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## Immunoglobulin E Reactivity of Recombinant Allergen Tyr p 13 from *Tyrophagus putrescentiae* Homologous to Fatty Acid Binding Protein

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The storage mite, *Tyrophagus putrescentiae*, is one of the important causes of allergic disorders. Fifteen allergenic components were demonstrated in storage mite by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, but only the group 2 allergen Tyr p 2 has been cloned and characterized. In this study, we attempted to identify and characterize new allergens from *T. putrescentiae*, which is a dominant species of storage mite in Korea. Expressed sequence tags were analyzed to identify possible storage mite allergens, and the cDNA sequence encoding a protein homologous to fatty acid binding protein, a mite group 13 allergen, was identified and named Tyr p 13. Its deduced amino acid sequence showed 61.1 to 85.3% identity with other mite group 13 allergens. The recombinant protein was expressed in *Escherichia coli* using a pET 28b vector system, and its allergenicity was investigated by enzyme-linked immunosorbent assay (ELISA). The recombinant allergen was detected in 5 of 78 (6.4%) *T. putrescentiae*-positive sera tested, and it inhibited 61.9% of immunoglobulin E binding to crude extract at an inhibitor concentration of 10 µg/ml by inhibition ELISA using serum from the patient who showed the strongest reaction by ELISA. In this study, a novel allergen was identified in *T. putrescentiae*. This allergen could be helpful for more-detailed characterizations of storage mite allergy.

The storage mite, *Tyrophagus putrescentiae*, is found in stored products such as dried eggs, ham, herring meal, cheese, and different kinds of nuts (17). Storage mites are the cause of occupational allergic diseases of bakers (21) and food industrial workers (2, 25) and among residents in agricultural environments (4). Koreans store uncooked rice in rice bins or chests in their homes, and thus the storage mite, *T. putrescentiae*, is often found on kitchen floors in urban areas (10). By crossed immunoelectrophoresis using the sera from occupationally exposed farmers, 14 different allergenic components were identified from the whole-body extract of *T. putrescentiae* (1). However, less is known about the molecular nature of the allergens derived from *T. putrescentiae*. Only the group 2 allergen Tyr p 2 has been cloned and characterized from *T. putrescentiae* (6), although 20 groups of mite allergens have been cloned to date (26).

cDNA library screening and phage display technology have recently been utilized to identify and clone new allergens (7). However, both of these strategies have obvious limitations, i.e., due to posttranslational modifications and improper protein folding. In a previous study, 15 allergenic components were

demonstrated in *T. putrescentiae* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, and a 16-kDa allergen was found to be most prevalent (52%) (18). In addition, considerable cross-reactivity between *T. putrescentiae* and *Dermatophagoides* species was demonstrated by inhibition enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis (16).

A cDNA sequence encoding a protein homologous to fatty acid binding protein, mite group 13 allergen, was identified by analyzing the expressed sequence tag data of *T. putrescentiae*. Mite group 13 allergens have been cloned and characterized from *Blomia tropicalis* (Blo t 13) (5), *Acarus siro* (Aca s 13) (8), and *Lepidoglyphus destructor* (Lep d 13) (7). The cross-reactivity of the Blo t 13 with other dust mites was also demonstrated using monoclonal antibodies (14).

In this study, we undertook to produce a recombinant fatty acid binding protein and then characterize the allergenic properties of this protein from *T. putrescentiae*.

### MATERIALS AND METHODS

**Preparation of crude extract.** The allergen extract was prepared by using phosphate-buffered saline (PBS) and protease inhibitors. Thirty grams of frozen mites which had been reared as described previously (20) was homogenized in liquid nitrogen. The sample was defatted in 200 ml of a 1:1 volume of ethyl ether and ethyl acetate, and 1 mg of 1-phenyl-3-(2-thiazolyl)-2-thiourea (Sigma, St. Louis, Mo.) was added to prevent melanization. The proteins were extracted with slow overhead stirring at 4°C overnight in phosphate-buffered saline (PBS), pH 7.4, containing 6 mM 2-mercaptoethanol and a 1/1,000 volume of protease inhibitor set III (Calbiochem, San Diego, CA). The extract was then centrifuged

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TABLE 1. Expressed sequence tags matched with allergenic clones

Allergens	Biochemical identity	Clones ( <i>n</i> = 22)	EST no.
Tyr p 13	Fatty acid binding protein	8	21, <sup>a</sup> 41, 58, <sup>a</sup> 366, <sup>a</sup> 433, 445, <sup>a</sup> 472, <sup>a</sup> 479, <sup>a</sup>
Tyr p 1	Cysteine protease	4	28, 115, <sup>a</sup> 161, 242
Tyr p 3	Serine protease	2	186, <sup>a</sup> 485
Tyr p 8	Glutathione-S-transferase	2	188, 513
Tyr p 10	Tropomyosin	1	174
Tyr p 20	Arginine kinase	1	360
	α-Tubulin	1	259, <sup>a</sup>
	Troponin C	3	22, 89, 606

<sup>a</sup> Full-length sequence.

at 10,000 × *g* for 30 min at 4°C, and the supernatant was filtered through a 0.22-μm syringe filter (Millipore, Bedford, MA). The protein content was determined by the Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. The extract was used for the ELISA and skin prick tests. For the skin prick test, it was dialyzed in modified Coca solution (0.9% NaCl, 0.25% NaHCO<sub>3</sub>, 0.04% phenol), and diluted extract at a final concentration of 0.2 mg/ml was used for the test. The tip of a 20-gauge needle was introduced through a drop of the extract into the superficial layers of the skin, which were gently lifted. Wheals larger than 3 mm after 15 min were considered indicative of a positive reaction. All tests included 1 mg/ml histamine dihydrochloride (Bayer) as a positive control and albumin-saline with phenol (Bayer) as a negative control.

**Subjects and serum samples.** A total of 456 patients participated in this study. Of these patients, 432 were 3 to 15 years old (male:female ratio = 223:209; average age, 8 years), and all were seen at the Pediatric Clinic at Inje University Seoul Paik Hospital, Seoul, Korea. Twenty-four patients (male:female ratio = 16:8, average age, 29 years) who attended the Allergy Clinic at Severance Hospital, Yonsei University, Seoul, Korea, were also included. All the subjects were examined by skin prick testing using extracts of *Dermatophagoides pteronyssinus*, *D. farinae*, *Blattella germanica*, *Periplaneta americana*, cat dander, dog hair, *Alternaria tenuis*, *Penicillium* mix, alder, oak, and ragweed (Bencard Co., Gaggenau, Germany). The extract of *T. putrescentiae* prepared as described in the above section was also included. All sera obtained from test participants underwent ELISA testing, and the serum samples obtained from subjects who showed a negative reaction both in the skin prick test and ELISA were used for the negative control experiments.

**cDNA cloning of fatty acid binding protein.** A cDNA sequence encoding a protein homologous to fatty acid binding protein was obtained by the analyzing *T. putrescentiae* expressed sequence tags. A cDNA library of *T. putrescentiae* was constructed using a ZAP Express cDNA Gigapack II gold cloning kit (Stratagene, La Jolla, CA).

**Expression and purification of recombinant allergen.** The open reading frame of fatty acid binding protein was amplified by PCR using a primer set (Tp13F [ATGGTTCAACTGAACGGCTC] and Tp13R [CTGTTTACTGGCGTTGTAG]), subcloned into pGEM T Easy vector (Promega, Madison, WI), and subsequently transferred into the EcoRI site of pET 28 b vector (Novagen, Madison, WI). The orientation of the insert was confirmed by PCR annealing T7 primer to the vector and annealing Tp13R primer to the insert. The expressed protein contained the additional 36 amino acids derived from pET 28b vector and 3 amino acids from pGEM T Easy vector (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPNSLVI) at its N terminus. Recombinant protein was expressed in *Escherichia coli* BL21(DE3) and purified by Ni-nitrilotriacetic acid agarose (QIAGEN, Valencia, CA) affinity column chromatography under denaturing conditions (9).

**IgE reactivity of the recombinant allergen.** The reactivities of specific immunoglobulin E (IgE) antibodies to the recombinant allergen were examined by ELISA. ELISA plates were coated with 100 μl (0.1 M sodium carbonate, pH 9.6) of 2 μg/ml of recombinant Tyr p 13, and plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with 3% skim milk in PBST for 1 h. Subsequently, the plates were incubated with 50 μl/well of 1:4 (in PBST containing 1% bovine serum albumin)-diluted sera for 1 h. IgE antibodies were detected using biotinylated goat anti-human IgE (epsilon chain specific; Vector, Burlingame, CA) and streptavidin-peroxidase (Sigma). The signal was developed using 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The mean absorbance plus 2 standard deviations of the sera of 18 healthy controls was used as a cutoff value.

For the inhibition test, 100 μl of 20 μg/ml of extract was coated overnight at

4°C. The 1:4-diluted serum was preincubated with various quantities of crude extract or recombinant protein for 2 h at room temperature and overnight at 4°C. IgE antibodies were detected as described above.

## RESULTS

**Skin prick test.** A total of 432 pediatric subjects (male:female ratio = 223:209, average age, 8 ± 3 years, mean ± 1 standard deviation) was enrolled in this study; 358 children suffered from asthma, 325 from allergic rhinitis, 17 from atopic dermatitis, and six children from urticaria. By skin prick testing, 69.5% had positive responses to *D. pteronyssinus*, 57.8% to *D. farinae*, 34.2% to *B. germanica*, 22.3% to *P. americana*, 16.3% to cat dander, 15.7% to *Alternaria*, 12.8% to dog hair, 12.7% to *T. putrescentiae*, 6.0% to *Penicillium*, 5.3% to oak, 4.4% to ragweed, and 4.2% to alder.

Among these, 55 children (male:female ratio = 26:29) showed positive reactions to *T. putrescentiae*. The mean total IgE level of these 55 children was 708.03 ± 522.79 (ranging from 11.2 to 2,960) IU/ml, and the mean eosinophil count was 493.07 ± 264.56/mm<sup>3</sup> (ranging from 82 to 1,430). Twenty-four adult patients (9 patients suffering from allergic asthma, 6 from urticaria, 3 from allergic rhinitis, 3 both from allergic asthma and allergic rhinitis, 2 from severe coughing, and 1 from atopic dermatitis) with a mean total IgE of 501.33 ± 645.82 (ranging from 13.9 to 4,530) IU/ml and a mean eosinophil count of 449.01 ± 283.13/mm<sup>3</sup> (ranging from 130 to 1,120) also showed a positive reaction to *T. putrescentiae*.

**Analysis of expressed sequence tag database.** A total of 382 readable amino acid encoding sequences were produced from 446 clones in the of *T. putrescentiae* cDNA library. A BLASTX search of 22 clones showed high homology to the previously known allergens. Seven different groups of mite allergens were identified, and three clones showed significant homology to cockroach group 6 allergen (Table 1).

**Homology to fatty acid binding proteins.** Eight clones out of 446 analyzed were found to be homologous to fatty acid binding protein, and 6 clones contained the full-length open reading frame. The complete sequence was found to be 643 nucleotides in length (Fig. 1) and included a 5' noncoding region of 59 bp, a 3' untranslated region of 184 bp, and an open reading frame of 396 bp. This open reading frame codes for a 132 amino acid protein with a calculated molecular mass of 14.57 kDa and an isoelectrical point of 7.22. A cytosolic fatty acid binding protein signature at amino acid residues 5 to 22 was detected using the PROSITE program (<http://www.isb-sib.ch/announce/>). The protein also contains a potential N-glycosyl-

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-59  GGCCAAATC GATCTCGACT TTTTCGGTCT TTCACCACCC CCTCAAACCTA AACCCCTCACC
  1  ATGGTTCAAC TGAACGGCTC CTACAAGCTC GAGAAGAGCG ACAACTTTGA TGCCTTCCTC
    M V Q L N G S Y K L E K S D N F D A F L 20
 61  AAGGAACTCG GCGTCAACTT TGTGACCCGA AACCTCGCCA AGTCGGCCAG CCCACCCGTG
    K E L G V N F V T R N L A K S A S P T V 40
 121 GAGGTGATCG TCGACGGCGA CAGCTACACC ATCAAGACCA GCTCCACGCT GAAGAACAGC
    E V I V D G D S Y T I K T S S T L K N S 60
 181 GAGATCAAGT TCAAGCTGGG CGAGGAGTTC GAGGAGGACC GCGCCGACGG CAAGAAGGTG
    E I K F K L G E E F E E D R A D G K K V 80
 241 CAGACCTCGG TCACCAAGGA GGGCGACAAC AAGCTGGTGC AGGTGCAGAA GGGCGACAAG
    Q T S V T K E G D N K L V Q V Q K G D K 100
 301 CCGGTGACCA TTGTGCGGGA GTTCAGCGAG GAGGGCTCA CCGTCACCGC CACCGTCAAC
    P V T I V R E F S E E G L T V T A T V N 120
 361 GGCGTACCT CCGTGCGATT CTACAAGCGC CAGTAAACAG AATGAACAGA ACAAAAAATT
    G V T S V R F Y K R Q *** 131
 421 CTCCTACAT TCCGGTAGAT TACTGTTC A TGTTTACCA TAAAAACAAC CCCACACCA
 481 CCAAGATCTT CTGAAAATCC AATTTTTCAT CTATCTATCA ATCAGTTATT TTTGATTGGC
 541 TAATAAATAA TTTATTGCAC TTCAAAAAAA AAAAAAATAA AAAAA
    
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FIG. 1. The nucleotide and deduced amino acid sequences of *T. putrescentiae* fatty acid binding protein, Tyr p 13 (GenBank accession number AY710432). Its estimated molecular mass and isoelectrical point are 14.57 kDa and 7.22.

ation site at position 5 to 7, and a putative polyadenylation signal was found between nucleotide residues 542 and 548.

The amino acid sequence of the putative fatty acid binding protein from *T. putrescentiae* shows 85.3% identity with Aca s 13 from *A. siro*, 62.3% identity with Blo t 13 from *B. tropicalis*, and 61.1% identity with Lep d 13 from *L. destructor* (Fig. 2). Due to its high homology with mite group 13 allergens and in accord with the allergen nomenclature (13), it was named Tyr p 13.

**Expression and IgE binding of recombinant Tyr p 13.** Recombinant Tyr p 13 with a short N-terminal fusion protein which includes 6-histidine was expressed in *E. coli* and metal affinity purified (Fig. 3). The yield of recombinant Tyr p 13 was 5.432 mg/liter bacterial culture as determined by Bradford assay. The recombinant allergen was detected by IgE antibodies in 5 of the 78 (6.41%) *T. putrescentiae*-positive sera tested (Fig. 4).

**Inhibition of IgE reactivity by the recombinant protein.** This inhibition study was performed using the serum from the pa-

tient who showed the strongest IgE reactivity to the recombinant allergen. The crude extract inhibited a maximum IgE reactivity by 95.7%, whereas the recombinant allergen inhibited it by 61.9% at an inhibition concentration of 10 µg/ml (Fig. 5).

DISCUSSION

Fatty acid binding proteins has been described as a putative vaccine candidate for some parasitosis-inducing species such as *Fasciola hepatica* (22), *Schistosoma japonica* (3), and *Schistosoma mansoni* (24). The first fatty acid binding protein reported as an allergen was the ABA-1 of *Ascaris suum* (23). It was also found that a small number of low-molecular-weight proteins which share the similarity with fatty acid binding proteins are highly up-regulated in psoriatic skin (15). Even though they bear no significant homology with ABA-1, several proteins homologous to fatty acid binding proteins from dust mites have been known to be allergenic (group 13). However,

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Tyr p 13  MVQLNGSYKLEKSDNFDAFLKELGVNFVTRNLAKSASPTVEVIVDGSYTIKTSSTLKNS 60
Aca s 13  --.I.....L.....T....S.N.....A....T 58
Lep d 13  .ANIA.Q...D..E...Q..DK...G.LVKTA..TVK..L..A...T.IFRSL..F..T 60
Blo t 13  .P-IE.K.....K..D...G.MVKTA..TLK..L..D.Q..T.VFRSL..F..T 59

Tyr p 13  EIKFKLGEFEEDRADGKVKVTSVTKEGDNKLVQVQKGDKPVTIVREFSEGLTVTATVN 120
Aca s 13  ..S.....A....T.K.V.N..S.T.F...Q...E.....D..... 118
Lep d 13  .....R.K.VIV.D...F..T.Y...E.KV...KGDEVE...S.D 120
Blo t 13  .....R.K.V.N.....FI.T.Y...E.K...D.QGDDVV...S.G 119

Tyr p 13  GVTSVRFYKRQ 131 <100.0%>
Aca s 13  ..... 129 <85.3%>
Lep d 13  .....P...A 131 <61.1%>
Blo t 13  D....T...I 130 <62.3%>
    
```

FIG. 2. Amino acid sequence alignment of four arthropod fatty acid binding proteins: Aca s 13 (*Acarus siro*, accession number AJ006774), Lep d 13 (*Lepidoglyphus destructor*, Q9U5P1), Blo t 13 (*Blomia tropicalis*, Q17284). Dots indicate amino acid identity with *T. putrescentiae*, and the percentage of each sequence identity is shown in parentheses.

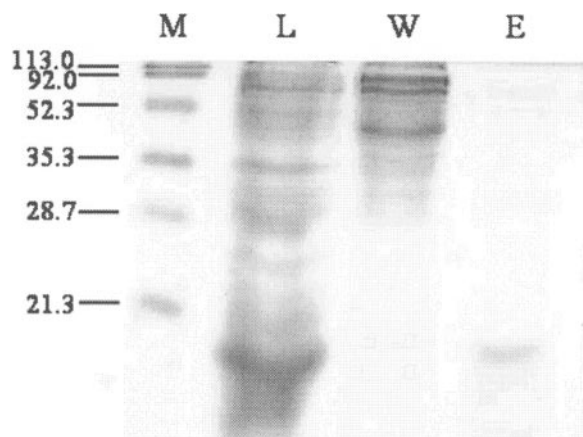


FIG. 3. Purification of recombinant Tyr p 13. Proteins were separated under reducing conditions on 15% acrylamide gels and stained with Coomassie brilliant blue. Lanes: M, molecular mass markers in kDa; L, total cell lysate of *E. coli* BL21 cells bearing rTyr p 13 after IPTG induction; W, fraction washed from the column; E, fraction eluted.

the frequencies of IgE binding to these allergens are low, suggesting that they are minor allergens, e.g., 11% (5 of 45) to Blo t 13 from *Blomia tropicalis* (5), 23% (3 of 13) to Aca s 13 from *Acarus siro* (8), and 13% (6 of 45) to Lep d 13 from *Lepidoglyphus destructor* (7). However, recombinant Blo t 13 inhibited 62% of IgE binding to crude extract according to the radioallergosorbent inhibition test using one patient's serum, which suggests that it may be clinically significant for some patients. In the present study, a 6.41% (5 of 78) IgE binding frequency to recombinant Tyr p 13 was observed and recombinant Tyr p 13 at 10  $\mu\text{g/ml}$  inhibited 61.9% of IgE binding to the crude extract by inhibition ELISA using the serum from the patient who showed the strongest reaction to recombinant Tyr p 13. However, more biochemical analysis is necessary because N-glycosylation of the allergen may have affected its allergenicity. Furthermore, patients from rural area or bakers who are more likely to be sensitized to storage mite allergens may show different allergic responses to this molecule.

Until now, the diagnosis of allergic disorders has mainly

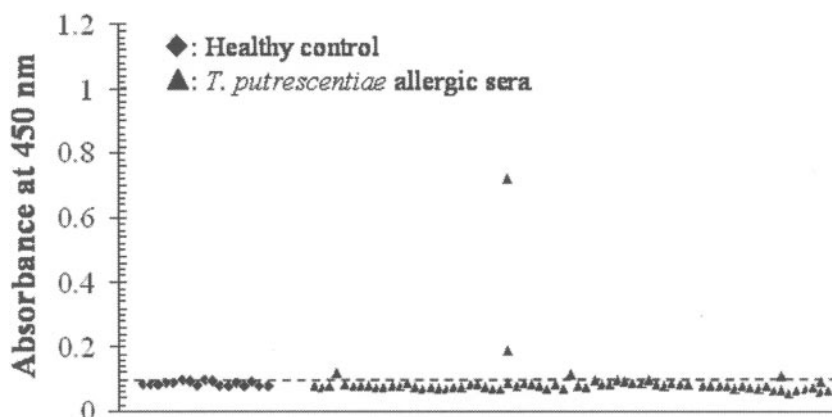


FIG. 4. IgE reactivity of human sera against recombinant Tyr p 13. The dotted line indicates the cutoff value.  $\blacklozenge$ , healthy control sera;  $\blacktriangle$ , *T. putrescentiae*-sensitized sera.

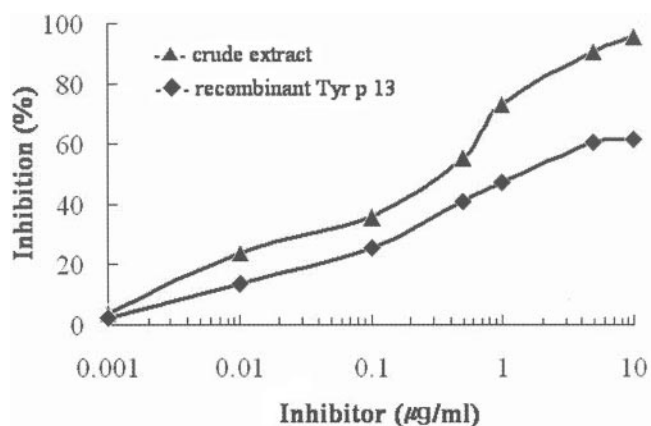


FIG. 5. ELISA inhibition analysis. IgE binding to crude extract was inhibited with crude extract ( $\blacktriangle$ ) and recombinant Tyr p 13 ( $\blacklozenge$ ).

been based on crude extracts. Allergen extracts have several limitations such as batch-to-batch variations and difficulty in standardization. Moreover, false-positive reactions may occur because of the nonallergenic compounds. In addition, skin prick testing using *T. putrescentiae* commercial allergen extract is not believed to be reliable for the detection of measurable levels of IgE antibodies (11). Thus, the cloning and genetic engineering of the allergens offers many advantages, and they are expected to replace crude extracts for diagnosis and treatment in the near future (12).

In a study using a panel of seven purified natural or recombinant allergens, mite-allergic patients who were polysensitized to various minor allergens, which included highly cross-reactive allergens and storage mite allergens, could be discriminated from those who were mainly sensitized to major allergens (e.g., Der p 1 and Der p 2) (19). This is important, as atopic individuals polysensitized to many minor allergens are less likely to benefit from immunotherapy based on crude allergen extracts. Therefore it is necessary to characterize minor allergens in more detail, especially with respect to allergenicity and cross-reactivity. Detailed characterization of the minor allergens may lead us to the more-beneficial immunotherapeutic approaches.

More clones are being analyzed in an effort to characterize *T. putrescentiae* allergens in detail, and possible allergens identified by expressed sequence tag analysis are now being investigated. Moreover, the clinical relevance of Tyr p 13 remains to be evaluated in vivo. It is hoped that the use of recombinant Tyr p 13 in combination with other allergens could improve the diagnostic specificity.

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## AUTHOR'S CORRECTION

### Immunoglobulin E Reactivity of Recombinant Allergen Tyr p 13 from *Tyrophagus putrescentiae* Homologous to Fatty Acid Binding Protein

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