

Immunologic Characterization and Allergenicity of Recombinant Tyr p 3 Allergen from the Storage Mite *Tyrophagus putrescentiae*

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Key Words

Basophil histamine release • IgE antibody • Mite group 3 allergens • Storage mite • Trypsin-like serine protease • Tyr p 3 allergen • *Tyrophagus putrescentiae*

Abstract

Background: The involvement of the storage mite *Tyrophagus putrescentiae* in allergies has been increasingly reported in many countries. Molecular analysis has shown that group 3 mite allergens are homologous to trypsin. Similar allergens have not been identified in *T. putrescentiae*. Our aims were to characterize group 3 allergens in *T. putrescentiae* and to investigate their significance in allergenicity. **Methods:** cDNAs of PreTyr p 3 and rTyr p 3 from *T. putrescentiae* were cloned and expressed in *Escherichia coli*. Native Tyr p 3 (nTyr p 3) was purified from spent growth medium with an affinity column coupling of antibody. Biological activities of rTyr p 3 were compared with nTyr p 3 in terms of IgE activity, enzymatic activity and histamine release. **Results:** Full-length cDNA of PreTyr p 3 encodes a 285-amino acid trypsin-like protease and acquires enzymatic activity after removing the pre- and pro-sequences. rTyr p 3 is a 26-kDa protein with equivalent IgE reactivity but weaker enzymatic activity than that of nTyr p 3. A limited level of cross-reactivity has been found between rTyr p 3, Der p 3 and *Blomia*. Eight of 10 *T. putrescentiae*-sensitized individuals showed >50% histamine release

after triggering with rTyr p 3. **Conclusions:** Our studies demonstrate that Tyr p 3 is a frequent allergen (58%) in *T. putrescentiae*-sensitized patients. Since rTyr p 3 displays equivalent biological activities as nTyr p 3, the role of group 3 allergens can be studied using rTyr p 3 to elucidate the pathogenic effects and diagnostic applications of Tyr p 3.

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Introduction

The storage mite *Tyrophagus putrescentiae* is distributed worldwide. It is commonly found in many contaminated foods, such as wheat flour, wheat germ, cheese, cereal, dried fruit and mushrooms [1]. It can tolerate both high and low temperatures, grows rapidly in moist environments [2] and has become a household pest in Asia [3]. Initially, storage mite-induced allergy was mainly found in farmers and grain workers, and is considered to be closely related to respiratory disease in these individuals [4]. Although house dust mites and storage mites cohabit in many situations, they may cause concomitant hypersensitivity, but monosensitivity to *T. putrescentiae* is dif-

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difficult to identify. However, storage mite sensitization has been noted in asthmatic patients of Mexico City, Mexico [5], and in allergic patients in Ohio, USA [6], and Silesia, Poland [7]. In both urban and rural areas, allergic patients sensitized to *T. putrescentiae* have been reported [8, 9]. Moreover, these investigations also showed that *T. putrescentiae* plays an important role in allergen-induced IgE-mediated reactions, and food contaminated with *T. putrescentiae* may lead to systemic anaphylaxis [10]. At least 20 IgE-binding allergenic components of *T. putrescentiae* have been distinguished previously [1]. So far, only four allergens with full-length sequences of *T. putrescentiae* have been characterized and published: Tyr p 2 [11], Tyr p 10 (tropomyosin) [12], Tyr p 13 (fatty acid-binding protein) [13] and Tyr p α (tubulin) [14]. However, many allergenic components of *T. putrescentiae* have not been clarified to date, especially those with protease activity.

Allergens in groups 3, 6 and 9 have been found to have potential serine protease activity [15]. Among them, group 3 allergens appear to be the major allergens that cause allergic reactions and play a significant role in allergic diseases [16, 17]. Group 3 allergen, Der f 3, was originally isolated from *Dermatophagoides farinae* by Heymann et al. [18], and similar allergens were subsequently isolated from *D. pteronyssinus* by Stewart et al. [19]. Biochemical studies have revealed that extracts of *D. pteronyssinus*, *D. farinae* and *Euroglyphus maynei* contain both trypsin and chymotrypsin enzymes [20]. Trypsin enzyme activities have been identified in the extract of *T. putrescentiae* [21]. Trypsin-like proteases play an important role as digestive enzymes and as zymogen activators [22]. Allergens with proteolytic activity have been shown to activate complement to generate anaphylatoxins C3a and C5a, via processing of C3 and C5 [23], and trigger the secretion of proinflammatory cytokines by bronchial epithelial cells or eosinophils and disrupt the epithelial permeability barrier of the lungs [24, 25]. These findings indicate that proteolytic activity appears to be the major contributor to their allergic potential [26]. In addition, in vitro studies have also indicated that Der p 3 can trigger GM-CSF and eotaxin release in pulmonary epithelium by activation of protease activation receptor-2 [27]. Due to the significant role of group 3 allergens in allergenic reactions, five recombinant group 3 mite allergens, Der s 3 (*D. siboney*) [16], Der p 3 (*D. pteronyssinus*) [28], Der f 3 (*D. farinae*) [29], Eur m 3 (*E. maynei*) and Blo t 3 [17], have been studied previously. However, group 3 allergens in *T. putrescentiae* have not been investigated to our knowledge. Nucleotide sequence analyses indicate that

the homology of the group 3 mite allergens is correlated with phylogenetic relationships between mite species: Blo t 3 has 41, 47 and 42% identity to Der p 3, Der f 3 and Eur m 3, respectively. In contrast, Der p 3, Der f 3 and Eur m 3 share approximately 80% homology [17]. The homology and cross-activity of group 3 allergens between *T. putrescentiae*, *Dermatophagoides* and *Blomia tropicalis* remain to be elucidate.

In this study, a novel *T. putrescentiae* allergen of trypsin-like component (rTyr p 3) was identified and characterized in terms of cDNA cloning, protein purification, enzymatic activity, IgE reactivity and histamine release. Cross-reactivity with the other group 3 mite allergens was also examined. The identification and immunologic characterization of rTyr p 3 may help to elucidate the role of storage mites in IgE-mediated allergic sensitization.

Materials and Methods

Mite Extract

T. putrescentiae was collected from contaminated food samples and carefully identified. The mites were cultured at 25°C/75% relative humidity in a covered plastic cup containing a medium consisting of yeast extract and mouse chow, then separated from the medium and stored at -80°C. Separation of the mites from the medium was achieved by gently stirring the medium with a glass rod following overnight culture. The mites which migrated to the cover were collected and frozen at -80°C. This collection included a great proportion (>90%) of mites with a minimum of feces and was almost free of medium. Frozen mites were homogenized and extracted with phosphate-buffered saline (PBS, pH 7.2). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, Calif., USA) using bovine serum albumin as standard.

Patient Sera

Sera were obtained from patients who visited the Division of Allergy, Immunology and Rheumatology in the Taichung Veterans General Hospital (TCVGH), Taichung, Taiwan, ROC. These patients were mostly sensitized in their residences and had been diagnosed with allergy if they had at least one of the following criteria: history of asthma, rhinitis, atopic dermatitis or eczema and a positive test for *T. putrescentiae* with ELISA screening. A total of 110 allergic patients were enrolled and 10 nonallergic individual sera were obtained. Collection of blood samples from the patients and volunteers was approved by the Institutional Review Board of TCVGH (TCVGH IRB No. 940628/C05110).

Cloning of Gene-Specific Fragments for PreTyr p 3 and Mature Tyr p 3

Total RNA was isolated with Trizol reagent and cDNA was synthesized with primer dT₁₇ and MMLV reverse transcriptase (Gibco-BRL, New York, N.Y., USA). The degenerate primers were designed based on the conserved region of group 3 allergens. Polymerase chain reaction (PCR) products were eluted using a gel

extraction kit (Viogen, Taipei, Taiwan, ROC) ligated into a pUC119 vector (Promega, Madison, Wisc., USA) and transformed into *Escherichia coli* XL1-Blue (Stratagene, Amsterdam, The Netherlands) competent cells.

The full-length cDNA of PreTyr p 3 was amplified by the 5' and 3' rapid amplification of cDNA end systems (Gibco-BRL) using PCR and screened by Southern hybridization with probes. The 3'-RACE-ready cDNA template was synthesized by reverse transcriptase using an adaptor primer (AP, 5'-GGCCACGCGTCGACTAGTACT₁₇-3'). This template was utilized for PCR using the primer: Tp-SP-F, 5'-CGTTGCAGCAAAGCTTCCTGCAAAGGGC-3' and AUAP (a part of primer AP). The 5'-RACE-ready cDNA template was prepared with a gene-specific primer: Tp-SP-3R, 5'-GGCAATGTCGTTGTCGCTTGT-3' designed by the sequences we obtained from 3'-RACE PCR. The 5'-RACE PCR was carried out with primers dG₁₂ and Tp-SP-3R. The full length of PreTyr p 3 was performed using the primers Tp-SP-EF, 5'-CGCGGATCCATGAAAATTTTAC TTTTGTG-3', and Tp-SP-ER, 5'-CGCGGATCCTTACCAAATTGCACCTATGAA-3', according to the RACE product sequences and containing a *Bam*H1 restriction enzyme site. The cDNA encoding for mature Tyr p 3 (rTyr p 3) was subcloned and amplified with primers containing *Bam*H1: Tp-SP-MF/Tp-SP-ER. PCR was carried out at 94°C for 10 s, 55°C for 20 s and 72°C for 3 min for 30 cycles. Amplicons were purified and analyzed as described above.

Expression of Recombinant PreTyr p 3 and rTyr p 3 and Preparation of Polyclonal Antibodies

The PCR products for PreTyr p 3 and rTyr p 3 were digested with *Bam*H1, ligated into a *Bam*H1-cut pQE30 vector and transformed into *E. coli*-M15. Transformants were selected by kanamycin (25 µg/ml) and ampicillin (100 µg/ml). Expression of rTyr p 3 was performed according to the methods described in the manufacturer's instructions to the QIA Expressionist™ Kit (Qiagen, Hilden, Germany). The recombinant proteins were expressed as a 6xHis-tagged protein using 1 mM isopropyl-β-D-thiogalactopyranoside (Promega) induction. The proteins were purified by nickel-nitrilotriacetic acid agarose metal affinity column chromatography under native conditions. Then, the purified rTyr p 3 was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Chelmsford, Mass., USA) for N-terminal sequencing by automated Edman degradation. This protein was also excised from the gel followed by digestion with trypsin protease, and then the generated peptides were identified by MALDI-TOF LC/MS/MS analysis. Polyclonal antibody anti-rTyr p 3 was obtained from a New Zealand rabbit, which was injected subcutaneously with rTyr p 3 emulsified with Freund's adjuvant.

Purification of nTyr p 3

nTyr p 3 was extracted using frozen spent growth medium (SGM) and purified with an affinity column coupling of anti-rTyr p 3 antibody. Briefly, 10 g SGM were homogenized with 5 ml of PBS (pH 7.2) for 10 min, sonicated in ice for 30 min and centrifuged at 13,000 g at 4°C for 30 min. The supernatant was filtered through a membrane filter (pore size: 0.45 µm) and loaded onto an affinity column. This affinity column was prepared using 1 g (dry weight) of resin containing cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden) with modifications of the methods [30].

Briefly, the resin was swollen and washed extensively in 1 mM HCl followed by equilibration with coupling buffer (100 mM NaHCO₃ and 0.5 M NaCl, pH 8.3); then 10 mg rabbit anti-rTyr p 3 serum was bound to the swollen cyanogen bromide-activated Sepharose 4B by incubation in coupling buffer overnight with continuous mixing at 4°C. The resin was washed thoroughly with coupling buffer to remove the excess antibody until OD₂₈₀ was <0.05. To block any excess binding sites, the resin was incubated with 1 M Tris-HCl, pH 8.0, for 2 h at 25°C. The resin was washed with three cycles of solution A containing 0.1 M CH₂NaO₂ and 0.5 M NaCl, pH 4.0, followed by solution B containing, 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0. The final column was 10 × 2 cm and contained 10 ml of gel. After loading the filtered SGM, the affinity column was incubated overnight at 4°C and subsequently washed with buffer (0.1 M borate, 0.5 M NaCl and 0.05% Tween-20) extensively. The bound nTyr p 3 was eluted from the affinity column with elution buffer (0.1 M glycine and 0.15 M NaCl, pH 2.6) and neutralized with 1/10 volume of 1 M Tris-HCl, pH 8.0. The column was then regenerated with loading buffer (50 mM Tris-HCl and 20 mM CaCl₂, pH 8.0).

Sequence Analyses

The amino acid sequences of PreTyr p 3 were deduced with Translate Tool software (www.expasy.org/tools/dna.html). The homology search of PreTyr p 3 was carried out with the BLAST network server in the National Center for Biotechnology Information. Multiple sequence alignment of PreTyr p 3 and other group 3 allergens was performed using the ClustalW Web Service (www.ebi.ac.uk/Tools/clustalw/index.html). The SignalP program was used to predict the signal peptide cleavage site (www.cbs.dtu.dk/services/SignalP).

SDS-PAGE and Immunoblotting for Human IgE-Binding Capacity

The extracts of mite bodies and SGM of *T. putrescentiae* or rTyr p 3 were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking with skimmed milk, membranes were reacted with serum samples from patients (1:5 dilution) or rabbits (1:2,500 dilution). Blots were then immersed with horseradish peroxidase-conjugated goat anti-human IgE antibodies (1:1,000 dilution; Biosource, Camarillo, Calif., USA) or goat anti-rabbit IgG antibodies (1:5,000 dilution; Chemicon, Temecula, Calif., USA) and washed with PBS after each treatment. Finally, blots were immersed with enhanced chemiluminescence reagent (Pierce, Rockford, Ill., USA) for 2 min and exposed to X-ray films.

IgE ELISA Reactivity and Inhibition Tests of rTyr p 3

IgE antibodies in serum samples were detected by ELISA. Our preliminary test showed that the optimal level of absorption could be obtained by coating a plate with 5 µg/ml rTyr p 3 in 0.1 M NaHCO₃ (pH 8.4); thereafter, ELISA plates were coated with 5 µg/ml of nTyr p 3 or rTyr p 3 for the entire experiment. One hundred microliters of diluted (1:5) human sera were added for overnight incubation at 4°C. Plates were washed and incubated with horseradish peroxidase-conjugated goat anti-human IgE (1:1,000) for 1 h at room temperature. The colorimetric reaction was performed using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (Zymed, San Francisco, Calif., USA). Absorbance at 405 nm was determined using a PowerWare-X340 ELISA reader (Bio-

TEK, Winooski, Vt., USA). For the inhibition test, serum samples were selected from 10 allergic patients (Pt1–Pt10) who had high titers of IgE antibodies against mite extracts of *T. putrescentiae*, but none to *D. pteronyssinus*. Each serum was preincubated, with various concentrations (0.01–100 µg/ml) of rTyr p 3 and subsequently reacted with 5 µg/ml of extract coated on the plate. The subsequent procedures for determining IgE antibodies by ELISA were the same as those described previously.

Basophil Histamine Release Assay

Washed polymorphonuclear cells were obtained from the venous blood of non-atopic donors using Polymorphprep™ solution (Axis-Shield PoC AS, Oslo, Norway). The cells were resuspended in RPMI 1640 (Invitrogen, Carlsbad, Calif., USA) by adjusting to 2×10^6 cells/ml using trypan blue assessment (0.4%; Invitrogen). Passive sensitization of basophils with specific IgE from allergic (Pt1–Pt10) or healthy (H1–H3) sera was performed. Following sensitization, cells were incubated with serial concentrations (0.01–100 µg/ml) of rTyr p 3 for 30 min at 37°C, and the supernatant was collected and reacted with *O*-phthalaldehyde (5 mM) for 7 min. The reaction was stopped by adding H₂SO₄ (0.04 M). The histamine released into the supernatant was measured by a fluorescent spectrophotometer (F4500; Hitachi, Tokyo, Japan) [31].

Serine Protease Activity

Enzyme substrates and inhibitors were purchased from Sigma (St. Louis, Mo., USA). Specific trypsin activity was determined using *p*-toluene arginine-methyl ester (TAME) and chymotrypsin activity using *N*-benzoyl-L-tyrosine ethyl ester (BTEE); absorption was measured at 405 nm [32]. The following protease inhibitors were used: phenylmethylsulfonyl for serine protease in general, soybean trypsin inhibitor and tosyllysine chloromethyl ketone for trypsin, and dithiothreitol (DTT) for cysteine protease in general [22]. Samples were pretreated with 10 mM of each inhibitor for 60 min at 25°C before addition of the specific substrate.

Statistical Analyses

Statistical analyses were carried out using SPSS (version 12; SPSS, Chicago, Ill., USA). Correlations were analyzed using Pearson's χ^2 test in the IgE-binding activity assay. Protease inhibition experiments were analyzed using one-way ANOVA and Dunnett's multiple comparison tests. $p < 0.05$ was considered statistically significant.

Results

Molecular Cloning and Sequence Analyses of PreTyr p 3 cDNA from T. putrescentiae

The full-length cDNA of the group 3 allergen from *T. putrescentiae* (accession No. EU302595) has 1,062 base pairs, including a 67-bp 5' nontranslated region, a ATG start codon at position 68–70 and a stop codon (TAA) at position 923–925, with an open reading frame coding for 285 deduced amino acids (fig. 1). We also identified the sequence of AATTAAAA present 103 nucleotides down-

stream of the termination codon that may serve as a poly(A) additional signal to the 3' terminus of mRNA for rTyr p 3. Furthermore, prediction with the sequence analysis tool showed that PreTyr p 3 possesses a 16-amino acid signal peptide (1–16, MKILLFLCFLVSVAF) with a high hydrophobic amino acid sequence, a typical feature of signal sequences for secretion. Data revealed that PreTyr p 3 has a serine protease with a premature protein structure and a mature protein which may start at the amino acid isoleucine (+1), which has been proven in nTyr p 3 by Edman degradation analysis. In comparison with other group 3 allergen sequences, Blo t 3 (AN-AAM10779), Der p 3 (AN-P39675), Der f 3 (AN-P49275) and Eur m 3 (AN-AF047615), all group 3 mite allergens shared 6 conserved cysteine residues (marked with ▲ in fig. 2) and 3 conserved histidine residues (marked with ■ in fig. 2) at respective locations. Moreover, the catalytic triad of the enzyme of the trypsin family, which is essential for enzymatic activity, was also conserved at His-73, Asp-212 and Ser-218 (labeled with ★ in fig. 2) as was a substrate binding site of trypsin at Asp-118 (labeled with ● in fig. 2). In addition, signature sequences for the active sites of histidine (ILTAAHC; amino acids 68–74) and the serine (DSCQGDSGG; amino acids 212–220) were also highly conserved (marked with a solid-line square and dashed-line square, respectively). The additional sequence information of rTyr p 3 was confirmed from LC/MS/MS analyses of peptides. Fifteen interpretable mass spectra were obtained from trypsin-digested peptides, and sequences of these tryptic fragments appeared to match the deduced amino acid sequence derived from the cDNA of PreTyr p 3.

Purification of nTyr p 3 from SGM of T. putrescentiae by Anti-rTyr p 3 Polyclonal Antibodies

The protein profiles of crude extracts on SDS-PAGE showed several protein bands existing in mite bodies and SGM (fig. 3a). Running the gel with the same amount of proteins, a stronger intensity of the 26-kDa protein reacting with rTyr p 3 antibody was observed in the extracts of SGM (fig. 3b). When SGM had been purified by the affinity column coupling of anti-rTyr p 3 antibody, a single band with a molecular weight of 26 kDa remained (fig. 3a), and this protein reacted perfectly with rTyr p 3 antibodies (fig. 3b). Further identification of nTyr p 3 was achieved by N-terminal sequencing. The result showed that nTyr p 3 has a sequence of IVGGTEAV, which was found in the deduced amino acid sequence of PreTyr p 3 cDNA starting from position '+1' (fig. 2).

Fig. 1. Nucleotides and deduced amino acid sequences of recombinant PreTyr p 3. The fragment used as a probe for Southern hybridization is marked with shading. The full nucleotide and deduced amino acid sequences have been submitted to the GenBank database [accession No. (AN)-EU302595].

8	CATTTTACTACA AAGAAGATTTT TTTATTAATTTT ACTTTATTTCAA GCACTTAATATC	67
	Tp-SP-EF	
68	<u>ATGAAAATTTTA CTTTTTTGTGT</u> TTTCTAGTCAGT GTGGCTTTTGCT AAGCCACCTACA	127
	M K I L L F L C F L V S V A F A K P P T	
	Tp-SP-MF	
128	ATTGAGCTCAAA AGTAACACCAAA TCTCAAAATGGT TTTATTGTTGGA <u>GGAACGGAGGCG</u>	187
	I Q L K S N T K S Q N G F I V G G T E A	
188	<u>GTAGACGGCGAT</u> GCACCTCACCAA GTTTCTCTTCAA CATACAAGCCAT TTTTGTGGTGGT	247
	V D G D A P H Q V S L Q H T S H F C G G	
248	TCTATCATCTCG GAAAGATGGATT CTCACTGCAGCT CAT <u>TGTATTGAT</u> <u>GCAGATGACCTA</u>	307
	S I I S E R W I L T A A H C I D A D D L	
308	<u>AGTAATCCAGGC</u> GGCATGAGCGTT CGTTATAACACC TTAATCTAAAA TCTGGCACTTTG	367
	S N P G G M S V R Y N T L N L K S G T L	
	Tp-SP-3R	
368	<u>GTAAAGGTGAAA</u> AGCATCAAAGTG CATGAACAATAC TCAAACGTGACA <u>AGCGACAACGAC</u>	427
	V K V K S I K V H E Q Y S N V T S D N D	
428	<u>ATTGCCCTCCTT</u> GAAACGGTGGCA TCTATGAATTTA AATCAGACAAAT <u>GCCGTTGCAGCA</u>	487
	I A L L E T V A S M N L N Q T N A V A A	
488	<u>AAGCTTCCTGCA</u> AAGGCAATGAT CCTCAGGATGGA GATCTTTTCTCA TCCGGTTGGGCG	547
	K L P A K G N D P Q D G D L F L S G W G	
548	<u>ACTTTGCACTCT</u> GGTGACACGACC ATTCCTACTAAT CTTCAGAAGGTC ACTGTGCCTCTC	607
	T L H S G D T T I P T N L Q K V T V P L	
608	<u>ACTAATCGAAGT</u> GTCTGCGCCGAA GCCTACACTGGA ATTGTGAACATC ACTGAGAACATG	667
	T N R S V C A E A Y T G I V N I T E N M	
668	<u>TTCTGTGCTGGA</u> AAGATGGGCATC GGAGGTGTCGAC AGCTGTCAGGGT GACAGCGGTGGA	727
	F C A G K M G I G G V D S C Q G D S G G	
728	GGTGCCATGCTT AACAAAGAGCTT GTAGGCGTTGTC AGTTTTGGCGTC GGCTGCGGTGAT	787
	G A M L N K E L V G V V S F G V G C G D	
788	CCTAAGTATCCA GGCCTCTACACC CGTGTAGTCAA TACCTTGACTGG ATTGAGCTGAGC	847
	P K Y P G V Y T R V S Q Y L D W I E L S	
848	GCTAAATCAAGT GCTACCACTTTG GTTGCACTTAAT ATTACATTGTTT CTGACGCTTTTC	907
	A K S S A T T L V A V N I T L F L T L F	
	Tp-SP-ER	
908	<u>ATAGGTGCAATT</u> TGGAATTGTAC CTTTAAAAACAA TGTCAAATTATT TATTCTGACTC	967
	I G A I W *	
968	CTGAAAAATTAA TGTATTTTTAGA CCACAGTTTATG TTTAAACAACAA GTGTACAAAACA	1027
1028	CAATTAAATATC TTAATAAAAAA AAAAAAAAAA	1062

Immunologic Characterization of rTyr p 3 and Cross-Reactivity with Other Group 3 Mite Allergens

Tests of IgE reactivity with the sera of allergic patients showed significant correlation between nTyr p 3 and rTyr p 3 ($R^2 = 0.94$; $p < 0.05$; fig. 4a), and a good correlation was also observed between crude extract and rTyr p 3 ($R^2 = 0.64$; $p < 0.05$, data not shown). Furthermore, individual differences of the specific IgE reactivity to rTyr p 3 in *T. putrescentiae*-sensitized patients existed, which resulted in various levels of inhibition (fig. 4b). When concentrations of rTyr p 3 reached 100 $\mu\text{g}/\text{ml}$, IgE inhibition ranged from 17 to 80%. rTyr p 3 displayed significant inhibition

(80%) in the serum of Pt-1 and Pt-2, moderate inhibition (40–60%) in Pt-3, Pt-4, Pt-9 and Pt-10, and inhibition $<20\%$ in Pt-5, Pt-6, Pt-7 and Pt-8.

Cross-reactivity of group 3 allergens with *Tyrophagus*, *Dermatophagoides* and *Blomia* was studied using *T. putrescentiae*-sensitized sera. IgE antibodies against rTyr p 3 were found in 58% (58/100) without absorption, in 48% (48/100) after absorption with Der p 3 and in 38% (38/100) after absorption with *Blomia* (data not shown). These results suggest that the group 3 allergen in *T. putrescentiae* may act as a major allergen triggering IgE-mediated hypersensitivity and has limited cross-reactivity

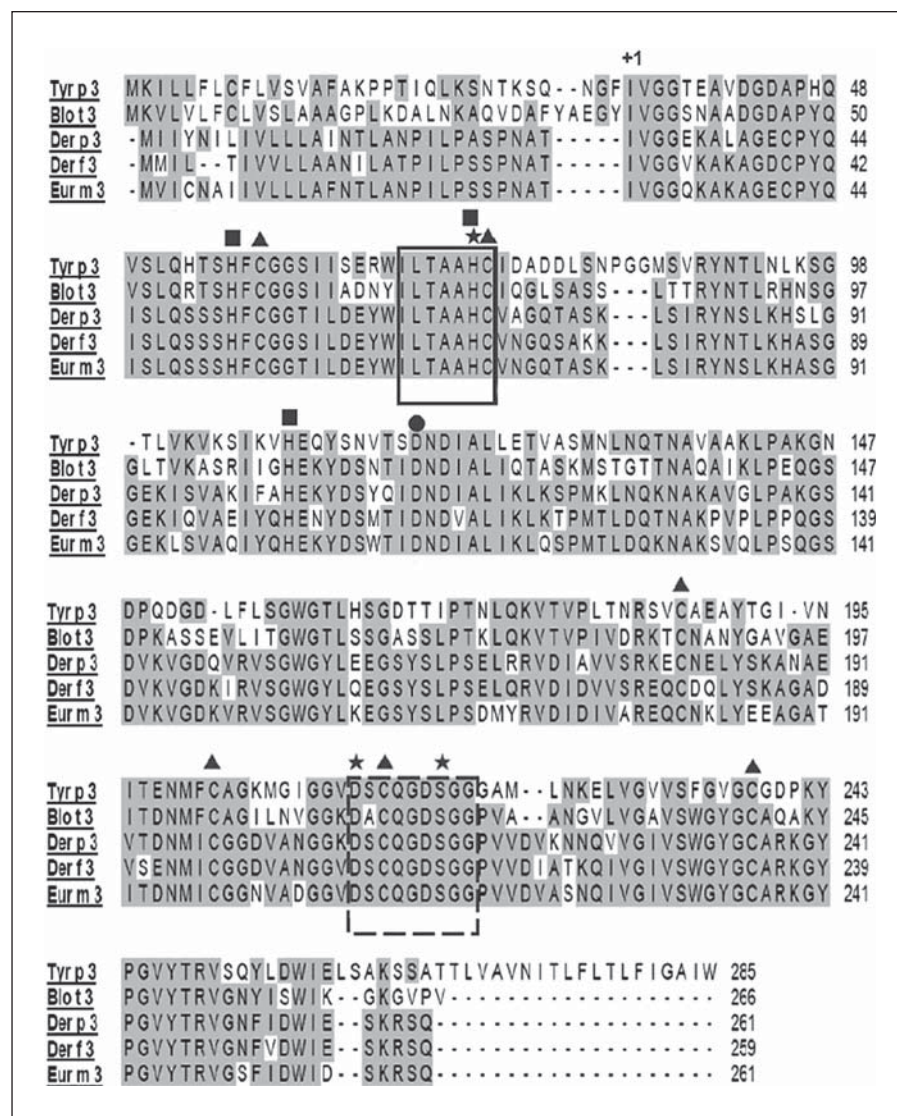


Fig. 2. Alignment of the amino acid sequences of recombinant PreTyr p 3 and other group 3 mite allergens. The conserved residues are shaded and gaps are indicated (-).

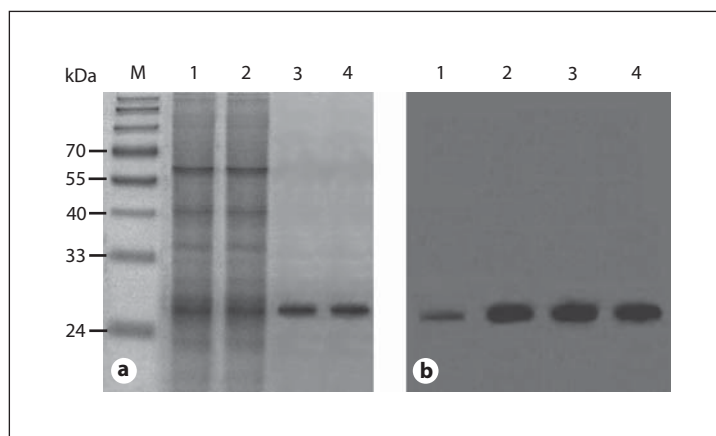
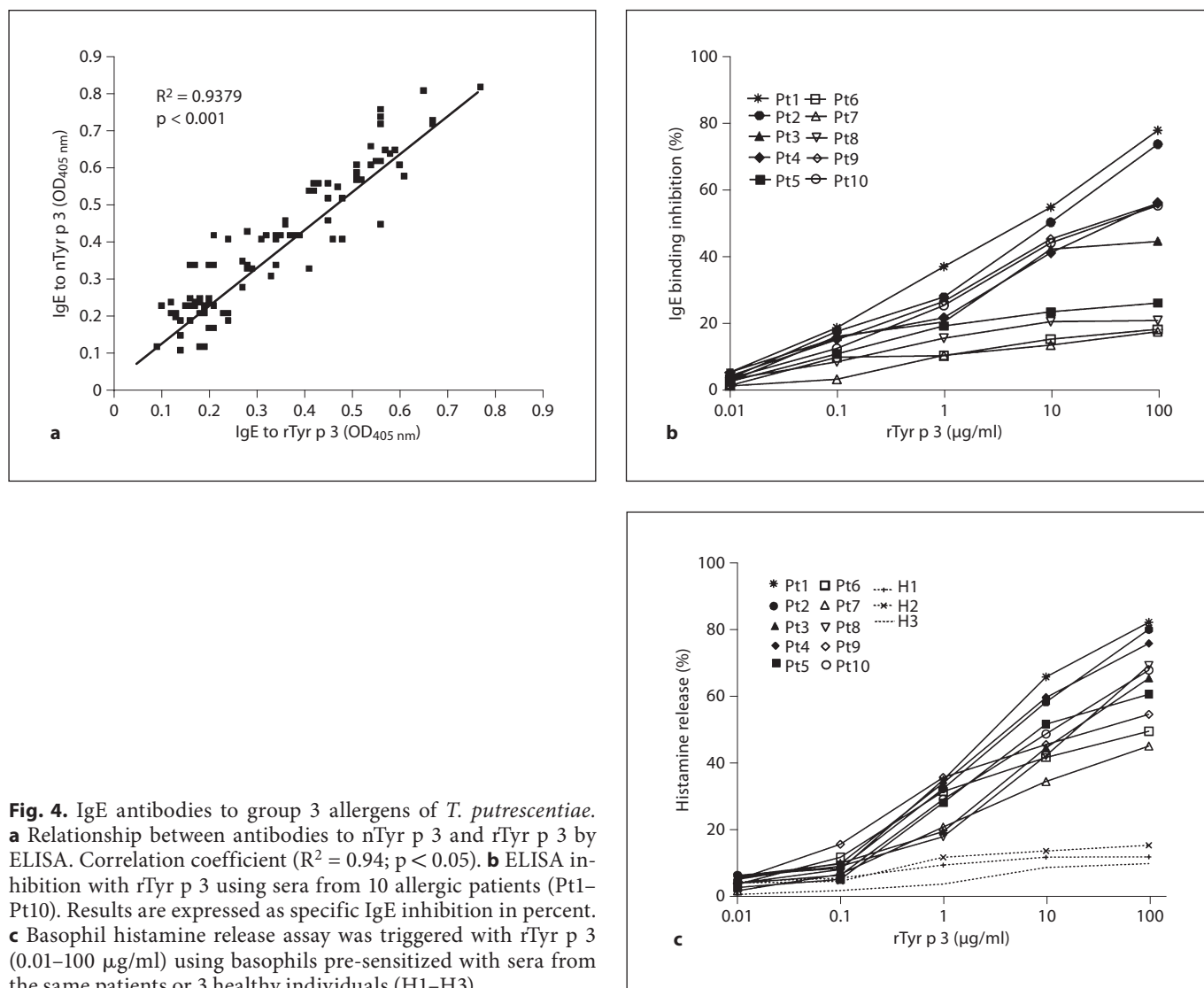


Fig. 3. Protein profiles of extracts of mite bodies (ME), SGM of *T. putrescentiae*, nTyr p 3 and rTyr p 3. **a** Proteins were analyzed on 12% SDS-PAGE. M = Molecular marker; 1 = ME (15 µg); 2 = SGM (15 µg); 3 = nTyr p 3 (10 µg); 4 = rTyr p 3 (10 µg). **b** Western blot analysis with rabbit anti-rTyr p 3 polyclonal antibodies.



with *Dermatophagoides*, although being slightly higher than that with *Blomia*.

Role of Tyr p 3 in Triggering Histamine Release in IgE-Mediated Hypersensitivity

In the presence of rTyr p 3, histamine release from basophils presensitized with serum from *T. putrescentiae*-sensitized subjects showed a dose-dependent change in general, but no significant difference compared with normal serum (<20%). Histamine release resulting from basophil degranulation was higher in allergic patients, ranging from 40 to 80%, especially in the serum from Pt-1 and Pt-2, amounting to 80%. In 8 of 10 *T. putrescentiae*-allergic patients, histamine release

was >50% after triggering with rTyr p 3 (100 $\mu\text{g/ml}$; fig. 4c).

Enzymatic Activity of nTyr p 3 and rTyr p 3

Enzymatic activity assays showed that both nTyr p 3 and rTyr p 3 could digest TAME but not rPreTyr p 3, and none of them could digest BTTEE. However, nTyr p 3 exhibited the twofold enzymatic activity of rTyr p 3 (fig. 5). The enzymatic activity of rTyr p 3 could be significantly inhibited by both serine protease (phenylmethylsulfonyl, 80%) and trypsin inhibitors (soybean trypsin inhibitor, 88%, and tosyllysine chloromethyl ketone, 82%), but not at all by cysteine protease (dithiothreitol). Similar inhibition was observed in nTyr p 3, too (data not shown).

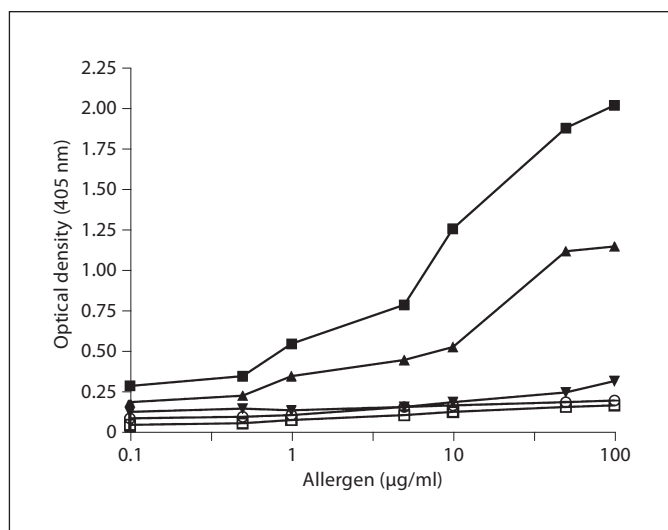


Fig. 5. Enzymatic activity assay for nTyr p 3, recombinant PreTyr p 3 (rPreTyr p 3) and Tyr p 3. The specific substrates TAME and BTEE were used for trypsin and chymotrypsin, respectively. TAME + nTyr p 3 (■), TAME + rTyr p 3 (▲), TAME + rPreTyr p 3 (▼), BTEE + nTyr p 3 (○) and BTEE + rTyr p 3 (□) were tested.

Discussion

Storage mites have been shown to cause allergic symptoms, which present as bronchial asthma, allergic rhinitis and conjunctivitis, similar to house dust mites. Among storage mites, the Glycyphagidae (*Blomia* spp.) and Acaridae (*Tyrophagus* spp.) families predominate over the others. In clinical medicine, the importance of house dust and storage mites is growing. It has been reported that the prevalence of *T. putrescentiae* sensitization is in the range of 22–43% for Korean and European allergic patients [7, 8, 33]. Similarly, around 30% (720/2,400) of allergic patient sera showed a positive reaction to *T. putrescentiae* in our allergy clinic (data not shown).

Trypsin is one of the most important endopeptidases and is found in the intestine of *Tyrophagus*, *Blomia* and *Dermatophagoides* [21, 22, 32]. In these mites, both extracellular and intracellular digestion occurs in the midgut as food is processed, and then the food and cells containing enzymes are passed into the posterior midgut where they are encased in a peritrophic membrane, subsequently compacted in the hindgut and excreted as fecal pellets [34]. Thus, our studies showed that SGM exhibited a higher concentration of trypsin than that of mite bodies, as reported for other mites [17, 29, 32]. During purification of nTyr p 3, nTyr p 3 activity in SGM was retained for weeks, as observed for other group 3 mite allergens,

possibly explaining why group 3 allergens accumulate in the environment and cause allergic reactions. These studies indicate that these enzymes are not only involved in the digestion of mites, but also act as potent inhaled allergens for hypersensitive patients [35].

In comparison with other group 3 allergen sequences, protein alignment analysis showed that rTyr p 3 had 51, 47, 47 and 45% homology with the group 3 mite allergen proteins of Blo t 3, Der p 3, Der f 3 and Eur m 3, respectively. Aligning the deduced amino acid sequence with those of other members of the group 3 allergens, rTyr p 3 appeared to display some conserved sequences reported in other species of mites except for the amino acids located at the ends of the N- and C-termini. In Der f 3 studies, two segments of the N-terminal cDNA have been found: a pre-sequence for secretion and a pro-sequence for folding [29]. ProDer f 3 appeared to be activated by another protease which converted ProDer f 3 to the active form. The sequence of the cDNA of Tyr p 3 supports this hypothesis by two facts. Firstly, cDNA of PreTyr p 3 had pre- and pro-sequences and the recombinant protein, rPreTyr p 3, did not show enzymatic activities to TAME. Secondly, the protein derived from the cDNA of rTyr p 3 (mature rTyr p 3) could digest the specific substrate TAME, but had lower activity than that of nTyr p 3. These results suggest that Tyr p 3 is produced in an inactive form in mites similar to that of ProDer f 3, and the pro-sequence may play a role in correct folding during activation. Maintaining Tyr p 3 in an inactive form can also protect the mite cell from this enzymatic attack under non-feeding situations.

A good correlation was found between mite extracts of *T. putrescentiae* and rTyr p 3, suggesting that group 3 allergens may play an important role in determining the allergenicity of *T. putrescentiae*. Since *Dermatophagoides*, *Blomia* and *Tyrophagus* cohabit in domestic environments, it is difficult to completely exclude the possibility of cross-reactivity from patients who are monosensitized to *T. putrescentiae*. Our observation demonstrated a limited level (10–20%) of cross-activity of group 3 allergens between *Tyrophagus*, *Dermatophagoides* and *Blomia*. In addition, the equivalent IgE binding activity between nTyr p 3 and rTyr p 3 has also been proven, indicating that the storage mite *T. putrescentiae* can act as a primary sensitizing mite independently of *D. pteronyssinus* in allergic patients naturally exposed to them.

Our studies showed that IgE activities to mite extracts of *T. putrescentiae* could not be absorbed by rTyr p 3 in 4 *T. putrescentiae*-sensitized patients (Pt-5, Pt-6, Pt-7 and Pt-8). We suspected that these patients might be sensi-

tized to other components, e.g. the group 2 allergen of *T. putrescentiae*. This was proven by the fact that 60–80% inhibition could be obtained by absorption with the recombinant Tyr p 2 (data not shown). Our results suggest that not only Tyr p 3 but also Tyr p 2 can act as major allergen in *T. putrescentiae*-sensitized patients, as reported for *Dermatophagoides* mites [15]. Since most sera can contribute to the histamine release of basophils after stimulation with rTyr p 3, these results suggest that a trypsin-like serine protease of group 3 allergen in *T. putrescentiae* may play a significant role (at least in part) in triggering IgE-mediated hypersensitive reactions.

In conclusion, rTyr p 3 displays considerable biological activities and allergenicity similar to that of nTyr p 3. A positive reaction in the histamine release assay suggests that rTyr p 3 plays a significant role in the pathogenesis

of allergic diseases. Our studies indicate that enzymatic activity of rTyr p 3 was obtained, but lower than that of nTyr p 3. The high expression level of rTyr p 3 in *E. coli* enables IgE epitope mapping in future studies. The limited level of IgE cross-reactivity between rTyr p 3 and other group 3 allergens suggests the applicability of rTyr p 3 as a suitable agent for further diagnostic and therapeutic applications.

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References

- Arlian LG, Geis DP, Vyszynski-Moher DL, Bernstein IL, Gallagher JS: Antigenic and allergenic properties of the storage mite *Tyrophagus putrescentiae*. *J Allergy Clin Immunol* 1984;74:166–171.
- Aspaly G, Stejskal V, Pekar S, Hubert J: Temperature-dependent population growth of three species of stored product mites (Acari: Acaridida). *Exp Appl Acarol* 2007;42:37–46.
- Jeong KY, Lee IY, Lee J, Ree HI, Hong CS, Yong TS: Effectiveness of education for control of house dust mites and cockroaches in Seoul, Korea. *Korean J Parasitol* 2006;44:73–79.
- Arlian LG, Vyszynski-Moher DL, Johansson SG, van Hage-Hamsten M: Allergenic characterization of *Tyrophagus putrescentiae* using sera from occupationally exposed farmers. *Ann Allergy Asthma Immunol* 1997;79:525–529.
- Prieto Ursua L, Sienra Monge JJ, del Rio Navarro BE, Mercado Ortiz VM: Skin tests for different species of acari in asthmatic children from Mexico City (in Spanish). *Rev Alerg Mex* 1995;42:49–53.
- Kondreddi PK, Elder BL, Morgan MS, Vyszynski-Moher DL, Arlian LG: Importance of sensitization to *Tyrophagus putrescentiae* in the United States. *Ann Allergy Asthma Immunol* 2006;96:124.
- Szilman E, Szilman P, Solarz K, Brewczynski P, Sieron AL: Sensitization to the storage mite *Tyrophagus putrescentiae* in urban population of Upper Silesia (Poland). *Wiad Parazytol* 2004;50:471–476.
- Park JW, Ko SH, Yong TS, Ree HI, Jeoung BJ, Hong CS: Cross-reactivity of *Tyrophagus putrescentiae* with *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* in urban areas. *Ann Allergy Asthma Immunol* 1999;83:533–539.
- Musken H, Fernandez-Caldas E, Maranon F, Franz JT, Masuch G, Bergmann KC: In vivo and in vitro sensitization to domestic mites in German urban and rural allergic patients. *J Investig Allergol Clin Immunol* 2002;12:177–181.
- Matsumoto T, Hisano T, Hamaguchi M, Miike T: Systemic anaphylaxis after eating storage-mite-contaminated food. *Int Arch Allergy Immunol* 1996;109:197–200.
- Eriksson TL, Johansson E, Whitley P, Schmidt M, Elsayed S, van Hage-Hamsten M: Cloning and characterisation of a group II allergen from the dust mite *Tyrophagus putrescentiae*. *Eur J Biochem* 1998;251:443–447.
- Jeong KY, Lee H, Lee JS, Lee J, Lee IY, Ree HI, Hong CS, Yong TS: Molecular cloning and the allergenic characterization of tropomyosin from *Tyrophagus putrescentiae*. *Protein Pept Lett* 2007;14:431–436.
- Jeong KY, Kim WK, Lee JS, Lee J, Lee IY, Kim KE, Park JW, Hong CS, Ree HI, Yong TS: Immunoglobulin E reactivity of recombinant allergen Tyr p 13 from *Tyrophagus putrescentiae* homologous to fatty acid binding protein. *Clin Diagn Lab Immunol* 2005;12:581–585.
- Jeong KY, Lee H, Lee JS, Lee J, Lee IY, Ree HI, Hong CS, Park JW, Yong TS: Immunoglobulin E binding reactivity of a recombinant allergen homologous to alpha-Tubulin from *Tyrophagus putrescentiae*. *Clin Diagn Lab Immunol* 2005;12:1451–1454.
- Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM: Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002;129:1–18.
- 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.
- Cheong N, Yang L, Lee BW, Chua KY: Cloning of a group 3 allergen from *Blomia tropicalis* mites. *Allergy* 2003;58:352–356.
- Heymann PW, Chapman MD, Aalberse RC, Fox JW, Platts-Mills TA: Antigenic and structural analysis of group II allergens (Der f II and Der p II) from house dust mites (*Dermatophagoides* spp.). *J Allergy Clin Immunol* 1989;83:1055–1067.
- Stewart GA, Lake FR, Thompson PJ: Faecally derived hydrolytic enzymes from *Dermatophagoides pteronyssinus*: physicochemical characterisation of potential allergens. *Int Arch Allergy Appl Immunol* 1991;95:248–256.
- Stewart GA, Bird CH, Krska KD, Colloff MJ, Thompson PJ: A comparative study of allergenic and potentially allergenic enzymes from *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei*. *Exp Appl Acarol* 1992;16:165–180.
- Ortego F, Sanchez-Ramos I, Ruiz M, Castanera P: Characterization of proteases from a stored product mite, *Tyrophagus putrescentiae*. *Arch Insect Biochem Physiol* 2000;43:116–124.
- Montealegre F, Quinones C, Torres N, Goth K: Detection of serine proteases in extracts of the domestic mite *Blomia tropicalis*. *Exp Appl Acarol* 2002;26:87–100.

- 23 Maruo K, Akaike T, Ono T, Okamoto T, Maeda H: Generation of anaphylatoxins through proteolytic processing of C3 and C5 by house dust mite protease. *J Allergy Clin Immunol* 1997;100:253–260.
- 24 King C, Brennan S, Thompson PJ, Stewart GA: Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998;161:3645–3651.
- 25 Miike S, McWilliam AS, Kita H: Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. *J Immunol* 2001;167:6615–6622.
- 26 Hewitt CR, Foster S, Phillips C, Horton H, Jones RM, Brown AP, Hart BJ, Pritchard DI: Mite allergens: significance of enzymatic activity. *Allergy* 1998;53:60–63.
- 27 Sun G, Stacey MA, Schmidt M, Mori L, Matoli S: Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* 2001;167:1014–1021.
- 28 Smith WA, Chua KY, Kuo MC, Rogers BL, Thomas WR: Cloning and sequencing of the *Dermatophagoides pteronyssinus* group III allergen, Der p III. *Clin Exp Allergy* 1994;24:220–228.
- 29 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;377:62–66.
- 30 Yang L, Resnick MI, Marengo SR: A simple procedure for isolating microgram quantities of biologically active bikunin from human urine. *BJU Int* 2005;96:647–653.
- 31 Yoshimura T, Kaneuchi T, Miura T, Kimura M: Kinetic analysis of the fluorescence reaction of histamine with orthophthalaldehyde. *Anal Biochem* 1987;164:132–137.
- 32 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;75:29–35.
- 33 Kanceljak-Macan B, Macan J, Buneta L, Milkovic-Kraus S: Sensitization to non-pyroglyphid mites in urban population of Croatia. *Croat Med J* 2000;41:54–57.
- 34 Brody AR, Wharton GW: *Dermatophagoides farinae*: ultrastructure of lateral opisthosomal dermal glands. *Trans Am Microsc Soc* 1970;89:499–513.
- 35 Ando T, Homma R, Ino Y, Ito G, Miyahara A, Yanagihara T, Kimura H, Ikeda S, Yamakawa H, Iwaki M: 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;23:777–784.