

IgE Binding Epitopes of Bla g 6 from German Cockroach

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Abstract: Bla g 6, a German cockroach allergen, 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 investigated. This study aimed to analyze the IgE binding epitopes from the five peptide fragments of Bla g 6. The full-length of three Bla g 6 isoallergens (Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301) 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 6.0101 were generated by polymerase chain reaction (PCR) and expressed in *Escherichia coli*. Enzyme-linked immunosorbent assay (ELISA) was performed on 24 patients' sera that adjusted the final concentration 10 mM of CaCl₂ 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 peptide fragments of Bla g 6. Based on IgE responses, one patient's serum exhibited the strongest IgE reactivity. We 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.

Keywords: Allergen, *Blattella germanica*, calcium binding protein, IgE, epitope, troponin C.

I. INTRODUCTION

It has been reported that about 3% to 17% of Korean allergic patients 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 allergen in the USA in 1964, 28% of 114 allergic patients was reported to be 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 Korean homes. *Blattella germanica* and *Periplaneta americana* are commonly found in human dwellings although the distribution of cockroach species varies

across different geographical regions [3]. Several studies have determined that cockroaches are important sources of allergens which are responsible for eliciting allergic diseases such as asthma, atopic dermatitis, and rhinitis [4]. These allergic disorders usually affect people who live in the inner city and urban areas and places where cockroach allergens are detectable in the kitchens and bedrooms. 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 characterize biological functions or allergenic properties of each allergen [9,10].

Hindley *et al.* [11] 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). An IgE binding frequency of 14% (n=104) was shown, indicating that it is a minor allergen. Interestingly, 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 [10,11]. Recently, Tyr p 24, troponin C from storage mite, *Tyrophagus putrescentiae*, was also shown to have Ca-dependent IgE reactivity [12].

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The synthesized or expressed overlapping peptides are very useful for mapping 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 the possible side effects that could be elicited during the long-term administration of drugs [13,14].

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 reactivities were examined. The information obtained in this study may helpful in developing strategies for component-resolved diagnoses and immunotherapeutics for cockroach allergies.

2. MATERIALS AND METHODS

2.1. Subjects and Serum Samples

Serum samples were obtained from the subjects who were found to be sensitized to German cockroach 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.

2.2. 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 being euthanized with carbon dioxide gas and kept at -70°C until use.

2.3. Molecular Cloning of the Full-Length and Five Peptide Fragments of Bla g 6 cDNA

Trizol reagent (GibcoBRL, Rockville, MD, USA) was applied to 10 adult German cockroaches right after freezing with liquid nitrogen to isolate total RNA. 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 µM 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 based on the Bla g 6 sequence (Accession no. DQ279092, DQ279093, and DQ279094) (Table 1). 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 vector (Promega, Madison, WI, USA) or pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and then the DNA sequences were determined at Solgent (Daejeon, Korea).

Table 1. Forward and Reverse Specific Primer Sequences of the Full-Length and Five Peptide Fragments of Bla g 6

Primers	*Specific oligonucleotide primer sequences
BG6.0101F	5'- <u>GGATCCA</u> ATGGATGAACTTCCTCCA-3'
BG6.0101R	5'- <u>CTCGAGT</u> TATTCTCCAGTCATGAC-3'
BG6.0201F	5'- <u>GGATCCA</u> ATGGATGAAATTCAGCA-3'
BG6.0201R	5'- <u>CTCGAGT</u> TATTCTCCAGTCATGAC-3'
BG6.0301F	5'- <u>GGATCCA</u> ATGGCAGATGAGCAACTT-3'
BG6.0301R	5'- <u>CTCGAGT</u> TAGCCAGTCATCATCTC-3'
BG6P1F	5'- <u>GGATCCA</u> ATGGATGAACTTCCTCCA-3'
BG6P1R	5'- <u>CTCGAGG</u> ATCTCGCGGAGGACATT-3'
BG6P2F	5'- <u>GGATCCA</u> ATGGATGAACTTCCTCCA-3'
BG6P2R	5'- <u>CTCGAGG</u> TACAATCTGAAGGCTTC-3'
BG6P3F	5'- <u>GGATCCA</u> GGCACCATCCTGGAGATG-3'
BG6P3R	5'- <u>CTCGAGG</u> ATCTCGCGGAGGACATT-3'
BG6P4F	5'- <u>GGATCCA</u> ATCTCTGGCCTCTAGGTTC-3'
BG6P4R	5'- <u>CTCGAGT</u> TATTCTCCAGTCATGAC-3'
BG6P5F	5'- <u>GGATCCA</u> GGCACCATCCTGGAGATG-3'
BG6P5R	5'- <u>CTCGAGT</u> TATTCTCCAGTCATGAC-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.

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 (Fig. (1)). The full-length Bla g 6.0101 ligated into pGEM-T Easy vector was used as template for the amplification of peptide fragments by PCR with appropriate primers. After PCR reactions, the PCR products were analyzed on 1.5% agarose gel and ligated into pCR4-TOPO vectors.

2.4. 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 vector or pCR4-TOPO vector, were digested with *Bam*HI and *Xho*I restriction enzymes. Those digested DNA fragments were subcloned into pET-28b expression vector (Novagen, Merck Biosciences, Darmstadt, Germany) and then transformed into the competent *E. coli* BL21 (DE3) pLysS. Transformed cells were grown in 1 l of Luria Bertani broth. One mM of isopropyl-1-thio-β-galactopyranoside (IPTG) was added when the absorbance at 600 nm reached 0.6 and incubated for an additional 4 hr. 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 4-20% Tris-Glycine gel (Invitrogen) and then visualized by Coomassie brilliant blue R250 (Sigma-Aldrich, Sydney, Australia). The concentration of purified proteins was assessed by Bradford Assay (Bio-Rad Laboratories, Reagent Park, Australia).

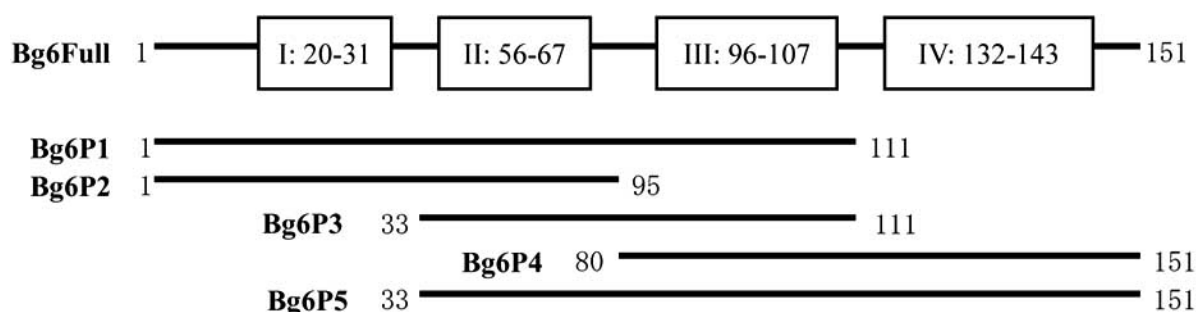


Figure 1. The scheme of the overlapping peptide fragments of Bla g 6. The full-length Bla g 6 has two EF-hand domains at which calcium ions binds. To analyze the IgE binding epitopes, each peptide fragments (P1 to P5) was designed to include one to three different EF-hand binding domains.

2.5. IgE Binding Reactivity and Epitope 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 $\mu\text{g}/\text{ml}$ of purified Bla g 6 and five peptide fragments were coated per well in the 96 well microtiter plates. The plates were washed with phosphate buffered saline containing 0.05% Tween-20 (PBST) between each incubation. The wells were incubated with 200 μl of blocking buffers (3% w/v skim milk in PBST) for 1 hr, 50 μl of human sera (diluted 1:4 in PBST containing 1% bovine serum albumin (BSA)) for 1 hr, 50 μl of biotinylated goat anti-human IgE (diluted 1:1000 in PBST containing 1% BSA) (Vector, Burlingame, CA, USA) to detect the IgE antibodies for 1 hr, 50 μl of streptavidin-peroxidase (diluted 1:1000 in PBST) (Sigma) for 30 min. For color development, 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 15 negative sera) + (standard deviations of 15 negative sera) $\times 2$.

3. RESULTS

3.1. Molecular Cloning of the Full-Length and Five Peptide Fragments of Bla g 6

Reverse transcriptase 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). The 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 masses were 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 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.

3.2. Production of 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 vector 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 apparent bands of about 21 kDa at the SDS-PAGE analysis (Data not shown). The five recombinant peptides fragments were separated in the gradient SDS gel and showed approximately 13 kDa to 17 kDa bands, as expected (Fig. (2)).

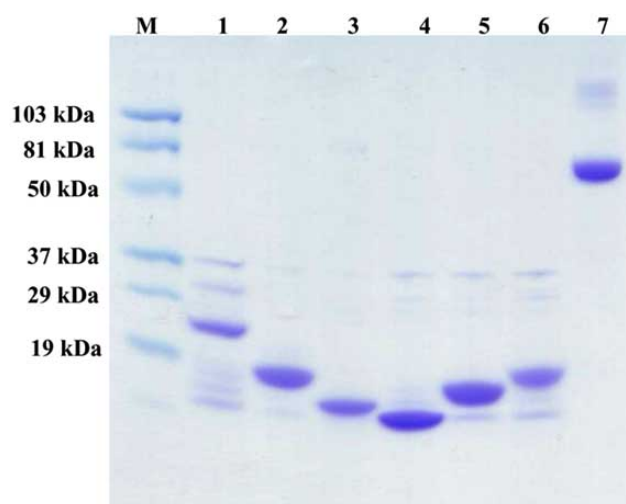


Figure 2. Purified proteins of recombinant Bla g 6 peptide fragments. The five recombinant fragments were separated in the 4-20% gradient SDS gel. M, molecular weight marker; 1, the full-length rBla g 6.0101; 2, recombinant peptide fragment 1 (P1); 3, recombinant peptide fragment 2 (P2); 4, recombinant peptide fragment 3 (P3); 5, recombinant peptide fragment 4 (P4); 6, recombinant peptide fragment 5 (P6); 7, BSA.

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.

3.3. IgE Reactivity to rBla g 6 Isoallergens

The cut-off values of ELISA 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. 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. 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. 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).

3.4. IgE Epitope Mapping of Recombinant Bla g 6

To identify the IgE binding regions of Bla g 6, three patient's sera that showed positive IgE reactivity to all rBla g 6 isoallergens were selected (S8, S14, and S23) and ELISA

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	<u>0.0930</u>	0.0575	<u>0.0970</u>
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	<u>0.1060</u>	<u>0.0710</u>	<u>0.1125</u>
S9	M	43	AA	<u>0.0915</u>	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	<u>1.6975</u>	<u>1.7010</u>	<u>1.6970</u>
S15	M	26	AA	0.0700	0.0580	0.0830
S16	F	16	AD	0.0825	<u>0.0775</u>	<u>0.1025</u>
S17	F	22	Dermatitis, AE	<u>0.0945</u>	0.0650	<u>0.0925</u>
S18	F	57	AA	0.0820	0.0605	<u>0.0920</u>
S19	M	30	Urticaria	0.0825	<u>0.0700</u>	<u>0.0945</u>
S20	M	24	AA	<u>0.0860</u>	<u>0.0735</u>	<u>0.1120</u>
S21	M	56	Asthma	0.0855	<u>0.0680</u>	0.0915
S22	M	45	AR, AA	<u>0.1025</u>	<u>0.0795</u>	<u>0.106</u>
S23	M	38	AR	<u>0.0905</u>	<u>0.0680</u>	<u>0.0990</u>
S24	F	58	Bronchitis	0.0850	<u>0.0750</u>	<u>0.0925</u>

^aTo 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, respectively.

was performed with the five recombinant peptide fragments (P1-P5) of Bla g 6.0101. Cut-off values of each fragment were determined to be 0.0663 for full-length rBla g 6.0101, 0.0801 for P1, 0.0680 for P2, 0.0829 for P3, 0.0708 for P4, and 0.0652 for P5. 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 (P1). 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).

4. 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 g 4), birch (*Be-*

tula 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 pratense*, Phl p 7). Their allergenicities and epitopes have been well investigated [15,16]. Bla g 6 from the German cockroach (*Blattella germanica*), Der f 17 from house dust mites (*Dermatophagoides farinae*), and Tyr p 24 from storage mite (*Tyrophagus putrescentiae*) have been characterized as the calcium binding proteins among indoor arthropods allergens [11,12,17]. Bla g 6 and Tyr p 24 belongs to the troponin C family [11,17].

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 are 14 allergens identified as troponin C from worms, German cockroaches, American cockroaches, American lobsters, and mites. Ani s 1 from *Anisakis simplex* was defined as troponin C that contains two EF-hand domains and reacted 20% of IgE antibodies from allergic patients. The troponin C proteins from various organisms have sequence homologies and troponin C proteins could be cross-reactive [18], although limited degree of cross-reactivity between Bla g 6 and Tyr p 24 was described [12].

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 than domains I and II (Fig. (3)). 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.* [11]. 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 of aller-

Table 3. IgE Reactivity of Recombinant Bla g 6 Peptide Fragments

Sera	Recombinant Proteins*					
	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
	0.0663	0.0801	0.0680	0.0829	0.0708	0.0652

*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 previous 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.

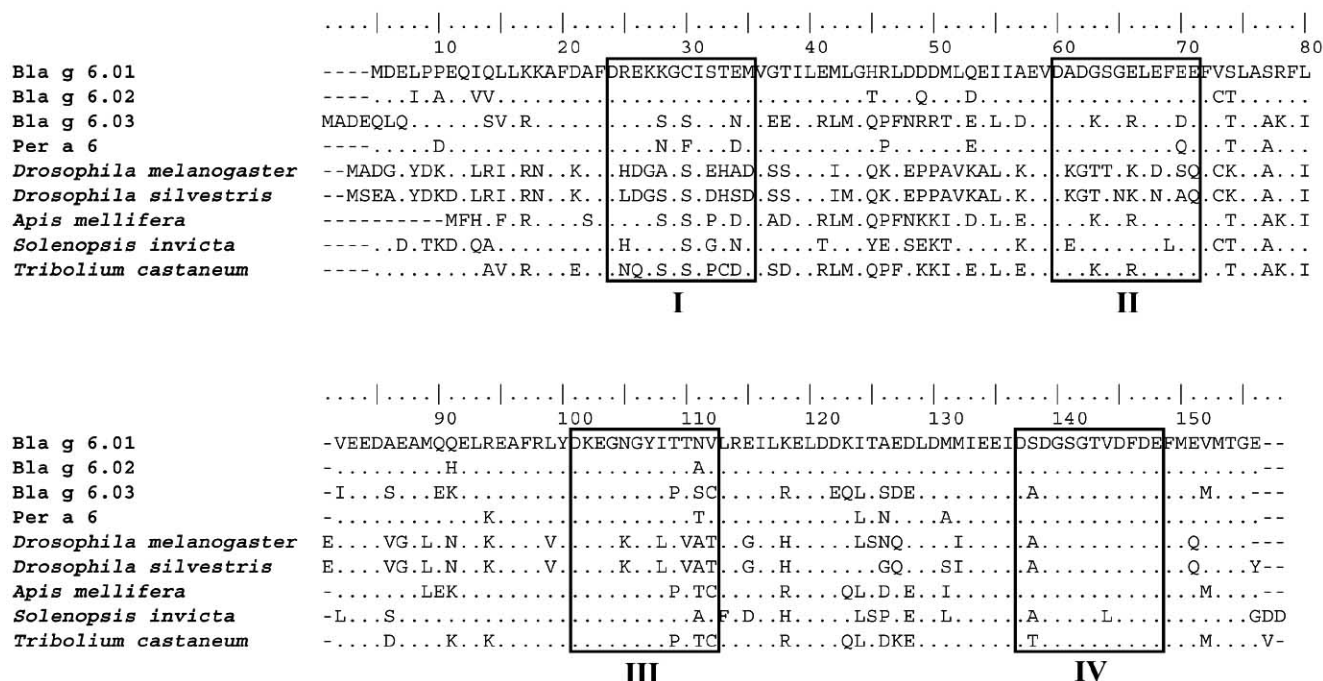


Figure 3. The sequence alignment of troponin C from different arthropods. Bla g 6.0101, Bla g 6.0201, and Bla g 6.0301, *Blattella germanica* (German cockroach, Accession no. DQ279092, DQ279093, and DQ279094), Per a 6, *Periplaneta Americana* (American cockroach, Accession no. AY792950), *Drosophila 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.

genicity 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 effects on IgE responses to the localized populations [19]. 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 house dust mite 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 [20]. 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 [21]. This study implies that different environmental conditions such as sanitation and health status of individuals 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 major 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 [22]. 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 [23].

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, we 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.

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