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IgE-binding Reactivity of Peptide Fragments of Bla g 1.02, a Major German Cockroach Allergen

Myung-hee Yi, Kyoung Yong Jeong, Chung-Ryul Kim and Tai-Soon Yong

SUMMARY Cockroaches cause allergic diseases and are closely linked with the development of asthma. Bla g 1 is one of the major allergen proteins produced by German cockroaches. It consists of tandem repeats of approximately 100 amino acids. The aim of the present study was to identify linear IgE-binding epitopes of Bla g 1.02. RT-PCR was used to clone a cDNA sequence encoding Bla g 1.02 (EF202179) which shared 98.6-99.8% identity with a previously reported Bla g 1.02 (AF072220). To investigate IgE binding regions, five separate but overlapping Bla g 1.02 peptide fragments (A: aa 1-111, B: aa 102-215, C: aa 206-299, D: aa 289-403, E: aa 394-491) were amplified and cloned. The full-length and five peptide fragments were overexpressed in *Pichia pastoris* and *E. coli*, respectively, and their IgE binding reactivities were measured by ELISA using 37 serum samples isolated from cockroach-sensitized patients. The sera of 24 patients (64.9%) recognized the full-length Bla g 1.02 recombinant protein. Among 19 selected serum samples, 11 sera (57.9%) reacted to peptide fragment A, 5 sera (31.3%) to B, 4 sera (21.1%) to C, 9 sera (47.4%) to D, and 10 sera (52.6%) to peptide fragment E. IgE-binding epitopes are found to be distributed to each tandem repeat of Bla g 1. The combination of peptide fragments A, D, and E may able to detect all Bla g 1-sensitized subjects. We suggest that these peptide fragments may be useful in allergy diagnosis and the design of novel immunotherapeutics.

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Cockroaches produce major indoor allergens that induce IgE-mediated allergic diseases such as allergic rhinitis and asthma.[1] Exposure to high levels of cockroach allergens in children who live in cities and in low-income housing has been reported to cause such respiratory problems.[2-3] Patients with cockroach allergies exhibited high levels of serum IgE antibodies as compared to general asthmatics and patients suffering from mite asthma.[4-5] Seven German cockroach (*Blattella germanica)* allergens have been thus far identified and characterized. These allergens are derived from the intestinal tract, ovaries, ootheca, exuvia, and feces of the cockroach.[6-7]

Bla g 1 is a German cockroach allergen that consists of several tandem repeats of approximately 100 amino acids and displays IgE-binding frequency in 30-77% of cockroach allergic patients.[7,8] It contains more than 2 duplexes, each containing 2 consecutive amino acid repeats that share 26-29% homology, and each duplex represents a distinct molecular unit of Bla g 1.[8] Bla g 1 isoallergens (cDNA clones Bla g 1.01 and Bla g 1.02) show 70-72% nucleotide sequence homology to the antigenically cross-reactive *Periplaneta americana* (American cockroach) group 1 allergen (Per a 1).[9] Exposure to

From the Department of Environmental Medical Biology and Institute of Tropical Medicine, Arthropods of Medical Importance Resource Bank, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea.
Correspondence: Tai-Soon Yong
E-mail: tsyong212@yuhs.ac

Bla g 1 is a high risk factor for sensitivity to German cockroaches.[10-11] Bla g 1 is located primarily within the cockroach alimentary tract, mainly the midgut, and indeed Northern hybridizations using various gut tissues have demonstrated that Bla g 1 is produced exclusively by midgut cells.[12] Quantitative analyses of Bla g 1 mRNA expression and Bla g 1 protein levels in adult females showed that both are closely linked to the reproductive cycle of female cockroaches. Large amounts of Bla g 1 are excreted in cockroach feces. Fecal production closely follows the food consumption patterns in *B. germanica*, with Bla g 1 content following a similar cyclic rise and fall as reflected by food intake and the production of feces.[13]

Bla g 1 could serve at least two physiological functions, both of which are associated with food intake. Firstly, Bla g 1 is secreted from the midgut epithelium *via* the rough endoplasmic reticulum and may play a role in digestion.[9] Bla g 1 shares 35–40% amino acid sequence identity with the *Anopheles gambiae* ANG12 and *Aedes aegypti* AEG12 proteins, both of which are produced in the female mosquito midgut and undergo temporal changes in expression relative to the acquisition of blood meal.[14] Secondly, Bla g 1 may have an important structural role in the midgut. A recent report showed that cockroach allergen-like microvillar membrane proteins from *Tenebrio molitor* possess a 37% amino acid sequence identity with Bla g 1.[15] Since Bla g 1 fecal concentrations are highest as feeding decreases, microvilli that contains Bla g 1 may be sloughed off after large food boluses pass through the midgut.

Diverse pharmacotherapeutic approaches are used in an attempt to alleviate allergic symptoms in patients. However, only allergen specific immunotherapy is believed to have continuous curative effects.[16] In order to precisely diagnose and treat allergic reactions, high quality cockroach extracts are necessary. However, extracts are prone to contamination with other allergens (that subjects are not sensitized to) and various proteolytic enzymes which can affect the stability of allergens and may cause inflammatory responses in their own right.[17-18] Such problems may be overcome by the use of highly purified recombinant allergens that are produced in sufficient quantities for treatment.[19]

B cell and T cell epitopes should be analyzed for immunotherapy using recombinant allergens. Following the identification of IgE-binding epitopes, allergens that exert a low IgE binding capacity can then be synthesized for immunotherapy by incorporating amino acids substitutions within epitope regions.[20] Although Bla g 1 is an important factor in patient allergies toward cockroaches, studies into B cell and T cell epitope recognition by Bla g 1 have not yet been carried out.

We have previously described the amino acid sequence polymorphisms of Bla g 1.02, with the major variant submitted to GenBank (accession number EF202179).[21] The objective of the present study was to determine the location of IgE-binding epitopes for the predominant form of Bla g 1.02 through the use of recombinant proteins. IgE-binding epitopes of Bla g 1 may assist in improving cockroach allergy diagnosis and in the development of novel therapeutic approaches.

MATERIALS AND METHODS

Total RNA isolation and cDNA synthesis

Total RNA was isolated from 10 adult female *B. germanica* using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, tissue from each cockroach was homogenized in 1 ml of TRIzol reagent and homogenates extracted using chloroform. The total RNA was precipitated with isopropyl alcohol, washed in 75% ethanol, and finally resuspended in RNase-free water. First strand cDNA was synthesized from 5 μg of total RNA using AMV reverse transcriptase (Promega, Madison, WI, USA) and an oligo(dT_{18}) primer.

RT-PCR and molecular cloning of Bla g 1.02 cDNA

RT-PCR was performed using a forward (5'-GCCATAGAATTCCTCAATAAC) and reverse primer (5'-GAAACTTTCTTAGATATGGAAC) which were designed based on the Bla g 1.02 cDNA sequence obtained from GenBank (accession number AF072220). RT-PCR was carried out under thermal cycling conditions as follows: an initial step at 95°C for 5 minutes, followed by 35 cycles of 94°C for 30

seconds, 58°C for 30 seconds and 72°C for 2 minutes, with a final extension of 9 minutes at 72°C. The amplified products were ligated into a pGEM-T Easy vector (Promega) and sequenced in both directions (Solgent Co. Ltd., Daejeon, Korea). All DNA sequences were aligned and compared with the GenBank Bla g 1.02 cDNA sequence and deduced into amino acid sequences, using the SDSC Biology Workbench program (http://workbench.sdsc.edu/).

P. pastoris expression of full-length Bla g 1.02

The cDNA sequence encoding Bla g 1.02 cloned in pGEM-T Easy vector[21] was amplified using PCR, forward primer Blag102FF (CTCGAGCATCATCATCATCATCATGCCATAGAATTCCTCAAT) and reverse primer Blag102RF (GCGGCCGCTGATGATGATGATGATGAGACAATCCAAAGAGT). The underlined sequences correspond to *Xho*I and *Not*I sites, respectively, and six histidine codons were incorporated into both primers. The PCR product was digested with *Xho*I and *Not*I and subcloned into pPIC9 (Invitrogen Life Technologies). GS115 cells were then transformed with *Sal*I-linearized plasmids using a *Pichia* EasyCom Kit (Invitrogen Life Technologies). His^+ transformants were selected on RDB plates (1.34% yeast nitrogen base without amino acids, 1 M sorbitol, 1% dextrose, 4 x 10^{-5}% biotin, and 0.005% each of L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine). Individual clones were grown in buffered methanol-complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acid, 4 x 10^{-5} % biotin, 0.5% methanol) for 5 days at 220 rpm, 30°C, with 0.05% methanol (v/v) added every 24 hours. Harvested supernatants were diluted with lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$, pH 8.0) and recombinant proteins purified using nickel nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Valencia, CA, USA), according to manufacturer's instructions.

Expression of Bla g 1.02 fragments in *E. coli*

Five overlapping Bla g 1.02 fragments were generated (A: aa 1-111, B: aa 102-215, C: aa 206-299, D: aa 289-403, and E: aa 394-491)(Fig. 1) using PCR with the specific primers shown in Table 1. PCR amplicons were ligated into the expression vector pET28b (Novagen, Madison, WI) following digestion with *Bam*HI and *Xho*I. Recombinant plasmids were transformed into *E. coli.* BL21 (DE3) cells and the expression of Bla g 1.02 fragments induced with 1 mM isopropyl thio-β-D-galactoside at 37°C. After 4 hours, cultures was harvested and cells resuspended in lysis buffer (6 M urea, 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) and the peptides purified using Ni-NTA resin according to the manufacturer's instructions.

SDS-PAGE and quantitative analyses

Protein measurements were conducted using the Bradford assay (Bio-Rad, Hercules, CA). Purified full-length recombinant Bla g 1.02 and the pro-

Table 1 Oligonucleotide primers used for the amplification of Bla g 1.02 gene fragments

Name	Sequence
Bla g 1.02 A	F: 5'-GGATCCGGCCATAGAATTCCTCAA
	R: 5'-CTCGAGAAATATCTGGTGGATGAG
Bla g 1.02 B	F: 5'-GGATCCGCACATAATTGAACTCAT
	R: 5'-CTCGAGTCTGCGGAGGTGTTTACG
Bla g 1.02 C	F: 5'-GGATCCGCCAGTCACAGCACGTAA
	R: 5'-CTCGAGGAAAATTTGGTGGATGAG
Bla g 1.02 D	F: 5'-GGATCCGCATATAATTGAACTCAT
	R: 5'-CTCGAGTCTGCGGACATGTTTGCG
Bla g 1.02 E	F: 5'-GGATCCGCCTGCCACAGGTCGCAA
	R: 5'-CTCGAGATGAGACAATCCAAAGAG

Underlined regions in forward (F) and reverse (R) sequences indicate *Bam*H1 and *Xho*I sites, respectively.

tein fragments were separated on 5-20% gradient SDS-PAGE gels (Invitrogen Life Technologies) and stained with Coomassie brilliant blue R250.

Serum samples

Sera were obtained from 37 patients (age range, 5-58 years; average age 32.7 years) (Table 2) at the Allergy Clinic at Yonsei University College of Medicine, Seoul, Korea. The diagnosis of allergic symptoms was based on a skin prick test and patient history. Normal serum samples were obtained from subjects who showed a negative reaction to skin prick tests. Informed consent was obtained from all individuals prior to blood collection.

Table 2 IgE reactivities of human sera against recombinant Bla g 1.02

Serial No.	Sex[a]	Age	Diagnosis	OD_{450}[b]
P1	M	24	Bronchial asthma	0.0750[c]
P2	F	52	Dermatitis	0.0780
P3	M	30	Urticaria	0.0615
P4	M	35	Anaphylatic shock	0.0650
P5	M	56	Asthma	0.0625
P6	F	9	Rhinitis	0.0645
P7	F	15	Rhinitis/dermatitis	0.0685
P8	M	20	Dermatitis	0.0800
P9	M	21	Dermatitis	0.1475
P10	M	48	Dyspnea	0.0730
P11	F	58	Bronchitis	0.0740
P12	M	52	Allergic asthma, rhinitis	0.1115
P13	F	46	Allergy cough	0.0830
P14	M	45	Asthma/rhinitis	0.1045
P15	M	14	Rhinitis	0.1095
P16	M	43	Allergic asthma	0.0720
P17	M	26	Allergic asthma	0.0690
P18	M	38	Allergic rhinitis	0.0690
P19	M	49	Conjunctivitis	0.0675
P20	F	16	Atopic dermatitis	0.0975
P21	M	49	Allergic asthma	0.0630
P22	F	15	Rhinitis/dermatitis	0.0655
P23	M	19	Dermatitis	0.0945
P24	M	38	Allergic rhinitis	0.0815
P25	M	26	Atopic dermatitis	0.0790
P26	M	33	Allergic rhinitis	0.1235
P27	M	24	Allergic asthma	0.0820
P28	M	13	Allergic rhinitis	0.0840
P29	F	45	Allergic asthma	0.0845
P30	M	20	Asthma	0.0730
P31	M	23	Allergic asthma, allergic rhiniits	0.0880
P32	M	22	Allergic asthma, rhiniits, cholinergic urticaria	0.0770
P33	M	51	Allergic asthma, allergic rhinitis	0.0730
P34	M	37	Allergic asthma, allergic rhinitis	0.0690
P35	F	26	Allergic asthma, allergic rhinitis, drug allergy	0.0670
P36	M	49	Cough	0.0685
P37	F	22	Allergic eczema	0.0775

[a]M, male; F, female; [b]The cut-off value was 0.070999; [c]Positives are indicated by underlined values.

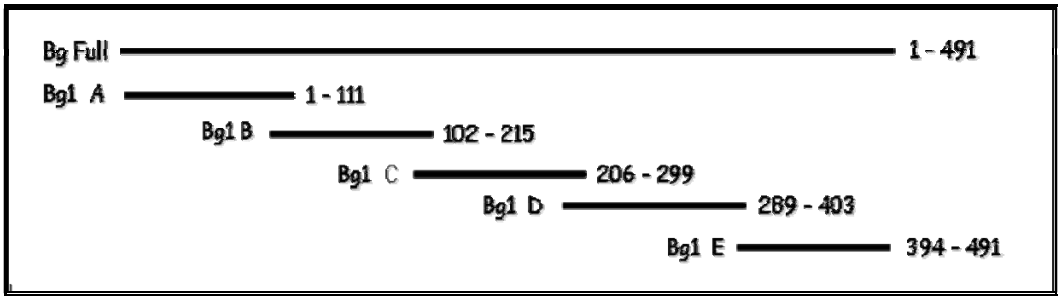

Fig. 1 Schematic representation of Bla g 1.02 fragments used for epitope analysis.

Enzyme-linked immunosorbent assay (ELISA)

Wells of microtiter ELISA plates were coated individually with 100 µl of purified full-length Bla g 1.02 and each protein fragment (10 µg/ml) and incubated at 4°C overnight. Plates were washed three times with 0.05% Tween-20 in PBS (PBST). Subsequently, wells were blocked with 3% skim milk in PBST for 1 hour at 37°C and then washed three times with PBST. Fifty microliters of a 1:4 diluted sera from cockroach sensitized and non-sensitized subjects were added to each well, and the plates were incubated at 37°C for 2 hours. After rinsing 3 times with PBST, 50 µl of biotinylated goat anti-human IgE (1:1,000 dilution) (Vector, Burlingame, CA) was added, and followed by incubation at 37°C for 1 hour. After rinsing with PBST, streptavidin-peroxidase was added and the plates were incubated at 37°C for 30 minutes. The plates were washed 5 times with PBST, and 100 µl of 3,3',5,5'-tetramethylbenzidine substrate solution (Kirkegaard Perry Laboratories, Gaithersburg, MD) was added. Following an incubation for 15 minutes in the dark, reactions were terminated by the addition of 100 µl of 0.5 M H_2SO_4. Absorbances at 450 nm were obtained using an ELISA reader. Cut-off values were calculated by following the equation: cut-off = (mean absorbance of 16 negative sera) + (2 × [standard deviations of 16 negative sera]).

RESULTS

Expression of recombinant Bla g 1.02 and peptide fragments

DNA sequence encoding full-length recombinant Bla g 1.02 containing 6 His codons were subcloned into pPIC9 for expression in *P. pastoris*, while five overlapping peptide fragments were expressed in *E. coli* (Fig. 1). Protein fragments (A, B, C, D, and E) were solubilized from the *E. coli* insoluble fractions using urea, and subsequently purified using Ni-NTA resin. The theoretical molecular weight of the full-length Bla g 1.02 is 57.5 kDa and the molecular weight of fragments A, B, C, D and E are 17.3, 17.6, 15.4, 17.6, 15.8 kDa, respectively (Fig. 2). The protein concentrations of the final purified preparations were as follows: full-length Bla g 1.02, 2.90 mg/l; fragment A, 14.69 mg/l; fragment B, 15.51 mg/l; fragment C, 1.83 mg/l; fragment D, 17.00 mg/l; and fragment E, 2.24 mg/l.

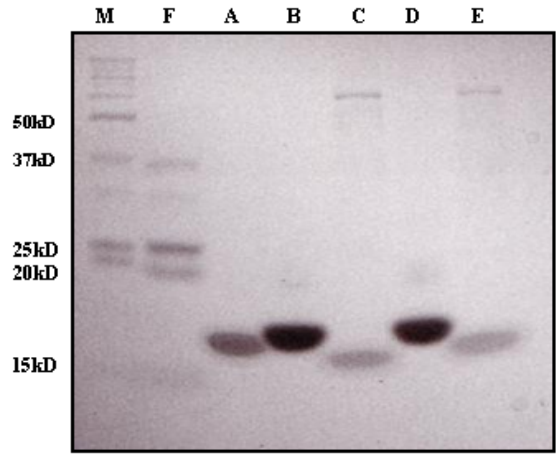

Fig. 2 Expression and purification of recombinant full-lenth and fragments of Bla g 1.02. M, molecular mass marker (Bio Rad marker No.0373); F, Bla g 1.02 full-length; A, Bla g 1.02 fragment A; B, Bla g 1.02 fragment B; C, Bla g 1.02 fragment C; D, Bla g 1.02 fragment D; and E, Bla g 1.02 fragment E.

IgE-binding reactivity of full-length recombinant Bla g 1.02

ELISA was used to test the reactivity of purified recombinant Bla g 1.02 with IgE antibodies from cockroach allergic patients. Among the 37 sera tested, 24 patients (64.9%) showed IgE reactivity to full-length recombinant Bla g 1.02. Sera from healthy subjects, which were used as negative controls, did not exhibit any IgE reactivity (Fig. 3).

IgE-binding reactivity of Bla g 1.02 peptide fragments and IgE epitope analysis of recombinant Bla g 1.02

Linear IgE-binding epitopes were studied using Bla g 1.02 fragments that were expressed as five separate recombinant overlapping peptides A, B, C, D, and E. As mentioned above, 24 from 37 patient serum samples demonstrated IgE reactivity to recombinant full-length Bla g 1.02 protein. Nineteen sera were then used to test IgE reactivities with the purified recombinant peptide fragments. Of these, 16 sera showed positive reactivity to at least one of the Bla g 1.02 fragments (Fig. 4). Heterogeneous patterns of IgE reactivities to the Bla g 1.02 allergen fragments were found. Only 1 serum sample showed IgE-binding reactivity to all five peptide fragments. Fragment A showed the highest IgE reactivity frequency (12/19, 63.2%), followed by fragments E (10/19, 52.6%), and D (9/19, 47.4%). Fragments B and fragment C exhibited low IgE binding frequencies, *i.e*. 5/19 (26.3%) and 4/19 (21.1%), respectively. The strongest IgE reactivity of all fragments among all patients tested was observed towards fragment B (Table 3).

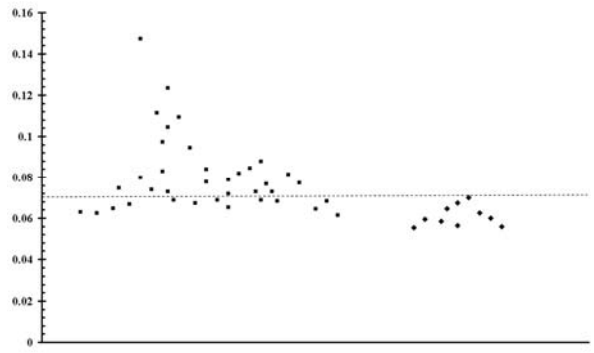

Fig. 3 IgE antibody reactivity of human sera against recombinant Bla g 1.02. The dashed horizontal line indicates the cut-off value. ■, Sera from subjects sensitized to German cockroach extract (Uni-CAP>0.7); ◆, Sera from healthy subjects.

DISCUSSION

Inhalation of cockroach allergens by sensitized atopic patients causes asthmatic responses. Measurements of Bla g 1 levels within the home has revealed environmental exposures at > 2 U/g is a strong risk factor for sensitization.[24-25] Despite its importance, the biological functions and mechanisms through which allergic reactions are induced to genetically sensitized individuals are not fully understood. In this study, IgE reactivities of Bla g 1 expressed as a recombinant protein in *P. pastoris* and the overlapping Bla g 1 fragments produced from *E. coli* were assessed in cockroach allergic patients. It is

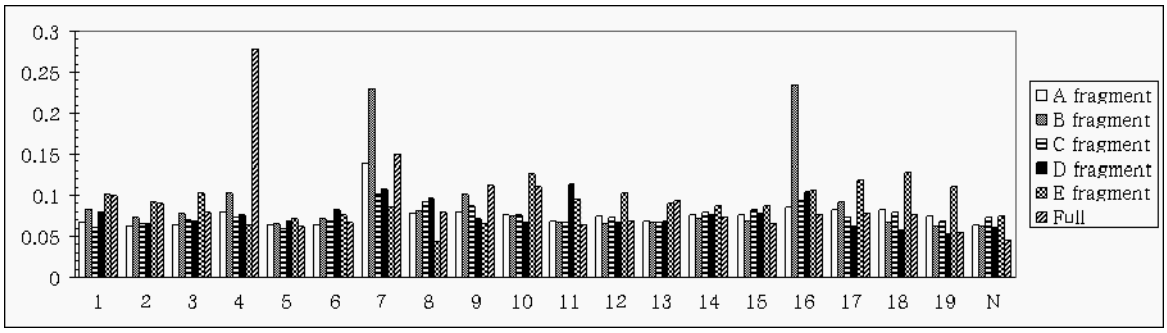

Fig. 4 Binding profile of IgE antibodies to recombinant Bla g 1.02 full length and fragment proteins. Data is from 19 serum samples selected from 37 allergic patients. N, pooled sera from 10 healthy non-allergic subjects.

important to note that *E. coli* has been found to be incapable of replicating a recombinant plasmid which harbors more than two Bla g 1 duplexes.[22] This observation is due to a *recA* independent process called "replication slippage," through which *E. coli* removes repeated DNA in plasmids.[23] Furthermore, yields of recombinant full-length Bla g 1 in the *E. coli* host are far inferior to the methanol-inducible *P. Pastoris* system. Therefore, *P. pastoris* was used to express the full length protein in the present study. However, the recombinant full-length Bla g 1 expressed in *P. pastoris* showed the multiple bands on the SDS-PAGE. Multiple forms of recombinant Bla g 1 were produced possibly by multiple integrations of expression cassettes in the yeast genome and subsequent recombinant events between repeats and loss of DNA repeat.[22] Another possibility is proteolytic degradation of the expressed proteins, as addition of PMSF and EDTA was reported to reduce slightly the production of the low molecular protein bands.

IgE binding reactivities to full-length and peptide fragments were determined by ELISA using 37 serum samples of cockroach allergic patients. Sera from 24 patients (64.9%) reacted with rBla g 1.02, however, the IgE binding intensity was low, a feature that has also been noted elsewhere.[22] In order to analyze linear IgE-binding epitopes, we designed five peptide fragments based on established tandem repeats of Bla g 1, and measured IgE responses of each fragment. Using ELISA, 12 (63.2%), 5 (26.3%), 4 (21.1%), 9 (47.4%), and 10 (52.6%) were reactive to fragments A, B, C, D, and E, respectively. Three sera (P2, P10, and P23) reacted to full-length proteins, but did not react to peptide fragments (Table 3). This observation may indicate the presence of conformational epitopes. Amino acids of fragment B may contain cryptic epitopes since three sera reacted with this protein B with a higher intensity than the full-length peptide. All serum samples that were positive to the individual peptide fragments were found to be reactive to at least one of the fragments A, D, or E.

Upon comparison to the amino acid sequences of fragments A, C, and E, twelve amino acid differences out of 83 conserved amino acids are observed (Fig. 5A). The IgE responses were similar for

Table 3 IgE binding reactivities of recombinant Bla g 1.02 peptide fragments

	A	B	C	D	E	F
P1	0.0670	0.0825	0.0610	0.0790	0.1010	0.100
P2	0.0625	0.0730	0.0660	0.0650	0.0925	0.0910
P8	0.0645	0.0785	0.0700	0.0685	0.1035	0.0790
P9	0.0790	0.1030	0.0735	0.0770	0.0635	0.2775
P10	0.0645	0.0660	0.0595	0.0690	0.0720	0.0620
P11	0.0635	0.0725	0.0695	0.0835	0.0760	0.0670
P12	0.1375	0.2300	0.1015	0.1075	0.0855	0.1505
P13	0.0780	0.0815	0.0920	0.0965	0.0435	0.0795
P14	0.0800	0.1010	0.0875	0.0720	0.0660	0.1125
P15	0.0770	0.0750	0.0760	0.0675	0.1265	0.1105
P16	0.0690	0.0670	0.0670	0.1135	0.0955	0.0645
P23	0.0695	0.0675	0.0675	0.0695	0.0910	0.0930
P25	0.0765	0.0720	0.0800	0.0770	0.0880	0.0730
P27	0.0830	0.0925	0.0740	0.0620	0.1185	0.0775
P28	0.0750	0.0650	0.0730	0.0670	0.1030	0.0695
P31	0.0825	0.0670	0.0800	0.0575	0.1280	0.0760
P32	0.0755	0.0630	0.0685	0.0535	0.1110	0.0540
P33	0.0770	0.0685	0.0830	0.0785	0.0870	0.0660
P37	0.0855	0.2340	0.0945	0.1045	0.1065	0.0770

Each cut-off value is A, 0.075148; B, 0.086673; C, 0.087476; D, 0.074573; E, 0.094261; full length Bla g 1.02 (F), 0.053648.

fragments A and E, whereas the fragment C exerted the lowest IgE reactivity. It is possible that the IgE response is altered by the Glu residue at position 266 of fragment C, which corresponds to Gly at residue 78 and Gln at residue 454 of the A and E fragments, respectively. Comparison of the B and D fragments reveals six amino acid differences. The IgE reactivity of the fragment D is slightly higher than that of the fragment B, which may be caused by the presence of an isoleucine (Ile) at position 321 for this fragment (Fig. 5B). The influences of a single amino acid substitution on IgE reactivity have been described for Der p 2 and Der f 2.[26-28]

Previous work has shown that single duplexes of Bla g 1 are sufficient in binding IgE, and that the presence of an N-terminus in the recombinant Bla g 1 did not affect IgE binding responses.[22] The present study demonstrated poor IgE reactivity to a tandem repeat of consecutive amino acids derived from the one duplex. Differential IgE-binding patterns to Bla g 1.02 in individual patient sera were also observed.

In summary, the peptide fragments A, D, and E may be suitable tools in the identification of Bla g 1-sensitized subjects. Information on Bla g 1.02 IgE-binding epitopes could be helpful in the diagnosis of allergies and may facilitate the design of new immunotherapeutic regimes.

ACKNOWLEDGMENTS

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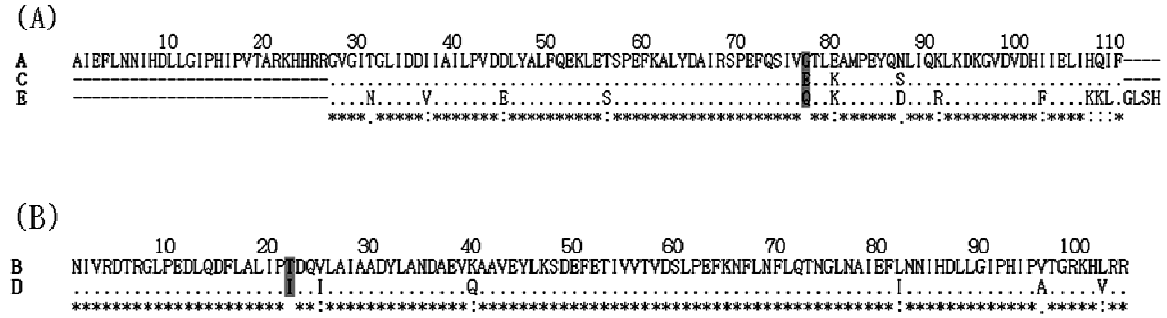

Fig. 5 Amino acid sequence alignments of Bla g 1.02 fragments. (A) Bla g 1.02 A, C and E fragments. (B) Bla g 1.02 B and D fragments. Amino acids are denoted as identical (*), highly conserved (:), weakly conserved (.) or non-conserved (shaded) residues.

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