Identification of the IgE binding epitopes of peptide fragments of Bla g 6 from German cockroach (*Blattella germanica*)

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Identification of the IgE binding epitopes of peptide fragments of Bla g 6 from German cockroach (*Blattella germanica*)

Directed by Profess Tai-Soon Yong

The Master's Thesis

submitted to the Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Master of Medical Science

Sunjin Un

June 2009

This certifies that the Master's Thesis
of Sunjin Un is approved.

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June 2009

Acknowledgements

제가 이 자리까지 올 수 있도록 지치고 힘들 때마다 항상 곁에서 사랑으로 위로하시며, 힘이 되어주신 하나님께 진심으로 감사를 드립니다.

이 논문이 나오기까지 격려와 자상함으로 지도해 주신 용태순 교수님, 김규언 교수님, 그리고 신명헌 교수님께 감사를 드립니다. 독일바퀴 알레르겐의 논문을 위한 실험을 세심하게 가르쳐주시고 옆에서 많은 도움을 주신 정경용 박사님께도 깊은 감사를 드립니다. 대학원 과정을 잘 마칠 수 있게 따스함을 보여주신 박순정 교수님과 김형표 교수님, 그리고 환경의생물학교실원 모두에게 감사를 표합니다. 특히 256 맴버 중에 날 가족만큼 소중하게 생각해주고, 믿어주며, 항상 어려움이 있을 때 도와준 명희에게 고맙고, 내가 256실험실에 잘 적응할 수 있게 도와준 광현과 경진이, 이인용 선생님, 이종원 선생님, 김충렬 선생님, 남성현 선생님, 그리고 새로운 맴버인 지나와 은주에게도 고마움을 전합니다.

날 이해해주고 묵묵히 곁에서 격려해 주고 기도해 준 모든 친구들에게 고마움을 표합니다.

제가 논문을 완성할 수 있도록 항상 곁에서 기도해 주시고 사랑으로 응원해주시고, 격려해주시고, 용기를 주신 부모님께 그리고 날 너무 사랑해주고, 항상 내편이 되어주고, 내가 석사과정을 마칠 수 있게 옆에서 도와주고 이해해준 선경언니, 선인언니, 선현언니, 선희, 그리고 두 형부들에게 이 논문을 바칩니다.

2009년 6월

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ABSTRACT

Identification of the IgE binding epitopes of peptide fragments of Bla g 6 from German cockroach (*Blattella germanica*)

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(Directed by Professor Tai-Soon Yong)

German cockroach allergens induce IgE-mediated hypersensitivity. Among the German cockroach allergens studied, Bla g 6 shows homology to muscle protein troponin C. It contains four calcium-binding domains at 20-30, 56-67, 96-107, and 132-143 amino acid (aa) residues, and its immunoglobulin E (IgE) reactivity is dependent upon calcium ion level. However, the IgE binding epitopes of Bla g 6 have not been studied. This study aimed to identify the IgE binding epitopes from the five linear peptide fragments of Bla g 6. The full-length and five peptide fragments (P1: aa 1-111, P2: aa 1-95, P3: aa 33-111, P4: aa 80-151, and P5: aa 33-151) of Bla g 6 were generated by polymerase chain reaction (PCR) and expressed in *Escherichia coli* (*E. coli*).

Enzyme-linked immunosorbent assay (ELISA) was performed on 24 patients' sera that adjusted the final concentration 10 mM of $CaCl_2$ to determine the IgE activities of Bla g 6. Eight sera (33.3%), 9 sera (37.5%), and 11 sera (45.8%) showed IgE reactivity to Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301, respectively. Among the sera from the positive IgE reactivity, three patients' sera were selected and the IgE reactivity was measured by ELISA with the five recombinant peptide fragments of Bla g 6. Based on IgE responses, one patient's serum exhibited the strongest IgE reactivity. I assumed that the aa between 96-151 residues, including the calcium binding domains III and IV, would be important for IgE binding. These results may provide information that will yield safe diagnostic methods and immunotherapeutics.

Key words: allergen, *Blattella germanica*, calcium binding protein, IgE, epitope, troponin C

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I. INTRODUCTION

It has been reported that about 3% to 17% of allergic patients in Korea are sensitized to outdoor allergens derived from pollens, trees, bushes, grass, etc. However, indoor allergens such as house dust mites (HDM), cockroaches, cat dander, dog hair, etc are mainly responsible for the allergic diseases. Approximately 60% of allergic patients and 17.2% of patients who attended the allergy clinic at Severance Hospital, Yonsei University College of Medicine, Seoul, Korea in 2005 showed positive skin prick test reactivity to HDM and cockroaches, respectively. This indicates that indoor arthropods

are important sources of allergens that induce type-I hypersensitivity to genetically predisposed persons, compared to outdoor allergens[1].

Cockroaches were first described as an indoor allergens in the USA in 1964, and as a result, 28% of 114 allergic patients were sensitized to the cockroach extracts, *Periplaneta americana* and *Blatta orientalis*[2]. Four species of cockroaches, *Blattella germanica*, *Periplaneta americana*, *Periplaneta fuliginosa*, and *Periplaneta japonica* are commonly found in human dwellings in Korea. The distribution of cockroach species varies across different geographical regions. For instance, *Blattella germanica* is present in the US and Europe, *Periplaneta americana* is present in South America and several Asian countires[3]. Several studies have determined that cockroaches are important sources that elicit allergic diseases such as asthma, atopic dermatitis, and rhinitis[4]. These allergic disorders are usually present people who live in the inner city and urban areas and places where cockroach allergens are detectable in the kitchens and bedrooms of suburban or inner city homes. Subjects showed different hypersensitivities to cockroach allergens according to social status and race, especially in children[5-7]. Saliva, fecal substances, secretions, cast skins, debris, and the dead bodies of cockroaches are known

to include several allergenic components[8].

Seven German cockroach allergens and six American cockroach allergens have been isolated, cloned, and the recombinant proteins have been expressed in order to identify and characterize biological functions or allergenic properties of each allergen[9,10]. The German and American cockroach allergens Bla g 1 and Per a 1 are midgut microvilli protein homologs[11,12], Bla g 2 is an inactive aspartic protease[13], Per a 3 is an arylphorin/hemocyanin[14], Bla g 4 is a lipocalin[15], Bla g 5 is a glutathione *S*-transferase (GST)[16], Bla g 6 and Per a 6 are troponin C[17], Bla g 7 and Per a 7 are tropomyosin[18,19], Bla g 8 is a myosin light chain[3], Per a 9 is an arginine kinase[20], and Per a 10 is a serine protease[21].

Hindley et al.[17] identified three isoallergen of Bla g 6 with 17 kDa molecular weights (Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301). A binding frequency of 14% (n=104) of IgE was shown, indicating that it is a minor allergen. However, the intensity of IgE reactivity is increased with the addition of calcium ions. Bla g 6 consists of two EF-hand calcium binding domains in the aa sequences. The 12 residues are directly involved for the EF-hand calcium binding domains and form a loop. Six aa of 12

residues are directly involved to bind calcium ions at the position 1, 3, 5, 7, 9, and 12[3,17].

IgE molecules bind to the specific binding sites of the allergens, which are called an epitope, and the epitopes can be classified into conformational epitopes and linear epitopes. The synthesized or expressed overlapping peptides are very useful for finding the linear epitopes of a specific allergen. A hypoallergen for immunotherapy could be generated based on the information obtained from B and T cell epitope mapping. Allergen specific immunotherapy could be used instead of medicinal therapy and could reduce several of the possible side effects that could be elicited during the long-term administration of drugs[22,23]. Among German cockroach allergens, Bla g 2, Bla g 4, and Bla g 5 were subjected to analyze the sequence variations and identify the IgE binding epitopes using recombinant peptide fragments of the predominant isoforms[24-27].

This study was undertaken to investigate the IgE binding epitopes of Bla g 6. Recombinant proteins of the three isoallergens and five peptide fragments derived from Bla g 6.0101 were expressed, and their IgE reactivity was examined. The information obtained in this study may helpful in developing strategies for component-resolved diagnoses and immunotherapeutics for cockroach allergies.

II. MATERIALS AND METHODS

1. Subjects and serum samples

Serum samples were obtained from the subjects who diagnosed with German cockroach allergies according to their case histories and skin prick testing at the Allergy Clinic of the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. These sera were examined for specific IgE reactivities to German cockroaches using the Uni-CAP system (Pharmacia, Uppsala, Sweden) and ELISA. Among the sensitized sera, 24 serum samples showed concentrations greater than 0.7 kU/l and were selected for the current study (n =24, aged 9-58 years, mean 36 years). Fifteen sera from healthy individual were used as negative controls.

2. German cockroaches

Adult cockroaches were reared at the Korea National Arthropods of Medical Importance Resource Bank, Department of Environmental Medical Biology, Yonsei University College of Medicine, Seoul, Korea. Cockroaches were fed with mouse food.

Among the cockroach colony, adult cockroaches were selected after euthanization with carbon dioxide gas and kept at -70°C until use.

3. Molecular cloning of the full-length and five peptide fragments of Bla g 6 cDNA

Trizol reagent (GibcoBRL, Rockville, MD, USA) was used on 10 adult German cockroaches in order to isolate total RNA right after freezing the cockroaches with liquid nitrogen. To synthesize the first strand of full-length Bla g 6 cDNA of the three isoallergens, a mixture of 5.0 μg of total RNA and 1 μg of oligo dT was reverse transcribed to cDNA and amplified by PCR. The specific oligonucleotide primer sequences were designed to contain the *Bam*HI and *Xho*I restriction sites and 6 histidine sequences based on the Bla g 6 sequence of the Genbank database (Accession no. DQ279092, DQ279093, and DQ279094) (Table 1). The 50 μl of PCR reaction mixture was composed of 5 μl of cDNA, 1 μl of 10 μM forward and reverse primers, 5 μl of 10X Taq buffer, 1 μl of 10 mM dNTP, 0.5 μl (2.5 units) of Taq DNA polymerase (Solgent, Daejeon, Korea), and 36.5 μl of the distilled water. The 35 cycles of PCR were

performed after 5 min of initial denaturation at 95°C. Each cycle was performed under the following conditions: 30 sec at 94°C, 30 sec at 52°C, and 1 min at 72°C. The final extension was carried out at 72°C for 9 min. The PCR products were analyzed in 1% agarose gel and purified with an UltraClean™ DNA Purification Kit (Mo Bio Laboratories, Inc., Solana Beach, LA, USA). The purified Bla g 6 DNA fragments were ligated into pGEM-T Easy vectors (Promega, Madison, WI, USA) or TOPO vectors (Invitrogen, Carlsbad, CA, USA) and then the DNA sequences were determined at Solgent.

For the IgE mapping of Bla g 6.0101, the full- length cDNA of Bla g 6.0101 was fragmented into five peptides (P1: aa 1-111, P2: aa 1-95, P3: aa 33-111, P4: aa 80-151, and P5: aa 33-151) based on the calcium binding domains (Figure 1). The specific primer sequences were designed to contain the *Bam*HI restriction sites for forward primers and the *Xho*I restriction sites and 6 histidine sequences for reverse primers (Table 1). To clone peptide fragments, plasmid containing full-length Bla g 6.0101 was amplified by 35 cycles of PCR with appropriate primers as described above except the annealing temperature (59°C). After PCR reactions, the PCR products were analyzed on

1.5% agrose gel and ligated into TOPO vectors.

Table 1. Forward and reverse specific primer sequences of the full-length and five peptide fragments of Bla g 6

Bla g 6	Specific oligonucleotide primer sequences
BG6.0101F	5’-GGATCCAATGGATGAACTTCCTCCA-3’
BG6.0101R	5’-CTCGAGTTATTCTCCAGTCATGAC-3’
BG6.0201F	5’-GGATCCAATGGATGAAATTCCAGCA-3’
BG6.0201R	5’- CTCGAGTTATTCTCCAGTCATGAC-3’
BG6.0301F	5’- GGATCCAATGGCAGATGAGCAACTT-3’
BG6.0301R	5’- CTCGAGTTAGCCAGTCATCATCTC-3’
BG6P1F	5’-GGATCCAATGGATGAACTTCCTCCA-3’
BG6P1R	5’-CTCGAGGATCTCGCGGAGGACATT-3’
BG6P2F	5’-GGATCCAATGGATGAACTTCCTCCA-3’
BG6P2R	5’- CTCGAGGTACAATCTGAAGGCTTC-3’
BG6P3F	5’- GGATCCAGGCACCATCCTGGAGATG-3’
BG6P3R	5’- CTCGAGGATCTCGCGGAGGACATT-3’
BG6P4F	5’- GGATCCATCTCTGGCCTCTAGGTTC-3’
BG6P4R	5’- CTCGAGTTATTCTCCAGTCATGAC-3’
BG6P5F	5’- GGATCCAGGCACCATCCTGGAGATG-3’
BG6P5R	5’- CTCGAGTTATTCTCCAGTCATGAC-3’

The primers were designed to obtain the full-length of Bla g 6 isoallergens based on the sequences enrolled in the Genbank database (Accession no. DQ279092, DQ279093, and DQ279094) and the full-length Bla g 6.0101 was used for generating five overlapping peptide fragments. These primers contained the *Bam*HI restriction sites for forward primers and *Xho*I restriction sites for reverse primers. The restriction sites are underlined.

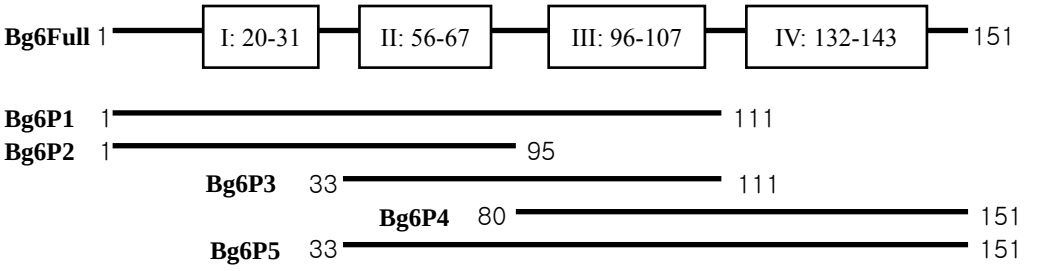

Figure 1. The scheme of the overlapping peptide fragments of Bla g 6.

The full-length Bla g 6 has two EF-hand domains at which calcium ions binds. To analyze the IgE binding epitopes, each peptide fragments (P1 to P5) was designed to include one to three different EF-hand binding domains.

4. Subcloning and expression of the full-length and five peptide fragments of Bla g 6 in *E. coli*

To prepare Bla g 6 recombinant proteins, all cDNA sequences encoding the full-length and five peptide fragments of Bla g 6, which were ligated into pGEM-T Easy or TOPO vectors, were digested with *Bam*HI and *Xho*I restriction enzymes. Those digested DNA fragments were subcloned into pET-28b expression vectors (Novagen, Merck Biosciences, Darmstadt, Germany) and then transformed into the competent *E. coli* BL21 (DE31) pLysS cells. A selected positive colony was inoculated into 10 ml of Luria Bertani (LB) broth and incubated overnight at 37°C in the shaking incubator between 210 and 230 rpm. These cultured cells were added to 1 L of LB broth and grown until the absorbance at 600 nm reached 0.6 under the same conditions. One mM of isopropyl-1-thio-β-galactopyranoside (ITPG) was added and incubated for an additional 4 hr. Cells were collected using centrifugation at 3,000 g for 15 min. In order to purify the recombinant proteins, cells were resuspended with the lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$) and lysed by French Pressure at 11,000 PSI and a sonicated at 5×10 sec with 10 sec pauses at 200-300 W. All of Bla g 6

recombinant proteins were purified from soluble fractions using nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA, USA). Purified proteins were run onto the Tris-Glycine gel (Invitrogen) and then visualized by Coomsassie brilliant blue R250 (Sigma-Aldrich, Sydney, Australia). The concentration of purified proteins was assessed by Bradford Assay (Bio-Rad Laboratories, Reagent Park, Australia).

5. IgE binding reactivity and eptiopes analysis of the full-length and five peptide fragments of Bla g 6

ELISA was performed not only to investigate the specific IgE binding response of Bla g 6 but also to identify the IgE binding epitopes of Bla g 6 with the five peptide fragments. Ten μg/ml of purified protein of Bla g 6 and five peptide fragments were coated per well in the 96 well microtiter plates and incubated at 4°C overnight. The plates were washed with phosphate buffered saline containing 0.05% Tween-20 (PBST) and then blocked with 200 μl of blocking buffers (3% w/v skim milk in PBST). After 1 hr of incubation at room temperature (RT), they were washed three times with PBST and then 50 μl of human sera (diluted 1:4 in PBST containing 1% bovine serum albumin

(BSA)) were added to wells and incubated for 1 hr at RT. Fifty μl of biotinylated goat anti-human IgE (diluted 1:1000 in PBST containing 1% BSA) (Vector, Burlingame, CA, USA) were added to detect the IgE antibodies and kept for 1 hr at RT after washing with PBST. Each well was incubated with 50 μl of streptavidin-peroxidase (diluted 1:1000 in PBST) (Sigma) for 30 min at RT. After washing five times with PBST, 3,3',5,5'-tetramethyl-benzidine (TMB, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added and placed in the dark for 15 min. Before detecting the signals, 100 μl of the stop solution (0.5 M H_2SO_4) was added and the signal was measured at an absorbance of 450 nm using the automatic microplate reader (TECAN, Salzburg, Austria). The cut-off values were determined by the following the equation: cut-off = (mean absorbance of 16 negative sera) + 2 × (standard deviations of 15 negative sera).

III. RESULTS

1. Molecular cloning of the full-lengths and five peptide fragments of Bla g 6

Reverse transcription polymerase chain reaction (RT-PCR) was performed with the specific primers (Table 1) to obtain the full-length cDNA sequences of the three isoallergens of Bla g 6 (Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301). All ten cloned sequences from each isoallergen contained 456 base pairs (bp) both for Bla g 6.0101 and Bla g 6.0201, and 465 bp for Bla g 6.0301. Those sequences encoded 151, 151, and 156 aa and their molecular mass was estimated to be 17,217 Da, 17,096 Da, and 17,963 Da for Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301, respectively. The deduced aa sequences of the three isoallergens of Bla g 6 showed no differences with the formerly reported Bla g 6 (Genbank accession No. DQ279092, DQ279093, and DQ279094).

PCR was carried out with the specific primers designed to amplify the overlapping five peptide fragments of Bla g 6.0101 (Table 1). The five peptide fragments (P1-P5) of Bla g 6.0101 of 333 bp, 285 bp, 237 bp, 246 bp, and 357 bp were

produced with the deduced aa residues 153, 137,115, 115, and 152, respectively. Their molecular masses were calculated to be 17.4 kDa for P1, 15.6 kDa for P2, 13.0 kDa for P3, 12.8 kDa for P4, and 17.0 kDa for P5.

2. Recombinant proteins of Bla g 6

To obtain the recombinant Bla g 6, cDNAs encoding Bla g 6 and five peptide fragments of Bla g 6 were subcloned into pET-28b vectors including 6 histidine-tagged proteins and then expressed in *E. coli*. All recombinant proteins were purified from soluble fractions. The purified recombinant proteins of Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301 showed 21 kDa bands at the SDS-PAGE analysis. The five recombinant peptides fragments were separated in the gradient SDS gel (Invitrogen) and showed approximately 13 kDa to 17 kDa bands, as expected (Figure 2).

The yield of each recombinant protein was 9.75 mg for Bla g 6.0101, 7.11 mg for Bla g 6.0201, 1.61 mg for Bla g 6.0301, 25.8 mg for P1, 18.1 mg for P2, 26.5 mg for P3, 17.2 mg of P4, and 18.0 mg for P5 from 1 L bacterial cultures.

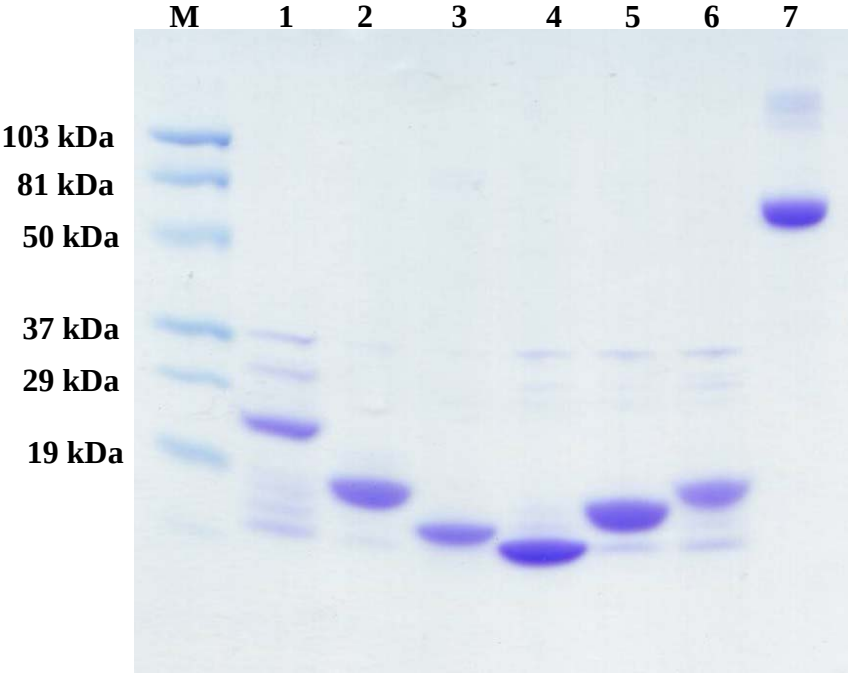

Figure 2. Purified proteins of recombinant Bla g 6 peptide fragments.

The five recombinant fragments were separated in the gradient SDS gel. Lane M corresponds to molecular weight marker, lane 1 to the full-length rBla g 6.0101, lane 2 to recombinant peptide fragment 1 (rP1), lane 3 to recombinant peptide fragment 2 (rP2), lane 4 to recombinant peptide fragment 3 (rP3), lane 5 to recombinant peptide fragment 4 (rP4), lane 6 to recombinant peptide fragment 5 (rP6), and lane 7 to BSA.

3. IgE reactivity to rBla g 6 isoallergens

The cut-off values were determined to be 0.0858, 0.0679, and 0.0919 of rBla g 6.0101, rBla g 6.0201, and rBla g 6.0301, respectively. However, only one patient's serum sample showed the significantly high IgE reactivity against rBla g 6 and other samples displayed IgE titers slightly higher than the cut-off values of the three rBla g 6 isoallergens. Of 24 patient sera, the IgE antibodies of 8 (33.3%), 9 (37.5%), and 11 (45.8%) patients' sera recognized as the recombinant Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301, respectively. Among 24 patients' sera, 5 patients' sera (S8, S14, S20, S22, and S23) showed the positive IgE reactivity to all of rBla g 6 isoallergens, two patients' sera (S2 and S17) showed positive IgE reactivity to rBla g 6.0101 and rBla g 6.0301, three patient sera (S16, S19, and S24) exhibited positive IgE reactivity to rBla g 6.0201 and rBla g 6.0301, and S9, S18, and S21 sera showed positive IgE reactivity to one of three isoallergens (Table 2).

Table 2. The IgE reactivity of recombinant Bla g 6 isoallergens

No.	Sex	Age	Diagnosis [a]	Absorbance at 450 nm		
				rBla g 6.0101	rBla g 6.0201	rBla g 6.0301
S1	M	49	AR	0.0760	0.0520	0.0845
S2	M	52	AR, AA	0.0930	0.0575	0.0970
S3	M	48	Dyspnea	0.0715	0.0545	0.0735
S4	M	14	Rhinitis	0.0760	0.0585	0.0875
S5	M	26	AD	0.0830	0.0555	0.0785
S6	F	46	Allergic cough	0.0765	0.0580	0.0805
S7	M	35	AS	0.0840	0.0565	0.0850
S8	M	47	Dermatitis	0.1060	0.0710	0.1125
S9	M	43	AA	0.0915	0.0555	0.0855
S10	M	13	AR	0.0745	0.0570	0.0755
S11	M	29	AR, AA	0.0595	0.0575	0.0665
S12	F	9	AR	0.0735	0.0625	0.0810
S13	M	49	Conjunctivitis	0.0725	0.0580	0.0785
S14	M	20	Dermatitis	1.6975	1.7010	1.6970
S15	M	26	AA	0.0700	0.0580	0.0830
S16	F	16	AD	0.0825	0.0775	0.1025
S17	F	22	Dermatitis, AE	0.0945	0.0650	0.0925
S18	F	57	AA	0.0820	0.0605	0.0920
S19	M	30	Urticaria	0.0825	0.0700	0.0945
S20	M	24	AA	0.0860	0.0735	0.1120
S21	M	56	Asthma	0.0855	0.0680	0.0915
S22	M	45	AR, AA	0.1025	0.0795	0.106
S23	M	38	AR	0.0905	0.0680	0.0990
S24	F	58	Bronchitis	0.0850	0.0750	0.0925

To investigate the IgE reactivity of three rBla g 6 isoallergens, ELISA was performed as duplication. Eight (33.3%), 9 (37.5%), and 11 (45.8%) sera from allergic patients were recognized recombinant Bla g 6 isoallergens. The underline indicates that human serum samples showed positive IgE reactivity to each rBla g 6 isoallergens. Allergic rhinitis,

allergic asthma, atopic dermatitis, anaphylactic shock and allergic eczema are abbreviated to AR, AA, AD, AS, and AE[a], respectively.

4. IgE epitope mapping of recombinant Bla g 6

To identify the IgE binding regions of Bla g 6, three patient' sera that showed positive IgE reactivity to all rBla g 6 isoallergens were selected (S8, S14, and S23) and ELISA was performed with the five recombinant peptide fragments (rP1-rP5) of Bla g 6.0101. Cut-off values of each fragment were 0.0663 for rBla g 6.0101 as the positive control, 0.0801 for rP1, 0.0680 for rP2, 0.0829 for rP3, 0.0708 for rP4, and 0.0652 for rP5. Sera from S8 and S14 showed positive IgE binding reactivity to all of the peptide fragments of rBla g 6. However, serum from S23 showed negative IgE binding reactivity only to the recombinant peptide fragment 1 (rP1). Compared to the positive IgE reactivity of two serum samples (S8 and S14) to each fragment, the serum from S14 showed much stronger reactivity than the sera from others. Absorbance at 450 nm measured to the recombinant full-length and five peptide fragment proteins of S14 were 2.0540, 1.1475, 0.7055, 1.0060, 1.8830, and 1.8910, respectively. The IgE reactivity of the recombinant peptide fragment 2 (0.7055), which consists of calcium binding domains I and II, was reduced approximately 3-fold compared to the full-length rBla g 6 (2.0540), even though recombinant peptide fragments 4 and 5 which contained calcium

binding domains III and IV, which retain about 95% IgE reactivity of the full-length rBla g 6. Based on these results, it is thought that the important IgE binding epitope(s) is located between calcium binding domains III and IV (Table 3, Figure 3).

Table 3. IgE reactivity of recombinant Bla g 6 peptide fragments

	Recombinant Protein of Bla g 6					
	Full-length	**P1**	**P2**	**P3**	**P4**	**P5**
S8	0.1670	0.1820	0.1815	0.1895	0.1585	0.1720
S14	2.0540	1.1475	0.7055	1.0060	1.8830	1.8910
S23	0.0720	0.0795*	0.0730	0.0870	0.0870	0.0755
BSA	0.0555	0.0675	0.0590	0.0680	0.0600	0.0525

Recombinant full-length Bla g 6.0101 was used for the positive control and BSA was used for the negative control. The IgE epitopes of Bla g 6 were analyzed using three sera samples that showed positive IgE responses to most of rBla g 6 isoallergens from the pervious ELISA study. Serum sample 8 and 14 exhibited the positive IgE reactivity to all of the five recombinant peptide fragments of Bla g 6.0101. However, Serum sample 23 showed negative IgE reactivity to rP1 that is indicated by the asterisk (*).

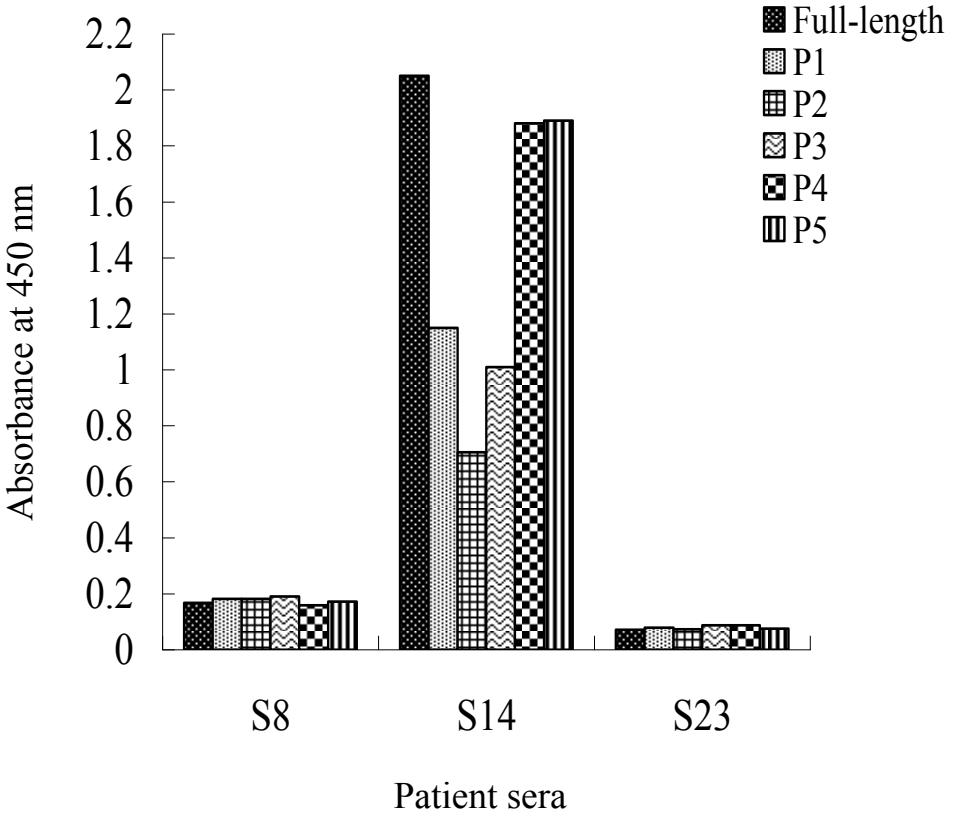

Figure 3. The IgE reactivity of recombinant Bla g 6 peptide fragments. Serum sample 14 (S14) maintains about 95% IgE reactivity to recombinant peptide fragment 4 (rP4) and 5 (rP5) including the calcium binding domains III and IV. It is indicated that calcium binding domains III and IV are involved with IgE binding to Bla g 6.

IV. DISCUSSION

Calcium is essentially involved in a diverse set of cell physiologies, such as nucleotide metabolism, muscle contraction, cell cycle control, and cell signal transduction in cellular processes. When the calcium ions bind to the EF-hand protein, the conformational structure of the protein will change and it will subsequently influence their functional roles. The calcium binding proteins include calmodulin, troponin C, myosin light chain, and parvalbumins. Calcium binding proteins are found in various sources such as plants, insects, fishes, food, and human beings and classified by the numbers of the EF-hand domains that bind with calcium ions and consist of helix-loop-helix as a pair. More than thousands of calcium binding proteins are defined from different sources, but only 73 calcium binding proteins were described as allergens (http://www.allergome.org). Among two, three, or four EF-hand domains of calcium binding proteins, especially the allergens with two EF hand motifs, usually exist in pollens such as alder (*Alnus glutinosa*, Aln 4), birch (*Betula verrucosa*, Bet v 4), rape (*Brassica rapa*, Bra r 1, Bra r 2, and *Brassica napus*, Bra n 1, Bra n 2), Bermuda grass

(*Cynodon dactylon*, Cyn d 7), olives (*Olea europaea*, Ole e 3), and timothy grass (*Phleum pretense*, Phl p 7). Their allergenicities and epitopes have been well investigated[28,29]. Two allergens have been isolated from the German cockroach (*Blattella germanica*, Bla g 6) and house dust mites (*Dermatophagoides farinae*, Der f 17) as the calcium binding proteins from indoor arthropods. Bla g 6, a German cockroach allergen, is known to belong to the troponin C family[17,30].

However, basic information regarding the IgE binding epitopes of troponin C, one of the calcium binding proteins has been not fully studied. Until now, there were 13 allergens identified as troponin C from worms, German cockroaches, American cockroaches, American lobsters, and mites. *Anisakis simplex* (Ani s 1) was defined as troponin C that contains two EF-hand domains and reacted 20% of IgE antibodies from allergic patients. Based on its biological function, the troponin C proteins have sequence homologies so that troponin C from different sources could be cross-reactive[31].

When the sequences of each of the two EF-hand domains were analyzed with other troponin C proteins from different arthropods (*Apis mellifera*, *Drosophila melanogaster*, *Drosophila silvestris*, *Periplaneta americana*, *Solenopsis invicta,* and

Tribolium castaneum), calcium binding domains III and IV are more strongly conserved sequences than domains I and II (Figure 4). As EF-hand domains III and IV showed strong similarities to the Bla g 6 calcium binding domains III and IV, it is necessary to investigate the cross-reactivity among troponin C proteins from these arthropods.

Bla g 6 was found to be the minor allergen based on the result of the 14% positive IgE reactivity in the study of Hindley et al.[17]. Even though there were no differences of aa sequence identity between the cloned Bla g 6 isoallergens in this study and the previously reported Bla g 6, the IgE titers of Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301 showed different the IgE prevalence. Also, as shown in Table 2, each patient generated the different patterns of IgE reactivity to rBla g 6 isoallergens. This discrepancy could be explained by several reasons. First it is assumed that subtle changes in the aa sequence, namely allergen variants, may affect the allergenicity. For instance, Blo t 12 isoallergens that collected and isolated from different continents (Singapore and Cartagena, Colombia) showed 92% of aa identity and showed the different IgE reactivity. The allergen polymorphism may have the effect on IgE responses to the localized populations[32]. However, it can not explain the different IgE

reactivities of Bla g 6 because recombinant proteins with identical aa sequences have been investigated. Secondly, it may reflect the different genetic predispositions of subjects. The polymorphic HLA molecules were subjected to affect T cell reactivity, which could induce allergic responses. For instance, specific IgE reactivity of HDM allergens was dependent on the predisposed HLA class II genes: predisposed HLA-DPB1*1301 alleles and HLA-DPB1*0501 alleles showed the positive IgE responses to *Dermatophagoides pteronyssinus* 5 (Der p 5) and Der p 1 in Taiwan, respectively, but the normal HLA-DPB1*0201 allele showed negative specific IgE reactivity to Der p 1[33]. Thirdly, allergen-specific IgE binding reactivity can vary among different populations. The study by Hales et al. showed that the Australian aboriginal population, who has a high incidence of scabies infection, had higher IgE binding responses against the minor house dust mite allergen, Der p 4, than the major allergens, Der p 1 and Der p 2[34]. This study implies that different environments may influence the immune response to the same allergens. Lastly, different methods to test IgE reactivity or different expression system (Pichia or *E. coli*), which may affect the quality of allergens, may partly account for the difference in allergenicity.

Even though ELISA was carried out to determine the IgE epitopes of Bla g 6, the IgE epitope of Bla g 6 could barely be predicted from the results. Moreover, ELISA inhibition using immunodominant peptide fragments could not be carried out because of insufficient patient sera to confirm the IgE epitope of Bla g 6. Based on the one significant result of IgE reactivity, the IgE epitope is thought to be mainly located in the calcium binding domains III and IV. The calcium binding domains III and IV are known to have higher affinity for calcium ions compared to calcium binding domains I and II[35]. It would be interesting to investigate the relationship between the affinity for calcium ions and allergenicity of troponin C allergens.

The IgE epitope mapping of allergens could eventually yield the ability to provide tailored examination and therapy for IgE-mediated allergies in each patient by the means of the production of recombinant proteins and hypoallergenic allergens[36].

In summary, Bla g 6 isoallergens were cloned and expressed to analyze the IgE binding epitopes. Based on IgE responses of serum from a patient who exhibited the strongest IgE reactivity, I assume that the aa between the 96-151 residues, including the calcium-binding domains III and IV, could be important for IgE binding. These results

may provide information that will yield safe and effective diagnostics and immunotherapeutics for cockroach allergies.

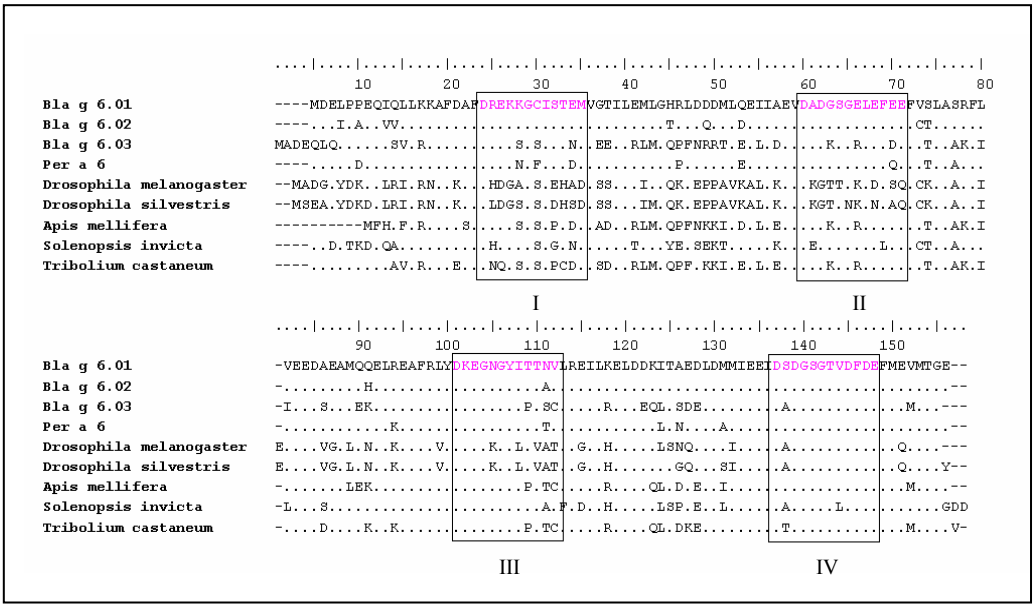

Figure 4. The sequence alignment of Troponin C from different arthropods. Bla g 6.0101, Bla g 6.0201, and Bla g 60301, *Blattella germanica* (German cockroach, Accession no. DQ279092, DQ279093, and DQ279094), Per a 6, *Periplaneta Americana* (American cockroach, Accession no AY792950), *Drosophila melanogaster* (Accession no NM078895), *Drosophila silvestris* (Accession no AF047329), *Apis mellifera* (Accession no NM001014430), *Solenopsis invicta* (Accession no AF432912), and *Tribolium castaneum* (Accession no XM968886). Calcium binding domain I, II, III, and IV of troponin C are boxed.

V. CONCLUSION

1. The cloned sequence of three Bla g 6 isoallergens, Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301, from Korea showed no variation with the formerly reported isoallergens (Genbank accession No. DQ279092, DQ279093, and DQ279094).
2. Even though the IgE prevalence of Bla g 6 was reported to be 14% in a previous study, 33.3%, 37.5%, and 45.8% of 24 patient sera showed the positive IgE reactivity to the recombinant proteins of Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301, respectively, in this study.
3. Based on the result of IgE epitope mapping with the five recombinant peptide fragments of Bla g 6.0101, I assume that the aa between the 96-151 residues, including the calcium-binding domains III and IV, could be important for IgE binding.

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ABSTRACT (in KOREAN)

독일바퀴(*Blattella germanica*)의 알레르겐, Bla g 6의 peptide 단편들의 IgE 반응 항원결정기(epitope)분석

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은 선 진

독일바퀴(*Blattella germanica*)은 천식과 같은 알레르기 질환을 유발하는 중요한 원인이며, 현재까지 7개의 알레르겐들이 밝혀졌다. 그중 Bla g 6는 근육수축에 관여하는 troponin C의 생물학적 특성을 가지고 있다. Bla g 6는 세 개의 isoallergens(Bla g 6.0101, Bla g 6.0201, Bla g 6.0301)이 보고되어 있으며, 2개의 EF-hand calcium binding domain을 포함하고 calcium 결합에 따라서 면역글로브린 E(IgE) 결합능이 2배정도 차이가 날 수 있다고 알려져 있지만 아직까지 IgE가 결합하는 epitope이 정확하게 밝혀지지 않았다. 본 연구에서는 독일바퀴의 알레르겐 Bla g 6의 전체서열에서 2개의 EF-hand calcium binding region이 서로 다르게 포함되도록 5개의 단편들로(P1: aa 1-111, P2: aa 1-95, P3: aa 33-111, P4: aa 80-151, P5: aa 33-151) 나누어

서 클로닝하고, expression vector에 과발현시킨 후 *E. coli*에서 재조합단백질을 얻었으며 그 후 재조합 Bla g 6 알레르겐에 반응하는 환자들의 혈청을 선별하여 효소결합면역흡착법(ELISA)을 수행하여 단편의 재조합 Bla g 6 단백질과 단편들과 반응하는 특이 면역글로브린 E 항체의 결합능을 살펴서 항원결정기(IgE binding epitope)을 규명하는 것을 목적으로 삼고 실험을 진행하였다. 24명의 환자들 중에 33.3%(8/24)가 Bla g 6.0101에, 37.5%(9/24)가 Bla g 6.0201에, 45.8%(11/24)가 Bla g 6.0301에 반응을 하였다. 24명중에 세 명의 환자혈청을 선택하여 항원 결정기를 규명하기 위한 실험한 결과, 전체 서열 중에 3번째와 4번째의 calcium-binding domains이 Bla g 6의 중요한 IgE 결합하는 항원결정기를 포함 할 것 이라는 것을 예측할 수 있었으며, 이 결과가 바퀴 알레르기 진단과 면역치료제 개발에 응용될 수 있을 것이다.

핵심되는 말: 알레르겐, 독일바퀴(*Blattella germanica*), calcium-binding protein, 면역글로브린 E, 항원결정기, troponin C

PUBLICATION LIST

Allergenicity of recombinant troponin C from *Tyrophagus putrescentiae*. Int Arch Allergy Immunol Aceepted.