

Peptide mapping of immunoglobulin E and immunoglobulin G immunodominant epitopes of an allergenic *Blomia tropicalis* paramyosin, Blo t 11

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Summary

Background The identification of immunodominant peptides containing the IgE and IgG epitopes on allergen molecules is an important step in understanding the interaction of the allergen with the immune system and, thus, essential for the development of effective immunotherapeutic and diagnostic reagents. The present study aimed to map the IgE and IgG immunodominant peptides of *Blomia tropicalis* (Bt) allergen Blo t 11, a high molecular weight allergen homologous to paramyosin, exhibiting important allergenic activity.

Methods Eleven overlapping fragments of Blo t 11 cDNA gene were expressed as glutathione s-transferase (GST) fusion peptides, which were affinity-purified using the glutathione-Sepharose column. Human IgE and IgG immunodominant peptides were determined by dot blot immunoassay using crude Bt extract-positive sera from asthmatic patients. Evaluation of allergenicity, specific hIgG subclass analysis, and cross- and self-inhibition studies were determined by enzyme-linked immunosorbent assay.

Results Blo t 11 contains multiple IgE and IgG immunodominant peptides scattered throughout the molecule. The dominant IgE and IgG peptides were mapped at amino acid positions 336–557 and 698–875, respectively. An immunodominant peptide (fD) registered a higher percentage of IgE and IgG reactivity compared to the rFL-Blo t 11. Significant serum levels of Blo t 11- and fD-specific IgG1, IgG2 and IgG4, but not IgG3 were detected in the Bt extract-positive sera tested. Cross-inhibition study revealed the rFL-Blo t 11 was significantly inhibited by fD.

Conclusion The IgE and IgG immunodominant peptides of Blo t 11 have been mapped. Our data suggest that utilization of Blo t 11 fragment(s) or chimeric fusion fragments containing IgE and IgG epitopes could be a better alternative in the development of diagnostic and therapeutic reagents for mite allergy.

Keywords *Blomia tropicalis*, Blo t 11, epitope mapping, house dust mite, mite allergen, paramyosin, peptide mapping

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Introduction

Allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis, have become increasingly prevalent over the past few decades in both developed and developing countries [1]. Epidemiological studies have revealed that 10–40% of population from industrialized countries suffers from allergic diseases and the costs to public health and the economy is substantial and rising [2].

The role of house dust mites in the pathogenesis of allergic asthma and atopic diseases has been well recognized [3]. The most frequently implicated indoor allergen source has been the house dust mite. Several clinically important mite species are distributed worldwide but the clinical significance of each

species varies from one region to another. In tropical countries like Singapore, the house dust mites *Blomia tropicalis* (Bt) and *Dermatophagoides pteronyssinus* (Dp) are of clinical importance [4].

Blo t 11 is a house dust mite allergen isolated from a Bt cDNA library [5]. The cDNA gene coding for Blo t 11 exhibits significant homology to paramyosin. Our previous studies showed that the recombinant Blo t 11 is an important allergen with an *in vitro* IgE reactivity of approximately 52% among the asthmatic patients' sera tested [5].

Paramyosin is a highly antigenic protein involved in parasitic infections. It is a candidate vaccine for filariasis and schistosomiasis, and is a prominent antigen (antigen B) in human cysticercosis [6–11]. In mite allergy, native paramyosin (nDer f 11) isolated from *D. farinae* (Df) has been found to be highly allergenic, with an IgE reactivity of 80% to mite-sensitive sera [12]. In addition to the highly immunodominant feature of paramyosin, it has been suggested that it may play an immunoregulatory function as it has been found to disrupt the

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complementary C1 activity [13]. Taken together, the utilization of paramyosin or its fragments as antigens may therefore contain both immunodominant and immunosuppressive factors.

The identification of immunodominant peptides containing the IgE and IgG epitopes on allergen molecules is an important step in understanding the interaction of the allergen with the immune system. This is important in the development of effective immunotherapeutic and diagnostic reagents for mite allergy. The present study aimed to determine the profile and distribution of the immunodominant peptides containing the human IgE and IgG epitopes of Blo t 11 using overlapping recombinant peptides.

Materials and methods

Human sera

Thirty-six human sera obtained from doctor-diagnosed asthmatic patients with positive skin prick test (SPT) to crude Bt extract were used for IgE and IgG epitope mapping. A panel of sera from 60 asthmatic patients (kindly provided by Prof. Sam Choon Kook and Prof. Liam Chong Kin of the University of Malaya, Malaysia) and 134 allergic rhinitis patients (kindly provided by Dr Wang DeYun of the National University of Singapore, Singapore) were used for allergenicity studies.

A subset of 40 IgG-positive and five IgE-positive sera to Blo t 11 and fD were used for the human IgG subclass analysis and inhibition study, respectively. Sera from non-atopic volunteers ($n = 4$) and cord blood sera ($n = 6$) were used as negative controls for the IgE and IgG immunoblot assays while four non-atopic and 41 cord blood sera were used for the IgE and IgG enzyme-linked immunosorbent assay (ELISA). All sera were stored at -80°C until use.

Construction of Blo t 11 overlapping fragments

Generation of Blo t 11 overlapping fragments was based on secondary structure predictions using the PHD secondary structure prediction program [5]. The different overlapping fragments were constructed without disrupting the predicted alpha helical structure of the allergen, thus, the N- and C-termini of the fragments were mostly located on the loops connecting the helical structures. Figure 1 shows the schematic diagram of the strategy for the generation of 11 Blo t 11 peptides. Seven overlapping fragments (f1–f7) were initially generated by PCR. Three additional shorter peptides (fA–fC) were subsequently constructed for more refined peptide mapping. Finally, the immunodominant fD was generated and its allergenicity was compared with the rFL-Blo t 11.

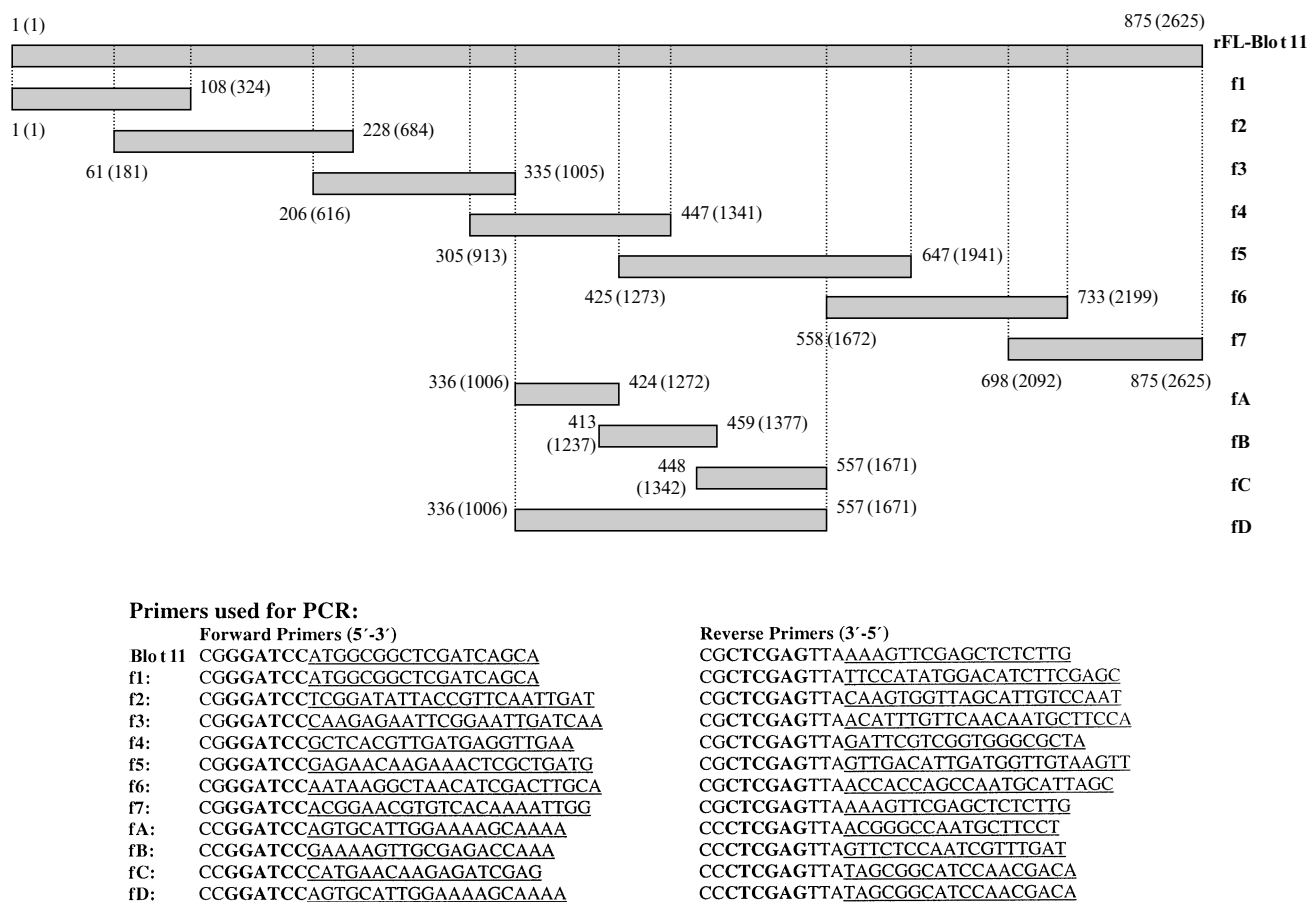


Fig. 1. Schematic diagram showing the strategy for generation of 11 Blo t 11 overlapping fragments. Numbers indicate amino acid residue of deduced protein while number in parenthesis indicate the nucleotide number of cDNA insert. f1–f7 and fA–fD indicate the 11 fragments generated by PCR using the set of primers listed.

Expression and purification of full-length Blo t 11 and its derived fragments

The full-length Blo t 11 cDNA gene and the 11 Blo t 11 overlapping fragments were amplified by PCR. Gel-purified PCR products were ligated into pGEX-4T-1 vector (Pharmacia Biotech, UK). The rFL-Blo t 11 and peptides were expressed as glutathione S-transferase (GST) fusion protein/peptides in *Escherichia coli* (BL21) by isopropyl- β -D-thiogalactopyranoside (IPTG) induction, as described previously [14]. Purification of recombinant proteins was performed by affinity chromatography using the glutathione-Sepharose column (Sigma, St Louis, MO, USA) according to the protocol described in the GST Purification Module (Pharmacia Biotech, Piscataway, NJ, UK). Purified protein samples, quantified by the DC Protein Assay (Bio-Rad, Hercules, CA, USA), were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [15] and by Western blot analysis as previously described [16].

Dot blot immunoassay

Human IgE and IgG immunodominant peptide mapping was performed *in vitro* by dot blot immunoassay using a panel of sera from mite-sensitive subjects following the protocol previously described [6]. Two micrograms of rFL-Blo t 11 or peptides were applied onto Hybond C nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, UK) in duplicates using a BioDot apparatus (Bio-Rad). Five micrograms of crude Bt extract and 2 μ g of purified GST were used as controls. The dot blots were blocked with 5% non-fat milk powder in PBST [phosphate-buffered saline (PBS) with 0.05% Tween-20] and incubated for 14–16 h at 4 °C with allergic sera diluted 5 $\times$ (for hIgE) or 20 $\times$ (for hIgG) in PBST. Incubation with secondary antibodies was performed with 1000 $\times$ dilution of biotinylated anti-human IgE (250 μ g/mL) or anti-human IgG (250 μ g/mL; Pharmingen, San Diego, CA, USA) in PBST for 1 h, followed by 1-h incubation with 5000 $\times$ dilution of alkaline phosphatase-conjugated ExtrAvidin (Sigma) in PBST. Signals were detected using AP-conjugate Substrate Kit (Bio-Rad). Densitometric analysis was performed using the GS 700 Densitometric Imaging System (Bio-Rad). Results were expressed as densitometric indices after subtracting the background and GST indices for each serum tested.

Human IgE and IgG ELISA

ELISA was used to evaluate the profile of sensitization of a panel of rhinitis and asthmatic patients' sera to rFL-Blo t 11 and fD; as well as the specific IgG subclasses to the allergens. Briefly, 5 μ g each of the rFL-Blo t 11, fD, and GST; and 100 μ g of Bt crude extract were coated onto ELISA plates overnight at 4 °C using 50 μ L of 0.1 M NaHCO₃. Plates were blocked with 1% bovine serum albumin (BSA; Sigma) in PBST for 1 h at room temperature (22 °C). ELISA plates were incubated for 1 h each at room temperature with human sera diluted in blocking buffer (5 $\times$ for IgE and IgG subclasses; and 20 $\times$ for total IgG) followed by 1-h incubation with biotinylated anti-human IgE, IgG or IgG subclasses (Pharmingen) diluted 1000 $\times$ in blocking buffer. Plates were incubated with 2000 $\times$ dilution of ExtrAvidin-AP conjugate (Sigma) for 1 h. Finally, colorimetric reaction was performed using p-nitrophenyl phosphate

(Sigma). Absorbance at 405 nm was determined using the Spectra & Rainbow ELISA reader (Tecan, Salzburg, Austria).

Inhibition study

Absorption assay was used to determine the cross- and self-inhibition capacity of the immunodominant fD with the rFL-Blo t 11 and *vice versa*. Five rFL-Blo t 11 and fD IgE-positive sera were pre-absorbed for 14–16 h at 4 °C with 40 μ g of fD, rFL-Blo t 11 or GST followed by human IgE ELISA on a fD or rFL-Blo t 11 solid-phase following the above protocol.

Results

Initial IgE immunodominant peptide mapping using f1–f7 revealed a significant reactivity of the 36 sera tested to f4 (86%) and f5 (83%), which corresponds to amino acid positions 305–447 and 425–647, respectively (Fig. 2a). Further IgE peptide mapping using smaller Blo t 11 fragments showed significant reactivity to fA (78%) and fC (75%), corresponding to amino acid positions 336–424 and 448–557, respectively. Reactivity to fB (41%) was comparatively lower as shown in Fig. 2(a). The high frequency of reactivity to f4 and f5 suggests the presence of different immunodominant epitopes in these two fragments. These separate IgE epitopes were likewise found in fA and fC but not on fB, which spans mostly of the overlapping region between f4 and f5.

Eight of the Blo t 11 fragments registered an equal or higher IgE reactivity compared to the FL-Blo t 11 (52%) except for f3 (44%), f6 (30%), and fB (41%). Interestingly, several rFL-Blo t 11-negative sera were positive to some of the fragments. Ninety-four percent (34/36) of the sera tested registered a positive IgE reaction to at least one of the Blo t 11 fragments.

The majority of the 36 sera used for IgG peptide mapping experiments reacted strongly to most fragments (Fig. 2b). Fragment 7 (f7), the peptide corresponding to the carboxyl terminus of the FL-Blo t 11, showed the highest frequency of IgG reactivity (94%). Moreover, 30 (83%) and 31 (86%) sera tested exhibited a positive reaction to the IgE-immunodominant peptides f4 and f5, respectively. All sera tested showed IgG-positive reactions to at least one Blo t 11 fragment. All but one (f2) Blo t 11 fragment tested displayed higher frequency of IgG reactivity compared to the FL-Blo t 11 (Fig. 2b).

In view of the observed higher frequency of IgE and IgG reactivity of fA and fC, fragment D (fD), encompassing fA–fC, was generated as shown in Fig. 1. The allergenicity of the rFL-Blo t 11 and fD was comparatively determined by ELISA as summarized in Table 1. A significant increase in the percentage of both IgE and IgG reactivity was observed with fD as compared to the rFL-Blo t 11. These significant increases in IgE and IgG reactivity were observed in both the asthmatic and rhinitis patients' sera tested. IgE reactivity was observed to have increased by almost fourfold (12% vs. 45%) with fD as compared to FL-Blo t 11 using 60 asthmatic patients sera. A twofold increase in IgE reactivity (18% vs. 36%) was also observed with the 134 rhinitis patients' sera used. Similarly, an increase of 1.7-fold (47% vs. 82%) and 1.4-fold (52% vs. 76%) in IgG reactivity was observed using the same asthmatic and rhinitis patients' sera, respectively. These data indicated that fD contains the immunodominant IgE and IgG epitopes.

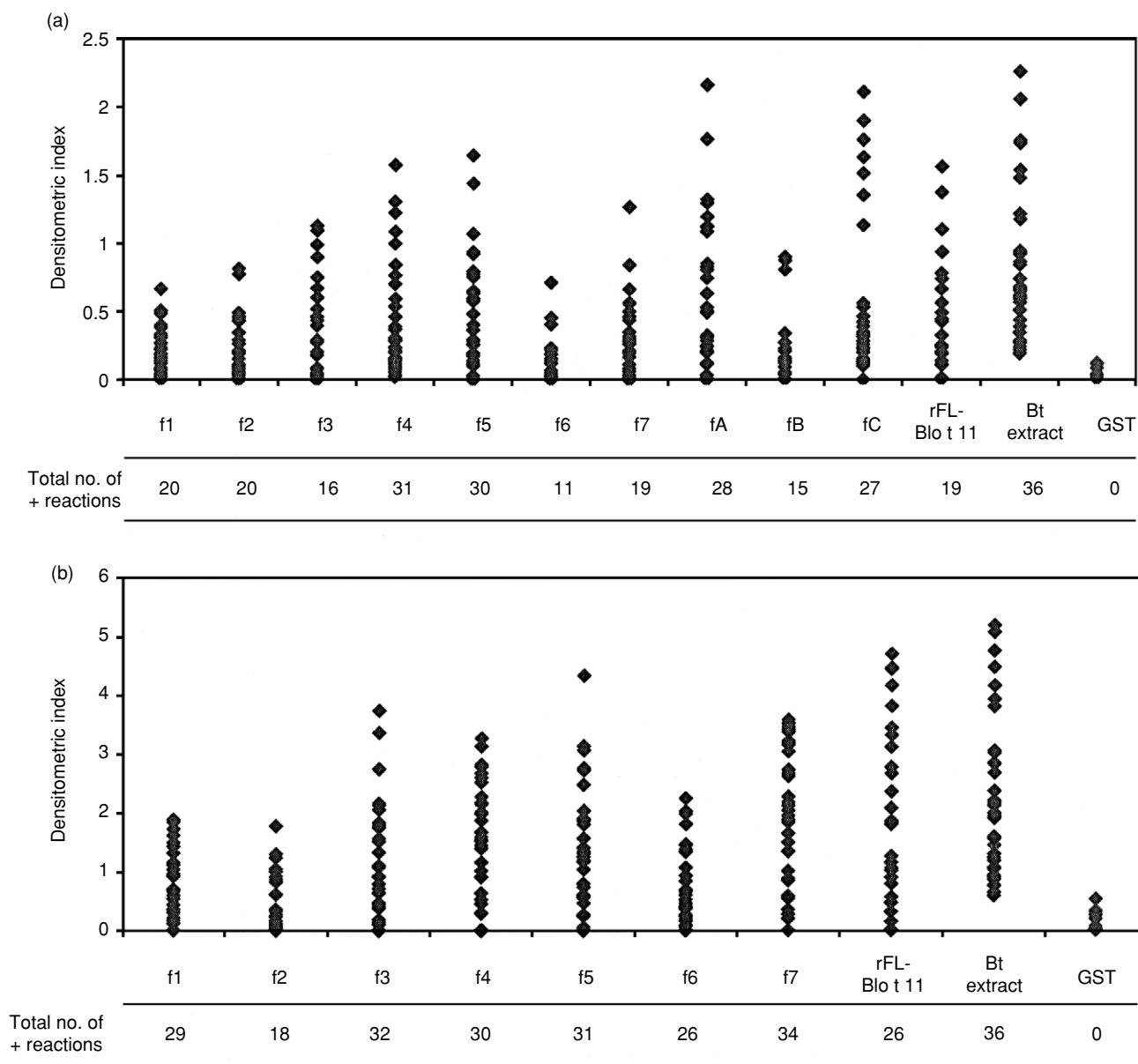


Fig. 2. Summary of human IgE (a) and IgG (b) immunodominant peptide mapping by dot blot immunoassay using 36 Bt extract-positive sera from asthmatic patients. Mean + 2SD (standard deviation) of the densitometric indices of cord blood and non-atopic sera controls were used as cut-off values (0.214 for IgE and 0.388 for IgG) in determining positive reactions.

Results of the *in vitro* determination of rFL-Blo t 11- and fD-specific human IgG subclasses are summarized in Fig. 3. Out of 40 Bt extract-positive sera tested, a significant percentage reacted to IgG1, IgG2, and IgG4 but not to IgG3 using both rFL-Blo t 11 and fD as antigens. The rFL-Blo t 11 exhibited 67.5% (27/40), 57.5% (23/40), and 87.5% (35/40) reactivity to IgG1, IgG2, and IgG4, respectively, while the immunodominant fD reacted positively at 95% (38/40) to both IgG1 and IgG4, and at 75% (30/40) to IgG2. Both antigens had low frequency of specific IgG3 at 30% (12/40) for rFL-Blo t 11 and 35% (14/40) for fD. Interestingly, for all four IgG subclasses, fD showed a higher percentage of reactivity than the rFL-Blo t 11, supporting the previous results that the fD is more immunogenic than the rFL-Blo t 11.

An inhibition study using five asthmatic patients' sera revealed a significant percentage of cross-inhibition between fD and the full-length allergen (Fig. 4a). An average of 59% inhibition was observed with fD against the rFL-Blo t 11. In contrast, a lower percentage of cross-inhibition was observed with the rFL-Blo t 11 against fD (Fig. 4b) at an average of 35% inhibition. Both antigens also showed significant self-inhibition (Figs 4a and b).

Discussion

Blo t 11 is the first high molecular weight allergen identified in the mite species *Blomia tropicalis* with important allergenic

Table 1. Summary of the profile of sensitization of asthmatic and rhinitis patients' sera against FL-Blo t 11, fD, and Bt extract by hIgE and hIgG ELISA

Antibody assayed	Sera used	Antigens	Positive	Negative	Percentage of reactivity
hIgE	Asthmatic (<i>n</i> = 60)	rFL-Blo t 11	7	53	12%
		fD	27	33	45%
		Bt extract	48	12	80%
	Rhinitis (<i>n</i> = 134)	rFL-Blo t 11	24	110	18%
		fD	48	86	36%
		Bt extract	82	66	61%
hIgG	Asthmatic (<i>n</i> = 60)	rFL-Blo t 11	28	32	47%
		fD	49	11	82%
	Rhinitis (<i>n</i> = 134)	rFL-Blo t 11	70	64	52%
		fD	102	32	76%

Results shown represent the optical density reading at 405 nm after 60 min incubation with pNPP substrate solution for IgE (converted as IgE concentration in ng/mL) or 20 min for IgG (plotted as optical density). Reactivity was considered positive if the OD₄₀₅ of the tested sera is higher than the IgE or IgG (in parenthesis) cut-off values of 4.9 (0.248), 5.0 (0.081), and 7.5 for fD, rFL-Blo t 11, and Bt extract, respectively (computed as mean $\pm$ 2 SD from OD₄₀₅ values of the negative controls).

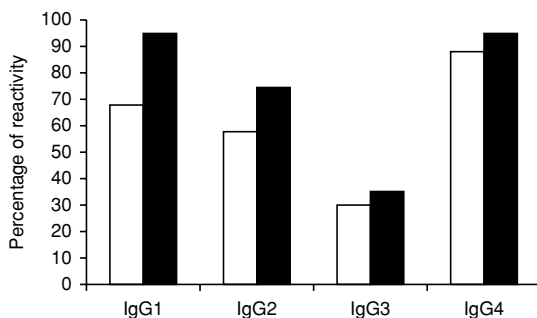


Fig. 3. Summary of the human IgG subclass analysis of the recombinant FL-Blo t 11 (□) and fD (■) by ELISA using 40 Bt extract-positive sera. The results represent the optical density reading at 405 nm after 20 min incubation with pNPP substrate solution. Mean $\pm$ 2SD (standard deviation) of the optical density at 405 nm of the cord blood and non-atopic sera controls were used (as) cut-off values for rFL-Blo t 11 and fD (in parenthesis)-positive reactions computed as 0.035 (0.036), 0.059 (0.061), 0.055 (0.063), and 0.058 (0.040) for IgG1, IgG2, IgG3, and IgG4, respectively.

activity [5]. The cDNA gene coding for Blo t 11 and its deduced amino acid sequence exhibits significant homology with different invertebrate paramyosins [7–12]. We previously reported the poor expression of the rFL-Blo t 11 in *E. coli* [5] and *Pichia pastoris* (unpublished data) expression systems; and its susceptibility to degradation [5], a condition observed in native Df paramyosin [6]. In this study, we report the expression of an immunodominant fragment of Blo t 11 (fD) with higher IgE and IgG reactivity against Bt extract-positive sera tested. Fragment D is highly expressed in *E. coli* and the recombinant peptide is highly stable and soluble, thus a better source of diagnostic and/or therapeutic reagent for mite allergy compared to the rFL-Blo t 11.

Our data revealed that the rFL-Blo t 11 contains multiple IgE and IgG immunodominant peptides. The immunodominant IgE peptides were mapped at amino acid positions 336–424 and 448–557. On the other hand, the most reactive IgG peptides were located at amino acid positions 698–875. The percentages of IgE and IgG reactivity of the different Blo t 11 fragments are significantly higher than the rFL-Blo t 11, indicating that the shorter peptides have higher IgE and IgG binding capacity. This observation could be explained by the potential exposure and accessibility of the IgE and IgG epitopes on the shorter

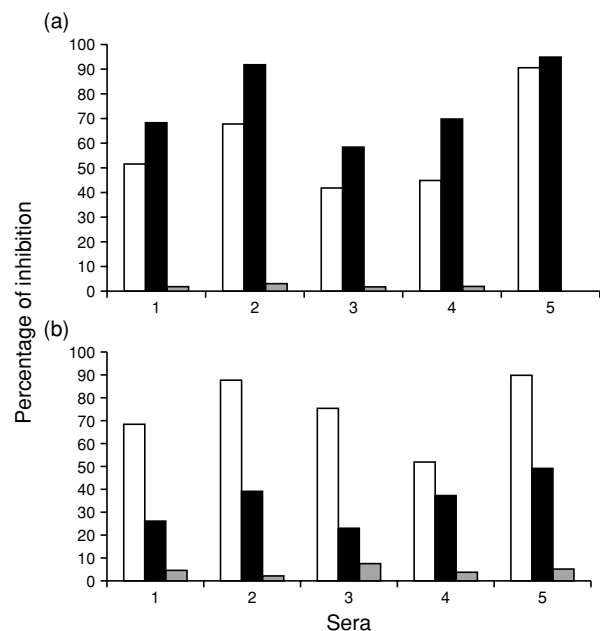


Fig. 4. Summary of ELISA results for cross- and self-inhibition analysis using five human asthmatic patients' sera. Sera were pre-absorbed 14–16 h at 4 °C with 40 µg/mL of fD (□); rFL-Blo t 11 (■) or GST (□) and were assayed on ELISA plates coated with rFL-Blo t 11 (a) or fD (b). Percentage inhibition was calculated as the difference in the OD₄₀₅ values between unabsorbed and absorbed sera divided by the former and expressed as percentage.

Blo t 11 peptides in contrast to a potential masking effect of the epitopes on the high molecular weight rFL-Blo t 11 as a result of the improper folding of the protein expressed in *E. coli*. The relatively large size of the rBlo t 11-GST fusion protein with a predicted molecular weight of 128 kDa (102 kDa + 26 kDa) might be a factor involved in the improper folding of the expressed protein. Our results concur with the observations of Tsai et al. that Der f 11 peptides are more allergenic than the whole rDf642 [6]. It has been proposed previously that the degradation products of Df paramyosin, which react strongly to serum IgE, cause sensitization among susceptible individuals. The observation that the native and recombinant Der f 11 are susceptible to degradation matches our results.

Furthermore, our initial results on the isolation of native Blo t 11 suggest that it is likewise highly degradable as shown by western blot analysis using Blo t 11-specific antibodies (unpublished data). Crossed radioimmuno-electrophoresis (CRIE) studies [17] and immunoblotting analysis [18] using the whole Bt extract revealed the presence of multiple species-specific IgE-binding antigens with molecular weights ranging from 11 to 85 kDa. The possibility that some of the high molecular weight antigens identified in the whole Bt extract are the degradation products of Blo t 11 cannot be ruled out.

The pattern of IgE and IgG reactivity to the different fragments are remarkably similar with peptides f4 and f5, both registering high frequency of positive IgE and IgG binding, and for peptide f2 which exhibited low frequency reaction to both IgE and IgG. A significant difference was observed, however, with f1, f3, f6, and f7 in terms of the total number of positive IgE and IgG reactivity (Fig. 2). Interestingly, f4 and f5 consistently registered significant IgE and IgG reactivity, indicating that both fragments contain IgE and IgG epitopes making them potential candidates in the development of diagnostic reagents for mite allergy. On the other hand, f6 and f7 are good candidates for the development of therapeutic reagent for their low IgE but high IgG reactivity. Overall, results of the epitope mapping suggest that serum IgE and IgG antibodies against Blo t 11 recognize common and distinct epitopes scattered throughout the entire FL-Blo t 11. These results were expected, considering that the entire molecule is largely hydrophilic as determined by hydrophobicity predictions.

The expression of the immunodominant fD was aimed to produce an alternative reagent to the rFL-Blo t 11 to circumvent the problem of low expression and susceptibility to degradation, but retaining, if not improving, its allergenicity. Fragment D contains the immunodominant IgE and IgG epitopes. The observed increase in the allergenicity of fD compared to the rFL-Blo t 11, as summarized in Table 1, implies that the utilization of Blo t 11 fragment(s) or chimeric fusion fragments containing IgE and/or IgG epitopes could be a better alternative for diagnostic and therapeutic purposes. Incidentally, the observed percentage of IgE reactivity of the immunodominant peptide fD presented in this study is comparable with that of the native Blo t 11 which was recently isolated in our laboratory (manuscript under preparation).

Evaluation of rFL-Blo t 11- and fD-specific IgG subclasses showed elevated serum levels of specific IgG1, IgG2, and IgG4 but not IgG3 (Fig. 3). IgG antibodies represent 75% of the total circulating immunoglobulins, and the detection of allergen-specific IgG suggests exposure and sensitization to such allergens. There have been few studies on specific IgG subclasses to Bt allergens. Of the four human IgG subclasses, IgG4 has been found to be elevated in patients with atopic diseases [19, 20] and among patients undergoing allergen-specific immunotherapy [21, 22]. It has been suggested that IgG4 aids immunotherapy by neutralizing antigens and blocking IgE production and it could be used as diagnostic marker for allergic sensitization [19]. Similarly, elevated serum levels of allergen-specific IgG1 and IgG2 have been shown among atopic dermatitis, atopic rhinitis, and asthmatic patients [20, 22]. The significant IgG1, IgG2, and IgG4 reactivity in our present study concurs with the above-mentioned studies. This result could be due to repeated and prolonged antigenic exposure. On the other hand, our observation that IgG3 showed low percentage of reactivity

agrees with the results of Armenaka et al. [23]. It should be noted that IgG3 is the human IgG subclass with the highest capacity to activate the complement cascade. In view of this, our finding of a lower percentage of rFL-Blo t 11-specific IgG3 reactivity compared to the other IgG subclasses might be due to the fact that paramyosin has been reported to inhibit the C1 complement [13].

The importance of fD as an alternative diagnostic and/or therapeutic reagent for mite allergy compared to the rFL-Blo t 11 was further validated with the results of the inhibition study. Our results indicate that the IgE epitopes found in fD were recognized by the serum IgE antibodies of the asthmatic and rhinitis patients tested. Furthermore, fD is capable of blocking the binding of serum IgE with the rFL-Blo t 11. The observed lower percentage of cross-inhibition of the rFL-Blo t 11 against fD as opposed to the higher percentage of cross-inhibition of fD against FL-Blo t 11 supports our claim that the IgE epitopes on the full-length allergen might be inaccessible to IgE antibodies in contrast to exposed IgE epitopes in the shorter fD.

The results presented in this paper add to the body of evidence that high molecular weight antigens like paramyosin are important for mite allergy. Such high molecular weight antigens have been overlooked previously and their examination is timely. In addition, our results add further strength to the possibility that the actual sensitizing specificities for high molecular weight antigens are degradation products. The description of the high IgE and IgG binding capacity of fD peptide could therefore help to evaluate the importance of this allergen.

Acknowledgements

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