

RESEARCH ARTICLE

Proteome mining for novel IgE-binding proteins from the German cockroach (*Blattella germanica*) and allergen profiling of patients

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Although cockroaches are known to produce allergens that can cause IgE-mediated hypersensitivity reactions, including perennial rhinitis and asthma, the various cockroach allergens have not yet been fully studied. Many proteins from the German cockroach show high IgE reactivity, but have never been comprehensively characterized. To identify these potential allergens, proteins were separated by 2-DE and IgE-binding proteins were analyzed by nanoLC-MS/MS or N-terminal sequencing analysis. Using a combination of proteomic techniques and bioinformatic allergen database analysis, we identified a total of ten new *B. germanica* IgE-binding proteins. Of these, aldolase, arginine kinase, enolase, Hsp70, triosephosphate isomerase, and vitellogenin have been reported as allergens in species other than *B. germanica*. Analysis of the Food Allergy Research and Resource Program allergen database indicated that arginine kinase, enolase, and triosephosphate isomerase showed significant potential cross-reactivity with other related allergens. This study revealed that vitellogenin is an important novel *B. germanica* allergen. Personalized profiling and reactivity of IgE Abs against the panel of IgE-binding proteins varied between cockroach-allergic individuals. These findings make it possible to monitor the individual IgE reactivity profile of each patient and facilitate personalized immunotherapies for German cockroach allergy disorders.

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1 Introduction

Asthma, allergic rhinitis, and atopic dermatitis are common allergic diseases, especially among children, and their

frequency has increased rapidly over the past decade in Taiwan [1]. Cockroaches, which are an important source of indoor allergens, produce potent allergens that trigger IgE antibody-mediated allergic responses and are reported to be an important cause of asthma in many regions of the world, including Europe, Latin America, North America, and South Africa, and, more recently, Far East Asia, including Taiwan [2]. During infestation, significant allergenic responses can be triggered by many cockroach allergens that may be found in house dust, including cast-off skin, feces, secretions, and even whole bodies and ootheca. The debris or excretions produced by casting or death can spread into the air and cause allergic symptoms after inhalation [3]. Such debris may increase allergic risk and can lead to acute asthma attacks and even hospitalization. Early exposure to cockroach allergens during infancy may be associated with recurrent wheezing and asthma in early childhood [3, 4].

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Abbreviations: aldolase, fructose 1,6-bisphosphate aldolase; CRD, component-resolved diagnosis; FARRp, food allergy research and resource program; IST, intradermal skin test; MAST-CLA, multiple allergen simultaneous test-chemiluminescent assay; RACK1, receptor for activated protein kinase C-like protein; SERCA, calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (calcium pump) isoform 1; TIM, triosephosphate isomerase; WBE-G, whole-body extract of German cockroach

Recent evidence also suggests that cockroach allergens cross-react with allergens present in arthropods and other invertebrates [5].

Among atopic patients in Taiwan, 50.7% of patients with allergic rhinitis and 57.5% of patients with asthma have been found to be sensitive to cockroach extracts [1]. More interestingly, 36.4% of asthmatic patients are hypersensitive to *B. germanica* [6]. Similarly, 36.8% of children with asthma living in inner-city areas of the United States have been found to be allergic to cockroaches, especially the German cockroach [4, 6, 7]. Different methods have shown that potential allergens with molecular masses ranging from 6 to 120 kDa are present in both whole-body extracts and feces [8]. The allergic components of the German cockroach have not yet been extensively studied, although seven *B. germanica* allergens (Bla g 1, 2, 4, 5, 6, 7, and 8) have been cloned and immunologically characterized. However, in a previous study, serum samples from 36% of cockroach-allergic patients failed to react to five of these allergens (Bla g 1, 2, 4, 5, and 7), even though the patients had positive CAP assay results against cockroach [9]. This, combined with a recent report of IgE binding to Bla g 6 in 14% of cockroach-allergic patients [10], shows that the known cockroach allergens clearly fail to cover the entire allergen repertoire of the German cockroach and that other allergens remain to be identified and characterized.

In recent years, proteomic strategies (allergenomics) have provided new opportunities for screening and identifying putative allergens [11]. Using 2-DE immunoblotting methods, researchers can quickly reduce sample complexity and resolve proteins based on their isoelectric points (pI) and relative masses, then protein spots corresponding to potential allergens can be detected by immunoblotting using sera from allergic patients and identified by N-terminal sequencing or MS. Furthermore, analysis of the various allergen bioinformatic databases can help researchers identify potentially homologous or cross-reactive allergens. The aim of this study was to develop an efficient platform for fully and rapidly identifying novel human IgE-binding allergen candidate proteins from *B. germanica*. A comprehensive panel of IgE-binding proteins may be useful for developing individual diagnostic and/or specific immunotherapeutic strategies for cockroach allergic diseases.

2 Materials and methods

2.1 Patient samples and measurement of specific IgE

The initial inclusion of cockroach-allergic patients was on the basis of a result greater than +2 for cockroach crude extract-specific IgE Ab in a multiple allergen simultaneous test-chemiluminescent assay (MAST-CLA) (Hitachi Chemi-

cal Diagnostics, Mountain View, CA), resulting in the identification of 213 positive patients. For this study, 44 of these allergic cockroach-allergic patients with positive results for recognition of whole-body extract of the German cockroach (WBE-G) proteins on immunoblot analysis were randomly selected from these 213 patients. We selected 12 of the allergic sera, which were age- and sex-matched to normal controls, for 1-DE and 2-DE screening of IgE-binding proteins. Equal volumes of serum from these patients were used separately on 1-DE immunoblots or pooled to constitute an atopic serum pool, which was used on the 2-DE immunoblots. Sera from 12 non-allergic individuals were used as controls. The samples from the remaining 32 patients were used for screening of IgE-binding prevalence.

2.2 Protein extraction and sample preparation

German cockroaches were obtained from the Department and Graduate Institute of Entomology, National Taiwan University. Specimens of *B. germanica* were taken from a colony and reared in a dark incubator at 30°C under 60–70% relative humidity. Adult male and female cockroaches were freeze-dried and the oothecae carefully separated from female nymphs. For preparation of a protein powder from WBE-G, the freeze-dried samples were ground in a liquid nitrogen-filled mortar and extracted for 1 h at 4°C with constant stirring with 50 mM PBS, pH 7.0, containing 0.2 mM DTT and 1 mM PMSF, then the mixture was filtered through a Whatman No. 1 filter and centrifuged at 15 000 × g for 20 min at 4°C. The protein-containing supernatant was collected and the proteins precipitated by rapid addition of 10% w/v TCA with gentle vortexing and incubation at 4°C for 90 min, then the mixture was centrifuged at 15 000 × g for 20 min at 4°C. The supernatant was removed, ice-cold acetone containing 0.07% β-mercaptoethanol was added to the pellet and the sample incubated on ice for 15 min and centrifuged as above, then the acetone-containing supernatant was removed and the precipitate (*i.e.* the protein sample) air-dried and stored at –70°C until use.

2.3 Electrophoresis

WBE-G proteins were separated by 1-DE or 2-DE as described previously [12]. For 1-DE, WBE-G protein powder (150 µg per gel) was incubated for 10 min at 95°C with 1-DE sample buffer, then loaded onto a 12.5% SDS-PAGE (6.5 × 8.5 cm). Molecular weight standards ranging from 97 to 14.4 kDa (GE Healthcare, NJ) were also included. For 2-DE, 1.2 mg of WBE-G protein powder was incubated with modified 2-DE sample buffer (8 M urea, 1 M thiourea, 0.5% pharmalyte, pH 3–10, 60 mM DTT, 4% CHAPS, bromophenol blue) and loaded through sample cups. The first

dimension of the 2-DE was run on IPG strips (Immobiline DryStrip pH 3–10, 11 cm; (GE Healthcare) on a horizontal electrophoresis system (IPGphor II system; GE Healthcare.). The IPG strips were focused using a four-step procedure in which the voltage was ramped to 500 V for 3 h, maintained at 1500 V for 3 h, focused at 2000 V over 8 h, then maintained at 2000 V for 4 h (all at 20°C). Prior to the second dimension, the focused strips were equilibrated for 15 min at room temperature in 50 mM Tris-HCl, pH 6.8, 8 M urea, 1% SDS, and 0.25% DTT. The strips were then placed on a 12.5% SDS-PAGE, and separation was performed at 20 mA for 4 h.

2.4 Protein staining and detection of IgE-binding proteins

The separated proteins were detected by in-gel staining with CBB or subjected to immunoblotting after transfer of the proteins on the 1-DE or 2-DE gels to PVDF membranes (Millipore, Bedford, MA) by semi-dry blotting (Nihon EIDO, Tokyo) for 4 h at 1 mA/cm². The membranes were then blocked with 1% skimmed milk in TBS and incubated for 16 h at 4°C with a 1:10 dilution of serum from the individual cockroach-hypersensitive patient (1-DE immunoblotting) or the serum pool from 12 cockroach-hypersensitive patients (2-DE immunoblotting) or pooled serum from 12 healthy controls in TBST (TBS containing 0.1% Tween 20) with 1% skimmed milk. Bound IgE was detected using a 1:1500 dilution of alkaline phosphatase-conjugated goat anti-human IgE antibody (BioSource International, Camarillo, CA) and BCIP/NBT as the substrate.

2.5 N-terminal sequencing

The 2-DE CBB-stained spots corresponding to proteins recognized by the serum samples from cockroach-hypersensitive patients were excised and subjected to N-terminal sequence analysis using a Procise 494 protein sequencer (Applied Biosystems, Foster City, CA) and the resulting peptide sequences identified by automated searching against the NCBI protein database (<http://www.ncbi.nlm.nih.gov/>).

2.6 In-gel digestion

The 2-DE CBB-stained spots corresponding to IgE-binding proteins were excised, destained with 1 mL of 40% ACN in digestion buffer (25 mM NH₄HCO₃, pH 7.8), and dried in a SpeedVac concentrator. Each protein was then digested for 16 h at 37°C with sequencing-grade trypsin (Promega, Madison, WI) in digestion buffer and the resulting peptides extracted sequentially with 50 µL of 1% TFA and 50 µL of 0.1% TFA/60% ACN. The combined extracts were vacuum-

dried and resuspended in 0.5% TFA for nanoLC ESI-MS/MS analysis (see the following section).

2.7 nanoLC-ESI-MS/MS analysis and protein identification

The selected protein spots were identified by nanoLC ESI-MS/MS analysis. Each trypsin-digested sample was loaded onto a C18 pre-concentration column (5 mm × 250 µm; Pep-Map C18, LC Packings, Amsterdam, The Netherlands) and separated on an RP C18 nano-column (15 cm × 75 µm; LC Packings) using a 60 min linear gradient of 5–95% ACN in 0.5% formic acid at a flow rate of 200 µL/min. The separated peptides were characterized using a Qstar XL Q-TOF MS (Applied Biosystems), and MASCOT software (Matrix Science, Boston, MA) was used to identify the corresponding protein by automated searching against the NCBI protein database. The following search parameters were used: trypsin was used as the digestion enzyme, the mass tolerance for the monoisotopic peptide window was set to ±1.0 Da, the MS/MS tolerance window was set to ±0.5 Da, one missed cleavage was allowed, and carbamidomethyl, deamidated, and oxidized methionine were chosen as variable modifications. The matching criteria used were that each identified peptide had to score more than 20 and at least two unique peptides had to be specific to the identified protein. To further verify the results of our protein identification, two of the protein spots (spots 4 and 5), identified, respectively, as enolase and arginine kinase were probed with polyclonal rabbit antibodies against enolase from Bermuda grass (*C. dactylon*) or arginine kinase from tiger shrimp (*P. monodon*), respectively [12, 13].

2.8 Full-length cDNA library construction and cloning of IgE-binding proteins

To construct a cDNA library for *B. germanica*, total RNA was isolated from *B. germanica* using TRIzol reagent (Life Technologies, Eggenstein) according to the manufacturer's instructions. Poly(A) RNA was purified by oligo(dT) cellulose chromatography, and a SMARTTM cDNA Library Construction kit (Clontech Laboratories, Palo Alto, CA) was used for the rapid amplification of full-length cDNA with complete 5' ends. For cloning of the IgE-binding proteins, degenerate oligonucleotides based on N-terminal or internal amino acid sequences were used to clone the internal sequences, while the 5' and 3' portions were cloned by RACE PCR, as described previously [12]. For RACE PCR, the gene-specific primers were based on an internal sequence, while the cloning vector-specific primers were based on the 5' or 3' lambda sequencing primers. The full coding sequences of the putative IgE-binding proteins were then amplified and cloned into the pGEM-T vector (Promega), and subjected to DNA sequencing.

2.9 Allergenicity assessment using comprehensive allergen databases

Allergenic molecules homologous to the identified IgE-binding proteins were used to search the Allergome database (<http://www.allergome.org>), which is an exhaustive repository of data related to all known IgE-reactive components. The Food Allergy Research and Resource Program (FARRP) Protein AllergenOnline Database (<http://www.allergenonline.org>) was used to examine both the short identified peptide matches and the cloned IgE-binding proteins for structural similarities to various allergens. According to the FAO/WHO guidelines [14], cross-reactivity between an expressed protein and a known allergen has to be considered in cases when there are at least six contiguous identical amino acids or when a segment of at least 80 residues shares more than 35% identity at the amino acid level. Here, we used search parameters suggested by FARRP to define short peptide sequence matches of eight or more contiguous identical amino acids or an overall protein sequence alignment with identity matches greater than 50% with a known allergen as indicating possible cross-reactivity.

2.10 Purification of natural allergens and IgE-binding proteins

In order to examine the prevalence of IgE-binding to Bla g allergens and the newly identified IgE-binding proteins in cockroach-allergic patients, two known German cockroach allergens (Bla g 1, Bla g 7) and one newly identified IgE-binding protein (enolase) were produced in *E. coli* as 6 × His fusion proteins (pQE30; Qiagen, Germany) [15, 16], while allergens Bla g 2 and Bla g 4 were expressed in a yeast expression system (*Pichia pastoris* pPIC9K; Invitrogen, San Diego, CA) [9]. Other natural IgE-binding proteins were purified from cockroaches as described previously [12], with some modifications. Briefly, Bla g 5 was purified from WBE-G using a glutathione-Sepharose column and elution with 10 mM reduced glutathione solution [17]. Arginine kinase was purified from WBE-G by 70–90% saturated ammonium sulfate precipitation, followed by purification on a HiTrap Q high-performance ion exchange column system (0.4 × 2 cm, GE Healthcare) [12]. For vitellogenin purification, proteins were extracted from lyophilized cockroach oothecae with buffer A (25 mM Na₂CO₃-NaHCO₃, 10 mM 2-mercaptoethanol, 1 mM EDTA, 5 μM NaN₃, and 25 μM PMSF, pH 10.0), then a 25–50% saturated ammonium sulfate cut was prepared, dissolved in buffer A, and dialyzed against the same buffer. After centrifugation, the supernatant was applied to a HiTrap Q HP column pre-equilibrated with buffer A and vitellogenin-containing fractions eluted with a 25 mL linear gradient of 0–1 M NaCl in buffer A, then, after dialysis, were further purified on a Superdex 200 × 5/150 GL size-exclusion column (GE Healthcare). The obtained proteins were used for dot blotting (see the following section).

2.11 Human IgE reactivity profile analysis

We used a solid-phase platform to determine the IgE reactivity patterns of sera from cockroach-allergic individuals. The platform was modified from a previously described protocol for an IgE dot-blot immunoassay [12]. Briefly, 0.5 μg per spot of each purified protein (Bla g 1, Bla g 2, Bla g 4, Bla g 5, Bla g 7, enolase, arginine kinase, and vitellogenin) was applied to a PVDF membrane using a Bio-Dot apparatus (Bio-Rad, Richmond, CA). To verify the identification of IgE-binding proteins and investigate IgE-binding prevalence and profiles, the serum samples from the 32 cockroach-allergic patients not used in the initial identification of IgE-binding proteins were used. Serum samples from 32 allergic and 12 non-allergic individuals were diluted 1:25, and incubated for 16 h at 4°C with the dot-blotted proteins, then the blots were incubated for 1 h at room temperature with a 1:50 000 dilution of HRP-conjugated goat anti-human IgE Abs (BioSource International) and the results visualized using an enhanced chemiluminescent detection reagent (Millipore) and autoradiography. Densitometric analysis was performed using ImageQuant TL 2007 software (GE Healthcare). The mean densitometric index + two SD (standard deviation) of the sera from 12 non-allergic controls for each test protein was used as the cut-off value for determining positive reactivity [18] and the results were expressed as the relative densitometric index, calculated as the absorbance using the patient's serum/mean + 2 SD for the normal sera [19].

2.12 Intradermal skin test (IST)

An IST was performed on one patient who had serum IgE reactive with cockroach in the MAST-CLA test. The intradermal skin test was performed as described previously [12], using 50 μL of purified German cockroach vitellogenin at concentrations of 2.5 μg/mL and 0.25 μg/mL in physiological saline (0.9% NaCl) or physiological saline solution alone as a negative control. Skin responses (wheals and erythema) were measured 15 min after injection. Wheals with diameters of at least 3 mm greater than that produced by the negative control were regarded as positive responses.

3 Results and discussion

3.1 Detection of IgE-binding proteins

Our approach allowed the characterization of previously undiscovered allergenic components of *B. germanica*. As shown in Fig. 1, cockroach-allergic patients (lanes 2–13) had highly complex IgE reactions to *B. germanica* proteins on Western blotting of 1-DE gels, as compared with a serum pool from 12 normal controls (lane 14). We detected more than 20 IgE-binding bands, ranging from 14 to 97 kDa in

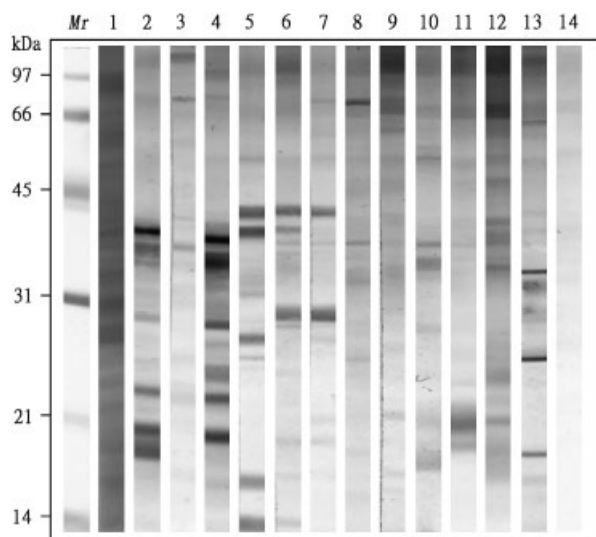


Figure 1. CBB-stained or IgE-reactive proteins in the WBE-G. Lane 1, CBB staining of the crude extract; lanes 2–13, IgE immunoblots using serum samples from different allergic patients; lane 14, IgE immunoblot using pooled sera from 12 non-allergic individuals (negative control).

size. Bands with approximate molecular weights of 35, 40, 60, 80, and 97 kDa showed strong reactivity and were frequently recognized by IgE from allergic patients. Table 1 summarized the demographic profile of the individuals evaluated according to the factors of age and the presence of allergic symptoms. These patients, who had allergic symptoms and high levels of specific IgE against cockroach (MAST-CLA class > +2), also reacted with allergens from other invertebrate species (data not shown), including mites and shrimp. This result supports the findings of previous studies [20–22] and suggests cross-reactivity between allergens in cockroaches and other invertebrate species.

The putative *B. germanica* allergens were characterized using 2-DE and IgE immunoblotting, which revealed that the most highly expressed proteins in the extract were ~40 kDa in size and had neutral pIs (Fig. 2A) and that at least 24 different IgE-binding spots with molecular weights from 16 to 97 kDa and pIs ranging from 4.0 to 9.0 were observed (Fig. 2B). The arrows in Fig. 2A indicate the protein spots of interest that correspond to the IgE-binding spots in Fig. 2B. Control experiments using pooled sera from non-allergic donors showed no significant reactivity with any of the proteins in the extract (Fig. 2C).

3.2 Protein identification

N-terminal sequence analysis and BLAST similarity searches revealed that, totally 24 protein spots were analyzed, but only five IgE-binding spots can be determined by N-terminal sequence analysis and belonged to four different proteins (Table 2). The N-terminal sequences of spots 4b, 9,

Table 1. Clinical characteristics of the 12 cockroach-allergic patients

Patient	Gender	Age	Total IgE (IU/ml)	MAST-CLA class for cockroach	Allergic symptoms
a	M	9	345	4	AR
b	F	58	389	4	AR, AS
c	M	8	3355	4	AS
d	M	12	2873	3	AS
e	M	37	628	2	ACE, AD
f	F	9	1463	2	AD, AR
g	M	7	925	2	AR
h	F	18	486	2	AR
i	F	11	2362	2	ACE, AD
j	M	29	456	2	AR, AS, UR
k	M	53	246	2	UR
l	F	26	352	2	AS

ACE, allergic chronic eczema; AD, atopic dermatitis; AR, allergic rhinitis; AS, asthma; UR, urticaria; MiteDF, *Dermatophagoides farinae*; MiteDP, *D. pteronyssinus*; N.T., not tested.

10, 12, and 16 showed high similarities (77–100%) with predicted proteins. The results from our MS/MS analysis are summarized in Table 3; the identified IgE-reactive peptides belonged to 13 different proteins (1–23 unique and specific peptides per protein, covering 4–48% of each protein). The identified protein spots all matched the criteria described in Section 2, except for spots 11a and 11b (see Supporting Information Table), for which only one unique peptide was identified; the low peptide identification for these spots may be due to species-specific amino acid sequence variations and the fact that the *B. germanica* genome has not yet been fully sequenced.

The identified proteins included three well-known *B. germanica* allergens, Bla g 7 (spot 7), Bla g 4 (spots 14 and 15), and Bla g 5 (spot 13). Spots 14 and 15 shared seven unique peptides, but had different pIs in the 2-DE map, suggesting that they may represent two different isoforms of Bla g 4 [23]. Notably, we also identified ten new IgE-binding proteins (Table 3), these being vitellogenin (spots 1a, 1b, 1c, and 16), an Hsp70 family protein (spot 2), GroEL-like chaperonin (Hsp60, spot 3), enolase (spots 4a and 4b), arginine kinase (spots 5a, 5b, 5c, and 5d), fructose 1,6-bisphosphate aldolase (aldolase, spot 6), receptor for activated protein kinase C (RACK1, spots 8a and 8b), calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (calcium pump) isoform 1 (SERCA, spots 9 and 10), phosphoglyceromutase (spots 11a and 11b), and triosephosphate isomerase (TIM) (spot 12). The protein identification results obtained by MS were consistent with those obtained by N-terminal sequencing analysis. In addition, in the case of enolase and arginine kinase, our peptide fingerprinting results were verified by Western blotting using specific anti-enolase or anti-arginine kinase antibodies (Fig. 3).

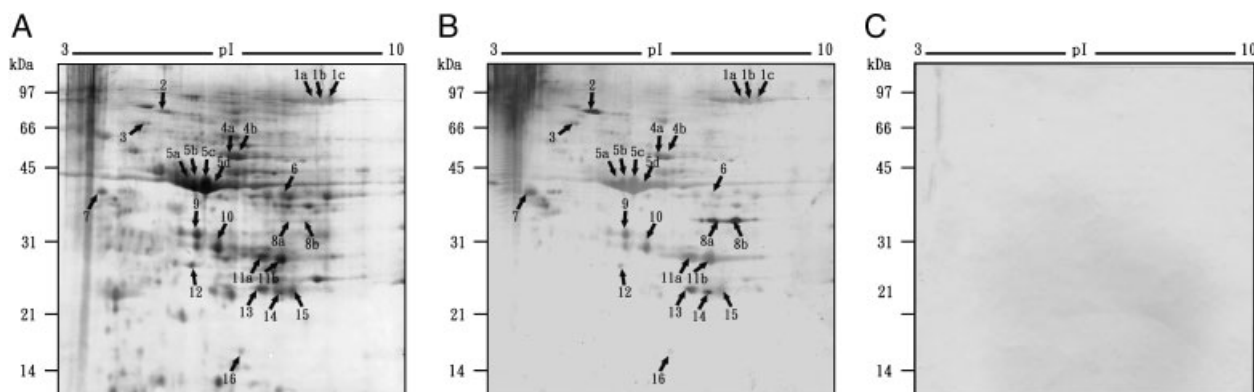


Figure 2. CBB-stained 2-DE maps of *B. germanica* proteins and the corresponding immunoblot using pooled sera from cockroach-allergic patients. The proteins were separated by 2-DE and stained with CBB (A) or probed with pooled sera from 12 allergic patients (B) or 12 non-allergic individuals (C). The IgE-binding protein spots were numbered (1–16) and collected for identification.

Table 2. N-terminal sequence analysis of IgE-binding proteins identified in WBE-G

Spot no.	Experimental results		Identification/accession no.	Identified N-terminal sequence	Theoretical		N-terminal similarity (%)
	pI	MW (kDa)			pI	MW (kDa)	
1a, 1c	8.27	50	Vitellogenin/CAA06379 ^{a)}	M.S.	8.17	213.5	–
4b	6.44	45	Enolase/AAU95200	PLQKLFARRI	5.07	39.55	90
5b, 5c	5.98	42	Arginine kinase/ABC86902 ^{a)}	N.D.	5.9	39.82	–
9	5.83	31	ER Ca ²⁺ transporting ATPase/XP_623125	SLPSVETLGC	5.38	112.09	100
10	6.27	28	ER Ca ²⁺ transporting ATPase/XP_623125	GTLTTNQ	5.38	112.09	100
12	5.33	25	Triosephosphate isomerase/XP_001600119	GRKFWVGGN	5.58	26.84	77
16	6.61	16	Vitellogenin/CAA06379 ^{a)}	DAFYTILDIP	8.17	213.50	100

a) Determined by LC-MS/MS analysis;
M.S., multiple signals; N.D., not determined.

Top-down proteomic methods (allergenomics) have been used to identify novel allergens in other species, but have not previously been used systemically in *B. germanica* [11, 13, 24–26]. In this study, we used allergenomics to identify ten new candidate allergens and three previously reported allergens (Bla g 4, Bla g 5, and Bla g 7) from *B. germanica*. Interestingly, we did not identify Bla g 1 or Bla g 2, two well-known allergens from *B. germanica*. However, these are believed to be involved in digestive function and are present in at least 4–10-fold higher levels in feces than in whole-body extracts [2, 27]. In our study, we collected the female insects at the oviposition stage, when the concentration of Bla g 1 is very low [28]. In addition, the insects were starved for 24 h before being collected, which may have decreased Bla g 1 [29] and Bla g 2 levels in the insect body. Since the protein samples used in the present study were prepared from whole bodies, levels of Bla g 1 and Bla g 2 may have been below the detection threshold of the techniques we used.

The genome of *B. germanica* has not been completely sequenced. In the case of the 16 IgE-binding spots, belonging to nine proteins, for which the sequence was not found in available databases, the first-run MS spectrometric

data were first used in searches against proteins from other species, then, to verify the results obtained, we cloned the corresponding cockroach genes. The genes encoding the IgE-binding proteins, *arginine kinase*, *enolase*, *RACK1*, and *TIM*, were fully sequenced and have been submitted to the GenBank (accession numbers ABC86902, ABC96322, ABI63548 and ABI63547). Predictably, second-run MS spectrometric data searches performed against the proteins translated from the newly cloned genes yielded higher scores and better protein coverage and fit more unique peptides (Table 3). However, several of the German cockroach IgE-binding spots could not be identified (Fig. 2B), even though they were clearly detected by 2-DE IgE immunoblotting. One possible explanation is that the amount of IgE-binding protein was not sufficient to generate meaningful data. Alternatively, it is possible that the databases used simply did not contain any sequence corresponding to the IgE-binding protein or its homologs. To overcome these obstacles, future studies on the identified proteins could make use of the complete German cockroach genome sequence (once published) or more sensitive Fourier Transform mass spectrometric analysis.

Table 3. LC-MS/MS identification of IgE-binding proteins obtained from the 2-DE gel

Spot no.	Protein ID Species/accession no.	Theoretical		LC-MS/MS analysis		
		Mass (kDa)	pI	Sequence coverage (%)	Score	No. of peptides (unique/matched)
1	Vitellogenin					
a	<i>B. germanica</i> /CAA06379	213.5	8.17	10	632	16/19
b	<i>B. germanica</i> /CAA06379	213.5	8.17	14	944	23/23
c	<i>B. germanica</i> /CAA06379	213.5	8.17	14	817	21/22
2	Hsp70 family member <i>Bombyx mori</i> /BAB92074	71.13	5.33	24	779	9/15
3	GroEL-like chaperonin <i>Blattabacterium</i> sp. (<i>Blattella germanica</i>) str. <i>Bge</i> /YP_003283834	57.8	5.09	48	1156	12/22
4	Enolase					
a	<i>Tomocerus</i> sp. <i>jcrjws1</i> /AAF72641	41.3	5.37	8	125	2/4
	<i>B. germanica</i> /ABC96322 ^{a)}	47.14	5.91	40	667	9/16
b	<i>Scolopendra polymorpha</i> /AAF72640	41.55	5.44	13	225	2/5
	<i>B. germanica</i> /ABC96322 ^{a)}	47.14	5.91	57	888	7/18
5	Arginine kinase					
a	<i>Homalodisca coagulata</i> /AAT01074	39.95	5.8	25	647	3/9
	<i>B. germanica</i> /ABC86902 ^{a)}	39.82	5.91	33	846	3/11
b	<i>Homalodisca coagulata</i> /AAT01074	39.95	5.8	22	585	3/8
	<i>B. germanica</i> /ABC86902 ^{a)}	39.82	5.91	38	914	4/14
c	<i>Homalodisca coagulata</i> /AAT01074	39.95	5.8	22	645	2/7
	<i>B. germanica</i> /ABC86902 ^{a)}	39.82	5.91	33	850	3/12
d	<i>Homalodisca coagulata</i> /AAT01074	39.95	5.8	22	658	4/8
	<i>B. germanica</i> /ABC86902 ^{a)}	39.82	5.91	33	828	4/11
6	Fructose 1,6-bisphosphate aldolase <i>Drosophila melanogaster</i> /CAA42666	42.94	7.59	11	234	3/4
7	Allergen Bla g 7 (tropomyosin) <i>B. germanica</i> /AAF72534	32.81	4.72	41	659	9/12
8	Receptor for activated protein kinase C (RACK1)					
a	<i>Apis mellifera</i> /XP_392962	35.75	7.19	19	159	4/5
	<i>B. germanica</i> /ABI63548 ^{a)}	35.73	7.61	21	159	5/5
b	<i>Nasonia vitripennis</i> /XP_001600152	35.73	7.16	16	143	4/4
	<i>B. germanica</i> /ABI63548 ^{a)}	35.73	7.61	29	291	7/8
9	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (calcium pump) isoform 1 <i>Tribolium castaneum</i> /XP_975930	109.81	5.33	5	317	5/6
10	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (calcium pump) isoform 1 <i>Tribolium castaneum</i> /XP_975930	109.81	5.33	5	324	5/6
11	Phosphoglyceromutase					
a	<i>Stomoxys calcitrans</i> /ACN69143	28.5	6.61	10	212	1/3
b	<i>Apis cerana</i> /AAQ67426	28.94	8.4	7	77	1/2
12	Triosephosphate isomerase <i>Loboptera decipiens</i> /CAE45561	22.86	5.02	13	162	2/3
	<i>B. germanica</i> /ABI63547 ^{a)}	26.82	5.56	21	291	2/5
13	Allergen Bla g 5 <i>B. germanica</i> /AAB72147	22.93	6.38	8	85	2/2
14	Allergen Bla g 4 <i>B. germanica</i> /P54962	20.91	6.47	48	382	7/7
15	Allergen Bla g 4 <i>B. germanica</i> /P54962	20.91	6.47	43	394	7/7
16	Vitellogenin <i>B. germanica</i> /CAA06379	213.5	8.17	4	260	7/7

a) The gene was cloned and the protein spot re-identified by nanoLC ESI-MS/MS.

3.3 Potential cross-reactivities with other allergens

To identify potential cross-reactivities with our allergen candidates, we used the identified IgE-binding proteins to search the Allergome and FARRp databases, which identify, respectively, molecules causing an IgE-mediated disease that have been selected from international scientific journals and from web-based resources or proteins that may present a potential risk of allergenic cross-reactivity. The Allergome database yielded six significant hits, showing that the homologs of aldolase, arginine kinase, enolase, Hsp70, TIM, and vitellogenin had been previously reported as allergens in other species (Table 4). However, none of these proteins have previously been described as allergens in *B. germanica*. The FARRp database search revealed that *B. germanica*

arginine kinase shares 87.3% identity with the Plo i 1 arginine kinase allergen from the Indianmeal moth, 83.4% identity with the Lit v 2 allergen from the Pacific white shrimp, and 83.1% identity with the Pen m 2 allergen from the tiger shrimp (Table 4). Similarly, the identified cockroach enolase (~50 kDa) shares 60.0–65.4% identity with various enolase allergens from plants and fungi, and TIM shares 60.7% identity with the bread wheat Tri a 19 allergen, which is also a TIM protein. These three cases fulfilled the FAO/WHO criteria for overall polypeptide sequence alignment with identity matches greater than 50%, indicating that there may be cross-reactivity between the identified IgE-binding allergen candidates and the known allergens (Fig. 4 and Table 4). We further used pairwise comparison to search for matches between short stretches (eight amino acids) of amino acids from the IgE-binding allergen candidates and the allergen sequences in the FARRp database. Interestingly, the three cross-reactive allergen candidates each yielded at least one matching peptide (one for TIM, five for enolase, and six for arginine kinase) in this analysis (Fig. 4), confirming the possibility of cross-reactivity between these three proteins and known allergens.

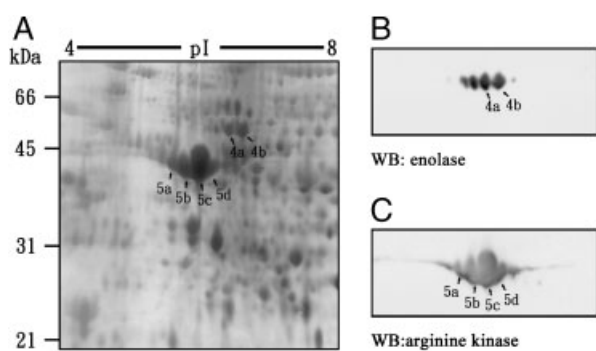


Figure 3. Validation of IgE-binding proteins using specific antibodies. (A) Locations of enolase (4a, 4b) and arginine kinase (5a to 5d) protein spots in the stained 2-DE gel. Individual spots probed with polyclonal antibodies against allergenic homologs of enolase (B) or arginine kinase (C).

3.4 IgE reactivity profiles of allergic patients

The solid-phase platform containing various German cockroach allergens (Bla g 1, Bla g 2, Bla g 4, Bla g 5, Bla g 7, enolase, arginine kinase, and vitellogenin) was used to determine the IgE reactivity patterns of sera from 32 allergic patients. The prevalence of IgE recognition was highest for Bla g 2 (62.5%), followed by vitellogenin (46.88%), arginine kinase (34.38%), enolase (25.0%), and other Bla g allergens

Table 4. Identity of IgE-binding proteins and their related homologous allergens

Protein ID	Allergen (identity)	Organism	Other related homologous allergens
Arginine kinase (Bla g 9)	Plo i 1 (87.3%)	<i>Plodia interpunctella</i> (Indianmeal moth)	Blo t 20, Bom m 9, Der f 20, Eri s 2, Hom g 2, Met e 2, Met t 2, Per a 9, Scy s 2
	Lit v 2 (83.4%)	<i>Litopenaeus vannamei</i> (Pacific white shrimp)	
Enolase	Pen m 2 (83.1%)	<i>Penaeus monodon</i> (Tiger shrimp)	
	Cand a (65.4%)	<i>Candida albicans</i> (Fungi)	Asp v, Cyn d 22, Sac c Enolase
	Hev b 9 (66.7%)	<i>Hevea brasiliensis</i> (Plant)	
	RHo m 1 (66.7%)	<i>Rhodotorula mucilaginosa</i> (Fungi)	
	Cla h 6 (62.3%)	<i>Davidiella tassiana</i> (Fungi)	
	Asp f 22 (61.9%)	<i>Aspergillus fumigatus</i> (Fungi)	
	Alt a 11 (61.5%)	<i>Alternaria alternata</i> (Fungi)	
	Cur l 2 (60.0%)	<i>Cochliobolus lunatus</i> (Fungi)	
	Pen c 22 (60.6%)	<i>Penicillium citrinum</i> (Fungi)	
TIM	Tri a 19 (60.7%)	<i>Triticum aestivum</i> (bread wheat)	For t 1, latex TPI, Lit c
Vitellogenin	Der p 14 (19.4%)	<i>Dermatophagoides pteronyssinus</i> (mite)	Der f 14, Eur m 14, Onc ke vitellogenin
Aldolase	— ^{a)}	— ^{a)}	For t 2, wheat-flour
Hsp70 family member	— ^{a)}	— ^{a)}	Blo t Mag29, Cla h 4, Der f HSP70, Pen c 19, Tox g HSP70

a) –, the gene sequence has not been cloned or registered in the databases used.

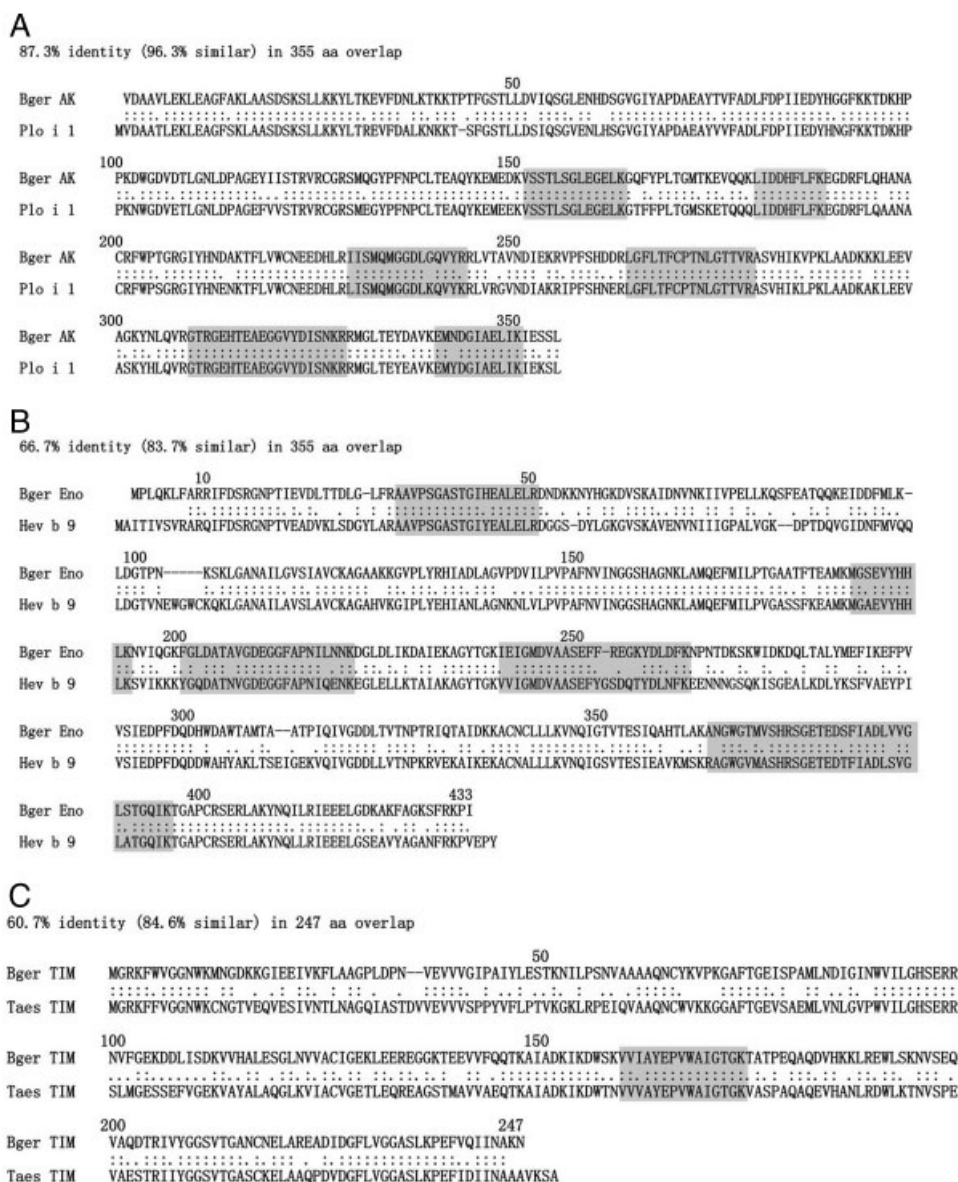


Figure 4. Protein sequence alignment results for cockroach arginine kinase (A), enolase (B), and TIM (C) with known related allergen proteins from the FARRp database. The colons indicate regions that are identical between the related allergens, while the gray squares indicate MS/MS-identified peptide regions that are identical across short spans.

(Fig. 5A). Significant differences in the prevalence of IgE reactivity to different allergens were found between the 32 allergic patients. Although most allergic patients displayed IgE reactivity to Bla g 2, the IgE reactivity profiles were found to be heterogeneous and differed substantially between allergic individuals (Fig. 5B). Two patients reacted with six allergens, four with five, five with four, and 21 with 1–3, resulting in a unique personalized IgE-reactivity profile for the different subjects, both in terms of quantity and reactivity. The concept of using purified allergens to determine a patient's sensitization profile has been called "component-resolved diagnosis" (CRD) [30] and its application allows the establishment of an individual reactivity for every patient. Cockroach crude extracts are used for allergy diagnosis and therapy, but allergen profiling is not

routinely carried out. The use of multiple allergen molecules and CRD offers many fundamental advantages. It allows the detection of specific IgE reactivities to different allergen components and makes it possible to distinguish various subtypes of German cockroach allergy. IgE reactivity profiles to multiple allergen molecules can be analyzed in a single test using minute amounts of serum. Personalized allergen-specific IgE profiles could be used to tailor medical care to the individual's needs.

It should be noted that two of the putative allergens, arginine kinase and vitellogenin, showed not only different IgE reactivity profiles, but also higher IgE-binding frequencies than the known Bla g 1 (34.38%), Bla g 7 (31.25%), and Bla g 5 (31.25%) cockroach allergens. Thus, our study showed that at least two of the newly discovered

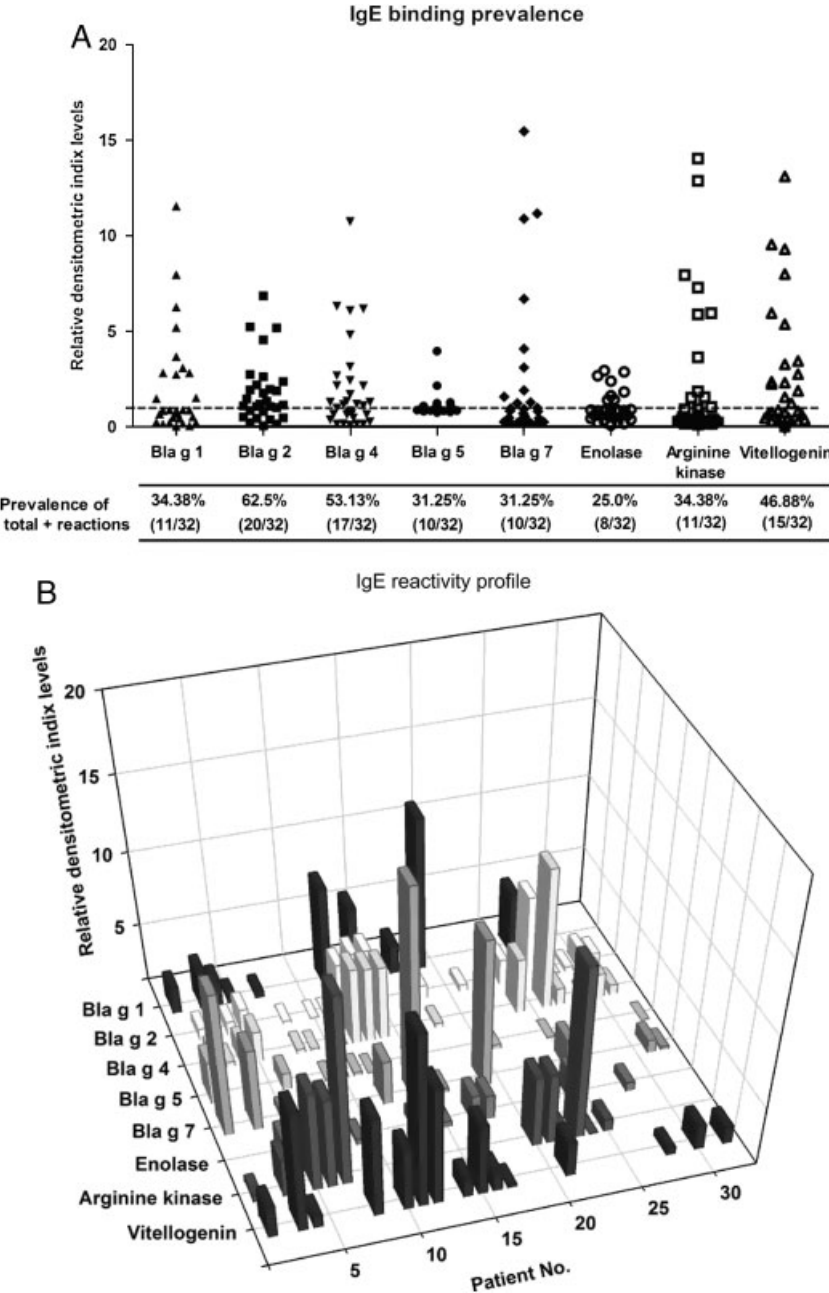


Figure 5. Summary of human IgE reactivity profiles obtained using the solid-phase platform and individual serum samples from 32 cockroach-allergic patients. (A) Human IgE-binding prevalence for each of the test proteins. The cut-off value used to classify reactions as “positive” is shown by the horizontal dotted line. (B) Relative densitometric index level in allergic individuals (no. 1–32), represented by different gray colors, plotted for every patient.

IgE-binding proteins, vitellogenin and arginine kinase, are important allergens. In particular, vitellogenin, which was recognized by 46.88% of cockroach-allergic patients (see the following section), should be considered a potential new major allergen of *B. germanica*.

3.5 Characteristics of the individual IgE-binding allergen candidate proteins

Vitellogenin is formed from a precursor form predicted to contain ~1863 amino acid residues and to be about

213.5 kDa in size [31]. The molecular weights of the vitellogenin protein spots observed in our 2-DE map were ~97 kDa (Fig. 2; spots 1a, 1b, 1c), 50 kDa (in oothecae; data not show), and 16 kDa (Fig. 2; spot 16). It is not yet known whether these spots represent isoforms or degraded forms of the protein. A previous study suggested that the 13 putative glycosylation and two putative endogenous subtilisin cleavage sites of vitellogenin would give rise to different isoforms with molecular weights of 50–95 kDa [32]; this may account for the high background and multiple signals found in the *N*-terminal sequences obtained from spots 1a and 1c. Vitellogenin has previously been described as an allergen of

chicken eggs (Gal d vitellogenin), dust mites (Der p 14), and salmonid fish roe (Table 4) [33–35]. However, the protein sequence identities between these proteins and *B. germanica* vitellogenin are all less than 20%, suggesting that cross-reactivity may not exist in this case. Instead, vitellogenin may act in a fashion similar to the mite vitellogenin allergen, Der p 14, which functions as an allergic adjuvant by enhancing IgE responses to itself and to other potential allergens [34]. Our immunodot results showed that the prevalence of IgE binding to *B. germanica* vitellogenin (46.88%) was greater than that to the pan-allergen, Bla g 7 (31.25%) (Fig. 5A). To further characterize the allergenicity of purified *B. germanica* vitellogenin *in vivo*, we performed a quantitative IST on a selected cockroach-allergic patient using vitellogenin. A dramatic wheal (>8 mm) and flare ($r > 17.5$ mm) reaction was observed in the sensitized patient, but no reaction was induced with physiological saline (Fig. 6) or using vitellogenin in a non-allergic individual (data not shown). These results show that vitellogenin can elicit specific IgE-mediated hypersensitivity responses in cockroach-allergic patients, indicating that it is an important allergen of *B. germanica*.

Another dominant IgE-binding protein identified in this study, arginine kinase, has been reported as an important inhalant and cross-reactive pan-invertebrate allergen from several invertebrate species, including the American cockroach, lobster, crab, crawfish, mite, moth, and shrimp [12, 36, 37]. Other studies on other allergens have also found cross-reactivity between cockroaches and other invertebrates [15, 20–22, 38]. For example, the German cockroach allergen Bla g 7 (tropomyosin) shows a high degree of sequence identity to its homologs in other invertebrates [15]. Thus, it is not surprising that the protein sequence of *B. germanica* arginine kinase shares >90% homology with its homologs in

other allergenic species. Arginine kinase from *B. germanica* was recognized by serum samples from 34.38% of the tested cockroach-allergic patients, indicating that it can be considered an immunodominant allergic antigen of *B. germanica*. We have therefore termed it Bla g 9 and submitted this name to the International Union of Immunological Societies (IUIS). The second cross-reactive allergen candidate identified was enolase, which is also highly conserved (> 80% homology) among homologs in other plants and fungi allergenic species. Enolase has previously been reported as a major cross-reactive allergen from various fungi and plants (Table 4) [39–42], but this is the first report of an enolase allergen in the cockroach. However, our IgE immunodot assay showed that only 25.0% of the patients' serum samples reacted with enolase and the relative densitometric index was low. This result may be due to amino acid sequence differences in the major part of the IgE-binding epitopes of enolase from *B. germanica* and enolase from other species [43]. The third potential cross-reactive allergen candidate identified was TIM, which is involved in glycolytic reactions and has previously been reported as an allergen in lychee fruit (Lit c), wheat flour (Tri a 19), latex, and the biting midge (*Forcipomyia taiwana*, For t 1) [44–47].

In addition, we identified two other allergen candidates in the Allome database search, namely aldolase and Hsp70, which have been previously reported as allergens in other species [47]. The aldolase protein family can be divided into two subtypes, class I aldolases, which are found in animals and higher plants and catalyze reactions *via* the formation of a Schiff base with the substrate, and class II aldolases, which are found in prokaryotes and fungi and require divalent cations for their activity [47]. The previously reported aldolase allergens, for t 2 from the biting midge and wheat aldolase allergen, belong to the class I aldolases [47, 48]. Some heat shock proteins (HSP) have been recognized as allergens or antigens in different diseases [49, 50] and some show IgE reactivity [49]. Numerous reports have identified Hsp70 allergens from a variety of sources, including mites, fungi, parasites, and latex (Table 4), but few of these studies have discussed allergenicity. In the present study, Hsp70 showed IgE reactivity, indicating that it might be a potential allergen in the German cockroach. Interestingly, another HSP protein, GroEL (Hsp60), identified as an IgE-binding protein in our study, which comes from *Blattabacterium* sp. (*Blattella germanica*) str. Bge, a primary endosymbiont of *B. germanica* [51], has never been previously suggested as an allergen. Furthermore, we identified three additional IgE-binding proteins, namely RACK1, SERCA, and phosphoglyceromutase, which have not previously been reported as allergens in any other species. To the best of our knowledge, this is the first report showing that these proteins can bind IgE from allergic patients.

Three of the candidate IgE-binding allergens from the German cockroach identified herein, namely arginine kinase, enolase, and TIM, have been reported as allergens in other species and showed potential cross reactivity in our

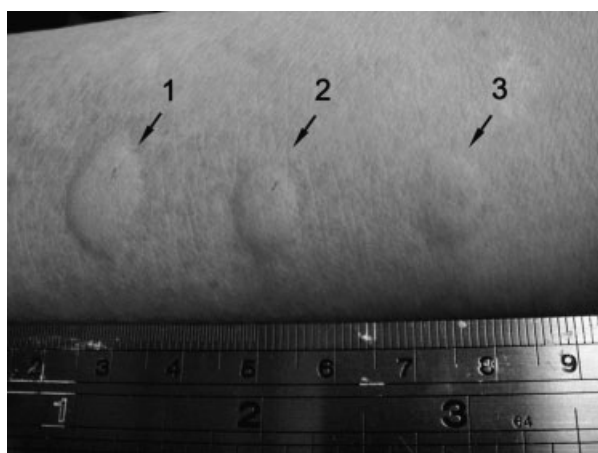


Figure 6. Skin test using purified vitellogenin. Purified German cockroach vitellogenin (50 μ L) at concentrations of 2.5 μ g/mL (spot 1) or 0.25 μ g/mL (spot 2) or physiological saline (spot 3) was injected intradermally into the forearm of a cockroach-allergic patient and wheal-and-flare responses assessed after 15 min.

analysis of the FARRp database. Moreover, arginine kinase has been reported as an important pan-invertebrate allergen in the American cockroach and other arthropod species [37]. Given that the German cockroach also produces another major pan-allergen, Bla g 7, we suggest that these cross-reactive allergens could be an important cause of German cockroach-allergic disease [15, 52]. As described previously [2, 15, 37, 53, 54], the characterization of cross-reactive allergens is important because cockroach-allergic patients usually produce IgE antibodies against some, but not all, of the components present in cockroach extracts. Studies on these cross-reactive allergens and the application of CRD to the German cockroach may help maximize efficiency of diagnosis of cockroach-allergic disease and/or minimize the components required for component-based immunotherapy in the future.

4 Concluding remarks

In conclusion, we used a combination of 2-DE immunoblotting, mass spectrometric analysis, and bioinformatic assessment that provides a fast and efficient platform for discovering novel allergens, especially potential cross-reactive allergens, in the *B. germanica* allergen repertoire. Analysis of individual IgE reactivity profiles may change established practice and personalize medicine allergy diagnosis, prevention, and therapy.

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Authors have declared no conflict of interest

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