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Analysis of Amino Acid Sequence Variations and Immunoglobulin E-Binding Epitopes of German Cockroach Tropomyosin

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The allergenicities of tropomyosins from different organisms have been reported to vary. The cDNA encoding German cockroach tropomyosin (Bla g 7) was isolated, expressed, and characterized previously. In the present study, the amino acid sequence variations in German cockroach tropomyosin were analyzed in order to investigate its influence on allergenicity. We also undertook the identification of immunodominant peptides containing immunoglobulin E (IgE) epitopes which may facilitate the development of diagnostic and immunotherapeutic strategies based on the recombinant proteins. Two-dimensional gel electrophoresis and immunoblot analysis with mouse anti-recombinant German cockroach tropomyosin serum was performed to investigate the isoforms at the protein level. Reverse transcriptase PCR (RT-PCR) was applied to examine the sequence diversity. Eleven different variants of the deduced amino acid sequences were identified by RT-PCR. German cockroach tropomyosin has only minor sequence variations that did not seem to affect its allergenicity significantly. These results support the molecular basis underlying the cross-reactivities of arthropod tropomyosins. Recombinant fragments were also generated by PCR, and IgE-binding epitopes were assessed by enzyme-linked immunosorbent assay. Sera from seven patients revealed heterogeneous IgE-binding responses. This study demonstrates multiple IgE-binding epitope regions in a single molecule, suggesting that full-length tropomyosin should be used for the development of diagnostic and therapeutic reagents.

The tropomyosins are a family of closely related proteins with multiple functions, including the regulation of the actin-myosin interaction, transport of mRNA (8), and mechanical support of the cytoplasmic membrane (19). Tropomyosin has been recognized as one of the most important allergens in crustacean foods (7, 20, 27). It is highly conserved, to the extent that tropomyosin may serve as a candidate marker for phylogenetic studies of mollusks by parsimony analysis (4). Allergic reactions to shellfish and mollusks are often cross-reactive, which may be explained by the highly conserved amino acid sequences of tropomyosins, but vertebrate tropomyosin is not known to be allergenic (2). Comparisons of the immunoglobulin E (IgE) epitope regions among tropomyosins from different molluscs by Ishikawa et al. (11) showed the presence of polymorphic sites, indicating that the oyster epitope is species specific (18). The presence of unique as well as shared epitopes in Blo t 10 and Der p 10 have also been described (34).

At least 18 different isoforms are known to be generated by alternative RNA splicing in mammalian cells. The synthesis of isoforms is developmentally regulated, and cells from different embryonic lineages express different isoforms (26). The alternate exon splicing patterns of *Drosophila melanogaster* were reported to involve 27 amino acids at the C terminus (3), which frequently contain IgE-binding regions (24). Specifically, eight different IgE-binding epitopes were identified in the American cockroach tropomyosin (Per a 7) by using a set of overlapping synthetic peptides (1).

The amino acid sequence diversity of individual allergens has been described in wild or cultured house dust mites (5, 29, 30, 32, 35) or storage mites (16). Small changes in the amino acid sequences of given allergens can influence their allergenicities (10). For example, certain natural isoforms of Bet v 1, the major birch pollen allergen, were found to have high T-cell reactivities and low or no IgE-binding activities (21). Analysis of these isoforms may lead us to a better understanding of the different allergenicities of many invertebrate tropomyosins and the development of immunotherapeutic strategies and products, such as hypoallergenic (low IgE-binding activity) products.

We have previously isolated the cDNA encoding German cockroach tropomyosin (15) and named it Bla g 7, according to the guidelines of the International Union of Immunological Societies Allergen Nomenclature Subcommittee (17). Recombinant tropomyosin expressed in *Escherichia coli* showed low levels of IgE-binding reactivity. Recombinant tropomyosin was also expressed as a nonfusion protein in *Pichia pastoris*, and its IgE reactivity was compared with that of its native counterpart. The structural differences of native and recombinant proteins did not seem to influence significantly the IgE reactivities of tropomyosins (14).

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

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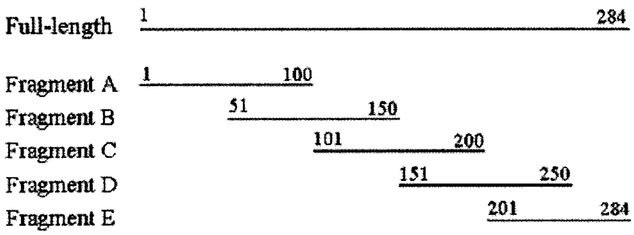

FIG. 1. Schematic representation of tropomyosin fragments for epitope analysis of German cockroach tropomyosin.

MATERIALS AND METHODS

Preparation of polyclonal anti-German cockroach tropomyosin antiserum. BALB/c mice were intraperitoneally injected with 30 μg of recombinant tropomyosin, which was expressed in *E. coli* BL21(DE3) and purified by Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen, Valencia, Calif.), according to the instructions of the manufacturer (15), in 100 μl of phosphate-buffered saline emulsified with an equal volume of alum adjuvant. Booster injections were given twice at 3-week intervals. The production of specific antibodies was monitored by enzyme-linked immunosorbent assay (ELISA), and the mice were killed 3 days after the second booster injection. The polyclonal antitropomyosin antiserum (1:1,000) was used to probe and identify the German cockroach tropomyosin.

Two-dimensional gel electrophoresis and immunoblotting. Two-dimensional gel electrophoresis was performed with precast gels (Invitrogen, Carsbad, Calif.), according to the instructions of the manufacturer. Cockroach extract was prepared as described previously (15). Fifty micrograms of whole-body extracts mixed with an equal volume of the sample buffer was loaded into the first-dimension gel. After isoelectric focusing (pH 3 to 10), second-dimension gel electrophoresis was carried out in a 4 to 20% gradient polyacrylamide gel containing sodium dodecyl sulfate. The proteins were then electrophoretically transferred onto a nitrocellulose membrane (pore size, 0.45 μm; Osmonics, Westborough, Mass.). After the membrane was blocked overnight with 3% skim milk, it was incubated for 1 h with mouse anti-recombinant Bla g 7 serum. The blots were then incubated with goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma, St. Louis, Mo.) for 1 h at room temperature and developed in a substrate solution of Nitro Blue Tetrazolium and 3-bromo-4-chloro-5-indolyl-phosphate (Promega, Madison, Wis.).

RT-PCR. cDNA encoding tropomyosin was amplified by using high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). A total of 150 mg of a German cockroach was pulverized in liquid nitrogen with a mortar and pestle, and total RNA was extracted by using the TRIzol reagent (Invitrogen), according to the manual prepared by the manufacturer. Reverse transcription was initiated with 6 μg of total RNA and an oligo(dT) (18 T residues) primer. Five microliters of single-stranded cDNA was used for each reaction. The primer sequences used were as follows: forward primer, 5′-ATGGATGCCATCAAGAAGAAG-3′; reverse primer, 5′-GTTTAGTTGCCAATAAGTTCGG-3′. The cDNA encoding tropomyosin from different cockroach species (*Periplaneta fuliginosa*), as well as *Blattella germanica*, was successfully cloned by RT-PCR with this specific primer set (13, 15). PCR was performed as follows: after an initial denaturation (5 min at 95°C), the samples were subjected to 35 cycles of amplification, each of which consisted of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. The final extension was performed at 72°C for 8 min. The DNA fragment so obtained was cloned into a pPCR Script Amp SK (Stratagene) vector.

Nucleotide sequence determination. A ThermoSequenase 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, Nebr.). All reactions (both forward and reverse) were performed in duplicate.

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

Subjects and serum samples. Sera from patients attending the Allergy Clinic of the Severance Hospital, Yonsei University, Seoul, Korea, were tested for the presence of IgE antibody against *B. germanica* by using the Uni-CAP system (Pharmacia, Uppsala, Sweden). Those with Uni-Cap results higher than 0.7 kU/liter were tested again for the presence of recombinant tropomyosin-specific IgE antibodies by ELISA, as described previously (15). Eight serum samples were selected for the epitope study (subject age range, 1 to 22 years; average age, 11 years). The diagnosis of allergy was based on case history and a skin-prick test.

IgE epitope analysis of subjects' sera. The reactivities of specific IgE antibodies to fragmented recombinant Bla g 7 were examined by ELISA with seven serum samples obtained from recombinant Bla g 7-positive patients. Purified recombinant proteins (0.2 μg/well) were coated (0.1 M sodium carbonate [pH 9.6]) onto a polystyrene microtiter plate (Corning, Corning, N.Y.) and incubated overnight at 4°C. After the plate was blocked with 3% skim milk for 1 h, the plate was washed with phosphate-buffered saline containing 0.05% Tween 20 and incubated for 1 h with human serum (1:4 dilution). After the plate was washed, IgE antibody was detected by using biotinylated goat anti-human IgE (epsilon chain specific; Vector, Burlingame, Calif.) diluted 1:1,000 with streptavidin-peroxidase (Sigma) diluted 1:1,000. The signal was developed by adding 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), and the optical density at 450 nm was determined after the addition of 1% H_2SO_4 on an automatic microplate reader (Tecan, Salzburg, Austria). The mean absorbance level plus 2 standard deviations for the sera from eight healthy controls was used as the cutoff value.

RESULTS

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

Sequence analysis of cDNA clones. A total of 11 different variants with amino acid variations were identified by determining the sequences of the 50 clones obtained by RT-PCR. Only slight differences were identified at seven locations in the deduced amino acid sequences. A previously described sequence (GenBank accession number AF260897) was found in 38 of the 50 clones, indicating that it is a major form. This major variant was used for further studies. The amino acid positions that were found to vary among the Bla g 7 variants were 81, 85, 89, 183, 234, 246, 278, and 284 (Fig. 3). The most

TABLE 1. Sequences of oligonucleotides used for production of fragmented tropomyosin

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

[a] The underlined nucleotides of each oligonucleotide represent the restriction enzyme site.

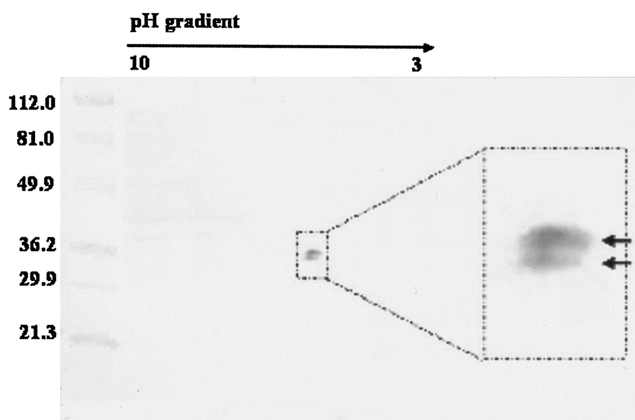

FIG. 2. Two-dimensional immunoblot analysis of German cockroach tropomyosin with mouse anti-rBla g 7 sera. The numbers on the left indicate the sizes of the molecular mass standards (in kilodaltons).

frequent amino acid change was observed at residue 284 (6 of 50 clones). This observation is in agreement with the findings from immunoblot analysis with a two-dimensional gel, which showed diffuse spots. The IgE epitopes identified from *Penaeus aztecus* tropomyosin are shown in Fig. 3. Only 2 of the 50 clones (clones pm043 and pm061) were found to have amino acid sequence variations in the IgE epitope regions.

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

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

DISCUSSION

Tropomyosin has been recognized as one of the most important allergens in crustacean foods (7, 20, 27). It is highly conserved, to the extent that tropomyosin may serve as a candidate marker for phylogenetic studies of mollusks by parsimony analysis (4). Allergic reactions to shellfish and mollusks

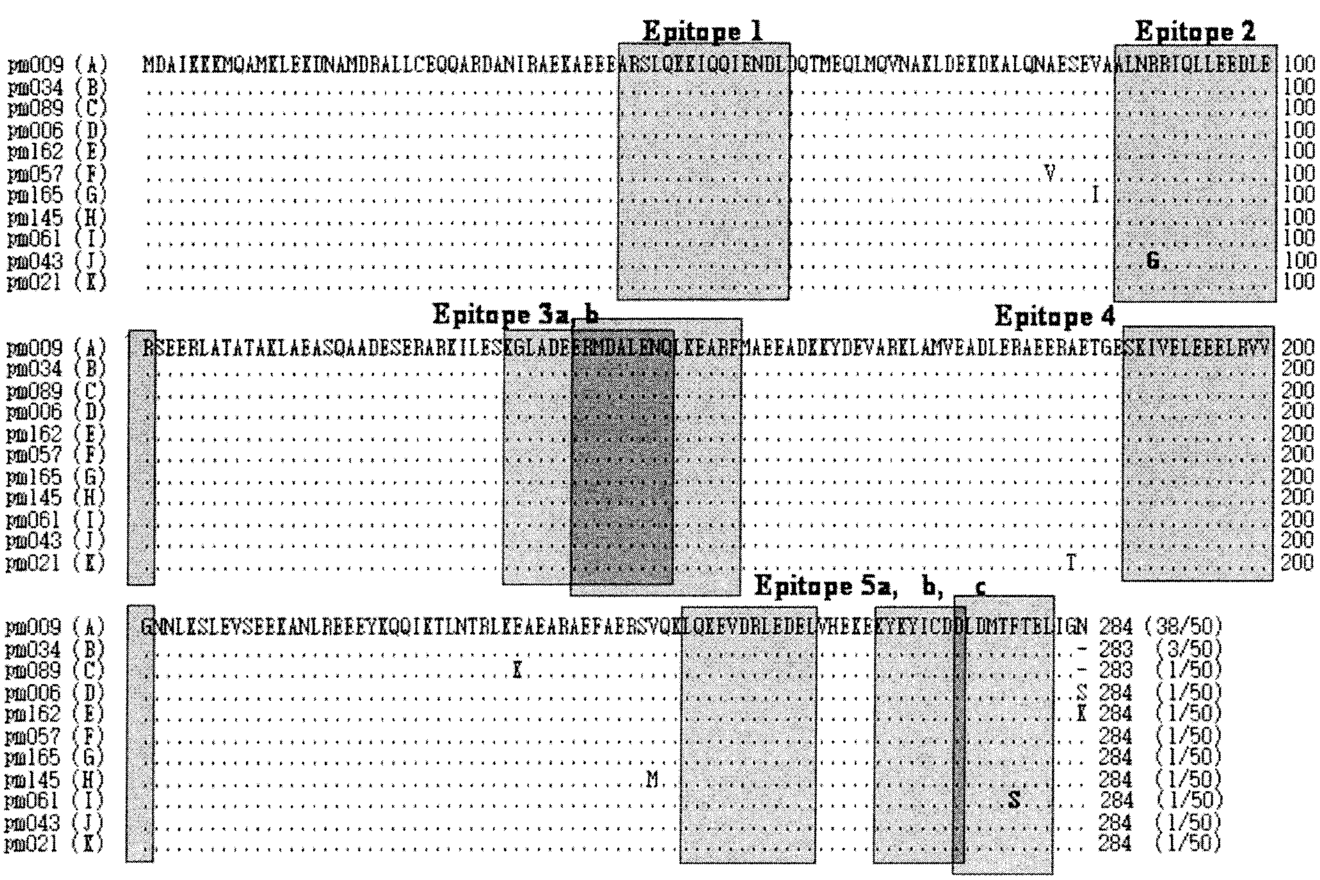

FIG. 3. Amino acid sequence variations in German cockroach tropomyosin identified by RT-PCR. The frequency of each sequence is shown in parentheses. Shaded areas indicate the IgE-binding epitopes identified from *P. aztecus* tropomyosin.

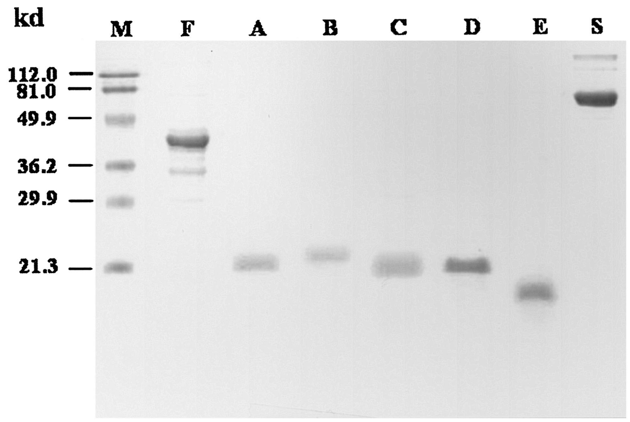

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of full-length and fragmented recombinant Bla g 7. Lanes: M, molecular mass standard; F, full-length fragment; A, fragment of 1 to 100 amino acid; B, fragment of 51 to 150 amino acids; C, fragment of 101 to 200 amino acids; D, fragment of 151 to 250 amino acids; E, fragment of 201 to 284 amino acids; S, bovine serum albumin.

are often cross-reactive, which may be explained by the highly conserved amino acid sequences of mollusk tropomyosin, but vertebrate tropomyosin is not known to be allergenic (2). Comparisons of the IgE epitope regions among tropomyosins from different mollusks by Ishikawa et al. (11) showed the presence of polymorphic sites, indicating that the oyster epitope is species specific (18). The presence of unique as well as shared epitopes in Blo t 10 and Der p 10 has also been described (34).

IgE is thought to be a key molecule in the mediation of many allergic diseases (22). It was reported that the IgE-binding capacity of the German cockroach extract was totally abolished by Atlantic shrimp extract, which was found to have strong IgE-binding components between 30 and 43 kDa (presumably tropomyosin) by IgE blot inhibition (6). However, in the previous study (6), recombinant German cockroach tropomyosin was able to inhibit only 32.4% of IgE binding to cockroach extract (15).

The first approach required in the study of the relationship

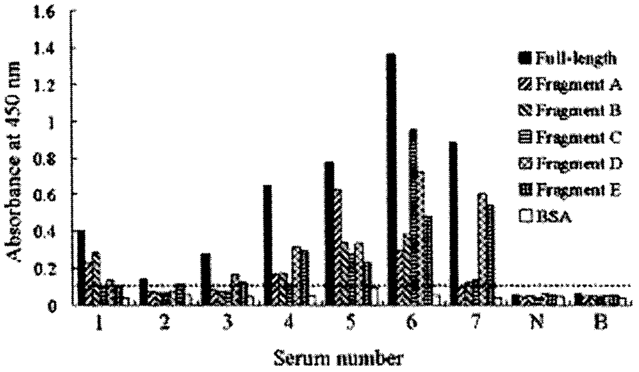

FIG. 5. Profiles of IgE antibody binding to recombinant Bla g 7 and relevant recombinant proteins obtained by ELISA. Dotted line, cutoff value (mean absorbance plus 2 standard deviations for the sera from eight healthy controls); 1 to 7, serum samples from seven allergic patients, respectively; N, Bla g 7-negative serum sample; B, buffer control.

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

Fragment	IgE binding in subject:						
	1	2	3	4	5	6	7
Full length	+	+	+	+	+	+	+
A	+	−	−	+	+	+	−
B	+	−	−	+	+	+	+
C	−	−	−	−	+	+	+
D	+	−	+	+	+	+	+
E	+	+	+	+	+	+	+
BSA[a]	−	−	−	−	−	−	−

[a] BSA, bovine serum albumin.

between structure and allergenicity is epitope identification. At present the SPOTs system (Genosys, The Woodland, Tex.) and the Novatope system (Novagen, Madison, Wis.) are extensively used to identify IgE-binding epitopes, and the results obtained with the two systems have been extensively compared (25). Moreover, fragmented peptides are reported to have higher IgE-binding capacities than whole molecules in the case of paramyosin, Der f 11 (33). These were not real peptide fragments presented by professional phagocytes of the immune system; however, these peptide fragments could have been made by the proteolytic enzymes derived from mites or the cockroaches themselves.

In the present study, we tried to determine whether the low allergenicity of German cockroach tropomyosin is affected or not by amino acid sequence variations of its isoforms. For convenience, the German cockroach tropomyosin amino acid sequences deduced from RT-PCR analysis were compared with those of *P. aztecus* tropomyosin (Fig. 3). Only two variant German cockroach tropomyosins resulting from amino acid substitutions in the IgE epitope regions were different from *P. aztecus* tropomyosin, which is one of the well-studied tropomyosin molecules (1), and 11 different amino acid sequence variations were identified (Fig. 3). The IgE-binding reactivities of intact or fragmented Bla g 7 were analyzed to investigate IgE epitopes in the Korean patient population (Fig. 5). All sera tested showed different patterns of IgE reactivity. Analyses of IgE epitopes from different patient groups or tropomyosin from different organisms showed that the epitopes exhibited different IgE-binding regions (1, 12, 23, 27), which implies the presence of various epitope regions, which are influenced by genetic backgrounds and environmental factors. The structural basis for bending tropomyosin around actin filaments is attributed to the structural regularity of the molecule (31). The tropomyosin coiled coil consists of two α-helices, which are characterized by the occurrence of tandem (heptad) repeats (28). The structural regularity of tropomyosin may be a possible explanation for the existence of multiple IgE-binding epitopes. Specific immunotherapy is an efficient treatment for subjects with IgE-mediated allergic reactions. Studies of IgE epitopes have led to a better understanding of the mechanisms underlying successful immunotherapy and the proposed use of hypoallergenic forms of allergens for immunotherapy (9).

In conclusion, the low allergenicity of previously reported German cockroach tropomyosin does not seem to be due to amino acid sequence variations. The IgE-binding epitope regions were found to be distributed over the whole molecule. It is not advisable to use a fragment for diagnostic or therapeutic purposes in case of tropomyosin. Invertebrate tropomyosin could provide a molecular model for investigation of the genetic and environmental factors affecting sensitization and the onset of allergic disorders.

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