

Expression of the recombinant Blo t 1 allergen from *Blomia tropicalis* in *Pichia pastoris* and its IgE reactivity with Vietnamese allergic sera

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Abstract

Blomia tropicalis, a common house dust mite, serves as a significant indoor allergen in tropical climates such as Vietnam. Precise identification of key allergens plays a crucial role in enabling component-resolved diagnostics and applying allergen-specific immunotherapies. In this study, we investigated the recombinant expression and immunological evaluation of Blo t 1, a cysteine protease allergen obtained from *B. tropicalis* isolated in Vietnam. The *Blo t 1* gene, after codon optimization and signal peptide removal, was cloned into the pPICZα A vector and expressed in *Pichia pastoris*. IgE-binding ability of the recombinant Blo t 1 was assessed using sera from 53 subjects (21 males, 32 females; aged 6–72 years), including 29 patients sensitized to *B. tropicalis* and 24 non-allergic controls. The purified recombinant protein with an approximate molecular weight of 47 kDa, including ~37 kDa from the truncated Blo t 1 sequence, a C-terminal His-tag, and ~10 kDa from the α-factor secretion signal, was purified using His-tag affinity chromatography. Dot blot results indicated that 23 out of the 29 allergic sera (79.3%) displayed IgE reactivity to the Blo t 1, which was further validated by western blot analysis. These findings support that Blo t 1 is a major allergen in the Vietnamese population and highlight its potential as a molecular tool for improving the accuracy of allergy diagnostics and the development of targeted immunotherapies in Vietnam. This work also adds to the limited data on *B. tropicalis* allergens expressed in yeast systems.

Keywords: Allergen, *Blomia tropicalis*, Blo t 1, IgE-binding reactivity, *Pichia pastoris*

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Introduction

House dust mites (HDMs) are recognized as among the most frequent indoor allergen sources and are strongly associated with allergic diseases such as asthma, allergic rhinitis and atopic dermatitis. In recent years, allergen-specific immunotherapy (AIT) has gained attention as an effective approach for HDM allergy, relying either on crude extracts or recombinant allergens. Recombinant allergens, compared to natural extracts, exhibit many benefits including consistency, defined composition and improved safety profile, thereby improving their applicability in both diagnostic and therapeutic purposes (Kim, 2023; Vrtala et al., 2014). Among heterologous expression systems, *Pichia pastoris* is widely employed as an excellent host for recombinant protein production due to its ability to perform efficient secretion and compatibility with eukaryotic post-translational processing, including glycosylation (Looser et al., 2015; Schmidt, 2004).

Blomia tropicalis is prevalent across tropical and subtropical zones globally. In Asian populations, sensitization to HDMs can be as high as 90%, which is considerably higher than that observed in Western countries (Tham et al., 2016). Particularly, in Vietnam, sensitization rate to *B. tropicalis* is notably prevalent. A recent study indicates that in southern Vietnam, 57.7% of allergic rhinitis patients had a positive skin prick test (SPT) to *B. tropicalis*, highlighting its dominant contribution to HDM-related allergies (Trinh et al., 2023). A genomic study identified 42 allergen groups in *B. tropicalis*, enhancing insights into its allergen profile (Xiong et al., 2024).

Blo t 1, a group - 1 allergen from *B. tropicalis*, is classified as a cysteine protease and has been recognized in many studies as a significant allergen. However, while Blo t 5 and Blo t 21 are often identified as dominant allergens, recent evidence indicates Blo t 1 also possesses high IgE reactivity in tropical populations. However, the prevalence and sensitization pattern of Blo t 1 may vary across regions due to differences in genetic polymorphisms, environmental factors and HDM biodiversity (Santos da Silva et al., 2017; Smith et al., 2001).

Although recombinant *B. tropicalis* allergens have been successfully expressed in *Escherichia coli* and *Pichia pastoris*, information specific to Vietnamese isolates, particularly Blo t 1 is still limited. Several studies note that Blo t 1 expressed in *E. coli* often leads

to inclusion bodies and absence of post-translational modifications (Pomes et al., 2018).

Accordingly, this study aimed to express and purify recombinant Blo t 1 from gene sequences amplified from *B. tropicalis* collected in Vietnam, using the *Pichia pastoris* system. We also examined its IgE-binding reactivity using serum samples from allergic patients and non-allergic controls. These findings not only enrich the allergen profile of *B. tropicalis* but also provide a foundation for component-resolved diagnostics and allergen-specific immunotherapy strategies adapted to the Vietnamese population.

Material and Methods

House dust mite sample, bacterial strains, vectors and reagents

Specimens of *Blomia tropicalis* were collected from indoor environments in Vietnam. Mites were identified based on morphological characteristics and confirmed by molecular methods. This identification was performed in our previous study on the characterization of *B. tropicalis* in Vietnam. All collected mite samples were stored at -80°C until experimental use (Chi et al., 2024b).

pPICZα A vector, strain X33 of *Pichia pastoris*, and other reagents were purchased from Invitrogen, Thermo Fisher Scientific, and Sigma.

Human sera

Serum samples were collected from 53 individuals (21 males, 32 females; aged 6 - 72 years) at the Allergy and Clinical Immunology Center of Bach Mai Hospital, Hanoi, Vietnam. Participants were divided into two groups: (i) Allergic group (n = 29): Patients with confirmed sensitization to *B. tropicalis*, defined as specific IgE $\geq$ 0.35 kU/L (measured by the ImmunoCAP™ system, Thermo Fisher Scientific); (ii) Control group (n = 24): Individuals with no history of atopic diseases and no detectable IgE to common aeroallergens. Individuals who had recently taken immunosuppressive drugs (e.g., corticosteroids) or antihistamines within four weeks before sampling were excluded. The Research Ethics Committee of Bach Mai Hospital granted ethical approval (Approval No.: BMH-REC/2023-045) and written informed consent was obtained from all participants or their legal guardians. All procedures were performed in accordance with the Declaration of Helsinki.

Expression and purification of the recombinant Blo t 1

Based on the gene sequence encoding *Blo t 1* allergen amplified from *B. tropicalis* specimens collected in Vietnam, as reported in our previous study on local mite allergen characterization (Chi et al., 2024a), codon usage was optimized by using GenScript Rare Codon Analysis Tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis), and the signal sequence (amino acids 1-18) was removed. The resulting truncated cDNA sequence was artificially synthesized and inserted into the pPICZ α A vector by using *EcoRI* and *NotI* restriction sites. Following sequence verification, the plasmid construct was linearized with *SacI* and transformed into *Pichia pastoris* X33 cells by electroporation (1.8 kV, 4.5ms-5ms pulse duration). Transformants were selected on YPD agar plates supplemented with Zeocin (1% yeast extract 2% peptone 2% dextrose (glucose) + 2% agar and 100 μ g/mL zeocin). Consequently, PCR clone screening was performed on the transformant, by using AOX1 universal forward primer (5'-GACTGGTTC CAATTGACAAGC-3') and an insert-specific reverse primer (5'-TAACGTTGCGTAGGTCACAAA-3'). The PCR cycling conditions were: 95°C for 5 min, 30 cycles of 95°C for 30 s, 62°C for 40 s, and 72°C for 1 min; followed by a final extension at 72°C for 10 min. The expected amplicon size was approximately 1304 bp, including 945 bp from the Blo t 1 insert and 359 bp from the vector backbone. Colonies with positive PCR results were selected for protein expression. The selected clones were inoculated into BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate, 1% glycerol, 0.4 mg/l biotin, and 0.1 M potassium phosphate, pH 6.0) and cultured overnight at 30°C with shaking. After harvesting by centrifugation at 1000 rpm for 5 minutes, the cells were resuspended in BMMY induction medium (same components as BMGY but without glycerol), and 1% (v/v) methanol was added every 24 hours for 4 days at 30°C (Francis and Rebecca, 2010). After induction, recombinant Blo t 1 expression was assessed in both culture supernatants and cell lysates by 12% SDS-PAGE stained with Coomassie Brilliant Blue G-250 and confirmed by Western blot using a monoclonal anti-His tag antibody (MA1-21315, Thermo Fisher Scientific). The culture supernatant was filtered through a 0.45 μ m PVDF membrane and loaded onto a 5 mL HisTrapTM Ni²⁺ affinity column (GE Healthcare). Elution was

performed with the denaturing buffer (45 mM sodium phosphate, 5.4 M guanidine-HCl, 270 mM NaCl, and 300 mM imidazole), and eluted fractions were analyzed by SDS-PAGE and further confirmed by Western blot using the anti-His tag antibody. The total protein concentration was measured using Lowry method. (Lowry et al., 1951).

Dot blotting

Approximately 5 μ g of purified recombinant Blo t 1 protein was applied as individual spots onto PVDF membranes. Blocking was carried out for 1 hour at room temperature with PBS containing 0.05% Tween-20 and 1% bovine serum albumin (BSA). After blocking, the membranes were incubated overnight at 4°C with serum samples from 53 individuals, including 29 allergic patients and 24 non-allergic controls.

After incubation, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated mouse anti-human IgE antibody (diluted 1:2000 in PBS-Tween) for 1 hour at 37°C. IgE-antigen interactions were visualized using TMB substrate (0.1 mg/mL). Dot intensities were captured using the ImageQuantTM LAS 500 imaging system (GE Healthcare).

To determine positive IgE reactivity, a cut-off value was calculated as the mean signal intensity of the 24 control sera plus two standard deviations (mean + 2SD), resulting in a threshold of 7700 arbitrary units. Based on the signal intensity, IgE responses were classified into four categories: negative (<8,000), low (8,000–14,000), medium (14,000–20,000), and high (>20,000). All dot blot experiments were performed in triplicate (Li et al., 2022).

Western blot

To confirm the IgE-binding reactivity observed in the dot blot assay, selected serum samples that tested positive were randomly pooled into four groups: three pools containing six sera each (Groups 1–3) and one pool containing five sera (Group 4). Additionally, a pooled sample from the 24 non-allergic control sera was used as a negative control.

Recombinant Blo t 1 protein was separated by 12% SDS-PAGE and transferred onto PVDF membranes using a standard wet transfer protocol. Blocking was performed in PBS containing 0.05% Tween-20 and 1% BSA for 1 hour at room temperature, followed by overnight incubation at 4°C with the pooled sera as primary antibodies. After washing, the membranes

were incubated for 1 hour at 37 °C with HRP-conjugated mouse anti-human IgE antibody (diluted 1:2000 in PBS-Tween). The signal was developed using TMB substrate and bands were visualized with the ImageQuant™ LAS 500 system. This assay served to confirm the specificity and molecular weight of IgE-reactive proteins (Li et al., 2022).

Statistical analysis

Data were presented as mean value $\pm$ standard error of the mean (SEM). Statistical analyses were performed using IBM SPSS Statistics 20 (IBM Corp., Armok, NY, USA). Group comparison were determined by the Student's T-test, and $P < 0.05$ was considered to be a statistically significant difference.

Results

Transformation of Blo t 1 into strain X33 of *Pichia pastoris*

The codon-optimized gene encoding the Blo t 1 allergen, excluding its native signal peptide (amino acids 1–18), was synthesized and cloned into the pPICZ α A expression vector using *Eco*RI and *Not*I

restriction sites. A C-terminal His-tag was incorporated to enable purification and detection. Rare codons were replaced with bias codons having a minimum usage frequency of 6.6% to improve translation efficiency and maximize recombinant Blo t 1 protein expression in *P. pastoris*. After transformation into *Escherichia coli* DH5 α , recombinant plasmids were isolated and verified by Sanger sequencing, confirming the correct insertion and orientation of the *Blo t 1* gene.

Before transformation into *Pichia pastoris* X33, the recombinant plasmid was linearized using *Sac*I to promote genomic integration at the AOX1 locus. The Mut⁺ phenotype of the transformants was confirmed (data not shown). The result of PCR clone screening was subsequently performed as described in the Methods section. A clear band of 1304 bp, comprising 945 bp from the Blo t 1 insert and 359 bp from the vector residues, was observed in all screened clones, indicating successful integration of the gene into the host genome. Clone No. 6, which exhibited a strong PCR signal and stable growth, was selected for subsequent protein expression experiments [Figure 1].

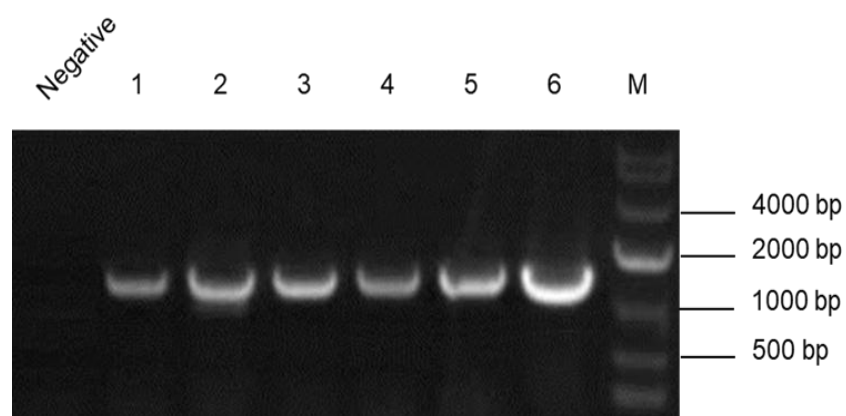


Figure-1. PCR clone screening of the *Blo t 1* gene by electrophoresis on 1% agarose gel.

1, 2, 3...: PCR product of respective clones, M: DNA marker; Negative: negative control, in which water was used instead of DNA template

Expression of recombinant Blo t 1

The selected *P. pastoris* clone (No.6) was cultivated in BMGY medium and induced with 1% methanol in BMMY medium for four consecutive days to promote expression of Blo t 1. Protein expression was analyzed in both the culture supernatant and intracellular fractions collected daily during the induction period. SDS-PAGE analysis showed a distinct protein band of approximately 47 kDa in the culture supernatant, corresponding to the expected molecular weight of the recombinant Blo t 1 protein, and no detectable band was observed in the intracellular fraction, suggesting efficient secretion of the protein on day 4 of induction. According to the *pPICZα A, B, and C Pichia Expression Vectors* manual (Invitrogen, 2010), recombinant proteins are expressed as fusions to the *S. cerevisiae* α -factor preproleader signal sequence,

which includes a 19-residue pre-region, a 64-residue pro-region, and a short spacer containing the KEX2 and STE13 protease cleavage sites. While this signal is designed to be cleaved by *Kex2* protease during secretion, incomplete cleavage can occur depending on the target protein's structure or sequence context (Aza et al., 2021; Invitrogen, 2010). In our case, the observed size (~47 kDa) suggests that the α -factor sequence was not fully removed, resulting in an additional ~10 kDa along with the His-tag [Figure 2A]. Western blot analysis using an anti-His tag monoclonal antibody confirmed the presence of the 47 kDa recombinant protein in the supernatant fraction, confirming effective secretion of the protein Blo t 1 in *P. pastoris*. [Figure 2B]. The recombinant protein Blo t 1 was expressed and secreted with a yield of 400 mg protein/L (data not shown).

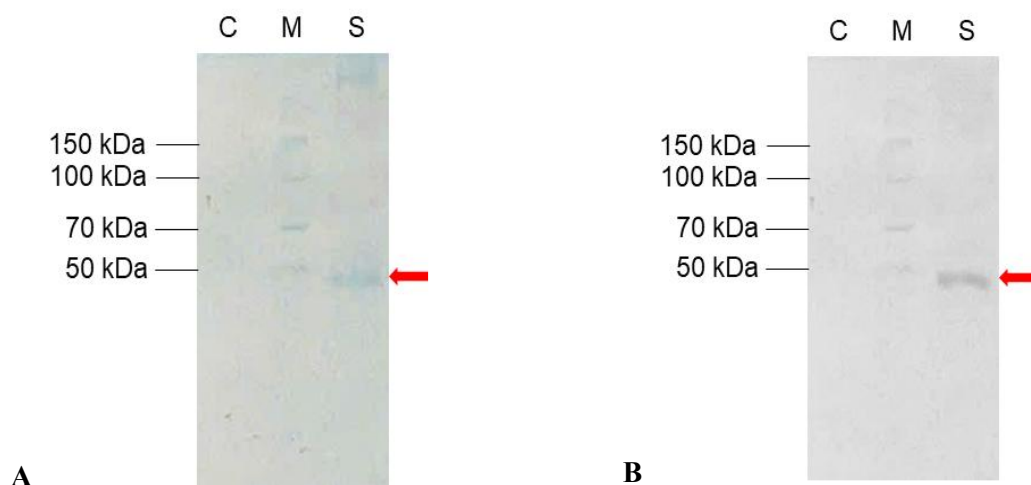


Figure-2. Electrophoresis analysis of expressed recombinant Blo t 1 in 12% SDS-PAGE, stained with Coomassie Brilliant Blue G 250 (A) and Western blot using His-tag antibody (B). Arrows indicated the recombinant Blo t 1 S: culture supernatant; C: cell pellet samples; M: PageRuler™ Unstained Broad Range Protein Ladder; (-): negative control, which is elution buffer.

Purification of recombinant protein Blo t 1

The recombinant protein was eluted with 300 mM imidazole in elution buffer. SDS-PAGE analysis of the eluted fractions showed a dominant single band at approximately 47 kDa, consistent with the predicted molecular weight of recombinant Blo t 1 [Figure 3A].

This band was confirmed by western blot using an anti-His tag monoclonal antibody, which clearly detected the His-tagged Blo t 1 protein [Figure 3B]. The result of the Western blot using the His-tag antibody confirmed that the recombinant protein Blo t 1 was successfully purified.

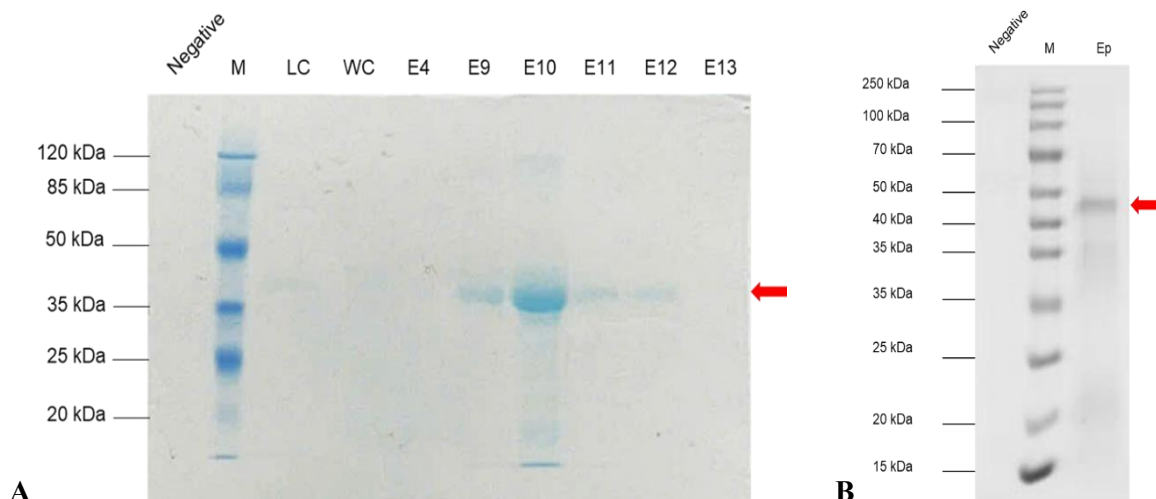


Figure-3. Purification of recombinant Blo t 1 in 12% SDS-PAGE, stained with Coomassie G 250 Brilliant Blue (A) and Western blot using His-tag antibody (B). Arrows indicated the recombinant Blo t 1

LC: the loading sample obtained from culture broth after centrifuging at 13000 rpm for 15 minutes and filtering via PVDF filter of 0.45 μ m; WC: last washing fraction; E2-E6: elution fractions; Ep: pooled elution fractions, containing the recombinant protein Blo t 1; M: PageRuler™ Unstained Broad Range Protein Ladder; Negative: negative control, which is elution buffer.

IgE reactivity of recombinant Blo t 1 with sera from allergic patients

Dot blot assays were performed using 29 serum samples from patients with IgE specific to *B. tropicalis* (≥ 0.35 kU/L) and 24 sera from non-allergic individuals. As shown in Figure 4A, 23 out of 29 allergic sera (79.3%) reacted positively with recombinant Blo t 1, while all control sera were negative. IgE signal intensities of the positive allergic group were significantly higher than those of both the negative allergic group and the non-allergic controls ($P < 0.0001$). A significant difference was also

observed between the positive and negative allergic subgroups ($P < 0.0001$) [Figure 4B]. To confirm the dot blot results, sera from 23 allergic individuals that showed positive IgE reactivity were randomly pooled into four groups, while one pooled sample from 24 non-allergic individuals served as the negative control. Western blot analysis showed a distinct band at ~ 47 kDa in all four allergic serum pools, corresponding to the recombinant Blo t 1 protein. No IgE reactivity was detected with the control serum pool. These results support the dot blot finding and confirm the IgE-binding capacity of recombinant Blo t 1 [Figure 4C].

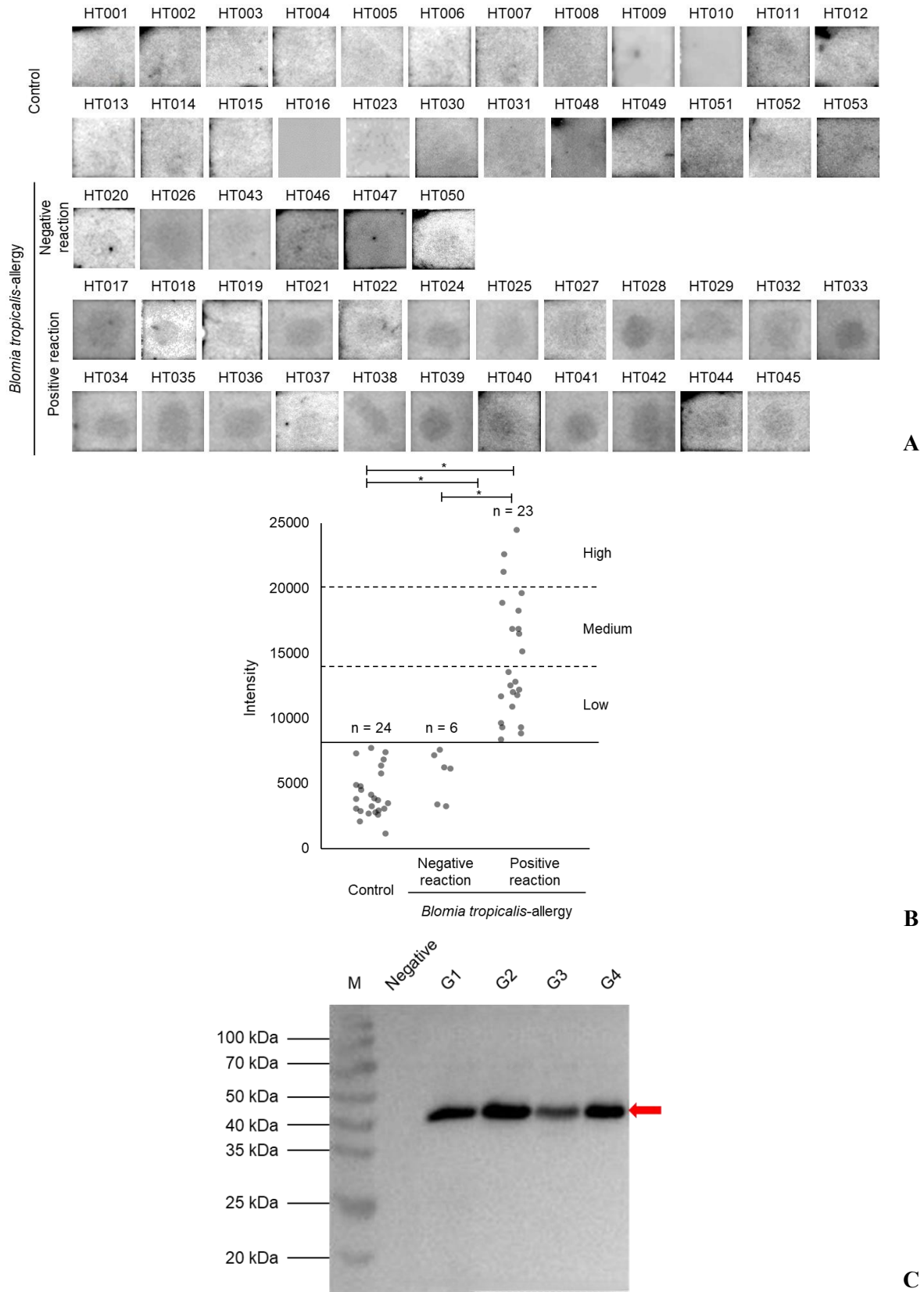


Figure-4. Dot blot and western blot analysis of the recombinant Blo t 1 with the serum samples.

(A) Dot blot of the recombinant Blo t 1 with 29 human sera, containing IgE positive to *B. tropicalis* and 24 sera of non-allergic control subjects; (B) Scatter plot of the densities of specific IgE-binding allergic sera to *B. tropicalis* (n = 29) and non-allergic control subjects (n = 24) to the recombinant Blo t 1. Data are presented as the mean $\pm$ standard error of the mean. *: $P < 0.0001$; (C) Western blot of the recombinant Blo t 1 with control sera and allergic sera, which showed positive reactions to the recombinant Blo t 1 determined by the dot blot technique. G1 – Group 1 (sera HT025, HT027-029, HT032, HT033); G2 – Group 2 (sera HT017-019, HT021, HT022, HT024); G3 – Group 3 (sera HT034-039); G4 – Group 4 (sera HT040-HT042, HT044, HT045); Negative: pooled sera of the control group.

Discussion

House dust mite (HDM) allergy is an increasing concern worldwide, as it can lead to various respiratory conditions such as asthma, allergic rhinitis and atopic dermatitis, which can significantly reduce quality of life and impose socioeconomic pressures on communities and national healthcare systems. Allergen-specific immunotherapy (AIT) has recently emerged as a promising therapeutic approach for managing HDM allergy. However, accurate identification of allergenic profiles is critical for the success of AIT, as it requires allergens of high purity and well-defined molecular characteristics. In this context, recombinant allergens clear benefits compared to crude extracts, including consistent purity, adequate yield and batch-to-batch reproducibility, making them suitable for component-resolved diagnostics while minimizing side effects and improving diagnostic accuracy (Vrtala et al., 2014; Xiong et al., 2023).

Among the allergen groups of *B. tropicalis*, Blo t 1 is classified as a cysteine protease, and it is considered an important allergen group, especially in countries with tropical and subtropical climates. Only a few studies have investigated the recombinant expression of Blo t 1 in *E. coli* and *P. pastoris* (Mora et al., 2003; Fonseca-Fonseca and Diaz, 2003; Cheong et al., 2003). While *E. coli* has been widely used for protein expression, its limitations, including the formation of inclusion bodies and lack of post-translational modifications, have prompted the use of *P. pastoris* as a more suitable alternative, especially for Group 1 and 2 HDM allergens (Vrtala et al., 2014). Unlike *S. cerevisiae*, *P. pastoris* can utilize methanol as a carbon source, achieve high cell density, and support correct protein folding with appropriate post-translational modifications (Vieira Gomes et al., 2018; Daly and Hearn, 2005). Additionally, the AOX1 promoter in *P. pastoris*, which drives high-level expression and is strongly inducible by methanol, is commonly used for producing recombinant protein (Turkanoglu Ozcelik et al., 2019; Vogl and Glieder, 2013).

In this study, the recombinant Blo t 1 protein was expressed in *P. pastoris* using a truncated gene lacking the native signal peptide, yielding a protein of approximately 37 kDa, which is comparable to that reported by Cheong et al. (2003). The expressed protein preserved key structural features of cysteine proteases, including conserved active site residues and six cysteine residues. Using the NetNGlyc 1.0 server, we identified 4 putative glycosylation sites, including 3 sites within the α -factor signal sequence and 1 site in the Blo t 1 region (Gupta and Brunak, 2002). Based on the predicted molecular weight (~1.08 kDa) of the carbohydrate side chains and the fact that the core amino acid sequence of Blo t 1 was preserved, the glycosylation was unlikely to markedly alter IgE-binding properties to Vietnamese allergic sera. In addition, our recombinant Blo t 1 protein was secreted into the culture medium at a yield of approximately 400 mg/L, significantly exceeding the 100 mg/L yield reported in Cheong's study (Cheong et al., 2003).

To assess the allergenic potential of the recombinant protein, we employed the dot blot technique, which offers simplicity, cost-effectiveness, reproducibility, and sensitivity. This method has been widely applied for evaluating IgE-binding and cross-reactivity of recombinant mite allergens (Fonseca-Fonseca and Diaz, 2003; Ramos et al., 2001). The dot blot and Western blot are only semi-quantitative techniques; therefore, we performed a comparison between dot blot intensities and clinical IgE titers measured by the ImmunoCAP™ system (Thermo Fisher Scientific) to give more quantitative data for Blo t 1 reactivity. However, the ImmunoCAP™ assay uses whole allergens or allergen mixes not derived from local *Blomia tropicalis*, which may differ in amino acid sequence and post-translational modifications from the *B. tropicalis* in Vietnam, thereby influencing IgE-binding epitopes. This was also a reason that commercially available Blo t 1 was not included in our experiments. After purification under denaturing conditions, our recombinant Blo t 1 protein reacted with 23 out of 29 sera (79.3%) from patients sensitized to *B. tropicalis*, while all 24 control sera were

negative. This reactivity rate was higher than the 71.74% previously reported by Fonseca-Fonseca and Diaz (2003) for Blo t 1 expressed in *E. coli*. Besides, our observed IgE reactivity was lower than in asthmatic children (90%) but higher than in allergic adults (65%) for Blo t 1 expressed in *P. pastoris* in Cheong's study (Cheong et al., 2003). These differences may be related to the age of the allergic patients, as our patient cohort ranged from 7 to 72 years. In addition, this sensitized rate of recombinant Blo t 1 in our study was comparable to the rates reported for recombinant Blo t 5 (82.9%) and Blo t 21 (80.0%) in sera from 35 Brazilian children with or at risk of asthma (Carvalho et al, 2013). While Blo t 5 and Blo t 21 are commonly identified as the major allergens of *B. tropicalis*, a study by Xiong et al. (2024) in Hong Kong showed that the sensitization rates of recombinant Blo t 26 and recombinant Blo t 18 displayed the highest positive values of 88.46% and 73.08%, respectively (Xiong et al., 2024). Differences in allergen sensitization could arise from genetic polymorphisms in allergen-encoding genes, fluctuations in mite species density, and environmental and genetic factors. The high rate of sensitization to recombinant Blo t 1 in our study further supports the hypothesis of regional prevalence in HDM allergen profiles (Xiong et al., 2024; Santos da Silva et al., 2017).

These findings suggest that Blo t 1 could be considered as a major allergen in the Vietnamese population, based on the high IgE reactivity observed in the patient sera (Lowenstein, 1978). Our results regarding the successful expression, purification, and IgE reactivity of recombinant Blo t 1 provide a basis for improving diagnosis and the development of targeted allergen-specific immunotherapy for sensitized patients in Vietnam. Furthermore, it expands the currently limited data of *B. tropicalis* in general and Blo t 1 in particular.

Conclusion

This study reports for the first time, the successful expression of recombinant Blo t 1 derived from *Blomia tropicalis* isolated in Vietnam using the *Pichia pastoris*, followed by purification through His-tag affinity chromatography. The purified Blo t 1 protein showed strong IgE-binding reactivity, with 79.3% (23/29) of sera from *B. tropicalis*-sensitized patients showing positive responses, while no reactivity was observed in non-allergic controls. Western blot analysis further verified the specificity of this IgE

recognition. The results highlight Blo t 1 as a major allergen in the studied Vietnamese cohort. In conclusion, this work contributes valuable data on local allergen profile of Blo t 1 and supports its potential application in improving diagnostic precision and guiding allergen-specific immunotherapy for *B. tropicalis* allergy in Vietnam.

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Declaration on use of generative AI tools

We hereby declare that AI tools were utilized during the manuscript preparation process only for the purposes of: (i) checking spelling and grammatical structure, (ii) verifying the consistency and format of reference citations.

The authors maintained full responsibility for all content, and every section of the manuscript was carefully reviewed and revised to match current academic publishing guidelines.

Contribution of Authors

Uyen NQ: Conceptualization, methodology, writing original draft and writing review and editing.

Chi NTL: Data curation, formal analysis, software and visualization.

Phuong VM: Data curation and formal analysis.

Truong NH & Phuong NH: Investigation and editing

Vinh HV: Supervision and writing, review and editing.

All authors read and approved the final draft.

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