

Short communication

Cloning of a group 3 allergen from *Blomia tropicalis* mites

Background: *Blomia tropicalis* is an important mite species in the tropics and subtropical regions of the world. It is well established that the allergen from this species of mite is one of the triggering factors for allergic asthma. The isolation and characterization of allergens in this mite species is desired to provide sensitive and specific reagents for diagnostic as well as therapeutic purposes.

Methods: The SMART (Clontech Laboratories, Palo Alto, CA, USA) rapid amplification of complementary DNA ends (RACE cDNA amplification) method was used to isolate the putative Blo t 3 gene. Polymerase chain reactions (PCR) were performed in the presence of specific gene primers to obtain the full-length gene, and were confirmed by DNA sequencing. The putative gene was cloned into *E. coli* expression vector GST-4T-1 and expressed as a fusion protein with glutathione-S-transferase (GST). The allergenicity of the GST-Blo t 3 recombinant protein was evaluated by human IgE enzyme-linked immunoassay (ELISA) and skin pricks tests.

Results: The full length Blo t 3 gene had 1138 base pairs, including a 105-bp long 5' nontranslated region, an ATG start codon at positions 106–108, and a stop codon TAA at positions 904–906, with an open reading frame coding for a polypeptide of 266 amino acids. Protein analysis revealed that it was a serine protease that had a prepro-mature structure that shared high sequence homology with group 3 dust mite allergens. The predicted molecular weight of the matured protein was approximately 23.8 kD with a theoretical pI of 8.87.

The frequency of IgE reactivity of the recombinant protein showed up to 50% of IgE reactivity with mite allergic subjects but IgE titer was generally low.

Conclusion: We had isolated and fully characterized the cDNA encoding an important *B. tropicalis* allergen that was highly homologous to Group 3 dust mite allergens and we proposed that it should be designated as Blo t 3. Its clinical importance was implicated by the high frequency of IgE reactivity with allergic sera.

N. Cheong¹, L. Yang², B. W. Lee²,
K. Y. Chua²

¹Bioprocessing Technology Center; ²Department of Paediatrics, Faculty of Medicine, National University of Singapore, Republic of Singapore

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Prof Chua Kaw Yan
Department of Paediatrics
Faculty of Medicine
National University of Singapore
Lower Kent Ridge
Singapore 119074

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The domestic mites, *Blomia tropicalis* classified under the family *Glycyphagidae* (1), is a main component of the house dust in the tropical and subtropical regions. It has been well documented that this mite is an important triggering factor for allergic asthma and rhinitis in the tropics (2–4, 6–8). Studies on the allergenicity of *B. tropicalis* revealed the presence of multiple allergens. Crossed radioimmuno-electrophoresis (CRIE) studies identified several species-specific IgE binding-antigens in *B. tropicalis* (6) while immunoblotting technique was able to reveal the presence of multiple components with molecular weights of 11–85 kD (8). To date, cDNA clones coding for five allergens have been characterized from this mite. These are Blo t 5 (5, 9), Blo t 10 (10) and Blo t 11 (11). Blo t 12 (which was designated as Blo t 11a) (3), Blo t 13 (previously known as Blo t 6) (12, 13).

This study reports the cloning a new *B. tropicalis* allergen that shares high sequence homology with group 3 mite allergens. The prototype group 3 allergen, Der f 3, was originally described by Heymann and coworkers (14).

It is a 29 kDa trypsin isolated from faecally enriched extracts of *Dermatophagoides farinae* by gel filtration. Steward et al. used benzamidine to affinity purified an approximately 30 kDa trypsin-like enzyme from spent mite medium of *Dermatophagoides pteronyssinus* (15) and Ando et al. had characterized a trypsin-like protease from faecal extract of *D. farinae* (16). Der p 3 was affinity purified from spent mite medium of *D. pteronyssinus* as a 31 kDa form predominantly, with occasionally 28 kDa and 21 kDa forms existing (17). Der p 3 and Der f 3 have been further characterized by *N*-terminal protein sequencing, nucleotide sequencing analysis and by monoclonal antibodies. These mite trypsins appeared to possess properties similar to those reported for other trypsins, particularly from insects. They were shown to be highly polymorphic and exhibit distinct species differences in the number and range of isoforms (15). Chromatofocusing studies revealed the presence of nine major isoforms in *D. pteronyssinus* with pI in the range 4 to > 8, but only two (range 4–5) in *D. farinae* (15, 18).

Material and methods

Sera sample

A total of 45 selected sera from subjects whom were tested positive to crude mite extract using Pharmacia CAP system (Pharmacia, Peapack, NJ, USA). These sera were kindly provided by Dr. Wang De Yun of the National University Hospital, Singapore.

5'-Rapid amplification of cDNA ends (5'-RACE) and 3'-RACE

The 5'-RACE Ready cDNA and 3'-RACE Ready cDNA (mite cDNA library) was generated by following the protocol described in the SMART RACE cDNA Amplification Kit User Manual, protocol number PT3269-1 version PR93377 (Clontech) with slight modifications. Briefly, total RNA was isolated from live *B. tropicalis* cultured mites (19) using commercial Trizol reagent (Gibco BRL, New York, NY). 1 µg of total RNA was utilized to generate either 5' or 3' RACE Ready cDNA products with specific primers and reagents provided by the kit in the presence of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. Generation of 5'-RACE fragment was performed as described earlier (11). In brief, a 50-µl PCR reaction mixture composed of 2.5 µl 5'-RACE-Ready cDNA, 5 µl of 10 x Universal Primer Mix (UPM), 1 µl of 10 µM Gene Specific Primer 1 (GSP 1) and 41.5 µl of Master Mix (Clontech) was prepared. Positive and negative controls were prepared according to the protocol of the manufacturer. GSP 1, 5'-ATA ACC CCA ACC AGA AAC ACG 3', was designed based on the nucleotide sequences of group 3 allergens of other mite species.

Clone 14, a truncated gene lacking the 3' region was isolated from the 5' RACE product with primer GSP 1, and its DNA sequence was confirmed by DNA sequencing. In order to generate the 3' region of Blo t 3, a 3'-RACE was performed. Gene Specific Primer 6 (GSP 6), 5'-CGC CAA CTA TGG TGC CGT TGG TGC TGA-3' was designed based on the known sequence of clone 14. A 50-µl PCR reaction mixture composed of 3'-RACE-Ready cDNA, primer GSP 6, UPM and Master Mix was set up as described earlier. Positive and negative controls were also included. The PCR product was designated as clone 15, which encoded the 3' end of the Blo t 3 gene with 280 overlapping nucleotides with the clone 14.

The full-length Blo t 3 cDNA gene was generated by long distance PCR using gene specific forward primer, 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA-3', and the reverse primer, 5'-ATA CGA CTC ACT ATA GGG CAA GCA GTG-3' based on the sequence of the clones 14 and 15, respectively. The PCR products were purified and confirmed by DNA sequencing.

Expression of Blo t 3 in bacteria

The Blo t 3 cDNA insert coding for the putative mature protein was amplified by PCR using primers 5'-CG GGA TCC ATT GTT GGT GGA TCG AAT-3' and 5'-G CGC GGC CGC TTA AAC TGG AAC TCC TTT-3', containing *Bam*HI and *Eco*RI restriction enzyme sites, respectively. Amplification was performed with 30 cycles as described earlier. The gel-purified PCR product was digested with *Bam*HI and *Eco*RI and was ligated into pGEX-4T1 vector (Pharmacia Biotech, UK). The recombinant protein was expressed as a glutathione S-transferase (GST) fusion protein in *E. coli* (BL21 strain) by 1 mM isopropyl- α -D-thiogalactopyranoside (Calbiochem-Novabiochem, Darmstadt, Germany) induction and purified using glutathione Sepharose column (Sigma, USA) as described earlier (20). Expressed protein was analyzed by SDS-PAGE according to the method of Laemmli (21) and by Western blot.

Skin prick tests

Skin prick tests were conducted according to the protocol described previously (10). Briefly, the skin of the volar forearm was pricked with a disposable lancet in the presence of a drop of allergen at a concentration of 25 µg/ml. The reaction was regarded as positive when the wheal diameter was 3 × 3 mm greater than the negative control. Glycerol buffer and GST (25 µg/ml), and 1 mg/ml of histamine were used as negative and positive controls, respectively.

ELISA for Human IgE reactivity to recombinant Blo t 3

50 µl of 5 µg/ml of each of the recombinant GST-Blo t 3 and GST were coated onto ELISA plates overnight at 4°C using 50 µl of 0.1 M NaHCO₃, pH 8.4. Plates were then washed three times with washing buffer (1 × phosphate-buffered saline (PBS) with 0.05% Tween 20) and blocked with 100 µl 1% bovine serum albumin (BSA) in PBS-Tween 20 for 60 min at 37°C. 50 µl human sera (preabsorbed with 10 µg/ml GST and diluted 1 : 5 in blocking buffer) were added with further overnight incubation at 4°C. Plates were then washed and 5/0 µl of 250 ng/ml of biotin-conjugated antihuman IgE (Southern Biotech, USA) were added and incubated for 60 min at 37°C. Plates were washed and 50 µl of 1 : 2000 dilution of ExtrAvidine Alkaline phosphatase-conjugated (Sigma, St. Louis, MO) were added for 1 h at 37°C in dark. After the final wash, colorimetric reaction was performed by adding 100 µl of paranitrophenyl phosphate (Sigma) and then incubated at 37°C in dark. Absorbance at 405 nm was determined using the Spectra & Rainbow ELISA reader (Tecan, Austria).

Results

Sequence analysis

The full length putative group 3 gene from *B. tropicalis* (designated as Blo t 3) had 1138 base pairs, including a 105-bp long 5' nontranslated region, a ATG start codon at positions 106–108, and a stop codon, TAA at positions 904–906, with an open reading frame coding for a polypeptide of 266 amino acids (Fig. 1).

Protein analysis revealed that it was a serine protease with a prepro-mature structure. Nucleotide sequence analysis showed that Blo t 3 had 41%, 47% and 42% homology with the group 3 of *D. pteronyssinus* (Der p), *D. farinae* (Der f) and *Euroglyphus maynei* (Eur m), respectively. Further, Blo t 3 had 47%, 48% and 47% protein sequence homology with Der p 3, Der f 3 and Eur m 3, respectively. In contrast, Der p 3, Der f 3 and Eur m 3 shared approximately 80% nucleotide and protein sequence homology with each other. Blo t 3, Der p 3, Der f 3 and Eur m 3 shared six conserved cysteine residues I, II, III, IV, V, and VI (Fig. 2) at the same respective locations in the molecules. The calculated molecular weights for Blo t 3, Der p 3, Der f 3 and Eur m 3 were 23.8, 24.5, 24.9 and 25, respectively, whereas the pI values were 8.87, 8.13, 5.36 and 5.8, respectively. The signature sequences for serine active site DACQGDSGGPY and the histidine active site LTAAHC were highly conserved. Further, residues

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taatacgcactcactatagggaagcagtggttaacaacgcagagtacgcgggacattcgag 60
                                                                 -35
aactatttttacacttcaaaaaataatagaatcatcttaccaaaatgaaggttctcggt 120
                                                                 M K V L V
ttattttgccttggttcattggccgctgctggaccattgaaagatgctttgaacaaagca 180
L F C L V S L A A A G P L K D A L N K A
                                                                 -1 +1
caggttgatgcttttctatgctgaaggatataattgttggtggatcgaatgctgctgatgg 240
Q V D A F Y A E G Y I V G G S N A A D G
gatgctccatatcaagtttcattacaacgaacatcacatttttgtggtggatcaatcatt 300
D A P Y Q V S L Q R T S H F C G G S I I
gcagacaactatattttgactgccgctcattgtattcaaggtttatcgacctcctctttg 360
A D N Y I L T A A H C I Q G L S T S S L
accattcgatacaataacttttacgacacaatcctgggtggtttgaccgtcaaagcatcacgt 420
T I R Y N T L R H N P G G L T V K A S R
atcattggacacgaaaaatacgaattcaaacactatcgataacgatattgctttgatacaa 480
I I G H E K Y D S N T I D N D I A L I Q
acggcttctaagatgtccaccggaacaactaacgctcaagcgattaaacttcagaacaa 540
T A S K M S T G T T N A Q A I K L P E Q
ggttcggaccctaaaagcaagttcaaaagttcttattactgggtgggtaccttgccttct 600
G S D P K A S S K V L I T G W G T L S S
ggtgctctctcattgccgaccaaattgcagaaagttaccgtaccgattgtcgatcgaaaa 660
G A S S L P T K L Q K V T V P I V D R K
acgtgtaacgcccaactatgggtgccgttggtgctgatattactgataacatgttctgtgct 720
T C N A N Y G A V G A D I T D N M F C A
ggatcttgaacgttggcggttaaggacgcttgccaagggtgattccggtggaccagttgct 780
G I L N V G G K D A C Q G D S G G P V A
gcgaatgggtgttctcggttggtgctgtctcatggggtatgggtgcgctcaagccaaatat 840
A N G V L V G A V S W G Y G C A Q A K Y
cctggtgtatacactcggtgcgaaactacatctcggtgattaaaggaaaaggagttcca 900
P G V Y T R V G N Y I S W I K G K G V P
gtttaaatcacatttgaacttaatatttaaattaattctaaactcaacaacaacaaactc 960
V -
aatccaattgtagatcaatcaatccaattgaatttgttattacacaatattgcaaacttt 1020
aaacaattaaaactatataagtcgccatctatcggtgacatatcaaaaaaaaaaaaaa 1080
aaaaaaaaaaagtactctgcgttggttaccactgcttgccctatagtgagtcgtattag 1138

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Figure 1. Nucleotide and deduced amino acid sequences of Blo t 3 allergen. The numbers on the right denote the nucleotide numbers. The start codon and the stop codon are underlined. Residues –1 to –35 denote the prepro-region. Residue +1 denotes the first amino acid of the mature protein.

comprising the active site of serine proteases, i.e. residues 75 His, 215 Asp, and 220 Ser were all conserved in the gene. Residue 120 Asp, which was essential for substrate binding and recognition, was also maintained (Fig. 2) (17, 22, 23).

Expression and allergenicity of recombinant Blo t 3 protein

Expression of recombinant fusion GST-Blo t 3 clone gave a single band at 54 kDa on a 7.5% SDS-PAGE. IgE

immunoblot analysis showed that the GST-Blo t 3 protein reacted with IgE in the mite-allergic pooled human sera. Recombinant Blo t 3 was also evaluated by skin prick tests in a small panel of mite allergic asthmatic patients and prominent wheal size and erythema were observed in at least ten patients (data not shown). *In vitro* IgE-binding activity was further evaluated by ELISA with 45 Blo t-extract positive sera. Figure 3 indicated that 51% (23/45) of these population showed positive IgE reactivity to recombinant mature GST-Blo t 3.

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Der p 3 -MIIYNILIVLLLLAINTLANPILPASPNAT-----IVGGEKALAGECPYQISLQSSSHFIC
Eur m 3 -MVICNAIIIVLLLLAFNTLANPILPSSPNAT-----IVGGQKAKAGECPYQISLQSSSHFC
Der f 3 -MMILT--IVVLLAANILATPILPSSPNAT-----IVGGVKAQAGDCPYQISLQSSSHFC
Blo t 3 MKVLVLFCLVSLAAGPLKDALNKAQVDAFYAEGYIVGGSNAADGDAPYQVSLQRTSHFC 60
      : : * * * . * . : : : * * * * : * * . * * * : * * *
      II
Der p 3 GGTILDEYWILTAAHCVAGQTASKLSIRYNSLKHSLGGEKISVAKIFAHEKYDSYQIDND
Eur m 3 GGTILDEYWILTAAHCVNGQTASKLSIRYNSLKHASGGEKLSVAQIYQHEKYDSWTIDND
Der f 3 GGSILDEYWILTAAHCVNGQSAKKLSIRYNTLKHASGGEKIQVAEYQHENYDSMTIDND
Blo t 3 GGSIIADNYILTAAHCIQGLSTSSLTIRYNTLRHNPGLTVKASRIIGHEKYDSNTIDND 120
      * * : : : * * * * : * : : : * * * * : * * * : : : * * : * * * * *
      III IV V
Der p 3 VVSRKECNELYSKANAEVTDNMICGGDVANGGKDSCQGDSGGPVVDVKNNQVGVISWGY
Eur m 3 IVAREQCNKLYEEAGATITDNMICGGNVADGGVDSCQGDSGGPVVDVANSQIVGVISWGY
Der f 3 VVSREQCDQLYSKAGADVSENMICGGDVANGGVDSCQGDSGGPVVDVATKQIVGVISWGY
Blo t 3 IVDRKTCNANYGAVGADITDNMFCAGILNVGGKDDACQGDSGGPVV--ANGVLVGAWSGY 238
      : * : * : * . * : : * * : * : * * : * * * : * * * : : * * * * *
      VI
Der p 3 GCARKGYPGVYTRVGNFIDWIESKRSQ-
Eur m 3 GCARKGYPGVYTRVGSFIDWIDSKRSQ-
Der f 3 GCARKGYPGVYTRVGNFVDWIESKRSQ-
Blo t 3 GCAQAKYPGVYTRVGNYSIWIKGKVPV
      * * : * * * * * : : : * * . *

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Figure 2. Amino acid sequence alignment of Blo t 3 with other group 3 mite allergens. *Dermatophagoides pteronyssinus* (Der p 3), *Dermatophagoides farinae* (Der f 3), *Euroglyphus maynei* (Eur m 3). The amino acid numbering is based on the Blo t 3 sequence. The signature sequences of the serine active site and the histidine active site are in bold, and the catalytic sites of the mature protein are underlined. * = identical, : = highly conserved, . = less conserved (CLUSTAL W MULTIPLE SEQUENCE ALIGNMENT PROGRAM; v1.8, 1999 [European Bioinformatics Institute, UK]).

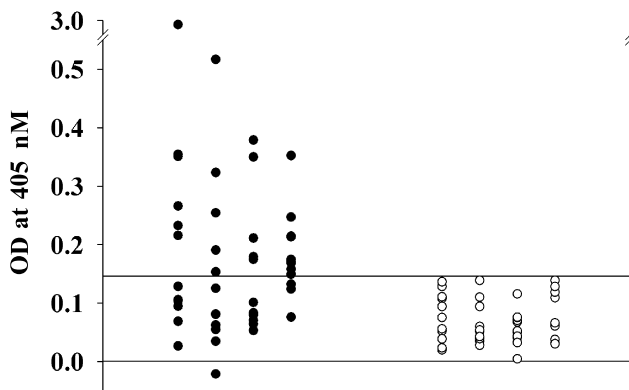


Figure 3. Human IgE ELISA assay using mite allergic sera with GST-Blo t 3 (●) and GST control (○) proteins. The cut-off value is the mean $\pm 2 \times$ standard deviation of the negative sera.

Discussion

In this study, we report for the first time the isolation of a full length cDNA clone encoding a trypsin-like serine protease from *B. tropicalis*. The deduced protein showed significantly high sequence and structural homology with the group 3 mite allergens.

Blo t 3 gene was expressed without its prepro regions as a fusion protein with GST in this study. Our ELISA and skin prick test data demonstrated that such recombinant Blo t 3 was allergenic. ELISA data indicated that the frequency of IgE reactivity in the tested population

was 50%, however, the IgE titer was generally low, except for a few sera. This low IgE titer could be attributed to improper folding of the protein when it was produced in *E. coli*. The ultimate solution will be the isolation of the native Blo t 3 from the mites so that its allergenicity can be assessed more fully. This is currently being carried out in our laboratory. So far, there was only one other published report on the expression of group 3 allergen from *D. farinae* (24). In that study, Der f 3 was expressed with its pro-sequence as a GST fusion protein in *E. coli*. The affinity purified Der f 3 protein failed to show protease activity. Subsequent alteration of an amino acid residue at the C-terminal end and then the removal of the pro-region rendered the recombinant protein enzymatically active, achieving almost the same specificity and activity as that of native Der f 3. The authors concluded that pro-Der f 3 did not have the ability to produce mature enzyme and that another protease which converted pro-Der f 3 to the active form was present in the mites. However, the allergenicity of the recombinant Der f 3 was not assessed in the study.

There is much controversy in defining the importance of the group 3 trypsin-like serine protease allergens. The IgE reactivity in the mite allergic patients ranges from as low as 16% (14) by the fluid phase assay to 100% (15, 25) by RAST. Intermediate result at 66% by RAST has also been demonstrated (26). A group 3 allergen from *D. siboney* was considered as a major allergen by the authors because of its high frequency.

(73%) of IgE reactivity (27). Unlike other studies mentioned above, our study was conducted using recombinant Blo t 3 that showed up to 50% frequency of reactivity with our tested population. The differences reported by the various studies could be attributed to the difference of the studied populations, the methods for the quantification of IgE antibody or in the purity and quantity of the allergen preparations being used. In order to have a proper evaluation of the clinical importance of Blo t 3, native Blo t 3 will be used in parallel with recombinant Blo t 3 for comparative

allergenicity studies using larger panels of allergic population in due course.

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References

- LIEBERS V, SANDER I, VAN KAMPER V, et al. Overview on denominated allergens. *Clin Exp Allergy* 1996;**26**:494–516.
- MARIANA A, HO TM, HEAH SK. Life-cycle, longevity and fecundity of *Blomia tropicalis* (Acaris: Glycyphagidae) in a tropical laboratory. *Southeast Asian J Trop Medical Public Health* 1996;**27**(2):392–395.
- PUERTA L, CARABALLO L, FERNANDEZ-CALDAS E, et al. Nucleotide sequence analysis of a complementary DNA coding for a *Blomia tropicalis* allergen. *J Allergy Clin Immunol* 1996;**98**(5):932–937.
- PUERTA L, FERNANDEZ-CALDAS E, MERCADO D, LOCKEY RF, CARABALLO LR. Sequential determinations of *Blomia tropicalis* allergens in mattress and floor dust samples in a tropical city. *J Allergy Clin Immunol* 1996;**97**(2):689–691.
- 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**(1–3):456–457.
- ARLIAN LG, VYZSENSKI-MOHER DL, FERNANDEZ-CALDAS E. Allergenicity of the mite, *Blomia tropicalis*. *J Allergy Clin Immunol* 1993;**91**(5):1042–1050.
- FERNANDEZ-CALDAS E. Allergenicity of *Blomia tropicalis*. *J Invest Allergol Clin Immunol* 1997;**7**(5):402–404.
- CARABALLO L, PUERTA L, MARTINEZ B, MORENO L. Identification of allergens from the mite *Blomia tropicalis*. *Clin Exp Allergy* 1994;**24**(11):1056–1060.
- CARABALLO L, AVJIOGLU A, MARRUGO J, PUERTA L, MARSH D. Cloning and expression of complementary DNA coding for an allergen with common antibody-binding specificities with three allergens of the house dust mite *Blomia tropicalis*. *J Allergy Clin Immunol* 1996;**98**(3):573–579.
- YI FC, CHEONG N, LYNETTE SHEK PC, 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**(8):1203–1210.
- 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**:286–293.
- CARABALLO L, PUERTA L, JIMENEZ S, et al. Sequence and IgE-binding of a new recombinant allergen from *Blomia tropicalis*. (Abstract 950). *J Allergy Clin Immunol* 1996;**97**(1:3):420.
- CARABALLO L, PUERTA L, JIMENEZ S, et al. 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**(4):341–347.
- HEYMANN PW, CHAPMAN MD, AALBERSE RC, FOX JW, PLATTSMILLS TA. Antigenic and structural analysis of group II (Der f II and Der p II) from house dust mites (*Dermatophagoides* spp.). *J Allergy Clin Immunol* 1989;**83**(6):1055–1067.
- STEWART GA, WARD LD, SIMPSON RJ, THOMPSON PJ. The Group III allergen from the house dust mite *Dermatophagoides pteronyssinus* is a trypsin-like enzyme. *Immunology* 1992;**75**(1):29–35.
- ANDO T, HOMMA R, INO Y, et al. Trypsin-like protease of mites-purification and characterization of trypsin-like protease from mite faecal extract *Dermatophagoides farinae*. Relationship between trypsin-like protease and Der f III. *Clin Exp Allergy* 1993;**23**(9):777–784.
- SMITH WA, CHUA KY, KUO MC, ROGERS BL, THOMAS WR. Cloning and sequencing of *Dermatophagoides pteronyssinus* group III allergen Der p III. *Clin Exp Allergy* 1994;**24**(3):220–228.
- STEWART GA, KOLLINGER MR, KING CM, THOMPSON PJ. A comparative study of three serine proteases from *Dermatophagoides pteronyssinus* and *D. farinae*. *Allergy* 1994;**49**:553–560.
- YI FC, CHEW FT, JIMENEZ S, CHUA KY, LEE BW. Culture of *Blomia tropicalis* and IgE immunoblot of its allergenicity. *Asian Pac J Allergy Immunol* 1999;**17**:189–194.
- CHUA KY, DILWORTH RJ, THOMAS WR. Expression of *Dermatophagoides pteronyssinus* allergen, Der p II in *Escherichia coli* and the binding studies with human IgE. *Int Arch Allergy Appl Immunol* 1990;**91**(2):124–129.
- LAEMMLI UK. Cleavage of structure proteins during assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.
- BRENNER S. The molecular evolution of genes and proteins: a tale of two serines. *Nature* 1988;**334**:528–530.
- RAWLINGS ND, BARRETT AJ. Families of serine peptidases. *Meth Enzymol* 1994;**244**:19–61.
- NISHIYAMA C, YASUHARA T, YUUKI T, OKUMURA Y. Cloning and expression in *Escherichia coli* of cDNA encoding house dust mite allergen Der f 3, serine protease from *Dermatophagoides farinae*. *FEBS Lett* 1995;**377**(1):62–66.
- KING C, SIMPSON RJ, MORITZ RL, THOMPSON PJ, STEWART GA. The isolation and characterization of a novel collagenolytic serine protease allergen (Der P 9) from the dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 1996;**98**(4):739–747.
- YASUEDA H, MITA H, AKIYAMA K, et al. Allergens from *Dermatophagoides* mites with chymotryptic activity. *Clin Exp Allergy* 1993;**23**:384–390.
- FERRANDIZ R, CASAS R, DREBORG S. Purification and IgE binding capacity of Der s 3, a major allergen from *Dermatophagoides siboney*. *Clin Exp Allergy* 1997;**27**:700–704.