

Allergenicity of Sigma and Delta Class Glutathione S-Transferases from the German Cockroach

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Key Words

Allergen · Bla g 5 · Cockroach glutathione S-transferase · IgE cross-reactivity

Abstract

Background: Cockroach glutathione S-transferases (GSTs) are known to elicit strong IgE responses. This study was undertaken to compare the IgE reactivity of German cockroach GSTs, Bla g 5 (σ class) and δ class GST (BgGSTD1). **Methods:** Full-length Bla g 5 and BgGSTD1 were cloned, and their recombinant proteins were expressed and purified. Their IgE reactivities and cross-reactivities were examined by ELISA using sera from cockroach-sensitized subjects. **Results:** A predominant variant of Bla g 5 cDNA has amino acid substitutions at positions 10 (C to F) and 42 (N to K). BgGSTD1 has substitutions at positions 27 (E to N) and 207 (K to R). Sera from cockroach-sensitized patients showed 20.5% IgE reactivity to Bla g 5 and 17.9% IgE reactivity to BgGSTD1. However, inhibition studies using 1 serum sample with the highest IgE reactivity showed limited cross-reactivity. **Conclusions:** IgE-binding frequency to the cockroach GSTs was low, but the titer of IgE reactivity was strong in some sera. The inclusion of different classes of GSTs could be helpful for the delicate diagnosis and immunotherapy of cockroach allergy.

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Introduction

Glutathione S-transferases (GSTs; EC2.5.1.18) are a superfamily of enzymes that play important roles in the detoxification of foreign compounds, particularly insecticides and plant allelochemicals, and the protection against oxidative stress [1]. More than 30 GST genes have been identified both from the fruit fly [2] and mosquito [3]. Insect cytosolic GSTs are classified into 6 groups by phylogenetic comparison of insect and mammalian GSTs [4].

GSTs from various arthropods can elicit allergic diseases. The group 8 allergen of the house dust mite (*Dermatophagoides pteronyssinus*) was shown to be homologous to GST [5]. GSTs from itch mites, which are the causative agent of scabies, have been documented as important allergens [6, 7]. The IgE cross-reactivity between American cockroach and mite GSTs has also been described [8]. The German cockroach Bla g 5, homologous to the σ class GSTs, is known to be important [9]. Interestingly, Bla g 5, along with Bla g 2, was shown to dominate the IgE antibody responses to cockroach [10]. Furthermore, no allergen was found to be immunodominant, and Bla g 5 was only 1 allergen (among 5 cockroach allergens tested) against which specific IgE levels were shown to be correlated with skin test reactivity [11].

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Three GSTs were identified by glutathione-agarose affinity chromatography from male adults of German cockroach [12]. The N-terminal amino acid sequence of one of the purified GSTs was identical to that of Bla g 5. More recently, a δ class GST (BgGSTD1), which has 15% amino acid sequence identity with Bla g 5, was purified from the German cockroach and its IgE reactivity was demonstrated [13]. Two IgE-reactive GSTs were also detected from 25 IgE-reactive spots by proteomic analysis, and one of them was found to be a Bla g 5 from the whole body extract of the German cockroach [14].

In this study, we have cloned 5'-regions of Bla g 5, because the N terminus of the previously reported sequence was truncated, and compared the allergenicity between Bla g 5 and BgGSTD1.

Material and Methods

Patients' Sera

Informed consent was obtained and blood collected from patients attending the Allergy Clinic of the Severance Hospital, Yonsei University, Seoul, Korea. Allergy was diagnosed based on case history and skin prick testing. All the sera obtained were tested for the German cockroach-specific IgE using the Uni-CAP system and ELISA. Those with CAP results higher than 0.7 kU/l were used for the following study ($n = 39$; age range 7–58 years, average 33 years). Fifteen serum samples obtained from subjects who showed negative reactions to both the skin prick test and Uni-CAP were used as negative controls.

Cloning of cDNAs Encoding Full-Length GST Sequences

Total RNA was isolated from adult female cockroaches that had been reared at the Arthropods of Medical Importance Resource Bank, Department of Environmental Medical Biology and Institute of Tropical Medicine, Yonsei University College of Medicine, using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA). For the cloning of BgGSTD1, a first-strand cDNA template was synthesized from 5 μ g of RNA. PCR was then performed using the following oligonucleotide primers: BgGSTD1F2 (forward), 5'-CCATGGGAATGACCATTGATTCTAT-3', and BgGSTD1R3 (reverse), 5'-GTGCGACTTAATGATGATGATGATGATGTTT-TGCGTGAGATT-3'. An *Nco*I restriction site was incorporated for the forward primer, and an *Xho*I restriction site and 6 histidine tag sequences for the reverse primer. The PCR products were purified with UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Inc., Solana Beach, Calif., USA), ligated into pGEM-T Easy vector (Promega, Madison, Wisc., USA) and analyzed by DNA sequencing at Solgent (Daejeon, Korea).

The 5'-end of Bla g 5 was amplified by BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, Calif., USA). 5'-RACE CDS Primer (adaptor primer, BD Biosciences) and Bla g 5 gene-specific primer (Bg5RC1, 5'-CCACTGAGGCCAACT-GCTTACC-3') designed according to the Bla g 5 internal sequence were utilized for 5'-RACE. The PCR product was purified, subcloned and analyzed as described above.

RT-PCR was carried out for the amplification of full-length cDNA encoding Bla g 5. The oligonucleotides used for the RT-PCR were as follows: Bg5NF (forward), 5'-GGATCCAATG-GCTCCTTCTTATAAAC-3', and BgBR (reverse), 5'-CTCGAG-CAGATCTGTAGGAGGA-3'. *Bam*HI and *Xho*I restriction sites were incorporated in the forward and reverse primers, respectively. The PCR product was analyzed as previously described.

Expression of GSTs in Escherichia Coli

Both cDNA sequences encoding Bla g 5 and BgGSTD1 were cloned into pGEM-T Easy vector and were then directionally subcloned into pET 28b expression vector (Novagen, Merck Biosciences, Darmstadt, Germany) and transformed into *E. coli* BL21 (DE3; Novagen). Subsequently, the expression of recombinant proteins was induced by the addition of 1 mM isopropyl-1-thio- β -galactopyranoside and purified from the insoluble fraction of the cell lysates by using a Ni-NTA agarose resin (Qiagen, Valencia, Calif., USA) under denaturing conditions (20 mM Tris, 500 mM NaCl, 1 M imidazole, 6 M urea, pH 7.9). Recombinant Bla g 5 protein was also purified under native conditions (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0), and it was used for the analysis of IgE-binding reactivity. The concentration of each of the proteins was determined by Bradford Assay (Bio-Rad Laboratories, Reagent Park, NSW, Australia). The purified proteins were also analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis using 15% reducing gels and stained with Coomassie Blue R250 (Sigma-Aldrich, Sydney, NSW, Australia).

Specific IgE Binding to Recombinant GSTs

The reactivity of specific IgE antibodies to recombinant proteins was examined by ELISA. In brief, microtiter plates were coated with 10 μ g/ml of recombinant protein and incubated with 1:4 diluted serum samples. Subsequently, IgE antibodies were detected using biotinylated goat anti-human IgE (ϵ chain specific; Vector, Burlingame, Calif., USA) and streptavidin-peroxidase (Sigma-Aldrich). The signal was developed using 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, Md., USA). The mean absorbance $\pm 2 \times$ standard deviations of the sera from 15 healthy controls was used as a cutoff value.

Inhibition Study

Cross-inhibition between Bla g 5 and BgGSTD1 was examined using ELISA. Diluted sera were incubated with various concentrations of GSTs overnight before adding to the GST-coated plates. Then IgE antibodies were detected as described above. The percentage of inhibition was calculated as $(1 - A_i/A_0) \times 100$, where A_i stands for absorbance at 450 nm with an inhibitor and A_0 for absorbance at 450 nm without an inhibitor.

Results

Isolation and Analysis of cDNA Clones

To obtain the full-length cDNA sequence of Bla g 5, RACE-PCR and sequencing were carried out using primers designed on the basis of the previously reported truncated sequence (GenBank accession No. EF202178). An

		10	20	30	40	50	60	70	80	90	100	110
Bla g 5	~~~~YKLT	YCVKAL	GEPIR	FLLSYGEKDF	EDYRFQEGDW	PNLKPSMPFG	KTPVLEIDGK	QTHQSVAISR	YLGKQFGLSG	KDDWENLEID	MIVDTISDFR	AAIANYHYDA
5-2	MAPS.....F											
5-1	MAPS.....F											
5-3	MAPS.....F											
5-4	MAPS.....F											
5-9	MAPS.....F											
5-11	MAPS.....F											
5-12	MAPS.....F											
5-17	MAPS.....F											
5-18	MAPS.....F											
5-22	MAPS.....F											
5-24	MAPS.....F											
5-25	MAPS.....F											
5-29	MAPS.....F											

		120	130	140	150	160	170	180	190	200	
Bla g 5	DENSKQKKWD	PLKKETIPYY	TKKFDEVVKA	NGGYLAAGKL	TWADFYFVAI	LDYLNHMAKE	DLVANQPNLK	ALREKVLGLP	AIKAWVAKRP	PTDL	
5-2											(15/27) [99.0%]
5-1											(1/27) [98.5%]
5-3											(1/27) [99.0%]
5-4											(1/27) [98.5%]
5-9											(1/27) [97.5%]
5-11	..S.....										(1/27) [98.5%]
5-12											(1/27) [98.5%]
5-17											(1/27) [98.5%]
5-18											(1/27) [98.5%]
5-22											(1/27) [98.0%]
5-24											(1/27) [98.5%]
5-25											(1/27) [98.5%]
5-29											(1/27) [98.0%]

Fig. 1. Amino acid sequence variations of Bla g 5 identified by RT-PCR. The frequency and the percent sequence identity of each variant are shown in parentheses and brackets, respectively. The predominant form of the full-length Bla g 5 sequence was deposited in GenBank under accession No. EU266077.

additional 4 amino acid residues were identified. Subsequently, RT-PCR was performed using primers designed to amplify the complete cDNA sequence which spans 615 bp. Translation with the putative initiation codon would produce a polypeptide of 204 amino acids with an estimated molecular mass of 23,393 Da and a predicted isoelectric point value (pI) of 7.593. Deduced amino acid sequences of full-length Bla g 5 showed 97.5–99.0% identity to the previously reported Bla g 5 (EF202178).

cDNA sequence-encoding BgGSTD1 was also isolated by RT-PCR. The cloned cDNA sequence contains an 648-bp open reading frame coding for 216 amino acids, with a calculated molecular mass of 24,645 Da and a pI of 7.15. Deduced amino acid sequences of BgGSTD1 identified in this study showed 99.0% identity to previously reported BgGSTD1 (AM778448).

Sequence Variations of Cockroach GSTs

Examination of 27 clones of full-length Bla g 5 revealed 13 variants (fig. 1). Sporadic amino acid changes at 15 positions were identified in 12 clones. All Bla g 5

cDNA sequences investigated had the same 2 amino acid substitutions at position 10 (Cys to Phe) and position 42 (Asn to Lys). The predominant form of full-length Bla g 5 sequence was deposited in GenBank under accession No. EU266077 which is a secondary number to EF202178 because these 2 sequences are essentially identical except for the primer sequences. Analysis of 3 BgGSTD1 clones showed polymorphisms at position 27 (Glu to Asn) and position 207 (Lys to Arg). This sequence was deposited in GenBank under accession No. EU266076.

Production of Recombinant Proteins

The cDNAs encoding Bla g 5 and BgGSTD1 were expressed as 6 His-tagged proteins in *E. coli*. The recombinant proteins were subjected to SDS-PAGE after purification (fig. 2a). Bla g 5 and BgGSTD1 showed bands of 30 and 27 kDa, respectively, as compared with their calculated molecular masses of 28.07 and 25.66 kDa, which included their tags (34 amino acids at the N terminus and 8 amino acids at the C terminus for Bla g 5, and 2 amino acids at the N terminus and 6 His at the C terminus for

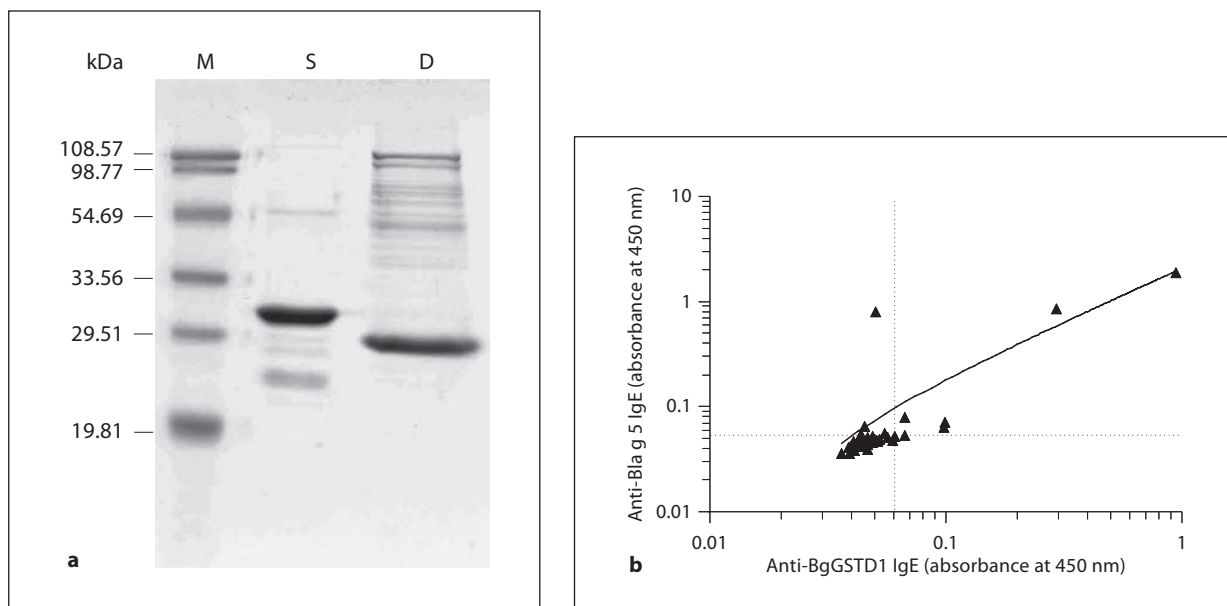


Fig. 2. IgE reactivity of human sera. SDS-PAGE analysis of purified recombinant GSTs (a). IgE reactivity of sera from cockroach-sensitized patients against recombinant cockroach GSTs (b). The dotted line indicates the cut-off values. M = Molecular mass standard; S = σ class recombinant GST; D = δ class recombinant GST.

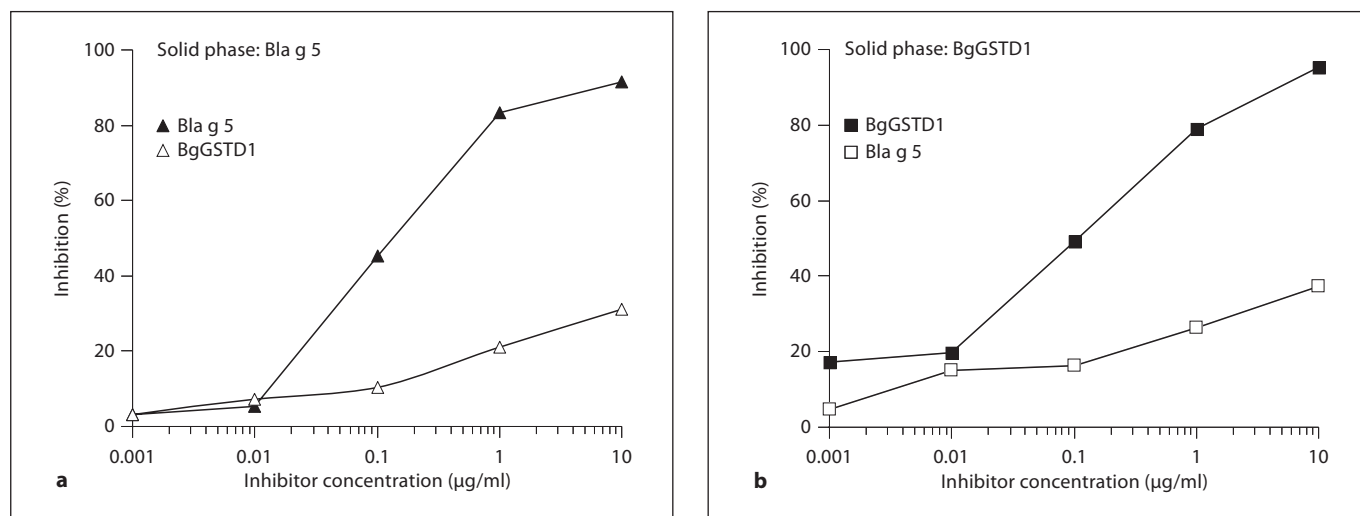


Fig. 3. Cross-reactivity between Bla g 5 and BgGSTD1. Inhibition of Bla g 5 with BgGSTD1 (a) and inhibition of BgGSTD1 with Bla g 5 (b).

BgGSTD1). The yield of purified proteins was 34.964 mg/l (22.508 mg/l from soluble fraction and 12.456 mg/l from insoluble fraction) of bacteria culture for Bla g 5 and 1.089 mg/l for BgGSTD1. The soluble fraction of the purified Bla g 5 was used for further analyses.

IgE Reactivity and Inhibition Analysis

IgE reactivity to Bla g 5 and BgGSTD1 was determined by ELISA (table 1; fig. 2b). Among 39 serum samples tested, 20.5% (8/39) and 17.9% (7/39) showed IgE reactivity to Bla g 5 and BgGSTD1, respectively. Ten out of 39 sera

Table 1. IgE reactivity of German cockroach-sensitized sera against recombinant GSTs

Serial No.	Sex	Age years	Diagnosis	Absorbance at 450 nm ^a	
				Bla g 5	BgGSTD1
P1	M	7	atopic dermatitis	1.8890	0.9440
P2	M	49	allergic asthma	0.0360	0.0360
P3	M	48	dyspnea	0.0360	0.0390
P4	M	14	allergic rhinitis	0.0385	0.0410
P5	F	46	allergy cough	0.0405	0.0385
P6	M	26	atopic dermatitis	0.0420	0.0395
P7	M	35	anaphylatic shock	0.0475	0.0510
P8	M	47	allergic dermatitis	0.0475	0.0595
P9	M	16	allergic dermatitis	0.0455	0.0490
P10	F	15	allergic rhinitis/dermatitis	0.0470	0.0515
P11	M	33	allergic rhinitis	0.0785	0.0670
P12	F	45	allergic rhinitis	0.0510	0.0465
P13	M	49	cough	0.0505	0.0565
P14	F	40	allergic asthma	0.0535	0.0670
P15	F	52	allergic dermatitis	0.0425	0.0420
P16	M	43	allergic asthma	0.0395	0.0465
P17	M	13	allergic rhinitis	0.0415	0.0445
P18	M	19	allergic dermatitis	0.0415	0.0435
P19	M	21	allergic dermatitis	0.0455	0.0460
P20	F	9	allergic rhinitis	0.0455	0.0430
P21	M	49	conjunctivitis	0.0465	0.0405
P22	F	9	allergic dermatitis	0.0560	0.0550
P23	F	49	allergic asthma	0.0480	0.0440
P24	M	20	allergic rhinitis	0.0710	0.0995
P25	M	20	allergic dermatitis	0.0515	0.0605
P26	F	42	allergic asthma	0.0655	0.0450
P27	F	17	atopic dermatitis	0.8050	0.0505
P28	F	11	allergic dermatitis	0.8540	0.2950
P29	F	16	atopic dermatitis	0.0635	0.0980
P30	F	22	allergic eczema	0.0475	0.0490
P31	F	20	allergic dermatitis	0.0485	0.0525
P32	M	30	allergic urticaria	0.0450	0.0440
P33	M	41	allergic rhinitis	0.0475	0.0445
P34	M	56	allergic asthma	0.0440	0.0465
P35	F	45	allergic asthma	0.0530	0.0435
P36	M	45	asthma/rhinitis	0.0505	0.0430
P37	M	38	allergic rhinitis	0.0520	0.0490
P38	M	52	allergic asthma	0.0490	0.0500
P39	F	58	bronchitis	0.0530	0.0440

^a The cutoff values were 0.062 for BgGSTD1 and 0.055 for Bla g 5, respectively.

showed IgE reactivity to cockroach GSTs. Five sera showed IgE reactivity to both Bla g 5 and BgGSTD1. Three and 2 sera exhibited IgE reactivity to Bla g 5 and BgGSTD1. The level of IgE antibodies against Bla g 5 was higher than that of IgE antibodies against BgGSTD1. Sensitization to Bla g 5 and BgGSTD1 does not seem to

correlate ($p > 0.099$). Therefore, 1 serum sample showing the strongest IgE reactivity to both GSTs was selected, and inhibition studies were conducted to investigate the degree of cross-reactivity between GSTs (fig. 3). Serum preabsorbed with 10 $\mu\text{g/ml}$ of BgGSTD1 inhibited 31.2% of IgE reactivity to Bla g 5, whereas serum preabsorbed with the same amount of Bla g 5 inhibited 91.6% of IgE reactivity to Bla g 5. Bla g 5 was able to inhibit 37.3% of IgE reactivity to BgGSTD1, whereas BgGSTD1 inhibited 95.2% of IgE reactivity to BgGSTD1.

Discussion

Small bugs infesting households can cause various health problems [15]. In particular, cockroach species produce potent allergens, and sensitization to their allergens causes severe allergic disorders [16]. In this study, we have described the cloning, expression and characterization of German cockroach GSTs. GSTs have been proposed as an arthropod panallergen [17] distinct from tropomyosin, an invertebrate panallergen [18].

Two GSTs of 23 kDa, pI 5.9, and 22 kDa, pI 6.8, were purified by glutathione-agarose column [14]. The 23-kDa GST reacted strongly with the IgE antibodies, whereas the 22-kDa GST exhibited weak immunogenicity. The N-terminal amino acid sequence of one of the purified Bla g 5 has Phe at position 10 and deletion of the first amino acid Met [9, 12]. For BgGSTD1, 2 amino acid substitutions were also found: Glu to Asn at position 27 and Lys to Arg at position 207. Moreover, polymorphisms could also contribute to the differences in isoelectric points and molecular masses with previously reported sequences. For Bla g 5, we have previously described the amino acid substitution of Lys for Asn at position 42 (EF202178), as compared with U92412 [19]. The cloning of the so-far missing cDNA sequence of the N-terminal region of Bla g 5 confirmed the N-terminal sequence obtained by protein sequencing by Arruda et al. [9]. Interestingly, the amino acid at position 10 of Bla g 5 was determined as Phe which is identical to the N-terminal protein sequence of Arruda et al. [9] and differs from Cys in their cDNA sequence (U92412) (fig. 1). Probably, an error in the primer design which was based on the U92412 sequence may result in Cys at position 10 in the previously reported sequence of Bla g 5 (EF202178).

Some GSTs may play a protective role against tissue damage from oxidative stresses [20]. Cockroaches that have been exposed to insecticide and other chemicals would express elevated levels of GSTs, which could re-

main in large amounts in the bodies of dead cockroaches. It is conceivable that GSTs released from the dead cockroaches sensitize genetically predisposed persons.

Sensitization to Bla g 5 and BgGSTD1 does not seem to have a statistically significant correlation ($p > 0.099$) (fig. 2b). Furthermore, the cross-reactivity between Bla g 5 and BgGSTD1 is found to be limited (fig. 3) when assessed by 1 serum sample which showed the strongest IgE reactivity to the recombinant GSTs. It is possible that various isoforms may elicit allergic responses at the same time. Cosensitization by the same allergen source organism and the same route may in part explain this observation.

Low amino acid sequence identity (14%) and low cross-reactivity of Bla g 5 and BgGSTD1 were observed in this study. Therefore, BgGSTD1 may represent a new group of cockroach allergens. Cross-reactivity with other GSTs from dust mites and from other species of cockroach needs to be investigated. It is also possible that other classes of GSTs such as μ class and/or ε class GSTs also

exist in the German cockroach. In addition, it would be interesting to see if anti-GST IgE antibodies are produced by the administration of allergen extracts for immunotherapy.

In summary, polymorphic residues of full-length Bla g 5 and BgGSTD1 were investigated, and recombinant proteins of predominant variant GSTs were produced. Recombinant proteins showed strong IgE reactivity from some serum samples tested. We have also described the limited degree of cross-reactivity between the recombinant GSTs of different classes from the tested serum sample. BgGSTD1 may be useful for diagnostic and therapeutic purposes in people allergic to cockroaches.

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