Sequence variation and IgE-binding reactivity of peptide fragments of Bla g 4, a major German cockroach allergen

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Directed by Professor Tai-Soon Yong

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This certified that the Master's Thesis of Kwang Hyun Shin is approved.

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It has been said that in the pursuit of scientific achievement, especially in the rapidly changing world of biology, graduate students will be constantly buffeted with new technologies and methods such that they would never be able to sit down and write a thesis that is on the cutting edge of their field. I can’t help reflecting for such efforts on myself.

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Cockroaches are a major cause of asthma. Recombinant cockroach allergens have been identified and characterized, and different polymorphisms of major allergens have been described in various geographical regions. A change in the amino acid sequence of cockroach allergens could affect IgE binding capacities and T cell responses. In this study, reverse transcriptase-PCR was carried out to examine the sequence variations of the major German cockroach allergen Bla g 4, a member of the calycin protein family. A total of 27 different variants of Bla g 4 were identified by analysis of 51 clones obtained by RT-PCR. A Bla g 4 variant sequence appeared in 14 of the 51 clones (27.5%), indicating that it is a dominant form (EF202172). Differences between the dominant form (27.5%) obtained in this study and previously reported Bla g 4 (U40767) forms were found at 13 amino acid positions: amino acid position 8 (T to S), 13 (N to M), 62 (V to Y), 64 (K to T), 69 (K to Q), 71 (K to N), 73 (K to N),

75 (T to A), 110 (E to D), 131 (I to L), 133 (F to L), 134 (S to T), and 135 (V to W). Of note, the amino acid residues at 51-75 and 132-155 showed a high degree of sequence variation. These data suggest that Bla g 4 is highly polymorphic. A multiple amino acid sequence alignment with eight allergenic lipocalin family proteins was carried out by using the Clustal X program. Despite the probable structural similarity, the identity between the sequences was very low, showing only the two identical residues, Gly33 and Trp35 to the main conserved segment of the lipocalin family. Their influence on IgE-binding capacity and T cell responsiveness will be investigated in order to better understand immune responses to Bla g 4. The aim of this study was to investigate IgE reactivity to recombinant Bla g 4 (rBla g 4) in the sera of allergic patients and identify linear IgE binding epitopes. For protein expression, full-length Bla g 4 (EF202172) was divided into five overlapping peptide fragments (E1~E5; E1: aa 1-100, E2: aa 34-77, E3: aa 74-117, E4: aa 114-156, E5: aa 153-182). The full-length and five peptide fragments of Bla g 4 were generated by PCR and over-expressed in *E. coli* BL21 (DE3). The IgE binding reactivities of the full-length and peptide fragments were measured by enzyme linked immunosorbent assay (ELISA) using 32 serum samples of from patients with cockroach allergies. The sera of 8 patients (25%) reacted with rBla g 4, a prevalence significantly lower than the 40~60% reported previously by Arruda et al (1996). Four sera (100%) showed IgE-binding reactivity to full-length and peptide fragment 4, 3 sera (80%) reacted with peptide fragment 2, one (20%) serum

sample reacted with peptide fragment 3. The ELISA results from the overlapping recombinant fragments localized the epitope region to amino acids 34~73 and 78~113, with the major IgE epitope of Bla g 4 localized to amino acid residues 118~152 of the C-terminus. Heterogeneity in IgE responses to Bla g 4 in individual patients was also observed, correlating with the finding that the most allergens have multiple IgE-binding sites which are at least partly due to the polyclonal nature of the immune response to protein allergens. Out results may provide fundamental bases for developing effective diagnostics and therapeutics for treating cockroach allergies.

Key words : allergen, *Blattella germanica*, variation, epitope, lipocalin

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I. INTRODUCTION

The treatment of allergic diseases in the past was largely dependent on medication.[1] However, the knowledge of the protein chemistry of allergens has improved during the past 20 years. Allergen standardization is no longer based on protein nitrogen units or weight/volume estimates. Biological and immunochemical procedures have been introduced by regulatory authorities in the United States and Europe to improve standardization of natural allergenic products.[2,3] However, allergens prepared from natural source materials remain heterogeneous products, containing many non-allergenic proteins and other macromolecules that are parenterally administered together with the few active components, the protein allergens.[2] Natural allergenic products are also at risk of being contaminated with allergens from other

sources and can contain proteolytic enzymes.[4] These enzyme may be either allergenic or non-allergenic and can decrease the stability of specific allergens when treated together with other allergens during immunotherapy.[5] Occasionally, standardized allergen vaccines contain endotoxins,[6,7] which can cause unpredictable effects such as enhanced T_H 1 responses or toxic and inflammatory responses.[8] It is difficult to standardize a specific allergen in a mixture of different proteins. These limitations can improve on natural allergenic products by use of recombinant allergens.[9] The use of recombinant allergens in structural and immunologic studies requires the production of large quantities of allergen that can be easily purified from suitable expression vectors. Recombinant allergens for immunotherapy may be used to alter immune responses or to reduce the allergenic activity of other allergens. Genetically modified recombinant allergens provide several advantages. These molecules preserve the repertoire of allergen-specific T cell epitopes and inhibit the binding of allergic patients' IgE antibodies to allergens represented by B cell epitopes.[10] Specially, hypoallergens with reduced allergenic activity could be obtained by site-directed mutagenesis of IgE binding epitopes. These studies are useful for the designing of specific immunotherapy.

Cockroaches are insects of the Order Blattodea. The name of the order is derived from the Greek word for "cockroach," blatta. There are roughly 3,500 species in six families. Cockroaches exist worldwide, except in polar regions. The most commonly found domestic

species of cockroach are *Blattella germanica* (German cockroach) and *Periplaneta Americana* (American cockroach). In Korea, cockroaches were found in 62% (107/174) of homes, i.e., 63 with *B. germanica*, 60 with *Periplaneta* spp., which included 16 homes infested with both.[11]

Exposure to high levels of cockroach allergens is an important factor for the production of IgE antibodies and development of allergic disease. Cockroaches have been reported to be associated with asthma in many regions of the world, including Taiwan, Japan, Thailand, and Singapore in the Pacific Rim; Costa Rica and Puerto Rico in Central America; India; South Africa; and parts of Europe.[12] These problems were caused by high levels of cockroach allergens in low-cost public housing,[13] which were associated with positive allergy skin test responses in a group of children with mild-to-moderate asthma.[14] Recently, among urban asthmatic subjects sensitized to cockroach allergens, exposure to high levels of cockroach allergens in the home was strongly associated with increased hospitalizations and other measures of asthma morbidity.[15] Patients with cockroach allergies had especially high levels of serum IgE antibodies compared to general asthmatics. Although the effect of professional extermination and house cleaning resulted in cockroach population decrease and substantial reduction of allergen levels in some homes, the allergen levels in most homes remained high enough to induce asthma symptoms in sensitized individuals.[16]

Several German cockroach allergens, which stimulate IgE production and cause IgE-mediated

diseases, have been identified. Six allergens have been cloned and immunologically characterized from *B. germanica*. These allergens differ in structure and function. Bla g 1 has variable molecular weights,[17-19] with a primary structure that consists of several tandem repeats of approximately 100 amino acids. The prevalence of IgE binding to purified protein was approximately 77%,[20] but the function of Bla g 1 remains unknown. Bla g 2 is 36 kDa and an inactive aspartic protease,[21] the prevalence of IgE antibodies was from 60% to 68%.[22] Bla g 4 has an estimated molecular weight of 21 kDa and belongs to the lipocalin super-family, which binds or transports small hydrophobic molecules.[23] IgE binding reactivity to recombinant protein was found in 40~60% of cockroach-allergic patients.[24] Bla g 5 has been identified as a 23 KDa glutathione S-transferase (GST). IgE antibodies against Bla g 5 were found in 70% of cockroach-allergic patients.[25] Bla g 6 encodes troponin C, an 18 kDa[26] calcium-binding subunit of troponin that regulates muscle contraction. The prevalence of IgE binding to recombinant protein was 14%.[27] Bla g 7 encoded the 33 KDa tropomyosin, which exhibited a IgE binding reactivity of 16.2% among patients with cockroach allergies.[28] In a recent study, the relative importance of cockroach allergens (Bla g 1, Bla g 2, Bla g 4, Bla g 5, and Bla g 7) was measured by IgE antibodies in over 100 sera from cockroach-sensitized subjects. Thirty-six of the sera did not react any of 5 the allergens, despite having a positive IgE CAP to *B. germanica*.[29] This result indicated that these 5 allergens do not represent the full repertoire of

German cockroach allergens, suggesting that other allergens exist in *B. germanica*.

Bla g 4 is a species-specific allergen produced by *B. germanica*[23] and expressed in a sex-specific manner in the reproductive system of adult male German cockroaches. It is associated with the spermatophore, transferred to the female during copulation and developmentally regulated by juvenile hormone.[30] Although the sequence identity between Bla g 4 and lipocalins is low (19~24%), it contains the three structurally conserved regions (SCR) of lipocalin. Structural homology between other allergenic lipocalins (Rat n 1, Can f 1, Can f 2, Bos d 2, Bos d 5, Equ c 1, Equ c 2) is very high. The structural model shows it to be cup-shaped with eight-stands and an anti-parallel β barrel shape.[31] Higher expression yields of Bla g 4 can be obtained in *Pichia pastoris* than in *Escherichia. coli*.[24] Accordingly, Bla g 4 warrants further investigation for its role in causing inflammation in patients with asthma. Various polymorphisms of major allergens have been described in different geographical regions. There could be predominant variants in different geographical regions. Different variants of Der p 2 showed 25~50% different immunoglobulin E binding activities,[32-34] and amino acid substitutions can render T cell epitopes active or inactive.[35] Also, the cytokine patterns of T cell responses induced by different variants of recombinant Der p 2 were found to differ even with a single amino acid substitution.[32] This demonstrates that the occurrence of even just a few amino acid substitutions could alter the immune responses.

Although Bla g 4 is a major allergen, it has not yet been reported among the major IgE binding epitopes. We are thus compelled to investigate their allergenicity and how they sensitize individuals. In this study, the sequence variations of Bla g 4 were determined and recombinant protein fragments were produced to investigate their IgE-binding reactivities.

Ⅱ. METERIALS AND METHODS

1. Patient sera

A total of 47 sera were included in this study. Thirty-two cockroach-allergic patients and fifteen healthy individuals without cockroach allergies were recruited, and their sera were studied.. The allergic patients (n = 32, ages ranging from 7 to 58 years) attended the Allergy clinic of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. All sera were confirmed for the presence of IgE antibodies > 0.7 kU/L against *B. germanica* using the Uni-CAP system (Pharmacia, Uppsala, Sweden). Table 1 shows the characteristics of the 32 allergic patients and 15 negative controls.

2. Molecular cloning of Bla g 4 cDNA

Total RNA was isolated from six adult *B. germanica* males using the Trizol reagent (GibcoBRL, Rockville, MD, USA) according to manufacturer's instructions. A cDNA encoding Bla g 4 was synthesized by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Based on the published Bla g 4 cDNA sequence in GenBank, the following sense and antisense primers were designed: 5'-GCAGTTTTGGCACTATGTGC-3'(sense) and 5'-TAGTGACATGTGGAGTG-3'(antisense). PCR was performed with 35 cycles of 30 sec at 94°C, 30 sec at 50°C, and 1 min at 72°C in a volume of 50 ul. An initial 5-min incubation at 95°C was performed, along with a final extension of 9 min at 72 °C. The PCR amplified products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into competent *E. coli* JM109. A total of 51 transformants were verified to contain the Bla g 4 DNA inserts by DNA sequencing.

3. Sequence analysis and database search

A ThermoSequence kit (Amersham Life Science, Cleveland, Ohio) was used for nucleotide sequence determination. Reaction mixtures were run on a Long ReadIR 4200 DNA sequencer (LI-COR Inc., Lincoln, NE, USA). All reactions (both forward and reverse) were performed in duplicate. Sequence alignments and database homology searches, multiple sequence alignments were performed using the BioEdit (www.Mbio.ncsu.edu/BioEdit) and CLUSTAL X programs (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX). Comparison of amino acid sequences was carried

out at the NCBI site using the BLAST network service. The predominant form was submitted to the GenBank database, under the accession number EF202172.

4. Expression and construction of Bla g 4 peptide fragments

To express the recombinant Bla g 4 in *E. coli*, the full-length Bla g 4 cDNA was amplified from pGEM-T Easy-Bla g 4. Bla g 4 (EF202172) was divided into five overlapping peptide fragments (E1~E5; E1: aa 1-100, E2: aa 34-77, E3: aa 74-117, E4: aa 114-156, and E5: aa 153-182) (Fig. 1). The specific primers used in PCR are listed in Table 2 and the PCR was carried out with an annealing temperature of 52°C. The amplified PCR products were ligated into pET-28b (Novagen, Madison, WI, USA) using *Bam*HI and *Xho*I restriction sites and transformed into *E. coli* BL21 (DE3).

For protein expression, the full-length and peptide fragments of Bla g 4 were over-expressed in *E. coli* BL21 (DE3). A single colony from the positive clones was inoculated into 10 mL of Luria Bertani broth and cultures were grown overnight at 37°C with shaking. After 16 h, the culture was further diluted into one liter and cultured at 37°C with vigorous shaking (210 rpm) until the OD_{600} reached 0.6, when Isopropyl-1-thio-β-galactopyranoside (IPTG) was added to a

final concentration of 1mM to induce expression. After 4 h, the cells were and harvested by centrifugation at 3000 × g for 20 min. The pellets of the full-length and peptide fragments (E1, E2, E4) were resuspended in 20 mM Tris, 500 mM NaCl, 5 mM imidazole, and 6 M urea, pH 7.9, and sonicated for 6 × 10 sec with 10 sec pause at 250W. The pellets expressing peptide fragments (E3, E5) were resuspended in 10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4 at pH 8.0 and lysed using a sonicator. Recombinant proteins were purified by using nickel-nitrilotriacetic acid resin (QIAGEN, Valencia, CA) according to the manufacturer's instructions under native conditions. Purified recombinant proteins were separated on 18% polyacrylamide gels containing sodium dodecyl sulfate under denaturing conditions and visualized by Coomassie brilliant blue.

5. IgE binding reactivity and IgE epitope analysis of recombinant Bla g 4

Purified recombinant Bla g 4 was diluted to 10 ㎍/mL in a coating buffer (0.1 M sodium carbonate, pH 9.6). Each well of a 96-well microplate was coated with 100 μl of the recombinant protein and incubated at 4°C overnight, followed by washing with phosphate-buffered saline containing 0.05% Tween 20 (PBST). For the blocking, 200 μl of blocking

solution (3% skim milk in PBS) was added individually and the plate was further incubated at room temperature (RT) for 1 h. Human serum (1: 4 dilutions) was then added for 2 h. The wells were washed as previously described. Fifty microliters of biotinylated goat anti-human IgE (1:1,000 dilution) (Vector, Burlingame, CA, USA) were added to each well and incubated for 1 h at RT. After washing with PBST, 50 µl of streptavidin-peroxidase (Sigma) diluted 1:1,000 in the diluent buffer was added to each well and incubated for 30 min. The plates were washed as described above with 3, 3', 5, 5'-tetramethyl-benzidine (TMB, KPL, and Gaithersburg, Maryland, USA) as a substrate solution added at 100 µl /well. The plate was kept in the dark for 20 min and the reaction stopped by addition of 100 µl of 0.5M H_2SO_4. The optical density (OD) was determined at 450 nm with the automatic microplate reader (TECAN, Salzburg, Austria). The mean absorbance level plus two standard deviations (SD) of 15 healthy controls was used as a cut-off value.

IgE binding reactivities of the peptide fragments were measured by enzyme-linked immunosorbent assay (ELISA) using four sera samples obtained from recombinant Bla g 4 positive patients. Purified recombinant peptide fragments (10μg/mL) in a coating buffer (0.1 M sodium carbonate, pH 9.6) were coated into each well of the microplate and the ELISA was performed as described above. The cut-off value was the absorbance level plus two standard deviations of four healthy controls. Each determination was done in duplicate.

Table 1. IgE reactivity of human sera against recombinant Bla g 4

Serum sample	Sex [a]	Age(yr)	Diagnosis	OD_{450} [b]	Serum sample	Sex [a]	Age(yr)	Diagnosis	OD_{450} [b]
P01	M	43	Allergic asthma	0.079	P21	M	26	Allergic asthma	0.072
P02	F	52	DF[c]	0.085	P22	M	33	Allergic rhinitis	0.083
P03	F	45	Allergic rhinitis	0.073	P23	F	17	Atopic dermatitis	0.082
P04	F	57	Allergic asthma	0.072	P24	F	36	Allergic asthma	0.073
P05	F	46	Allergic rhinitis	0.074	P25	M	30	Allergic rihinitis	0.073
P06	M	49	Allergic asthma	0.077	P26	M	7	Atopic dermatitis	0.081
P07	F	22	Atopic dermatitis	0.078	P27	M	20	Allergic asthma	0.072
P08	M	35	Allergic rhinitis	0.074	P28	M	24	Allergic asthma	0.103
P09	M	49	Allergic asthma	0.089	P29	M	41	Allergic asthma	0.068
P10	M	48	Allergic asthma	0.086	P30	M	14	Atopic dermatitis	0.069
P11	M	11	Atopic dermatitis	0.215	P31	F	15	Atopic dermatitis	0.104
P12	F	45	Allergic asthma	0.090	P32	M	53	Allergic rhinitis	0.068
P13	M	26	Atopic dermatitis	0.079					
P14	M	45	Allergic rhinitis	0.091					
P15	M	56	Allergic asthma	0.086					
P16	M	47	Atopic dermatitis	0.163					
P17	F	37	Allergic asthma	0.080					
P18	F	58	Allergic asthma	0.079					
P19	M	52	Allergic asthma	0.072					
P20	M	38	Allergic rhinitis	0.076					

a M, male; F, female.

b The cut-off value was 0.086.

c DF, Dermatofiroma.

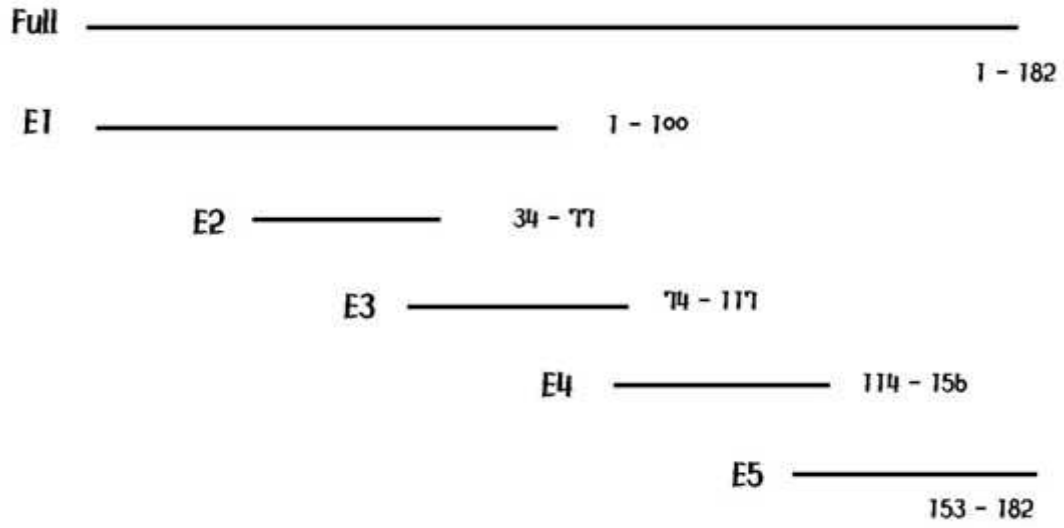


Figure 1. Schematic representation of Bla g 4 fragments for epitope analysis.

Table 2. Sequences of oligonucleotides used for the production of fragments of Bla g 4

Oligonucleotides	Sequences
Bg4-E1F	5'-GGATCCGGCAGTTTTGGCACTA-3'
Bg4-E1R	5'-CTCGAGGTGCTGTATCATTTCT-3'
Bg4-E2F	5'-GGATCCGTGGATTATTGCAGCC-3'
Bg4-E2R	5'-CTCGAGTCTTATAGCAGTCTTA-3'
Bg4-E3F	5'-GGATCCGGCTATAAGAGGACGA-3'
Bg4-E3R	5'-CTCGAGGCCTTCCACAATTGCG-3'
Bg4-E4F	5'-GGATCCAGTGGAAGGCTGTCCC-3'
Bg4-E4R	5'-CTCGAGCACCTGATCCAAAGTG-3'
Bg4-E5F	5'-GGATCCGGATCAGGTGAATCAA-3'
Bg4-E5R	5'-CTCGAGTTAGTGACATGTGGAG-3'

The underlined bases denote the restriction enzyme site.

Ⅲ. RESULTS

1. Sequence analysis of Bla g 4 cDNA

The full-length of Bla g 4 cDNA clone was obtained by RT-PCR using the specific primers based on the previously reported sequence (GenBank accession number U40767). The complete sequence was 548 nucleotides (Fig. 2) with 39 nucleotide differences compared with the previously reported Bla g 4 (U40676). These nucleotide changes resulted in 13 polymorphic amino acid residues located at positions 8 (T to S), 13 (N to M), 62 (V to Y), 64 (K to T), 69 (K to Q), 71 (K to N), 73 (K to R), 75 (T to A), 110 (E to D), 131 (I to L), 133 (F to L), 134 (S to T), and 135 (V to W). A total of 27 amino acid variations were identified from the sequences of the 51 clones. The Bla g 4 variant identified in this study (EF202172) was found in only 14 of the 51 clones (27.5%), indicating that it is the major form (SV08) (Fig.3). This major variant was used further studies. The previously reported version of Bla g 4 was found in 4 of the 51 clones (7.8%). The most common amino acid sequence variations were at positions 28 to 38, 48 to 84, and 132 to 157.

The evolutionary relationships between the variants are shown in Fig.3, and were further analyzed using phylogenetic tree analysis. These analyses showed that the variants belonged

to five groups.

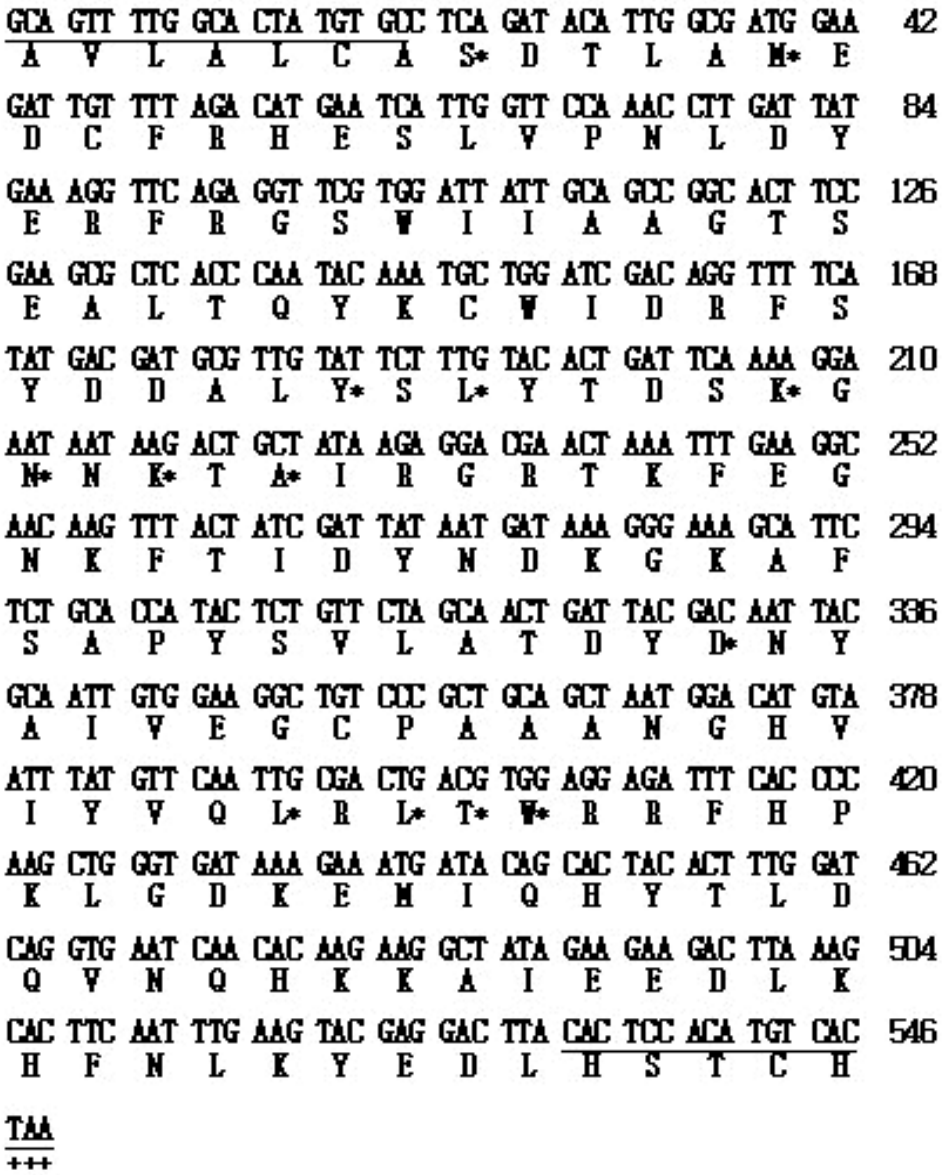


Figure 2. Nucleotide sequence and deduced amino acid sequence of Bla g 4. The initiation methionine is lacking from the deduced sequence. Differences between the cDNAs of the present Bla g 4 (EF202172) and previously reported Bla g 4 were found at 13 amino acid positions (*). The stop codon TAA is shown (+). Underlines indicate the primer binding sites.

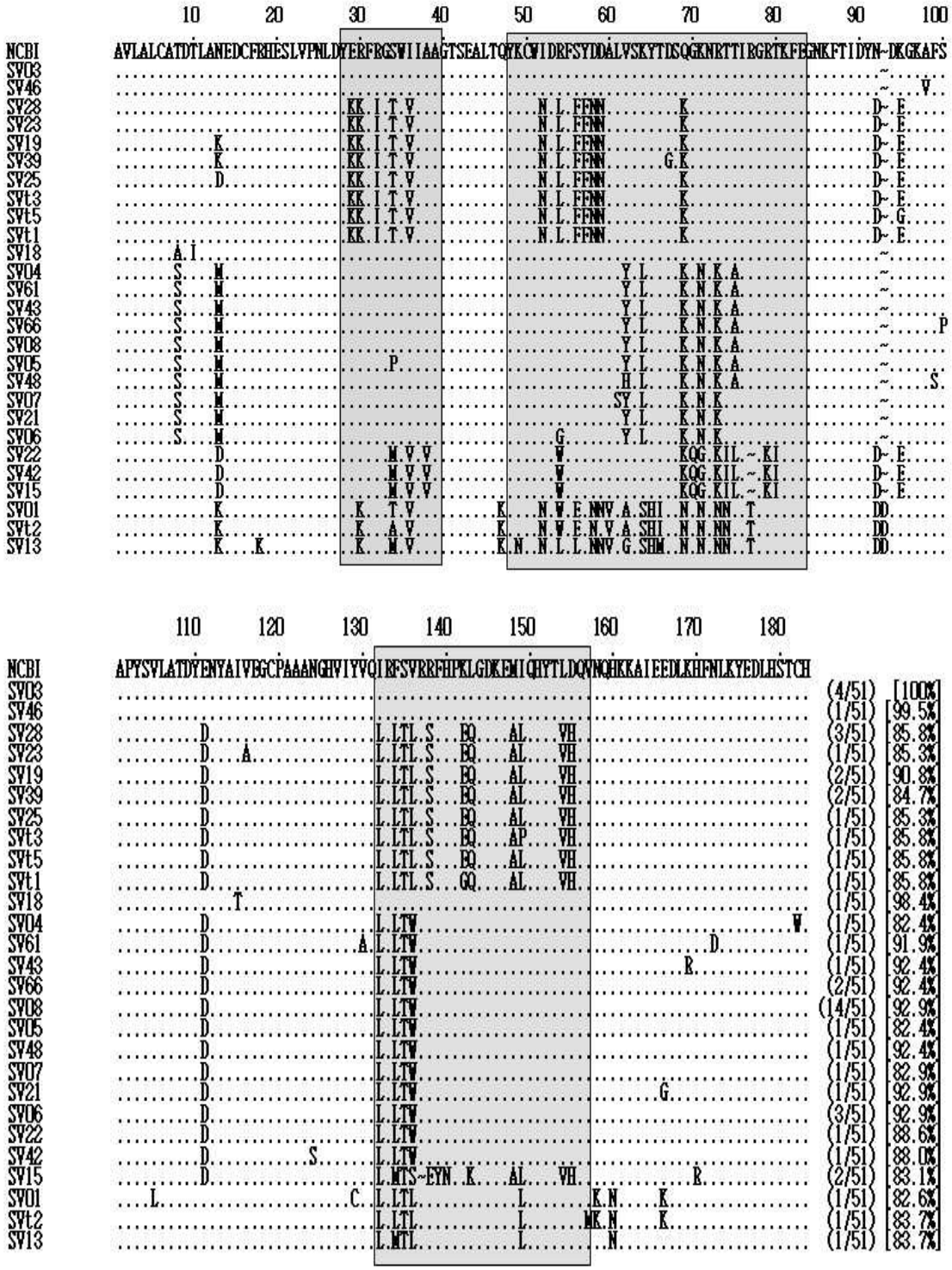


Figure 3. Amino acid sequence variations in German cockroach allergen Bla g 4 identified by RT-PCR. The frequency of each sequence is shown in parentheses. Shaded areas indicate a high degree of sequence variation.

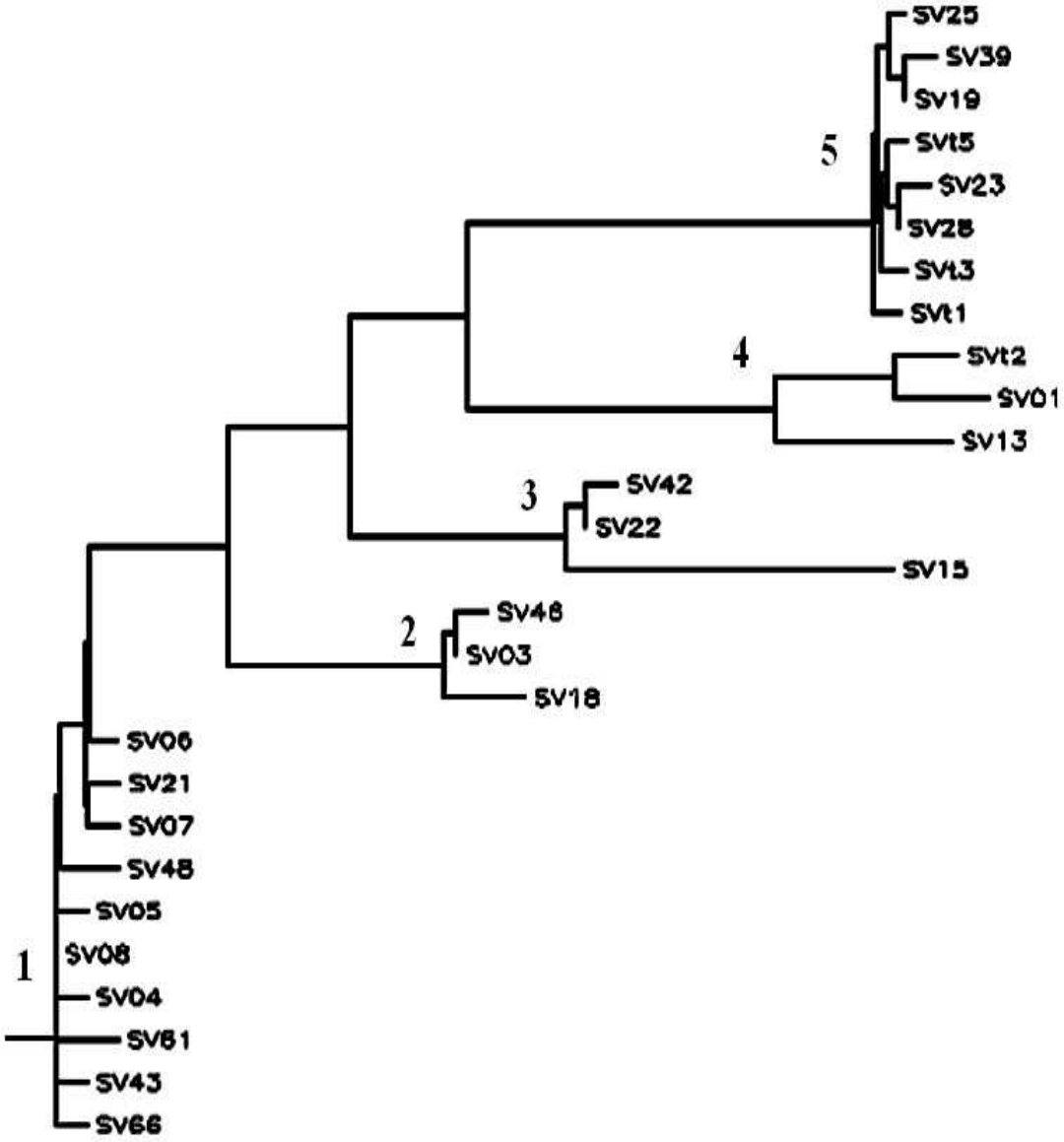


Figure 4. Phylogenetic relationship between Bla g 4 variants. Analysis was performed with the Clustal W program at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw/) and the resulting tree was visualized with the Tree View program.

2. Homology of allergenic lipocalins

A multiple amino acid sequence alignment with eight allergenic lipocalin family proteins was carried out by using the Clustal X program (Fig. 5). Despite the identity between the sequences being very low, the similarity of the overall structures was expected to be very high. The two identical residues, Gly33 and Trp35 (Bla g 4 numbering) in the main conserved segment of the lipocalin family are shown.

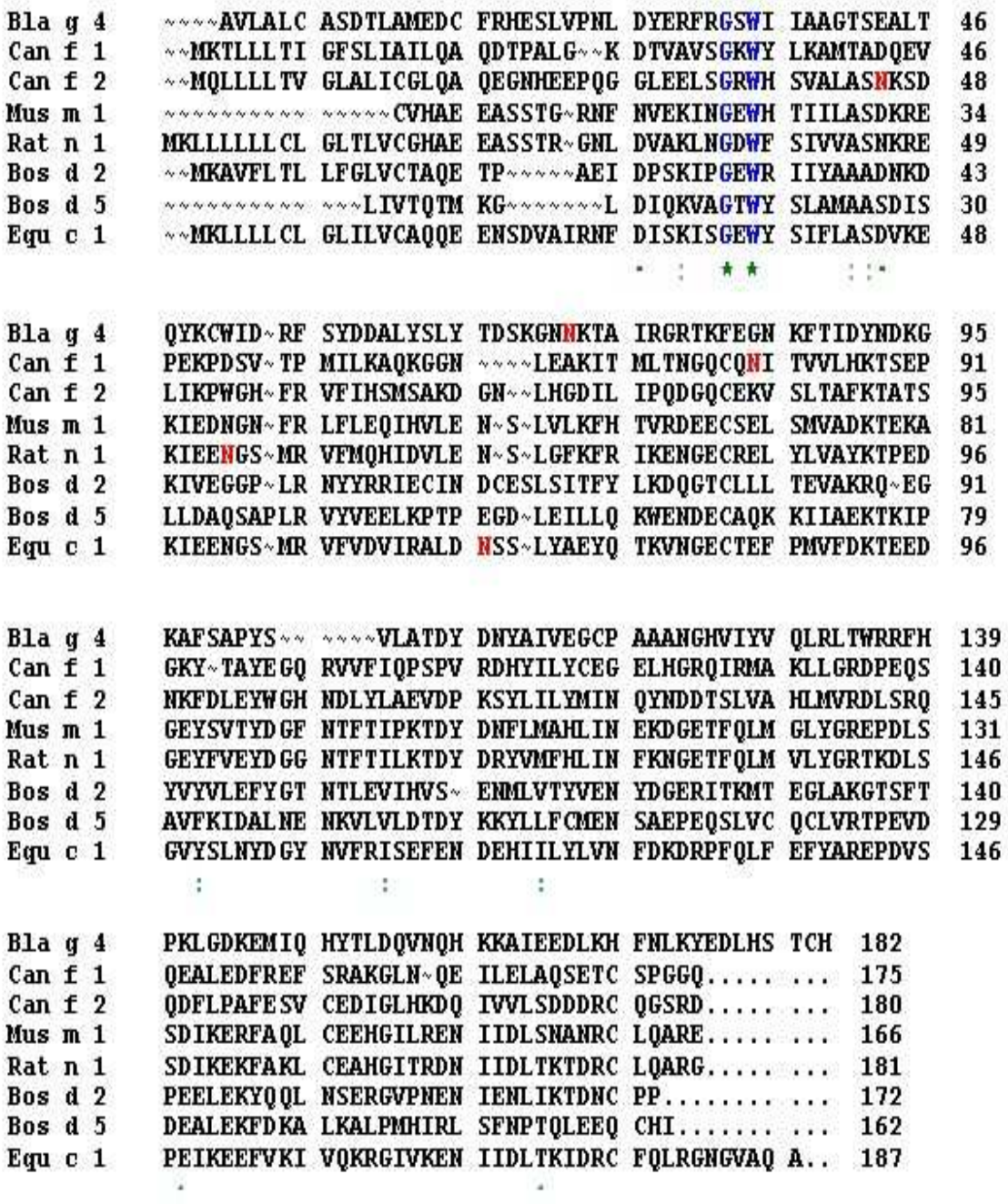
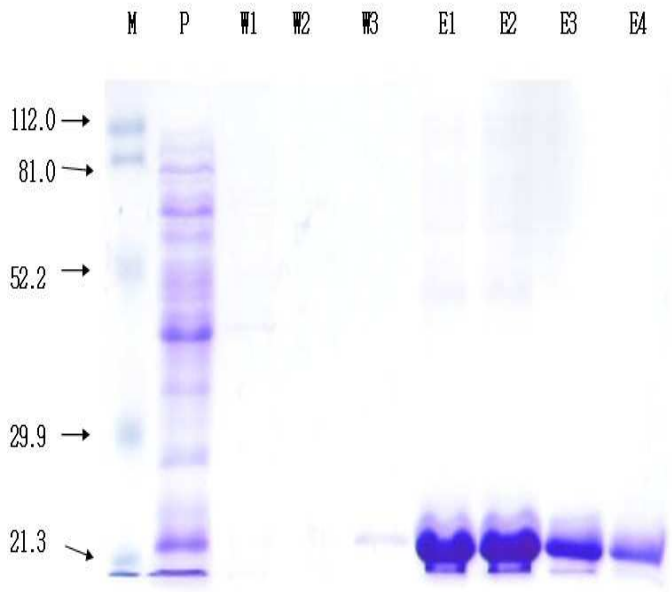


Figure 5. Sequence alignment of allergenic lipocalins: Bla g 4 (*Blattella germanica*, EF202172), Can f 1 (C*anis familiaris*, AF027177), Can f 2 (C*anis familiaris*, AF027178), Mus m 1 (*Mus musculus*, X03208), Rat n 1 (*Rattus norvegicus*, M26835), Bos d 2 (*Bos domesticus*, L42867), Bos d 5 (*Bos domesticus*, X14712), Equ c 1 (*Equus caballus*, U70823). Putative *N*-glycosylation sites are shown in red; *****, fully conserved residues; **:** , conservation of strong groups; **·**, conservation of weak groups.

3. Expression of recombinant Bla g 4 and peptide fragments

Full-length and peptide fragments containing *Bam*HⅠ – *Xho*I were subcloned into the pET-28b expression vector. The recombinant proteins were expressed as N-terminal fusion proteins with six histidine residues. The full-length and fragments 1, 2, and 4 were obtained from an insoluble fraction, while fragments 3 and 5 were obtained from a soluble fraction. The full-length protein purified by Ni-nitrilotriacetic acid (NTA)-agarose migrated as a single band with a molecular weight somewhat higher than the predicted 24 kDa (Fig.6, A). The protein yield was 2.072 mg/L as measured by Bradford assay (Bio-rad, Hercules, CA). Peptide fragments migrated at molecular weights higher than their predicted sizes on a 5~20% gradient SDS gel (Invitrogen).

(A)



(B)

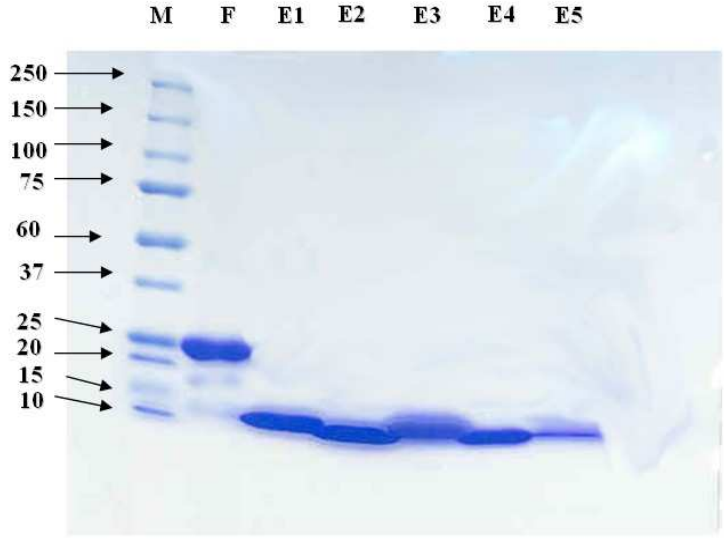


Figure 6. (A) Expression and purification of recombinant Bla g 4. Proteins run on 10% SDS PAGE and stained with Coomassie brilliant blue. Line : M, MW markers in kd; P, fraction passed through the column; W1~W3, fractions washed through the column; E, fraction eluted. (B) SDS-PAGE of full-length and peptide fragments. F, full-length; E1, 1~100 amino acid; E2, 34~77 amino acid; E3, 74~117; E4, 114~156; E5, 153~182.

4. IgE-binding reactivity of recombinant Bla g 4

The purified recombinant Bla g 4 was tested for its reactivity with IgE antibodies by ELISA in 32 sensitized sera to German cockroach extract. Among the 32 sera tested, 25% of the samples exhibited IgE reactivity to recombinant Bla g 4. Healthy human sera used as the negative control did not show any IgE reactivity.

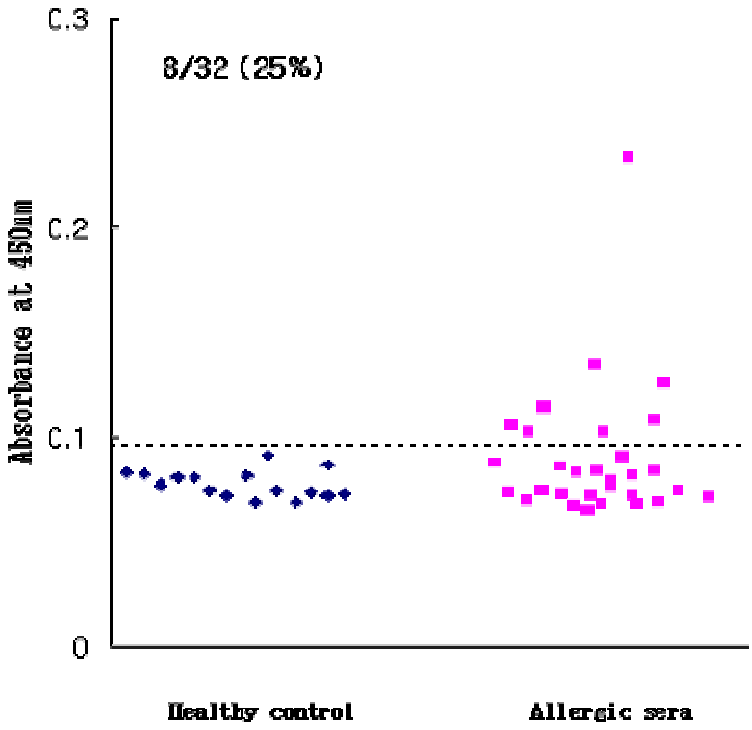


Figure 7. IgE reactivity of human sera against recombinant Bla g 4. The horizontal line indicates the cut-off value. ◆, Healthy control sera; ■, Sensitized sera to German cockroach extract (Uni-CAP > 0.7).

5. Determination of the IgE epitope

For the determination of IgE epitopes, Bla g 4-derived peptides were expressed as five overlapping recombinant peptides E1, E2, E3, E4 and E5. Four sera showing high level absorbance to recombinant Bla g 4 were selected for IgE epitope analysis. The IgE epitope study from Bla g 4-sensitized sera was performed by ELISA (Fig. 8) and the results summarized in Table 3. The results showed that IgE–binding sites were heterogeneously located among the different sera of the protein. IgE antibodies from patients 1, 2 and 4 were able to recognize fragments 2 and 4. However, fragments 1 and 3 were only recognized by the IgE antibodies of patients 2 and 1, respectively. All four sera recognized fragment 4. These results indicated that there were at least three IgE-binding antigenic determinants.

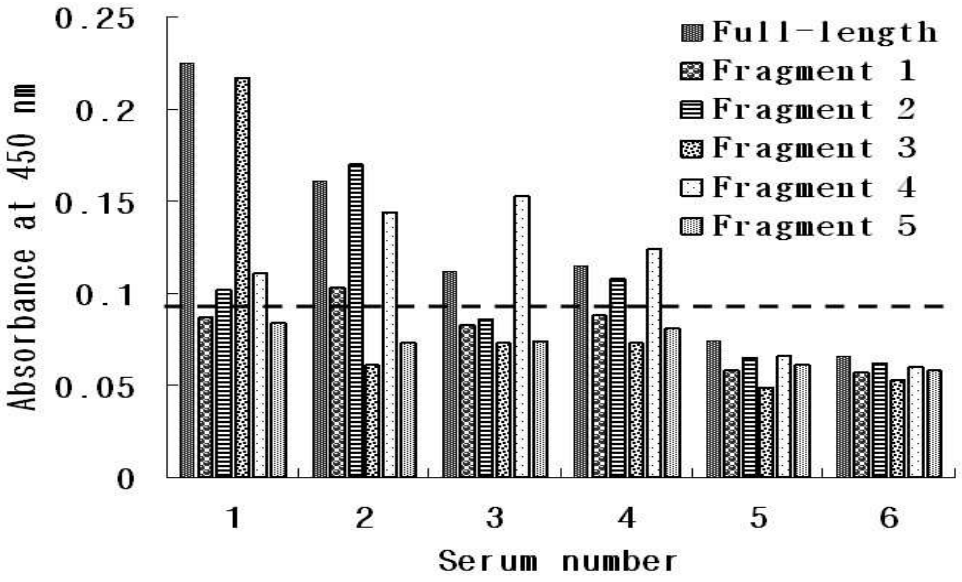


Figure 8. Binding profiles of IgE antibodies to recombinant Bla g 4 and relevant recombinant proteins by ELISA. 1~7, allergic sera; 5, pooled sera from healthy control; 6, bovine serum albumin.

Table 3. IgE binding reactivities of peptide fragments of German cockroach allergen, Bla g 4

Patients	1	2	3	4
Full-length	+	+	+	+
Fragment 1	–	–	–	–
Fragment 2	–	+	–	+
Fragment 3	+	–	–	–
Fragment 4	+	+	+	+
Fragment 5	–	–	–	–

Ⅳ. DISCUSSION

The immunological consequences of the sequence variation in cockroach allergens has been shown by IgE binding, T cell response to peptides, and the induction of cytokines.[32] The variation was first reported for a cDNA encoding Bla g 4 where 13 sequences of the 182 amino acids differed from previously reported Bla g 4. A mammalian lipocalin allergen, Bos d 2 showed only one amino acid substitution for each variant.[36] But, the frequency of Bla g 4 substitutions was remarkably high compared with other allergenic lipocalins. There was a high degree of coordinated sequence variation, especially for residues 29 to 39, 52 to 80 and 132 to 155, and to a lesser degree at 13, 92 and 111. Bla g 4 is known to be produced in the cockroach male reproductive organs and transferred to females during copulation.[30] The function of Bla g 4 in German cockroach is expected to play a role as a pheromone-binding protein.[23] The pheromone-binding protein in other insects was reported to show high levels of sequence variation.[37] The nature of these substitutions, thus, may appear to result from the protein's function in insects. This present data also indicates that the Bla g 4 variants could provide a good representation of divergence in the sequences.

Sequence comparison of lipocalin allergens reveals a family where homology is very low, with only residues G and W in the GXW triplet of the signature sequence located in the β-strand A

being clearly conserved.[38] These result confirmed again that G and W were the conserved residues in the allergenic lipocalins.

The relationships between Bla g 4 variants were phylogenetically analyzed based on sequence similarity. Figure 4 shows the phylogenetic relationship between these variants. This analysis suggests that Bla g 4 has at least 5 isoforms in the related pair of the variants. The presence of Bla g 4 isoforms in cockroach could be demonstrated by two-dimensional IgE immunoblots of purified recombinant Bla g 4. The presence of multiple isoforms for other major allergens has also been reported.[39] The isoforms of Birch pollen allergen Bet v 1 and mite allergen Der p 2, which have 10 and 8 isoforms respectively, were reported to differ not only in the extent of IgE-binding activity but also in T cell activation.[39,40] Moreover, isoforms of Der p 2 could induce different cytokine levels in allergic subjects.[39] It will be interesting to perform further studies to investigate the IgE binding to Bla g 4 isoforms.

The IgE-binding reactivity of recombinant Bla g 4 was assessed by ELISA, and 25% of German cockroach-sensitized Korean subjects were found to have positive IgE reactivity to recombinant Bla g 4. This prevalence was significantly lower than the 40~60% previously reported by Arruda et al.[13] Bla g 4 has been defined as the major allergen in allergic patients. but these results suggest a possibility of a minor allergen, a result supported by Satinover et al. (prevalence 17.4%).[29] The discrepancies in the IgE-binding reactivity in the results obtained

could be explained from two assumptions. One is to start with minor modifications of the complete molecule by amino acid substitution. If the modifications cause changes in the three-dimensional structure, they could also be expected to affect the IgE binding. Intramolecular disulfide bridges are one characteristic of lipocalins.[41] Disruption of similar bridges in mite allergens has been shown to interfere with the IgE binding.[42-45] Another factor is a glycosylation. The allergenic lipocalins, Bla g 4, Can f 1, Can f 2, Rat n 1, and Equ c 1 each have one N-glycosylation site. On the other hand, Bos d 2, Bos d 5, and Mus m 1 are not glycosylated (Fig. 5). This finding suggests that glycosylation is not critical for allergenicity. However, it is obvious that the glycan moiety could affect the IgE binding of the allergen.[46] Deglycosylation of Can f 2 would reduce IgE binding but more studies are needed to confirm and explain these results.[47] Thus, it is quite possible that glycosylation may affect the binding of IgE.

To determine the IgE-binding epitope, it is necessary to assess the results of the immunological studies with reference to the molecular characteristics. Especially in the case of B-cell epitopes, three-dimensional structures are essential both for the localization of the IgE-binding regions and for the comparative structural analyses of lipocalin allergens.[38] This study is the first report of B-cell epitopes for Bla g 4. All the sera tested showed different patterns of IgE-reactivity (Table 3). These results showed that although human IgE antibodies reacted with antigenic determinants distributed throughout the whole molecule, a major IgE-binding region was

located at residues 118 to 152, close to the C-terminus of Bla g 4. The B-cell epitopes of Bos d 5 have been studied mostly using protein fragments, where residues 41 to 60, 102 to 124, and 149 to 162 were identified as the major epitopes,[48] a somewhat similar result in Bla g 4. Therefore, comparison between other allergenic lipocalins needs to be done to determine whether all lipocalins are similar. Heterogeneity in IgE responses to Bla g 4 in individual patients was also observed, which correlated with the finding that the most allergens have multiple IgE-binding sites which is at least partly the result of the polyclonal nature of the immune response to protein allergens.[49-51]

In summary, the predominant variants and allergenicity of the allergen in different regions can be different. We investigated the predominant variant and linear IgE binding epitope of Bla g 4. Determination of allergenicity of the predominant variant might generate new information for developing more effective and safe materials for immunotherapy as well as specific diagnostics for local patients. Also, analysis of B cell epitopes may provide new information for manipulation of allergens and production of safer forms of specific immunotherapy with reduced allergenic molecules.

Ⅴ. CONCLUSIONS

1. Bla g 4 variant identified in this study (EF202172) was found in only 14 of the 51 clones (27.5%), indicating that it is the major form.

2. The evolutionary relationships between the variants by phylogenetic tree analysis showed that the variants belonged to five groups.

3. Showing only the two identical residues, Gly33 and Trp35 (Bla g 4 numbering) in the main conserved segment of the lipocalin family are shown.

4. Among the 32 sensitized sera to German cockroach extract tested, IgE reactivity to recombinant Bla g 4 was 25%.

5. The epitopes regions are found to be located at 34~73, 78~113 and 118~ 152 positions.

REFERENCES

1. Creticos PS. Managing asthma in adults. Am J Manag Care 2000;6:940-63.
2. American Academy of Allergy, Asthma, and Immunology. The use of standardized allergen extracts [position statement]. J Allergy Clin Immunol 1997;99:583-6.
3. Bousquet J, Lockey RF, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases [WHO position paper]. Allergy 1998;53:1-42.
4. Van der Veen MJ, Mulder M, Witteman AM, et al. 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;98:1028-34.
5. Esch RE. Role of proteases on the stability of allergenic extracts. Arb Paul Ehrlich Inst Bundesamt Sera Impstoffe Frankf AM 1992;85:171-77.
6. Wolff SM. Biological effects of bacterial endotoxins in man. J Infect Dis 1973;128:259-64.
7. Miller MA, Hjelle JT. Endotoxins and their clinical significance. Clinical applications of the Limulus amoebocyte lysate test Boston: CRC Press; 1990.
8. Liu AH. Endotoxin exposure in allergy and asthma: reconciling a paradox. J Allergy Clin Immunol 2002;109:379-92.

9. Niederberger V, Valenta R. Recombinant allergens for immunotherapy. Where do we stand?. Curr Opin Allergy Clin Immunol 2004;4:549-54.

10. Larche M, Wraith DC. Peptide-based therapeutic vaccines for allergic and autoimmune disease. Nat Med 2005;11:69-76.

11. Jeong K.Y, Lee I.Y, Lee J, Ree H.I, Hong C.S. and Yong TS. Med. Effectiveness of education for control of house dust mites and cockroaches in Seoul, Korea. Korean J Parasitol 2006;44:73-79.

12. Chapman MD, Vailes LD, Hayden ML, Platts-Mills TAE, Arruda LK. Cockroach allergens and their role in asthma. J Allergy Clin Immunol 1996;942-51.

13. De Blay F, Sanchez J, Hedlin G, et al. Dust and airborne exposure to allergens derived from cockroach (Blattella germanica) in low-cost public housing in Strasbourg (France). J Allergy Clin Immunol 1997;99:107-112.

14. Huss K, Adkinson 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;107:48-64.

15. Litonjua AA, Carey VJ, Burge HA, Weiss ST, Gold DR. Exposure to cockroach allergen in the home is associated with incident doctor-diagnosed asthma and recurrent wheezing. J Allergy Clin Immunol 2001;107:41-47.

16. Arruda LK, Chapman MD. The role of cockroach allergens in asthma. Curr Opin Pul Med 2001;7:14-19.

17. Melen E, Pomes A,Vailes LD, Arruda LK, ChapmanMD. Molecular cloning of Per a 1 and definition of the cross-reactive Group 1 cockroach allergens. J. Allergy Clin. Immunol 1999;103:859-64.

18. Pollart SM, Mullins DE, Vailes LD, Hayden ML, Platts-Mills TAE. Identification, quantitation, and purification of cockroach allergens using monoclonal antibodies. J. Allergy Clin. Immunol 1999;87:511-21.

19. Schou C, Lind P, Fernandez-Caldas E, Lockey RF, Lowenstein H. Identification and purification of an important cross-reactive allergen from American (Periplaneta americana) and German (Blattella germanica) cockroach. J. Allergy Clin. Immunol 1990;86:935-46.

20. Pomes A, Melen E, Vailes LD, Retief JD, Arruda LK, Chapman MD. Novel allergen structures and tandem amino acid repeats derived from German and American cockroach. J. Biol. Chem 1998;273:30801–7.

21. Pomes A, ChapmanMD,Vailes LD, Blundell TL, Dhanaraj V. Cockroach allergen Bla g 2: structure, function, and implications for allergic sensitization. Am. J. Respir. Crit. Care Med 2002;165:391–97.

22. Arruda LK, Vailes LD, Mann BJ, Shannon J, Fox JW. Molecular cloning of a major

cockroach (Blattella germanica) allergen, Bla g 2. J. Biol. Chem 1995;270:19563–68.

23. Arruda LK, Vailes LD, Hayden ML, Benjamin DC, Chapman MD. Cloning of cockroach allergen, Bla g 4, identifies ligand binding proteins (or calycin) as a cause of IgE antibody responses. J Biol Chem 1995;270:196-201.

24. Vailes LD, Kinter MT, Arruda LK, Chapman MD. High-level expression of cockroach allergen, Bla g 4, in *Pichia pastoris*. J Allergy Clin Immunol 1998;101:274-80.

25. Arruda LK, Vailes LD, Platts-Mills TA, Hayden ML, Chapman MD. Induction of IgE antibody responses by glutathione S-transferase from the German cockroach(*Blattella germanica*). J Biol Chem 1997;272:20907-12.

26. Arruda LK, Vailes LD, Benjamin DC, Chapman MD. Molecular cloning of German cockroach (*Blattella germanica*) allergens. Int. Arch. Allergy Immunol 1995;107:295–97.

27. Hindley J, Wunschmann S, Satinover SM, Woodfolk JA, Chew FT. Bla g 6: a troponin C allergen from Blattella germanica with IgE binding calcium dependence. J. Allergy Clin. Immunol 2006;117:1389–95.

28. Jeong KY, Lee J, Lee IY, Ree HI, Hong CS,Yong TS. Allergenicity of recombinant Bla g 7, German cockroach tropomyosin. Allergy 2003;58:1059–63.

29. Satinover SM, Reefer AJ, Pomes A, Chapman MD, Platts-Mills TA, Woodfolk JA. Specific IgE and IgG antibody-binding patterns to recombinant cockroach allergens. J Allergy

Clin Immunol 2005;115:803-9.

30. Fan Y, Gore JC, Redding KO, Vailes LD, Chapman MD, Schal C. Tissue localization and regulation by juvenile hormone of human allergen Bla g 4 from the German cockroach, *Blattella germanica*. Insect Mol Biol 2005;14:45-53.

31. Flower DR, North AC, Attwood TK. Structure and sequence relationships in the lipocalins and related proteins. Protein Sci 1993;2:753-61.

32. Hales BJ, Hazell LA, Smith W, Thomas WR. Genetic variation of Der p 2 allergens: effects on T cell responses and immunoglobulin E binding. Clin Exp Allergy 2002;32:1461-67.

33. 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;32:1042-47.

34. Van Ree R. Isoallergen: a clinically relevant phenomenon or just a product of cloning? Clin Exp Allergy 2002;32:975-78.

35. 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;107:985-92.

36. Jaakko Rautiainen, Seppo Auriola, Anita Konttinen, Tuomas Virtanen , Marja Rytkonen-

Nissinen , Thomas Zeiler , Rauno Mantyjarvi. Two new variants of the lipocalin allergen Bos d 2. J Chromatogr B Biomed Sci Appl 2001;5;763(1-2):91-8.

37. Christopher SW, Richard GH. Insights Into Genome Differentiation: Pheromone-Binding Protein Variation and Population History in the European Corn Borer (*Ostrinia nubilalis*). Genetics 1999;153: 1743–51.

38. Juha Rouvinen , Tuomas Virtanen , Rauno Mantyjarvi. Search for the determinants of allergenicity in proteins of the lipocalin family. J Chromatogr B Biomed Sci Appl 2001;756:199–206.

39. Hales BJ, Hazell LA, Smith W, Thomas WR. Genetic variation of Der p 2 allergens: effects on T cell responses and immunoglobulin E binding. Clin Exp Allergy 2002;32:1461–7.

40. Ferreira F, Hirtenlehner K, Jilek A. 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.

41. Skerra A. Lipocalins as a scaffold. Biochim Biophys Acta 2000;18;1482(1-2):337-50.

42. Olsson S, van Haqe-Hamsten M, Whitley P. Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite Lepidoglyphus destructor. Mol Immunol 1998;35(16):1017-23.

43. Hakkaart GA, Aalberse RC, van Ree R. Epitope mapping of the house-dust-mite allergen

Der p 2 by means of site-directed mutagenesis. Allergy 1998;53(2):165-72.

44. Nishiyama C, Fukada M, Usui Y, Iwamoto N, Yuuki T, Okumura Y, Okudaira H. Analysis of the IgE-epitope of Der f 2, a major mite allergen, by in vitro mutagenesis. Mol Immunol 1995;32(14-15):1021-29.

45. Kauppinen J, Zeiler T, Rautiainen J, Rytkonen-Nissinen M, Taivainen A, Mantyjarvi R, Virtanen T. Mutant derivatives of the main respiratory allergen of cow are less allergenic than the intact molecule. Clin Exp Allergy 1999;29(7):989-96.

46. van Ree R, Aalberse RC. Specific IgE without clinical allergy. J Allergy Clin Immunol 1999;103(6):1000-1.

47. Virtanen T, Zeiler T, Rautiainen J, Mantyjarvi R. Allergy to lipocalins: a consequence of misguided T-cell recognition of self and nonself?. Immunol Today 1999;20(9):398-400.

48. Selo I, Clement G, Bernard H, Chatel J, Creminon C, Peltre G, Wal J. Allergy to bovine beta-latoblobulin: specificity of human IgE to tryptic peptides. Clin Exp Allergy 1999;29(8):1055-63.

49. Stanley JS, King N, Burks AW. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. Arch Biochem Biophys 1997;342:244-53.

50. Wang F, Robotham JM, Teuber SS, Tawde P, Sathe SK, Roux KH. Ana o 1, a cashew

(*Ananardium occidental*) allergen of the vicilin seed storage protein family. J Allergy Clin Immunol 2002;110:160-66.

51. Bredehorst R, David K. What establishes a protein as an allergen?. J Chromatogr B Biomed Sci 2001;756:33-40.

ABSTRACT (IN KOREAN)

독일바퀴 주 알레르겐, Bla g 4 의 서열 다형성 및 펩티드 단편들의 IgE 반응성

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신 광 현

바퀴는 천식이나 여러 호흡기 알레르기 질환을 일으키며, IgE와 결합하는 항원을 가지고 있다. 알레르겐은 환경적, 유전적 요인에 따른 지역적인 차이에 따라 단백질 서열에 다양한 변이가 있다. 이러한 지역적 차이에는 우세한 변이체를 관찰할 수 있다. 소수의 아미노산 치환이라도 면역반응에 상당한 영향을 주기 때문에, 우세한 변이체의 알레르기 항원성을 결정하는 것은 대단히 중요하다. 따라서 본 연구를 통해 한국에서 우세한 Bla g 4의 변이체를 찾아서 아미노산 서열을 분석하고, *E.coli* 에서 재조합 단백질을 생산하여 바퀴에 감작된 환자 혈청으로부터 IgE 결합능을 조사하였다. 또한, 면역치료에 중요한 IgE binding epitope를 규명하기 위해 Bla g 4의 펩티드 단편들을 합성하여 ELISA (Enzyme-linked immunosorbent assay)를 수행하였다. 독일바퀴의 수컷 성충으로부터 RT-PCR (reverse transcriptase polymerase chain reaction)을 이용해 Bla g 4 유전자를 클로닝하였다. 기존의 Bla g 4 DNA서열 (U40767)과 다른 변이체(EF202172)가 조사되어 아미노산 서열을 분석

한 결과, 다음과 같이 13개의 아미노산 서열이 치환되어 있었다: 8 (T→S), 13 (N→M), 62 (V→Y), 64 (K→T), 69 (K→Q), 71 (K→N), 73 (K→R), 75 (T→A), 110 (E→D), 131 (I→L), 133 (F→L), 134 (S→T), and 135 (V→W). 51개의 클론중에 총 27개의 변이체가 조사되었으며, 가장 빈도수가 높은 변이체(14/51; 92.9%)를 한국에서 우세한 변이체(EF202172)로 결정하였다. 이를 *E.coli* (DE3)에서 발현시켜 재조합 단백질을 생산하고, 세브란스 병원의 알레르기 클리닉을 찾은 바퀴알레르기 환자 32명의 혈청을 연구에 이용하였다. 그 결과 총 32명의 환자들 중 8명이 재조합 Bla g 4에 강하게 반응하여 IgE 결합능은 25%을 나타내었다. 클로닝한 Bla g 4를 각각 5개의 단편으로 나누어 발현하고 재조합 단백질을 생산하였다. Bla g 4에 강하게 반응하는 4명의 환자혈청을 이용하여 이들의 IgE 결합능을 조사한 결과, 단편1은 0명(0%), 단편2는2명(50%), 단편3은1명(25%), 단편4는 4명(100%), 단편5는 0명(0%)으로 조사되었다. 따라서 IgE-binding epitopes 부위는 아미노산 서열위치 34~73, 78~113이며, 주 결합부위는 118~152에 위치한 것으로 나타났다.

재조합 알레르겐을 이용한 Bla g 4의 linear B cell epitope에 대한 연구는 본 실험을 통하여 처음으로 보고된 것이며, 이는 알레르기 환자의 정확한 진단과 면역치료에 대한 기초자료로 활용될 것이다.

핵심이 되는 말 : 알레르겐, 독일바퀴, 변이체, 항원결정부위