

Purification and cloning of a Delta class glutathione S-transferase displaying high peroxidase activity isolated from the German cockroach *Blattella germanica*

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A highly active glutathione S-transferase was purified from adult German cockroaches, *Blattella germanica*. The purified enzyme appeared as a single band of 24 kDa by SDS/PAGE, and had a different electrophoretic mobility than, a previously identified Sigma class glutathione S-transferase (Bla g 5). Kinetic study of 1-chloro-2,4-dinitrobenzene conjugation revealed a high catalytic rate but common substrate-binding and cosubstrate-binding affinities, with $V_{\max}$, k_{cat} , K_m for 1-chloro-2,4-dinitrobenzene and K_m for glutathione estimated to be $664 \mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, 545 s^{-1} , 0.33 mM and 0.76 mM , respectively. Interestingly, this enzyme possessed the highest activity for cumene hydroperoxide among insect glutathione S-transferases reported to date. Along with the ability to metabolize 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane and 4-hydroxynonenal, this glutathione S-transferase may play a role in defense against insecticides as well as oxidative stress. On the basis of the amino acid sequences obtained from Edman degradation and MS analyses, a 987-nucleotide cDNA clone encoding a glutathione S-transferase (*BggstD1*) was isolated. The longest ORF encoded a 24 614 Da protein consisting of 216 amino acid residues. The sequence had close similarities ($\sim 45\text{--}60\%$) to that of Delta class glutathione S-transferases, but had only 14% identity to Bla g 5. The putative amino acid sequence contained matching peptide fragments of the purified glutathione S-transferase. ELISA showed that BgGSTD1 bound to serum IgE obtained from patients with cockroach allergy, indicating that the protein may be a cockroach allergen.

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a ubiquitous superfamily of enzymes that play key roles in detoxification of xenobiotic and endogenous electrophiles [1]. They catalyze the conjugation of the tripeptide glutathione (GSH) to electrophilic centers of lipophilic compounds via a nucleophilic substitution/addition reaction, thus forming more soluble con-

jugates that can be readily excreted from the cells. GSTs display remarkably broad substrate specificities, including unsaturated carbonyls, electrophilic aldehydes, epoxides, and organic hydroperoxides. The majority of GSTs identified are cytosolic, but a few members have been identified in microsomes as well as mitochondria/peroxisomes. Cytosolic GSTs are

Abbreviations

5-ADO, 5-androstene-3,17-dione; BSP, bromosulphophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene; DDT, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene; EA, ethacrynic acid; ENPP, 1,2-epoxy-3-(4-nitrophenoxy)propane; GST, glutathione S-transferase; GSH, reduced glutathione; 4-HNE, 4-hydroxynonenal; 4-NBC, 4-nitrobenzyl chloride; 4-NPA, 4-nitrophenol acetate; 4-NPB, 4-nitrophenethyl bromide; *t*-PBO, *trans*-4-phenyl-3-buten-2-one.

heterodimeric or homodimeric proteins. Each subunit is approximately 24–28 kDa in size. Phylogenetic analysis has revealed the presence of at least six classes of cytosolic GSTs in insects [2]. The majority of GSTs are in the Delta and Epsilon classes, and the remaining enzymes are in the Omega, Sigma, Theta and Zeta classes.

The German cockroach (*Blattella germanica*) is an economically important pest that is commonly found in human dwellings worldwide. Like many other insects, the German cockroaches have been studied extensively for their resistance to insecticides [3–6]. Elevated levels of GST activity have been observed in cockroach strains that have developed resistance to organophosphates, carbamates and pyrethroids. However, information about the enzymatic activities of cockroach GST is scarce. To date, no cockroach GST has been shown to metabolize any insecticide. Biochemical studies have been conducted to characterize GSTs of the German cockroach, but they have been limited to enzymes partially purified using native PAGE [7]. Only one Sigma class GST (BgGSTS1) has been identified by molecular cloning [2,8]. The recombinant enzyme exhibits very low activity toward 1-chloro-2,4-dinitrobenzene (CDNB), a typical substrate of GSTs. Interestingly, BgGSTS1 is a potent cockroach allergen [8] and is commonly known as Bla g 5 – the fifth protein allergen isolated from *B. germanica* [8,9]. Bla g 5 is such a potent allergen that as little as 3 pg of recombinant protein is sufficient to cause positive immediate skin tests in cockroach-allergic patients. Subsequent *in vitro* immunologic experiments have indicated that more than one GST exhibits serum IgE-binding activity, indicating that more GST members may also be allergens [7,10]. We now report the purification, characterization and molecular cloning of a Delta class GST from the German cockroach. The potential roles of this GST in defense against insecticides, as well as serum IgE-binding activity, are discussed.

Results

GST purification

Ultracentrifugation removed ~40% of cellular protein present in the whole body homogenate while preserving ~91% of the GST activity (Table 1). Affinity chromatography using a GSH column was used to further purify the cytosolic fraction. A small proportion (< 10%) of the GST activity was detected in the flow-through fraction. Further experiments confirmed that the lack of binding was not due to overloading of the column matrix. SDS/PAGE of the affinity-purified

Table 1. Purification summary of *B. germanica* GST. Activity was determined with CDNB as substrate at room temperature.

Fraction	Total protein (mg)	Total activity ($\mu\text{mol}\cdot\text{min}^{-1}$)	Specific activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	Yield (%)	Purification (x)
Homogenate	92.9	208.1	2.2	100	1
Cytosolic fraction	55.6	189.5	3.4	91	1.5
GSH-affinity column	1.27	129.2	102	62	45
Phenyl HP column	0.09	45.36	508	22	227

fraction revealed two major protein bands and several faint bands (Fig. 1). The affinity-purified proteins were then separated using hydrophobic interaction chromatography. One major peak exhibiting enzyme activity was observed in the final 30% ethylene glycol elution (Fig. 2A), resolving as a single band on SDS/PAGE with a molecular mass of ~24 000 Da (Fig. 1). HPLC analysis of the purified GST confirmed the presence of a single protein of ~95% purity (Fig. 2B), suggesting that the enzyme exists as a homodimer. This GST had an electrophoretic mobility slightly greater than that of a previously cloned Sigma class GST (Bla g 5), indicating that the GST identified in this study is unlikely to be Bla g 5. A summary of purification data for *B. germanica* GST is presented in Table 1. It is important to

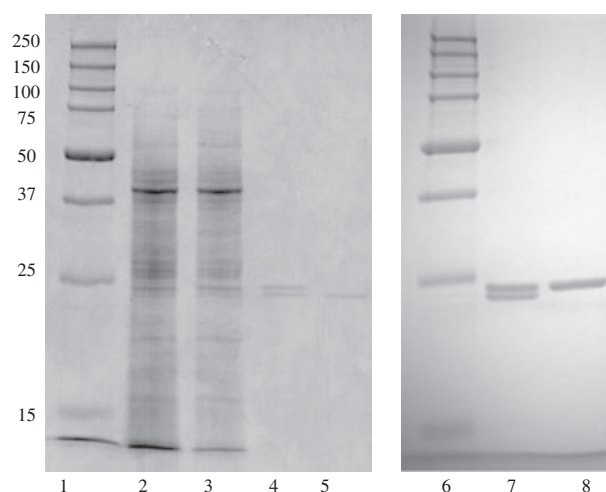


Fig. 1. SDS/PAGE analysis of *B. germanica* GSTs. Electrophoresis was performed in a 12% gel. Lanes 1 and 6: molecular mass markers, as indicated by the scale (in kDa) on the left. Lane 2: crude homogenate. Lane 3: cytosolic fraction. Lanes 4 and 7: affinity-purified fraction. Lane 5: purified enzyme collected from phenyl column. Lane 8: recombinant Sigma class cockroach GST (Bla g 5).

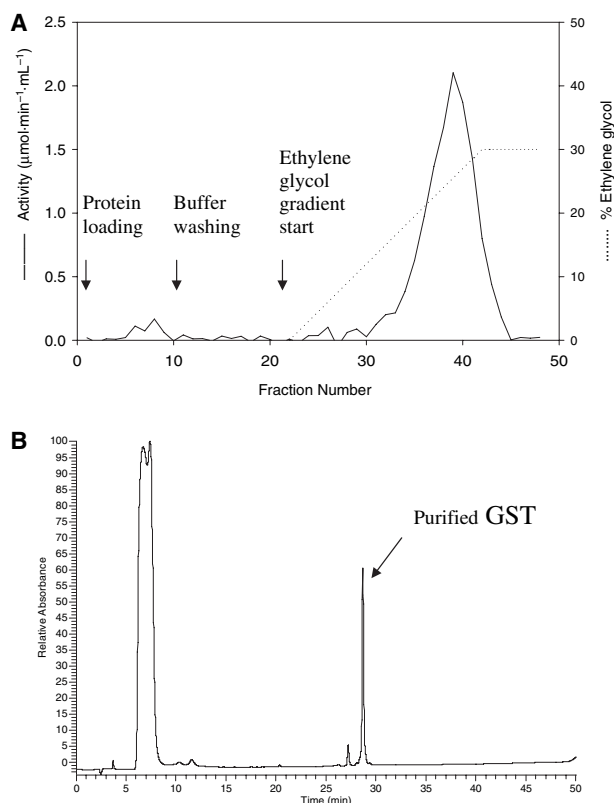


Fig. 2. Purification of cockroach GST by phenyl-Sepharose chromatography. (A) The elution profile for GST activity using phenyl-Sepharose chromatography (fraction size, 1 mL). (B) An HPLC chromatogram of cockroach GST isolated by phenyl-Sepharose chromatography. GST activity was determined with CDNB, and units are given in $\mu\text{mol CDNB conjugate}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$. Protein content was measured after fractions showing enzyme activity were pooled, because of the limited amount of protein applied to the column. HPLC separation of the purified GST was performed using a C18 column, with acetonitrile content being increased linearly from 10% to 90% over 40 min. Protein effluents were detected using UV absorbance at 220 nm.

note that the majority of the enzyme activity ($\sim 60\%$) applied to the phenyl column was lost in this procedure, with less than 5% of enzyme activity being recovered in the unbound fraction. No enzyme activity was recovered by eluting the phenyl column with a higher concentration of ethylene glycol.

Substrate specificities and kinetic properties of purified cockroach GST

The purified *B. germanica* GST exhibited unusually high activity ($508 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) towards the general substrate CDNB (Table 1). Kinetic studies of the purified enzyme were carried out with various concentrations of GSH and CDNB. Enzyme activities

conformed to Michaelis–Menten kinetics, with K_m CDNB, K_m GSH, $V_{\max}$ and k_{cat} values estimated to be 0.33 mM, 0.76 mM, $664 \mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ and 545 s^{-1} , respectively. In addition to CDNB, the cockroach GST also catalyzed the conjugation of many substrates that are commonly metabolized by other insect GSTs (Table 2). The purified GST exhibited high activity for CDNB, 1,2-dichloro-4-nitrobenzene (DCNB) and cumene hydroperoxide (CHP), as compared to GSTs isolated from *Drosophila melanogaster* (DmGSTD1) [22], *Nilaparvata lugens* (NlGST1-1) [17] and *Anopheles gambiae* (AgGSTD6) [23]. It is interesting to note that the purified cockroach GST has the highest cumene peroxidase activity among insect GSTs reported to date.

Amino acid sequencing

The N-terminal amino acid sequence of the cockroach GST was determined to be TIDFYYPGSVDCRSV-LLAA by Edman degradation. Additional sequence information was obtained from LC/MS/MS analyses of peptides generated from digestions using trypsin and V8 acid protease. Four interpretable mass spectra were obtained from collision-induced dissociation of molecular ions formed from protease-digested peptides. The length of these peptides was six to eight amino acid residues. The deduced amino acid sequence of one

Table 2. Substrate specificities of purified cockroach GST compared with those of other insect GSTs. Values are the means $\pm$ SE from three separate experiments. Substrate specificities of Delta class GSTs from *D. melanogaster* D1 [22], *N. lugens* 1–1 [17] and *A. gambiae* D6 [23] are given for comparison. ND, activity was not detected.

Substrate	Activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)			
	BgGST	DmGSTD1	NlGST1-1	AgGSTD6
CDNB	508 ± 49	58.1	141	195
DCNB	0.91 ± 0.15	0.13	0.09	0.64
CHP	3.2 ± 0.1	0.27	0.51	0.98
4-NPA	0.63 ± 0.01		0.05	
EA	0.34 ± 0.07		ND	
4-HNE	1.06 ± 0.03			
4-NPB	ND			< 0.15
ENPP	ND			0.102
4-NBC	ND			
BSP	ND			
5-ADO	ND			
t-PBO	ND			
DDT ^a	144	864 ^b		7.71

^a Activity in $\text{nmol}\cdot\text{mg}^{-1}$ after a 2 h incubation at room temperature.

^b Value was calculated on the basis of the reported DDTase activity of $7.2 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein obtained at 37°C .

tryptic peptide [SV(L/I)(L/I)AA(K/Q)] resembled the later part of the sequence obtained by Edman degradation, suggesting that the two may be overlapping sequences. Another tryptic peptide, with a deduced sequence of DDS(L/I)YP(K/Q), appeared to be closely related to the peptide DDSLYPK identified previously in Delta class GSTs of *Manduca sexta* and *D. melanogaster* [19,24]. The deduced sequences of two other peptides were WFENA(K/Q) and (L/I)NHS-GC(L/I)E. The N-terminal sequence of the purified cockroach GST was very similar (~80% identical) to that of Delta class GSTs from *N. lugens* and *Bombyx mori* [17,18]. These results indicated that the cockroach GST may belong to the Delta class.

Cloning of a Delta class GST from *B. germanica*

The cloning of cDNA encoding the 24 kDa protein was accomplished using degenerate primers for Delta class insect GSTs and modified RACE techniques. The full-length sequence of *BgGSTD1* was 987 nucleotides long, and the longest ORF encoded a protein of 216 amino acids (Fig. 3). A putative polyadenylation sequence AATAAA was detected 219 nucleotides downstream of the stop codon TGA. The predicted M_r of the translated protein was 24 614, which is in good agreement with results obtained from SDS/PAGE of the purified protein (Fig. 1). Peptide sequences determined by Edman degradation and LC/MS/MS were observed in the cloned enzyme. Two potential N-glycosylation sites, Asn199-His200-

Ser201 and Asn212-Leu213-Thr214, were identified near the C-terminal end of the protein. On the basis of amino acid sequence alignments with other insect GSTs using the CLUSTAL W program, the cloned cockroach GST was determined to be more closely related to GSTs of the Delta class (~42–60% identical) than to those of other classes (Table 3). Hence, the enzyme is classed as a Delta class enzyme and

Table 3. Percentage identity of the deduced amino acid sequence of BgGSTD1 with other insect GSTs.

GST family	Identity (%)	Species	Gene name	GenBank accession number
Delta	60.2	<i>Drosophila melanogaster</i>	<i>DmGSTD1</i>	NM_079602
Delta	59.3	<i>Nilaparvata lugens</i>	<i>NIGST1-1</i>	AF448500
Delta	57.9	<i>Lucilia cuprina</i>	<i>LcGST1</i>	L23126
Delta	56.9	<i>Anopheles dirus</i>	<i>AgGSTD3</i>	AF273039
Delta	54.6	<i>Bombyx mori</i>	<i>BmGST1</i>	AB176691
Delta	45.4	<i>Manduca sexta</i>	<i>MsGSTof1</i>	AF133268
Delta	45.4	<i>Anopheles gambiae</i>	<i>AgGSTD7</i>	AF071161
Sigma	14.8	<i>Anopheles gambiae</i>	<i>AgGSTS1</i>	AF513639
Sigma	13.9	<i>Blattella germanica</i>	<i>Bla g 5</i>	U92412
Sigma	13.0	<i>Drosophila melanogaster</i>	<i>DmGSTS1</i>	NM_166217
Sigma	13.9	<i>Manduca sexta</i>	<i>MsGST2</i>	L32092
Epsilon	36.6	<i>Anopheles gambiae</i>	<i>AgGSTE2</i>	AF316636
Epsilon	34.7	<i>Anopheles gambiae</i>	<i>AgGSTE1</i>	AF316635
Omega	10.6	<i>Anopheles gambiae</i>	<i>AgGSTO1</i>	AY255856
Theta	26.4	<i>Anopheles gambiae</i>	<i>AgGSTT1</i>	AF515526
Zeta	9.7	<i>Anopheles gambiae</i>	<i>AgGSTZ1</i>	AF515522

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1  CCCCCTATTCAATTGAAGAGTGCAGCTGCTTCTTTGTCTCCTCAAGTTCAAGA
61  AAACGCTAACCTCAAGATGACCATGATTCTTACTTGCCCGCAGTGCTCCATGCCGC
    M T I D F Y Y L P G S A P C R
121 TCAGTCCTGCTGGCAGCTAAAGCTTTGGGGTTGATCTAAACCTCAAGTGACTAACCTG
    S V L L A A K A F G V D L N L K V T N L
181 ATGGCTGGAGAACATCTCACACCAGAAATTTTGAATGAATCTCAACATACAATTCCT
    M A G E H L T P E F L K M N P Q H T I P
241 ACTGTAATGATAATGGCTTTTGCCTCTGGGAAAGCCGAGCTATCCTCTCATATTGGCW
    T L N D N G F C L W E S R A I L S Y L A
301 GACCAATATGGCAAAGATGACTCATGTACCCCAAGGATCCCAAGAAGCGTGCTCTTGTA
    D Q Y G K D D S L Y P K D P K K R A L V
361 GACCAGAGATTGTATTTTGAATCTGGGAAGTCTGTACAGAGATTGGTGATTATTATTAT
    D Q R L Y F D L G T L Y Q R F G D Y Y Y
421 CCAATCATGTTTGCAGAAAGCATCTCCTGATGCAGAAAAATGAAGAACTTGAAGAAGCC
    P I M F A K A S P D A E K M K K L E E A
481 TACCAGTTTTTGGATAAATTTCTGGAAGGACAAAAGTTTGTGTGGAAATCTTTGACC
    Y Q F L D K F L E G Q K F V A G N S L T
541 ATTGCTGATATTGCTACCATTTGCTTCAAGTGTCAACAGCAGCAATTTGGCTTTGACATC
    I A D I A T I A S V S T A A I L G F D I
601 ACCAGATACCCCAACGTGAACAAGTGGTTCGAAAATGCAAGAAGGTGATTCCTGGATAC
    T R Y P N V N K W F E N A K K V I P G Y
661 GATGAACCAACCATCTGGCTGCCTTGAATTTAAGAAATGTGGGATAATCTCACGCAA
    D E L N H S G C L E F K K M W D N L T Q
721 AAATGAAAAGTTGTAAAGATTCTGCTTGACAATCAAAACAATTTTGTCTGTGCTAAGT
    K *
781 GTTGTGACTGGCATTCTCCATCTAAAGTATTTACCTCAATGTTTTCGAAAATTTGCATT
841 AATTATATATGGCTGTGCAACAGTGCAGGTTTAAAGGAAGGAAGCTATTTTCCATT
901 TTGTATTATATAATGTATTAGGCTCTTTGCTTAGAAAATTTTATAATAAATTTAGAAA
961 AAAAAAAAAAAAAAAAAAAAAAAAAA

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Fig. 3. Nucleotide and deduced amino acid sequence of *B. germanica* GSTD1. The putative polyadenylation sequence AATAAA is underlined. The potential N-glycosylation sites have white letters on a black background. Amino acid sequences matched with those identified by Edman degradation and MS are in bold letters and boxes, respectively.

Detection of IgE against GSTs from the German cockroach

A pooled serum sample obtained from a panel of 16 patients allergic to the German cockroach was used to determine the specific IgE binding to different cockroach GSTs (Fig. 5). Both BgGSTD1 and Bla g 5 showed significant binding to IgE in the patient's sera as compared to the negative control BSA. This result indicated that BgGSTD1 is potentially another

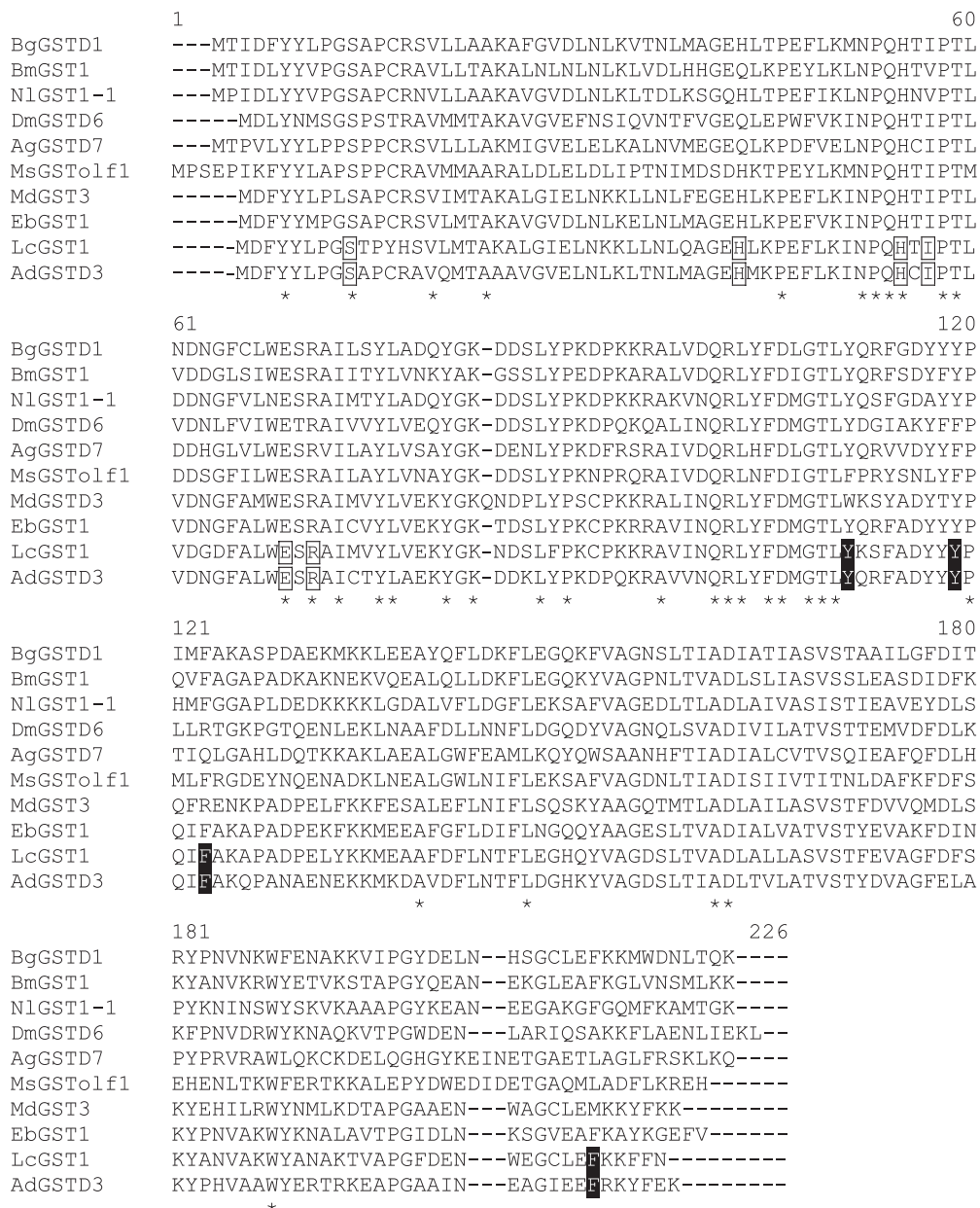


Fig. 4. Alignment of the deduced amino acid sequence of BgGSTD1 with other insect Delta class GSTs. Identical amino acids are marked with asterisks. G-site residues are boxed. H-site residues have white letters on a black background.

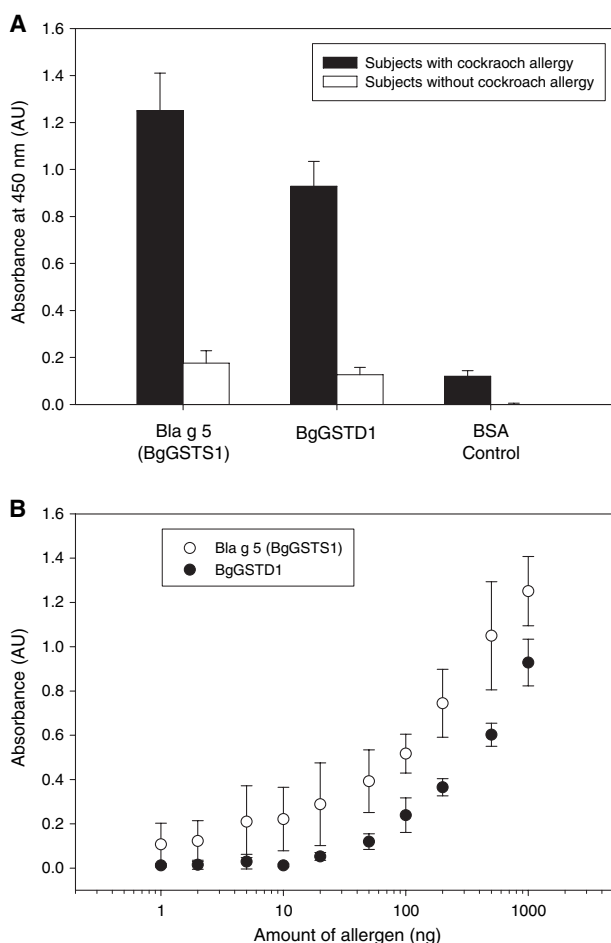


Fig. 5. The IgE binding of GSTs present in the German cockroach assayed by ELISA. (A) The binding of 1 μ g of allergen or BSA control with sera obtained from subjects who have cockroach allergy (solid bar) and healthy controls (open bar). (B) Titration curve of Bla g 5 (open circle) and BgGSTD1 (solid circle) against IgE obtained from patients with cockroach allergy. Data represent the mean and standard deviation determined from triplicate experiments.

cockroach-derived allergen. However, the response of BgGSTD1 was determined to be lower than that of Bla g 5 ($P < 0.025$) using student's *t*-test. Subsequent titration curves of Bla g 5 and BgGSTD1 revealed that both GSTs bind to patient's serum IgE in a concentration-dependent manner (Fig. 5B). Neither of the IgE-binding curves reached saturation at the level of 1 μ g per well.

Discussion

A novel GST has been identified and purified from the German cockroach in this study. Amino acid sequences obtained from the purified GST as well as from cDNA

clones suggested that the enzyme is a member of the Delta class GSTs. This enzyme, BgGSTD1, catalyzes GSH conjugation of CDNB effectively, with specific activity exceeding $500 \mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$. Previous attempts to purify GSTs from the German cockroach resulted in three protein bands isolated from native PAGE [7]. All three of the partially purified GSTs turned over CDNB at a rate of less than $2 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein. It was not certain whether any one of the three protein bands consisted of GSTD1, whereas the enzyme activity was substantially reduced during the purification process using PAGE. Alternatively, GSTD1 may have been lost at the ammonium sulfate precipitation stage. In the purification scheme established by Yu & Huang [7], proteins that precipitated at 45–75% saturation were collected. Phenyl-Sepharose chromatography performed in this study indicated that GSTD1 was rather hydrophobic, requiring 30% ethylene glycol to be eluted from the column. It is possible that GSTD1 may have precipitated at a saturation level below 45% and therefore not have been recovered in the previous study.

To date, CDNB conjugation catalyzed by BgGSTD1 is the highest among Delta class GSTs with known sequences. Kinetic studies revealed that the K_m CDNB, K_m GSH and $V_{\max}$ values were 0.33 mM, 0.76 mM and $664 \mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, respectively. The affinities for CDNB and GSH were within the range observed in Delta class GSTs of other insect species [17,22,23,25–27], indicating that the unusually high catalytic rate is not a reflection of the binding of substrate and cosubstrate. Previously reported X-ray crystal structures of Delta class GSTs revealed the amino acid residues involved in pocket formation for the binding of GSH (G-site) and substrate (H-site) [27–29]. GSH was surrounded by amino acids corresponding to Ser11, His40, His52, Ile54, Glu66 and Arg68 in BgGSTD1, whereas the H-site consisted of Tyr107, Tyr115, Phe119 and Phe206 (Fig. 5). The presence of these conserved residues in BgGSTD1 was consistent with the observation that the GSH-binding and CDNB-binding affinities of BgGSTD1 fell within the ranges determined for other insect Delta class GSTs. Further experiments may provide insights into the mechanism by which BgGSTD1 metabolizes CDNB at such a high rate. It is possible that the amino acid sequence and/or the three-dimensional conformation of the enzyme may facilitate catalysis by lowering the activation barrier of the reaction [30]. In addition, the rate of product release may contribute to the efficiency of the reaction [31].

Functionally, BgGSTD1 may play an important role in the resistance to insecticides. Like many Delta class GSTs [22,23], BgGSTD1 metabolized

1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) to 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene (DDE) (Table 2). Elevated levels of Delta class GSTs in *D. melanogaster* and *A. gambiae* were detected in the DDT-resistant strains. The possible role of BgGSTD1 in DDT resistance remains to be determined. BgGSTD1 also exhibited high peroxidase activity, using CHP as a model substrate. Vontas *et al.* demonstrated that the peroxidase activity was a vital antioxidant defense that conferred resistance to pyrethroid insecticide in the brown planthopper, *N. lugens* [32]. Two pyrethroids, λ -cyhalothrin and permethrin, induced oxidative stress and lipid peroxidation in planthoppers. The reduction in pyrethroid-induced lipid peroxidation and mortality in the resistant strains was associated with the increased GST activity. Thus, BgGSTD1 can contribute to defense against insecticides both directly and indirectly.

BgGSTD1 exhibited the highest peroxidase activity among all GSTs reported to date, turning over $3.2 \mu\text{mol CHP}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ (Table 2). Peroxidase activities of GSTs are of particular importance to insects, because they do not possess selenium-dependent glutathione peroxidase. A survey of reported GST-mediated peroxidase activity across insect species revealed that Delta class GSTs probably have higher activity than the enzymes in the Sigma and Epsilon classes [17,22,23, 25–27,33–35]. In addition to peroxides, Delta class GSTs also metabolize lipid peroxidation products such as 4-hydroxynonenal (4-HNE) [36]. 4-HNE is one of the several reactive α,β -unsaturated aldehydes formed from the breakdown of long-chain lipid hydroperoxides [37]. The ability to metabolize peroxides and lipid peroxidation products suggested that Delta class GSTs may play a pivotal, and possibly primary, role in the survival of insects under oxidative stress. As mentioned earlier, introduction of pyrethroids to brown planthoppers induced oxidative stress and the formation of lipid peroxides [32]. The authors suggested that reactive oxygen species may be generated from P450-mediated oxidation of the pyrethroids. It is possible that P450s oxidize the phenyl group of pyrethroids, yielding quinone metabolites that in turn generate reactive oxygen species. Apart from insecticides, many natural products in plants can be metabolized by mammalian P450s to form reactive quinones [38]. Similar reactions can be expected to occur in insects. For scavengers such as cockroaches, it is quite possible that the dietary constituents are metabolized to form reactive quinones, along with reactive oxygen species and peroxides. The unusually high peroxidase activity of BgGSTD1 would aid the survival of cockroaches under the potential oxidative stress arising from their scavenger diet.

The amino acid sequences of several peptide fragments obtained by Edman degradation and LC/MS/MS analysis of the purified BgGSTD1 were crucial for the isolation of cDNA. The N-terminal amino acid sequence provided essential information to indicate that the purified GST was probably a member of the Delta class. As the amino acid sequences at the N-terminal region of many Delta class GSTs across species are very similar, degenerate primers were designed to clone the conserved region. The 5'-end and 3'-end of the sequence were then determined using RACE techniques. Earlier studies usually relied on using antisera raised against the purified enzyme to confirm that the isolated clone(s) encoded for the corresponding protein. However, the GSTs cloned were not the same as the purified enzymes anticipated [25,39]. With the determination of the genome sequences for *D. melanogaster* and *A. gambiae*, it is now known that the Delta class GSTs consist of many members with high sequence homology [2]. The use of a polyclonal antibody for the cloning of a particular GST enzyme is limited by the antibody's cross-reactivity. Amino acid sequences obtained from LC/MS/MS analysis provided much needed information for cloning a specified protein. This approach is especially useful in distinguishing splice variants of Delta class GSTs, when amino acid sequence information is obtained towards the C-terminal end.

Like Bla g 5, BgGSTD1 bound to serum IgE obtained from cockroach-sensitized patients (Fig. 5), indicating that BgGSTD1 may also be a protein allergen from the German cockroach. The results confirmed previous findings that more than one GST has IgE-binding activity [7,10]. Future experiments, e.g. skin prick tests, could provide further information on the *in vivo* allergenicity of BgGSTD1. *In vitro* ELISA conducted using $1 \mu\text{g}$ of protein showed that BgGSTD1 elicited $\sim 70\%$ of the IgE-binding activity of the recombinant Bla g 5 (rBla g 5). One possible explanation for the lower binding activity of BgGSTD1 is that the protein may not be as widely recognized as rBla g 5 by patients allergic to cockroaches. The number of epitopes may be another contributory factor, as BgGSTD1 may have fewer epitopes than rBla g 5.

It has been well documented that patients allergic to birch pollen show hypersensitivity to fresh fruits or vegetables [40,41]. Structural similarities of homologous allergens between birch pollen and fruits (or vegetables) led to IgE-mediated cross-reactivity. Several classes of proteins, such as pathogenesis-related proteins and profilins, have been identified as contributing to the cross-reactivity. Results obtained from immunoblotting and site-directed mutagenesis studies indicated

that the conformational epitopes were more important than the linear epitopes in IgE binding. In the case of birch allergen Bet v 1a, a single point mutation (Ser112 to Pro) disrupted the three-dimensional structure and drastically reduced IgE-binding activity and cross-reactivity [42]. The amino acid sequences of BgGSTD1 and Bla g 5 were quite different, sharing only 14% sequence identity (Table 2). The IgE binding of BgGSTD1 may have resulted from cross-reactivity, possibly due to the presence of shared conformational epitope(s) with Bla g 5. The potential cross-reactivity among GSTs may broaden the enzyme's role in cockroach allergy.

In conclusion, a novel Delta class GST (BgGSTD1) has been purified and cloned from the German cockroach. This GST catalyzed the metabolism of CHP, DDT and 4-HNE, suggesting that the enzyme may contribute to the cockroach's defense against insecticide and oxidative assaults. Interestingly, BgGSTD1 showed IgE reactivity with serum obtained from cockroach-sensitized patients, indicating that this protein may potentially be another cockroach allergen. Future experiments will be needed to examine potential IgE cross-reactivity between BgGSTD1 and the known cockroach allergen Bla g 5 (BgGSTS1).

Experimental procedures

Purification of cockroach GST

Whole body extracts of adult German cockroach were prepared as described by Duong & Chang [10], with modifications. Briefly, 10 g of German cockroach was homogenized in 20 mL of 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM EDTA, 10 mM dithiothreitol and 25 μ M phenylmethanesulfonyl fluoride at 4 °C using a ceramic mortar and pestle. After centrifugation at 10 000 *g* for 5 min at 4 °C using a Sorvall RC-5B centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) with a Sorvall SS34 rotor, the supernatant fraction was collected as a soluble body extract. The extract was ultracentrifuged at 105 000 *g*_{max} for 60 min using a Beckman Optima XL-100K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) with a Beckman type 50.2 Ti rotor, and the supernatant (cytosolic) fraction was harvested. The sample was then applied to a 10 mL GSH agarose column (Sigma-Aldrich, St Louis, MO, USA) equilibrated with 50 mM imidazole/HCl buffer with 1 mM dithiothreitol (pH 7.4) (buffer A), at 4 °C. The affinity column was washed with 50 mL of buffer A containing 0.2 M NaCl, and the bound protein was eluted with 50 mM Tris/HCl buffer with 1 mM dithiothreitol, 0.2 M NaCl, 5 mM GSH, and 2 mM *S*-hexylglutathione (pH 8.5). The effluent was concentrated, and then washed twice with

buffer A using an Amicon ultracentrifugation unit (*M*_r cut-off = 10 000; Millipore Corp., Billerica, MA, USA). The concentrated fraction containing GST activity was loaded onto a 1 mL HiTrap phenyl HP column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM potassium phosphate buffer containing 1 mM dithiothreitol (pH 6.5), at a flow rate of 1 mL·min⁻¹ at room temperature. The column was then equilibrated with 25 mM Tris/HCl buffer containing 1 mM dithiothreitol (pH 7.4) (buffer B). Protein was eluted with a linear gradient to 30% ethylene glycol over 20 min. Fractions showing GST activity were pooled and concentrated as stated above. SDS/PAGE was performed using a 12% SDS-polyacrylamide gel in a Bio-Rad Mini Protean II cell (Bio-Rad Laboratories, Hercules, CA, USA). HPLC analysis of purified protein was performed on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler, a binary pump, and a photodiode array detector. Separation was performed on a Phenomenex Jupiter C18 column (2.0 × 250 mm, 5 μ m; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) at a constant flow rate of 0.25 mL·min⁻¹. The solvent gradient increased linearly from 10% solvent B to 90% solvent B over 40 min, and then returned to 10% solvent B in 1 min. The column effluent was monitored by UV absorbance at 220 nm. Recombinant protein of Bla g 5, a Sigma class GST [8], was purchased from Indoor Biotechnologies, Inc. (Charlottesville, VA, USA).

Biochemical assays

Spectrophotometric assays were used to measure GST activity with CDNB, DCNB, CHP, 4-nitrobenzyl chloride (4-NBC), 4-nitrophenethyl bromide (4-NPB), 4-nitrophenol acetate (4-NPA), 1,2-epoxy-3-(4-nitrophenoxy)propane (ENPP), bromosulphophthalein (BSP), ethacrynic acid (EA), 4-HNE, 5-androstene-3,17-dione (5-ADO), and *trans*-4-phenyl-3-buten-2-one (*t*-PBO), as described previously [11–15]. Dehydrochlorination of DDT to form DDE was determined using the method of Ranson *et al.* [16]. The protein content was measured using the Pierce Coomassie Plus protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA), with BSA as the protein standard. Kinetic parameters of the Michaelis–Menten equation (*V*_{max} and *K*_m) were estimated using SIGMAPLOT (Systat Software Inc., Point Richmond, CA, USA).

Amino acid sequencing

Edman degradation, performed by Proteos, Inc. (Kalamazoo, MI, USA), was used to determine the amino acid sequence of 20 N-terminal residues. The amino acid sequence of peptides generated after protease digestion was

obtained using HPLC-ESI tandem MS. Purified German cockroach GST (20 µg) was digested with 0.2 µg of trypsin or V8 acid protease in 0.2 mL of 0.1 M Tris/HCl buffer (pH 8.3) overnight at 37 °C. Chromatographic separation of peptides was carried out on an Agilent 1100 HPLC system using a Phenomenex Jupiter Proteo column (2.0 × 250 mm, 4 µm). The mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) at a constant flow rate of 0.2 mL·min⁻¹. The solvent gradient increased linearly from 10% solvent B to 90% solvent B over 50 min; this was followed by re-equilibration for 10 min. MS analysis was performed on a Thermo Electron Deca XP ion trap mass spectrometer (Thermo Fisher Scientific). ESI was operated in a positive mode, with a spray voltage of 4.0 kV, a sheath gas flow of 60 AU, an auxiliary gas flow of 10 AU, and a capillary temperature of 270 °C. Collision-induced dissociation was performed with normalized collision energy, activation *Q*-value and activation time set at 25%, 0.25 and 30 ms, respectively.

Extraction of total RNA and cDNA synthesis

Total RNA was isolated from adult German cockroaches using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Removal of contaminating agents from the crude RNA extract was performed using a Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA, USA). First-strand cDNA synthesis was carried out using a BD SMART RACE cDNA amplification kit (BD Bioscience Clontech, Mountain View, CA, USA).

Isolation of *BgGSTD1* cDNA

A degenerate PCR strategy was employed for cloning the 5'-coding region of *B. germanica* GST. The degenerate primers were designed on the basis of the amino acid sequence obtained by Edman degradation and from reported GST sequences of *N. lugens*, *Bo. mori* and *M. sexta* [17–19]. The 50 µL PCR reaction mixture contained 20 ng of first-strand cDNA, 0.5 nmol of forward primer [5'-CTGCCCGGATCTGCTCCCTGC(A/C)G(C/G/T)TC(A/G/C)GT-3'], 0.5 nmol of reverse primer [5'-CTCTGGTA CAGAGTTCC(C/G)AT(A/G)TC(A/G)AA-3'], 0.3 mM dNTPs, 1 mM MgSO₄, 2.5 units of Invitrogen *Pfx* DNA polymerase, and 5 µL of the manufacturer's amplification buffer. Amplification (94 °C for 0.25 min, 55 °C for 0.5 min, and 68 °C for 3 min) was performed for 35 cycles. The 305 bp PCR product was subcloned into Invitrogen One Shot competent cells using a Zero Blunt TOPO PCR cloning kit. Sequences of the cDNA clones were obtained using an Applied Biosystems 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The 3'-end of the cDNA was amplified by PCR with a specific forward primer (5'-CCTGATGGCTGGAGAACATCTCACACC-3')

and the adaptor primer for 3'-RACE provided in the kit. The 5'-end of the cDNA sequence was obtained using a modified 5'-RACE system (Invitrogen). Reverse transcription was performed using a specific backward primer R1 (5'-GGTGTGAGATGTTCTCCAGCCATCAGG-3'). The first-strand cDNA was tailed using terminal deoxytransferase in the presence of dCTP. The PCR reaction was carried out using the backward primer R1, the abridged anchor primer, and *Pfx* DNA polymerase, under the conditions described earlier. A second round of PCR was performed with a specific backward primer R2 (5'-GAG GATAGCTCGGCTTTCCCAGAGGCA-3') and the abridged universal amplification primer provided in the kit. PCR products were cloned and sequenced in both directions as described above.

ELISA

The ELISA developed to measure the IgE-mediated allergen binding was adapted from Beezhold *et al.* [20]. Briefly, the wells of a high-capacity ELISA assay plate (Corning Inc., Acton, MA, USA) were coated with 1 µg of cockroach GST diluted in 100 µL of 50 mM sodium carbonate buffer (pH 9.6). BSA was used as a negative control. The plate was incubated at 37 °C for 30 min, and then at 4 °C overnight. After being washed three times with 150 µL of NaCl/P_i containing 0.05% Tween-20 (T-NaCl/P_i), the wells were blocked with 250 µL of 5% nonfat skimmed milk in T-NaCl/P_i at 4 °C overnight, and then washed another three times with T-NaCl/P_i. Human sera collected from 16 cockroach-sensitized patients were kindly provided by J. Slater (US Food and Drug Administration, Bethesda, MD, USA) [21]. Control sera were collected from three healthy volunteers who had no history of cockroach allergy. These serum samples were provided with the full knowledge and consent of the patients. Sera were diluted 1 : 10 with T-NaCl/P_i, aliquoted (100 µL) into the wells, and incubated for 2 h at room temperature. After washing five times with T-NaCl/P_i, aliquots of 100 µL of T-NaCl/P_i-diluted (1 : 10 000) horseradish peroxidase-labeled anti-human IgE (Sigma-Aldrich) were added to the wells and incubated at room temperature for 1 h. Finally, the wells were washed five times as described before, and the peroxidase reactivity was detected by the addition of 3,5,3',5'-tetramethylbenzidine (Ultra TMB solution; Pierce). The incubation was stopped at 15 min by the addition of 2 M sulfuric acid. The absorbance at 450 nm was recorded using a SpectraMax Plus 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

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