

Immunoglobulin E reactivity of native Blo t 5, a major allergen of *Blomia tropicalis*

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Summary

Background Blo t 5 is a major allergen of *Blomia tropicalis* and its complementary DNA (cDNA) has been expressed in both prokaryotic and eukaryotic expression systems. Although the recombinant Blo t 5 has been well characterized, relatively less is known about its native counterparts and the allergenicity comparison of the native and recombinant Blo t 5 allergens has not been reported.

Objective The aims of this study are to characterize the native counterpart of Blo t 5, and compare the allergenicity of native and recombinant Blo t 5 by *in vivo* and *in vitro* assays.

Methods Native Blo t 5 were purified by immuno-affinity chromatography and characterized by proteomic means. The allergenicity of the allergen was evaluated by skin prick tests, human IgE ELISA, ELISA inhibition and histamine release assays.

Results Native Blo t 5 consists of at least five distinct isoforms, ranging from pI 3 to 5.5. Allergenicity assessment of recombinant and native Blo t 5 based on skin reaction, IgE-binding capacity and histamine release in allergic individuals indicated that there was a good correlation between both forms of Blo t 5 in general. However, data from IgE ELISA inhibition assay revealed the presence of additional unique IgE epitopes in native Blo t 5.

Conclusions At least five distinct isoforms of Blo t 5 have been identified. Comparative assessment of native and recombinant Blo t 5 revealed that the allergenicity of these two forms was similar but not completely identical suggesting that the various isoforms of native Blo t 5 may exhibit additional unique IgE epitopes.

Keywords allergenicity, Blo t 5, *Blomia tropicalis*, native, recombinant

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Introduction

Allergic diseases have increased in prevalence during the past 30 years. Epidemiological studies show that house dust mite (HDM) allergy is the major health problem worldwide [1–3]. It is well established that the common HDMs of the genus *Dermatophagoides* are the source that involved in triggering of allergic symptoms [4, 5]. However, in many tropical and subtropical regions, allergens from *Blomia tropicalis* are the main allergic triggering factor [6, 7].

Previous study had shown that there are at least 21 IgE binding proteins present in *B. tropicalis* [8]. At least nine *B. tropicalis* allergens have been identified and characterized with known complementary DNA (cDNA) sequences [9–14]. The frequency of human IgE reactivity to these allergens has been reported, ranging from 11% to 70% [9–15]. Among *B. tropicalis* allergens cloned and characterized to date, 14 kDa Blo t 5 is a major allergen with reactivity in at least 70% of allergic subjects [10, 15–18].

The use of recombinant allergens for diagnostic and therapeutic purposes is an attractive proposition as it allows component resolved evaluation and allergen standardization [19]. However, it is also well known that recombinant allergens with improper protein folding and the lack of post-translational modifications could lead to a potential alteration or masking effect of IgE epitopes thereby affecting the allergen–antibody recognition. Therefore, the comparison of native and recombinant allergens is essential. Recombinant protein of Blo t 5 allergen has been expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion protein [10, 20] and in *Pichia pastoris* KM71 [21] and has been found to exhibit up to 70% of IgE reactivity [10, 15, 17]. To date there was no published report on the comparative assessment of allergenicity of recombinant and native Blo t 5.

This study describes the identification and characterization of the native Blo t 5. The comparative allergenicity assessment of native and recombinant Blo t 5 was made by evaluating their ability to elicit skin responses and trigger histamine release in sensitized subjects. *In vitro* human IgE binding and IgE inhibition assays were also performed to further elucidate the IgE epitopes in native and recombinant Blo t 5.

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Methods

Human sera

Sera were obtained from 25 children (aged 4–15 years old) seen at the National University Hospital's Children's Specialist Clinic, with clinically diagnosed allergic disorders (asthma, rhinitis and/or eczema). All had positive skin prick test (SPT) reactions to *B. tropicalis* and *D. pteronyssinus*. Serum from a non-sensitized subject was used as control.

Expression and purification of glutathione S-transferase-Blo t 5

The Blo t 5 cDNA insert was amplified from *B. tropicalis* cDNA library [12] using specific primers. The insert was ligated to pGEX-4T1 vector with *Bam*HI and *Xho*I restriction enzyme sites, and transformed to *E. coli* strain BL21 (DE3). The fusion protein was produced by 1 mM isopropyl β -D-thiogalactoside (Calbiochem-Novabiochem, Darmstadt, Germany) induction and purified using glutathione sepharose (Amersham Biosciences, Buckinghamshire, UK) as described previously [12]. Purified GST-Blo t 5 was cleaved by thrombin (Sigma, St Louis, MO, USA) and followed by purification via hydrophobic interaction and ion exchange chromatography.

Native Blo t 5

B. tropicalis extract were prepared from cultured mites [22] by grinding in a mortar, suspended in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride (Sigma). Native Blo t 5 was immuno-affinity purified from the extract as previously described [23] with the use of Blo t 5-specific monoclonal antibody (clone 4A7), generated in our laboratory [24]. Blo t 5 from extract was eluted with acidic buffer 0.1 M glycine, 0.15 M NaCl, pH 2.6, and neutralized with Tris-HCl, pH 8. The purified native Blo t 5 was dialysed against deionized water and kept at -80°C until further analysis.

N-terminal microsequencing

Ten micrograms of purified native Blo t 5 were run on 10% of Tricine polyacrylamide gels at a constant voltage of 100 V/gel using Hoefer TM SE250 electrophoresis unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). The gel was then electroblotted onto polyvinylidene difluoride (Millipore, Bedford, MA, USA) membranes at constant current at 100 mA for 45 min and visualized with Coomassie brilliant blue G-250. Stained bands were cut and subject to automatic Edman degradation analysis according to the manufacturer's standard protocol.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis

Ten microlitres of dialysed recombinant Blo t 5 (0.2 $\mu\text{g/mL}$) was dried in a Savant Automatic Environmental Speed-Vac AES2010 Centrifugal Concentrator (Savant, Holbrook, NY, USA). The dried sample was digested overnight at 37°C with 20 μL of tryptic digestion buffer (0.02 $\mu\text{g}/\mu\text{L}$ trypsin in 25 mM ammonium bicarbonate, pH 8.5) and was then passed through a C18 ZipTip (Millipore). One microlitre of the

sample was loaded onto matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) sample plate along with 1 μL of matrix solution (10 mg/mL α -cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid, 50% acetonitrile). The sample was allowed to dry under ambient condition. Mass analyses were carried out using the Voyager-DE STR MALDI-TOF mass spectrometer (Perseptive Biosystem, Framingham, MA, USA). The computed monoisotopic peptide masses were calculated using Peptide-Mass [25]. The protein identification based on the MALDI-TOF peptide masses was performed with ProFound [26].

Two-dimensional electrophoresis and western blotting

Two-dimensional electrophoresis was performed according to manufacturer's instruction. Briefly, 1 μg native Blo t 5 was loaded per gel. The first dimensional isoelectric focusing (IEF) was performed on 7 cm, pH 3–10 linear IPG strips (Amersham Biosciences) at 20°C with maximum current setting of 50 $\mu\text{A}/\text{strip}$ using IPGphor IEF unit (Amersham Biosciences). SDS-PAGE was performed on 1.0 mm thick 15% glycine polyacrylamide gels at a constant current of 20 mA/gel at 15°C using electrophoresis units. The gels were then silver stained [27]. For western blotting, the separated proteins were transferred onto nitrocellulose membrane (Amersham Biosciences) at 100 mA/gel for 1 h at room temperature. The membranes were blocked with 3% non-fat milk in 0.05% PBS-Tween 20 (PBST) for 1 h, before incubating with five pooled sera of Blo t 5-sensitized allergic children ($5 \times$ dilutions) at 4°C , overnight. After washing, the blots were incubated with horseradish peroxidase-conjugated mouse-anti human Ig (Sigma) ($10\,000 \times$) 1 h at room temperature. The signals were detected by autoradiography using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

Skin prick tests

SPTs of native and recombinant Blo t 5 was performed with 38 skin tested *B. tropicalis*-positive subjects (28 females and 10 males, aged 10–49 years old). Three non-atopic subjects were included as negative control. SPTs were conducted according to the protocol described previously [12]. Twenty-five micrograms per millilitre of purified allergen was used in this study.

Human immunoglobulin E enzyme-linked immunosorbent assay and dose-dependent inhibition assay

ELISA was performed by coating each well with 250 ng of fusion proteins in 0.1 M sodium bicarbonate buffer, pH 8.3, overnight at 4°C . The wells were blocked with 1% bovine serum albumin in PBST for 1 h at room temperature, followed by incubation overnight at 4°C with diluted mite-allergic children sera. Specific IgE was detected by 0.25 $\mu\text{g/mL}$ biotinylated mouse anti-human IgE (Southern Biotechnology Associates Inc., Birmingham, AL, USA) followed by 1 : 2000 diluted ExtrAvidin-alkaline phosphatase (Sigma) each for 1 h. Signal was developed by addition of *p*-nitro-phenylphosphate (Sigma). For standard curve, the wells were coated with purified mouse anti-human IgE (Southern Biotechnology Associates Inc.) as capture antibody, and semi-purified

human IgE from human IgE myeloma (Chemicon International Inc., Temecula, CA, USA) was used as standard.

For dose-dependent ELISA inhibition assays, various concentrations (1 pg/mL–100 µg/mL) of recombinant and native Blo t 5 were used to absorb diluted pooled serum of five Blo t 5-sensitized allergic children at 4°C for 16 h. The sera were then reacted to either recombinant or native Blo t 5 coated wells at 4°C, 16 h. The IgE binding was detected by the ELISA protocol described above. The percentage of inhibition was calculated as $(1 - \text{OD } 405 \text{ nm with inhibitor} / \text{OD } 405 \text{ nm without inhibitor}) \times 100$.

Histamine release assay

Native and recombinant Blo t 5 were diluted in a histamine release reaction buffer to concentration ranging from 0.001 pg/mL to 10 µg/mL. Histamine release was performed with histamine release in heparinized whole blood kit (IBL, Hamburg, Germany), according to the manufacturer's recommendations. Briefly, 200 µL heparinized whole-blood samples were incubated at 37°C for 1 h with different concentrations of the allergen. Release of histamine will occur upon stimulation of basophilic granulocytes depending on their sensitivity to the allergen. The released histamine in the supernatant was subsequently determined using a specific plasma immunoassay, the histamine ELISA (IBL), according to the manufacturer's instructions.

Results

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and N-terminal amino acids sequencing of native Blo t 5

The protein profile of immuno-affinity purified native Blo t 5 is shown in Fig. 1a. This showed the presence of protein band with molecular weights about 14 kDa (arrow indicated). To confirm the identity of the immuno-affinity purified native

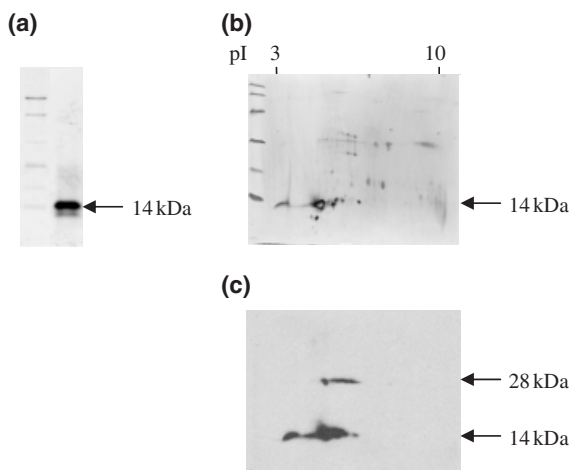


Fig. 1. Protein profile and human IgE immunoblots of native Blo t 5. (a) SDS-PAGE analysis after Coomassie Brilliant Blue staining, arrows indicated the bands of native Blo t 5, (b) silver-stained two-dimensional SDS-PAGE, pH 3–10. (c) Analysis of native Blo t 5 isoforms by two-dimensional western blotting using pooled sera from five Blo t 5 allergic subjects.

Table 1. N-terminal peptide sequence of native Blo t 5

Peptide	Sequence	Position
1	EHKPKKDD	2–9
2	KPKKDDFRNE	4–13
3	NEFDHLLIEQ	12–21
4	FDHLLIEQAN	14–23

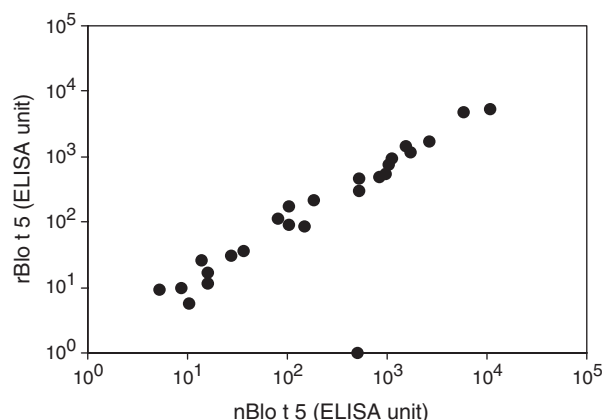


Fig. 2. Relative human IgE reactivity for native and recombinant Blo t 5 was assessed by ELISA and shown as correlation curve. One ELISA unit equals to 1 ng/mL. The ELISA unit was determined by a semi-purified human IgE as standard curve.

Blo t 5, N-terminal amino acids sequencing and mass spectrometry analysis were performed. The N-terminal amino acids sequences of the native Blo t 5 were summarized in Table 1. Most of the sequences obtained are a mixture of N-terminal peptide starting at position Glu+2 to Phe+14, indicating the purified native Blo t 5 was partially truncated.

Peptide mass fingerprinting was conducted using the tryptic peptides generated in non-reduced condition. Six peptides masses were found to match with the protein sequence, representing 62% of the full-length amino acid sequence (data not shown).

Two-dimensional western blotting analysis of native Blo t 5

Two-dimensional SDS-PAGE showed at least five protein spots at 14 kDa (Fig. 1b) with pI values ranging from 3 to 5.5, suggesting at least five distinct isoforms of Blo t 5.

These isoforms are allergenic as IgE immunoblots (Fig. 1c) show that IgE from sensitized subjects binds these isoforms. The blot also showed that IgE bound to protein at approximately 28 kDa, suggesting the formation of dimer in native protein. In contrast, non-atopic serum did not show any IgE binding (data not shown).

Allergenicity comparison between native and recombinant Blo t 5

In vitro IgE-binding activity was evaluated by ELISA (Fig. 2) with the sera of 25 mite-allergic children. The results revealed that IgE reactivity of both native and recombinant Blo t 5 correlates well, with only one child who showed high IgE binding to native Blo t 5 but not to recombinant Blo t 5.

Enzyme-linked immunosorbent assay inhibition

To compare the equivalent of protein IgE epitopes represented in the recombinant and native Blo t 5, IgE ELISA inhibition experiments were carried out. Sera from five Blo t 5-sensitized allergic children were used. As shown in Fig. 3, when the recombinant Blo t 5 was used as antigen, both native and recombinant displayed superimposable inhibition patterns on the recombinant molecule (Fig. 3a), suggesting the two molecules present comparable protein epitopes. However, inhibition by recombinant Blo t 5 was not complete (Fig. 3b) when native Blo t 5 was used as antigen, indicating that native molecule possess additional unique epitopes.

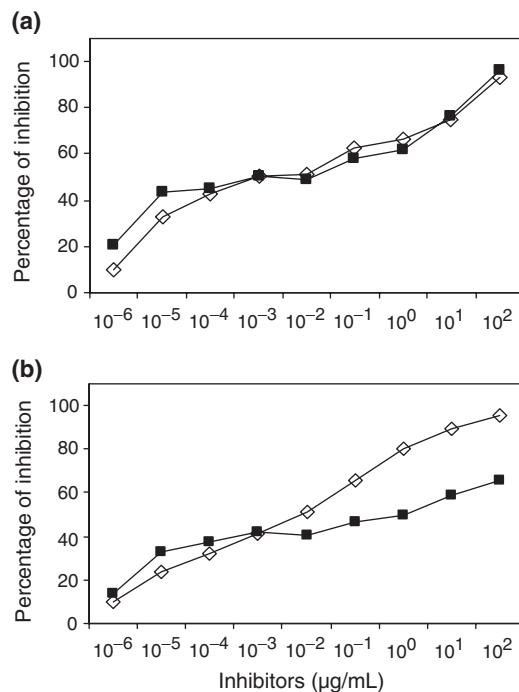


Fig. 3. Dose-dependent inhibition assay. Five Blo t 5 allergic patients sera were pooled and pre-absorbed with different concentration of recombinant (■) and native (◇) Blo t 5, before incubating with (a) recombinant and (b) native Blo t 5. The results are expressed as percentage of inhibition.

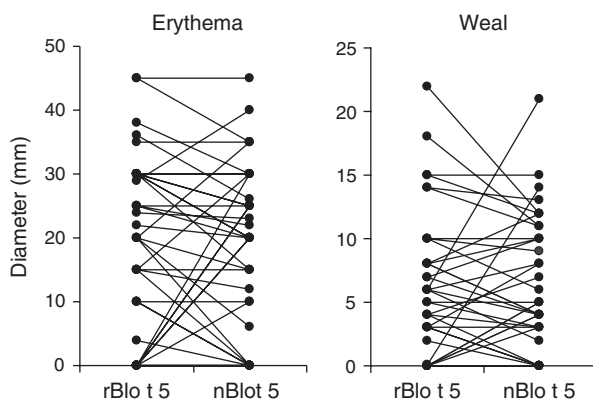


Fig. 4. The comparative assessment of the allergenicity of recombinant and native Blo t 5 based on the erythema and weal sizes by skin prick tests on 38 *Blomia tropicalis* skin test-positive subjects. The results are shown as the diameter of the skin reaction in millimetres.

Skin prick test responses

Allergenicity of native and recombinant Blo t 5 was evaluated by SPTs (Fig. 4) in 38 *B. tropicalis*-sensitive subjects and 16 of them showed comparable skin reaction (weal size) to both proteins. The other nine subjects had larger skin test reactions to native allergen, with six of them showing no reaction to recombinant Blo t 5. However, there were 11 subjects that showed larger skin reactions to recombinant Blo t 5. Two of the *B. tropicalis*-positive subjects were negative to Blo t 5. No positive reaction was observed in non-atopic subjects.

In vitro basophils histamine release

To evaluate the capacity of recombinant and native Blo t 5 to induce histamine release, whole blood from *B. tropicalis*-allergic subjects were challenged with various concentrations (0.001 pg/mL–10 µg/mL) of recombinant and native Blo t 5. A typical of dose-dependent histamine release (Fig. 5) from basophils was observed in all allergic subjects. There was no significant difference in histamine release between native and recombinant protein. All five subjects showed histamine release at 1 ng/mL of native and recombinant Blo t 5. No histamine release in response to native and recombinant Blo t 5 was triggered when basophils from non-atopic subject were used (data not shown).

Discussion

To date, only crude extracts have been used as diagnostic reagents. However, difficulties with standardization have been a major drawback. With the improvement in recombinant DNA technology, production of pure recombinant allergens is a promising step to circumvent this problem. Thus, the question arises as to whether this recombinant material could replace the laboriously purified natural allergens for diagnostic or therapeutic applications.

A diagnostic or potential therapeutic application of recombinant allergens requires the availability of recombinant protein preparations with properties comparable with those of the purified natural counterpart. Many [28–30], but not all [31] allergens expressed from cDNA so far have shown a considerable IgE binding reactivity that seems to be comparable with their natural counterpart. The immunogenicity of recombinant and native Blo t 5 was compared using direct human IgE ELISA (Fig. 2) and Blo t 5-specific two-site ELISA assay (data not shown) developed in our laboratory. The results showed that both native and recombinant Blo t 5 were very similar, suggesting a native-like folding of recombinant Blo t 5. However, the results of our absorption study with native and recombinant Blo t 5 (Fig. 3b) revealed that there was a significant difference between the two forms of allergen in IgE inhibition, suggesting that there are some antigenic structural differences between them. Further studies are necessary to address this question.

This study also showed that the recombinant and native Blo t 5 are able to bind to IgE receptors on basophils from allergic subjects and trigger them to release similar levels of histamine (Fig. 5). Recombinant group 2 allergens expressed in *E. coli* have been reported to show comparable IgE binding

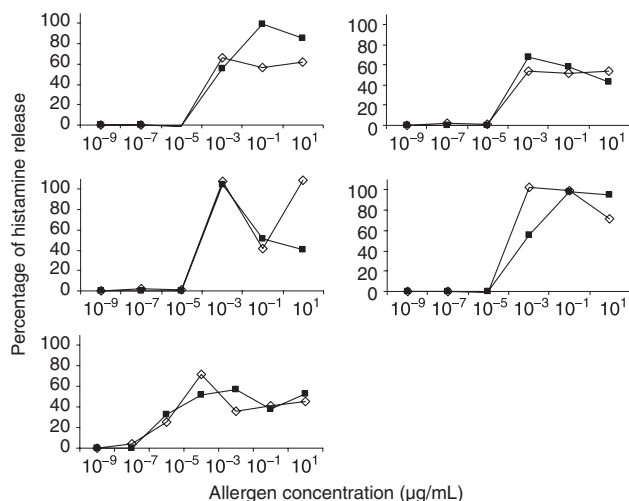


Fig. 5. Histamine release on challenge with different concentrations of recombinant (■) and native (◇) Blo t 5 in five Blo t 5-allergic subjects. The results are expressed as a percentage of total histamine.

activity to the native counterparts [30, 32]. Taken together, the post-translational modifications of these two mite allergens (Blo t 5 and Der p 2) probably do not play an important role in conferring allergenicity of these major mite allergens.

It has been known that allergens from various sources have been shown to comprise several isoforms [32–37], with different degrees of allergenicity [33, 37, 38]. The significantly greater skin reaction to native Blo t 5 in two subjects (Fig. 4) and incomplete ELISA inhibition (Fig. 3), could be explained by the presence of isoforms in the purified native protein, shown by heterogeneity on isoelectric points (Fig. 1). This observation could also be explained by differences in carbohydrate content, conformational changes, and differences in amide content. Recently we have isolated and expressed a number of cDNA coding for Blo t 5 variants (unpublished data), and the SPT results (data not shown) revealed that the isoforms have differential allergenicity in *B. tropicalis*-allergic subjects. Similar observations have been reported for food, mite and tree pollen allergens. Four variants of major allergen in hazelnut (Cor a 1.04) have been isolated and these variant showed modest differential IgE reactivity, ranging from 74% to 95% in hazelnut-allergic patients [33]. In contrast, the IgE binding activity was generally high in all Der p 2 variants; some slight differences were observed [38]. Furthermore, a report by Nishiyama et al. [30], indicated the presence of equal IgE recognition to three variants of Der f 2 in mite-sensitive patient sera. Unlike Cor a 1.04 and Der p 2, Bet v 1 consists of 11 isoforms [34] with three of the isoforms failed to recognize or showed remarkable low IgE binding [37]. Both Ferriera et al. and Thomas et al. [37–39] showed that the isoforms not only affect IgE binding capability but also T cell responses. Interestingly, these reports showed that the less allergenic isoforms of Bet v 1 and Der p 2 are the most potent activators of allergen-specific T lymphocytes and vice versa. The weaker or no skin reaction (Fig. 4) and lower levels of histamine release (Fig. 5) with native Blo t 5 in some of the subjects tested can also be explained by the varying contribution of other less allergenic isoforms besides the recombinant isoform

that used in this study, suggesting that these less allergenic isoforms may represent candidates for safe specific immunotherapy. However, for this, investigations on the antigenicity of the isoforms would be needed.

In summary, we have purified and identified at least five isoforms of native Blo t 5. Comparative allergenicity study of recombinant and native Blo t 5 revealed that among the *B. tropicalis* skin test-positive children, 24 out of 25 could be detected with recombinant Blo t 5 by *in vitro* IgE binding assay. SPT showed greater than 80% of the *B. tropicalis* skin test-positive subjects reacted with the recombinant Blo t 5 used in this study. It appears that the recombinant Blo t 5 allergen used in this study is probably representing one of the most allergenic isoforms of Blo t 5. Therefore, the current form of recombinant Blo t 5 can be used for diagnostic purposes. However, since a small subset of allergic subjects failed to react with this recombinant Blo t 5, evaluation of additional unique IgE epitopes exhibit by other isoforms is necessary for the development of better component-resolved diagnostic reagents.

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References

- Holgate ST. The epidemic of allergy and asthma. *Nature* 1999; 402:B2–4.
- Platts-Mills TAE, de Weck AL. Dust mite allergens and asthma – a worldwide problem. *J Allergy Clin Immunol* 1989; 83:416–27.
- Indoor allergens and asthma. Report of the 3rd International Workshop, Cuenca, Spain, June 30–July 2, 1995.
- Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002; 129:1–8.
- Ferrandiz R, Casas R, Dreborg S, Einarsson R, Ferrandiz B. Cross-reactivity between *Dermatophagoides siboney* and other house dust mite allergens in sensitized asthmatic patients. *Clin Exp Allergy* 1995; 25:929–34.
- Zhang L, Chew FT, Soh SY, Yi FC, Law SY, Lee BW. Prevalence and distribution of indoor allergens in Singapore. *Clin Exp Allergy* 1997; 27:876–85.
- Chew FT, Zhang L, Ho TM, Lee BW. House dust mite fauna in tropical Singapore. *Clin Exp Allergy* 1999; 29:201–6.
- Arlian LG, Vyszenski-Moher DL, Fernandez-Caldas E. Allergenicity of the mite, *Blomia tropicalis*. *J Allergy Clin Immunol* 1993; 91:1042–50.
- Cheong N, Yang L, Lee BW, Chua KY. Cloning of a group 3 allergen form *Blomia tropicalis* mites. *Allergy* 2003; 58:352–6.
- Arruda LK, Fernandez-Caldas E, Naspitz CK, Montealegre F, Vailes LD, Chapman MD. Identification of *Blomia tropicalis* allergen Blo t 5 by cDNA cloning. *Int Arch Allergy Immunol* 1995; 107:456–7.
- Puerta L, Caraballo L, Fernandez-Caldas E, Avjioglu A, Marsh DG, Lockett RF. Nucleotide sequence analysis of a complementary DNA coding for *Blomia tropicalis* allergen. *J Allergy Clin Immunol* 1996; 98:932–7.
- Yi FC, Cheong N, Shek LPC, Wang DY, Chua KY, Lee BW. Identification of shared and unique IgE epitopes of the highly

- conserved tropomyosins in *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. Clin Exp Allergy 2002; 32:1203–10.
- 13 Ramos JDA, Cheong N, Lee BW, Chua KY. cDNA cloning and expression of Blo t 11, the *Blomia tropicalis* allergen homologous to paramyosin. Int Arch Allergy Immunol 2001; 126:286–93.
 - 14 Caraballo L, Puerta L, Jimenez S, Martinez B, Mercado D, Avjiouglu A. Cloning and IgE binding of a recombinant allergen from the mite *Blomia tropicalis*, homologous with fatty acid-binding proteins. Int Arch Allergy Immunol 1997; 112:341–7.
 - 15 Kuo IC, Cheong N, Trakultivakorn M, Lee BW, Chua KY. An extensive study of human IgE cross-reactivity of Blo t 5 and Der p 5. J Allergy Clin Immunol 2003; 111:603–9.
 - 16 Kuo IC, Yi FC, Cheong N, Shek LPC, Chew FT, Lee BW. Sensitization to *Blomia tropicalis* and *Dermatophagoides pteronyssinus*: a comparison study between Singapore and Taiwan. Asian Pacific J Allergy Immunol 1999; 17:179–88.
 - 17 Shek LPC, Chua KY, Kuo IC, Huang CH, Chew FT, Lee BW. Pattern of allergic sensitization to recombinant mite allergens of *Dermatophagoides pteronyssinus* and *Blomia tropicalis* in Singapore. J Allergy Clin Immunol 1999; 103:S26.
 - 18 Jimenez S, Caraballo LR, Chua KY, Mercado D, Puerta L, Mercado D. IgE antibody responses to recombinant allergens of *Blomia tropicalis* (Bt) and *Dermatophagoides pteronyssinus* (Dp). J Allergy Clin Immunol 1999; 103:S185.
 - 19 Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gronlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). Clin Exp Allergy 1999; 29:896–904.
 - 20 Arruda LK, Vailes LD, Platts-Mills TAE et al. Sensitization to *Blomia tropicalis* in patients with asthma and identification of allergen Blo t 5. Am J Respir Crit Care Med 1997; 155:343–50.
 - 21 Goh LT, Kuo IC, Luo S, Chua KY, White M. Production and purification of recombinant *Blomia tropicalis* group 5 allergen from *Pichia pastoris* culture. Biotechnol Lett 2001; 23:661–5.
 - 22 Yi FC, Chew FT, Jimenez S, Chua KY, Lee BW. Culture of *Blomia tropicalis* and IgE immunoblot characterization of its allergenicity. Asian Pacific J Allergy Immunol 1999; 17:189–94.
 - 23 Yang L, Cheong N, Wang DY et al. Generation of monoclonal antibodies against Blo t 3 using DNA immunization with in vivo electroporation. Clin Exp Allergy 2003; 33:663–8.
 - 24 Yi FC, Lee BW, Cheong N, Chua KY. Quantification of Blo t 5 in mite and dust extracts by two-site ELISA. Allergy 2004; in press.
 - 25 Wilkins MR, Lindskog I, Gasteiger E et al. Detailed peptide characterization using PEPTIDEMASS – a World-Wide-Web-accessible tool. Electrophoresis 1997; 18:403–8.
 - 26 Zhang W, Chait BT. ProFound: an expert system for protein identification using mass spectrometric peptide mapping information. Anal Chem 2000; 72:2482–9.
 - 27 Seow TK, Ong SE, Liang RC et al. Two-dimensional electrophoresis map of the human hepatocellular carcinoma cell line, HCC-M, and identification of the separated proteins by mass spectrometry. Electrophoresis 2000; 21:1787–813.
 - 28 Zargari A, Schmidt M, Lundberg M, Scheynius A, Whitley P. Immunologic characterization of neutral and recombinant Mal f 1 yeast allergen. J Allergy Clin Immunol 1999; 103:877–84.
 - 29 Schmid-Grendelmeier P, Holzmann D, Himly M et al. Native Art v 1 and recombinant Art v 1 are able to induce humoral and T cell-mediated in vitro and in vivo responses in mugwort allergy. J Allergy Clin Immunol 2003; 111:1328–36.
 - 30 Nishiyama C, Yuuki T, Usui Y, Iwamoto N, Okumura Y, Okudaira H. Effects of amino acid variations in recombinant Der fII on its human IgE and mouse IgG recognition. Int Arch Allergy Immunol 1994; 105:62–9.
 - 31 Greene WK, Cyster JG, Chua KY, O'Brien RM, Thomas WR. IgE and IgG binding of peptides expressed from fragments of cDNA encoding the major house dust mite allergen Der p 1. J Immunol 1991; 147:3768–73.
 - 32 Olsson S, Van Hage-Hamsten M, Whitley P et al. Expression of two isoforms of Lep d 2, the major allergen of *Lepidoglyphus destructor*, in both prokaryotic and eukaryotic systems. Clin Exp Allergy 1998; 28:984–91.
 - 33 Luttkopf D, Muller U, Skov PS et al. Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. Mol Immunol 2002; 38:515–25.
 - 34 Swoboda I, Jilek A, Ferreira F et al. Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. J Biol Chem 1995; 270:2607–13.
 - 35 Chatel JM, Bernard H, Orson FM. Isolation and characterization of two complete Ara h 2 isoforms cDNA. Int Arch Allergy Immunol 2003; 131:14–8.
 - 36 Le Mao J, Mayer CE, Peltre G et al. Mapping of *Dermatophagoides farinae* mite allergens by two-dimensional immunoblotting. J Allergy Clin Immunol 1998; 102 (Part 1):631–6.
 - 37 Ferreira F, Hirtenlehner K, Jilek A et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 1996; 183:599–609.
 - 38 Hales BJ, Hazell LA, Smith W, Thomas WR. Genetic variation of Der p 2 allergens: effects on T cell responses and immunoglobulin E binding. Clin Exp Allergy 2002; 32:1461–7.
 - 39 Ferreira F, Hirtenlehner K, Briza P et al. Isoforms of atopic allergens with reduced allergenicity but conserved T cell antigenicity: possible use for specific immunotherapy. Int Arch Allergy Immunol 1997; 113:125–7.