Immunoglobulin E Binding Activity of Recombinant and Native Blo t 11 Allergens

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Abstract. The full-length Blo t 11 allergen was expressed as a GST-fusion protein in *E. coli* by IPTG induction and purified by affinity chromatography using an Agarose-GLutathione column. Fractions containing recombinant Blo t 11, resolved as 128 kD protein, were pooled and quantified using the BioRad Protein Assay. Native Blo t 11 was purified from a *Blomia tropicalis* (Bt) aqueous extract using a Blo t 11 monoclonal antibody affinity column. Two milliliters of the Bt extract yielded 467 ng of native Blo t 11 which was resolved as ~60 kD in an SDS-PAGE. The immunoglobulin E binding activity of the purified allergens was determined by enzyme-linked immunosorbent assay using a panel of 110 atopic Filipino patients' sera and 85 non-atopic sera. The native Blo t 11 allergen registered an IgE reactivity of 63% (69/110) while the recombinant Blo t 11 registered 57% (63/110). The Blo t 11 allergen can inhibit up to 63% IgE binding activity of Bt aqueous extract. Results presented herein demonstrate the clinical importance of Blo t 11 allergen as a triggering factor for allergies among Filipino atopic patients thus a potential reagent for diagnosis and immunotherapy of house dust mite allergy in the local setting.

Keywords: Blo t 11, house dust mite, *Blomia tropicalis*, allergen

INTRODUCTION

Immediate hypersensitivity reactions to substances in the environment can cause a number of common allergic diseases including allergic asthma, allergic rhinitis, and atopic dermatitis. Allergies are chronic inflammatory diseases, with periods of remission and flare-ups that adversely affects the quality of life of patients and their families. Epidemiological studies show that 10-40% of the world population is afflicted with allergic diseases [1] and the costs to public health and the economy is substantial and growing [2]. Most often, allergies are caused by the immune reaction to common inhaled proteins called allergens. The most frequently implicated indoor allergen sources are the house dust mites (HDM). HDM have been well recognized to play an important role in the pathogenesis of allergic diseases [3].
Blot 11 is a 2625 bp paramyosin homolog allergen gene isolated from *Blomia tropicalis* (Bt) [4], an HDM species ubiquitously found in tropical and subtropical regions [5]. Allergens isolated from Bt exhibit significant immunoglobulin E (IgE)–binding activities demonstrating the clinical importance of this HDM species as an important source of major allergens that trigger allergic reactions among atopic individuals [6]. Recombinant and native Blot 11 exhibits IgE reactivity in more than 50% of Singaporean and Malaysian allergic patients’ sera tested [7-9] but its clinical importance has never been shown among Filipino allergic patients. The IgE-binding activity of the recombinant and native Blot 11 allergen among Filipino allergic patients is comparatively shown in this study.

**EXPERIMENTAL**

**Study Subjects.** The study subjects included 110 doctor-diagnosed Filipino Bt-allergic patients with allergic asthma, atopic dermatitis and/or allergic rhinitis seen at the University of Santo Tomas Hospital’s Allergy and Clinical Immunology and Otorhinolaryngology Sections of the Out-Patient Department (OPD) and Dermatology clinics; and 85 healthy, non-atopic Filipino subjects. Subjects were asked to fill out a Patient Information Sheet that asked for basic information and their medical history with allergy. The patients’ phenotypes considered were based on the answers to questions from international standardized questionnaires of the International Study of Asthma and Allergy in Childhood (ISAAC) and the International Primary Care Airways Group (IPAG) core questions. Control subjects include individuals without history of allergy and neither do have immediate relatives with it.

**Collection of Blood Samples.** An average of 5 mL whole blood sample was collected from each subject by the Medical Technologists of the OPD, UST Hospital using a sterile 5 mL Terumo syringe with a gauge 21 needle. Blood samples were allowed to stand for one hour at room temperature. Serum samples were isolated by centrifugation and transferred into tubes in aliquots and stored at –20°C until use.

**Expression and Purification of rBlot 11.** The recombinant Blot 11 allergen was produced as previously described [4]. In brief, a clone of *E. coli* (BL21) containing the Blot 11 gene in pGEX-4T-1 vector was cultured in LB broth with Ampicillin. The allergen was expressed as a glutathione S-transferase (GST) fusion protein by isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Purification of recombinant proteins was performed by affinity chromatography using a Glutathione-Agarose column (Sigma, USA) according to the protocol described in the GST Purification Module (Pharmacia Biotech, UK). Purified protein sample was quantified by the BioRad Protein Assay (BioRad, USA), and was analyzed by SDS-PAGE.

**HDM Aqueous Extract Preparations.** Ten grams (wet weight) of frozen Bt was wrapped in aluminum foil and stored in a -80°C for 20 minutes. The frozen mites were mechanically ground using a precooled mortar and pestle for 30 minutes. Extraction was performed using a total volume of 50 mL of 1X TBS with 2 mM PMSF (Sigma-Aldrich, Saint Louis, MO, USA) and 1 mM EDTA (BioRad, Hercules, CA, USA) added slowly while grinding the mites. The mite extract suspension was incubated for 16-20 hours at 4°C with constant shaking. After centrifugation at 17,000 rpm for 20 minutes using the Sorvall® Ultra Pro 80 centrifuge (Kendro Lab. Products, Newtown, CT, USA), the supernatant was collected and quantified by BioRad Protein Assay as described below. Aqueous Bt protein extracts were stored in aliquots at -80°C until use.

**Isolation of Native Blot 11.** Purification of the native Blot 11 allergen from an aqueous Bt extract was performed using an immuno-affinity column chromatography as previous described [10]. Briefly, a column of Sepharose beads coupled with P2E7-G1 Blot 11 monoclonal antibody was thoroughly washed with 1x TBS until the OD at 280 nm was 0. A 500 mg Bt aqueous extract was then
gently passed through the column followed by another wash as described. Bound nBlo t 11 was eluted in 1.0 mL fractions using 5 mM Glycine, 50% Ethylene Glycol (Merck, Darmstadt, Germany), pH 11. Six hundred mL of each fraction were precipitated using trichloroacetic acid, TCA, (Merck, Darmstadt, Germany) and analyzed by denaturing SDS-PAGE followed by silver staining. Fractions with native Blo t 11 were pooled and quantified using the BioRad Protein Assay as described below.

**BioRad DC Protein Assay.** The recombinant Blo t 11 allergen and the Bt aqueous extract used in this study were quantitated by BioRad DC Protein Assay (BioRad, Hercules, CA, USA). Protein samples and diluted BSA (Sigma-Aldrich, Saint Louis, MO, USA) standards in duplicates (160 µL) and 40 µL of BioRad dye reagent were mixed in microtiter plates; incubated for 15 minutes in the dark; followed by optical density reading at 600 nm using a UV/VIS Spectrophotometer. A standard curve was prepared and was used as a basis in the calculation of total protein content of each aqueous extract.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.** Recombinant Blo t 11 and Bt aqueous extract were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein samples were mixed 1:1 with 2X SDS-PAGE sample buffer and were boiled for 10 minutes. Samples were separated on a 15% Tris-Glycine gel using the Mini PROTEAN electrophoresis system (BioRad, Hercules, CA, USA). Gel was run at 110 Volts for 90 minutes. Broad Range Marker (BioRad, Hercules, CA, USA) was used as standard. SDS-PAGE gel was stained with Coomassie brilliant blue R-250 (BDH laboratory Supplies, Poole, England).

**Western Blot Analysis.** The reactivity of the purified recombinant and native Blo t 11 allergens against the Blo t 11 monoclonal antibody P2E7-G1 [10] was determined by Western blot analysis. In brief, rBlo t 11-GST (0.4 µg), nBlo t 11 (0.4 µg), and GST (0.4 µg) were electrophoresed on a 15% Tris-Glycine gel and electroblotted onto Hybond C nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, England) using the MiniProtean 3 cell (BioRad, Hercules, CA, USA) at 110 V for 1 hour. The membrane was blocked with 5% skimmed milk in PBS-T. After overnight incubation with P2E7-G1 monoclonal antibody (1000x dilution) in blocking buffer at 4°C, the membrane was incubated with biotinylated anti-mouse IgG (Sigma-Aldrich, Saint Louis, MO, USA). Finally, the membrane was incubated with peroxidase-conjugated ExtrAvidin (Sigma-Aldrich, Saint Louis, MO, USA). The membrane was washed 6x with PBS-T between steps. Results were detected incubation with Alkaline Phosphatase Color Development Solution (BioRad).

**Enzyme-Linked Immunosorbent Assay.** Enzyme linked immunosorbent assay (ELISA) was used to evaluate the profile of sensitization of a panel of Filipino allergic and non-allergic subjects’ sera to the recombinant and native Blo t 11 allergens. Briefly, 10 mg/mL concentration of the allergens were prepared and coated onto ELISA plates overnight at 4°C using 50 µL of 0.1M NaHCO₃, pH 8.3. Plates were blocked with 1% BSA (Sigma) in PBS-T for 1 hour at room temperature. ELISA plates were incubated overnight with 5x diluted human sera followed by 1 hour incubation at room temperature with biotinylated anti-human IgE (Pharmingen, CA, USA) diluted 1000x in blocking buffer. Plates were incubated with 2000x dilution of ExtrAvidin-alkaline phosphatase conjugate (Sigma) for 1 hour. Finally, colorimetric reaction was performed using p-nitrophenyl phosphate (Sigma). Absorbance at 405 nm was determined using an ELISA reader. Human IgE (Pharmingen) was used as a standard per plate for the calculation of IgE concentration.

**Inhibition Assay.** Absorption assay by ELISA was performed to determine the percentage of inhibition of the Blo t 11 allergens and GST against the IgE reactivity of the Bt aqueous extract. A 96-well ELISA plate (Corning, NY, USA) was coated with Bt aqueous extract (10
µg/mL) as described above. Five selected human serum samples were pre-absorbed (14 hours) separately with rBlo t 11-GST, nBlo t 11 or GST to a final concentration of 10 µg/µL. ELISA was performed as described above. Percentage inhibition was computed using the following formula: \[
\text{% inhibition} = \left(\frac{A_{\text{unabsorbed}} - A_{\text{absorbed}}}{A_{\text{unabsorbed}}}\right) \times 100;
\]
where \(A_{\text{unabsorbed}}\) is the OD_{405} of serum samples not pre-absorbed with the Blo t 11 allergens or GST and \(A_{\text{absorbed}}\) is the OD_{405} of serum samples pre-absorbed with the Blo t 11 allergens or GST.

RESULTS AND DISCUSSION

Recombinant and Native Blo t 11.
Recombinant Blo t 11 allergen was expressed as a GST-fusion protein in \(E. coli\) by IPTG induction for three hours. A two-liter culture of \(E. coli\) containing the Blo t 11-pGEX-4T-1 vector was prepared, cells collected by centrifugation and lysed using SDS Lysis buffer. A total of 0.7 mg rBlo t 11 per liter of \(E. coli\) culture was isolated by affinity chromatography using a Glutathion-Agarose column. The purified rBlo t 11-GST allergen migrated at 128 kDa on an SDS-PAGE gel (Figure 1a) in line with its predicted molecular weight (102 kDa + 26 kDa GST).

The purified rBlo t 11 reacted positively to the Blo t 11 monoclonal antibody P2E7-G1 (Figure 1c) confirming the identity of the purified allergen. No reactivity was observed with the GST. Low yield of insoluble recombinant Blo t 11-GST fusion protein were obtained as previously reported [4]. The relatively large size of the recombinant protein might have contributed to the observed low expression. Furthermore, the recombinant protein was highly susceptible to degradation and the GST failed to be cleaved off after thrombin digestion. The susceptibility of a paramyosin homolog protein was likewise reported in both the native and recombinant Der f 11 expressed in \(E. coli\) [11] and in \(E. coli\)-expressed \(Sarcoptes scabei\) paramyosin [12]. Expression of recombinant protein in \(E. coli\) has been a successful and convenient method for the production of large amounts of many HDM allergens. Recombinant allergens expressed in \(E. coli\) as fusion proteins offer the advantages of ease of manipulation, short time of expression, ease in obtaining large quantities of recombinant proteins, and the availability of fast and efficient purification systems.

Native Blo t 11 was purified from a Bt aqueous extract using a Blo t 11 monoclonal antibody affinity column. Two milliliters of

Figure 1. SDS-PAGE profile of the recombinant Blo 11-GST (a) and native Blo t 11 (b). Western Blot analysis of the purified Blo t 11 allergens using monoclonal antibody P2E7-G1 (c).
the Bt extract yielded 467 ng of native Blo t 11. The purified nBlo t 11 was partially degraded; the major derived peptide was approximately 60 kD (Figure 1b). Similarly to the recombinant Blo t 11, the purified native allergen reacted positively to monoclonal antibody P2E7-G1 (Figure 1c). The results presented herein are consistent with previous studies showing the isolation of a native paramyosin with a molecular weight of -60 kDa [10]. The presence of high molecular weight allergens in B. tropicalis was first observed in the results of crossed radioimmuno-electrophoresis (CRIE) studies [13] and immunoblotting experiments [14] using the whole Bt extract, which revealed the presence of multiple species-specific IgE-binding antigens with molecular weights ranging from 11-85 kD. It is possible that some of the high molecular weight antigens previously identified in the whole Bt extract are the degradation products of the nBlo t 11.

The IgE-binding capacity of the recombinant and native Blo t 11 allergens were determined by ELISA using a panel of sera from 110 Filipino Bt-allergic subjects and 85 Filipino non-allergic subjects. A relatively low IgE concentration of the 85 non-allergic sera against the native and recombinant Blo t 11 allergens and GST was detected (Figure 2). None of the non-allergic sera used in this study registered an allergen-specific IgE concentration above 100 ng/mL. The mean ± the standard deviation of the IgE concentration observed among the 85 non-allergic sera was used as a cut-off value in determining positive reactions among the allergic sera used in this study.

Results of the IgE ELISA using the panel of sera from 110 Bt-allergic subjects showed that the native Blo t 11 allergen registered an IgE reactivity of 63% (69/110) while the recombinant Blo t 11 registered 57% (63/110) (Figure 3). The IgE-binding capacity of the recombinant Blo t 11 was comparable to that of the purified nBlo t 11 although a slightly lower percentage of IgE reactivity was observed with the recombinant allergen. As previously reported, it is not unusual for the biological activity of a recombinant protein produced in E. coli to exhibit lower IgE reactivity compared to the native protein [15-16].

![Figure 2](image-url)

**Figure 2.** IgE reactivity profile of the control sera from 85 non-allergic Filipino subjects as determined by ELISA. The mean ± the standard deviation (SD) of the IgE concentration of the 85 control sera were used as a cut-off value in determining a positive reaction for the allergic sera.
Despite the potential degradation of the nBlo t 11 allergen, it exhibited a significant IgE binding activity. In contrast, only 13 sera registered positive IgE reactivity to GST. This further validates the IgE reactivity of the recombinant Blo t 11-GST fusion protein, implying that the observed reactivity is due to IgE epitopes present in the Blo t 11 and not on the GST protein. Detailed analysis of the IgE reactivity of the allergic sera showed that 57 and 35 double positive and double negative, respectively, to both native and recombinant Blo t 11 allergens. These results will show that the forms of the allergens exhibits very similar IgE-binding activity. Despite the absence of post-translational modifications of proteins expressed in E. coli, the recombinant Blo t 11 expressed in E. coli exhibited comparable IgE-binding activity to its native counterpart. This can be explained by the flexibility of IgE secreting B cells to recognize both linear and conformation-dependent epitopes during antigen recognition. It is also interesting to note that 11 and 7 of the sera tested exhibited more than 1000 ng/mL IgE concentration to the native and recombinant Blo t 11 allergen, respectively. These results further support the clinical importance of Blo t 11 as a strong inducer of IgE among atopic persons. In contrast, none of the positive sera to GST registered above the 1000 ng/mL IgE concentration.

To further validate the importance of Blo t 11 as a major component of Bt aqueous extract as a sensitizing agent for allergic reactions, inhibition assay was performed. The Blo t 11 allergens can inhibit up to 63% IgE binding activity of Bt aqueous extract (Figure 4). Using five sera that reacted positively to the three allergens used, 40.8%, 44.4% and 9.8% mean inhibition was observed with the recombinant Blo t 11, native Blo t 11 and GST, respectively, against the IgE reactivity of the Bt aqueous extract. The comparable inhibition capacity of the recombinant and the native Blo t 11 allergens against the IgE reactivity of the Bt aqueous extract further supports the presence of similar IgE binding capacity of the forms of the allergen.

Considering the comparable IgE-binding capacity of the recombinant Blo t 11 to its native counterpart, the recombinant Blo t 11 is good candidate substitute reagent for HDM allergy diagnosis and immunotherapy. Added to this, recombinant Blo t 11 yields higher allergen concentration compared to
Thus far, Blo t 5 allergen, a 14 kDa protein from *Blomia tropicalis* [17] have been reported as the major allergen on this species of HDM. In addition eight minor allergens from Bt have also been cloned, expressed and characterized [18-19]. This results presented in this study add to the growing evidence of the importance of Bt as a major source of sensitizing allergens among atopic individuals. This study further supports the claim that high molecular weight allergens like paramyosin are important for mite allergy [17-18]. Such high molecular weight antigens have been previously overlooked and their examination is timely. In addition, the results obtained herein add further strength to the possibility that the actual sensitizing specificities for high molecular weight antigens are degradation products.

This study demonstrates the clinical importance of Blo t 11 allergen as a triggering factor for allergies among Filipino atopic patients thus a potential reagent for the diagnosis and immunotherapy of house dust mite allergy in the local setting. The recombinant and native forms of Blo t 11 exhibits comparable IgE-binding activity.

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