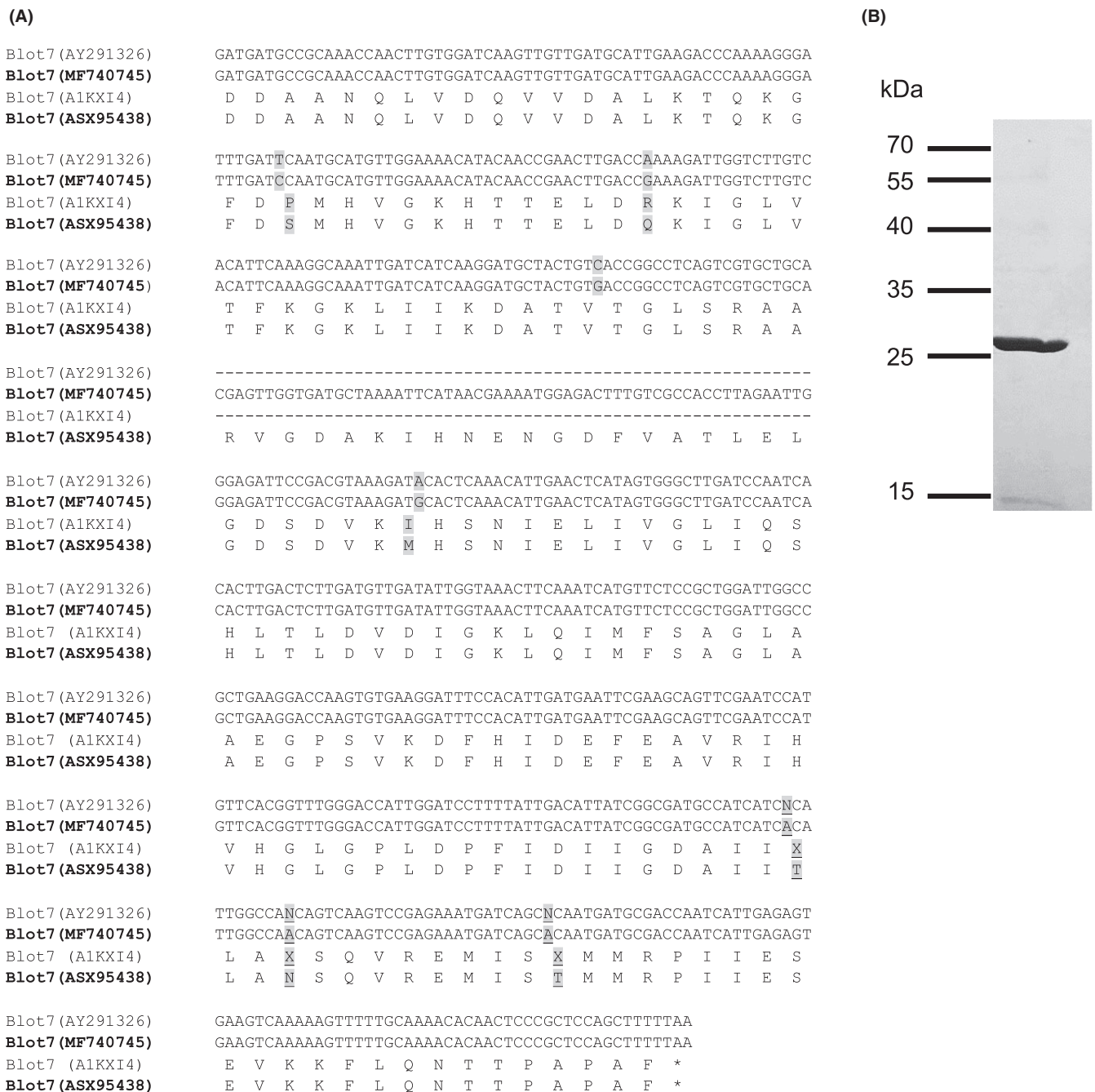


FIGURE 1 Cloning and production of mature rBlo t 7. Panel A: Comparison of the cloned cDNA and the deduced amino acid sequences of mature Blo t 7 (Genbank accession number MF740745 and protein identifier number ASX95438) to the corresponding ones submitted to EMBL (accession number AY291326) and UniprotKB (accession number A1KXI4). The changes in the sequences are highlighted in grey. The unidentified bases and amino acids in the AY291326 and A1KXI4 sequences (grey highlighted and underlined), respectively, were identified as A bases and Thr140, Asn143 and Thr152 (mature Blo t 7 numbering). Panel B: SDS-PAGE analysis of purified rBlo t 7 under reducing conditions

sequence, one of them was silent whereas the three others induced the point mutations Ser23Pro, Gln35Arg and Ile87Met (mature Blo t 7 numbering). All the three unknown nucleotides from the registered sequence were identified as A nucleotide in the Blo t 7 amplicon to deduce the nature of amino acids 140, 143 and 152 as Thr, Asn and Thr, respectively. Finally, the mature Blo t 7 sequence contained a putative N-glycosylation motif Asn168-Thr169-Thr170 (mature

Blo t 7 numbering). This new cDNA sequence encoding mature Blo t 7 was submitted to GenBank with the accession number MF740745. The corresponding protein identifier number was ASX95438. Moreover, Blo t 7 was acknowledged by the WHO/IUIS Allergen Nomenclature Sub-Committee.

Multiple amino acid sequence alignment using ClustalO was performed to evidence sequence similarities between Blo t 7 and the

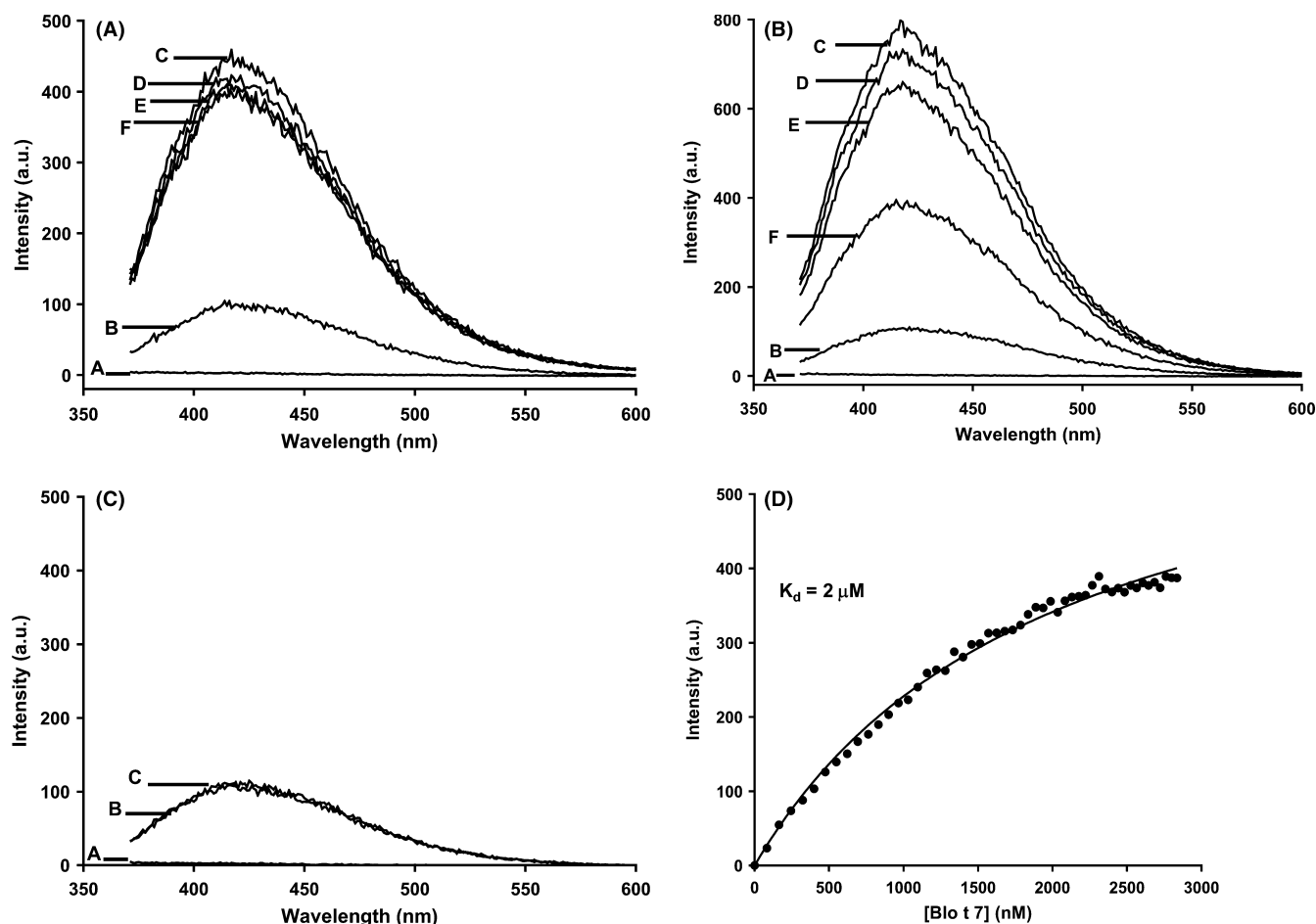


FIGURE 2 Hydrophobic ligand binding activity of rBlo t 7. Panel A: Fluorescence emission spectra of the cis-parinaric acid (cPNA, $\text{Ex}_{\text{max}} = 319 \text{ nm}$) binding to purified rBlo t 7. Panel B: Binding of cPNA to beta-lactoglobulin. Panel C: Binding of cPNA to rDer p 23. Curve A: PBS, curve B: cPNA alone, curve C: cPNA + protein. The concentration of rBlo t 7, beta-lactoglobulin, rDer p 23 and cPNA used in the assays was $0.1 \mu\text{mol/L}$, $0.067 \mu\text{mol/L}$, $0.45 \mu\text{mol/L}$ and $30 \mu\text{mol/L}$, respectively. The competitive binding of oleic acid used at different concentrations ($0.079 \mu\text{mol/L}$, $0.79 \mu\text{mol/L}$, $7.90 \mu\text{mol/L}$) is also shown for rBlo t 7 and beta-lactoglobulin. Curve D: cPNA: protein complex + $0.079 \mu\text{mol/L}$ oleic acid, curve E: cPNA: protein complex + $0.79 \mu\text{mol/L}$ oleic acid, curve F: cPNA: protein complex + $7.90 \mu\text{mol/L}$ oleic acid. One representative experiment out of three is shown. Panel D: titration curve of cPNA binding to rBlo t 7. Change in relative fluorescence intensity of cPNA following addition of increasing rBlo t 7 concentrations (from 0 to $3 \mu\text{mol/L}$). The solid line was fitted by non-linear regression techniques to a single non-competitive site model for the calculation of K_d

other members of the group 7 mite allergens as Der p 7, Der f 7, Tyr p 7, Aca s 7, Lep d 7 and Gly d 7 (Figure S1). Although the overall level of identities shared by this group of mite allergens is not high, such alignment confirms that the Blo t 7 protein sequence deduced from our cloned cDNA correspond to the correct one. Indeed, the identified insertion of 20 amino acids in Blo t 7 shared 55% identities with the corresponding ones from other group 7 mite allergens.

3.2 | Physico-chemical analysis of purified rBlo t 7

The secreted protein, following methanol induction of yeast transformed with pPICZ α A -Blo t 7, was purified to homogeneity by a combination of cation exchange and gel filtration chromatographies. It must be pointed that the size exclusion profile suggested that rBlo t 7 is monomeric. SDS-PAGE analysis showed that the purified rBlo

t 7 was homogeneous and migrated as a 25kD band (Figure 1B). This band was subsequently excised for in-gel trypsin digestion and the identification of tryptic peptides by mass spectrometry (MS) confirmed the nature of the protein band as rBlo t 7, covering 62% of the allergen sequence (Figure S2).

As Der p 7 and Der f 7 displayed structural homologies with lipid-binding proteins (LPS-binding protein (LBP) and bactericidal permeability-increasing (BPI) protein, respectively)²³, we first examined the fatty acid-binding activity of rBlo t 7 with the environment-sensitive fluorescent fatty acid cis-parinaric acid (cPNA) at pH 7.3. Under the same experimental conditions, we used the lipid-binding protein beta-lactoglobulin (BLG) and rDer p 23 as positive and negative controls, respectively. Binding of cPNA to rBlo t 7 was accompanied by a substantial increase in its fluorescence intensity which is indicative of an interaction with a hydrophobic environment (Figure 2A). Similar results were observed with BLG (Figure 2B) whereas

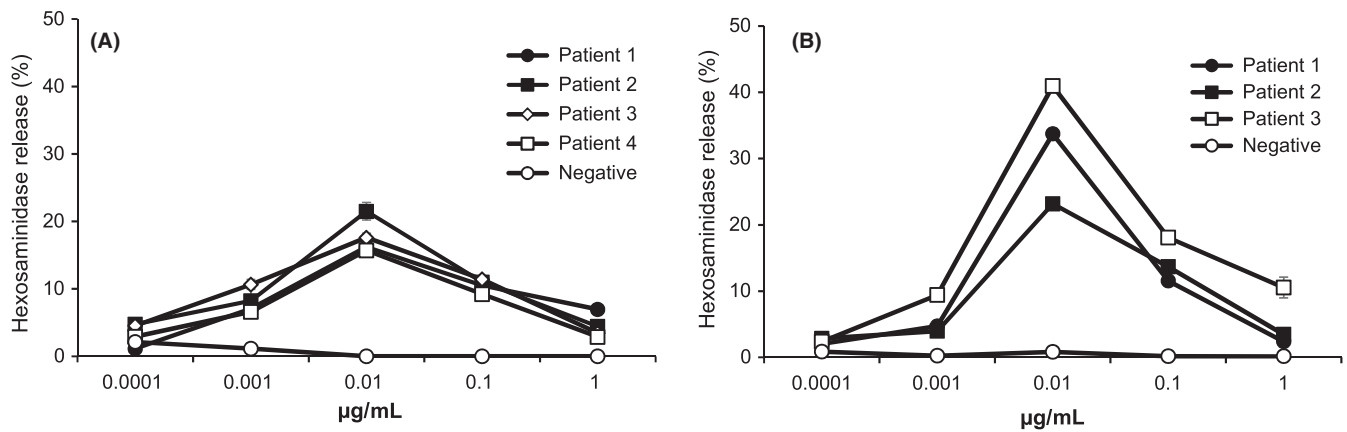


FIGURE 3 Allergic activity of rBlo t 7. RBL-SX38 cells were loaded with sera from patients sensitized to *B. tropicalis* and containing specific IgE to rBlo t 7 (N = 4, Panel A). As controls, cells were also primed with sera containing specific IgE to rBlo t 5 (N = 3, Panel B) or with 1 serum seronegative to *B. tropicalis* (N = 1, Panels A and B). Basophil degranulations were triggered by incubation with serial concentrations of allergens. The release of β -hexosaminidase activity is shown as percentages of total β -hexosaminidase obtained with Triton X-100

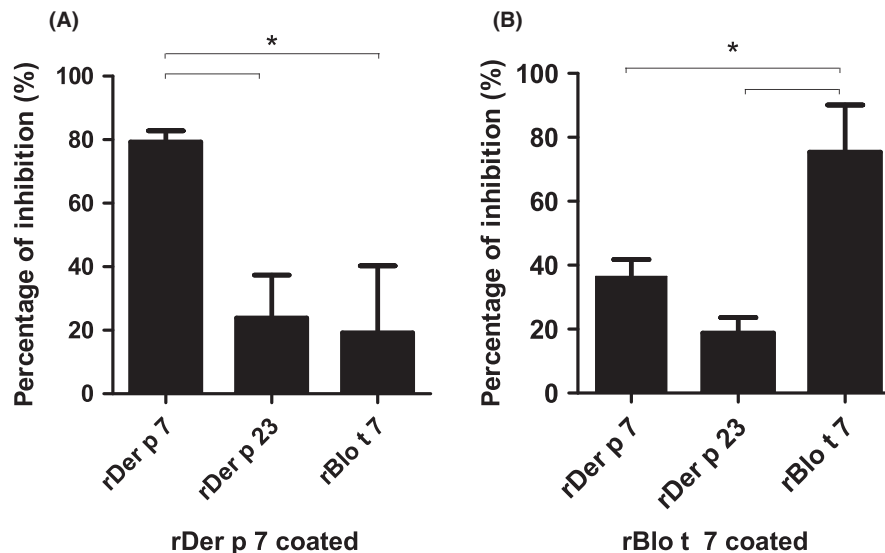


FIGURE 4 Cross-reactivity between rBlo t 7 and rDer p 7. Competitive IgE inhibition ELISA was conducted using sera containing IgE to rBlo t 7 (N = 6) or rDer p 7 (N = 25) at 1/10 dilution and pre-absorbed with 10 μ g/mL of rBlo t 7, rDer p 7 or rDer p 23 and with plates coated with rDer p 7 (Panel A) or rBlo t 7 (Panel B). The percentage of inhibition was calculated as $(1 - A_i/A_o) \times 100$ where A_i and A_o correspond to OD_{450 nm} with and without inhibitor, respectively. Data show mean percentage of inhibition + SD and are representative of three independent experiments. * $P < .05$

no significant fluorescence enhancement of cPNA was evidenced in the presence of rDer p 23 (Figure 2C). We did not observe significant fluorescence enhancement of the fluorescently labelled lipid probes DAUDA, DHE, DACA and Bodipy-C16 in the presence of rBlo t 7 compared to the intrinsic fluorescence of free ligands (data not shown). Displacement assays were performed with increasing concentrations of oleic acid. Whereas oleic acid, was unable to significantly displace cPNA from the binding site of rBlo t 7, efficient concentration-dependent competitive displacement was shown for BLG.

Finally, fluorescence titration curves for the binding of cPNA to rBlo t 7 clearly indicated that the binding was saturable (Figure 2D). We found an apparent dissociation constant K_d of 2 μ mol/

L, the stoichiometry being consistent with 1:1 binding. This value being within the range of K_d values calculated for other lipid-fatty acid-binding proteins.

3.3 | IgE reactivity

To determine the IgE-binding frequency of rBlo t 7, we needed to obtain serum samples which contain specific IgE to *B. tropicalis*. Unfortunately, ImmunoCap or skin-prick testing is not routinely performed in hospitals from Thailand to detect sensitizations to *B. tropicalis*. Consequently, we first performed an IgE serum screening by ELISA assay and using *B. tropicalis* mite bodies extracts. The serum

samples were obtained from 2 cohorts of individuals. The first cohort consisted in a group of patients suffering from allergic rhinitis or asthma and regularly followed at different hospitals from greater Bangkok and showing Dpt ImmunoCap positivity ($N = 171$) whereas the second one was a non-selected cohort of volunteers without information about allergy history ($N = 184$). The frequency of *B. tropicalis* sensitization reached 12.3% (21/171) and 6.5% (12/184) in these 2 respective populations, representing an overall prevalence of 9.3% in the tested sera (33 positive sera of 355). It must be pointed out that, among the twelve non-selected volunteers sensitized to *B. tropicalis*, only 33% were also sensitized to *D. pteronyssinus*.

Using the sera with IgE antibodies against *B. tropicalis*, the IgE reactivity to rBlo t 7 was determined by indirect ELISA and compared with the IgE-binding capacity of rBlo t 5, considered as a major *B. tropicalis* allergen. The tested sera displayed IgE reactivity percentage of 24% (8 sera of 33) for rBlo t 7 and 36% (12 sera of 33) for rBlo t 5, suggesting that Blo t 7 can be classified as an intermediate allergen.

We next compared the allergenicity of rBlo t 7 with the one displayed by rBlo t 5 in a RBL SX-38 degranulation assay using sera containing IgE to rBlo t 5 or rBlo t 7. rBlo t 7 was able to trigger basophil degranulation following a typical allergen concentration-dependent bell-shaped curve, with a maximum reached with 0.01 $\mu\text{g/mL}$ (Figure 3). The level of degranulation was quite similar (17%–21%) whatever the tested sera. Using the same allergen concentrations, the degree of basophil degranulation induced by rBlo t 5 was more variable, with values ranging from 22% to 42% suggesting that Blo t 5 exhibits more allergenic activity than Blo t 7.

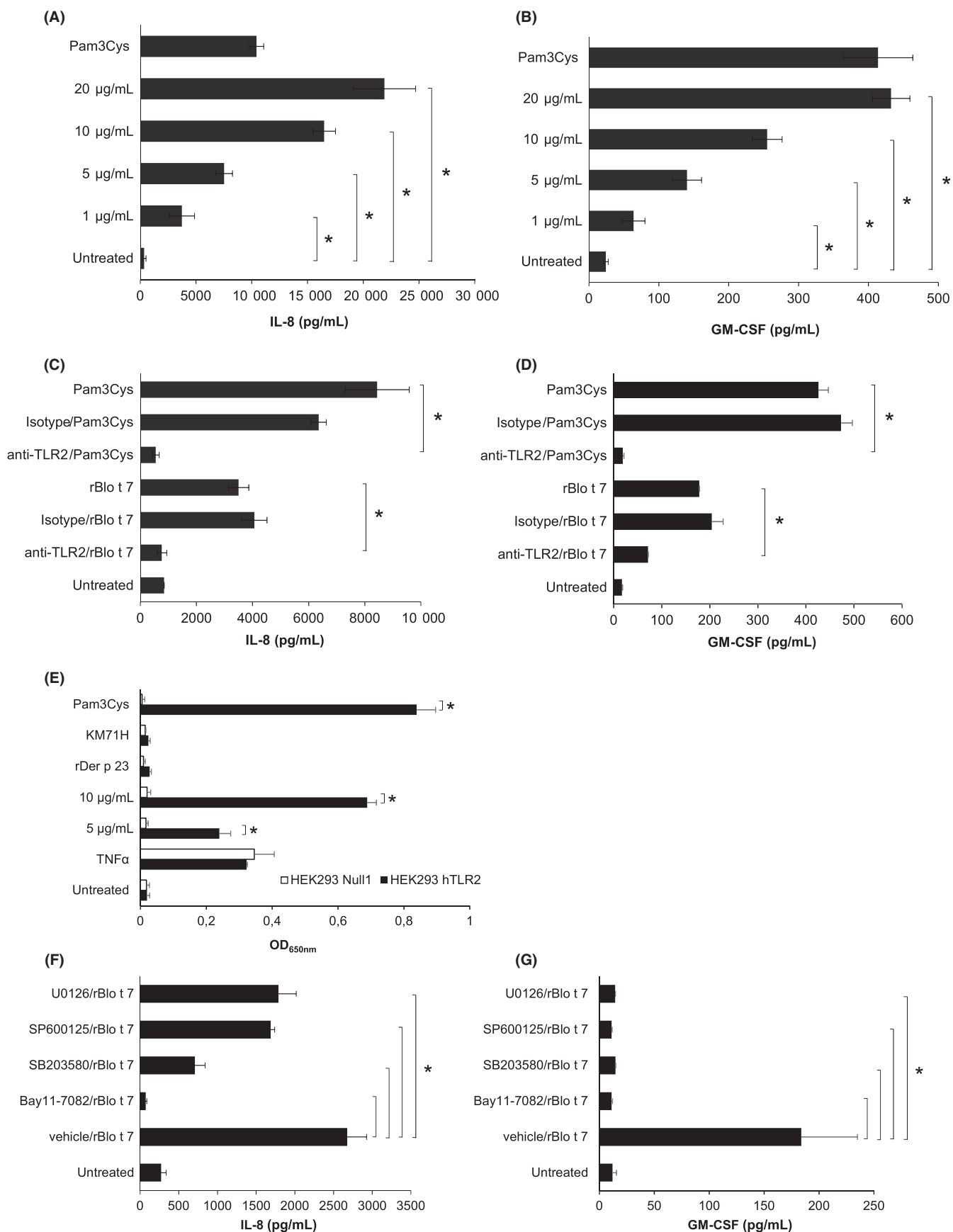
Finally, to determine whether Blo t 7 displays cross-reactivity with Der p 7, cross-inhibition IgE-binding assays were performed using sera displaying specific IgE to rDer p 7 ($N = 25$), rBlo t 7 ($N = 3$) or both allergens ($N = 3$). In these assays, rDer p 7- or rBlo t 7-positive sera were pre-incubated with 10 $\mu\text{g/mL}$ inhibitors (rDer p 7, rBlo t 7 or rDer p 23 used as negative control) before specific IgE ELISA testing (Figure 4). Whereas rDer p 7 could inhibit more than 80% of IgE-binding to itself, pre-incubation with rBlo t 7 could only prevent 20% of the IgE-binding to rDer p 7. Similar level of inhibition was observed with the pre-treatment with rDer p 23 (23%, $P = .27$ Wilcoxon test). Equivalently, more than 77% of the binding between coated rBlo t 7 and IgE from sera positive for rBlo t 7 could be inhibited by pre-incubation with soluble rBlo t 7, the IgE reactivity to rBlo t 7 was inhibited by only 36% by rDer p 7

($P = .0001$ Wilcoxon test) and by 20% by rDer p 23. In both assays, similar levels of inhibition were obtained using sera from Blo t 7-, Der p 7-monosensitized or double-sensitized patients. Taken together, all results suggested that the cross-reactivity between Der p 7 and Blo t 7 is limited.

3.4 | Blo t 7 is capable to activate airway epithelium through TLR2

As we recently evidenced that the fatty acid binding protein Der p 13 can stimulate TLR2-dependent signalling pathways in airway epithelial cells,²¹ we hypothesized that rBlo t 7 displays similar propensities. In cell activation assays, rBlo t 7 was shown first to trigger the production of IL-8 and GM-CSF in the BEAS-2B human bronchial epithelial cell line in a concentration-dependent manner (Figure 5A, B). Such cytokine productions were dependent on the stimulation of TLR2 as cell pre-incubation with anti-TLR2 blocking antibodies drastically reduced the rBlo t 7-induced IL-8 or GM-CSF secretion (Figure 5C, D). The ability of rBlo t 7 to stimulate TLR2 was confirmed using the HEK-Blue hTLR2 reporter cell line. As shown in Figure 5E, rBlo t 7 was able to activate HEK cells expressing TLR2 whereas the cell activation was abolished in the HEK parental cell line. The absence of TLR2 activation when cells were incubated with supernatants from methanol-induced wild-type KM71H cells that had been treated as for the rBlo t 7 purification protocol or with purified rDer p 23, a HDM allergen without lipid-binding properties, confirmed that the rBlo t 7-mediated TLR2 stimulation was not due to trace contaminants in the protein preparation. Finally, we used different pharmacological inhibitors to determine the requirement of NF- κ B and MAPK in the up-regulation of IL-8 and GM-CSF in rBlo t 7-treated BEAS-2B cells (Figure 5F, G). We observed that inhibition of I κ B α drastically reduced the production of IL-8 or GM-CSF following the treatment of BEAS-2B cells with rBlo t 7. Moreover, whereas the rBlo t 7-induced GM-CSF release was fully inhibited by any of the three tested MAPK inhibitors, the stimulation of IL-8 production by rBlo t 7 was essentially impaired by the p38 inhibitor SB203580 and to a lesser extent by UO126 and SP600125. Nevertheless, the reduction of IL-8 production by UO126 and SP600125 reached statistical significance. Altogether, our results clearly evidenced that rBlo t 7 can stimulate TLR2-, NF- κ B- and MAPK-dependent signalling pathways in airway epithelial cells.

FIGURE 5 Stimulation of TLR2 signalling pathways by rBlo t 7. Panel A and B: Production of IL-8 and GM-CSF by rBlo t 7-treated BEAS-2B cells in a concentration-dependent manner. The TLR2 agonist Pam₃Cys was used as positive control. Panel C and D: TLR2-dependence of IL-8 and GM-CSF stimulation triggered by rBlo t 7. BEAS-2B cells were pre-incubated with blocking anti-TLR2 antibody or isotype control (Panel C) prior to allergen (10 $\mu\text{g/mL}$) or Pam₃Cys treatment. Panel E: HEK-hTLR2 or the parental HEK-Null1 cells, stably transfected with plasmids encoding an NF- κ B/AP-1-dependent SEAP reporter gene, were stimulated with rBlo t 7, TNF- α , rDer p 23, Pam₃Cys or a BMMY from cultured wild-type KM71H and purified according to the purification protocol of rBlo t 7. The SEAP activity was determined 16 h post-activation. Panel F and G: BEAS-2B cells were pre-incubated with SP600125, SB203580, UO126 (MAPK pathway inhibitors) or BAY 11-7082 (NF- κ B pathway inhibitors) before treatment with rBlo t 7 (10 $\mu\text{g/mL}$). Data show mean cytokine concentrations or SEAP activity + SD and are representative of three independent experiments. * $P < .05$



4 | DISCUSSION

This article presents the correct nucleotide and deduced amino acid sequences of the *B. tropicalis* group 7 mite allergen Blo t 7, an allergenic protein which was fully underrated up to now. The measured IgE reactivity to rBlo t 7, compared with the IgE-binding capacity of the major *B. tropicalis* Blo t 5 allergen, in indirect ELISA as well as RBL assays, suggested that Blo t 7 can be classified as an intermediate allergen, similarly to the other members of the group 7 mite allergens.^{17, 18} The IgE-binding frequency of Blo t 7 in Thailand (24%) is apparently lower than the one previously reported in Singapore, which reached 44% in cohorts of allergic children. However, it must be pointed that the rBlo t 7 used in that study was produced in *E. coli* and based on an incorrect sequence.¹⁶ Our results could also confirm that, compared with neighbouring countries including Singapore, frequencies of sensitizations to *B. tropicalis* are unexpectedly low in Thailand, as was initially reported in Thai children or adults asthmatics cohorts.²⁴ More extensive studies using larger populations of allergic patients from different regions of Thailand are needed to support our conclusions. Our data showed as well that Blo t 7, exhibits low levels of IgE cross-reactivity with the homologous Der p 7. Such limited cross-reactivities among *B. tropicalis* and *Dermatophagoides* mite allergens belonging to the same group were previously observed, notably between Blo t 5 and Der p 5 or Blo t 1 and Der p 1,^{5, 13} strengthening the notion that *B. tropicalis* is distinct from the *Dermatophagoides* mite species.

Despite the molecular characterization of, up to now, more than 12 allergens from the *Blomia tropicalis* mite, we can consider that their capacity to stimulate innate immune signalling pathways remains fully unknown. In this study, to our knowledge, we showed for the first time that rBlo t 7 activates TLR2 signalling in both airway epithelial cells and HEK cells expressing TLR2. As TLR2 recognizes mainly lipopeptides/lipoproteins,²⁵ such innate immune activation triggered by rBlo t 7 is very likely mediated by its capacity to bind microbial lipids and to transfer their lipid cargo directly to TLR2 or to a co-receptor as CD14. Although the present study did not evidence any lipid transfer between rBlo t 7 and TLR2, the propensity of this *B. tropicalis* allergen to bind selectively fatty acids/lipids was, in contrast, clearly demonstrated. Together with the fatty acid binding protein Blo t 13,¹⁵ Blo t 7 represents, so far, the second *B. tropicalis* allergen displaying such lipid-binding activity. The two most studied group 7 mite allergens are the highly homologous Der p 7 and Der f 7 (>85% sequence identity) which exhibit a similar overall crystal structure, consisting in antiparallel beta-sheets wrapped around a helix to create a lipid-binding domain.^{19, 20} Surprisingly, the fatty acid/lipid-binding activities of these 2 allergens were poorly characterized. The unique ligand identified until now is the lipopeptide polymyxin which weakly binds to both Der p 7 and Der f 7. The identification of the Der f 7 amino acids involved in the PB binding revealed that most of these residues are hydrophobic in nature and a similar binding site is present into the Der p 7 structure.²⁰ Remarkably, whereas the overall identity between Blo t 7 and Der p 7/Der f 7 does not reach 30%, most of hydrophobic amino acids forming the Der f 7 binding site

(Ala15, Ile16, Leu64 and Gly80) are conserved in Blo t 7 (Leu15, Val16, Leu64 and Gly80). This suggested not only that Blo t 7 could also interact with polymyxin B but that, in spite of low sequence identities, members of the group 7 mite allergen share a common hydrophobic binding site. It must be pointed out that *D. pteronyssinus* mite allergens Der p 2, Der p 13 and Der p 21 were shown to exhibit a similar capacity to activate TLR2.^{21, 26, 27} Taken together, our data suggest that mite allergens from groups 2, 7, 13 and 21, although they are structurally distinct, are members of lipid/fatty acid binding protein families which share a common TLR2-dependent mechanism to stimulate innate immunity.

Little is known about the mechanism of the allergic response triggered by *B. tropicalis* allergens and particularly about the role of TLR2. Using two different animal models of *B. tropicalis* allergen extract-induced asthma (intranasal sensitizations and challenge using *Blomia* extracts or subcutaneous sensitization with the allergen extract plus aluminium hydroxide and intranasal challenge through the extracts alone),^{28, 29} it was evidenced that heat-resistant allergenic proteins are capable to stimulate the development of the typical cardinal features of airway inflammation in a similar way to *D. pteronyssinus*. Such lung inflammation, dominated by large eosinophil infiltrates together with a modest neutrophilia was shown to be TLR2-independent.²⁹ The relevance of this TLR2 independence must be however questionable. Indeed, it must be pointed out that the extensive delipidation of the *B. tropicalis* allergen extracts used in that study could mask the contribution of microbial ligands and TLR2 in the *B. tropicalis*-induced allergic response. Of note, the same lipid-free allergen extracts co-administrated with unpurified LPS or Pam3Cys trigger TLR2-dependent airway neutrophilia.²⁹ Importantly, the GM-CSF produced derived from lung epithelial cells during the *B. tropicalis* sensitization was shown to represent a key factor for the initiation of the Th2 priming.²⁸ By its capacity to stimulate the TLR2-dependent production of IL-8 and GM-CSF, two important cytokines for the recruitment of neutrophils and dendritic cells, respectively, Blo t 7 could represent one of the key allergens involved in the initiation of the *B. tropicalis*-induced airway inflammation.

In conclusion, we not only deciphered the allergenic determinant of Blo t 7 as its propensity to transport fatty acids/lipids but clearly demonstrated the capacity of Blo t 7 to stimulate TLR2 signalling pathways in airway epithelial cells. Although the ligand(s) of natural Blo t 7 remain(s) to be identified, we assume that it (they) could similarly activate TLR2, according to the large diversity of identified TLR2 activators. It would be important to evaluate the role of Blo t 7 in the development of the allergic airway inflammation *in vivo*. Nevertheless, the *in vitro* IL-8 and GM-CSF production by Blo t 7-stimulated airway epithelial cells suggests that Blo t 7 can be considered as one of the key allergens for the initiation of pro-inflammatory signalling pathways leading to *B. tropicalis*-induced airway inflammation. Our results demonstrate as well that future strategies for optimized immunotherapeutic treatments of *B. tropicalis* sensitizations could also take into consideration the blockade of signalling pathways triggered by *B. tropicalis* lipid-binding proteins, even if their IgE-binding capacities are low.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Derp7(P49273)	MMKLLLI AAAAFVAVSAD ---PIHYDKITEEINKAVDEAVAAIEKSETFDPMKVDPHSDK
Derf7(Q26456)	MMKFLLI AAVAFVAVSAD ---PIHYDKITEEINKAIDDAIAAIEQSETIDPMKVDPHADK
Blot7(A1KXI4)	-MKSTIVLLACFVG-----IAF-SD--DAANQLVDQVVDALKTQKGFDSMHVGKHTTE
Blot7(ASX95438)	-----D--DAANQLVDQVVDALKTQKGFDPMHVGKHTTE
Tyrp7(B0KZL1)	MKSAVLVLVACFA-----GIAVADN--GNANQFVDQIVTALKTQKNFDPLVIPPHHMN
Acas7(B0KZJ0)	MKSFALIAIACFV-----GMAVADD--GNANQFVDQIVNALKSQKNFDPLVIAEQHTN
Lepd7(Q9U1G2)	MQYLAI AIVIVALAGLSAAAHKPAYYDD--NMNQMVDQIVKSLTTKKELDPFKIEQTKVP
Glyd7(Q1M2M5)	MQYLSF SLLVALVGLTVA-VGPVFYDD--NMNQMVDQIIQSLTTKKELDPFKIESKTIP
	. * : * : : : . : * : :
Derp7(P49273)	FERHIGIIDLKGE LDMRNIQVRGLKQMKRVGDANVKSE DG VVKAHLLVGVHDDVVSMEYD
Derf7(Q26456)	FERHVGIVDFKGE LAMRNI EARGLKQMKRQGDANVKGEE GIVKAHLLIGVHDDIVSMEYD
Blot7(A1KXI4)	LDQKIGLVTFKGLKLIKD ATVTGLSRAA-----GDS DVKIHSNIE
Blot7(ASX95438)	LDRKIGLVTFKGLKLIKD ATVTGLSRAARVGD AKIHNENGDFVATLELGDS DVKMHSNIE
Tyrp7(B0KZL1)	IDRKIGAIHLKGTADLK ETKITGLSHVRRVGD ALLKNENG SFTAKLHLGDDNVKLFSDIS
Acas7(B0KZJ0)	FDRKLGALHIK GKIDLKNTRITGLSHVQRVGD AILENKNGSFQAKLHLGDNNVKLFSDIT
Lepd7(Q9U1G2)	IDKKIGLIHIKGSATIK NAVITGLSHISR RGD AKIDTDGGAF AATLKLGDKNIRIKTDLH
Glyd7(Q1M2M5)	IDKKVGLIHLKGSITIKNAEITGLSHIARNGDAKIDTANGGFAALLKLGDKNIRVKTDLI
	: : : : * : : * : : : * : : :
Derp7(P49273)	LAYKLGDLHPNTHVISDIQDFVVELSLEVSEEGNMTLTSFEVRQFANVVNHIGGLSILDP
Derf7(Q26456)	LAYKLGDLHPTTHVISDIQDFVVALSLEISDEGNITMTSFEVRQFANVVNHIGGLSILDP
Blot7(A1KXI4)	L--IVGLIQSHLTLDVDIGKLQIMFSAGLAAEG-PSVKDFHIDEFEAVRIHVHGLGPLDP
Blot7(ASX95438)	L--IVGLIQSHLTLDVDIGKLQIMFSAGLAAEG-PSVKDFHIDEFEAVRIHVHGLGPLDP
Tyrp7(B0KZL1)	LHFLHNIIHPNLKVEIDIGNIGVGFVTIGADGKPALKD FDI EEFKHVKI HVHGLGPLDH
Acas7(B0KZJ0)	IHLLSNIIHPHLKVEADIGNIGVKFGVAIGADGKPALKD FVEKLEHVKI HVHGLGPLDP
Lepd7(Q9U1G2)	LD-LGKIIHPNLKFEGHIGDIDMKLKLKLD AEGKPSLDQFEIDEFEQVELFIHGLGPLDP
Glyd7(Q1M2M5)	VN-IGKILHPHLKLESEIGDIDMKLKLALV-DGKPELDQSRLTNSSTFICRFTVWDHSIP
	: : : . . * : : : : * : . : : .
Derp7(P49273)	IFAVLSDVLT AIFQDTVRAEMTKVLAP-----AFKKELERNNQ-----
Derf7(Q26456)	IFGVLSDVLT AIFQD TVRKEMTKVLAP-----AFKRELEKN-----
Blot7(A1KXI4)	FIDIIGDAIIXLAXSQVREMISXMMRP-----IIESEVKKFLQNTTPAPAF
Blot7(ASX95438)	FIDIIGDAIITLANSQVREMISTMMRP-----IIESEVKKFLQNTTPAPAF
Tyrp7(B0KZL1)	LVDLIGEAYISLANTQARHMITGIVRP-----ILDQELKNFKMGGF-----
Acas7(B0KZJ0)	LVDLVADAFIAIGNTQARHLVTGIVRP-----IIESELKNFKLGG-----
Lepd7(Q9U1G2)	LVDVIADSFVKYFNPQARKLVTDMLKP-----ILVEEIKKLKLN-----
Glyd7(Q1M2M5)	LVDVIADAFVKYFNPQARKLVTDAMKPIAMKPILVEEIKKLKMG-----
	: . : : : . * : : : * : * : :

Fig.S1

EAEA	DDAANQLVDQ	VVDALKTQKG	FDPMHVGKHT	TELDRKIGLV	TFKGKLIKD
	ATVTGLSRAA	RVGDAKIHNE	NGDFVATLEL	GDSDVKMHSN	IELIVGLIQS
	HLTLDVDIGK	LQIMFSAGLA	AEGPSVKDFH	IDEFEAVRIH	VHGLGPLDPF
	IDIIGDAIIT	LANSQVREMI	STMMRPIIES	EVKKFLQNTT	PAPAF

Fig.S2

Supplementary informations

1)Supplementary methods

rBlo t 7 identification by mass spectrometry (MS)

Recombinant Blo t 7 sample (1.5 μ g) was separated using reducing SDS-PAGE onto a 4-12% polyacrylamide NuPAGE Novex Bis-Tris precast gel (Life Technologies). Next, band corresponding to rBlo t 7 was excised and subjected to “in-gel” digestion. Briefly, protein band was destained for 1 min with 50% acetonitrile (ACN) in 50 mM NH_4HCO_3 , then dehydrated with 100% ACN. The reduction step was performed with 25 mM dithiothreitol at 50°C for 20 min followed by alkylation step carried out with 55 mM iodoacetamide for 20 min at room temperature in the dark. Next, gel pieces were dehydrated with 100% ACN and dried before the addition of 2 μ L of trypsin prepared at 1 ng/ μ L (Promega). Then, 30 μ L of 0.01% ProteaseMAX surfactant (Promega) prepared in 50 mM NH_4HCO_3 was added to cover the gel pieces and the sample was incubated at 37°C for 105 min. Finally, enzymatic digestion was stopped with 1% formic acid.

Extracted peptides (15 μ L) were loaded on an Accucore C_{18} column (C_{18} , 2.6 μ m, 150 Å, 15 cm x 75 μ m i.d., Thermo Fischer Scientific) with a flow rate of 450 μ L/min and eluted with a gradient of ACN. MS analysis was performed using an Impact HD mass spectrometer (Bruker Daltonics) equipped with a Captive Spray source (Bruker Daltonics). Acquisitions were performed in positive mode with capillary voltage set at 1300 V. MS spectra were acquired over the m/z range 110-2000 with a scan rate of 2 Hz. MS/MS spectra were acquired using the Intensity Dependent Acquisition Speed (IDAS) mode with a MS-MS/MS cycle time fixed at 2 s, a MS/MS trigger threshold set at 4.3×10^3 counts (cts) and a MS/MS acquisition speed defined as follow: 4 Hz for precursor intensity below 2.5×10^3 cts and 16 Hz for precursor intensity above 2.5×10^3

cts. Active exclusion list was enabled and precursor ions were excluded after one spectrum for 0.20 min.

Protein/peptide identification was performed with PEAKS software v8 (Bioinformatics Solutions Inc.) with the following parameters: peptide tolerance of 10 ppm (0.05 Da for fragment ions), trypsin enzyme, one non-specific cleavage and 3 missed cleavages allowed, carbamidomethylation of cysteine as a fixed modification, methionine oxidation and asparagine deamidation as variable modifications. Protein identifications were confirmed with the application of a 0.1% peptide-spectrum match false discovery rate (estimated with the decoy fusion method) and manual inspection of MS/MS data.

2)Supplementary Figure legends

Figure S1.

The amino acid sequence alignment of the cloned mature Blo t 7 (Blot7 ASX95438) and the Blo t 7 reference clone (Blot7 Ref UniprotKB A1KXI4 accession number) with other group 7 mite allergens: Tyr p 7 (UniprotKB B0KZL1) , Aca s 7 (UniprotKB B0KZJ0) , Lep d 7 (UniprotKB Q9U1G2), Gly d 7 (UniprotKB Q1M2M5), Der p 7 (UniprotKB P49273) and Der f 7(UniprotKB Q26456) by using clustalO. * represents identities among the sequences

Figure S2

MS sequence coverage of rBlo t 7 tryptic digestion

Amino acid sequence of expressed rBlo t 7, the residues EAEA in bold corresponding to the C-terminus of the mating factor leader sequence. Grey highlighted sequence represents peptides unambiguously identified by MS/MS.