# Characterization of allergenic properties of German cockroach tropomyosin using recombinant proteins

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# Characterization of allergenic properties of German cockroach tropomyosin using recombinant proteins

Directed by Professor Chein-Soo Hong

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Kyoung Yong Jeong

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ABSTRACT

# Characterization of allergenic properties of German cockroach tropomyosin using recombinant proteins

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Cockroach infestation may sensitize and elicit allergic responses to genetically predisposed individuals. Invertebrate tropomyosins are a frequent cause of allergy and highly cross-reactive in nature. For the initial characterization of German cockroach tropomyosin (Bla g 7), cDNA cloning, expression of recombinant protein, and investigation of its allergenicity were performed. German cockroach tropomyosin was cloned by reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers designed on the basis of known tropomyosins. The cloned Bla g 7 shared up to 98.5% amino acid sequence identity with other allergenic tropomyosins. The cloned cDNA was over-expressed in *Escherichia coli* and purified by affinity chromatography using Ni-nitrilotriacetic acid (NTA) resin. The allergenicity of the recombinant tropomyosin was

examined by enzyme-linked immunosorbent assay (ELISA). ELISA showed a recombinant Bla g 7 sensitization rate of 16.2% to German cockroach allergic sera. Recombinant Bla g 7 was able to inhibit 32.4% of the specific IgE binding to cockroach extract. Tropomyosin represents a minor allergen in cockroach extracts.

The allergenicity of the recombinant tropomyosins derived from various arthropods are not coherent. To gain a deeper insight into the allergenicity, the IgE-binding reactivities of native and recombinant German cockroach tropomyosins (*E. coli*-expressed and *Pichia*-expressed) were compared. Native tropomyosin was purified by ammonium sulfate fractionation, hydroxyapatite column chromatography, and electroelution. Recombinant tropomyosin was expressed in *Pichia pastoris* in an attempt to produce the recombinant protein which is comparable to natural counterpart in allergenicity. The allergenicity of native and recombinant tropomyosins was compared by ELISA inhibition study. Native German cockroach tropomyosin showed 17.6% IgE binding reactivity from German cockroach sensitized sera. Recombinant tropomyosin was produced without fusion protein and the N-terminus was blocked as a native counterpart. Its IgE binding reactivity was comparable to that of native tropomyosin over the concentration range of 1 to 1000 ng/mL in the ELISA inhibition test. Besides the structural differences of native and recombinant proteins, other factors may also influence on the various IgE

reactivities of tropomyosins.

Diverse amino acid sequences have been described for individual allergens from various sources. The effects of such sequence diversity need to be considered with respect to IgE binding reactivity of an allergen. Two-dimensional (2-D) gel electrophoresis and immunoblot analysis using mouse anti-recombinant Bla g 7 serum were performed to investigate isoforms at the protein level. RT-PCR was applied to examine the sequence diversity. Eleven different variants of deduced amino acid sequences were identified. German cockroach tropomyosin has only minor sequence variations, which do not seem to affect the allergenicity significantly. These results support the molecular basis underlying the cross-reactivity of arthropods tropomyosins. Recombinant fragments were also generated by PCR and IgE-binding epitopes were assessed by ELISA. The sera of seven patients' revealed heterogeneous IgE-binding responses. This study demonstrates multiple IgE-binding epitope regions in a single molecule.

A two-site ELISA was developed using monoclonal antibodies (mAbs) raised against recombinant German cockroach tropomyosin, and was applied to assess the invertebrate tropomyosin level in house dust samples. The detection limit of the developed two-site ELISA was determined to be about 8 ng/mL for recombinant German cockroach tropomyosin and 1 $\mu$g/mL for German cockroach whole body extract. Tropomyosin was detected in three samples

(24.20 ± 32.11 ㎍/g) from nine bedding samples (33.3%) and in only one (6.80 ㎍/g) of 13 kitchen dust samples (7.7%). These data support the low rate of sensitization to cockroach tropomyosin in Korean respiratory allergy patients.

The results of the present study led us to conclude that the most important determinant of the sensitization rate and allergenicity of German cockroach tropomyosin is not the structural characteristics or amino acid sequence variations, but the environmental factors such as distribution in house dust.

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Key words : allergen, *Blattella germanica*, epitope, tropomyosin

PREFACE

# Characterization of allergenic properties of German cockroach tropomyosin using recombinant proteins

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The thesis has four chapters, each describing the different aspects of German cockroach tropomyosin.

First chapter describes the molecular cloning and production of recombinant protein in *Escherichia coli* and its allergenicity.

In the second chapter, native tropomyosin was purified and recombinant tropomyosin was expressed in *Pichia pastoris* as a non-fusion protein. Allergenicity of native, yeast-expressed recombinant protein, and bacteria-expressed recombinant protein was compared to investigate the influence of structural differences on IgE-binding reactivity.

In the third chapter, two dimensional gel electrophoresis and RT-PCR were applied to investigate amino acid sequence variations. Five peptide fragments were expressed to examine IgE-binding epitopes.

In the last chapter, a two-site ELISA was developed using

monoclonal antibodies raised against recombinant tropomyosin and tropomyosin levels in house dust were investigated.

# Chapter I

## Allergenicity of recombinant Bla g 7, German cockroach tropomyosin

# Ⅰ. INTRODUCTION

Cockroaches are known to produce several IgE binding proteins which are an important cause of asthma.[1] Exposure to low levels of cockroach allergens is known to be associated with wheezing among infants in the first three months of life.[2,3] The principal domiciliary cockroaches in Korea are *Blattella germanica* and *Periplaneta americana*,[4] and cross-reactivity between these and other animals, possibly caused by tropomyosin has been reported.[5,6] In particular, IgE antibodies to tropomyosin, which possibly induce food allergies during mite immunotherapy have been described.[7] Several lines of evidence have described cockroach antigens, but no direct experiment was performed in a study about German cockroach tropomyosin as a highly cross-reactive antigen. Only two cDNA clones of cockroach tropomyosin were identified from American cockroach to date.[8,9]

Tropomyosin, a kind of muscle protein, is a highly cross-reactive allergen, which sensitizes predisposed individuals via food or house dust. It has been suggested that tropomyosin is an invertebrate pan-allergen due to its high cross-reactivity.[10] More than 80% of patients allergic to house dust mites are reported to be sensitized to tropomyosin,[11] and that is highly cross-reactive among invertebrate species, which is probably due to their conserved structure.

Molecular cloning and the characterization of the tropomyosins

of domestic insects are inevitably required to investigate their allergenicity and how they sensitize individuals. This study was designed to clone German cockroach tropomyosin and to characterize its allergenic properties.

# Ⅱ. MATERIALS AND METHODS

## 1. Subjects and serum samples

Human allergic sera were obtained from patients attending the Allergy Clinic of the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. The diagnosis of allergy was followed the revised nomenclature[12] and based on case history and skin prick testing. Sera from patients were tested for the presence of IgE antibodies against *Dermatophagoides farinae* and *B. germanica* using the Uni-CAP system (Pharmacia, Uppsala, Sweden). Those with CAP results higher than 0.7 kU/L were used for the following study (n = 37, ages ranging from 9 to 75 years, average age 33 years).

## 2. Preparation of extract

Thirty gram of live or frozen cockroaches was pulverized in liquid nitrogen. The defatted sample in 200 mL of a 1:1 volume of ethyl ether:ethyl acetate was extracted with slow overhead stirring at 4℃ overnight in phosphate buffered saline (PBS), pH 7.4 containing 6 mM of 2-mercaptoethanol, 1/1000 volume of protease inhibitor set III (Calbiochem, San Diego, CA, USA) and 1 mg/mL of 1-phenyl-3-(2-thiazolyl)-2-thiourea (Sigma, St Louis, MO, USA) to prevent melanization.[13] The extract was then centrifuged

at 10000 g for 30 min at 4℃ and the supernatant was finally filtered through a 0.22 ㎛ filter (Millipore, Bedford, MA, USA).

### 3. Molecular cloning of tropomyosin

Total RNA was prepared from female German cockroaches with TRIzol reagent (GibcoBRL, Rockville, MD, USA) according to the manufacturer's instructions. First strand cDNA was synthesized from 5 ㎍ of total RNA, and PCR was performed using a pair of degenerate primers designed on the basis of known allergenic tropomyosins: TrpmF1 (forward primer): GGABGCBATCAAGAADAARAYT, (where B stands for C, G, or T; R for A or G; D for A, T, or G and Y denotes C or T), TrpmR1 (reverse primer): CAACCATDGCYAAYTTACGT. After 5 min of initial denaturation at 95℃, 35 cycles of PCR (each of 1 min at 94℃, 1 min at 55℃, and 2 min at 72℃) were performed and this was followed by 8 min at 72℃ for a final extension. The PCR amplified DNA fragment was gel-purified and subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) before sequence determination. Nested PCR was performed with two specific primers corresponding to the subcloned cDNA sequence (TrpmF2: GGCCTGGCAGATGAAGAGCG, TrpmF3: CGCACGTAAATTAGCAATGG).

### 4. DNA sequence analysis

Nucleotide sequences were determined using a ThermoSequenase kit (Amersham Life Science, Cleveland, Ohio, USA) and run on a Long ReadIR 4200 DNA sequencer (LI-COR, Inc., Lincoln, NE, USA). Sequence alignments were performed using the CLUSTAL X program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX).[14]

## 5. Expression of tropomyosin

The open reading frame of tropomyosin was obtained by RT-PCR using two primers (TrpmF4: ATGGATGCCATCAAGAAGAAG, TrpmR2: GTTTAGTTGCCAATAAGTTCGG) using the same conditions, cloned into pGEM-T easy vector, and subsequently transferred into the *Eco*R I site of pET28b vector (Novagen, Madison, WI, USA). The orientation of the insert was confirmed by PCR using T7 primer annealing to the vector and TrpmR2 primer annealing to the tropomyosin. The resultant sequence had an additional 37 amino acids (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPNWI) at the N-terminus.

The recombinant tropomyosin was over-expressed in *E. coli* BL21 (DE3). The cells were grown at 37℃ in Luria Bertani broth to 0.5 $OD_{600}$. Isopropyl-1-thio-β-galactopyranoside (1 mM) was then added to the bacteria culture to induce the expression of

recombinant protein. The culture was harvested four hours after induction. The cellular pellet was resuspended in lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM $NaH_2PO_4$, pH 8.0), and lysed using a French presser. Recombinant tropomyosin was purified by using Ni NTA-agarose (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions under native condition. Purified recombinant tropomyosin was analyzed by 10% polyacrylamide gel containing sodium dodecyl sulfate.[15]

## 6. Specific IgE-binding to recombinant tropomyosin

The prevalence of specific IgE antibodies against recombinant tropomyosin was determined by ELISA. Briefly, each well of the microtiter plates was coated with 200 ng of recombinant tropomyosin. The 1:2 diluted serum was incubated for one hour. Alkaline phosphatase-conjugated mouse monoclonal anti-human IgE (Sigma) was diluted 1:1000 in PBS-0.05% Tween 20 containing 1% bovine serum albumin (BSA) (Sigma). For color development, 100 μL of para-nitrophenyl phosphate (mg/mL) (Sigma) was added and the absorbance was measured with an automatic microplate reader (TECAN, Salzburg, Austria).

The assays were done in triplicate. The mean absorbance level plus two standard deviations (SD) of the sera from 13 healthy controls was used as the cut-off value.

## 7. ELISA inhibition test

Polystyrene microtiter plates were coated with 100 ㎕ of 20 ㎍ /mL of *B. germanica* extract 0.05 mM of carbonate buffer (pH 9.6) overnight at 4℃. The 1:4 diluted serum was preincubated with various quantities of the crude extract or recombinant protein for two hours at room temperature and overnight at 4℃. After washing, each well of the microtiter plate was blocked with 200 ㎕ of 1% BSA in PBS for one hour at room temperature. Fifty ㎕ of the preincubated serum was then incubated for one hour at room temperature. Specific IgE was detected using 50 ㎕ of 1:1000 diluted biotinylated goat anti-human IgE (Vector, Burlingame, CA, USA) followed by streptavidin-peroxidase (Sigma) diluted 1:1000 and incubated for one hour and 30 min, respectively. The signal was developed by adding 3,3′,5,5′-tetramethyl-benzidine (TMB, KPL, Gaitherburg, Maryland, USA)[16] at a concentration of 0.4 g/L.

# Ⅲ. RESULTS

## 1. Molecular cloning of tropomyosin

The cloned sequence encoded 284 amino acids with an estimated molecular mass of 32.836 kd and an estimated isoelectrical point of 4.57. There were no potential *N*-linked glycosylation sites (NXT/S) and no proline or tryptophan residues in the amino acid composition. This sequence, Genbank accession number AF260897, was designated as Bla g 7 according to the guidelines of the International Union of Immunological Societies Allergen Nomenclature Subcommitee[17] (Fig. Ⅰ-1). The deduced amino acid sequence of the cloned cDNA showed 79-98.5% identity with previously reported allergenic tropomyosins (Fig. Ⅰ-2).

```
   1 ATGGATGCCATCAAGAAGAAGATGCAGGCGATGAAGCTGGAGAAGGACAACGCGATGGAT
   1   M  D  A  I  K  K  K  M  Q  A  M  K  L  E  K  D  N  A  M  D
  61 CGCGCCCTTCTCTGCGAACAGCAGGCCCGCGACGCCAACATCCGGGCCGAGAAGGCTGAG
  21   R  A  L  L  C  E  Q  Q  A  R  D  A  N  I  R  A  E  K  A  E
 121 GAGGAGGCCCGATCCCTGCAGAAGAAGATCCAGCAGATTGAGAATGATCTTGATCAGACC
  41   E  E  A  R  S  L  Q  K  K  I  Q  Q  I  E  N  D  L  D  Q  T
 181 ATGGAGCAGTTGATGCAAGTCAACGCCAAGCTGGACGAGAAGGACAAGGCCCTGCAGAAT
  61   M  E  Q  L  M  Q  V  N  A  K  L  D  E  K  D  K  A  L  Q  N
 241 GCTGAGAGTGAGGTCGCTGCCCTCAACCGCCGAATCCAACTGCTGGAGGAGGATCTTGAG
  81   A  E  S  E  V  A  A  L  N  R  R  I  Q  L  L  E  E  D  L  E
 301 AGGTCTGAGGAACGTTTGGCCACAGCCACCGCCAAGTTGGCTGAGGCTTCCCAGGCTGCC
101   R  S  E  E  R  L  A  T  A  T  A  K  L  A  E  A  S  Q  A  A
 361 GATGAGTCAGAGCGAGCTCGTAAGATTCTTGAATCCAAAGGCCTGGCAGATGAAGAGCGT
121   D  E  S  E  R  A  R  K  I  L  E  S  K  G  L  A  D  E  E  R
 421 ATGGATGCTTTGGAGAACCAGCTGAAGGAAGCCAGGTTCATGGCTGAGGAAGCTGACAAG
141   M  D  A  L  E  N  Q  L  K  E  A  R  F  M  A  E  E  A  D  K
 481 AAATATGATGAGGTCGCACGTAAATTAGCAATGGTTGAGGCCGACTTGGAAAGAGCAGAA
161   K  Y  D  E  V  A  R  K  L  A  M  V  E  A  D  L  E  R  A  E
 541 GAGCGTGCCGAGACTGGTGAATCCAAGATTGTGGAGCTTGAGGAAGAACTGCGCGTTGTC
181   E  R  A  E  T  G  E  S  K  I  V  E  L  E  E  E  L  R  V  V
 601 GGCAACAACCTGAAGTCCCTTGAGGTGTCTGAAGAGAAGGCCAACCTGCGTGAGGAAGAG
201   G  N  N  L  K  S  L  E  V  S  E  E  K  A  N  L  R  E  E  E
 661 TACAAGCAACAGATTAAGACTCTGAATACCAGGCTAAAGGAGGCTGAAGCTCGTGCTGAG
221   Y  K  Q  Q  I  K  T  L  N  T  R  L  K  E  A  E  A  R  A  E
 721 TTCGCTGAAAGATCCGTGCAGAAATTGCAGAAGGAGGTTGACAGGCTTGAGGATGAATTG
241   F  A  E  R  S  V  Q  K  L  Q  K  E  V  D  R  L  E  D  E  L
 781 GTACACGAGAAGGAGAAGTACAAGTACATTTGTGACGATCTTGATATGACTTTCACCGAA
261   V  H  E  K  E  K  Y  K  Y  I  C  D  D  L  D  M  T  F  T  E
 841 CTTATTGGCAACTAAACTACTACAACTGTTCTCTGGATCTTGTTCAGTTGGAACCCTGAG
281   L  I  G  N
 901 TTCACATAGCAGACCATGGCAGACAATGAACTACTTCTTGCCTCATTGTAAAGGTGCTAT
 961 AATAAAGTTGGTGATATTTTAAAGGTTGCATTTAAAAATCACCAAAGTATTTTAAACCCA
1021 CAGTGCTATGTGGACTCACTGTGTAGTTTGGTCAACAACAATTTCAAAAGTGTAAAGAAA
1081 TTAAAATAAACATCTTATTTACTAAAAAAAAAAAA
```

Figure Ⅰ-1. Nucleotide and deduced amino acid sequences of Bla g 7. This sequence was submitted to Genbank under accession number AF260897.

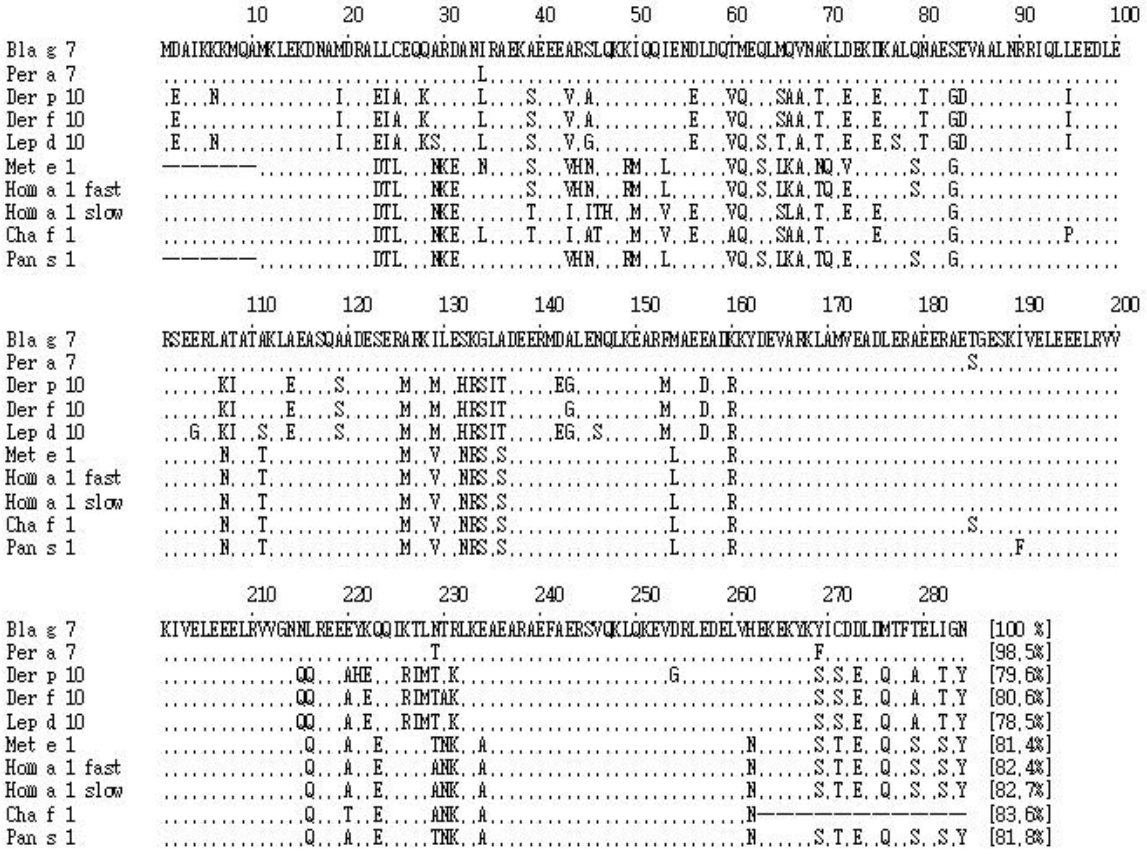
10 20 30 40 50 60 70 80 90 100
Bla g 7 MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANIRAEKAEEEARSLQKKIQQIENDLDQTMEQLMQVNAKLDEKIKALQNAESEVAALNRRIQLLEEDLE
Per a 7
Der p 10
Der f 10
Lep d 10
Met e 1
Hom a 1 fast
Hom a 1 slow
Cha f 1
Pan s 1
110 120 130 140 150 160 170 180 190 200
Bla g 7 RSEERLATATAKLAEASQAADESERARKILESKGLADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAEERAETGESKIVELEEELRVV
Per a 7
Der p 10
Der f 10
Lep d 10
Met e 1
Hom a 1 fast
Hom a 1 slow
Cha f 1
Pan s 1
210 220 230 240 250 260 270 280
Bla g 7 KIVELEEELRVVGNNLREEEYKQQIKTLNTRLKEAEARAEFAERSVQKLQKEVDRLEDELVHEKEKYKYICDDLDMTFTELIGN [100 %]
Per a 7 [98,5%]
Der p 10 [79,6%]
Der f 10 [80,6%]
Lep d 10 [78,5%]
Met e 1 [81,4%]
Hom a 1 fast [82,4%]
Hom a 1 slow [82,7%]
Cha f 1 [83,6%]
Pan s 1 [81,8%]

Figure I-2. Sequence alignment of Bla g 7 with other tropomyosins: Bla g 7 (*Blattella germanica*, AF260897), Per a 7 (*Periplaneta americana,* AF106961), Der p 10 (*Dermatophagoides pteronyssinus*, Y14906), Der f 10 (*Dermatophagoides farinae,* D17682), Lep d 10 (*Lepidoglyphus destructor*, AT250096), Met e 1 (*Metapenaeus ensis*, U08008), Hom a 1 fast (*Homarus americanus* fast muscle, AF034954), Hom a 1 slow (*Homarus americanus* slow muscle, AF034953), Cha f 1 (*Charybdis feriatus*, AF061783), Pan s 1 (*Panulirus stimpsoni*, AF030063). The percentage of each sequence identity was given in the parenthesis.

## 2. Expression and purification of recombinant tropomyosin

The open cDNA reading frame was subcloned into pET-28b expression vector. The molecular weight of the expressed protein was 42 kd by SDS-PAGE. The yield of the purified protein was 5.495 mg/L as measured by the Bradford assay (Bio-rad, Hercules, CA) (Fig. Ⅰ-3).

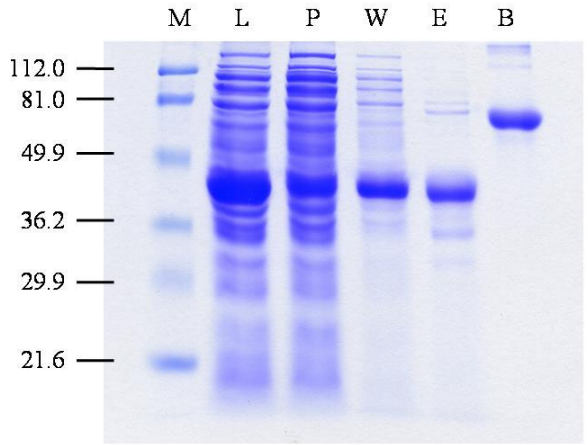


Figure Ⅰ-3. Expression and purification of recombinant tropomyosin. Proteins were run on 10% acrylamide gel and stained with Coomassie brilliant blue. Lanes: M, MW markers in kd; L, total cell lysate of *E. coli* BL21 cells bearing rBla g 7 after IPTG induction; P, fraction passed through the column; W, fraction washed the column; E, fraction eluted; B, bovine serum albumin.

### 3. IgE reactivity of the recombinant tropomyosin

Specific IgE against recombinant tropomyosin was determined by ELISA. The results showed that six of the 37 sera tested (16.2%) could be considered positive (Fig. Ⅰ-4).

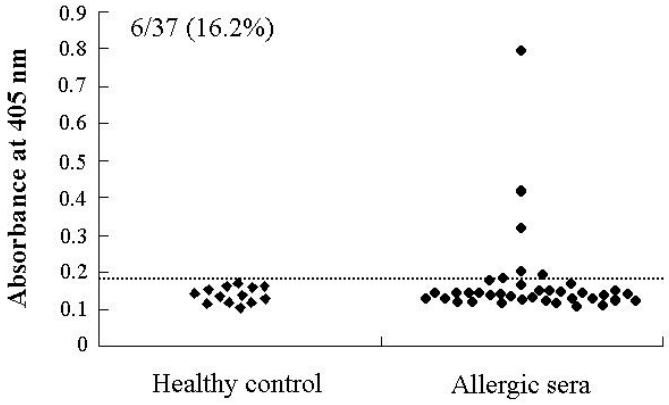


Figure. Ⅰ-4. IgE reactivity of human sera against recombinant Bla g 7. The horizontal line indicates the cut-off value. ◆: Healthy control sera; ●: German cockroach sensitized sera (Uni-CAP > 0.7 kU/L).

## 4. Inhibition of specific IgE binding to crude extract

The whole body extract of German cockroach reached a maximum of 92.2% at an inhibitor concentration of 10 ㎍/mL and recombinant tropomyosin inhibited IgE binding by 32.4% at an inhibitor concentration of 10 ㎍/mL on ELISA (Fig. Ⅰ-5).

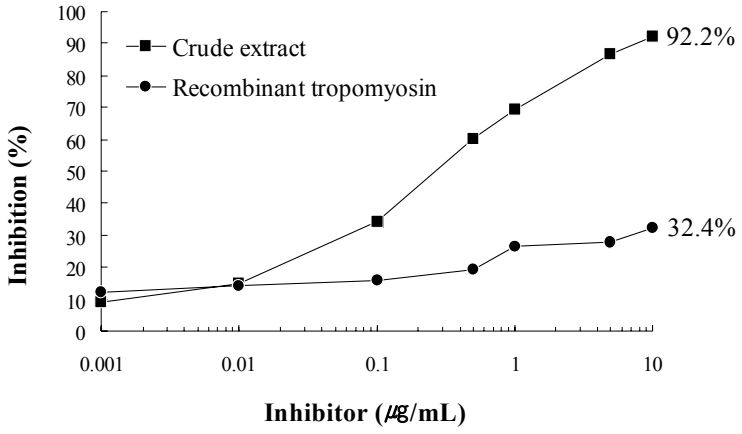


Figure Ⅰ-5. ELISA inhibition of *B. germanica* specific IgE with whole body extract and recombinant tropomyosin.

## Ⅳ. DISCUSSION

Tropomyosin is suggested to be a cross-reacting allergen between foods and inhalant allergens originating from animals. Sensitization to a particular food allergen may ultimately lead to sensitization to certain inhalant allergens[18] and vice versa. [7,19]

Various IgE reactivities to tropomyosin has been reported. There could be three possible reasons for various IgE reactivities to invertebrate tropomyosins. First, IgE reactivity could be affected by the structural differences between native tropomyosin and recombinant counterpart. The studies using purified native tropomyosin found that the frequencies of IgE reactivity to *D. farinae* and *P. americana* were 80.6% (25/31)[11] and 41.4% (12/29),[8] respectively. In a previous study of recombinant Der f 10, it is reported that the IgE-binding response of recombinant fusion protein was at least 25 times weaker than that of the purified native tropomyosin,[11] and that recombinant Der p 10 reacted with only 5.6% (4/71) of house dust mite allergic sera even though it contains non-fusion protein.[20] Recombinant *Blomia tropicalis* tropomyosin showed a IgE reactivity of 29% (27/93).[21] In our study, 16.2% (6/37) of the sera allergic to German cockroach showed positive IgE reactivity to recombinant Bla g 7.

Post-translational modifications may reflect the reduced allergenicity of bacteria-expressed tropomyosin. The N-terminus of vertebrate tropomyosin is known to be acetylated and that this

influences its biological activity.[22,23] Moreover, phosphorylation is thought to influence head to tail interaction and substantially increases viscosity at low ionic strength.[24] Recombinant Bla g 7 has an additional 37 amino acids at N-terminus instead of acetylation. Second, cross-reactivity with other invertebrates in polysensitized populations, and genetic variants affecting the specific sensitizations could contribute to the different allergenicities. Lastly, the little amino acid change in the epitope region of tropomyosin could be the other explanation, because isoforms in different tissues and at different developmental stages have been observed in another insect, *Locusta migratoria*[25] and these isoforms could have different allergenicities.

In summary, the cDNA encoding tropomyosin was isolated and sequenced to elucidate its role in cockroach allergy. Tropomyosin showed 79-98.5% amino acid sequence identity to the other invertebrate tropomyosins. Six (16.2%) of the 37 subjects' sera showed positive IgE reactivity to recombinant Bla g 7, which is not thought to be a major allergen of the German cockroach, but the importance of invertebrate tropomyosin should not be underestimated because of its strong cross-reactivity. However, much remains to be clarified about the cross-reactivity of invertebrate tropomyosins. Attempts to produce the recombinant protein in the eukaryotic system and to purify native tropomyosin are in progress to further understand their allergenicities.

# Chapter Ⅱ

# Expression of tropomyosin from Blattella germanica as a recombinant non-fusion protein in *Pichia pastoris* and comparison of its IgE reactivity its with native counterpart

# Ⅰ. INTRODUCTION

Allergen standardization is essential for diagnostic and immunotherapeutic purposes.[26] However, the protein contents of even commercially available, natural allergen extract are highly variable especially in case of the cockroach extract.[27] Recombinant allergens are useful for the identification and evaluation of the important allergens from the different allergen sources. Microarray diagnostic assays using various recombinant allergens could simplify the future diagnosis of allergic diseases.[28-30] Genetic engineering of the allergen could possibly allow the development of a safe and effective therapeutic approach.[31-34]

Tropomyosin is one of the important respiratory allergens of house dust mite, *Dermatophagoides farinae*[11] and cockroach, *Periplaneta americana*[9] (50 - 80.6% IgE binding frequencies). However, the recombinant tropomyosin expressed in *Escherichia coli* showed only a low degree of IgE binding reactivity (5.6 - 29% IgE binding frequencies) (*D. pteronyssinus,*[20] *Anisakis simplex*,[35] *Blomia tropicalis*,[21] *Lepidoglyphus destructor*.[36] Specifically, IgE reactivity of recombinant tropomyosin from *D. farinae* showed at least 25 times weaker than that of the native counterpart.[11] Native tropomyosin is known to be acetylated at the N-terminus,[37] which is a major determinant for function.[22] Structurally unmodified and fully functional recombinant skeletal muscle α-tropomyosin which is acetylated at N-terminus was

produced in methylotrophic yeast *Pichia pastoris*[38] and *Saccharomyces cerevisiae*[39] at a high level (20 mg/mL).

A cDNA encoding tropomyosin from German cockroach, *Blattella germanica*, which is a dominant species in Korea[4] was cloned and designated as Bla g 7 according to the guidelines of the International Union of Immunological Societies of Allergen Nomenclature Subcommitee and produced a recombinant tropomyosin in *E. coli* showing low IgE binding reactivity in a previous study.[40] However, it is uncertain whether or not this is because of structural differences between recombinant tropomyosin and native protein.

In the present study, we describe the production of recombinant German cockroach tropomyosin in *Pichia pastoris* in an attempt to produce the recombinant protein which is comparable to natural counterpart in allergenic activity. The IgE-binding reactivity of recombinant and native German cockroach tropomyosins were compared by ELISA inhibition test.

## Ⅱ. MATERIAL AND METHODS

### 1. Preparation of polyclonal anti-Bla g 7 antiserum

BALB/c mice were intraperitoneally injected with 30 ㎍ of recombinant tropomyosin expressed in *E. coli*[40] in 100 μL of PBS emulsified with an equal volume of alum adjuvant. Booster injections were given twice at three week intervals. The production of specific antibodies was monitored by ELISA and mice were sacrificed three days after the second booster injection. The polyclonal anti-tropomyosin anti-serum (1:1000) was used to probe and assay for the purification of native and *Pichia*-expressed tropomyosin.

### 2. Purification of native Bla g 7

Cockroach (60 g) were homogenized in 200 mL of 6 mM 2-mercaptoethanol in a Waring blender at 4℃. All subsequent steps were performed at 4℃ unless indicated otherwise. The homogenate was pressed through three layers of cheese cloth. The filtered homogenate was mixed with 20 volumes (four liters) of ethanol. The solution was allowed to stand for one hour and centrifuged for 30 minutes at 3,000 rpm to collect the sediment. The supernatant was discarded and the sediment was resuspended in ether and air-dried. The dried residue was extracted with gentle

overhead stirring overnight in 1 M KCl, 10 mM Tris, 6 mM 2-mercaptoethanol, 1 mg/mL of 1-phenyl-3-(2-thiazolyl)-2-thiourea (Sigma, St. Louis, MO, USA), 0.5 mM EDTA, and protease inhibitor set III (Calbiochem, San Diego, CA, USA), at pH 7.5. The insoluble residue was removed by centrifugation at 20,000 g for 30 minutes. The clear supernatant was fractionated with 40 - 55% ammonium sulfate saturation. The precipitate recovered by centrifugation at 30,000 g for 30 minutes was extracted in 1 M KCl, 6 mM 2-mercaptoethanol, and 10 mM phosphate, at pH 7.1 and subsequently concentrated in the same buffer using stirred cell (Model 8200, Millipore, Bedford, MA, USA) under pressure of $N_2$ gas. The concentrated sample was applied to a hydroxyapatite column to remove the possible troponin contamination[41] which is known as another important German cockroach allergen.[42] The proteins were eluted with a linear gradient generated from 10 mM phosphate and 250 mM phosphate in 1 M KCl, and 6 mM 2-mercaptoethanol, at pH 7.1. Fraction of 2 mL were collected at a flow rate of 0.5 mL/minute. The fractions which contained tropomyosin were subjected to be separated on 4-20% SDS-PAGE (Invitrogen, Carsbad, CA, USA) and then isolated by electroelution (Model 422 electr-eluter, Biorad, Hercules, CA, USA).

### 3. Expression of Bla g 7 in *Pichia pastoris*

The open reading frame of Bla g 7 was obtained by RT-PCR

and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) as described previously (Genbank accession number AF260897).[40] The coding region of Bla g 7 was amplified by PCR using cDNA cloned in pGEM-T Easy vector as the template with primers TmpF (5′-CGGGATCCACCATGGATGCCATCAAG-3′) and TpmR (5′CTTATTGGCAACTAAGCGGCCGCATTC-3′). The underlined sequences correspond to *Bam* HI and *Not* I sites, respectively. TmpF primer contains an optimum translational initiation sequence (ACCATGG).[43,44] The amplified cDNA was digested with *Bam* HI and *Not* I, and subcloned in pPIC9 (Invitrogen). GS115 cells were transformed with *Stu* I-linearized plasmid using a *Pichia* EasyComp Kit (Invitrogen). $His^+$ transformants were selected on RDB plates (1M sorbitol, 1% dextrose, $4\times10^{-5}$% biotin, 1.34% Yeast Nitrogen Base without amino acids, 0.005% each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine). Genomic DNA of individual colonies was isolated and analyzed by PCR using 5′*AOX* primer and 3′*AOX* primer to ascertain the correct integration of cDNA into the *Pichia* genome according to the protocol suggested by the manufacturer.

The clone with the correct integration was selected and the cells were grown for three days at 30℃, 250 rpm, and 0.75% methanol (v/v) was added every 24 hours. The harvested cells were resuspended in 1 M KCl, 10 mM Tris, 6 mM 2-mercaptoethanol, 0.5 mM EDTA, and protease inhibitor set Ⅲ, at

pH 7.5 and subjected to ammonium sulfate fractionation, hydroxyapatite column chromatography, and electroelution as described above (Purification of native Bla g 7).

## 4. Protein sequence analysis

Purified proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (0.45 μm, Millipore) and subjected to Edman degradation using Procise 491HT protein sequencer (Applied Biosystems, USA) at the Korea basic science institute in order to ascertain the N-terminal blocking.

## 5. Serum samples

Sera from allergic patients with Uni-CAP values (Pharmacia, Uppsalsa, sweden) higher than 0.7 kU/L to *B. germanica* (21 males and 13 females, mean age 29 years, ranging from 6 to 58 years) were used to assess the allergenicity of tropomyosin. Ten healthy control sera, Uni-CAP negative to *B. germanica* and *D. farinae*, were used as negative controls in the ELISA test.

## 6. IgE binding reactivity of native Bla g 7

Purified Bla g 7 (2.0 ㎍/mL) in coating buffer (0.1 M sodium carbonate, pH 9.6) was coated onto the microtiter plate (100 μ

L/well) and incubated at 4℃ overnight. The plate was washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and incubated with 3% skim milk in PBS for the blocking. Then, the plate was incubated with human sera (1:4 dilution) for one hour. After washing, antibody was detected using biotinylated goat anti-human IgE (1:1000 dilution) (Vector, Burlinggame, CA, USA) and streptavidin-peroxidase (1:1000 dilution) (Sigma). Signals were developed using 3,3′,5,5′-tetramethyl-benzidine (TMB, KPL, Gaitherburg, Maryland, USA) as a substrate and optical density was determined at 450 nm on an automatic microplate reader (TECAN, Salzburg, Austria). The mean absorbance level plus five SD of 10 healthy control sera was used as the cut-off value.

## 7. ELISA inhibition

Cockroach whole body extract (20 ㎍/mL) in coating buffer was coated onto the each well (0.1 mL/well) in duplicate and incubated at 4℃ overnight. After blocking with 3% skim milk, the wells were incubated with patient serum pool (1:4) which have been pre-incubated for two hour at room temperature with the solution of various concentrations of native protein, recombinant proteins or crude extract. Then the plate was processed as standard ELISA (described above). Results were presented as the percent inhibition of the reaction without inhibitor.

## 8. SDS-PAGE and immunoblotting

Proteins were loaded on 5% polyacrylamide stacking gel above a 10% separating gel, and run with a discontinuous buffer system.[15] After electrophoresis, gel were stained with 0.2% Coomassie Brilliant Blue R-250 or transferred electrophoretically to nitrocellulose membranes (OSMONICS, Westborough, MA, USA). Immunodetection was performed at the every purification step using sera raised against *E. coli*-expressed recombinant (ER) tropomyosin and NBT/BCIP (Promega), as previously described.

# Ⅲ. RESULTS

## 1. Purification of native tropomyosin and *Pichia*-expressed recombinant tropomyosin

Native tropomyosin was purified from whole body extract by ammonium sulfate fractionation, hydroxyapatite column chromatography, and electroelution. The yield of native allergen was 0.08% (w/w) (Table Ⅱ-1). Recombinant tropomyosin was expressed as a non-fusion protein in *Pichia pastoris* and purified by the same procedure used for the purification of native tropomyosin. The yield of purified recombinant tropomyosin was approximately 7.2 mg/L of yeast culture. N-terminal sequencing of either native or recombinant tropomyosins could not be performed, indicating that the N-termini of tropomyosins were blocked as expected. The similar pattern of elution profile from the native and recombinant tropomyosins was observed on the hydroxyapatite column (Fig. Ⅱ-1). Both recombinant and native tropomyosin were eluted at 111 mM phosphate concentration (fraction number 31-39). The purified proteins gave a single homogeneous band on Coomassie blue stained SDS-PAGE (Fig. Ⅱ-2). The native tropomyosin and *Pichia*-expressed recombinant (PR) tropomyosin showed the same mobility. The molecular weight of tropomyosin expressed from *E. coli* is much higher than those of the native or *Pichia*-expressed protein, because it has an additional 37 amino

acids (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPNWI) at the N-terminus.

Table II-1. Purification of German cockroach tropomyosin

| Step | Native tropomyosin | | Recombinant tropomyosin | |
|---|---|---|---|---|
| | Total (mg) | *Yield (%) | Total (mg) | *Yield (%) |
| Crude extract/Cell lysate | 594 | 100 | 409 | 100 |
| 40-55% $(NH_4)_2SO_4$ | 93 | 16 | 165 | 40 |
| Hydroxyapatite column | 57 | 9.6 | 49 | 12 |
| Electroelution | 0.49 | 0.08 | 7.2 | 1.8 |

*Yield of preparation

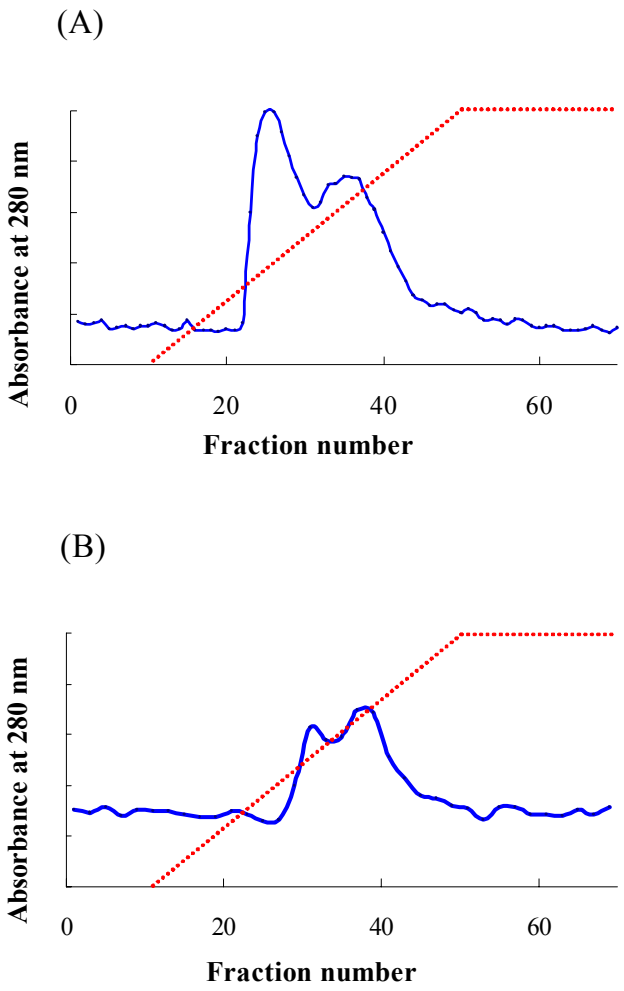


Figure. Ⅱ-1. Chromatographic purification of native tropomyosin (A) and *Pichia*-expressed recombinant tropomyosins (B) on hydroxyapatite column. Dotted line indicates the concentration of elution buffer.

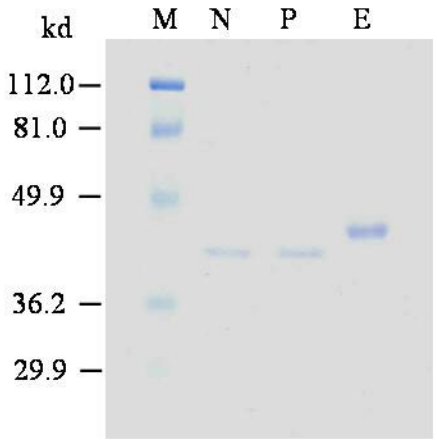


Figure Ⅱ-2. SDS-PAGE of native and recombinant tropomyosins. M, molecular mass standard; N, native tropomyosin; P, *Pichia*-expressed recombinant tropomyosin; E, *E. coli*-expressed recombinant tropomyosin.

## 2. Comparison of IgE binding to native and recombinant *Blattella germanica* tropomyosins

The frequency of IgE binding to tropomyosin was examined by ELISA. IgE-reacting antibody to the native tropomyosin was detected in six of 34 sera tested (17.6%) (Fig. Ⅱ-3). All six sera which considered to have IgE antibodies to the native tropomyosin also showed positive reactivities to the ER and PR tropomyosin (Data not shown). ELISA inhibition experiment were carried out to compare the allergencities of different tropomyosin molecules, the ER tropomyosin, the PR tropomyosin, and native tropomyosin showed a maximum of 26.5%, 40.8%, and 60.5% at an inhibitor concentration of 10 ㎍/mL to the whole body extract of German cockroach. At the inhibitor concentration of 0.01 to 1 ㎍/mL, PR tropomyosin and native tropomyosin showed a similar pattern of inhibition. An abrupt increase of percent inhibition was observed from the native tropomyosin at inhibitor concentrations of above 1 ㎍/mL (Fig. Ⅱ-4).

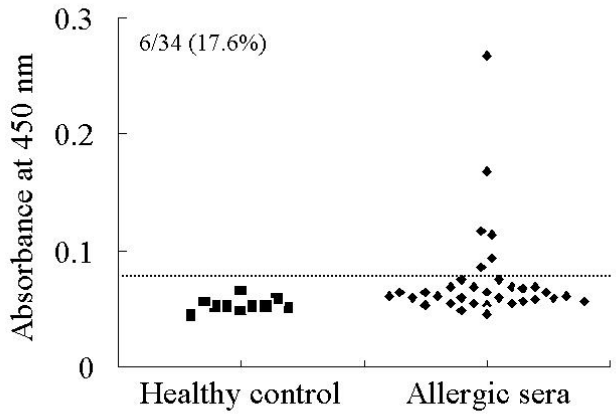


Figure Ⅱ-3. IgE reactivity of human sera against native German cockroach tropomyosin. The horizontal line indicates the cut-off value. ■, Healthy control sera; ◆, German cockroach sensitized sera.

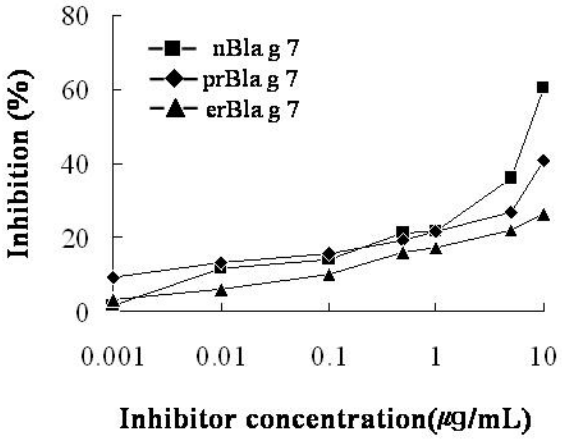


Figure Ⅱ-4. Binding inhibition of IgE to German cockroach extract by native tropomyosin (■), *Pichia*-expressed recombinant tropomyosin (◆), and *E. coli*-expressed recombinant tropomyosin (▲).

# Ⅳ. DISCUSSION

Various expression systems have been applied to produce recombinant allergens for clinical use to overcome the limitations of natural allergen extracts such as stability, contamination and variation in composition and quantity.[45-48] The methylotrophic yeast *P. pastoris* is one of several valuable tools which are capable of performing post-translational modification such as disulfide bond formation, glycosylation, acetylation and proper folding.[49] For example, biological activity of some allergen such as mite group 1 allergen is critical for the allergenicity.[50] Much effort has been made to produce enzymatically active, recombinant mite group 1 allergen and it was eventually rewarded in *P. pastoris*.[51,52]

Tropomyosin is one of the allergen for whom molecular mimicry between the allergen and endogenous proteins of the host is thought to be important.[53] Recombinant tropomyosin was produced as a non-fusion protein and compared its allergenicity with native counterpart in this study. Phosphorylated tropomyosin is known to have a higher propensity for head-to-tail polymerization. Anion exchange column chromatography such as Mono Q column in the presence of 9 M urea is required to separate the phosphorylated and non-phosphorylated tropomyosin.[24] However, this step was omitted because proteins purified by hydroxyapatite column produced a single band on SDS-PAGE. Native and PR tropomyosin was 40 kd as determined by

SDS-PAGE (Fig. Ⅱ-2), which is larger than the one calculated from the nucleotide sequence.

Native tropomyosin showed 17.6% of IgE-binding frequency, which is largely in agreement with previous data on *D. pteronyssinus* (5.6%),[20] *B. tropicalis* (29%),[21] and *L. destructor* (13%)[36] (Fig. Ⅱ-3). However, *D. farinae* tropomyosin showed high IgE-binding frequency among tropomyosins which are respiratory allergens.[11] It may be reasonable to hypothesize that food allergens (shellfish tropomyosins) which are introduced by the oral route and respiratory allergens (mite or cockroach tropomyosins) which are introduced by respiration would elicit different immune responses. These observations suggest that pathogenicity of a particular allergen could be determined by different genetical or environmental factors.[54,55] None of the cockroach allergens studied so far is a proteolytic enzyme, although the proteolytic activity of some allergens is thought to enhance their allegenicity.[50] The comparative modeling and experimental studies of Bla g 2, which has sequence similarity to the aspartic proteases, led to the speculation that key elements affecting the production of IgE antibodies appear to be route of exposure, allergen dose, and the genes that regulate the host immune responses.[56] The age of allergen exposure is also an important factor for the allergic diseases.[57] In addition, in a study using sera from 191 pairs of Australian twins, genetic control of specific IgE responses to rye grass pollen allergens was found to be limited and determined

largely by environmental factors.[58] Tropomyosin could be a molecular model to investigate the factors affecting the sensitization and the onset of allergic disorders.

The abrupt increase of the percentage inhibition of IgE-binding by native tropomyosin above 1 ㎍/mL compared to recombinant troppomyosin may imply the presence of isoforms with different IgE-binding reactivity. Multiple isoforms having little amino acid sequence variations may show cooperative IgE binding. In particular, natural isoforms of Bet v 1, the major birch pollen allergen, were found to have various IgE binding reactivities.[59] Alternatively, it may be influenced by the aggregation of tropomyosin with other proteins which is known to occur even in the absence of actin than in presence (Fig. Ⅱ-4).[60] Further investigation on polymorphism will be required to confirm this observation.

Recombinant German cockroach tropomyosin from *Pichia pastoris* that was improved in allergenicity was successfully produced. The IgE binding frequency of the native and PR troppomyosins were comparable to that of ER tropomyosin, even though the IgE binding affinity or intensity was improved.[40] This recombinant tropomyosin would be useful for the diagnosis of tropomyosin-specific IgE and is important because of its high cross-reactivity.[10] The clinical relevance of the allergen remains to be evaluated in vivo in a large scale.

# Chapter Ⅲ

## Analysis of amino acid sequence variations and IgE binding epitopes of German cockroach tropomyosin

# Ⅰ. INTRODUCTION

Tropomyosin is a family of closely related proteins with multiple functions, including the regulation of the actin-myosin interaction, transport of mRNA,[61] and mechanical support of the cytoplasmic membrane.[62] At least 18 different isoforms are known to be generated by alternative RNA splicing in mammalian cells. The synthesis of isoforms is developmentally regulated and cells from different embryonic lineages express different isoforms.[63] The alternate exon splicing patterns of *Drosophila melanogaster* were reported to involve 27 amino acids at the C-terminus,[64] which frequently contain IgE-binding regions.[10] Specifically, eight different IgE binding epitopes were identified in the American cockroach tropomyosin (Per a 7) by using a set of overlapping synthetic peptides.[65]

Amino acid sequence diversity of individual allergens has been described in wild or cultured house dust mites[66-70] or storage mites.[71] Small changes in the amino acid sequences of given allergens can influence their allergenicity.[59] For example, certain natural isoforms of Bet v 1, the major birch pollen allergen, were found to have high T cell reactivity and low or no IgE binding activity.[72] Analysis of these isoforms may lead us to a better understanding of the different allergenicities of many invertebrate tropomyosins, and the development of immunotherapeutic strategies, such as of hypoallergenic products.

Cross-reactivity by tropomyosin between invertebrates is well documented.[73] It has also been reported that although Blo t 10 and Der p 10 shared 95% identity, unique IgE epitopes do exist.[21] Therefore, it is not unadvisable to use one of the highly conserved allergens for diagnostic or therapeutic purpose.

In order to better understand the different allergenicities of German cockroach tropomyosin, the cDNA sequence variations of German cockroach tropomyosin were investigated by RT-PCR. Fragmented recombinant proteins were also produced and their IgE-binding reactivities were examined.

## Ⅱ. MATERIAL AND METHODS

### 1. Two-dimensional gel electrophoresis and immunoblotting

Two dimensional gel electrophoresis was performed using precast gels (Novex), according to the manufacturer's instructions. Cockroach extract was prepared as described previously.[40] Fifty μg of whole body extracts mixed with an equal volume of the sample buffer was loaded into the first dimension gel. After isoelectric focusing, the second dimension gel electrophoresis was carried out in a 4-20% gradient polyacrylamide gel containing sodium dodecylsulfate. Proteins were then electrophoretically transferred onto a nitrocellulose membrane (0.45 ㎛, OSMONICS, Westborough, MA, USA). After blocking overnight with 3% skim milk, the membrane was incubated for one hour with mouse anti-recombinant Bla g 7 serum. The blots were then incubated with goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) for one hour at room temperature and developed in a substrate solution of NBT/BCIP (Promega).

### 2. Reverse transcriptase-polymerase chain reaction

cDNA encoding tropomyosin was amplified using high fidelity *Pfu* DNA polymerase (Stratagene). After extracting total RNA from 150 mg of German cockroach using TRIzol reagent

(Invitrogen), reverse transcription was initiated using six ㎍ of total RNA and oligo-dT (T18) primer. Five μL of single stranded cDNA was used for each reaction. The primer sequences used were as follows: forward primer; 5′-ATGGATGCCATCAAGAAGAAG-3′, reverse primer; 5′-GTTTAGTTGCCAATAAGTTCGG-3′. The cDNA of different cockroach species, *Periplaneta fuliginosa*, tropomyosin was successfully cloned by RT-PCR using this specific primer set.[74] PCR was performed as follows: after an initial denaturation (5 min at 95℃), samples were subjected to 35 cycles of amplification, each of which consisted of 1 min at 95℃, 1 min at 55℃, and 2 min at 72℃. The final extension was performed at 72℃ for 8 min. The DNA fragment so obtained was cloned into pPCR Script Amp SK (Stratagene) vector.

### 3. Nucleotide sequence determination

A ThermoSequenase kit (Amersham Life Science, Cleveland, OH) was used for the nucleotide sequence determination. Reaction mixtures were run on a Long ReadIR 4200 DNA sequencer (LI-COR, Inc., Lincoln, NE). All reactions (both forward and reverse) were performed in duplicate.

### 4. Generation of fragments by PCR amplification

For epitope analysis, rBla g 7 was divided into five fragments containing 50 overlapping amino acids, i.e., A (residues 1-100), B (residues 51-150), C (residues 101-200), D (residues 151-250), and E (residues 201-284) (Fig. Ⅲ-1). The oligonucleotides used in the PCR are listed in Table Ⅲ-1. Each cDNA fragment was amplified by PCR and ligated into pGEM-T Easy vector. cDNA of Bla g 7 cloned in pET-28b was used as template, and restriction enzyme cleavage sites were incorporated in each oligonucleotide primer to create restriction sites for subcloning (*Bam*H I for forward primers and *Xho* I for reverse primers). The PCR was carried out with an initial denaturation at 95℃ for 5 min, then 35 cycles of denaturation at 94℃ for 30 sec, annealing at 52℃ for 30 sec, and extension at 72℃ for 1 min. The PCR product was cloned into pGEM-T Easy vector (Promega) and subsequently into pET-28b vector after restriction digestion. Recombinant proteins were expressed in *E. coli* BL21 (DE3) and purified by Ni-NTA agarose affinity column chromatography (Qiagen).

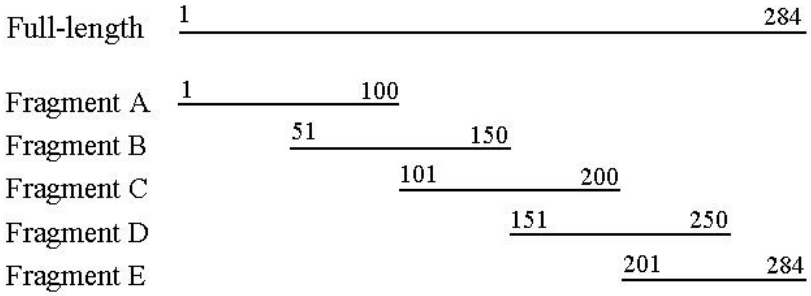


Figure Ⅲ-1. Schematic representation of PCR fragments for the epitope analysis of German cockroach tropomyosin.

Table Ⅲ-1. Sequences of oligonucleotides used for the production of fragmented tropomyosin

| Oligonucleotides | Sequence |
|---|---|
| Bg7AF | 5′-GGATCCAATGGATGCCATCAAGAAG-3′ |
| Bg7AR | 5′-CTCGAGCTCAAGATCCTCCTCCAG-3′ |
| Bg7BF | 5′-GGATCCACAGCAGATTGAGAATGAT-3′ |
| Bg7BR | 5′-GGATCCACAGCAGATTGAGAATGAT-3′ |
| Bg7CF | 5′-GGATCCAAGGTCTGAGGAACGTTTG-3′ |
| Bg7CR | 5′-CTCGAGGACAACGCGCAGTTCTTG-3′ |
| Bg7DF | 5′-GGATCCAGCCAGGTTCATGGCTGAG-3′ |
| Bg7DR | 5′-CTCGAGCCTGTCAACCTCCTTCTG-3′ |
| Bg7EF | 5′-GGATCCAGGCAACAACCTGAAGTCC-3′ |
| Bg7ER | 5′-CTCGAGGTTGCCAATAAGTTCGGA-3′ |

The underlined letters of each oligonucleotide represent the restriction enzyme site.

## 5. IgE-epitope analysis of the subjects' sera

The reactivities of specific IgE antibodies to fragmented recombinant Bla g 7 were examined by ELISA using seven sera samples obtained from recombinant Bla g 7 positive patients. Purified recombinant proteins (0.2 ㎍/well) were coated (0.1 M sodium carbonate, pH 9.6) onto a microtiter plate and incubated overnight at 4℃. The plate was washed with PBS containing 0.05% Tween 20 and incubated for one hour with human sera (1:4 dilution). After washing, IgE antibody was detected using 1:1000 diluted biotinylated goat anti-human IgE (Vector, Buringame, CA) with 1:1000 diluted streptavidin-peroxidase (Sigma). The signal was developed by adding 3,3′,5,5′-tetramethyl-benzidine (TMB, KPL, Gaithersburg, MD), and optical density was determined at 450 nm after adding 1% $H_2SO_4$ on an automatic microplate reader (TECAN, Salzburg, Austria). The mean absorbance level plus two standard deviations of the sera from eight healthy controls was used as the cut-off value.

# Ⅲ. RESULTS

## 1. Two-dimensional immunoblot analysis

In an attempt to investigate the isoforms of German cockroach tropomyosin at the protein level, whole body extract proteins were subjected to 2-dimensional electrophoresis and followed by immunoblotting using mouse anti-Bla g 7 sera (Fig. Ⅲ-2). Diffused spots with molecular masses of 34-40 kd and isoelectric point values ranging from 4.5 to 5.5 were observed. These spots suggested the existence of many isoforms with subtle amino acid variations.

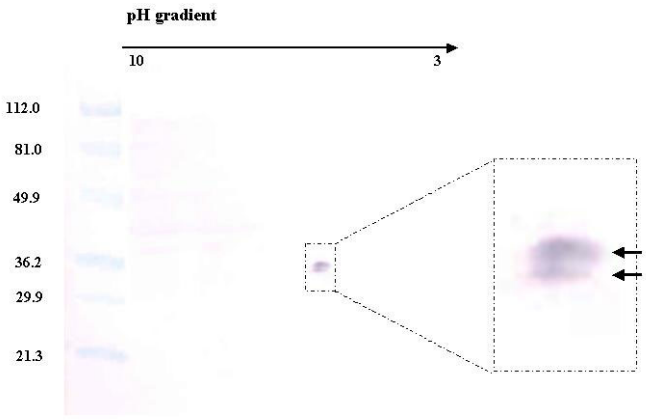


Figure Ⅲ-2. 2D-immunoblot analysis of German cockroach tropomyosin using mouse anti-rBla g 7 sera.

## 2. Sequence analysis of cDNA clones

A total of 11 different variants with amino acid variations were identified by determining the sequences of 50 clones obtained by RT-PCR. Only slight differences were identified at seven locations in the deduced amino acid sequences. A previously described sequence (Genbank accession number AF260897) was found in 38 of the 50 clones, indicating that it is a major form. This major variant was used for the further studies. The positions that were found to vary among the Bla g 7 variants were amino acids of 81, 85, 89, 183, 234, 246, 278, and 284 (Fig. Ⅲ-3). The most frequent amino acid change was observed at residue number 284 (6/50). This observation is in agreement with immunoblot analysis in 2-D gel, which showed diffused spots. The IgE epitopes identified from *Penaeus aztecus* tropomyosin are shown in Figure Ⅲ-3. Only two (pm043 and pm061) of the 50 clones were found to have amino acid sequence variations in the IgE epitope regions.

An analysis of IgE epitopes was carried out, because these variations were not thought to significantly influence IgE binding reactivity.

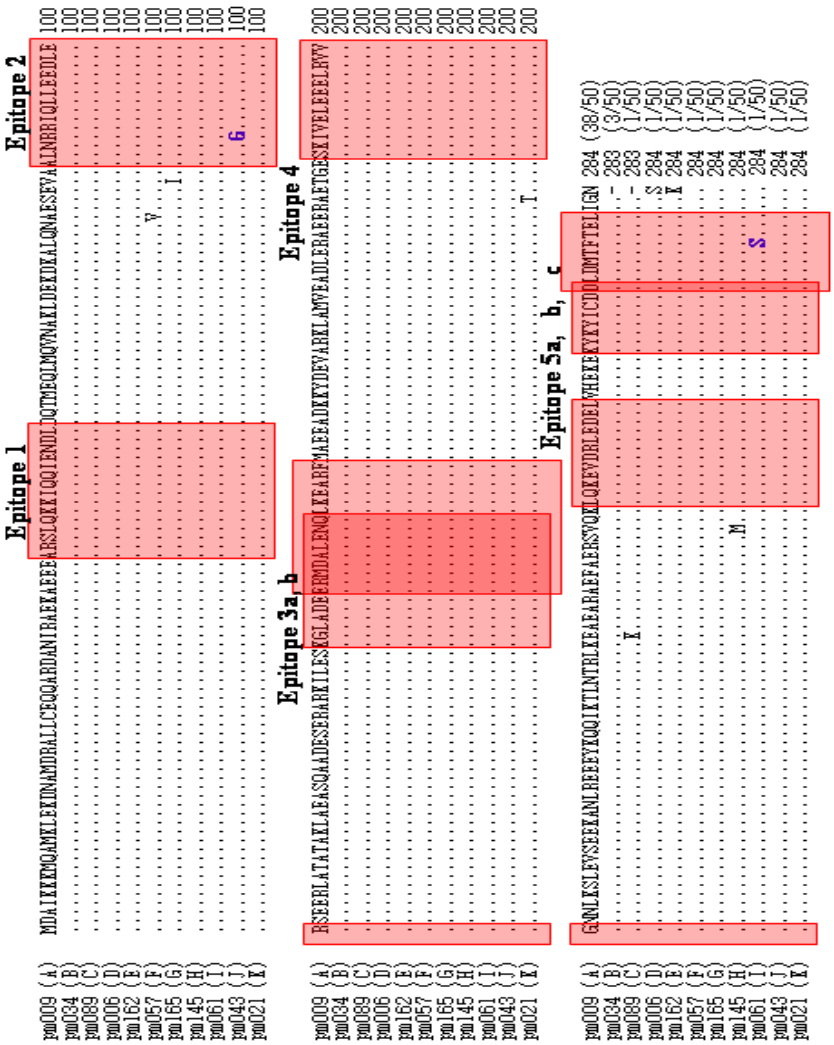


Figure Ⅲ-3. Amino acid sequence variations of German cockroach tropomyosin identified by RT-PCR. The frequency of each sequence is shown in the bracket. Shaded areas indicate the IgE-binding epitopes identified from *Penaeus aztecus* tropomyosin.

### 3. IgE reactivities of recombinant peptides by ELISA

Recombinant fragments of Bla g 7 were expressed in *E. coli* and assayed for IgE reactivity (Fig. Ⅲ-4). IgE reactivities to intact Bla g 7 and to recombinant proteins were determined by ELISA, using Bla g 7-sensitized sera (Fig. Ⅲ-5), and are summarized in Table Ⅲ-2. The results obtained showed that IgE-binding regions were heterogeneously distributed among the different sera. The IgE antibodies from patients 5 and 6 were able to bind all recombinant proteins tested, indicating that there are at least three different IgE-binding epitopes recognized by the serum of a single patient. IgE antibodies from patients 1, 4, 5, and 6 were able to recognize the fragments A and B, and the IgE antibodies of patients 5, 6, and 7 recognized fragment C. Fragments D and E were recognized by the IgE antibodies of patients 1, 3, 4, 5, 6, and 7. The IgE antibody from patient 2 recognized fragment E only.

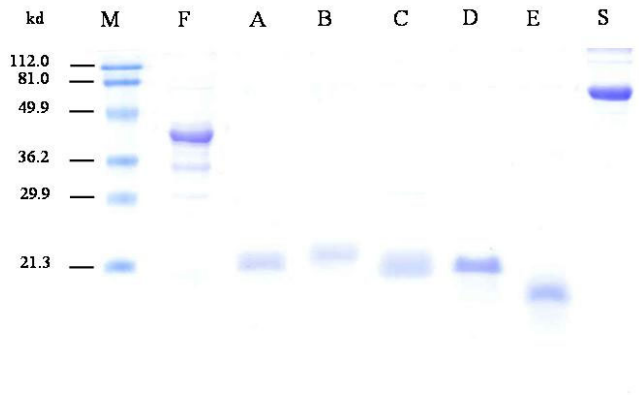


Figure Ⅲ-4. SDS-PAGE of full-length and fragmented recombinant Bla g 7. M, molecular mass standard; F, full-length; A, fragment of 1-100 amino acid; B, fragment of 51-150 amino acid; C, fragment of 101-200 amino acid; D, fragment of 151-250 amino acid; E, fragment of 201-284 amino acid; S, bovine serum albumin.

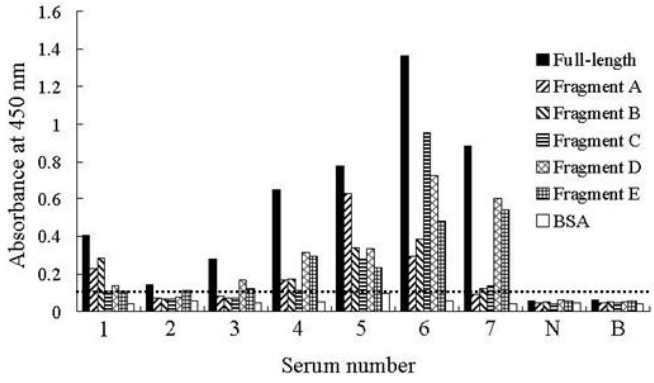


Figure Ⅲ-5. Binding profiles of IgE antibodies to recombinant Bla g 7 and relevant recombinant proteins by ELISA. Seven allergic sera (1-7), serum samples from Bla g 7-negative (N), and buffer control (B).

Table Ⅲ-2. IgE-binding reactivities of peptide fragments of German cockroach tropomyosin

| Patients | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Full-length | + | + | + | + | + | + | + |
| Fragment A | + | − | − | + | + | + | − |
| Fragment B | + | − | − | + | + | + | + |
| Fragment C | − | − | − | − | + | + | + |
| Fragment D | + | − | + | + | + | + | + |
| Fragment E | + | + | + | + | + | + | + |
| BSA | − | − | − | − | − | − | − |

## Ⅳ. DISCUSSION

Tropomyosin is highly conserved, to an extent that enables its use as a phylogenetic marker.[73] It also has been recognized as one of the most important allergens in crustacean food.[75-77] Allergic reactions to shellfish and molluscs are often cross-reactive, but vertebrate tropomyosin is not known to be allergenic.[78] Comparisons of the IgE epitope regions among tropomyosins from different molluscs by Ishikawa et al,[79] showed the presence of polymorphic sites, indicating that the oyster epitope is species-specific.[80] The presence of unique as well as shared epitopes in Blo t 10 and Der p 10 have also been described.[21]

Immunoglobulin E is thought to be a key molecule in mediating many allergic diseases.[81] It was reported that the IgE binding capacity of German cockroach was totally abolished by atlantic shrimp extract, which was found to have strong IgE-binding components between 30 and 43 kd (presumably tropomyosin) by IgE blot inhibition.[5] However, in the present study, recombinant German cockroach tropomyosin was able to inhibit only 32.4% of the IgE binding to cockroach extract.[40]

The first approach required in the study of the relationship between structure and allergenicity is epitope identification. Currently the SPOTs system (Genosys, The Woodland, TX) and the Novatope system (Novagen, Madison, WI) are extensively used and compared to identify IgE-binding epitopes.[82] Moreover,

fragmented peptides are reported to have higher IgE binding capacities than whole molecules in case of paramyosin, Der f 11.[83] These were not real peptide fragments presented by professional phagocytes of the immune system, however, these peptide fragments could have been made by the proteolytic enzymes derived from mites or the cockroaches themselves.

In the present study, I have tried to determine whether the low allergenicity of German cockroach tropomyosin is affected or not by amino acid sequence variations of its isoforms. Only two variants resulting from amino acid substitutions in the IgE epitope regions different from *Penaeus aztecus* tropomyosin, were identified among the 11 different amino acid sequence variations (Fig. Ⅲ-3). IgE-binding reactivities of intact or fragmented Bla g 7 were analyzed to investigate IgE epitopes in the Korean patient population (Fig. Ⅲ-5). All the tested sera showed different patterns of IgE-reactivity. IgE epitope analyses of different patient groups or tropomyosin from different organisms exhibited different IgE binding regions,[65,77,84,85] which implies the presence of various epitope regions, which are influenced by genetic backgrounds and environmental factors. The structural basis for bending tropomyosin around actin filaments is attributed to structural regularity of the molecule.[86] The tropomyosin coiled-coil consists of two α-helices, which are characterized by the occurrence of tandem (haptad) repeats.[87] The structural regularity of tropomyosin may explain the possible reason for the existence of multiple

IgE-binding epitopes. Specific immunotherapy is an efficient treatment for subjects suffering from IgE-mediated allergic reactions. The studies of IgE epitopes have led to a better understanding of mechanisms underlying successful immunotherapy and the proposed use of hypoallergenic (low IgE binding activity) forms of allergens for immunotherapy.[88]

In conclusion, the low allergenicity of previously reported German cockroach tropomyosin does not seem to be due to its amino acid sequence variations. The IgE-binding epitope regions were found to be distributed over the whole molecule. Invertebrate tropomyosin could provide a molecular model for investigating the genetic and environmental factors affecting sensitization and the onset of allergic disorders.

# Chapter Ⅳ

# Tropomyosin level in the house dusts measured by a two-site ELISA

# Ⅰ. INTRODUCTION

Monoclonal antibody based immunoassays have been widely used to quantitate or monitor specific allergen levels.[89] Mite group 1[90] and mite group 2[91] allergen measurements are considered as a gold standard for monitoring dust mite allergen levels. However the ratio of mite group 1 to group 2 varies by up to tenfold.[92] Moreover, Bla g 1 and Bla g 2 have been used to monitor the cockroach allergens.[93,94]

These assays are particularly useful tools in allergen standardization and allergen avoidance studies. In terms of allergen avoidance, the monitoring of allergen levels after chemical treatment to reduce the allergen of interest, is essential to fully evaluate the efficiency of the treatment. The avoidance of allergen exposure is thought to be a critical element in the prevention and management of allergic diseases.[95] In particular, allergen avoidance in childhood is known to effectively reduce the prevalence of sensitization.[96,97] The allergen content of crude extract varies markedly for different source materials or different extraction procedures. Inconsistent protein contents and relative potencies have been reported even in the commercially available cockroach extract.[27] The measurement of the allergen contents of crude extracts is a necessity for analyses performed for diagnostic or therapeutic purposes.[98,99]

Tropomyosin is a well characterized food allergen, but its

characteristics as a respiratory allergen have been poorly examined. Several tropomyosins from domestic arthropods have been reported to be allergenic, however, it is uncertain whether their allergenicities are due to cross-reactivity or poly-sensitization. Most dust samples are reported to contain comparable ratios of tropomyosin to Der p 2, but some dust samples have a high concentration of tropomyosin but low levels of Der p 2.[100] Furthermore, Orthodox Jews strictly prohibiting the consumption of shellfish according to Kosher dietary laws, are indirectly sensitized by cross-reacting tropomyosins.[101]

In this study, I attempted to develop a two site ELISA and to assess the tropomyosin level in the house dust, which might reflect overall arthropods infestation levels.

## Ⅱ. MATERIAL AND METHODS

### 1. Production of monoclonal antibodies to recombinant Bla g 7

Mouse myeloma cells Sp 2.0-Ag 14 were fused with spleen cells from BALB/c mice immunized with recombinant Bla g 7 mixed with alum adjuvant three times at 3-week intervals. Supernatants were screened for antibody production by ELISA. The hybridoma producing antibodies were cloned by limiting dilution. Monoclonal antibodies from expanded clones were purified by protein G affinity column chromatography (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The isotype of antibodies were determined using a Sigma ImmunoType$^{TM}$ Kit (Sigma).

### 2. Biotinylation of monoclonal antibodies

Monoclonal antibody 2G32 was labeled with biotin according to the procedure described by Hnatowich et al.[102] with slight modification. In brief, purified monoclonal antibodies were extensively dialyzed against 0.1 M sodium bicarbonate buffer (SBB), pH 8.6. Then 0.25 mg of sulfosuccinimidyl-6-biotinamido hexanoate (NHS-LC-biotin, Pierce) dissolved in dimethylsulfoxide (10 mg/mL) was added to 5 mg of the monoclonal antibodies and placed on ice for four hours. Unreacted NHS-biotin was removed

by extensive dialysis against SBB. Aliquoted antibodies were stored at -20℃ until required.

### 3. Two-site mAb ELISA

The optimal mAb combination was established by comparing titration curves of recombinant Bla g 7 (0.15 to 10 ㎍/mL) obtained, using the three different mAbs at 1 ㎍/well; biotinylated 2G32 at a 1: 1000 dilution was used for detection. The combination containing capture mAb 2G25 and biotinylated 2G32 gave optimal binding. Microtiter plates were coated with 10 ㎍/mL of mAb 2G25 on 0.05 M carbonate-bicarbonate buffer, at pH 9.6 overnight at 4℃.

### 4. House dust samples

Sampling visits were made in Seoul between October 8 and October 22, 2001. Dust samples were obtained by vacuuming bedding or kitchen floors with a household electronic vacuum cleaner (LV-582T, 520W, LG, Seoul, Korea). Samples (5 mg) of fine dust were extracted overnight in 0.5 mL of PBS containing 0.05% Tween 20, pH 7.4 (PBST) at room temperature with vigorous shaking. The samples were then centrifuged and 0.1 mL of the supernatant was used for allergen detection.

## 5. Measurement of tropomyosin levels in the house dust

Purified 2G28 antibody (10 $\mu$g/mL) in coating buffer (0.1 M sodium carbonate, pH 9.6) was used to coat the microtiter plate. In general, three washings were performed between each step. Sample extracts in PBST (0.1 mL) were added to the plates and incubated for one hour at room temperature. Then 0.1 mL of biotinylated antibody 2G32 diluted 1:1000 in PBST containing 1% bovine serum albumin was added and incubated for one hour at room temperature. The wells were then incubated with streptavidin-peroxidase (Sigma) diluted 1:1000 for 30 min. The signal was developed with 0.05% orthophenylenediamine and 0.006% hydrogen peroxide in 0.1 M phosphate-citrate buffer, pH 5.0. The reaction was stopped with 0.1 mL of 0.5% $H_2SO_4$ and absorbance was measured at 492 nm.

# Ⅲ. RESULTS

## 1. Specificities of monoclonal antibodies

Immunization with recombinant Bla g 7 resulted in the production of three monoclonal antibodies (2G25, 2G28, and 2G32) with specificity for Bla g 7, as determined by ELISA. All were found to be IgG1 according to the Sigma ImmunoType$^{TM}$ Kit (Sigma). For epitope analysis, the IgG reactivities of fragmented recombinant proteins (Fig. Ⅲ-1, 4) were measured by ELISA.

The mAb 2G25 reacted only with full-length protein, while both mAb 2G28 and 2G32 reacted with peptide A and full-length proteins (Fig. Ⅳ-1). These results indicate that the epitopes of mAb 2G28 and 2G32 are located between amino acid residues 1 to 50 amino acid residues. Reactivity of the antibody to the native proteins was confirmed by immunoblotting with house dust mite and cockroach extracts (Fig. Ⅳ-2). Dimer and monomer bands were detected in *B. germanica* and *D. farinae* extracts.

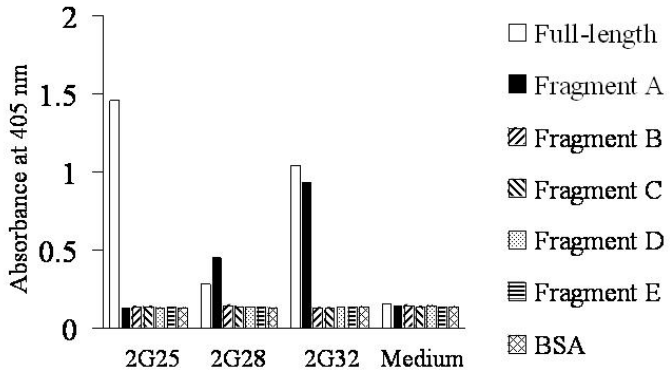


Figure Ⅳ-1. Epitope analysis of monoclonal antibodies raised against recombinant Bla g 7. Fragment A, 1-100 amino acid; fragment B, 51-150 amino acid; fragment C, 101-200 amino acid; fragment D, 151-250 amino acid; fragment E, 201-284 amino acid.

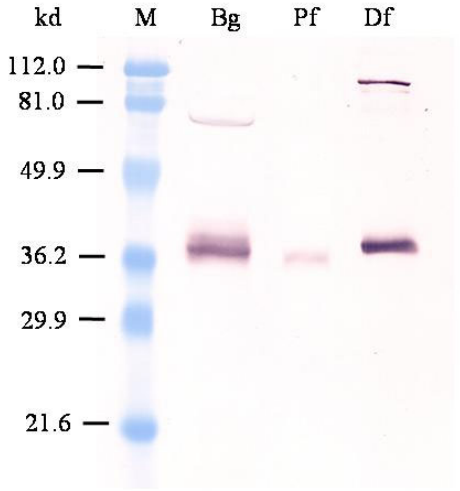


Figure Ⅳ-2. Cross-reactivity of monoclonal antibody 2G32. M, molecular mass standard; Bg, *Blattella germanica*; Pf, *Periplaneta fuliginosa*; Df, *Dermatophagoides farinae*.

## 2. Sensitivity of two-site ELISA

The applicability of two-site ELISA was tested using mAb 2G32 as a detection antibody (Fig. Ⅳ-3). MAb 2G25 exhibited the highest allergen binding ability, however, it showed poor reactivity with German cockroach crude extract (Data not shown). The pair mAb 2G28 as a coating antibody, and 2G32 as a detection antibody, were selected for further optimization.

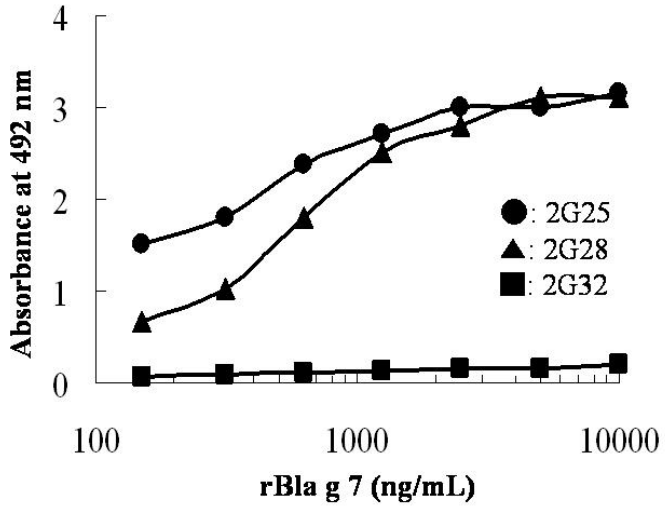


Figure Ⅳ-3. Binding of monoclonal antibodies to recombinant German cockroach tropomyosin. A two-site ELISA with biotinylated 2G32 and two different capture antibodies.

The dose-dependent curve of German cockroach extract was quantified to examine the sensitivity with respect to native proteins. The detection limit was determined to be approximately 1 ㎍/mL with crude extract (Fig. Ⅳ-4) and approximately 8 ng/mL by doubling the recombinant Bla g 7 dilution (Fig. Ⅳ-5).

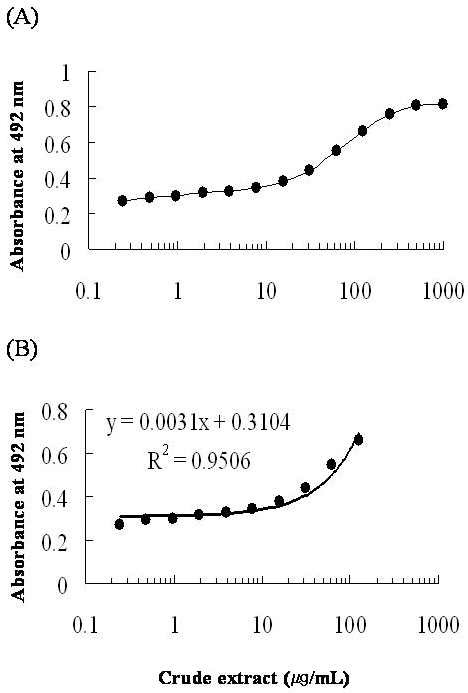


Figure Ⅳ-4. Dose-dependent curve of German cockroach extract by two-site ELISA. Two-site ELISA was quantified using doubling dilutions of cockroach extract from 1 mg/mL (A). The control curve was produced using doubling dilutions from 125 ㎍ /mL of recombinant protein (B).

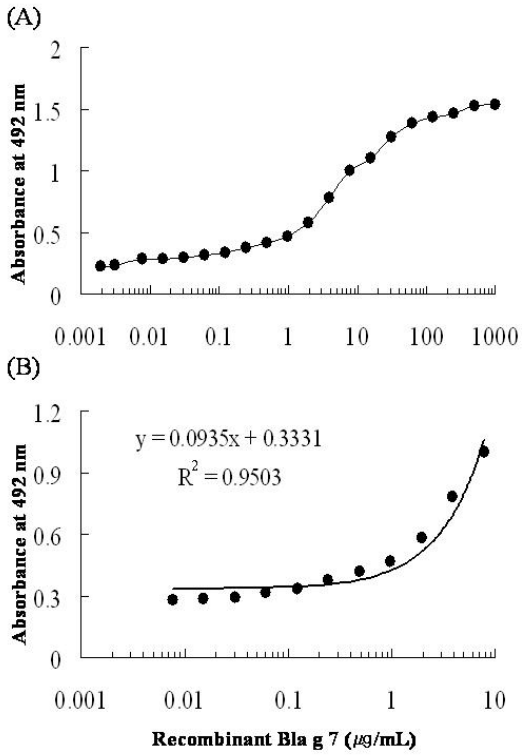


Figure Ⅳ-5. Dose-dependent curve of recombinant German cockroach tropomyosin by two-site ELISA (A). The control curve was produced using doubling dilutions from 7.81 μg/mL of recombinant protein (B).

### 3. Tropomyosin levels in dust samples

The developed ELISA was used to measure tropomyosin levels in the dusts from nine bedding samples and 13 kitchens (Fig. Ⅳ-6). Tropomyosin was detected in only three samples (24.20 ± 32.11 ㎍/g) from bedding (33.3%) and in one of kitchen dust (6.80 ㎍/g) (7.7%).

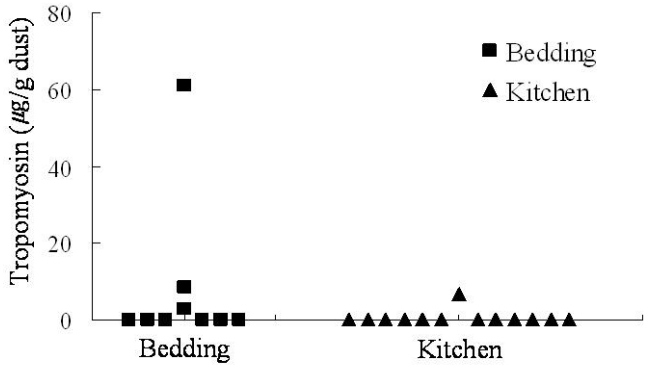


Figure Ⅳ-6. Tropomyosin levels in 22 dust samples. The tropomyosin concentrations in bedding and kitchen samples were compared by a two-site ELISA.

# Ⅳ. DISCUSSION

Cockroach is considered an important pest in daycare centers and elementary schools.[103] Appropriate care and measures should be undertaken, because early exposure to cockroach allergens is known found to be strongly associated with repeated wheeze during the first year of life.[57] Moreover, low-level cockroach exposure has been described as a risk factor of cockroach sensitization.[104]

Allergen avoidance is one of the best treatments available for the allergic diseases. Moreover, the avoidance of dust mite allergen at high altitude was reported to lead to a reduced airway inflammation, and a consequent improvement in non-specific bronchial hyperreactivity and in symptoms. In addition, re-exposure resulted in a rapid relapse.[105] And it was found possible to significantly reduce cockroach infestation (allergen source) in multifamily dwellings in a recent study.[106] Effective approaches combine the pesticide application, family education, and the physical elimination of hiding places.[92]

The two-site ELISA developed in this study was able to detect not only cockroach tropomyosin but also mite tropomyosin (Fig. Ⅳ-2). Tropomyosin was detected in four of 22 dust samples (18.18%) at a concentration of 3.61 ± 12.75 ㎍/g dust (Fig. Ⅳ-6). This observation differs from a previous report in Netherlands.[100] However it is in agreement with the results of sensitization studies

to cockroach tropomyosin using recombinant or native Bla g 7 in the Korean populations,[40] and the studies of cross-reactivities in Taiwanese populations which showed higher competitive inhibition of IgE binding between shrimp and crab but the inhibitions between either cockroach and shrimp or between cockroach and vrab were only partial.[107] It probably reflects regional and environmental differences. Higher tropomyosin levels in bedding samples may imply that dust mites are the major source of tropomyosin. It is also noteworthy that bedding is heavily contaminated with cockroach allergens.[106] The proposed explanation is that cockroach allergen is passively distributed, and that allergens are carried into the bed on feet and clothes. The results indicate that the allergens in bedding samples are not derived only from dust mites, but also emanate from other sources. A recent study of IgE reactivity to shrimp tropomyosin in unexposed Orthodox Jews implied that the tropomyosin in dust could be a source of allergen sensitization, and that contact may elicit the allergic reactions to the cross-reactive allergens.[101]

Many species of arthropods are known sources of potent allergens that sensitize and elicit IgE-mediated allergic reactions in humans.[108] The tropomyosin level could directly reflect the rate of arthropod infestation, because all invertebrate animals contain the tropomyosin. Moreover, it is not an excreted allergen like mite group 1[109] and group 2,[110] but it is a component of dead debris. Tropomyosin levels in house dust could indicate infestation by not

only of cockroach species but also of dust mites (the house dust mite and storage mite), because of its cross-reactivity (Fig. Ⅳ-2).

In this study, a two-site ELISA was developed to assess arthropod tropomyosin. However, the method requires further development before it can be used by patients themselves not by investigastors to make decisions about exposure and about allergen avoidance something like lateral flow test.[92] Monoclonal antibodies could also be used to measure tropomyosin specific IgE responses by development of a chimeric ELISA.[111]

# CONCLUSION

Cockroach is one of the main sources of indoor allergens. Recent evidences suggest that tropomyosin is also one of important allergens in German cockroach which is a dominant species in Korea. This study describes cloning, allergenicity, epitope analysis and distribution of tropomyosin in house dust in an attempt to elucidate the allergenic properties of German cockroach tropomyosin.

## 1. Allergenicity of recombinant Bla g 7, German cockroach tropomyosin

(1) cDNA encoding tropomyosin was cloned by RT-PCR and its deduced amino acid sequence was found to share up to 98.5% identity with previously reported allergenic tropomyosins.

(2) Recombinant tropomyosin expressed in *E. coli* showed a 16.2% (6/37) IgE binding frequency to German cockroach allergic sera.

(3) Recombinant tropomyosin expressed in *E. coli* was able to inhibit 32.4% of IgE binding to the crude extract at a concentration of 10 $\mu$g/mL.

## 2. Expression of tropomyosin from *Blattella germanica* as a recombinant non-fusion protein in *Pichia pastoris* and comparison of its IgE reactivity with its native counterpart.

(1) Native German cockroach tropomyosin showed 17.6% (6/34) IgE binding frequency to German cockroach sensitized sera.

(2) Recombinant tropomyosin expressed in *Pichia pastoris*, was able to inhibit 40.8% of IgE binding, whereas native tropomyosin was able to inhibit 60.5% of IgE binding in the crude extract at a concentration of 10 $\mu$g/mL.

(3) The German cockroach tropomyosin represents a minor allergen in cockroach extract but its allergic relevance cannot be underestimated due to its cross-reactivity.

## 3. Amino acid sequence variations of German cockroach tropomyosin and IgE-binding reactivity

(1) Eleven variants of German cockroach tropomyosin with subtle amino acid substitutions were identified by RT-PCR.

(2) Multiple IgE-binding epitope regions were found to exist in a single molecule recognized even in a single patient's serum tested.

(3) Seven serum samples revealed heterogeneous IgE-binding

responses, indicating the need for tailored allergen-specific immunotherapies for individuals.

## 4. Tropomyosin level in house dust measured by a two-site ELISA

(1) A two-site ELISA was developed to estimate the tropomyosin levels in house dust samples. The detection limit was determined to be about 8 ng/mL for rBla g 7 and 1 ㎍/mL for German cockroach crude extract.

(2) Tropomyosin was detected in only three samples (24.20 ± 32.11 ㎍/g) from nine bedding samples (33.3%) and in one (6.80 ㎍/g) of 13 kitchen dust samples (7.7%).

(3) The low level of tropomyosin in dust samples may support the low rate of sensitization to German cockroach tropomyosin in Korean populations.

In summary, tropomyosin is found to be a minor allergen in German cockroach. The low rate of sensitization is not by structural differences from native protein or by its amino acid sequence variations. The low level of tropomyosin in house dust may explain the low rate of exposure, sensitization and allergenicity.

The key elements affecting the production of IgE antibodies and the onset of allergic disorders, are thought to be the route of exposure, the allergen dose, age at sensitization, and the genes regulating host immune responses, rather than the intrinsic properties of a given allergen. The clinical relevance of the Bla g 7 remains to be evaluated in vivo by large-scale study. Tropomyosin could be a good molecular model for the investigation of factors affecting the sensitization and the onset of allergic disease.

It is believed that the results of the present study could facilitate the development of diagnostic and immunotherapeutic strategies based on the recombinant proteins obtained during the course of this study. In addition, the developed two-site ELISA could aid allergen standardization, allergen avoidance procedures, the assessment of environmental allergen exposure, and the further characterization of tropomyosin.

# REFERENCES


1. Arruda LK, Chapman MD. The role of cockroach allergens in asthma. Curr Opin Pul Med 2001; 7: 14-19.
2. Finn PW, Boudreau JO, He H, Wang Y, Chapman MD, Vincet C, Burge HA, Weiss ST, Perkins DL, Gold DR. Children at risk for asthma: home allergen levels, lymphocyte proliferation, and wheeze. J Allergy Clin Immunol 2000; 105: 933-942.
3. Huss K, Adikinson NF, Eggleston PA, Dawson C, Van Natta ML, Hamilton RG. House dust mite and cockroach exposure are strong risk factors for positive allergy skin test responses in the childhood asthma management program. J Allergy Clin Immunol 2001; 107: 48-54.
4. Jeong KY, Lee IY, Lee J, Ree HI, Hong CS, Yong TS. Effectiveness of health education for the control of dust mites and cockroaches in Seoul, Korea. Med Vet Entomol 2004, In press.
5. Crespo JF, Pascual C, Helm R, Sanchez-Pastor S, Ojeda I, Romualdo L, Martin-Esteban M, Ojeda JA. Cross-reactivity of IgE-binding components between boiled Atlantic shrimp and German cockroach. Allergy 1995; 50: 918-924.
6. Pascual CY, Crespo JF, San Martin S, Ornia N, Ortega N, Caballero T, Munoz-Pereira M, Martin-Estaban M. Cross-reactivity between IgE-binding proteins from *Anisakis*,

German cockroach, and chironomids. Allergy 1997; 52: 514-520.

7. van Ree R, Antonicelli L, Akkerdaas JH, Garrtani MS, Aalberse RC, Bonifazi F. Possible induction of food allergy during mite immunotherapy. Allergy 1996; 51: 108-113.
8. Asturias JA Gomez-Bayon N, Arilla MC, Martinez A, Palacios R, Sanchez-Gascon F, Martinez J. Molecular characterization of American cockroach tropomyosin (*Periplaneta americana* Allergen 7), a cross-reactive allergen. J Immunol 1999; 162: 4342-4348.
9. Santos ABR, Chapman MD, Aalberse RC, Vailes LD, Ferriani VP, Oliver C, Rizzo MC, Naspitz CK, Arruda LK. Cockroach allergens and asthma in Brazil: Identification of tropomyosin as a major allergen with potential cross reactivity with mite and shrimp allergens. J Allergy Clin Immunol 1999; 104: 329-337.
10. Reese G Ayuso R, Lehrer SB. Tropomyosin: an invertebrate pan-allergen. Int Arch Allergy Immunol 1999; 119: 247-258.
11. Aki T, Kodama T. Immunochemical characterization of recombinant and native tropomyosin as a new allergen from the house dust mite, *Dermatophagoides farinae.* J Allergy Clin Immunol 1995; 96: 74-83.
12. Johansson SGO, Hourihane JO'B, Bousquet J, Bruijnzeel-Koomen C, Dreborg S, Haahtela T, Kowalski ML, Mygind N, Ring J, van Vauwenberge P, van Hage-Hamsten

M, Wuthrich B. A revised nomenclature for allergy. An EAACI position statement from EAACI nomenclature task force. Allergy 2001; 56: 813-824.

13. Johnson GA, Boukma SJ, Kim EG. In vivo inhibition of dopamine-hydroxylase by 1-phenyl-3-(2-thiozolyl)-2-thiourea. J Pharmacol Exp Ther 1970; 171: 80-87.
14. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25: 4876-4882.
15. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.
16. Adams JC. Stabilizing and rapid thionin staining of TMB-based HRP reaction product. Neurosci lett 1980; 17: 7-9.
17. King TP, Hoffman D, Lowenstein H, Marsh DG, Platts-Mills TAE, Thomas W. Allergen nomenclature. Int Arch Allergy Immunol 1994; 105: 224-233.
18. De Maat-Bleeker F, Akkerdaas JH, van Ree R, Aslberse RC. Vineyard snail allergy possibly induced by sensitization to house dust mite (*Dermatophagoides pteronyssinus*). Allergy 1995; 50: 438-430.
19. Witteman AM, Akkerdaas JH, van Leeuen J, van der Zee JS, Aalberse RC. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. Int Arch, Allergy Immunol 1994; 105: 56-61.

20. Asturias JA, Arilla MC, Gomez-Bayon N, Martinez A, Martinez J, Palacios R. Sequencing and high level of expression in *Escherichia coli* of the tropomyosin allergen (Der p 10) from *Dermatophagoides pteronyssinus*. Biochem biophys Acta 1998; 1397: 27-30.

21. Yi FC, Cheong N, Shek PCL, Wang DY, Chua KY, Lee BW. Identification of shared and unique immunoglobulin E epitopes of the highly conserved tropomyosins in *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. Clin Exp Allergy 2002; 32: 1203-1210.

22. Cho YJ, Liu J, Hitchcock-DeGregori SE. The amino terminus of muscle tropomyosin is a major determinant for function. J Biol Chem 1990; 265: 538-545.

23. Urbancikova M, Hitchcock-DeGregori SE. Requirement of amino-terminal modification for striated muscle α-tropomyosin function. J Biol Chem 1994; 269: 24310-24315.

24. Heeley DH, Watson MH, Mak AS, Dubord P, Smillie LB. Effect of phosphorylation on the interaction and functional properties of rabbit striated muscle αα-tropomyosin. J Biol Chem 1989; 264: 2424-2430.

25. Krieger J, Raming K, Knipper M, Grau M, Mertens S, Breer H. Cloning, sequencing and expression of locust tropomyosin. Insect Biochem 1990; 20: 173-184.

26. Platts-Mills TA, Chapman MD. Allergen standardization. J Allergy Clin Immunol 1991; 87: 621-625.

27. Patterson ML, Slater JE. Characterization and comparison of commercially available German and American cockroach allergen extracts. Clin Exp Allergy 2002; 32: 721-727.
28. Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM, Twardosz A, Barletta B, Becker WM, Blaser K, Breiteneder H, Chapman M, Crameri R, Duchene M, Ferreira F, Fiebig H, Hoffmann-Sommergruber K, King TP, Kleber-Janke T, Kurup VP, Lehrer SB, Lidholm J, Muller U, Pini C, Reese G, Scheiner O, Scheynius A, Shen HD, Spitzauer S, Suck R, Swoboda I, Thomas W, Tinghino R, Van Hage-Hamsten M, Virtanen T, Kraft D, Muller MW, Valenta R. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. FASEB J 2002; 16: 414-416.
29. Harwanegg C, Laffer S, Hiller R, Mueller MW, Kraft D, Spitzuer S, Valenta R. Microarrayed recombinant allergens for diagnosis of allergy. Clin Exp Allergy 2003; 33: 7-13.
30. Jahn-Schmid B, Harwanegg C, Hiller R, Bohle B, Ebner C, Scheiner O, Mueller MW. Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E. Clin Exp Allergy 2003; 33: 1443-1449.
31. Pomes A, Chapman MD. Can knowledge of the molecular structure of allergens improve immunotherapy. Curr Opinion Allergy Clin Immunol 2001; 1: 549-554.
32. Rolland JM, O'Hehir RE. Allergen immunotherapy: Current and

new therapeutic strategies. Allergol Int 2002; 51: 221-231.

33. Kazemi-Shirazi L, Verena Niederberger V, Linhart B, Lidholm J, Kraft D, Valenta R. Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. Int Arch Allergy Immunol 2002; 127: 259-268.
34. Ferreia F, Wallner M, Breiteneder H, Hartl A, Thalhamer J, Ebner C. Genetic engineering of allergens: future therapeutic products. Int Arch Allergy Immunol 2002; 128: 171-178.
35. Asturias JA. Eraso E, Moneo I, Martinez. Cloning and high level expression in *Escherichia coli* of an *Anisakis simplex* tropomyosin isoform. Mol Biochem Parasitol 2000; 108: 263-267.
36. Saarne T, Kaiser L, Rasool O, Huecas S, van Hage-Hamsten M, Gafvelin G. Cloning and characterization of two IgE-binding proteins, homologous to tropomyosin and α-tubulin, from the mite *Lepidoglyphus destructor*. Int Arch Allergy Immunol 2003; 130: 258-265.
37. Hodges RS, Smillie LB. The histidine and methionine sequences of rabbit skeletal tropomyosin. Can J Biochem 1972; 50: 312-329.
38. Hilario E, Lataro RC, Alegria MC, Lavarda SCS, Ferro JA, Bertolini MC. High-level production of functional muscle α-tropomyosin in *Pichia pastoris*. Biochem Biophys Res Commun 2001; 284: 955-960.
39. Alegria MC, Lavarda SCS, Lataro RC, Hilario E, Ferro JA,

Bertolini MC. Conditions affecting production of functional muscle recombinant α-tropomyosin in *Saccharomyces cerevisiae*. Protein Expr Purif 2003; 30: 105-111.

40. Jeong KY, Lee J, Lee IY, Ree HI, Hong CS, Yong TS. Allergenicity of recombinant Bla g 7, German cockroach tropomyosin. Allergy 2003; 58: 1059-1063.
41. Smillie LB. Preparation and identification of α and β tropomyosins. Methods Ezymol 1982; 85: 234-241.
42. Arruda LK, Vailes LD, Benjamin DC, Chapman MD. Molecular cloning of German cockroach (*Blattella germanica*) allergens. Int Arch Allergy Immunol 1995; 107: 295-297.
43. Kozak M. Compilation and analysis of sequences upstream from the translational start PG in eukaryotic mRNAs. Nucleic Acids Res 1984; 12: 857-872.
44. Kozak M. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 1987; 15: 8125-8148.
45. Valenta R, Kraft D. Recombinant allergen molecules: Tools to study effector cell activation. Immunol Rev 2001; 179: 119-127.
46. van der Veen MJ, Mulder M, Witteman AM, van Ree R, Aalberse RC, Jansen HM, van der Zee JS: False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (*Dermatophagoides pteronyssinus*) allergens. J Allergy Clin

Immunol 1996; 98: 1028-1034.

47. Nelson HS, Ilke D, Buchmeier A. Studies of allergen extract stability: The effect of dilution and mixing. J Allergy Clin Immunol 1996; 98: 382-388.

48. Schneiner O, Kraft D. Basic and practical aspects of recombinant allergens. Allergy 1995; 50: 384-391.

49. Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 2000; 24: 45-66.

50. Shakib F, Schulz O, Sewell H. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. Immunol Today 1998; 19: 313-316.

51. Best EA, Stedman KE, Bozic CM, Hunter SW, Vailes L, Chapman MD, McCall CA, McDermott MJ. A recombinant group 1 house dust mite allergen, rDer f 1, with biological activities similar to those of the native allergen. Protein Expr Purif 2000; 20: 462-471.

52. Yasuhara T, Takai T, Yuuki T, Okudaira H, Okumura Y. Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding Clin Exp Allergy 2001; 31: 116-124.

53. Pomes A. Intrinsic properties of allergens and environmental exposure as determinants of allergenicity. Allergy 2002; 57: 673-679.

54. Parronchi P, Brugnolo F, Sampognaro S, Maggi E. Genetic and environmental factors contributing to the onset of allergic disorders. Int Arch Allergy Immunol 2000; 121: 2-9.

55. Otsu A, Shirakawa T. Genetic and environmental factors of atopy. Allergol Intl 2002; 51: 213-219.

56. Pomes A, Chapman MD, Vailes LD, Blundell TL, Dhanaraj V. Cockroach allergen Bla g 2: structure, function, and implications for the allergic sensitization. Am J Respir Crit Care Med 2002; 165: 391-397.

57. Gold DR, Burge HA, Carey V, Milton DK, Platts-Mills T, Weiss STl. Predictors of repeated wheeze in the first year of life. Am J Respir Crit Care Med 1999; 160: 227-236.

58. Sluyter R, Tovey ER, Duffy DL, Britton WJ. Limited genetic control of IgE responses to rye grass allergens in Australian twins. Clin Exp Allergy 1998; 28: 322-331.

59. Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, Ebner C. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 1996; 183: 599-609.

60. Heald RW, Hitchcock-DeGregori SE. The structure of the amino terminus of tropomyosin is critical for binding to actin in the absence and presence of troponin. J Biol Chem 1988;

263: 5254-5259.

61. Erdelyi M, Michon AM, Guichet A, Glotzer JB, Ephrussi A. Requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization. Nature 1995; 377: 524-527.
62. Luna EJ, Hitt AL. Cytoskeleton-plasma membrane interactions. Science 1992; 258: 955-964.
63. Rethinasamy P, Muthuchamy M, Hewett T, Boivin G, Wolska B, Evans C, Solaro R, Wieczoreck D. Molecular and physiological effects of α-tropomyosin ablation in mouse. Circ Res 1997; 82: 116-123.
64. Basi GS, Boardman M, Storti RV. Alternative splicing of a *Drosophila* tropomyosin gene generates mucsle tropomyosin isoforms with different carboxy-terminal ends. Mol Cell Biol 1984; 4: 2828-2836.
65. Ayuso R, Reese G, Leong-Kee S, Plante M, Lehrer SB. Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosin. Int Arch Allergy Immunol 2002; 129: 38-48.
66. Thomas WR, Chua KY, Smith WA. Molecular polymorphisms of house dust mite allergens. Exp Appl Acarol 1992; 16: 153-164.
67. Chua KY, Huang CH, Shen HD, Thomas WR. Analysis of sequence polymorphism of a major mite allergen Der p 2. Clin Exp Allergy 1996; 26: 829-837.

68. Smith WA, Thomas WR. Sequence polymorphisms of the Der p 3 house dust mite allergen. Clin Exp Allergy 1996; 26: 571-579.

69. Yuuki T, Okumura Y, Okudaira H. Genomic organization and polymorphisms of the major house dust mite allergen Der f 2. Int Arch Allergy Immunol 1997; 112: 44-48.

70. Smith WA, Hales BJ, Jarnicki AG, Thomas WR. Allergens of wild house dust mites: environmental Der p 1 and Der p 2 sequence polymorphisms. J Allergy Clin Immunol 2001; 107: 985-992.

71. Kaiser L, Gafvelins G, Johansson E, van Hage-Hamsten M, Rasool O. Lep d 2 polymorphisms in wild and cultured *Lepidoglyphus destructor* mites. Eur J Biochem 2003; 270: 646-653.

72. Park JW, Kim KS, Jin HS, Kim CW, Kang DB, Choi SY, Yong TS, Oh SH, Hong CS. Der p 2 isoallergens have different allergenicity, and quantification with 2-site ELISA using monoclonal antibodies is influenced by the isoallergens. Clin Exp Allergy 2002; 32: 1042-1047.

73. Chu KH, Wong SH, Leung PSG. Tropomyosin is the major mollusks allergen: reverse transcriptase polymerase chain reaction, expression and IgE reactivity. Mar Biotechnol 2000; 2: 499-509.

74. Jeong KY, Hwang H, Lee J, Lee IY, Kim DS, Hong CS, Yong TS. Allergenic characterization of the dusky brown cockroach,

*Periplaneta fuliginosa* tropomyosin. In submission.

75. Daul CB, Morgan JE, Waring NP, McCants ML, Hughes J, Lehrer SB. Immunologic evaluation of shrimp-allergic individuals. J Allergy Clin Immunol 1987; 80: 716-722.
76. Nagpal S, Rajappa L, Metcalfe DD, Rao PV. Isolation and characterization of heat-stable allergens from shrimp (*Penaeus indicus*). J Allergy Clin Immunol 1989; 83: 26-36.
77. Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. J Immunol 1993; 151: 5354-5363.
78. Ayuso R, Lehrer SB, Tanaka L, Ibanez MD, Pascual C, Burks AW, Sussman GL, Goldberg B, Lopez M, Reese G. IgE antibody response to vertebrate meat proteins including tropomyosin. Ann Allergy Asthma Immunol 1999; 83: 399-405.
79. Ishikawa M, Ishida M, Shimakura K, Magashima Y, Shiomi K. Tropomyosin, the major oyster *Crassostrea gigas* allergen and its IgE binding epitopes. J Food Sci 1998; 63: 44-47.
80. Leung PSC, Chu KH. cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster *Crassostrea gigas*. Clin Exp Allergy 2001; 31: 1287-1294.
81. Platts-Mills TAE. The role of immunoglobulin E in allergy and asthma. Am J Respir Crit Care Med 2001; 164: S1-5.
82. Reese G, Ayuso R, Leong-Kee SM, Plate MJ, Lehrer SB.

Characterization and identification of allergen epitopes: recombinant peptide libraries and synthetic, overlapping peptides. J Chromatogr B Biomed Sci Appl 2001; 25: 157-163.

83. Tsai LC, Chao PL, Hung MW, Sun YC, Kuo IC, Chua KY, Liaw SH, Chua KY, Kuo IC. Protein sequence analysis and mapping of IgE and IgG epitopes of an allergen 98-kDa *Dermatophagoides farinae* paramyosin, Der f 11. Allergy 2000; 55: 141-147.
84. Reese G, Jeoung BJ, Daul CB, Lehrer SB. Characterization of recombinant shrimp allergen Pen a 1 (tropomyosin). Int Arch Allergy Immunol 1997; 113: 240-242.
85. Ishikawa M, Ishida M, Shimakura K, Nagashima Y, Shiomi K. Purification and IgE-binding epitopes of a major allergen in the gastropod *Turbo cornutus*. Biosci Biotechnol Biochem 1998; 62: 1337-1343.
86. Stewart M. Structural basis for bending tropomyosin around actin in muscle thin filaments. Proc Natl Acd Sci USA 2001; 98: 8165-8166.
87. Smillie LB. Structure and functions of tropomyosins from muscle and non-muscle sources. Trends Biochem Sci 1979; 4: 151-155.
88. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger H-J, Scheiner O. Modulation of IgE reactivity of allergens by site-directed mutagenesis:

potential use of hypoallergenic variants for immunotherapy. FASEB J 1998; 12: 231-242.

89. Chapman MD, Vailes LD, Ichikawa K. Immunoassays for indoor allergens. Clin Rev Allergy Immunol 2000; 18: 285-300.

90. Luczynska CM, Arruda LK, Platts-Mills TAE, Miller JD, Lopez M, Chapman MD. A two-site minoclonal antibody ELISA for the quantification of the major *Dermatophagoides* spp. allergens, Der p I and Der f I. J Immunol Meths 1989; 118: 227-235.

91. Ovsyannikova IG, Vailes LD, Li Y, Heymann PW, Chapman MD. Monoclonal antibodies to Group II *Dermatophagoides* spp. allergens: murine immune response, epitope analysis and development of a two-site ELISA. J Allergy Clin Immunol 1994; 94: 537-546.

92. Pollart SM, Smith TF, Morris EC, Gelber LE, Platts-Mills TA, Chapman MD. Environmental exposure to cockroach allergens: analysis with monoclonal antibody-based enzyme immunoassays. J Allergy Clin Immunol 1991; 87: 505-510.

93. Tsay A, Williams L, Mitchell EB, Chapman MD, a multi-centre study group. A rapid test for detection of mite allergens in homes. Clin Exp Allergy 2002; 32: 1596-1601.

94. Pollart SM, Mullins DE, Vailes LD, Hayden ML, Platts-Mills TA, Sutherland WM, Chapman MD. Identification, quantitation, and purification of cockroach allergens using monoclonal antibodies. J Allergy Clin Immunol 1991; 87:

511-521.

95. Tovey E, Mark G. Methods and effectiveness of environmental control. J Allergy Clin Immunol 1999; 103: 179-191.

96. Arshad SH, Matthws S, Gant C, Hide DW. Effect of allergen avoidance in early infancy. Lancet 1992; 339: 1493-1497.

97. Wahn U, Lau S, Bergmann R, Kulig M, Forster J, Mergmann K, Bauer CP, Guggenmoos-Holzmann I. Indoor allergen exposure is a risk factor for sensitization during the first three years of life. J Allergy Clin Immunol 1997; 763-769.

98. Bousquet J, Lockey RF, Mallling HJ. Allergen immunotherapy: therapeutic vaccines for allergic disease. J Allergy Clin Immunol 1998; 102: 558-562.

99. Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. J Allergy Clin Immunol 2000; 106: 409-418.

100. Witteman AM, Voorneman R, van den Oudenrijn S, van Leeuwen J, Akkerdaas J, van der Zee JS, Aalberse RC. Silverfish protein in house dust in relation to mite and total arthropod level. Clin Exp Allergy 1996; 26: 1171-1176.

101. Fernandes J, Reshef A, Patton L, Ayuso R, Reese G, Lehrer SB. Immunoglobulin E antibody reactivity to the major shrimp allergen, tropomyosin, in unexposed Orthodox Jews. Clin Exp Allergy 2003; 33: 956-961.

102. Hnatowich DJ, Virzi F, Rusckowski M. Investigations of avidin and biotin for imaging applications. J Nucl Med. 1987;

28: 1294-1302.

103. Rullo VEV, Rizzo MC, Arruda LK, Sole D, Naspitz CK. Daycare centers and schools as sources of exposure to mites, cockroach, and endotoxin in the city of Sao Paulo, Brazil. J Allergy Clin Immunol 2002; 110: 582-588.

104. Matsui EC, Wood RA, Rand C, Kanchanaraksa S, Swartz L, Curtin-Brosnan J, Eggleston PA. Cockroach allergen exposure and sensitization in suburban middle-class children with asthma. J Allergy Clin Immunol 2003; 112: 87-92.

105. Piacentini GL, Martinati L, Fornari A, Comis A, Carcereri L, Boccagni P, Boner AL. Antigen avoidance in a mountain environment influence on basophil releasability in children with allergic asthma. J Allergy Clin Immunol 1993; 92: 644-650.

106. Arbes SJ Jr, Sever M, Archer J, Log EH, Gore JC, Schal C, Walter M, Nuebler B, Vaughn B, Mitchell H, Liu E, Collette N, Adler P, Sandel M, Zeldin DC. Abatement of cockroach allergen (Bla g 1) in low-income, urban housing: a randomized controlled trial. J Allergy Clin Immunol 2003; 112: 339-345.

107. Chiou YH, Tuo CY, Wang LY, Huang SP. Detection of cross-reactivity for atopic immunoglobulin E against multiple allergens. Clin Diag Lab Immunol 2003; 10: 229-232.

108. Arlian LG. Arthropod allergens and human health. Annu Rev Entomol 2002; 47: 395-433.

109. Tovey ER, Chapman MD, Platts-Mills TAE. Mite faeces are a major source of house dust allergens. Nature 1981; 289: 592-593.

110. Jeong KY, Lee IY, Ree HI, Hong CS, Yong TS. Localization of Der f 2 in the gut and fecal pellets of *Dermatophagoides farinae*. Allergy 2002; 57: 729-731.

111. Trombone APF, Tobias KRC, Ferriani VPL, Schuurman J, Aalberse RC, Smith AM, Chapman MD, Arruda LK. Use of a chimeric ELISA to investigate immunoglobulin E antibody responses to Der p 1 and Der p 2 in mite-allergic patients with asthma, wheezing and/or rhinitis. Clin Exp Allergy 2002; 32: 1323-1328.

ABSTRACT (IN KOREAN)

# 재조합단백질을 이용한 바퀴트로포마이오신의 알레르겐 특성 규명

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정 경 용

바퀴는 유전적 소인이 있는 사람들을 감작시켜서 알레르기 반응을 유발한다. 무척추동물의 트로포마이오신은 종종 알레르기 원인이 되며 교차반응성이 강하다. 바퀴 트로포마이오신 (Bla g 7)의 특성을 규명하기 위하여, cDNA 클로닝 후 재조합단백질을 합성하여 알레르기 발현성을 조사하였다. 기존에 밝혀진 트로포마이오신 서열에 근거하여 제조한 degenerate primers를 이용하여 역전사효소중합효소연쇄반응을 수행하여 바퀴 트로포마이오신의 cDNA를 클로닝하였다. 클로닝한 Bla g 7은 알레르겐으로 알려진 트로포마이오신의 아미노산 서열과 최고 98.5%의 상동성을 나타냈다. 클로닝한 cDNA를 이용하여 대장균에서 과발현시킨 후, Ni-nitrilotriacetic acid (NTA) agarose resin를 사용한 친화크로마토그래피의 방법으로 분리하였다. 효소면역흡착법 (ELISA)으로 재조합 트로포마이오신의 알레르기 발현성을 조사하였다. 효소면역흡착법 조사결과 재조합 Bla g 7의 감작율은 바퀴 알레르기환자 중에서 16.2%였다. 재조합 트로포마이오신은 바퀴조항원 특이 IgE 반응을 32.4%까지 억제시킬 수 있었다. 트로포마이오신은 바퀴조항원에서 minor allergen으로 판명되었다.

재조합 트로포마이오신의 알레르기 발현성에 관한 보고는 일관적이지 못하다. 알레르기 발현성을 보다 잘 이해하기위하여, native와

재조합 바퀴트로포마이오신 (*E. coli* 발현 및 *Pichia* 발현)의 IgE 결합능을 비교분석하였다. Native 트로포마이오신은 ammonium sulfate 분획, hydroxyapatite column 크로마토그래피와 electroelution의 방법을 이용하여 분리하였으며, 알레르기 발현성이 native 트로포마이오신과 상응하는 재조합단백질을 합성하기 위하여 *Pichia pastoris*에서 발현시켰다. Native와 재조합 트로포마이오신의 알레르기 발현성은 효소면역흡착억제법으로 비교하였다. Native 바퀴트로포마이오신은 바퀴감작환자 혈청에서 17.6%의 IgE 반응을 나타냈다. 재조합 트로포마이오신은 융합단백질 없이 발현하였으며, N-말단은 native 단백질과 마찬가지로 blocking되었다. 면역효소흡착억제실험에서 재조합단백질은 1-1000 ng/mL의 농도에서 native 단백질과 비슷한 수준의 IgE 결합능을 가졌다. Native와 재조합단백질의 구조적 차이점 이외의 다른 요소가 여러 트로포마이오신의 상이한 IgE 반응성에 영향을 주는 것으로 사료된다.

다양한 원천으로부터 유래한 각각의 알레르겐은 아미노산서열이 다양성을 갖는다고 보고되었다. 알레르겐의 IgE 반응성을 연구하기 위해서는 서열의 다양성이 미치는 영향을 고려하여야 한다. 재조합 Bla g 7을 면역한 마우스혈청을 이용하여 2차 전기영동과 면역이적법을 수행하여 단백질 수준에서의 isoforms에 관한 연구를 하였다. 역전사효소중합효소연쇄반응을 수행하여 아미노산 서열이 다른 11개의 variants를 찾았다. 바퀴트로포마이오신은 아미노산 서열의 변이가 심하지 않아서 알레르기 발현성에 큰 영향을 주지 않을 것으로 사료된다. 본 연구결과는 절지동물 트로포마이오신의 교차항원성에 관한 분자생물학적 기초를 제시한다. 중합효소연쇄반응의 반응산물을 이용하여 재조합단백질 단편을 합성하고, 면역효소흡착법으로 IgE 결합능을

조사하였다. 실험에 사용한 7명의 환자혈청은 모두 상이한 IgE 결합 반응을 나타냈다. 본 연구결과는 하나의 단백질이 여러 개의 IgE-항원결정부위를 갖는 것으로 판명되었다.

재조합바퀴트로포마이오신을 면역하여 생산한 단클론항체를 이용하여 two-site ELISA를 개발하여, 집먼지내의 트로포마이오신 농도를 측정하였다. Two-site ELISA의 detection limit는 재조합트로포마이오신은 8 ng/mL, 바퀴조항원은 1 $\mu$g/mL이었다. 9 곳의 침구먼지로부터 단지 3 곳에서 트로포마이오신이 검출 (24.20 ± 32.11 $\mu$g/g dust)되었으며, 13 곳의 부엌먼지로부터 단지 한 곳 (6.80 $\mu$g/g dust)에서 트로포마이오신이 검출되었다. 본 실험결과는 한국인의 낮은 트로포마이오신 감작율과 일치한다.

본 연구결과 바퀴트로포마이오신의 감작율과 알레르기 발현성을 결정하는 가장 중요한 요소는 구조적 특성이나 아미노산 서열의 변이가 아닌 집먼지내 알레르겐 농도와 같은 환경적 요인으로 생각된다.

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핵심되는 말 : 알레르겐, 바퀴, 항원결정부위, 트로포마이오신