

Original article

Lack of human IgE cross-reactivity between mite allergens Blo t 1 and Der p 1

Background: The group 1 mite allergens are the most significant indoor allergens and they belong to the papain-like cysteine protease family. To date there is only one published report on the isolation and characterization of group 1 allergens from *Blomia tropicalis* mites. The aims of the study are to determine the cross-reactivity between group 1 allergens and to evaluate their clinical importance in allergic patients.

Methods: The full-length Blo t 1 gene was obtained by SMARTTM RACE cDNA amplification method using gene-specific primers. The sequence alignment was performed using LOOK followed by three-dimensional homology modeling. The cDNA was expressed in *Pichia pastoris* as a secretory protein. Identification of native Blo t 1 in crude mite and spent mite medium extracts was done by Western immunoblot using monoclonal antibody. Allergenicity of recombinant Blo t 1 and native Der p 1 was examined by human IgE ELISA with 80 asthmatic sera.

Results: The cDNA sequence consists of 1105 base pairs, including 5'- and 3'-untranslating regions, encoding an open reading frame of 330 amino acid residues. The predicted molecular weight of the deduced protein was approximately 38 kDa. Blo t 1 shared 53 and 34% nucleotide and amino acid, respectively, sequence homology with Der p 1. Native Blo t 1 was detected in both crude mite and spent mite medium extracts, and its estimated molecular weight was about 26 kDa. The recombinant Blo t 1 reacted positively with IgE in 90 and 65% of sera from asthmatic children and adults, respectively, indicating that it is a major allergen. The correlation of human IgE reactivity between Blo t 1 and Der p 1 was low in these sera.

Conclusion: The full-length cDNA encoding group 1 *Blomia tropicalis* mite allergen (designated as Blo t 1) has been characterized and expressed from local mites in Singapore. This fecal allergen showed high frequency of human IgE reactivity with asthmatic sera in the tropics and there was a low correlation of IgE reactivity between Blo t 1 and Der p 1.

N. Cheong¹, S. C. Soon²,
J. D. A. Ramos³, I. C. Kuo³,
P. R. Kolortkar⁴, B. W. Lee³,
K. Y. Chua³

¹Bioprocessing Technology Centre, A*STAR, Singapore; ²Institute of Postgraduate Studies, University of Malaya, Malaysia; ³Department of Paediatrics, Faculty of Medicine, National University of Singapore, Singapore; ⁴Genome Institute of Singapore, A*STAR, Singapore

Key words: *Blomia tropicalis*; cysteine protease; Group 1 mite allergen gene; human IgE cross reactivity.

Associate Professor Kaw Yan Chua
Department of Paediatrics,
Faculty of Medicine
National University of Singapore
5 Lower Kent Ridge Road
Singapore 119074

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It has been well established that mite species such as *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Euroglyphus maynei* from the family Pyroglyphidae are important clinical species (1–4). In recent years, *Blomia tropicalis* mite classified under the family Glycypagidae (5) is an important source of indoor allergens that associate with allergic asthma and rhinitis in the tropics (6–11). The first major *B. tropicalis* allergen cloned was Blo t 5 (12, 13). Subsequently, cDNA encoding for Blo t 3, Blo t 4, Blo t 6, Blo t 10, Blo t 11, Blo t 12, Blo t 13, and Blo t 19 were isolated by us and others (7, 14–17). The estimated frequency of human IgE reactivity to Blo t 3, Blo t 5, Blo t 10, Blo t 11, Blo t 12, and Blo t 13 with mite allergic sera was about 51, 70, 20–29, 52, 50, and 11%, respectively.

A number of group 1 allergens have been isolated in various mites including *D. pteronyssinus* (Der p 1) (18–20), *D. farinae* (Der f 1) (21–23) and *E. maynei* (Eur m 1) (24). Der p 1, Der f 1, and Eur m 1 shared up to 80% sequence homology and they showed between 80 and 100% frequencies of IgE reactivity (25). The full-length cDNA encoding the group 1 *B. tropicalis* allergen has been reported very recently (26) and 13 out of 21 patients (62%) skin test positive to Blo t extracts demonstrated strong IgE binding to this *Escherichia coli* expressed recombinant Blo t 1. This study describes the expression of the full-length cDNA encoding group 1 allergen from *B. tropicalis* mite expressed in yeast. The allergenicity of the recombinant proBlo t 1 was assessed and compared with Der p 1 by *in vitro* IgE binding

assay using a larger panel of sera from asthmatic patients.

Materials and methods

Sera samples

Sera obtained from 40 children (aged 2–15 years, mean age 8.7 years) and 40 adults (aged 18–50 years, mean age 29.3 years), asthmatic patients attending the Allergy Clinics in the University Hospital, National University of Malaya, Malaysia, were used in this study. All the patients had strong positive skin prick test reactions to *B. tropicalis* and *D. pteronyssinus* extracts.

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

Total RNA was isolated from live cultured *B. tropicalis* mites (27) with Trizol reagent (Gibco BRL, Life Technologies, New York). Generation of RACE fragments were performed as described earlier (16). In brief, cDNA products were generated with primers and reagents provided by the kit in the presence of SUPERScript™ II, RNase H⁻ reverse transcriptase (Gibco BRL, Life Technologies). The 5'-RACE was performed using the 5' Universal Primer Mix (Clontech Laboratories, Palo Alto, CA) and a gene specific primer, 5'-CCA ACT GTT TCG TAC GAT CCA ATA ATC-3', which was designed based on the consensus nucleotide sequences of other group 1 mite allergens (corresponding to residues Asp184 to Trp192 in Der p 1). The 3'-RACE was performed with another gene specific primer: 5'-GA GGC TGT GGT TCA GGT TGG GCT TT-3', which was also designed based on the consensus nucleotide sequences of other group 1 mite allergens (corresponding to residues Gly29 to Ser38 in Der p 1) and the 3' Universal Primer Mix (Clontech Laboratories). 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., Waltham, MA). Amplicons were analyzed by agarose electrophoresis, cloned and sequenced to facilitate the isolation of the positive Blo t 1 gene fragments.

Generation of the full-length cDNA

Long-distance PCR reaction was performed to generate the full-length Blo t 1 cDNA according to manufacturer's instructions (Clontech Laboratories). The template DNA was from the Blo t cDNA library of the 5'- and 3'-RACE products generated earlier from the total mRNA of the mites. The sequences from the 5'- and 3'-RACE products were used to design the forward primer: 5'-TGT GGA ACG CAA ACC CAA ATC CAA AAA ACG-3' and the reverse primer: 5'-AGG GCA AGC AGT GGT AAC AAC GCA GAG TAC-3', respectively. The PCR products were purified with the QIAquick nucleotide removal kit (QIAGEN Inc., Valencia, CA) and ligated into pCR2.1-TOPO cloning vector (Invitrogen Corporation, Carlsbad, CA). The full-length Blo t 1 cDNA was determined using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA) with the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Kit.

Expression of proBlo t 1 in *Pichia pastoris* using the pPIC9 expression vector

The cDNA encoding for the putative proBlo t 1 protein was amplified by PCR using the forward primer: 5'-CCG CTC GAG

AAA AGA GAG AAA CCA ACT CGT GAA GAG ATC AAA ACA-3' and reverse primer: 5'-AAG GAA AAA AGC GGC CGC TTA AAG TGT GGC ATA GGT GAC ATA GTT-3', containing the *Xho* I and *Not* I restriction enzyme sites, respectively. In addition, the KEX2 recognition site, Leu-Glu-Lys-Arg-Glu, has been introduced for efficient cleavage. The expression of the PCR product in *P. pastoris* was performed according to methods described in manual version E (Invitrogen Corporation). Briefly, the gel purified PCR product was digested with *Xho* I and *Not* I, ligated to pPIC9 vector, and transformed to DH5α. 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 the minimal methanol (MM) plates. In order to identify the high yielding clones, an individual colony was inoculated in 50 ml of buffered glycerol-complex medium (BMGY, 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 1500 g for 5 min at room temperature when the OD_{600nm} reached 2.0–6.0. The cell pellet was resuspended in 10 ml of buffered methanol-complex medium (BMMY, replacing the glycerol in BMGY with 0.5% methanol) for induction of the recombinant expression. The recombinant protein expression was analyzed on 12% SDS-PAGE.

Sequence analyses

The amino acid sequence was translated from the Blo t 1 nucleotide sequence using the Translate Tool software (<http://www.expasy.org/tools/dna.html>) at the ExpASY Molecular Biology Server. The homology search of the Blo t 1 nucleotide sequence was carried out with the BLAST network server in the National Centre for Biotechnology Information. Multiple sequence alignment was done using the multiple sequence alignment program ClustalW WWW Service (<http://www.ebi.ac.uk/clustalw>) at the European Bioinformatics Institute. The nucleotide sequence homology comparison of Blo t 1 with other group 1 allergens was performed using the cDNA sequences encoding the open reading frames for these allergens. The SignalP V1.1 program from server in Center for Biological Sequence Analysis was used to predict the signal peptide cleavage site (28).

Molecular modeling of Blo t 1 and Der p 1

The molecular modeling program LOOK (part of Genemine) (29) was used to model Blo t 1 and Der p 1 based on structures of actinidin and papain, respectively. Coordinates for actinidin (2ACT) and papain (1MEG) were obtained from the Research Collaboratory for Structural Bioinformatics resource at the University of California, San Diego. Sequence alignment was carried out using LOOK followed by homology modeling. LOOK was used for modeling of the side chains based on packing considerations followed by energy minimization. WebLabViewerLite (Molecular Simulations Inc., San Diego, CA) was used to visualize the coordinates and to make the final figures.

Western blot analysis

Recombinant proBlo t 1 (0.4 µg), crude *B. tropicalis* mite extract (20 µg), spent mite medium extract (20 µg) and glutathione-S-transferase (GST, 0.4 µg) were electrophoresed on 12% Tris-glycine gel and electroblotted onto Hybond C membrane (Amersham

Biosciences AB, Uppsala, Sweden) using the MiniProtean II cell (BioRad Laboratories, Hercules, CA). The membrane was blocked with 5% skimmed milk in PBS-T (0.05% Tween-20). After overnight incubation of the membrane with 1000 × dilution of anti-Blo t 1 monoclonal antibody (clone 11F1, generated in our laboratory by DNA immunization) at 4°C with constant shaking followed by 6× wash with PBS-T, the membrane was incubated with 5000× dilution of biotinylated anti-mouse immunoglobulins (BD PharMingen, San Diego, CA). Prior to detection using the SuperSignal® WestPico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min, the membrane was incubated with 5000× dilution of peroxidase conjugated ExtrAvidin (Sigma, St Louis, MO) followed by 6× wash with PBS-T. Results were detected by autoradiography using the Kodak Biomax ML film.

Human IgE enzyme-linked immunosorbent assay (ELISA)

Recombinant proBlo t 1, GST, and native Der p 1 (isolated by affinity purification with mAb 4C1 from *D. pteronyssinus* spent mite medium) (30) were coated onto ELISA plates with 50 µl of 5 µg/ml protein in 0.1 M NaHCO₃, pH 8.4, overnight at 4°C. Plates were then washed three times with PBS-T and blocked with 100 µl 2% BSA in PBS-T for 2 h at 37°C. Diluted human sera, 50 µl (1 : 10 in blocking buffer) were added with further overnight incubation at 4°C. Plates were then washed and 50 µl of 250 ng/ml of peroxidase conjugated anti-human IgE (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added and incubated for 2 h at 37°C. Plates were washed and 50 µl of 1 mg/ml of o-phenylenediamine (Sigma) in citrate-phosphate buffer pH 5.6 containing 1 µl/ml of 30% H₂O₂ were added. Absorbance at 490/630 nm was determined 15 min later using the Spectra & Rainbow ELISA reader (Tecan, Austria Ges.m.b.H.). The mean plus threefold standard deviation of the OD reading for GST was set as the cut-off value for positive IgE reactivity.

Statistical analyses

Statistical analyses were carried out using SPSS, Version 11.0.1 (SPSS Inc., Chicago, IL). Correlations were analyzed using the Spearman's rank method. A *P* value of less than 0.05 was considered statistically significant.

Results

DNA sequence analysis of Blo t 1 cDNA

The full-length cDNA of Blo t 1 consisted of 1105 base pairs including the 5'- and 3'-untranslated regions. There was an open reading frame of 1002 base pairs encoding for a polypeptide of 330 amino acid residues (Fig. 1A). The calculated molecular weight of the deduced protein was 38 077.49 Da. Comparison of the sequence homology with the group 1 mite allergens indicated that the putative Blo t 1 nucleotide sequence had 53% homology with Der p 1 from *D. pteronyssinus*, 50% homology with Der f 1 from *D. farinae*, and 51% homology with Eur m 1 from *E. maynei*.

Protein sequence analysis

The deduced amino acid sequence of Blo t 1 had 34, 32 and 36% identity with Der p 1, Der f 1, and Eur m 1

respectively. The identity was low compared to the overall identity of 76% observed among the group 1 mite allergens from *D. pteronyssinus*, *D. farinae* and *E. maynei* (1). However the catalytic triad of Cys137, His281, and Asn301 in Blo t 1 was conserved with Der p 1, Der f 1, actinidin, and papain (Fig. 2) (20, 31). The residues Gln131, Gly135, Trp138, Ser302, and Trp303 involved in the active sites of the papain-like cysteine protease family were also conserved in Blo t 1. The conserved residues in the enzymatic active site indicated that Blo t 1 is a member of the cysteine protease family. Beside the Cys137 in the active site, Cys134, Cys168, Cys182, and Cys214 were also conserved with Der p 1, actinidin, and papain. In actinidin and papain, these cysteine residues formed two pairs of disulfide linkages. The cysteine residues involved in the third disulfide bond in actinidin and papain were not present in Blo t 1 and Der p 1. However, Blo t 1 had a unique Cys179 while the Der p 1 had the unique Cys4 and Cys117. The Blo t 1 contained a predicted signal peptide of 17 or 18 residues but the cleavage site between Cys18 and Lys19 gave a higher score than Ser17–Cys18. This indicated that Blo t 1 has a preprostructure as other cysteine proteases. As the N-terminal amino-acid sequencing was not done, the exact first amino acid residue of mature Blo t 1 could not be determined. The Blo t 1 protein sequence had one potential N-glycosylation site at Asn93 (Asn-Phe-Thr), which is most probably located at the pro-region. However, the conserved potential N-glycosylation site at position Asn53 in Der p 1, Der f 1, and Eur m 1 was not present in Blo t 1.

Molecular modeling of Blo t 1 and comparison with Der p 1

The structures of Blo t 1 and Der p 1 were modeled on the crystal structure of actinidin and papain, respectively, according to their sequence homology (Fig. 3A and B). The Blo t 1 showed the two-domain structure, as the Der p 1; domain I consisting mostly of the N-terminal residues was formed primarily by the α-helices, whereas domain II consisted mostly of the C-terminal residues and was formed predominantly with antiparallel β-sheet and short helices. A groove of similar dimension as that of Der p 1 was located between the two domains forming the catalytic site. The cysteine residues involved in the formation of the two putative disulfide bonds (Cys134–Cys182 and Cys168–Cys214) were in equivalent positions, as in Der p 1. However, minor differences such as the length of α-helices and β-strands, angles between the α-helices, and the loop regions were observed.

Identification of native Blo t 1

In order to confirm that the isolated Blo t 1 cDNA indeed encoded for a protein in the natural sources, Western immunoblot was performed to identify the native Blo t 1

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atg aaa ttt tta ttg gta gcg gca tta tgt gcc ttg gta gcc att ggc tca tgc a aaa cca	98																																		
M K F L L V A A L C A L V A I G S C K P																																			
act cgt gaa gag atc aaa acata ttt gaa caa ttc aag aaa gta ttt ggc aaa gtc tac cga	158																																		
T R E E I K T F E Q F K K V F G K V Y R																																			
aat gct gaa gag gag gct cgt gct gaa cac cat ttc aag gaa caa ttg aaa tgg gtg gat	218																																		
N A E E E A R R E H H F K E Q L K W V E																																			
gaa cac aat gga atc gat ggt gtc gaa tat gca atc aat gaa tat tcc gat atg tcc gaa	278																																		
E H N G I D G V E Y A I N E Y S D M S E																																			
caa gag ttc tcg ttc cat ctt ggt ggt ggc ctt aac ttt acc tac atg aaa atg gag	338																																		
Q E F S F H L S G G G L N F T Y M K M E																																			
gca gca aag gaa cca ttg atc aac acata tat gga tca ctt cca caa aac ttt gat tgg cga	398																																		
A A K E P L I N T Y G S L P Q A N F D W R																																			
caa aag gca cga ttg acc cga atc cga caa ggt gca tgc ggt tcg tgt tgg gca ttt	458																																		
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tct gaa caa gaa ttg gtt gat tgt acc tac aat cga tat gat ccg tcc tat caa tgc aat	578																																		
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Gga tgt ggc tca gga tac tca acc gaa gca ttc aaa tat atg atc cga acc ggt ttg gtt	638																																		
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atg tac acg atc caa caa cac ggt cca gtt gtg atc tac atg cac gga tgc aac aac tat	818																																		
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Present study	GTQTQIQKTKTTMKFLLVAALCALVAIGSCKPTREEIKTFEQFKKVFVKVYRNAEEEAR	60
Published	-----	
Present study	EHHFKEQLKWVEEHNGIDGVEYAINEYSDMSEQEFSFHLGGGLNFTYMKMEAAKEPLIN	120
Published	-----	
Present study	TYGSLPQNFDRQKARLTRIRQQGACGSCWAFAAAGVAESLYSIQKQSIGLSEQELVDC	180
Published	---IPANFDWRQKTHVNPIRQGGCSCWAFASSVAETLYAIHRHQNIILSEQELLDC	56
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Present study	TYNRYDPSYQCNCGSGSYSTAEFKYMIRTLGLVEERNYPYNMRTQWCDPDVEGQRYHVS	240
Published	TYHLYDPTYKCHGCGSGMSPEAFKYMKGGLLEESHYPYMKMLNQCQANARGTRVHVS	116
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Present study	QQLRYHSSDEDVMYTIQQHGPPVIYMHGSNNYFRNLGNGLRVGVAYNDAYTDHAVILV	300
Published	NSLRYRAGDQEIQAAMNHGPVVIYIHGTEAHFRNLRKGIILRGAGYNDQIDHAVVLV	176
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Present study	GTVQGVVDYWIIRNSWGTGWNGGYYVERGHNSLGINNYVTYATL	345
Published	GTQNGIDYWIVRTSWGTQWGDAGYGFVERHHNSLGINNYPIYASL	221
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Figure 1. Group 1 cysteine protease sequence. (A) Nucleotide and the deduced amino acid sequence of Blo t 1 allergen. Nucleotide sequence residues are numbered at the end of each row. The residues in italic and bold form indicate the proposed predicted signal peptide cleavage site. The beginning of the proBlo t 1 sequence is indicated with an arrow. The underlined residues indicate the potential *N*-glycosylation sites; – is a stop codon. (B) Comparison of the amino acid sequences of the present study and the published Blo t 1 gene. Gaps, –, are introduced for optimal alignment; * indicates identical residues; : indicates highly conserved residues; • indicates less conserved residues.

from the *B. tropicalis* mite extract (27) as well as the spent mite medium extract. Significant amounts of native Blo t 1 protein was detected with the Blo t 1 specific mAb 11F1 generated in our laboratory. The native Blo t 1 protein migrated at about 26 kDa present in both crude mite extract and spent mite medium (Fig. 4).

Allergenicity of recombinant proBla t 1 produced in *Pichia pastoris*

The proBlo t 1 protein was expressed and secreted into the *P. pastoris* culture medium. The protein migrated at about 36 kDa with a yield of around 100 mg/l. The allergenicity of recombinant proBlo t 1 was tested against

Blo t 1	-----MKFLLVAALCALVAIGSCKPTREE-----IKTFEQFKKVFGKVYR	40
Der p 1	-----MKIVLAIASLLALSAVYARPSS-----IKTFEYKKAFNKSYA	-61
Actinidin	MGLPKSFVMSLLFFSTLLILSLAFNAKNTLQRTNDEVK-----AMYESWLIKYGKSYN	-73
Papain	MAMIPSISKLLFVAICLFVYMGLSFGDFSIVGYSQNDLTSTERLIQLFESWMLKHNKIYK	-74
	. : : :	
Blo t 1	NAEEEEARREHHFKEQLKWVEEHNGIDG--VEYAINESDMSEQEFS--FHLGGGLNFTY	096
Der p 1	TFEDEEAARKNFLESVKYVQSNNG-----AINHLSDLSLDEFKNRFLMSAEAFEHLK	-9
Actinidin	SLGEWERRFEIFKETLRFIDEHNADTNRSYKVLNQFADLTDEEFSTYLRFTSGSNKTK	-13
Papain	NIDEKIYRFEIFKDNLYIDEETNKNN--SYWLGLNVFADMSNDEFKEKYTGSIAG--NYTT	-16
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Blo t 1	MKMEAAKEPLINTYG--SLPQNFDWRQKARLTRIRQQGACGSCWAFAAAGVAESLYSIQK	154
Der p 1	TQFDLNAETNACSIN--GNAPAEIDLQRMRTVTPIRMQGGCGSCWAFSGVAATESAYLAYR	51
Actinidin	VSNRYEPRVG-----QVLPSYVDWRSAGAVVDIKSQGECGSCWAFSAIATVEGINKVT	42
Papain	TELSYEEVLND--GDVNIPEYVDWRQKAVTPVKNQSGCGSCWAFSAVVTIEGIIKIRT	42
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Blo t 1	QSIGLSEQLVDCYNNRYDPSYQCNGCGSGYSTFAFKYMIRTG--LVEERNYPYNNMRTQW	213
Der p 1	NQSLDLAEQELVDCASQ-----HGCHGDTIPRGIEYIQHNG--VVQESYRYVAREQS	102
Actinidin	GVILSLSEQLIDCGRTQ-----NTRGCGGGYITDGFQFIINNGGINTENYPYTAQDGE	97
Papain	GNLNEYSEQLLDCDR-----RSYGCNGGYPSALQLVAQYG--IHYRNTYPEGVQR	94
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Blo t 1	CDPDVEGQRYHVSQYQLRYHSSDEDVMTIQQHGPVVIYMHGSNN--YFRNLGNGVLRGV	272
Der p 1	CR-RPNAQRFGISNYCQIYPPNVNKIREALAQTHSAIAVIGIKDLDAFRHYDGRITIIQR	161
Actinidin	CNVDLQNEKYVTITDYENVPYN--NEWALQTAVTYQPVSVLDAAGD--AFKQYSSGIFTGP	156
Papain	CRSREKGPYAAKTDGVRQVQPY--NEGALLYSIANQPVSVVLEAAGK--DFQLYRGGIFVGP	152
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Blo t 1	AYNDAYTDHAVILVWGVTQGVVDYWIIRNSWGTGWNGGGYGYVERGHN----SLGINNYV	328
Der p 1	DNGYQPNYHAVNIVGYSNAQGVVDYWIRNSWDTNWGDNGYGYFAANID----LMMIEEYP	217
Actinidin	CG--TAVDHAVTIVGYGTGGIDYWIIVKNSWDTTWGEEGYMRILRNVG--GAGTCGIATMP	212
Papain	CG--NKVDHAVAAGVYGPV----YILIKNSWGTGWNGGYIRIKRGTGNSYGVCGLYTSS	206
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	▲ ▲ ▲	
Blo t 1	TYATL-----	333
Der p 1	YVVIL-----	222
Actinidin	SYPVKYNQNHKPYSSLINPPAFSMSKDGPGVDDGQRYSA	254
Papain	FYPVKV-----	212

Figure 2. Comparison of the prepropeptide sequences of Blo t 1 with Der p 1 (accession no. P08176), actinidin (accession no. Q9AXD2), and papain (accession no. P00784). Amino acid sequence of Blo t 1 is numbered from the first methionine and the rest of the peptide sequences are numbered according to the standard protease numbering. The symbol ▲ is used to denote amino acid residues in the active sites of the cysteine proteases. The conserved cysteine residues forming disulfide linkages are shown in *bold*. Gaps, –, are introduced for optimal alignment; * indicates identical residues; : indicates highly conserved residues; • indicates less conserved residues.

a panel of sera from allergic asthma patients (40 children and 40 adults). All these sera showed positive IgE reactivity with the both crude *B. tropicalis* and *D. pteronyssinus* mite extracts (data not shown). Out of the 40 sera from both groups, 24 adult sera (60%) and 37 sera of children (92.5%) reacted with proBlo t 1 protein (Fig. 5A and B). Although the children's sera showed a higher frequency of IgE reactivity to Blo t 1, the adults' sera had higher Blo t 1 specific IgE titers than that of the children's sera ($P < 0.001$). The high frequency of Blo t 1 specific IgE reactivity among the asthmatic patients indicated that Blo t 1 is probably a major allergen from *B. tropicalis*.

Correlation of IgE reactivity of proBlo t 1 with native Der p 1

Information concerning the correlation and cross-reactivity of IgE reactivity within the same group of allergens is useful in terms of the understanding of the primary

sensitization and development of diagnosis and treatment of allergic diseases. In this study the correlation of IgE reactivity to proBlo t 1 and native Der p 1 was evaluated with sera from the same panel of 40 asthmatic children. There were 35 (87.5%) sera that showed positive IgE reactivity to native Der p 1 and such frequency was similar to that of proBlo t 1 (92.5%). There was no significant correlation of IgE reactivity to Blo t 1 and Der p 1 ($P > 0.5$) (Fig. 6).

Discussion

Genes encoding several allergens from *B. tropicalis* mites have been isolated, characterized and published in recent years (13–17). However, unlike the scenario in *Dermatophagoides* species, the major *B. tropicalis* allergen identified and shown to be clinically important was Blo t 5 (12, 13, 32) and little is known about the group 1 allergen

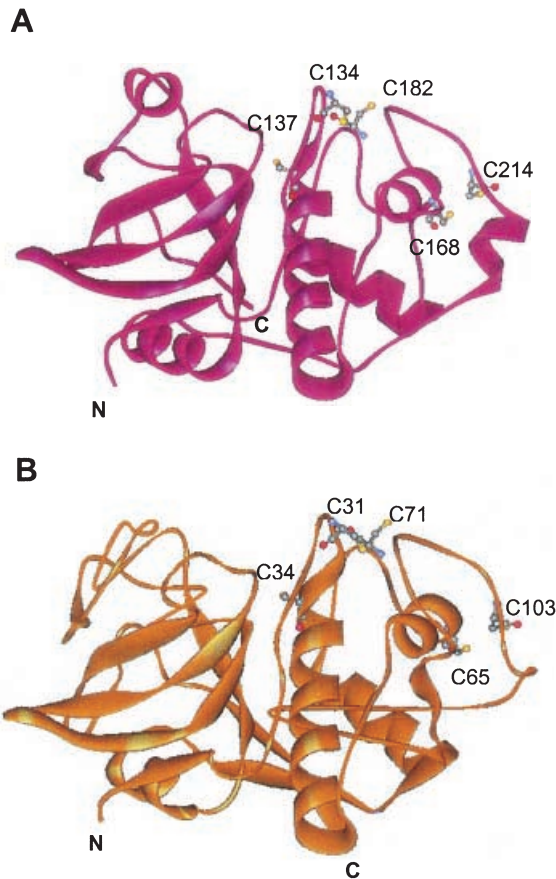


Figure 3. The molecular models of Blo t 1 (A) and Der p 1 (B). The conserved cysteine residues forming putative disulfide linkage as well as the active site cysteine are shown. The N- and C-terminals are indicated in both structures.

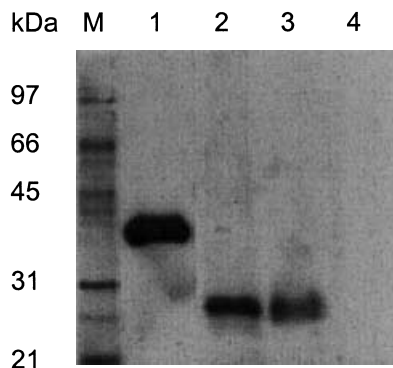


Figure 4. Detection of native Blo t 1 protein by Western blot with anti-Blo t 1 monoclonal antibody. Lane 1: recombinant proBlo t 1 expressed from *P. pastoris*; Lane 2: *B. tropicalis* crude mite extract; Lane 3: spent mite medium; Lane 4: glutathione-S-transferase. Molecular weight markers are shown (M).

in this mite species. This study describes the nucleotide and the deduced amino acid sequences derived from the full-length cDNA clone encoding Blo t 1. Sequence

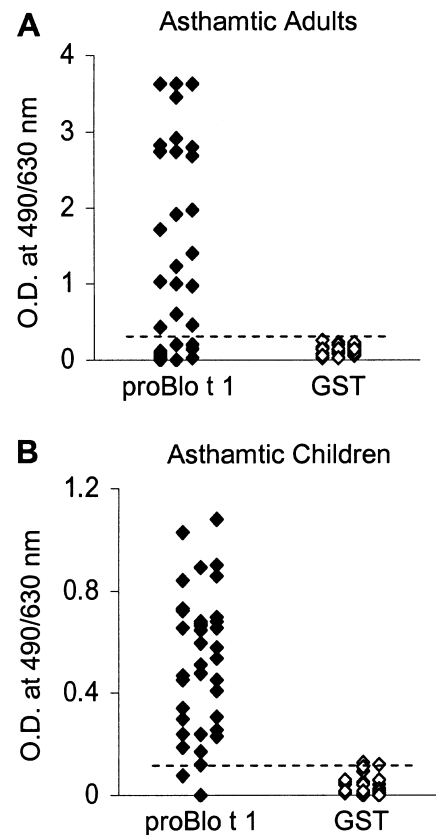


Figure 5. Human IgE reactivity to proBlo t 1 and GST with sera from 40 asthmatic adults (A) and 40 asthmatic children (B). Sera with high total IgE titers were selected for the Blo t 1 specific IgE reactivity evaluation. The cut-off values (denoted by the dotted line) are set at mean plus threefold standard deviation of the reactivity to GST.

alignment with group 1 homologs from other mite species revealed that Blo t 1 only shared up to 53% nucleotide sequence homology and less than 36% protein sequence homology with Der p 1, Der f 1 and Eur m 1. It has been reported that Blo t 5 and Blo t 3 shared less than 50% protein sequence homology with their respective homologs from other mite species (13, 14); it appears that the non-structural allergenic proteins of mites in the Glycyphagidae family share relatively low sequence homologies with homologs from mites in the Pyroglyphidae family.

Despite the low primary sequence homology with other group 1 mite allergens and other papain-like cysteine proteases, all the classical catalytic triads found in the enzyme active site of cysteine proteases are fully conserved in Blo t 1 reported in this study. In addition, our molecular modeling based on the three-dimensional structure of actinidin revealed that Blo t 1 has a two-domain structure with the enzyme active site formed between the two domains. The cysteine residue pairs that are involved in the formation of the two highly conserved disulfide bonds in actinidin and papain are also conserved in Blo t 1 (Fig. 3). However, this predicted

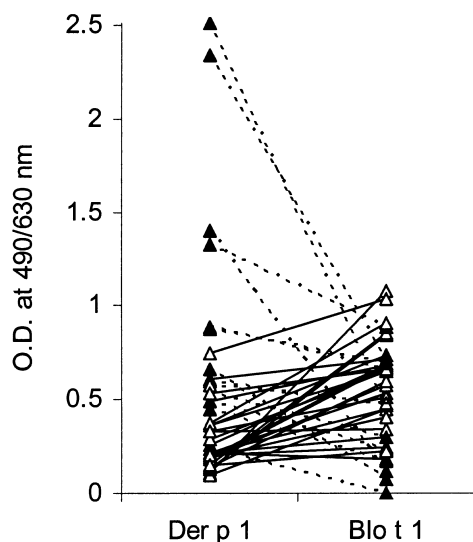


Figure 6. Comparison of Der p 1 and Blo t 1 reactivity of sera from asthmatic children (C). The human IgE reactivity of native Der p 1 and proBlo t 1 were measured with ELISA. Sera with higher reactivity to proBlo t 1 are indicated with symbol 6 and solid lines, whereas symbol 4 and dotted lines indicate sera with higher reactivity to native Der p 1.

structure of Blo t 1 protein should be validated experimentally.

The Western immunoblot analysis using anti-Blo t 1 mAb showed that a significant quantity of native Blo t 1 protein was present in spent mite medium extract suggesting that, like other group 1 mite allergens, Blo t 1 is a digestive enzyme associated with mite fecal pellets (33). It is worth noting that the putative conserved N-glycosylation site from residues 53–55 in the mature proteins of Der p 1, Der f 1, and Eur m 1 is absent in Blo t 1. Incidentally, the molecular weight of native Blo t 1 estimated on the SDS-PAGE was smaller than that observed for native Der p 1 (34). This reduction in molecular weight of native Blo t 1 could be accounted for by the absence of the conserved N-glycosylation site in mature Blo t 1 protein.

Recombinant Blo t 1 reacted with high frequency with tested sera from both children and adult asthmatics from tropical Malaysia (Fig. 5A and B) suggesting that it is probably a major allergen for asthmatic patients in the tropics. It was noted that although up to 90% of the sera of children asthmatics reacted with Blo t 1, the levels of Blo t 1 specific IgE in these sera were generally lower than that detected in adult sera. The clinical significance of these data deserves further investigation with a larger panel of allergic sera from the tropical and subtropical regions. Our previous studies showed that *D. pteronyssinus* and *B. tropicalis* mites are present in large numbers in the home environment (35). The mite allergic subjects in Singapore and Malaysia generally exhibit dual sensitization to *D. pteronyssinus* and *B. tropicalis* mite allergens

(32, 36). Our unpublished data showed that the prevalence of sensitization to Der p 1 in asthmatic children in these countries was very high. In this study sensitization to Blo t 1 and Der p 1 allergens was comparatively assessed for the first time using a panel of sera from asthmatic children from Malaysia. The data revealed a low correlation between IgE reactivity of Blo t 1 and Der p 1 implying that the degree of IgE cross-reactivity between the two group 1 allergens is low. Such an observation is not surprising given the fact that Blo t 1 shared less than 35% protein sequence homology with Der p 1. The recent paper on Blo t 1 also reported the absence of cross-reactivity from Der p positive serum to Blo t 1 as shown by immunoblotting, though with a small sample size of 27 patients. However, the degree of IgE cross-reactivity between these two group 1 allergens has to be addressed quantitatively with inhibition studies using both native and recombinant allergens. It is well established that there was little IgE cross-reactivity between Blo t 5 and Der p 5 (13, 36). We had also identified unique IgE epitopes in highly conserved tropomyosins (group 10 mite allergen) from *D. pteronyssinus* and *B. tropicalis* mites (15). Therefore, the lack of IgE cross-reactivity between homologous allergens from *D. pteronyssinus* and *B. tropicalis* mites is a common observation.

Recently, Mora et al. published the sequence for a truncated cDNA clone encoding for the truncated mature Blo t 1 protein without the pre and pro sequences as well as the 5' untranslated sequences. Interestingly, the Blo t 1 gene of the present study shares only 61% amino acid sequence homology with the published Blo t 1 sequence (Fig. 1B) (26). Furthermore, the authors reported that the predicted secondary and three-dimensional structures of their putative Blo t 1 protein share similar structural features with human cathepsin L and porcine cathepsin H, whereas our protein modeling results obtained using the LOOK program revealed that the predicted 3D structure of our Blo t 1 protein is highly homologous to that of actinidin, instead of cathepsins. Taken together, the data from both studies suggest that the cDNA clone isolated in this study is not an isoform of the one published by Maro et al. It is conceivable that our cDNA clone is encoding for a papain-like cysteine protease whereas the published cDNA clone is encoding for cathepsin-like cysteine protease. Both these cDNA sequences share only less than 35% amino acid sequence identity with Der p 1, Der f 1, and Eur m 1. Further studies are required to evaluate and elucidate the nature of the proteins encoded by these two cDNA clones.

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