

Identification of Per a 13 as a novel allergen in American cockroach

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ABSTRACT

Background: Cockroaches are an important source of indoor allergens. Environmental exposure to cockroach allergens is closely associated with the development of immunoglobulin E (IgE)-mediated allergic diseases. However, the allergenic components in the American cockroaches are not fully studied yet. In order to develop novel diagnostic and therapeutic strategies for cockroach allergy, it is necessary to comprehensively investigate this undescribed allergen in the American cockroach.

Methods: The full-length cDNA of the potential allergen was isolated from the cDNA library of the American cockroach by PCR cloning. Both the recombinant and natural protein molecules were purified and characterized. The allergenicity was further analyzed by enzyme linked immunosorbent assay, immunoblot, and basophil activation test using sera from cockroach allergic patients.

Results: A novel allergen belonging to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was firstly identified in the American cockroach and named as Per a 13. The cDNA of this allergen is 1255 base pairs in length and contains an open reading frame of 999 base pairs, encoding 332 amino acids. The purified Per a 13 was fully characterized and assessed to react with IgEs from 49.3 % of cockroach allergic patients, and patients with allergic rhinitis were more sensitized to it. Moreover, the allergenicity was further confirmed by immunoblot and basophil activation test.

Conclusions: We firstly identified GAPDH (Per a 13) in the American cockroach, which is a novel type of inhalant allergen derived from animal species. These findings could be useful in developing novel diagnostic and therapeutic strategies for cockroach allergy.

Abbreviations: RACE, rapid amplification of cDNA ends; nPer a 13, natural Per a 13; rPer a 13, recombinant Per a 13; IAC, immunoaffinity chromatography; SEC, size exclusion chromatography; IEC, ion exchange chromatography; HPLC, high performance liquid chromatography; AR, allergic rhinitis; AS, allergic asthma.

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1. Introduction

Cockroaches are an important source of indoor allergens in many parts of the world (Peden and Reed, 2010). Environmental exposure and sensitization to cockroach allergens are strongly associated not only with the development of immunoglobulin E (IgE)-mediated allergic diseases but also with the aggravation of severity and mortality of asthma (Do et al., 2016; Huang et al., 2021). Both German and American cockroaches are the predominant species in human dwellings and have become the focus of cockroach allergen-related research (Fukutomi and Kawakami, 2021).

At present, cockroach allergy is usually diagnosed by skin testing and/or specific IgE (sIgE) testing based on cockroach crude extracts (Do et al., 2016). Moreover, immunotherapy with cockroach extracts is a promising treatment strategy and has shown significant improvement in the clinical and immunological status of asthma and rhinitis patients in a very limited number of studies (Pomés and Arruda, 2014; Srivastava et al., 2011). However, the cockroach extracts may contain both allergenic and non-allergenic components (Sookrung et al., 2019). The levels of allergen components in extracts are also highly variable based on different source materials and extraction methods (Glesner et al., 2019; Kleine-Tebbe et al., 2019; Mindaye et al., 2020). These properties of cockroach extracts limit their sensitivity and specificity in diagnosis as well as optimal clinical efficacy in therapy. Therefore, a clear understanding of actual disease-eliciting allergens in cockroaches will effectively improve the diagnostic testing and immunotherapy for cockroach allergy.

Currently, 12 groups of allergens belonging to different protein families in cockroaches have been characterized and are officially listed in the Allergen Nomenclature database (<http://www.allergen.org/>), including microvilli-like protein (Bla g 1 and Per a 1) (Melén et al., 1999; Pomés et al., 1998), inactive aspartic protease (Bla g 2 and Per a 2) (Arruda et al., 1995b; Lee et al., 2012), hemocyanins (Bla g 3 and Per a 3) (Khurana et al., 2014; Wu et al., 1996), lipocalin (Bla g 4 and Per a 4) (Arruda et al., 1995a; Tan et al., 2009), glutathione S-transferase (Bla g 5 and Per a 5) (Arruda et al., 1997; Wei et al., 2014), troponin C (Bla g 6 and Per a 6) (Hindley et al., 2006), tropomyosin (Bla g 7 and Per a 7) (Asturias et al., 1999; Jeong et al., 2003; Santos et al., 1999), myosin light chain (Bla g 8 and Per a 8), arginine kinase (Bla g 9 and Per a 9) (Chuang et al., 2010; Sookrung et al., 2006), serine protease (Per a 10) (Sudha et al., 2008), alpha-amylase (Bla g 11 and Per a 11) (Fang et al., 2015; Jeong et al., 2013) and chitinase (Bla g 12 and Per a 12) (Fang et al., 2015; Pomés et al., 2020). These fundamental findings of the allergenic components have facilitated the development of component-resolved diagnosis for cockroach allergy (Pomés et al., 2019; Sookrung et al., 2019). However, the understanding of the allergenic components in cockroaches is still insufficient since 22 IgE-binding components in the American cockroach were found and new proteins belonging to novel allergen groups in the German cockroach were identified by immunoproteomic analysis a decade ago (Chuang et al., 2010; Thangam Sudha et al., 2007). Until now, the potential allergens in the American cockroach have not been well studied. We had screened several IgE-binding spots by 2-dimensional immunoblotting assays, which showed positive reaction with the serum from cockroach allergic patients (unpublished data). Accordingly, three peptides belonging to a novel protein family of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were identified by mass spectrometry. In the present study, we cloned and purified this GAPDH, and the allergenicity of this molecule was also characterized. It is now listed as Per a 13 in the Allergen Nomenclature database maintained by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee.

2. Material and methods

Detailed materials and methods are available in the supplement.

2.1. Patient's sera

Sera samples were obtained from 140 patients with positive serum sIgE to cockroach measured by ImmunoCAP (Thermo Fisher Scientific/Phadia, Uppsala, Sweden). The basic information and sensitization profiles were summarized in Table S1. Patients had a mean age of 37.97 years (range 2–72). Among them, 47.9 % were males and 52.1 % were females. Allergic rhinitis (40.7 %), urticaria (35 %), and asthma (20.7 %) were the most prominent clinical manifestations among these patients, followed by dermatitis (15.7 %) and anaphylaxis (3.6 %). All participants provided written informed consent, including parental consent for those less than 18 years of age. Ethics approval for this study was granted by the Ethics Committee in the First Affiliated Hospital of Nanjing Medical University.

2.2. Cloning of the full-length cDNA encoding Per a 13

Cloning of the full-length cDNA encoding Per a 13 was performed as described in the supplement. Briefly, the cDNA library was generated from the purified mRNA of the American cockroach and was then used as a template for amplification of the partial cDNA sequences using degenerate primers (GAPDH-F: 5'-GTNCCNGTNCNAAAYGT-3' or GAPDH-R: 5'-ACRTTNGGNACNGGNAC-3') and the vector-derived primers (M13F: 5'-TGTAACGACGCGCCAGT-3' or M13R: 5'-CAG-GAAACAGCTATGACC-3'). The 5' and 3' end of the Per a 13 cDNA was further amplified using semi-nested primers (GAPDH GSP-1F: 5'-CTTGCCAAAGTAATCCACGAC-3', GAPDH GSP-2F: 5'-GAAAGCTGTGGCGTGATGG-3', GAPDH GSP-1R: 5'-GAA-TACCCAACTCGTTGTCAT-3', GAPDH GSP-2R: 5'-CATCAA-GATGGAAGAGTGAG-3') and vector-derived primers, which were cloned into T vector. Sequencing results of the colonies were assembled to get the full-length cDNA encoding Per a 13. Multiple alignments of the amino acid sequence with two homologous allergens (Pan h 13 and Tri a 34) (Ruethers et al., 2021; Sander et al., 2011) listed in the IUIS database were performed by ClustalX2 (Larkin et al., 2007) and displayed by ESPript 3.0 (Robert and Gouet, 2014). The phylogenetic tree was generated based on the sequences of homologous proteins using the neighbor-joining algorithm in MEGA7 (Kumar et al., 2016) and visualized using Figtree software (<http://tree.bio.ed.ac.uk/software/figtree/>).

2.3. Purification and characterization of recombinant and natural Per a 13

The protein-coding region of Per a 13 was subcloned into the Nde I/Xho I sites of the pET21a vector. The recombinant plasmid was transformed into *E. coli* strain Rosetta (DE3) and protein expression was induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant Per a 13 (rPer a 13) was purified using Ni-NTA resin (GenScript, Nanjing, China) and refolded according to the published methods (Zhu et al., 2021).

Natural Per a 13 (nPer a 13) in American cockroach was purified using two different methods: immunoaffinity method and a traditional method combining ammonium sulfate precipitation, gel filtration, ion exchange and high-performance liquid chromatography. In the first method, the polyclonal antibodies against rPer a 13 (anti-rPer a 13 pAb) were produced in New Zealand rabbits, the purified anti-rPer a 13 pAb were coupled to CNBr-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden) and incubated with the extracts of the American cockroach. The pAb-bound nPer a 13 was eluted and lyophilized. The detailed procedures of combined chromatographies were described in the supplement. Briefly, total proteins in the extracts were precipitated with 40 %–60 % and 60 %–80 % saturation of ammonium sulfate, respectively. The 60 %–80 % fraction was pooled and loaded on a Superdex G75 increase 10/300 GL column (GE Healthcare, Uppsala, Sweden), and the resulted fraction positive to anti-rPer a 13 pAb was separated by using HiTrap Q HP column (GE Healthcare, Uppsala, Sweden). The nPer a 13

in the eluted fraction was further purified by loading it onto a reverse C8 HPLC column (Elite, Dalian, China). The purified nPer a 13 was verified by SDS-PAGE and immunoblot. Moreover, the nPer a 13 was confirmed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA) and the internal peptides were *de novo* analyzed by pNovo software (Chi et al., 2010). N-terminal sequence of nPer a 13 was determined using a PPSQ amino acid sequencer system (Shimadzu, Kyoto, Japan). The results were compared with the amino acid sequence of homologous proteins from the NCBI nr database. Glycosylation modification of nPer a 13 was detected with the Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's protocol.

2.4. Immunoreactivity of human sera IgE with rPer a 13

The IgE-binding reactivity of rPer a 13 was evaluated by indirect enzyme-linked immunosorbent assay (ELISA) according to the published method with slight modifications (Zhu et al., 2021). Briefly, 96-well plates were coated with rPer a 13 and blocked with 1 % BSA. Then, the wells were incubated with individual cockroach allergic patient's serum (1:5 dilution). The IgE binding was detected using HRP-conjugated anti-human IgE antibody (KPL, Maryland, USA) and 3, 3', 5, 5'-tetramethylbenzidine substrate (Beyotime, Shanghai, China). The reaction was then stopped and the absorbance value was recorded at 450 nm. The cut-off value was calculated as the average of the absorbance values plus 3 times the standard deviation from 20 healthy controls.

2.5. Immunoblot analysis of nPer a 13 and rPer a 13

Both nPer a 13 and rPer a 13 were separated by SDS-PAGE and electrotransferred onto PVDF membranes (Merck Millipore, Massachusetts, USA) as described in the supplement. Membranes were blocked and subsequently incubated with individual patient's serum (1:5 dilution) overnight at 4 °C. The bound IgE antibodies on the membranes were detected with HRP-conjugated anti-human IgE antibody.

2.6. Basophil activation test

The allergenicity of Per a 13 was assessed by basophil activation test (BAT) as described previously with some modifications (Wei et al., 2014). Peripheral blood mononuclear cells collected from healthy donors were passively sensitized by incubation with cockroach allergic patient's sera at 37 °C for 90 min, and cells from cockroach-negative donor without passive sensitization were used as control group. The cells were washed and stimulated with rPer a 13, anti-IgE antibody, and buffer alone at a final concentration of 10 µg/mL at 37 °C for 30 min. The cells were pelleted and mixed with the antibody cocktail consisted of FITC-conjugated anti-CD63 mAb and PE-conjugated anti-CCR3 mAb (Biolegend, California, USA) and incubated for 20 min on ice. The expressions of CCR3 and CD63 on basophils were evaluated using a FACSCanto Plus and analyzed using the build-in Diva software (BD Bioscience, California, USA).

3. Results

3.1. Cloning of the full-length cDNA encoding Per a 13

The full-length cDNA encoding Per a 13 was isolated from the cDNA library of American cockroach by PCR as described in the online supplement. The cDNA of Per a 13 is 1255 base pairs (bp) in length and contains an open reading frame of 999 bp (Fig. 1A). The nucleotide sequence of Per a 13 has been submitted to the GenBank under the accession number MG775273. Multiple alignments of the amino acid sequence with other homologous allergens recorded in the IUIS database

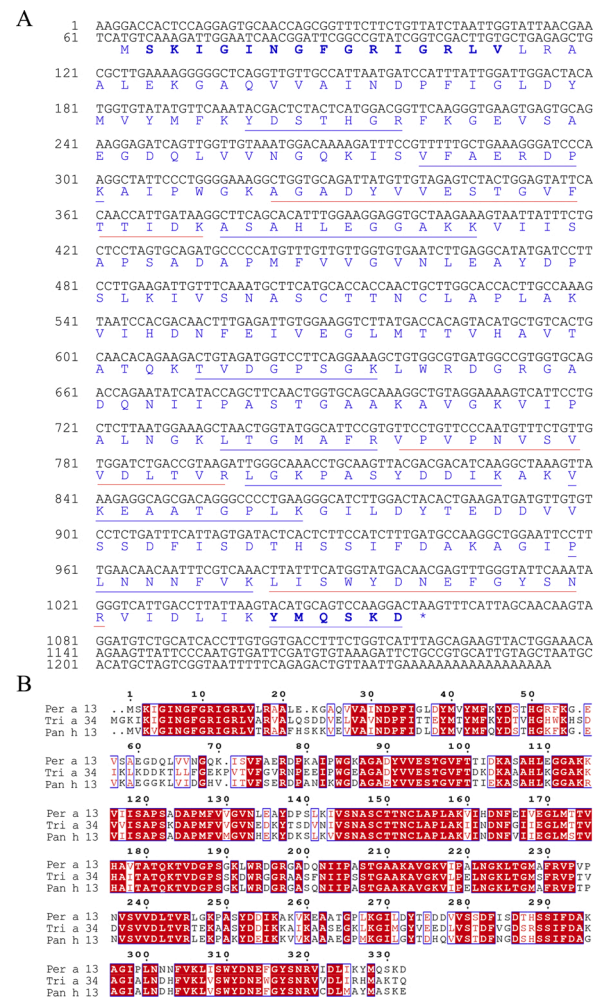


Fig. 1. cDNA sequence of Per a 13 and alignment with homologous allergens. Complete nucleotide sequence and amino acid sequence of Per a 13. Red lines indicated the peptides identified from the extracts of the American cockroach, and blue lines indicated the internal peptides identified from purified natural Per a 13 by *de novo* peptide sequencing. The N- and C-terminal sequences of nPer a 13 were shown in bold (A). Multiple alignment of amino acid sequence of Per a 13 and other homologous allergens. Identical residues were shaded in red, and conserved residues were boxed (B).

showed that Per a 13 shared 69 % sequence identity with Tri a 34 in wheat flour (Fig. 1B), an inhalant allergen from plant species that is associated with baker's asthma. Moreover, Per a 13 shared 80 % sequence identity with Pan h 13 (Fig. 1B), a food allergen from animal species that is associated with fish allergy. Both Tri a 34 and Pan h 13 had farther evolutionary relationship with Per a 13 from the evolutionary tree (Figure S5).

3.2. Purification and characterization of rPer a 13 and nPer a 13

The sequence coding region of Per a 13 was subcloned into the pET21a vector. SDS-PAGE analysis revealed that the rPer a 13 was expressed as inclusion bodies (aggregated protein in *E. coli*) (Fig. 2A, lane 4), which were purified using Ni-NTA resin under denaturing conditions followed by refolding (Fig. 2B, lane 3–4). The rPer a 13 was used to immunize rabbits and the titers of anti-rPer a 13 pAb was determined to be at least 1:320,000, the anti-rPer a 13 pAb was purified and confirmed a heavy chain band at approximately 55 kDa and a light chain band at approximately 25 kDa (Figure S6). The nPer a 13 was also successfully purified from the American cockroach extracts and migrated as a single band in SDS-PAGE (Fig. 2C, Figure S1D). Both of the nPer a 13 and rPer a

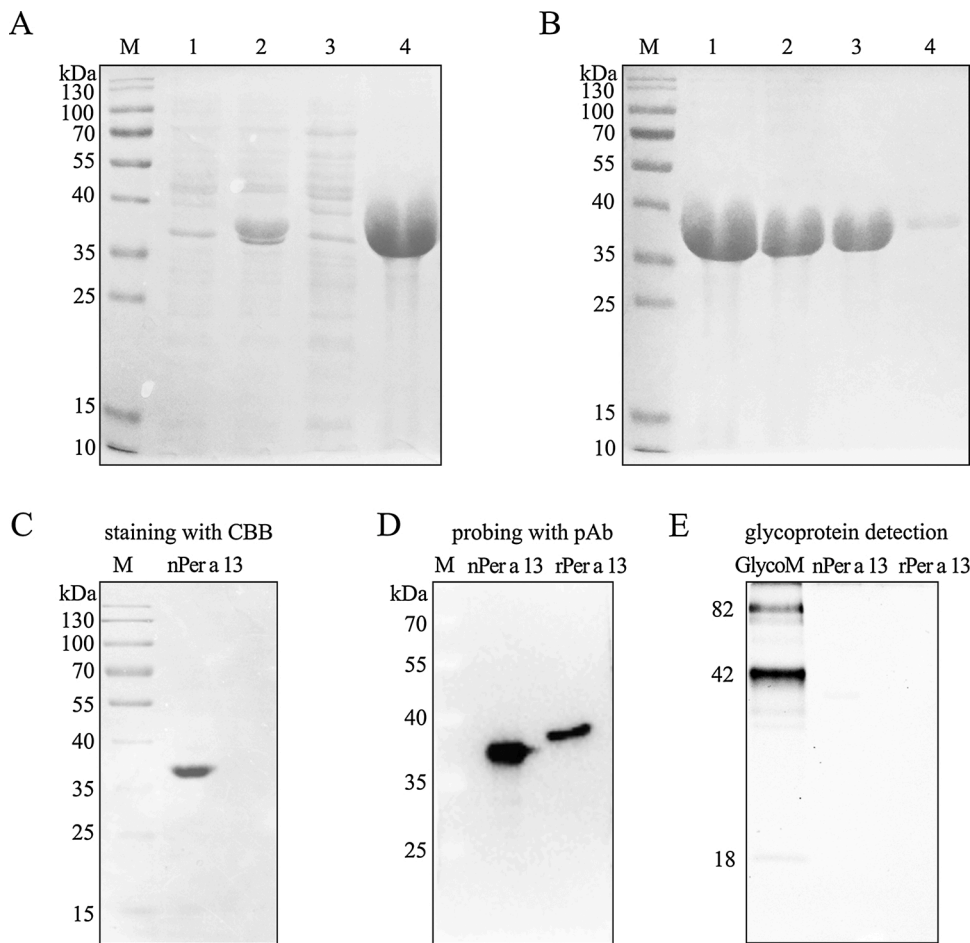


Fig. 2. Purification and characterization of recombinant and natural Per a 13. Expression of rPer a 13 in *E. coli*. Lane M, standard marker; Lane 1–2, lysate of *E. coli* induced with 0 mM, 1 mM IPTG, respectively; lane 3, the supernatant of *E. coli* lysate after ultrasonication; Lane 4, the precipitant of *E. coli* lysate after ultrasonication (A). Purification of rPer a 13 using Ni-NTA resin under denaturing conditions. Lane M, standard marker; Lane 1, denatured rPer a 13; Lane 2, washing fraction with 10 mM imidazole; Lane 3, elution fraction with 250 mM imidazole. Lane 4, elution fraction with 500 mM imidazole (B). Purification of nPer a 13 from the American cockroach extracts (C). Probing of purified nPer a 13 and rPer a 13 by anti-rPer a 13 polyclonal antibodies (D). Detection of glycosylation in nPer a 13. GlycoM, indicate the MW of the proteins in the marker that are glycosylate (E).

13 could be recognized by anti-rPer a 13 pAb, and nPer a 13 had a slightly lower molecular weight than rPer a 13 due to a His-tag at the C-terminal of rPer a 13 (Fig. 2D). *De novo* peptide sequencing of the nPer a 13 produced 11 peptides based on the LC-MS/MS raw data (Figure S2) and the peptides exhibited 22.9 % sequence coverage to the full-length amino acid sequence of Per a 13 (Fig. 1A). The first 15 N-terminal residues of nPer a 13 were determined as SKIGINGFGRIGRLV according to the results of Edman amino acid sequencing and BLAST search (Fig. 1A). The C-terminal sequence was YMQSKD according to the results of *de novo* peptide sequencing (Fig. 1A, Figure S2, No.11). In addition, the nPer a 13 was not glycosylated based on the result of glycosylation modification detection (Fig. 2E).

3.3. Analysis of IgE reactivity to Per a 13

Specific IgE against rPer a 13 was analyzed by ELISA using 140 sera from cockroach-allergic patients. As shown in data, 9 (49.3 %) of 140 sera showed positive IgE-binding to rPer a 13 (Fig. 3A). In the 69 positive patients' sera, 19 had an absorbance value great than 1.5-fold cutoff value in ELISA and were selected to further confirm the IgE-binding ability of Per a 13. Immunoblots showed that nPer a 13 and rPer a 13 exhibited the same IgE-binding pattern among the 19 patients' sera, in which 17 had distinct or slight IgE binding bands to Per a 13 and only 2 (No. 30 and 65) had negative results (Fig. 3B).

3.4. Basophil activation test

For *in vitro* basophil activation test, different groups of positive patients' sera in ELISA were selected for passive sensitization of basophils. In comparison with buffer alone, rPer a 13 at 10 μ g/mL induced

approximately 2.0-fold to 9.8-fold increase in the proportion (%) of CCR3 and CD63 double-positive cells (Fig. 4). However, basophils from cockroach-negative donors without passive sensitization could not be activated by Per a 13, which only showed 0.7-fold increase in the proportion (%) of CCR3 and CD63 double-positive cells (Figure S3).

3.5. Clinical symptoms in patients sensitized to Per a 13

For all tested patients, cockroach sIgE values measured by ImmunoCAP in patients with allergic rhinitis (AR) ($n = 38$) were higher than that in patients without AR or allergic asthma (AS) ($n = 73$) ($P < 0.01$) (Fig. 5A). Similarly, the absorbance values of IgE-binding to rPer a 13 measured by ELISA in patients with AR were higher than that in patients without AR or allergic asthma (AS) ($P < 0.05$) (Fig. 5B), IgE binding to rPer a 13 measured by ELISA in patients with both AR and AS were also higher ($P < 0.01$) (Fig. 5B). Moreover, IgE-binding to Per a 13 was more frequent in AR group than that in patients without AR or AS ($P < 0.05$) (Fig. 5C).

4. Discussion

Cockroach allergen exposure and sensitization have been recognized as an important cause of allergic disease morbidity (Huang et al., 2021). Until now, up to 12 allergens (i.e., Per a 1 to Per a 12) have been identified in the American cockroach. However, there still exist undescribed allergens in the American cockroach according to previous studies (Chuang et al., 2010; Thangam Sudha et al., 2007), which prompt us to further investigate it. In this study, we first identified and characterized a novel inhalant allergen belonging to GAPDH, which was assigned as Per a 13.0101 by the WHO/IUIS Allergen Nomenclature

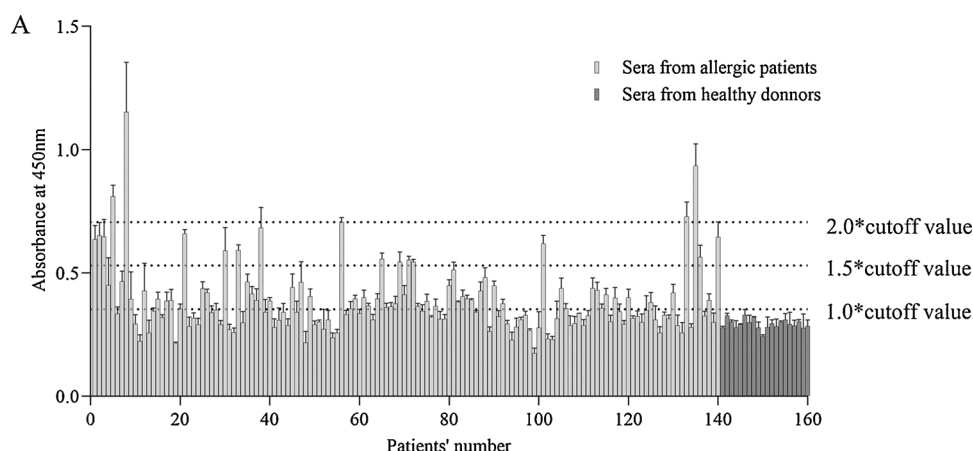
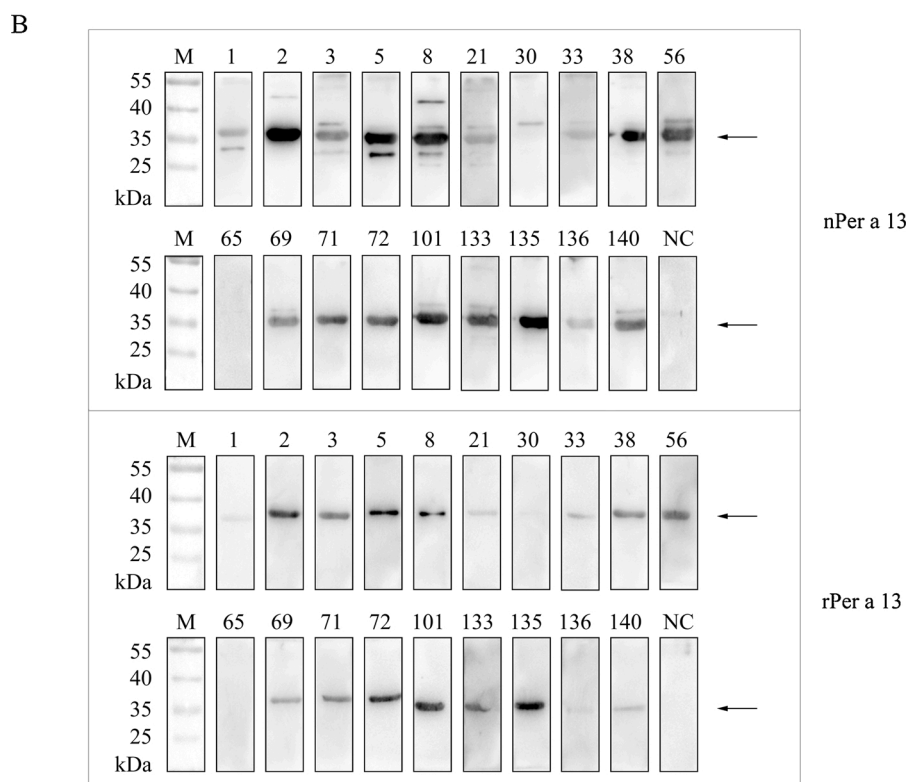


Fig. 3. IgE reactivity of Per a 13. Assessment of IgE-reactivity of rPer a 13 by ELISA using sera from 140 cockroach allergic patients and 20 healthy subjects. The cut-off value was calculated as the mean absorbance value of the 20 negative samples plus 3 standard deviations. The data are presented as mean $\pm$ SD of three replicates (A). Confirmation of the IgE-reactivity by immunoblot analysis. Both nPer a 13 and rPer a 13 were incubated with the same sera samples from 19 cockroach-allergic patients and 1 healthy subject. M, standard marker; Numbers referred to that in ELISA (B).



Sub-committee.

Initially, Per a 13 was identified from the extracts of the American cockroach by proteomics study. The cDNA encoding Per a 13 was isolated from the cDNA library by PCR strategy and was 1255 bp in length with an open reading frame of 999 bp. It was deposited in GenBank under the accession number MG775273. Through BLAST search, two nucleotide sequences named “putative Per a allergen” (GenBank: KY661337) and “glyceraldehyde-3-phosphate dehydrogenase” (GenBank: JN411914) in American cockroach were found with high sequence identity to Per a 13 in the NCBI nr database. Unfortunately, both of the two genes were not full-length cDNA and not investigated further. Our work was the first study that not only gave the full-length Per a 13 cDNA but also comprehensively characterized this novel allergen. Based on the immunoaffinity method, nPer a 13 was firstly purified from the crude extracts of the American cockroach. Considering the possibility of degradation and/or cross-linking with other proteins during affinity chromatography, we adapted a traditional method combining ammonium sulfate precipitation, size-exclusion

chromatography, ion-exchange chromatography and high-performance liquid chromatography to purify the nPer a 13. The obtained nPer a 13 was analyzed by SDS-PAGE and migrated as a single band at the molecular weight of $\sim$ 36 kDa, which was consistent with the theoretical molecular weight based on the amino acid sequence of Per a 13. *De novo* peptide sequencing and N-terminal characterization confirmed the internal peptides of purified nPer a 13, including the presence of full N- and C-terminal segments. These results confirmed that the purified nPer a 13 remained intact without any degradation. The possible amino acid difference between nPer a 13 and rPer a 13 was a His-tag at the C-terminal of rPer a 13, which made rPer a 13 had a slightly higher molecular weight than nPer a 13 in SDS-PAGE analysis. The difference of His-tag did not appear to affect the overall antigenicity because both the nPer a 13 and rPer a 13 could be recognized by anti-rPer a 13 pAb in immunoblot and immunoaffinity chromatography. The analysis of potential glycosylation in natural allergen is necessary because carbohydrates present on allergens have been considered to be highly cross-reactive determinants which are known to bind IgE (Malandain et al.,

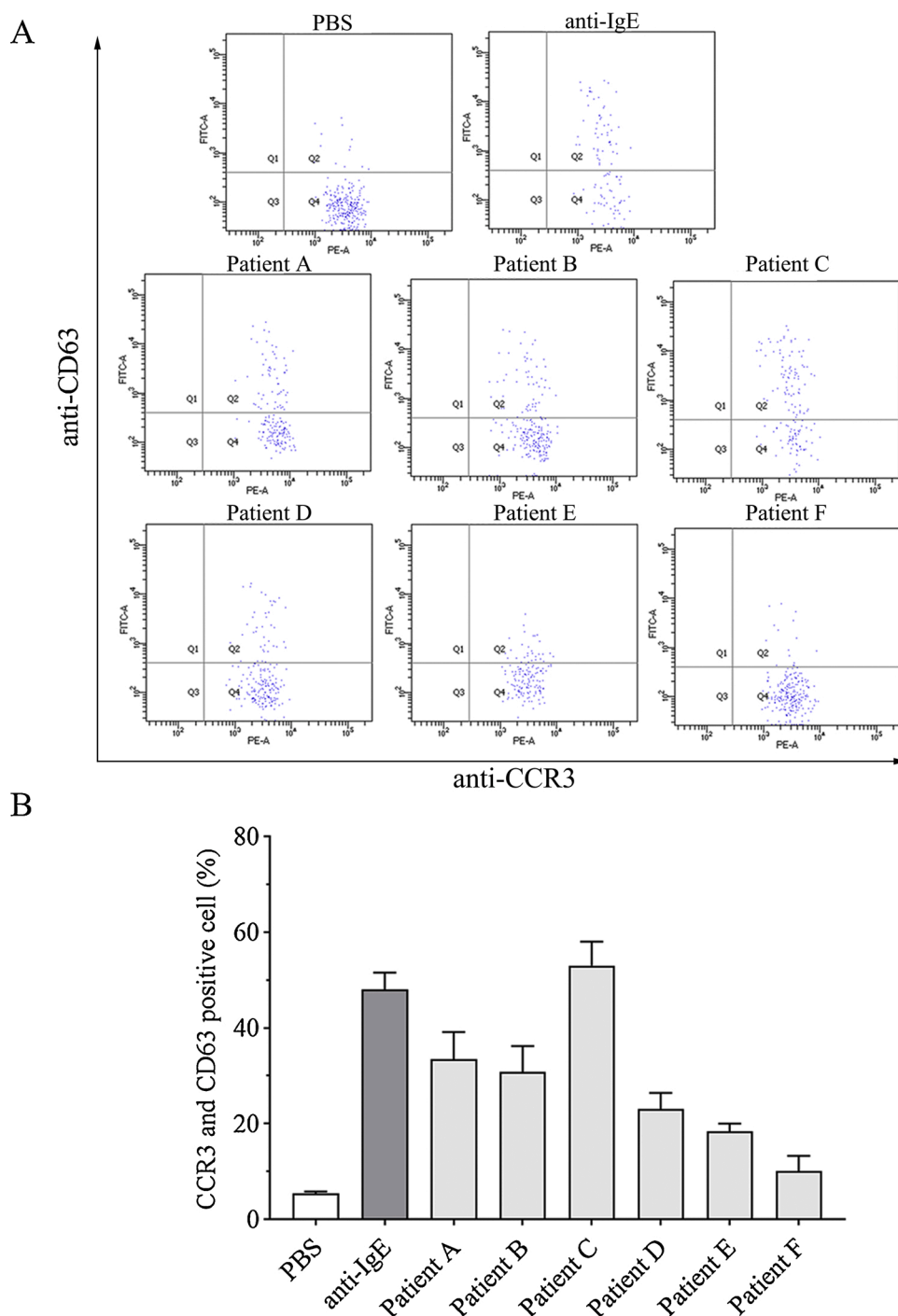


Fig. 4. Induction of basophil activation by Per a 13. Representative flow cytometry plots of basophils sensitized with sera from different cockroach-allergic patients and stimulated with PBS, anti-IgE and Per a 13, activation was assessed by monitoring the proportion of CCR3 and CD63 positive cells. PBS and anti-IgE were used as negative and positive controls, respectively. CCR3, C-C chemokine receptor 3 (A). The calculated proportion (%) of CCR3 and CD63 positive cells after stimulated with PBS, anti-IgE, and Per a 13. Data are presented as mean $\pm$ SD of three replicates (B).

2007), this may cause misleading results of IgE test (Gattinger et al., 2021). In our study, the nPer a 13 was not glycosylated as determined by the Pro-Q® Emerald 300 glycoprotein gel and blot stain kit, which suggested the IgE-binding activity of nPer a 13 was independent of carbohydrate moieties since nPer a 13 did not contain carbohydrate moieties.

While in 2018, the allergenicity of Per a 13 was assessed by using sera from 30 cockroach allergic patients and submitted to WHO/IUIS for nomenclature (data not shown). The committee of WHO/IUIS advised it would be more convincing to investigate the allergenicity of rPer a 13 since the nPer a 13 may bind with other associated proteins that would have contribution to IgE-binding activity. In order to confirm the allergenicity of Per a 13, a total of 140 sera samples from cockroach

allergic patients were collected in the past three years and 69 (49.3 %) had positive IgE reactions to rPer a 13 in ELISA, and the capacity of rPer a 13 to activate blood basophils was confirmed by basophil activation test. In addition, IgE immunoblot analysis showed the similar pattern of IgE-binding to rPer a 13 and nPer a 13 among 19 patients' sera with high IgE reactivity to rPer a 13 measured by ELISA, which indicated the natural and recombinant Per a 13 had comparable IgE-binding activity. It should be noted that subtle differences may still exist and may affect the allergenicity between nPer a 13 and rPer a 13, such as complete refolding of recombinant protein, potential modification on natural protein. However, this does not seem to be important for the overall allergenicity according to our experimental results. The allergenicity of Per a 13 (49.3 %) was comparable to those of Per a 3 (47.7 %–82.6 %)

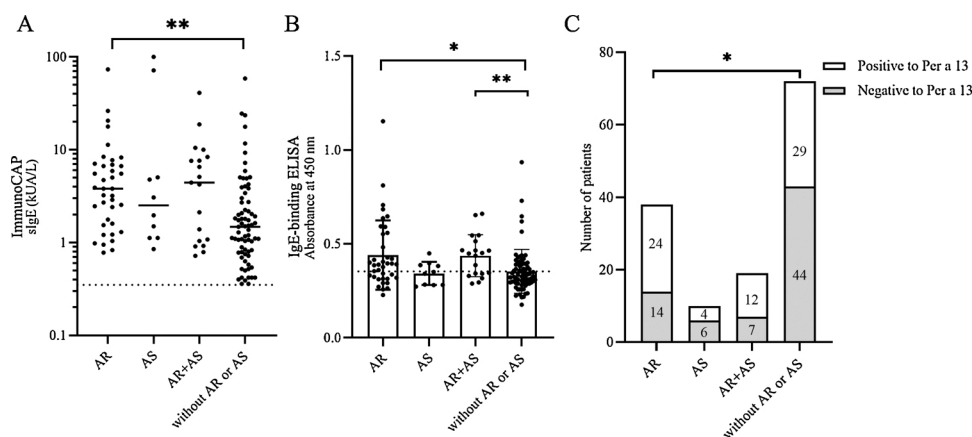


Fig. 5. Clinical symptoms in patients sensitized to Per a 13. Cockroach-specific IgE (sIgE) measured by ImmunoCAP in patients with allergic rhinitis (AR), allergic asthma (AS), in combination (AR + AS) and without AR/AS. Data are presented as sIgE content determined by ImmunoCAP. The dotted line indicates 0.35 kU/L cutoff level (A). The absorbance value of IgE-reactivity with Per a 13 in AR, AS, AR + AS and without AR/AS group. Data are presented as the mean value of three replicates. The dotted line indicates the cutoff value calculated in IgE-ELISA (B). The number of patients' sera that had positive and negative IgE-binding reactions to Per a 13 measured by IgE-ELISA in different patient groups. Numbers were showed in the corresponding column (C). Differences of cockroach specific-IgE value measured by ImmunoCAP and Per a 13 specific IgE absorbance value in IgE-ELISA between groups were

calculated by the Kruskal-Wallis test. Frequencies of IgE-binding between groups were analyzed by chi-square test. * $P < 0.05$, ** $P < 0.01$.

(Wu et al., 1996) and Per a 5 (25–100 %) (Sookrung et al., 2018; Wei et al., 2014), and was higher than Per a 4 (14.5 %), Per a 8 (19.7 %), Per a 7 (41.3 %) (Asturias et al., 1999), but was lower than Per a 1 (54.4 %–100 %) (Diraphat et al., 2003; Schou et al., 1990; Wang et al., 1999), Per a 2 (63.4 %) (Lee et al., 2012), Per a 9 (53.7 %–100 %) (Lee et al., 2012; Sookrung et al., 2006), Per a 10 (82.2 %) (Sudha et al., 2008), Per a 11 (83.0 %) and Per a 12 (63.8 %) (Fang et al., 2015). Notably, studies on allergenicity have shown manifold differences in IgE-reactivity of the same allergen, which should be due to geographic variations, distinct allergen isoforms involved in the allergenic response, the existence of difference between recombinant allergen and their natural counterpart that used in those studies. All the above factors should be taking into account when comparing allergenicities between allergens.

Per a 13 belongs to the GAPDH protein family which is first recognized as inhalant allergen from animal source in our study. Besides, GAPDH has been reported to be food allergens in both plant and animal species, such as manioc (Santos et al., 2011), mango (Tsai et al., 2017), rambutan (Jirapongsananuruk et al., 2011), shrimp (Khanaruksombat et al., 2014), prawn (Srinroch et al., 2015), and fish (Ruethers et al., 2021). Moreover, it was identified as inhalant allergens in wheat flour (Sander et al., 2011), indoor mould (Benndorf et al., 2008), and also reported as a contact allergen in latex (D'Amato et al., 2010). However, only GAPDH in wheat flour (Tri a 34) and fish (Pan h 13) were further investigated and listed in WHO/IUIS. Multiple sequence alignment revealed that Per a 13 shared 69 % and 80 % sequence identity to Tri a 34 and Pan h 13, respectively. However, they were not related to each other by phylogenetic analysis. GAPDH is a key glycolytic enzyme and responsible for energy production. Nevertheless, whether the enzyme activity contributed the overall allergenicity of Per a 13 is unknown. According to the previous study, such enzymatic activity may play a subsidiary but not critically important role in the allergenicity of a given protein (Huby et al., 2000). Currently, the connection between biochemical function and allergenicity is best understood in the case of proteases, such as Der p 1 and Per a 10 (Wan et al., 1999) (Sudha et al., 2009). In addition, many families of allergens that have LPS binding (such as Fel d 1) or lipid binding (such as Der p 2) could also contribute to their allergenicity (Herre et al., 2013; Trompette et al., 2009). However, the link between other biochemical functions and allergenicity is less clear (Radauer et al., 2008), which is worthy of further investigation.

It is worth noting that Per a 13 also shared 79 % sequence identity to human GAPDH (GenBank: P04406), which raised the question whether IgE cross-reactivity existed between them. Nevertheless, a previous study expected that similarity in short linear amino acid sequence was unlikely to result in cross-reactivity between two proteins unless they

shared similarities in the protein 3-D folding (Aalberse et al., 2001). Accordingly, we built the 3-D model of Per a 13 by SWISS-MODEL, the residue solvent accessibility and 3-D folding pattern were analyzed (Figure S4). A total of 28 and 26 residues with high solvent accessible score were obtained from Per a 13 and human GAPDH, respectively. Amino acid alignment showed that 13 solvent accessible residues were shared between both molecules (13 residues were highlighted with bold font in Table S2). However, the 13 identical solvent accessible residues belonging to different surface patches, and the alignment showed 20 gaps existed among the 28 residues in Per a 13 and the 26 residues in human GAPDH (Table S2), which could contribute to the difference of surface patch and protein 3-D folding (Figure S4). These properties made it quite difficult to calculate the exact identity of the solvent accessible residues to analyze the possible cross-reactivity. Moreover, the phylogenetic analyses also demonstrated human GAPDH exhibited a distant relationship with Per a 13 (Figure S5). All these features gave a hint that it may have a limited probability of cross-reactivity between these molecules, but this demands eventually experimental confirmation.

In conclusion, we identified and characterized Per a 13 as a novel allergen belonging to the GAPDH protein family in the American cockroach. To our knowledge, this is the first study that identified GAPDH as an inhalant allergen derived from animal species. Both of the nPer a 13 and rPer a 13 would be useful in developing novel diagnostic and therapeutic strategies for cockroach allergic patients. Identification of the IgE-binding epitopes and elucidation of the difference in immuno-reactivity between Per a 13 and human GAPDH remain our goals in the future.

Data availability

Data used during this study are included in this published article (and its supplementary information files).

Author contributions

JFW, JLS and MT designed and supervised the study. ZQX, LXZ and CL performed the experiments and analyzed the data. LXZ, YXJ, DXZ, MG undertook samples preparation, data collection and were involved in data analysis. YSY, MDC and LSZ were involved in the clinical study of patients. ZQX prepared this manuscript and JFW revised it. All of the authors approved the final version of this article.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2022.01.004>.

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