

Mite Amylase from *Blomia tropicalis* (Blo t 4): Differential Allergenecity Linked to Geographical Regions

Nge Cheong^a John Donnie A. Ramos^{a,c} Chwee Ying Tang^b Hiok Hee Chng^b
Rong Yao^d Zongan Liang^d Bee Wah Lee^a Kaw Yan Chua^a

^aDepartment of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, ^bRheumatology, Allergy and Immunology, Tan Tock Seng Hospital, Singapore; ^cDepartment of Biological Sciences, College of Science, University of Santo Tomas, Manila, Philippines; ^dDepartment of Respiratory Medicine, Sichuan University West China Hospital, Chengdu, PR China

Key Words

Skin prick test • Blo t 4 sensitization, prevalence • Immunoglobulin E reactivity

Abstract

Background: *Blomia tropicalis* is an important domestic dust mite in the tropics and subtropics. This study describes cDNA cloning of the group 4 allergen of *B. tropicalis*, and the evaluation of the sensitization of this allergen in atopic populations from 2 geographic regions. **Methods:** cDNA cloning was carried out using the Smart RACE cDNA amplification kit. The full-length Blo t 4 cDNA was isolated by cDNA library screening, 5' and 3' rapid amplification of cDNA ends and long-distance PCR. Sequence analysis was performed with a combination of the Clustal W, CGC and Blast program packages. The cDNA was expressed in *Pichia pastoris* yeast. The skin prick test was used to evaluate the sensitization profile of recombinant Blo t 4, crude dust mite allergen extracts and major *B. tropicalis* recombinant allergen Blo t 5. **Results:** The cloned Blo t 4 had a molecular weight of 56 kDa and had 68% amino acid homology with group 4 allergens of *Dermaphagoides pteronyssinus* and 65% with those of *Euroglyphus maynei*. A sensitization profile to the expressed recom-

binant Blo t 4 allergen (28%) showed an unusually higher frequency than to the major allergen Blo t 5 (22%) in allergic subjects from Chengdu, PR China. In comparison, the subjects from Singapore showed very low sensitization to Blo t 4 (4%) compared with Blo t 5 (84%). **Conclusions:** Group 4 allergens of *B. tropicalis* may be an important dust mite allergen in certain distinct populations.

Copyright © 2008 S. Karger AG, Basel

Introduction

Group 4 mite allergen was initially isolated as an amylase by its substrate affinity [1]. α -Amylase is an enzyme which catalyzes the hydrolysis of starch and related dextrans. It is one of the most common enzymes in nature, being present in bacteria, fungi, plants, insects and mammals. Amylase proteins from these species have been sequenced and show that there are several areas of high sequence similarity. These areas are important for both structural and functional activity [2]. α -Amylases from cereals and fungi have been shown to be major causes of occupational asthma and dermatitis [3–6]. The 3-dimensional structures of α -amylases from mammals, insects,

bacteria, plants and fungi have been determined [2]. These structures contain a barrel of 8 parallel β -strands surrounded by 8 α -helices, with a large loop between strand β 3 and helix α 3. α -Amylases contain several well-defined regions in their amino acid sequences that are highly conserved across a broad spectrum of organisms that are of evolutionary distinct origins. Group 4 allergens from *Dermatophagoides pteronyssinus* and *Euroglyphus maynei* have been shown to contain some of these conserved regions [7]. The 10 recognized invariant residues within these conserved regions include 7 thought to be involved in catalysis and substrate binding in the active site and 3 that are structurally important, and they are present in both species. Furthermore, α -amylases have also been found to bind to calcium ions. This has been postulated to preserve the structural integrity of the active site of the α -amylases [8].

α -Amylases have been divided by their related evolutionary paths into 3 main groups: fungi and yeast, plants alone, and insects and mammals (with microbes scattered among these groups). Within these groups a much higher degree of similarity is exhibited than with organisms from the other groups [9].

Porcine α -amylases have 12 cysteine residues which form 5 disulphide bonds. These 5 bonds are generally conserved in mammals [10]. The group 4 allergens in *D. pteronyssinus* and *E. maynei* possess 8 cysteine residues, which could form 4 of these disulphide bonds [7]. The maintenance of the tertiary structure is important for the allergenicity of some α -amylases. It has been previously shown that the reduction of disulphide bonds of group 4 allergen in *Dermatophagoides* diminishes the allergenicity of the protein [1]. However, α -amylases in cereal and fungus have been shown to be allergens in both their nonreduced and reduced forms [3].

Recently, by using the Smart Race cDNA amplification kit, we have isolated a cDNA clone coding for a group 4 allergen from *B. tropicalis*. This report describes the isolation and sequence characterization of this new allergen, designated as Blo t 4, and the sensitization profiles to this allergen in 2 geographically distinct populations: Chengdu, PR China, and Singapore.

Materials and Methods

Human Subjects

One hundred patients (aged 3–48 years, mean 26.48 years) with doctor-diagnosed asthma ($n = 65$) and asthma and rhinitis ($n = 35$) who attended the allergy clinics at Sichuan University West China Hospital, Chengdu, PR China, were recruited for skin

prick tests. For comparison between populations, 49 patients (aged 22–59 years, mean 37 years) with doctor-diagnosed asthma ($n = 8$), allergic rhinitis ($n = 26$) and patients with asthma and allergic rhinitis ($n = 15$) who attended the allergy clinic at Tan Tock Seng Hospital, Singapore, were also recruited. The Research Ethics Committee of both universities approved the protocol for the skin prick tests and the patient recruitment procedures.

Written consent was obtained before the skin prick tests were carried out. Subjects were skin prick tested to recombinant dust mite allergens Blo t 4 and Blo t 5. Der p and Blo t crude extracts were also included. Histamine was used as a positive control and PBS was used as a negative control. The concentration of the recombinant allergens was 25 μ g/ml, whereas the crude extract was 100 μ g/ml, and a volume of between 2 and 5 μ l was dropped on the volar aspect of the forearm. The skin was then gently pricked with a disposable lancet. Subjects were required to discontinue antihistamines at least 72 h prior to the test. The resulting weal and erythema reactions were studied after 20 min. Mean weal and erythema diameters were calculated from the largest and perpendicular diameters. A mean weal size of 3 mm or more or a weal greater in size than the negative control was considered a positive result.

5' and 3' Rapid Amplification of cDNA Ends

The 5' rapid amplification of cDNA ends (RACE)-Ready cDNA and 3' RACE-Ready cDNA (mite cDNA library) were generated by following the protocol described in the Smart RACE cDNA amplification kit's user manual (with slight modifications). Briefly, total RNA was isolated from the live cultured *B. tropicalis* mites [11] with Trizol reagent (Gibco BRL, Life Technologies, New York, N.Y., USA). 1 μ g of total RNA was utilized to generate either 5' or 3' RACE-Ready cDNA products, as described earlier [12, 13], with specific primers and reagents provided by the kit in the presence of Moloney murine leukemia virus (MMLV) reverse transcriptase. Subsequently, the 5' RACE was performed using the 5' Universal Primer Mix (Clontech Laboratories, Palo Alto, Calif., USA) and a gene-specific primer 1, 5'-TTG TTC ACC ACT ACG TGT TCG TAT ATC-3', which was designed based on the consensus nucleotide sequences of other group 4 mite allergens (corresponding to Asp67 to Gln76 in Der p 4). However, 2 more gene-specific primers (GSP 2 and GSP 3) were utilized for the generation of 3' RACE fragments. Positive and negative controls were prepared according to the protocol of the manufacturer. GSP 2, 5'-GGT TAT GGT GGT GTA CAG GTT TCA CC-3' (corresponding to residues Gly37 to Pro45 in Der p 4), and GSP 3, 5'-GGC CGC ATT ATT GAA TTC CGT TTT TAT AAA-3' (correspond to residues Gly248 to Lys257 in Der p 4), were designed based on the nucleotide sequences of Group 4 allergens of other mite species. The polymerase chain reaction (PCR) was carried out at 94°C for 5 s, 55°C for 10 s and 72°C for 3 min for 30 cycles using the PTC-100™ programmable thermal controller (MJ Research Inc., USA). Amplicons were analyzed by agarose electrophoresis, cloned and sequenced. Clone 1 was isolated from the 5' RACE product with GSP 1, whereas clones 2 and 3 were isolated from the 3' RACE product with GSPs 2 and 3. Their DNA sequences were confirmed to be the 3' end, the middle fragment and the 5' end of Blo t 4 gene, with overlapping homologous sequences between these clones.

Generation of Full-Length Blo t 4

Long-distance PCR reaction was carried out according to manufacturer's protocols (Clontech Laboratories) to generate the full-length Blo t 4 cDNA. The template DNA was the 5' and 3' RACE products previously generated from the total mRNA of the mites. The sequences of clones 1 and 3 were used to design the forward primer: 5'-CTT ACC AAT TAA TAA ACC AAC ATC TCA ACC-3' and the reverse primer: 5'-TTT AGA CAT TTC ATT TTA TTT ATT TTT TCA-3', respectively. Subsequently, the PCR products were purified with the QIAquick nucleotide removal kit (Qiagen Inc., Valencia, Calif., USA) and ligated into pCR2.1-TOPO cloning vector (Invitrogen Corp., Carlsbad, Calif., USA). The full-length Blo t 4 cDNA was confirmed using an ABI Prism® 377 DNA sequencer (Applied Biosystems, Foster City, Calif., USA) with the ABI Prism dRhodamine terminator cycle sequencing Kit.

Expression of Blo t 4 in *Pichia pastoris* Using the pPIC9 Expression Vector

The cDNA encoding the matured Blo t 4 protein was amplified by PCR using the forward primer 5'-CG CTC GAG AAA AGA TCC CTA TAG TAA TCC ACA TTT-3' and the reverse primer 5'-CG GCG GCC GCT TAA ACT CTA GCA TCA ACA CTA-3' containing the *Xho* I and *Not* I restriction enzyme sites, respectively.

The expression of the PCR product in *Pichia pastoris* was performed according to methods described in the manufacturer's manual, version E (Invitrogen). Briefly, the gel-purified PCR product was digested with *Xho* I and *Not* I, ligated to pPIC9 vector, and transformed to DH5 α . The plasmid was isolated and linearized with *Bgl* II and then transformed into the yeast strain GS115 by lithium chloride method for protein expression. Transformants were selected by the slow-growing phenotype (Mut-) on minimum methanol plates. In order to identify the highly expressed clones, an individual colony was inoculated in 50 ml of buffered glycerol complex medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulfate, 1% glycerol, 0.4 mg/l biotin and 0.1 M potassium phosphate, pH 6.0) and grown at 30°C in a rotary-shaking incubator at 250 rpm. The cells were harvested by centrifuging at 1,500 g for 5 min at room temperature when the OD_{600 nm} reached 2.0–6.0. The cell pellet was resuspended in 10 ml of buffered methanol complex medium (which is identical to buffered glycerol complex medium except that glycerol is replaced with 0.5% methanol) for induction of the recombinant expression. The expression kinetics indicated that optimum expression of this protein was about 12 h after induction. Thereafter, the medium containing the expressed recombinant protein was spun down at 10,000 rpm for 30 min with a Sorvall centrifuge using rotor SS-34. The supernatant was collected and the recombinant protein was quantitated using standard DC protein assay (Biorad, USA). The collected recombinant protein was analyzed on 12% SDS-PAGE by loading 2 μ g of protein per lane.

Western Blot Analysis and Absorption Assay

The IgE reactivity of Blo t 4 was analyzed by Western blot analysis using a positive serum. In brief, 2 μ g of Blo t 4, 1.0 μ g of glutathione S-transferase and 1.0 μ g of Blo t 5 were electrophoresed on a 12% tris-glycine gel and electroblotted onto nitrocellulose membrane (Hybond C, Amersham Life Sciences, Amersham, UK). The membrane was blocked with 5% bovine serum

albumin in 1 $\times$ TBS-T for 1 h at room temperature. After overnight incubation with 1 $\times$ dilution of the Blo t 4-positive human sera in blocking buffer at 4°C, the membrane was incubated with 250 $\times$ dilution of biotinylated mouse anti-human IgE, 250 μ g/ml (Pharmingen, San Jose, Calif., USA). Prior to detection using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill., USA) for 5 min, the membrane was incubated with 10,000 $\times$ dilution of peroxidase-conjugated ExtrAvidin (Sigma, St. Louis, Mo., USA). The membrane was washed 6 times with TBS-T between steps. Results were detected by autoradiography using Kodak Biomax Light ML film (Eastman Kodak Company, Rochester, N.Y., USA). For absorption assay, a final concentration of 5 μ g/ml of Blo t 4 was included in the diluted individual serum (1:1 in blocking buffer) and incubated at 4°C for 16 h before it was used in the subsequent Western blot, as described above.

Results

DNA Sequence Analysis

The Blo t 4 clone is 1,607 bp in length, it contains a 5' non-translating region of 27 bp, an ATG start codon at nucleotide positions 28–30 and a stop codon TAA at nucleotide positions 1,573–1,575, coding for a mature protein of 495 amino acids with a predicted molecular weight of approximately 56 kDa (fig. 1).

Protein Sequence Analysis

A protein sequence homology search was performed using the Advance Blast search. The search results revealed that the deduced amino acid sequence of Blo t 4 has 68 and 65% sequence homology with the group 4 allergen of *D. pteronyssinus* (Der p 4) and *E. maynei* (Eur m 4), respectively. The open reading frames of Blo t 4, Der p 4 and Eur m 4 contain 517, 519 and 518 amino acids, respectively. Figure 2 shows the amino acid sequences of Blo t 4, Der p 4 and Eur m 4. The N terminus of the mature enzyme is labeled +1 and the amino acid numbering is based on the Blo t 4 sequence. The underlined regions are recognized as conserved sequence regions of α -amylase. These regions are believed to be involved in either substrate binding or catalysis in the active site. I to VIII are the 8 conserved cysteine residues in these allergens (bold letters).

The similarities among these group 4 allergens are the presence of 8 conserved cysteine residues and 7 conserved regions of α -amylase (underlined regions in fig. 2). However, in Blo t 4, the β 3 region consists of amino acids VVLNH instead of IVLNH, the β 4 region contains GFRFDASKH instead of GFRSDASTH, the β 5 region contains HEVIY instead of HETIY and the β 7 region contains MVDSHD instead of MIDSHD. Moreover, the

acc a a t t a a t a a a c c a a c a t c t c a a c c a t g a t t g t a g a a t t a a t g t t t t t c a t t g g c c 60
M I V E L I V F S L A
t t g a c c a t a c c a t a t c a a a c c t t g g c a a g t a g t c c c t a t a t g t a t c c a c a t t t t c a a c a t 120
L T I P Y Q T L A S S P Y S D P H F Q H
a a t c g a a a g g t c a t t a c c c a t t g a t g a a t t t g t g g a c a t t g c c g a t g a a t g t 180
N R K V I T H L M Q W K F V D I A D E C
g a a c g t t t c t t g g t c c a t a t g t t a t g t t g g t t c a a g t t t c c c a g t t a a t g a a c a t 240
E R F L G P Y G Y G G V Q V S P V N E H
g c t c a t t g g a t c g a c a t c c a t g g t a t g a a c t a t a c a c c g g t a a g t t a t c g a a t c g t t 300
A S L D R H P W Y E L Y Q P V S Y R I V
a g t c g a t c t g g t a c a g a a t c c c a a t t t c g t g a t a t g g t t c a t c g t t g t a a t a a g t g g c 360
S R S G T E S Q F R D M V H R C N K A G
g t a c g a a t c t a t g t c a c g t t g t a c t a a t c a t a t g a c t g g t c c a c a a t c g g g c g t a g g a 420
V R I Y V D V V L N H M T G P Q S G V G
a t t g a t g g a a c a c a t t a c g a t g g t a a c t c a a t g a a t a t c t g g c a t a c c a t t t g g t c c a 480
I D G T H Y D G N S M Q Y P G I P F G P
a a t g a t t t c a t g g a c a t a a t c a a t c a a t t a g a t a t a c a a a t t a t g a t 540
N D F H G H E S C P T S N L D I Q N Y D
g a t c c a a c a a g c a c g a a a t t g t c g g t a t c a g g t t a c g t a t c a a a c a a c a t c a g c c 600
D P T Q A R N C R L S G L R D L K Q S A
g a t t a t g t a c g t a c a a a c a a g t g a t t t t t a a t c a t t t g a t g a t t g g t g t g c c 660
D Y V R T K Q A D F L N H L I D I G V A
g g t t t t c t g a t c a t c a a a c a t a t g t g g c a g g t g a c c t t c a a g c a a t c a t t c g 720
G F R F D A S K H M W P G D L Q A I Y S
a a a c t t c a t t t a a t g a g a a a t t t c c a t c a a a t t c a a t c c a t t c a t c a t a t c a t 780
K L H H L N E K Y F P S N S N P F I Y H
g a g g t t a t c a t t a c a a a a t a a t g c a a t t t c a a t t t c c g a t t a c g a a a t t g g g t c g t 840
E V I Y S N N N A I S I S D Y T K L G R
t c a a t t g a a t t c a t t a t a t c a t g a a t t g t a a t g a a t t c g t g g t a a t a a a a t t a 900
S I E F H Y Y H E L C N V I R G N N K L
a a a t g g t a c a t a a t t t g g t c a a c c a t g g g a a t g g t a c c a a t g a t g a t g a t t g a t a 960
K W L H N F G Q P W G M V P N D D A L I
a t g g t c g a t c a c a t g a t c t a c a a c g t g g a c a t a c t g g t c a a t t g g g t t g a a t a t c a a t 1020
M V D S H D L Q R G H T G Q L G L N I N
t a t t t t g a t c a a g a t t a t t g a a g t a g t a c c g a t t a t g t t g g t t g g c c t t a t g g a 1080
Y F E S R L L K V A T A F M L A W P Y G
a t t c c c g a g t c a t g a g t c a t a t c g a t g g a a t c a a a g a t t g t c a a t g g a a a g a t g a a 1140
I P R V M S S Y R W N Q K I V N G K D E
a a c g a t t g g a t t g g a c c g c c a a c c g a t g g t t c a g g a t c g a t a t t a t c a g t g a a a c c a a t 1200
N D W I G P P T D G S G S I L S V K P N
t c c a t c t t a c g t g a a t c a a g a a t g g a t t g t g a a c a t c g a t g g a g a c a a a t c a t a g t 1260
S D L T C N Q E W I C E H R W R Q I Y S
a t g g t a c a a t t a g g a a t a c g c c g g t g a t g a a c c a g t t a a g a a t t g g t g g g a a t a t g g a 1320
M V Q F R N T A G D E P W D N G
g a t t a t c a a a t t g c a t t c a g t c g t g a g a c a a c a t t a t t g c a a t c a a t c t a c a a a c 1380
D Y Q I A F S R G A K T F I A I N L Q N
g g g c a a c a t t t g a a c a a a t c t a c a c g g g t t a c c c a g t g g a a a t t a t t g t a t c t t 1440
G Q H L K Q N L H T G L P S G N Y C D L
g t t a c g g g a a t g t a a a t a a t g g a a a t g t t c t g g a c a a a t g g t t c a t g t c g a t g g a t c g 1500
V T G N V N N G K C S G Q M V H V D G S
g g t c g a a t g a t t c a a t t g g t g c a a t g c c g a c g a t c a t t a t a g c a a t t a g t g t t 1560
G H A M I S I G A N A D D P F I A L S V
g a t g c t c g a g t t a a g t t g a a a a a a a a a t a a a t g a a a t g t c t a a a 1607
D A R V -

Fig. 1. The amino acid sequence of Blo t 4. Nucleotide sequence residues are numbered at the end of each row. The start and stop codons are underlined.

β8 region for Blo t 4 is homologous to Eur m 4 and is different from that of Der p 4. Besides the 8 conserved cysteine residues, there is an additional unique cysteine residue found at position 260 in Blo t 4 and at position 310 in both Der p 4 and Eur m 4. Eur m 4 has another cysteine residue at position 12 (fig. 2). The estimated molecular weight of Blo t 4 is 56 kDa, for Der p 4 it is 57.2 kDa and for Eur m 4 it is 57.3 kDa. The isoelectric point for Blo t 4 is 6.41, for Der p 4 it is 6.7 and for Eur m 4 it is 7.0.

Expression of Recombinant Blo t 4 in *P. pastoris*

The recombinant Blo t 4 was expressed as a highly soluble protein and secreted into the culture medium. The

Table 1. Allergen sensitization profiles with recombinant Blo t 4 and Blo t 5 in allergic subjects

Allergen	China (n = 100)	Singapore (n = 49)	p value
<i>D. pteronyssinus</i> (crude)	42 (42)	38 (77)	n.s.
<i>B. tropicalis</i> (crude)	45 (45)	33 (67)	n.s.
Blo t 4 (recombinant)	28 (62) ¹	2 (6) ¹	<0.0001
Blo t 5 (recombinant)	22 (49) ¹	28 (84) ¹	<0.003

The data are numbers of patients positive for each allergen, with percentages in parentheses. n.s. = not significant. $p > 0.05$.

¹ These percentages are based on the total number of patients positive to *B. tropicalis* crude and not on the total number of patients tested in each population, i.e. n = 45 in the Chinese population and n = 33 in the Singaporean population.

estimated yield of the recombinant protein ranged from 20 to 30 mg/l of the yeast culture. The protein expression kinetics study indicated that the optimum expression of this protein was at about 12 h after induction. The recombinant Blo t 4 protein resolved at approximately 60 kDa on an SDS-PAGE gel (fig. 3).

Western Blot Analysis and Absorption Assay

The serum used for the Western blot and absorption assays had been previously tested and shown to be positive for IgE reactivity to both Blo t 4 and Blo t 5 allergens (fig. 3b). Only a single band was obtained for each of the tested allergens. Glutathione S-transferase served as a negative control for IgE reactivity. When the Western blot was repeated with the same serum but which had been absorbed overnight with Blo t 4, it was observed that the IgE-reactive Blo t 4 band was so much reduced that it was almost invisible, indicating that the adsorption was specific to the Blo t 4 allergen (fig. 3c).

Allergen Sensitization Studies

The sensitization profile of Blo t 4 was studied in 2 populations with asthma and/or rhinitis, from Chengdu in midwest China and Singapore. These profiles were also compared with Blo t 5, the major allergen of *B. tropicalis*. We observed that 28 (62%) of the tested asthmatic patients in China were sensitized to Blo t 4, and this frequency was higher than that for Blo t 5, for which it was 22 (49%; table 1). In contrast, the sensitization profile of Singaporean allergic patients was the inverse, with only 2 patients (6%) sensitized to Blo t 4 and 28 (84%) patients

		+1	I	
Blo t 4	--MIVELIVFSLALTIPYQTLASSPYSDPHFQHNKRVITHLMQWKFVDIAD	CERFLGP	35	
Der p 4	MLPKLYSKFMILVLTFLVCSFFTNVESKYHNPFIHGRSVITHLMEWKYDDIGDE	CERFLGP		
Eur m 4	MLPKIYLKLITLVLFISLQVNNVHCKYHNPFIHGRSVITHLMEWKYDDIGDE	CERFLGP		
		* * * * *		
		β2	II	
Blo t 4	YGGGVQVSPVNEHASLDRHPWYELYQPVSYRVSRSGETSQFRDMVHR	CNKAGVRIYVD	95	
Der p 4	YGGGVQVSPVNEHAILDRRPWYERYQPVSYDIRTSQDEQQFRMVKR	CNKAGVRIYVD		
Eur m 4	YGGGVQVSPVNEHAILDRRPWYERYQPVSYDLRTRSGDEQQFRMVKR	CNKAGVRIYVD		

		β3	III	
Blo t 4	VVLNHTGPQSGVGIDGTHYDGNMQYPGIPFGPNDFHGHESCPTSNLDIQNYDDPTQAR		155	
Der p 4	IVLNHMTGAQSGKGTNGHHYDGNLTQYPGVFPFPNDFHGHESCPTQDLEIHDYTNPKEAR			
Eur m 4	IVLNHMTGGQSGQGTNGNHYNGITMQYPGVFPFPNDFHGHESCQTKDLEIHNSYDRNEAR			

		IV loop 3	β4	
Blo t 4	NCRLSGLRDLKQSADEVRTKQADFLNHLIDIGVAGFRFDASKHWWPGDLQAIYSKLHLHN		215	
Der p 4	NCRLSGLRDLKQSEYVRQKQVDFLNHLIDIGVAGFRSDASTHQWPDLLRSIYSRLNLHN			
Eur m 4	NCRLSGLRDLKQSEYVRKQVDFLNHLIDIGVAGFRSDASTHQWPDLLRSIYSRLNLHN			

		β5		
Blo t 4	EKYFSPNSNPFYHEVIYSNNNAISIDYTKLGRSIEFHYYHEL	CNVIRGNKLNKLWLNHF	275	
Der p 4	KEFFPENSQPFYHETIYGGNGINSNEYTSLGRIIEFRFYKEITNVFRGNPLHLWLNKF			
Eur m 4	KEFFPENSHPFIYHETIYGGNGINSNEYTSLGRIIEFRFYKEITNVFRGNQLRWLNKF			

		β7	β8	
Blo t 4	GQPWGMVPNDALIMVDSHDLQRGHTGQLGLNINIFESRLKVATAFMLAWPYGIPRVMS		335	
Der p 4	GTEWGLVPSGDALVMDSDHDLRVGHTGKLGFINCFCGRLLKAATAFMLAWNYGVPRVMS			
Eur m 4	GTEWGLVPSNDALVMDSDHDLRVGHTGKLGFINCFEARLLKASTAFMLAWNYGIPRVMS			

		V	VI	
Blo t 4	SYRWQKIVNGKDENDWGPPTDGSGLSVKPNSDLTCNQEWICEHRWRQIYSMVQFRN		395	
Der p 4	SYFQWQI IKDGKDVNDWVGPPSDKNGNLSVHPNPDMTCNHEWICEHRWREIYNMVKFRM			
Eur m 4	SYFQWQI IRDGKDVNDWVGPPDQHGNI LSVHPNADLT CNHEWICEHRWRQIYNMVKFRM			

		VII		
Blo t 4	TAGDEPVKNWWDNGDYQIAFSRGAKTFIAINLQ-NG-QHLQNLHTGLPSGNYCDLVTGN		455	
Der p 4	IAGQEPVHNWWDNGDYQIAFSRGNRAFTAINLQKNQ-QNLQQLHTGLPAGTYCDIISGN			
Eur m 4	IAGQEPVRNWWWDNGDYQIAFSRGSHTAFTAINLQKNQKGNLRQLRHTGLPAGTYCDIISGD			

		VIII		
Blo t 4	VNNGKCSGQMVHVDGSGHAMISIGANADDPFIASVSDARV--		495	
Der p 4	LIDNKCCTGKSIHVDKNGQADVVGHDFAFVAYHIGARIVS			
Eur m 4	LIHNKCCTGKSIQVDKNGLADIYVGHDELDAFVAYHIGARIE-			

Fig. 2. Amino acid sequence alignment of group 4 from various mite species, *B. tropicalis* (Blo t 4), *D. pteronyssinus* (Der p 4) and *E. maynei* (Eur m 4). I–VIII are the 8 conserved cysteine residues. β2, β3, loop 3, β4, β5, β7 and β8 are the conserved regions and are proposed to be involved in catalysis and substrate binding (residues are underlined). The N terminus of the mature enzyme is labeled +1 and the amino acid numbering is based on the Blo t 4 sequence.

sensitized to Blo t 5. Amongst the 100 Chinese asthmatic patients, 16 patients showed positive skin prick tests to both allergens. The weal sizes to Blo t 4 and Blo t 5 were comparable, ranging from 3 to 4.5 mm for both allergens (table 2). Seven patients had larger weals in Blo t 4 skin responses compared to those for Blo t 5, with only 3 patients showing larger weal sizes with Blo t 5.

Discussion

In 1991, Lake et al. [1] purified natural α-amylase from *D. pteronyssinus* mites by affinity to oyster glycogen, gel filtration and chromatofocusing. The house dust mite α-amylase showed positive IgE reactivity with 46% of mite-allergic adults and 25% of mite-allergic children. In 1999, Mills et al. [7] cloned the genes encoding for the α-amylase from *D. pteronyssinus* and *E. maynei*, respectively. The mature Der p 4 and Eur m 4 proteins share about 90%

amino acid sequence homology. However, while recombinant Der p 4 produced in *Escherichia coli* bound IgE from 3/10 of the sera tested, no reactivity was found for Eur m 4, despite its strong similarity to Der p 4 [7]. To date, the question on IgE cross-reactivity between Der p 4 and Eur m 4 remains unresolved. Comparison of Der p 4, Eur m 4 and Blo t 4 protein sequences with published insect and mammalian α-amylases indicated that they contained the conserved regions typical of α-amylases, as reviewed by Janecek in 1997 [2]. In particular, the amino acid residues Asp194, Glu230 and Asp295 of these 3 allergens correspond to the 3 catalytic residues [14]. Asn99, Arg155, Asp164 and His198 correspond to the residues identified as calcium-binding ligands [15, 16]. Arg192 and Arg332 correspond to the chloride-binding site, but the third residue, normally an asparagine, is substituted by a serine at position 293. Other residues, such as Asp95, His100 and His294, are thought to be important for either substrate-binding or catalysis. Further, the residues

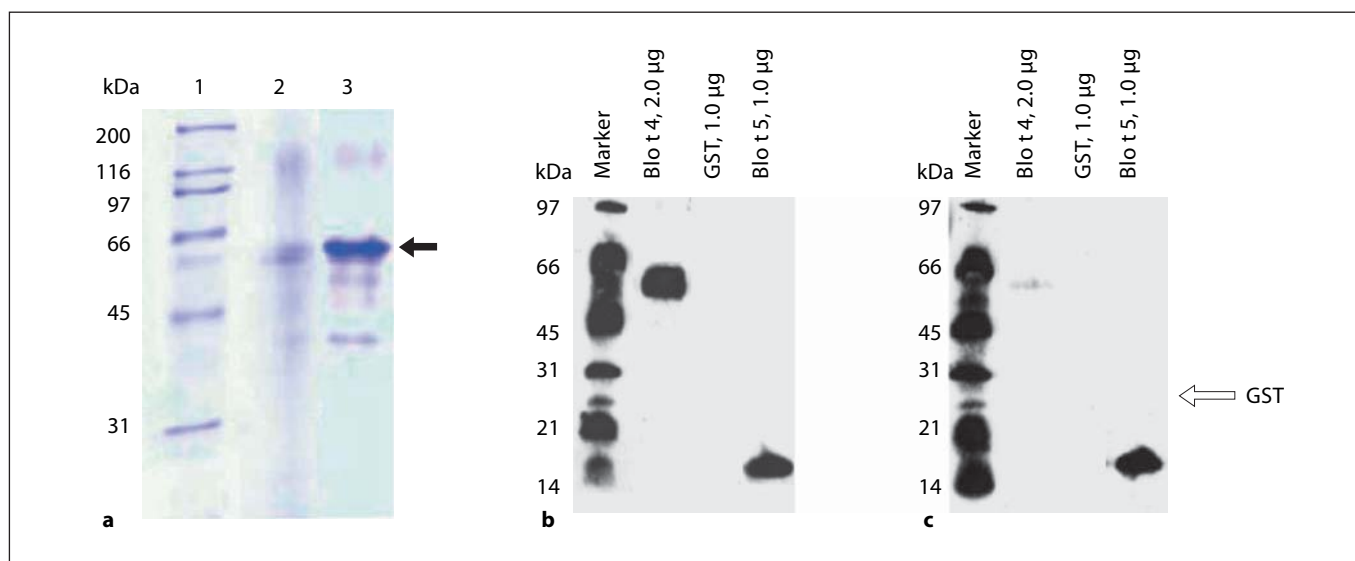


Fig. 3. **a** SDS-PAGE of the kinetics of expression of recombinant Blo t 4 in *P. pastoris*. Lane 1 shows the marker in kDa, lanes 2 and 3 show expression at 0 and 12 h, respectively, after induction with 2 µg of protein per lane. The arrow indicates that the recombinant Blo t 4 migrates to position at 60 kDa. **b** Western blot showing the IgE reactivity of a serum to both Blo t 4 and Blo t 5. Glutathione

S-transferase (GST) served as a negative control. **c** The serum (1:1 dilution with blocking buffer) had been absorbed with Blo t 4 (5 µg/ml of serum) overnight. Note the decrease in the reactivity of Blo t 4 after the absorption. However, there is no change in the reactivity with Blo t 5.

Gly37, Pro45 and Gly329 may have a structural role [2]. The glycine-rich flexible loop corresponds to residues 299–304 (GHGAGG) and is thought to be crucial for substrate-binding in mammals [17]. The sequence is GHTGKL in the house dust mite. It should be noted that both Blo t 4 and Der p 4 have 9 cysteine residues while Eur m 4 has 10. Eight of these cysteines are highly conserved among the α -amylases and are likely to be involved in forming disulphide bridges. This has been described in porcine α -amylase [10]: Cys29 links to Cys85, Cys138 to Cys157, Cys374 to Cys380, and Cys448 to Cys460. Further, a single potential N-glycosylation site is present in Eur m 4 at residues 147–149 (NYS) and in Der p 4 at residues 10–13 (NRS). Blo t 4 does not possess any predicted glycosylation site according to the European Bioinformatics Institute's PPSearch tool. Phosphorylation is another possible post-translational modification of these 3 allergens as there are phosphorylation sites scattered throughout these molecules. Though there is no direct evidence derived from the present study that these 3 allergens were phosphorylated, there is a possibility that the difference in their IgE reactivity could be due to the alteration of the IgE epitopes as a result of phosphorylation.

Table 2. Comparison of skin prick test wheal sizes in subjects from China with positive responses to both Blo t 4 and Blo t 5

Subject	Wheal size, mm	
	Blo t 4	Blo t 5
1	4	3.5
2	3.5	4
3	3.5	3
4	4	3.5
5	3.5	3.5
6	4.5	3
7	3.5	3.5
8	3.5	3
9	3.5	3.5
10	4	3.5
11	3.5	3.5
12	3.5	3.5
13	3	3.5
14	3	3.5
15	4.5	4
16	3.5	3.5

A number of epidemiologic studies indicated that atopic populations in the tropical and sub-tropical regions were sensitized by both *D. pteronyssinus* and *B. tropicalis* [18, 19]. Skin prick tests and in vitro IgE sensitivity confirmed that *B. tropicalis* is one of the dominant species of clinical importance in Latin America [20–24], Florida [1, 2] and Asia [11, 12, 25–27]. Blo t 5, the major allergen from *Blomia* mites, exhibited 70% of IgE sensitivity in patients with asthma [19, 20, 27] using in vitro IgE-binding assay and skin prick tests. In the present study, however, we have found that Blo t 4 reactivity in subjects from China was higher in frequency compared to the major allergen, Blo t 5. The high frequency of Blo t 4 sensitization compared to Blo t 5 in the Chinese subjects was unusual and not observed in other tested populations in south and southeast Asia [28]. Recently, Hales et al. [29] discovered that the IgE binding activity in the

sera from the aboriginal community in Australia was not directed to the expected major groups 1 and 2 mite allergens but instead to the group 4 amylase (Der p 4) allergen. They had also ruled out cross reactivity from parasite infections or from food contamination like mollusc amylase. The reasons for the high reactivity to group 4 allergens in some populations have yet to be ascertained, and the effect of these unique sensitization profiles on disease outcome will also require further investigation.

Acknowledgements

This work was supported by grants from the Academic Research Fund (R-178-000-074-112), National University of Singapore, Singapore and the National Medical Research Council (NMRC/0951/2005), Singapore.

References

- Lake FR, Ward LD, Simpson RJ, Thompson PJ, Steward GA: House dust mite derived amylase: allergenicity and physicochemical characterization. *J Allergy Clin Immunol* 1991;87:1035–1042.
- Janecek S: α -Amylase family: molecular biology and evolution. *Progr Biophys Mol Biol* 1997;67:67–97.
- Sandiford CP, Tee RD, Taylor AJN: The role of cereal and fungal amylase in cereal flour hypersensitivity. *Clin Exp Allergy* 1994;24: 549–557.
- Brisman J, Belin L: Clinical and immunological response to occupational exposure to α -amylase in the baking industry. *Br J Ind Med* 1991;48:604–608.
- Baur X, Posch A: Characterised allergens causing bakers' asthma. *Allergy* 1998;53: 562–566.
- Morren MA, Janssens V, Doomsgoossens A, Vanhoeyveld E, Cornelis A, Dewolfpeeters C, Heremans A: α -Amylase, a flour additive: an important cause of protein contact dermatitis in bakers. *J Am Acad Dermatol* 1993; 29:723–728.
- Mills KL, Hart BJ, Lynch NR, Thomas WR, Smith W: Molecular characterization of the group 4 house dust mite allergen from *Dermatophagoides pteronyssinus* and its amylase homologue from *Euroglyphus maynei*. *Int Arch Allergy Immunol* 1999;120:100–107.
- Buisson G, Duee E, Haser R, Payan F: Three-dimensional structure of porcine pancreatic α -amylase at 2.9 Å resolution: role of calcium in structure and activity. *EMBO J* 1987;6: 3909–3916.
- Janecek S: Sequence similarities and evolutionary relationships of microbial, plant and animal α -amylase. *Eur J Biochem* 1994;224: 519–524.
- Pasero L, Mazzei-Pierron Y, Abadie B, Chicheportiche Y, Marchis-Mouren G: Complete amino acid sequence and location of the five disulfide bridges in porcine pancreatic α -amylase. *Biochim Biophys Acta* 1986;869:147–157.
- 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.
- 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.
- Cheong N, Yang L, Lee BW, Chua KY: Cloning of a group 3 allergen from *Blomia tropicalis* mites. *Allergy* 2003;58:352–356.
- Hasegawa K, Kubota M and Matsuura Y: Role of catalytic residues in α -amylase as evidenced by the structures of the products: complexed mutants of a maltotetraose-forming amylase. *Protein Eng* 1999;12:819–824.
- Janecek S: New conserved amino acid region of α -amylase in the third loop of their (β/α) 8-barrel domains. *Biochem J* 1992;288:1069–1070.
- Janecek S: Close evolutionary relatedness among functionally distantly related members of the (α/β) 8-barrel glycosyl hydrolases suggested by the similarity of their fifth conserved sequence region. *FEBS Lett* 1995;377: 6–8.
- D'Amico S, Gerday C, Feller G: Structural similarities and evolutionary relationships in chloride-dependent α -amylase. *Gene* 2000;253:95–105.
- Caraballo L, Puerta L, Jimenez S, Martinez B, Mercado D, Avjioglu A, Marsh D: Cloning and IgE binding of a recombinant allergen from the mite *Blomia tropicalis*, homologous with fatty acid-binding proteins. *Int Arch Allergy Immunol* 1997;112:341–347.
- 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: 573–579.
- Arruda LK, Vailes LD, Platts-Mills TAE, Fernandez-Caldas E, Montealegre F, Lin KL, Chua KY, Rizzo MC, Naspietz CK, Chapman MD: Sensitization to *Blomia tropicalis* in patients with asthma and identification of allergen Blo t 5. *Am J Respir Crit Care Med* 1997;155:343–350.
- Lin KL, Hsieh KH, Thomas WR, Chiang BL, Chua KY: Characterization of Der p V allergen, cDNA analysis, and IgE-mediated reactivity to the recombinant protein. *J Allergy Clin Immunol* 1994;94:989–996.
- Tovey ER, Johnson MC, Roche AL, Cobon GS, Baldo BA: Cloning and sequencing of a cDNA expressing a recombinant house dust mite protein that binds human IgE and corresponds to an important low molecular weight allergens. *J Exp Med* 1989;170:1457–1462.

- 23 Von Heijne G: A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* 1986;14:4683–4690.
- 24 Perlman D, Halvorson H: A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptidase. *J Mol Biol* 1983;167:391–409.
- 25 Cheong N, Soon SC, Ramos JDA, Kuo IC, Kolartkar PR, Lee BW, Chua KY: Lack of human IgE cross-reactivity between mite allergens Blo t 1 and Der p 1. *Allergy* 2003;58: 912–920.
- 26 Yeoh SM, Kuo IC, Wang DY, Liam CK, Sam CK, De Bruyne JA, Lee BW, Cheong N, Chua KY: Sensitization profiles of Malaysian and Singaporean subjects to allergens from *Der-matophagoides pteronyssinus* and *Blomia tropicalis*. *Int Ach Allergy Immunol* 2003; 132:215–220.
- 27 Kuo IC, Cheong N, Trakultivakorn M, Lee BW, Chua KY: An extensive study of human IgE crossreactivity of Blo t 5 and Der p 5. *J Allergy Clin Immunol* 2003;111:603–609.
- 28 Lim DL, Shek LPC, Shaik WA, Baratawidjaja K, Trakultivakorn M, Pakit V, Cheong N, Chua KY, Lee BW: Pattern of sensitization to *Blomia tropicalis* and its recombinant allergens in four tropical Asian populations. *J Allergy Clin Immunol* 2002;109:S179 (abstract).
- 29 Hales BJ, Laing IA, Pearce LJ, Hazell LA, Mills KL, Chua KY, Thornton RB, Richmond P, Musk AW, James AL, LeSouef PN, Thomas WR: Distinctive immunoglobulin E anti-house dust allergen-binding specificities in a tropical Australian Aboriginal community. *Clin Exp Allergy* 2007;37:1357–1363.