Molecular cloning, expression, IgE binding activities and *in silico* epitope prediction of Per a 9 allergens of the American cockroach

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Abstract. Per a 9 is a major allergen of the American cockroach (CR), which has been recognized as an important cause of imunoglobulin E-mediated type I hypersensitivity worldwide. However, it is not neasy to obtain a substantial quantity of this allergen for use in functional studies. In the present study, the Per a 9 gene was cloned and expressed in *Escherichia coli* (*E. coli*) systems. It was found that 13/16 (81.3%) of the sera from patients with allergies caused by the American CR reacted to Per a 9, as assessed by enzyme-linked immunosorbent assay, confirming that Per a 9 is a major allergen of CR. The induction of the expression of CD63 and CCR3 in passively sensitized basophils (from sera of patients with allergies caused by the American CR) by approximately 4.2-fold indicated that recombinant Per a 9 was functionally active. Three immunoinformatics tools, including the DNASTAR Protean system, Bioinformatics Predicted Antigenic Peptides (BPAP) system and the BepiPred 1.0 server were used to predict the potential B cell epitopes, while Net-MHCIIpan-2.0 and NetMHCII-2.2 were used to predict the T cell epitopes of Per a 9. As a result, we predicted 11 peptides (23-28, 39-46, 58-64, 91-118, 131-136, 145-154, 159-165, 176-183, 290-299, 309-320 and 338-344) as potential B cell linear epitopes. In T cell prediction, the Per a 9 allergen was predicted to have 5 potential T cell epitope sequences, 119-127, 194-202, 210-218, 239-250 and 279-290. The findings of our study may prove to be useful in the development of peptide-based vaccines to combat CR-induced allergies.

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Introduction

Cockroach (CR) allergies have been recognized as an important cuase of imunoglobulin E (IgE)-mediated type I hypersensitivity since 1964 (1). In a previous study, it was found that 44% of 755 allergic patients who were treated at the allergy clinics in New York had positive reactions to CR extract in the skin prick test (SPT), and 13% of those subjects were allergic to CR alone (1). German CR (*Blattella germanica*; Bla g), American CR [*Periplaneta americana (P. americana)*; Per a] and smoky brown CR (*Periplaneta fuliginosa*) are the dominant indoor CR species which cause allergies among populations worldwide (2). In China, a total of 25.7% of patients with allergies are found to be positive for American CR allergens and 18.7% are found to be positive for German CR allergens by the SPT (3).

There are 22 IgE binding components in *P. americana*, including the proteins of 23, 28, 35, 38, 40, 49, 72, 78 and 97 kDa as major allergens (4), but only a few of these allergens namely, Per a 1 (5), Per a 2 (previousy known as Cr PI) (6), Per a 3 (7), Per a 4 (8), Per a 5 (9), Per a 6 (10), Per a 7 (11), Per a 9 (previousy known as *Periplaneta americana* arginine kinase) (12) and Per a 10 (13) have been characterized. Per a 9 is an arginine kinase, purified from American CR extract by monoclonal antibody based-affinity chromatography reacted with IgE in sera of all CR allergic Thai patients (12). Attempts to isolate Per a 9 from the American CR extract have been extremely laborious and have resulted in low yields. To day, only 3 groups of researchers have submitted these allergen genes (AY563004.1, GU301882.1 and EU429466) in GenBank. The availability of the sequence makes it possible to produce recombinant Per a 9 in large amounts to study both its physiologic role and its implications in allergic reactions.

CR immunotherapy is uncommonly used and reports on its effectiveness are very limited (14,15). The elucidation of B and T cell epitopes of allergens broaden our understanding of the structure-function relationship and predict the basis of cross-reactivity. The cross-reactive epitopes may be useful in reducing the number of allergens without compromising the

efficacy of therapy (16). B cell epitopes can be applied in the diagnosis, therapy and development of effective vaccines for immunotherapy. They can be identified by a number of methods, particularly computational tools, which provide a promising and rapid alternative. The predicted B cell epitopes may be modified to reduce the allergenicity of an allergen (17). T cell epitopes have been successfully identified based on computer simulation over the past decade. Extracellular peptides have to bind to major histocompatibility complex (MHC) class II to stimulate T lymphocyte responses. Thus, T cell epitopes have been predicted indirectly by the identification of MHC-binding molecules (18). In the present study, we firstly cloned and expressed the American CR major allergen, Per a 9, and subseqently identified the B and T cell epitopes of the Per a 9 allergen using an *in silico* approach. Our findings provide evidence of their potential use in the development of peptide-based vaccines for combating CR allergies.

Materials and methods

Ethics statement. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. Written informed consent for the use of blood samples was obtained from all participants prior to study entry according to the declaration of Helsinki.

Patients and samples. A total of 16 patients with allergic rhinitis with positive SPT results (allergens were supplied by ALK-Abelló, Inc., Hørsholm, Denmark) and with positive serum IgE test results to American CR extract [by using ImmunoCAP assay (Pharmacia Diagnostics AB, Uppsala, Sweden)], and 6 healthy controls (HC) were recruited in this study. Serum (4 ml) from peripheral venous blood was collected from each patient and the healthy controls for western blot analysis.

Cloning of cDNA encoding the full length of Per a 9 gene. Total RNA was isolated from adult female CRs reared at our institute using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified by measuring the absorbance ratios at 260/280 nm. cDNA was prepared by reverse transcriptase using a commercial RNA-PCR kit according to the manufacturer's instructions (Takara Biotech Co., Ltd., Dalian, China). For each reaction, 1 μg of total RNA was reverse transcribed using oligo-d(T). cDNA encoding Per a 9 was amplified by PCR using primers based on the no-coding sequence of the Per a 9 gene (AY563004.1; forward, 5'-TACA GCAAGTGGGACAGCAG-3' and reverse, 5'-ATATGGGCAT CAAAGATATA-3'). The PCR conditions were 95˚C/5 min (1 cycle), 95˚C/1 min, 55˚C/1 min and 72˚C/1 min (30 cycles), and 72˚C/5 min (1 cycle). The purified PCR product was cloned into the pMD18-T vector (Takara Biotech Co., Ltd.), before being transformed into *Escherichia coli* (*E. coli*) strain DH5α. The inserts were sequenced on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequence data were translated to amino acid sequence using the Show Translation tool in the SMS software package (http://www.bioinformatics.org/SMS/).

Expression and purification of Per a 9 in E. coli. The Per a 9 gene was subcloned into the pET15b vector (Novagen, Madison, WI, USA) using the *Nde*I and *Bam*HI sites and verified by DNA sequencing. The recombinant pET15b-Per a 9 plasmid was transformed into the ArcticExpress™ (*DE3*) RP host strain. A colony of the selected transformed ArcticExpress™ (*DE3*) RP *E. coli* on an overnight LB-ampicillin agar plate was inoculated into 5 ml of LB-ampicillin broth, and incubated at 15, 25 or 37˚C, respectively overnight. One milliliter of the culture was inoculated into 50 ml of fresh LB-ampicillin broth and incubated at 37˚C with shaking at 250 rpm until the optical density (OD) at A_{600nm} reached 0.6. Subsequently, IPTG was added to a final concentration of 1 mM and the culture was incubated for a further 4 h. The bacterial cells were harvested by centrifugation at 4,000 x g at 4˚C for 20 min, and were lysed in lysis buffer by sonication at 20 kHz, 2 min pulse-on, 3 min pulse-off. Cell debris was removed by centrifugation at 12,000 x g at 4˚C for 20 min. The supernatant was loaded on a Nickel column (Genscript, Nanjing, China), washed with running buffer containing 50 mM Tris-HCl, 300 mM NaCl and 5% glycerol (pH 8.0), and eluted with elution buffer containing 50 mM Tris-HCl, 300 mM NaCl, 50 and 250 mM imidazole and 5% glycerol (pH 8.0). The eluted fractions washed with 250 mM imidazole were obtained and identified as Per a 9.

Immunoreactivity of human sera with recombinant Per a 9. A 96-well plate was coated with purified recombinant Per a 9 at 10 μg/ml in carbonate-bicarbonate buffer (0.05 M, pH 9.6) overnight at 4˚C, 100 μl/well. Human serum samples [1:20 dilution in phosphate-buffered saline (PBS)-Tween-20 with 2% BSA] were then added to the plates for 2 h at room temperature. Following IgE binding, the plates were incubated with horseradish peroxidase-labeled goat anti-human IgE (1:2,500 dilution) (KPL, Inc., Gaithersburg, MD, USA), and the color was developed with tetramethylbenzidine peroxidase substrate. The plates were read on a microplate reader (Eon; BioTek, Winooski, Vermont, USA) at an absorbance of 405 nm. The cut-off of the enzyme-linked immunosorbent assay (ELISA) was calculated as the mean of the negative controls plus 2 standard deviations (SDs).

Western blot analysis of IgE reactivity. Immunoblots for the detection of serum specific IgE were performed using recombinant Per a 9 as previously described (19,20). Recombinant Per a 9 (5 μg) was added to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (gel concentration of 15%) under reducing conditions and then transferred onto nitrocellulose membranes. The nitrocellulose membranes were incubated with the sera of the patients with American CR allergies (1:5 to 1:20 in PBS-Tween-20 with 1% BSA, 10% normal goat serum) for 90 min. Following rinsing with PBS, the membranes were incubated with peroxidase-labeled anti-human IgE monoclonal antibody. The positive protein bands were visualized by incubating the membranes with tetramethylbenzidine peroxidase substrate. Sera from 2 non-atopic subjects were used as negative controls.

Basophil activation test. The expression of CD63 and CCR3 on the basophil surface is considered as an indicator of basophil activation (21,22). Briefly, peripheral blood mononucleated cells (PBMCs) from 20 ml blood donated by 4 healthy volunteers were separated by Ficoll-Paque density gradient, and treated with 10 ml LS (a solution containing 1.3 M NaCl,

0.005 M KCl and 0.01 lactic acid, pH 3.9) for 2 min at 8˚C. Following neutralization with 12% Tris (pH 10.9), non-specific IgE on the basophils was stripped off and the cells were passively sensitized with the sera of patients with American CR allergies or the healthy controls (n=4, 1 in 10 dilution, 2 h at 37˚C) (same patients and controls as mentioned above) as previously described (22). The cells were then challenged with Per a 9 (1.0 μg/ml) for 15 min at 37˚C. A goat anti-human IgE antibody (Serotec, Kidlington, UK) was used as a positive control. CCR3-PE-labeled antibody (85-12-1939-42; eBioscience Inc., San Diego, CA, USA) and anti-human CD63-FITC antibody (HH-MHCD63014; Invitrogen) were added to the cells for 15 min at 37˚C. Flow cytometric analysis of surface markers was performed at 488 nm on a FACSAria flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed by FACSDiva software.

Sequence retrieval and phylogenetic analysis. The complete amino acid sequence of the cloned Per a 9 gene was used as query to search for homologous sequences through the Swiss-Prot/TrEMBL (Uniprot; http://www.uniprot.org/) and tBLASTn in NCBI (blast.ncbi.nlm.nih.gov/Blast.cgi) (10,23-25). The phylogenetic tree was obtained by using the maximum-likelihood (ML) method on the basis of the JTT amino acid sequence distance implemented in MEGA 5.1, and the reliability was evaluated by the bootstrap method with 1,000 replications (24-32).

Physiochemical analysis and post-translational patterns and motifs. Physiochemical analysis, including molecular weight, theoretical pI, amino acid composition, instability index, aliphatic index and the grand average of hydropathicity (GRAVY) of Per a 9 was performed using the ProtParam tool (http://web.expasy.org/protparam/), as previously described (33). The Per a 9 characteristic pattern was examined for the original sequence and further analysis was performed to highlight the presence of functional motifs using the Prosite database (http://prosite.expasy.org/), as previously described (34).

Secondary structure prediction. Per a 9 secondary structural elements recognition was assessed by PSIPRED (bioinf.cs.ucl.ac.uk/psipred), which threads sequence segments through Protein Data Bank (PDB) library (http://www.pdb.org/) to identify conserved substructures (35). Furthermore, the secondary structure elements were also identified and compared with the results obtained with NetSurfP ver. 1.1 (www.cbs.dtu.dk) (36).

Homology modeling and validation. The Per a 9 protein sequence was searched for homology in the PDB (http://www.rcsb.org/). In addition, the homologous templates suitable for Per a 9 were selected using the PSI-BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the NCBI and Swiss-model server (http://swissmodel.expasy.org/). The best templates were retrieved from the results of PSI-BLAST and used for homology modeling. The Der f 25 modeled protein structure was built through Alignment Mode in SWISS-MODEL (http://swissmodel.expasy.org/) using the complete amino acid sequence. An initial structural model was generated and checked for recognition of errors in 3D structure by PROCHECK (37), ERRAT (verification of protein structures: patterns of nonbonded atomic interactions) (33,38) and VERIFY_3D (a method to identify protein sequences that fold into a known three-dimensional structure. Assessment of protein models with three-dimensional profiles) programs in Structural Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/) (33,38).

In silico prediction of B cell epitopes. Three immunoinformatics tools, including the DNASTAR Protean system (39), Bioinformatics Predicted Antigenic Peptides (BPAP) system (http://imed.med.ucm.es/Tools/antigenic.pl) and the BepiPred 1.0 server (http://www.cbs.dtu.dk/services/BepiPred/) were used to predict the B cell epitopes of Per a 9, as previously described (40). The ultimate consensus epitope results were obtained by combining the results of the three tools together with a previously published method (41). If the results of all 3 methods were non-epitope, then the consensus result was 0% epitope. Similarly, if the predicted results had only one or no non-epitope, the consensus result was 67 or 100% epitope, respectively. Finally, the regions whose consensus epitope result was 67 or 100% were selected as the final potential epitope regions. In the DNAStar Protean system, 4 properties (hydrophilicity, flexibility, accessibility and antigenicity) of the amino acid sequence were selected as parameters for epitope prediction. The peptide regions with good hydrophilicity, high flexibility, surface accessibility and high antigenic index were selected as candidate epitopes for further investigation. The BPAP system and BepiPred 1.0 server only need the amino acid sequence and provide more straightforward results, which combined with the physicochemical properties of amino acids such as hydrophilicity, flexibility, accessibility, turns and exposed surface (42).

In silico prediction of T cell epitopes. T cell epitopes are principally predicted indirectly by identifying the binding of peptide fragments to the MHC complexes. However, the binding grooves of MCH-II molecules are open at both ends allowing various lengths of peptides to bind. On the other hand, the same MHC molecule can accommodate a variety of binding sequences. These two properties make the development of accurate predictive algorithms for MHC-class II binding complex. For HLA-DR-based T cell epitope prediction, the artificial neural network-based alignment (NN-align) method NetMHCIIpan-3.0 (http://www.cbs.dtu.dk/services/NetMHCIIpan/) was applied (43). For HLA-DQ alleles, NetMHCII-2.2 (http://www.cbs.dtu.dk/services/NetMHCII/) was used (44). Although HLA-DQ provides limited binding-affinity data, it was recently reported to provide the optimal performance in predicting this locus (33). The binding significance of each peptide to the given MHC molecule is based on the estimated strength of binding exhibited by a predicted nested core peptide at a set threshold level. For HLA-DR-based T cell epitope prediction, HLA-DR 101, HLA-DR 301, HLA-DR 401 and HLA-DR 501 were used. The ultimate HLA-DR-based T cell epitope results were obtained by combining those 4 results together and if 3 of these were shown to be epitope, the consensus result was then considered epitope. This method was also used in HLA-DQ-based T cell epitope prediction. For HLA-DQ-based T cell epitope prediction, only HLA-DQA10501-DQB10201,

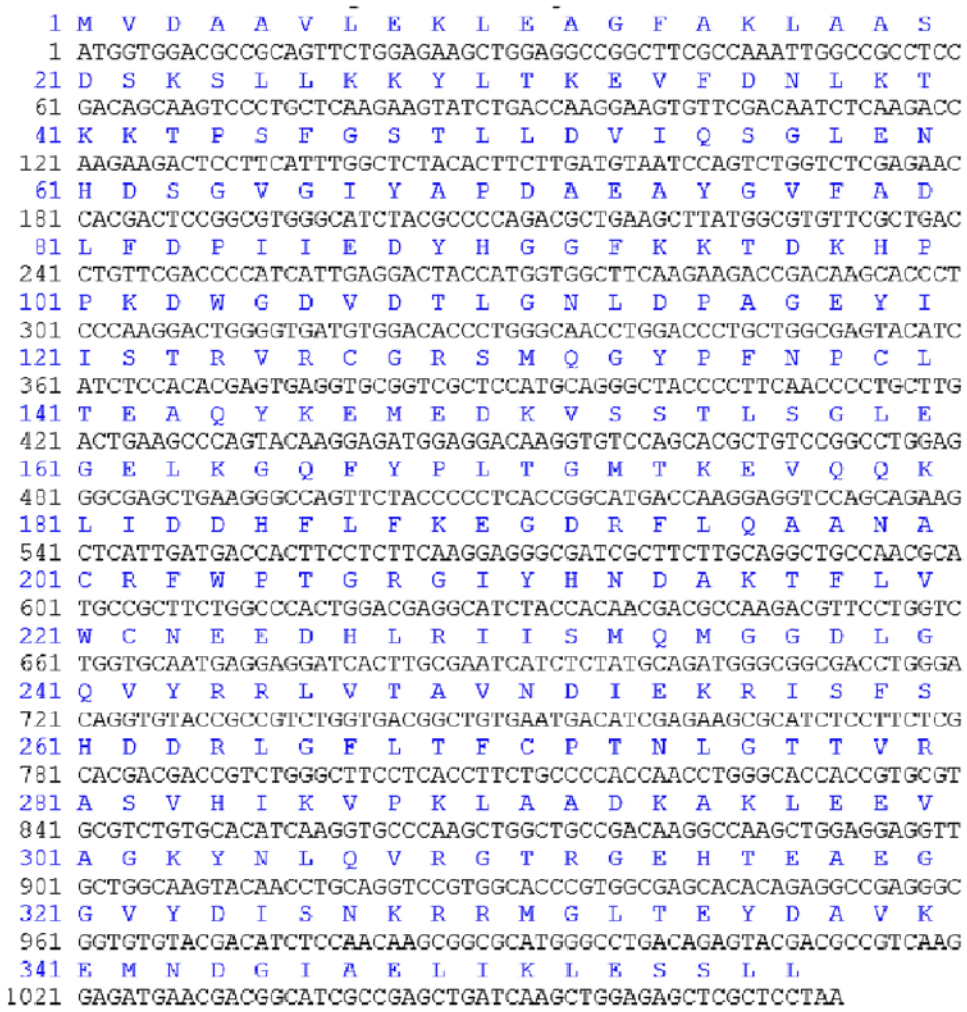

Figure 1. cDNA sequence encoding Per a 9 and the deduced amino acid sequence. The first 3 bases ATG represent the start code. The last 3 bases indicate the stop codon.

HLA-DQA10301-DQB10302, HLA-DQA10401-DQB10402 and HLA-DQA10102-DQB10602 were used. As a result, the ultimate consensus epitope results were obtained by combining the results of the HLA-DR-based T cell epitope and HLA-DQ-based T cell epitope. B cell and T cell epitopes identified by computational tools were mapped onto linear sequence and on the 3 dimensional model of Per a 9 in order to determine their position and secondary structure elements involved.

Statistical analysis. Data are expressed as the means ± SE for the indicated number of independently performed duplicated experiments. Statistical significance between means was analyzed by one-way ANOVA or the Student's t-test utilizing the SPSS 13.0 version. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cloning of cDNA encoding full length Per a 9 sequence. cDNA encoding Per a 9 was amplified by PCR using primers based on the no-coding sequence of Per a 9 gene. It is a 1,074 bp gene and encodes a 356 amino acid protein (Fig. 1). The sequence homology with the published one (Accession no. AY563004) was 99% (353/356) at the protein level.

Expression and purification of Per a 9 in E. coli. The Per a 9 gene was subcloned into the pET15b vector and transformed into the ArcticExpress™ (*DE3*) RP host strain. Per a 9 was expressed at 15, 25 or 37˚C following induction. Since Per a 9 was expressed in a soluble form in the supernatant at 15˚C (Fig. 2A), the condition of 1 mM IPTG and 15˚C was selected as the final condition throughout the study. The Per a 9 protein was purified by Ni column. More than 8.69 mg recombinant Per a 9 was obtained from 1 liter of cell culture. The purity of the purified Per a 9 was identified by SDS-PAGE. It showed a single band with an apparent molecular weight of 40 kDa (Fig. 2B).

Immunoreactivity to IgE. In order to determine the allergenicity of Per a 9, we examined the ability of Per a 9 to bind IgE in the sera of the patients with American CR allergies using a direct ELISA technique. The sera from all patients apart from patients 4, 6 and 12 exhibited a positive IgE reactivity to Per a 9. The results revealed that 13/16 (81.3%) sera from these patients reacted to Per a 9 (Fig. 3A). The IgE binding activity of Per a 9 in a representative group of 3 patients and 2 healthy

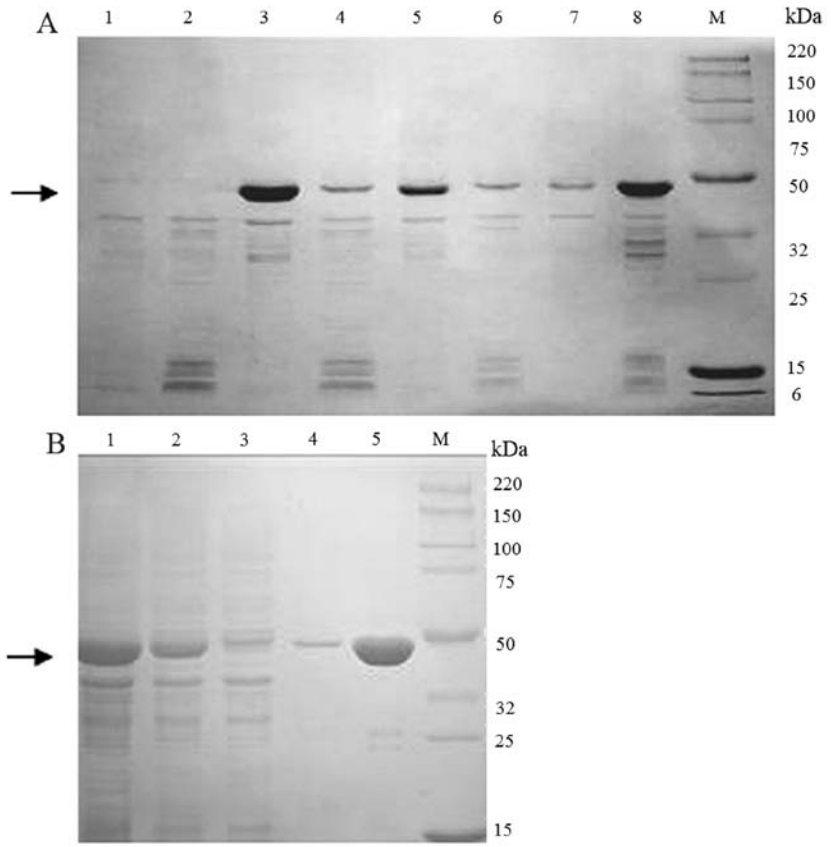

Figure 2. Expression and purification of Per a 9 in *E. coli*. (A) Per a 9 expressed at 15, 25 or 37˚C was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane M, Smart Broad-Range protein standard (Genscript, Nanjing, China); lane 1, the supernatant of un-induced cells; lane 2, the precipitant of un-induced cells; lane 3, the supernatant of the cells induced at 15˚C; lane 4, the precipitant of the cells induced at 15˚C; lane 5, the supernatant of the cells induced at 25˚C; lane 6, the precipitant of the cells induced at 25˚C; lane 7, the supernatant of the cells induced at 37˚C; lane 8, the precipitant of the cells induced at 37˚C. The arrow represents Per a 9 protein. (B) SDS-PAGE analysis of purified Per a 9 expressed in *E. coli*. Lane M, protein standard; lane 1, total protein after sonication; lane 2, the supernatant after centrifugation; lane 3, flow through; lane 4, washing with 50 mM imidazole; lane 5, washing with 250 mM imidazole. The arrow represents Per a 9 protein.

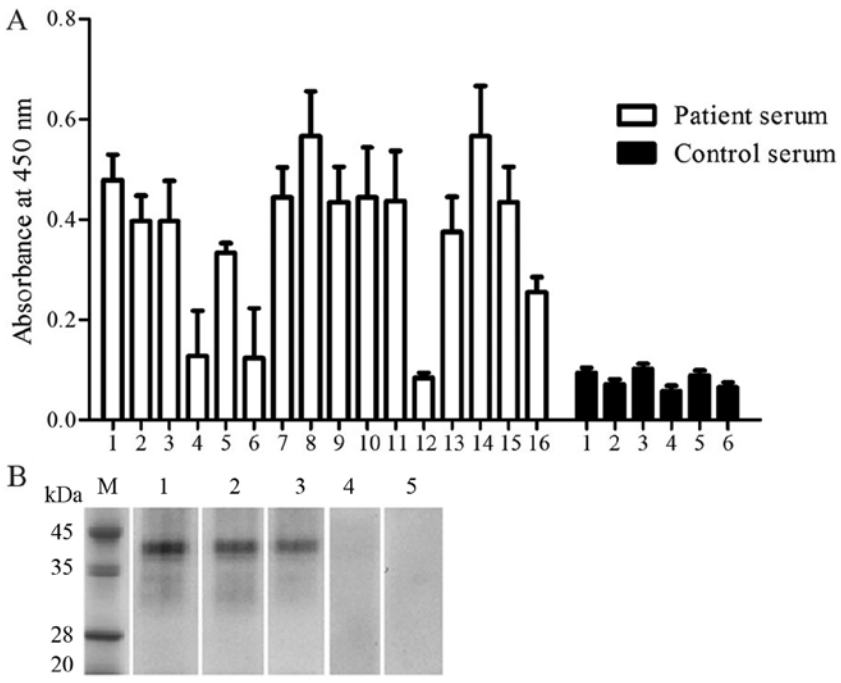

Figure 3. (A) Analysis of specific IgE reactivity of recombinant Per a 9 by direct enzyme-linked immunosorbent assay (ELISA). Sera were collected from patients with American cockroach (CR) allergies and healthy controls. The values are shown as the means ± SD from triplicate experiments. (B) Western blot analysis of IgE reactivity to Per a 9 sera from the patients with American CR allergy. Lanes 1-3, Per a 9 reacted with the sera from patients 8, 9, 10; lanes 4-5, Per a 9 reacted with sera from control subjects 1 and 2.

Table I. The predicated secondary structure of Per a 9.

Secondary structural prediction methods	α-helices	β-sheets
PSIPRED	3-12, 25-18, 32-39, 50-56, 73-89, 142-156, 174-183, 193-197, 240-255, 294-303, 35-354	66-68, 210-213, 218-223, 229-234, 260-261, 266-268, 279-286, 322-324
NetSurfP ver1.1	2-12, 25-28, 32-40, 50-56, 74-89, 142-157, 175-183, 193-197, 239-256, 294-303, 333-354	65-69, 120-128, 209-213, 217-223, 227-233, 267-269, 279-286, 307-310, 322-326
PredictProtein	4-13, 25-28, 32-39, 50-53, 78-89, 142-157, 175-183, 239-256, 294-303, 335-355	65-69, 167-170, 208-213, 217-222, 232-234, 267-270, 279-287, 307-311, 322-326
Overall results	3-12, 25-28, 32-39, 50-56, 74-89, 142-157, 175-183, 193-197, 239-256, 294-303, 335-354	65-69, 120-123, 210-213, 218-223, 228-234, 260-261, 280-286, 307-310, 322-326

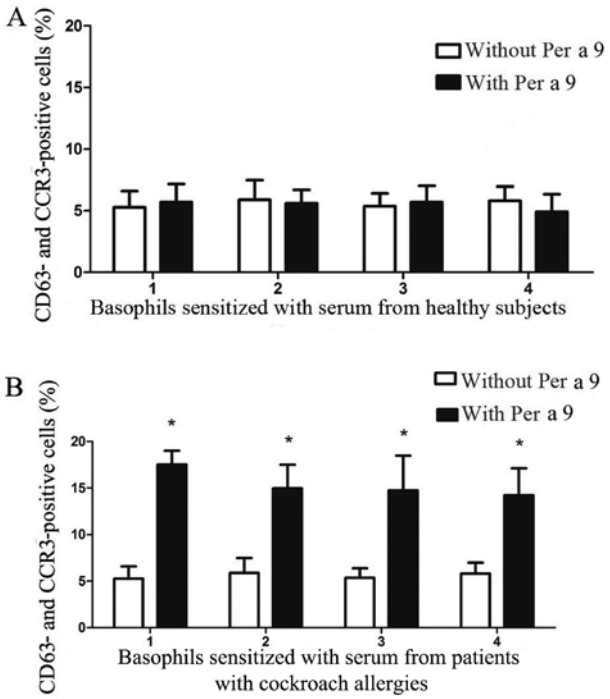

Figure 4. Induction of basophil activation by recombinant Per a 9. After non-specific IgEs on basophils being stripped off, cells from each donor were passively sensitized with sera from 4 different healthy subjects (A) or from 4 different patients with American cockroach (CR) allergies (B), and were then challenged with Per a 9 at 1.0 μg/ml. The values are shown as the means ± SE for the sera from 4 different subjects. *P<0.05 in comparison with the corresponding carrier alone control.

controls were assessed by western blot analysis and the results are illustrated in Fig. 3B.

Per a 9 induces basophil activation. Per a 9 at 1.0 μg/ml induced approximately up to a 4.2-fold increase in the number of CD63 and CCR3 double-positive cells when incubating with passively sensitized basophils from the sera of patients with American CR allergies. Per a 9 had no effect on the basophils sensitized by the sera from HC (Fig. 4).

Sequence retrieval and phylogenetic analysis. Uniprot and tBLASTn were used to search the homologous sequences of Per a 9. As a result, 47 sequences were obtained. In order to determine the relationships between Per a 9 and its homologous sequences, phylogenetic analysis was performed and the evolutionary tree inferred by the ML method is presented in Fig. 5.

Physiochemical analyses, post-translational patterns and motifs. The primary structure of Per a 9 contained 356 amino acids and the molecular weight was 39,735. The theoretical pI was 5.58 and the aliphatic index was 80.56. The GRAVY was -0.413, which incidated that Per a 9 exhibited a hydrophilic character. The instability index was 31.36 (<40), indicating that the Per a 9 protein was stable. Per a 9 is an arginine kinase and the results from PROSITE showed that Per a 9 had two obvious characteristic patterns of phosphagen kinase, including PS51509 (phosphagen kinase N-terminal domain profile) and PS51510 (phosphagen kinase C-terminal domain profile). Moreover, PS00112 was contained in PS51510 and consisted of CPTNLGT, which was the active center of phosphagen kinase. The ATP-binding sites were His (185) and Arg (229).

Structural analysis of Per a 9. Secondary structure prediction with PSIPRED identified 11 α-helices and 8 β-sheets in Per a 9. PredictProtein predicted 10 α-helices and 9 β-sheets. Alternatively, NetSurfP v1.1 predicted 11 α-helices and 9 β-sheets. The predicated secondary structure of Per a 9 is presented in Table I.

Homology modeling and validation. The search for the proteins of Per a 9 with known tertiary structure in the PDB yielded *Litopenaeus vannamei* arginine kinase (PDB Accession no. 4BG4) showing the highest sequence identity (83% with Per a 9). As a result, the 4BG4 template was used for homology modeling. The homology model that matched the aforementioned structures is shown in Fig. 6A. As indicated by the Ramachandran plot (Fig. 6B), 89.4% of the residues in the model structure were within the most favored regions, 10% of the residues were in the additional allowed region, 0.6% of the residues were in the generously allowed regions and 0% of the residues were in the disallowed region. As indicated by the ERRAT program, the results (Fig. 6C) revealed that the overall quality factor was 100, which indicated that the structure had a high resolution. As indicated by the VERIFY_3D program, the results revealed that 99.72% of the residues had an average 3D (atomic model)-1D (amino acid sequence) score of >0.2, which also indicated that

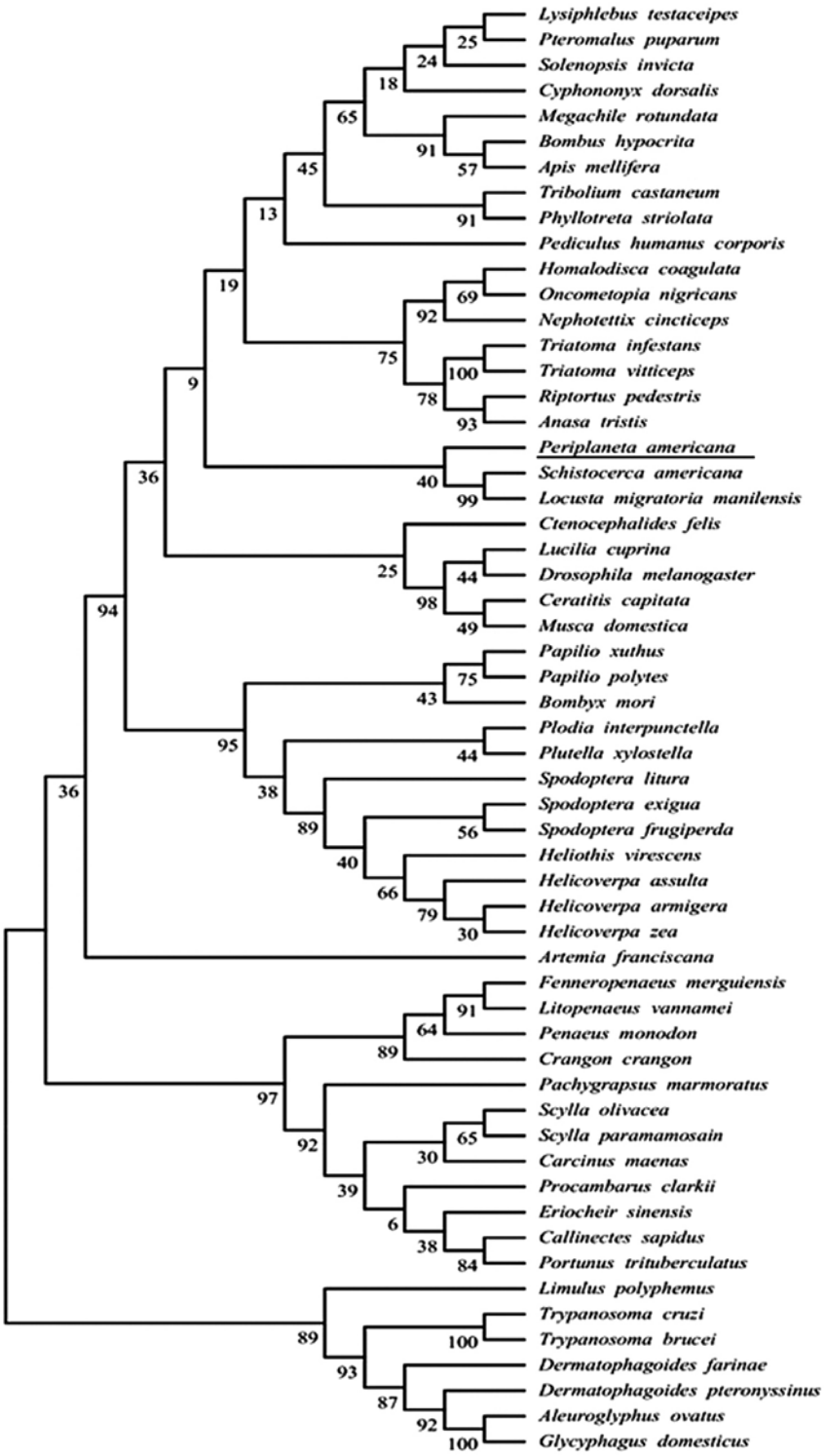

Figure 5. Phylogenetic relationship of Per a 9 allergen amino acid sequence with other homologs.

the structure was good. Based on these validations, it is shown that the homology model was adopted for this study.

B cell epitope prediction. Surface accessibility and fragment flexibility are important features for predicting antigenic epitopes. In addition, the existence of regions with high hydrophobicity also provides strong evidence of epitope identification. The antigenic index directly revealed the epitope forming capacity of the Per a 9 sequence. Based on these sequence properties, the final predicting regions of Per a 9 by DNASTAR were obtained as: 23-31, 38-46, 57-64, 70-72, 91-117, 143-154, 159-165, 174-183, 189-19, 222-227, 292-302, 309-32 and 337-345. The predicted results of the BPAP system were 19-20, 39-46, 58-74, 90-118, 131-136, 145-154, 157-165, 175-176, 275-278, 290-292, 295-299, 309-325 and 338-344. The predicted results of the BepiPred 1.0 server were 11-19, 21-28, 46-55, 115-127, 134-142, 152-158, 176-187, 193-203, 216-222, 238-251, 264-273, 277-293 and 296-308. Furthermore, the final

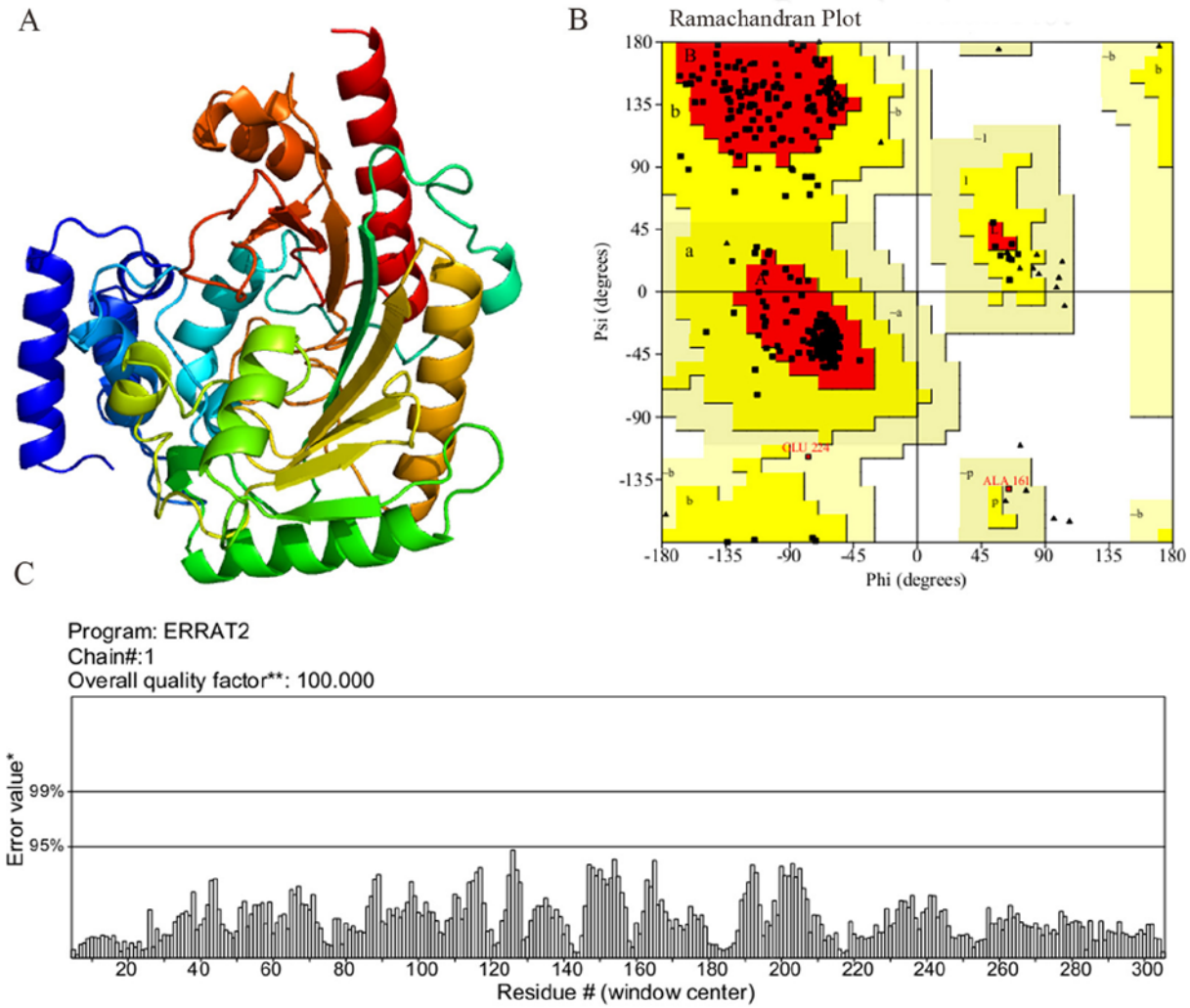

Figure 6. Three-dimensional structure and validation of Per a 9 homology model. (A) Protein structure of Per a 9 homology model. (B) Ramachandran plot of the generated homology model. The residures in most favored regions, additional allowed regions, generously allowed regions and disallowed regions are shown in red, yellow, grey and white in the Ramachandran plot, respectively. (C) Validation of the generated homology model by ERRAT program.

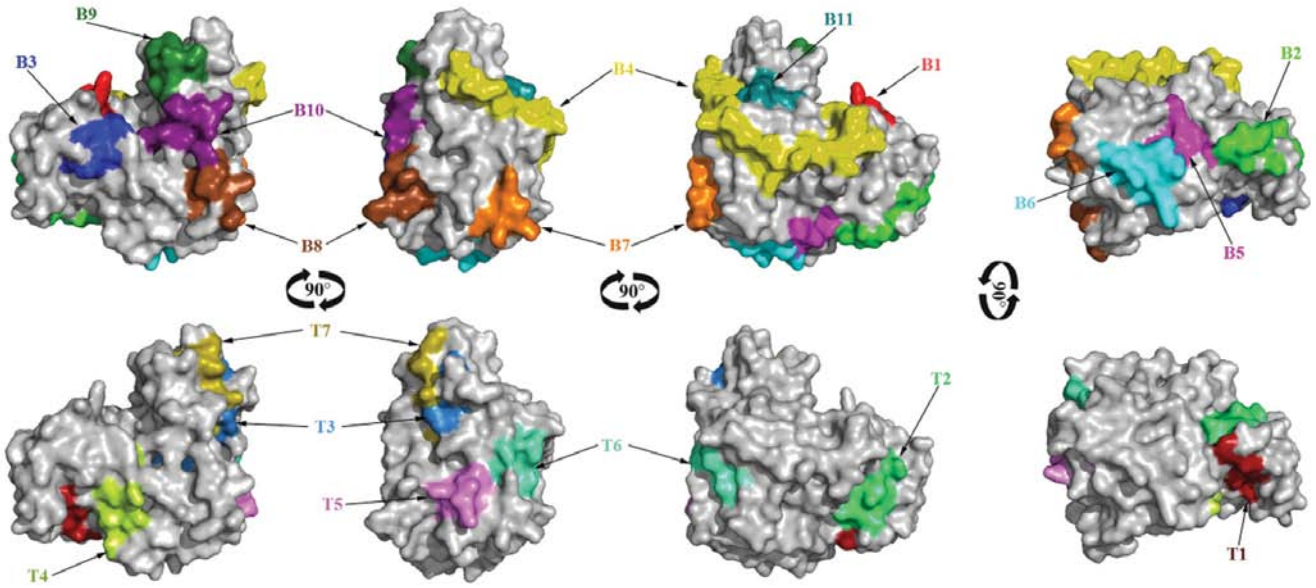

Figure 7. B cell and T cell epitopes superimposition on the surface of Per a 9 allergen structure. B1-B7 are the predicted B cell epitopes; T1-T7 are the predicted T cell epitopes.

Table II. The T cell epitope prediction of Per a 9.

HLA types	Location of the prediction results
HLA-DR 101	7-15, 14-22, 17-25, 23-31, 26-34, 29-37, 35-43, 40-48, 45-53, 46-54, 48-56, 50-58, 67-75, 78-86, 100-108, 117-125, 119-127, 123-131, 128-136, 136-144, 153-161, 156-164, 166-174, 167-175, 168-176, 186-194, 192-200, 194-202, 195-203, 201-209, 210-218, 227-235, 231-239, 238-246, 242-250, 267-275, 268-276, 273-281, 275-283, 277-285, 278-286, 279-287, 282-290, 287-295, 289-297, 290-298, 303-311, 304-312, 305-313, 308-316, 342-350, 348-356,
HLA-DR 301	68-76, 77-85, 180-188, 211-219, 210-218, 259-267
HLA-DR401	26-34, 29-37, 48-56, 49-57, 119-127, 120-128, 170-178, 194-202, 227-235, 228-236, 231-239, 239-247, 242-250, 245-253, 279-287, 282-290, 283-291, 346-354
HLA-DR501	8-16, 14-22, 19-27, 22-30, 24-32, 35-43, 118-126, 119-127, 167-175, 185-193, 186-194, 194-202, 195-203, 200-208, 210-218, 239-247, 243-251, 267-275, 278-286, 282-290, 283-291, 284-292, 286-294, 288-296, 289-297, 304-312, 322-330,
HLA-DQA10501-DQB10201	46-54, 67-75, 65-73, 74-82, 77-85, 186-194, 218-226, 220-228
HLA-DQA10301-DQB10302	65-73, 71-79, 72-80, 73-81, 79-87, 154-162, 214-222
HLA-DQA10501-DQB10301	2-10, 7-15, 10-18, 12-20, 13-21, 16-24, 44-52, 46-54, 61-69, 66-74, 71-79, 72-80, 89-97, 115-123, 125-133, 169-177, 194-202, 195-203, 212-220, 234-242, 237-245, 243-251, 274-282, 279-287, 283-291, 292-300, 308-316, 218-226, 242-250
HLA-DQA10102-DQB10602	12-20, 45-53, 48-56, 62-70, 72-80, 120-128, 154-162, 189-197, 192-200, 195-203, 214-222, 225-233, 228-236, 274-282, 347-355
The final predicted T cell epitopes	46-54, 65-80, 119-127, 194-202, 210-218, 239-250, 279-290

potential B cell epitopes of Per a 9 were selected on the basis of the results of these 3 tools. The ultimate results of the 3 immunoinformatics tools finally predicted 11 peptides (23-28, 39-46, 58-64, 91-118, 131-136, 145-154, 159-165, 176-183, 290-299, 309-320 and 338-344) and these peptides are shown in Fig. 7.

T cell epitope prediction. For the HLA-DR-based T cell epitope prediction of Per a 9, the final predicting regions of HLA-DR 101, HLA-DR 301, HLA-DR 401 and HLA-DR 501 are shown in Table II and the ultimate results of HLA-DR-based T cell epitope prediction finally predicted 5 peptides (119-127, 194-202, 210-218, 239-250 and 279-290). For HLA-DQ alleles, the final results of HLA-DQA10101-DQB10501, HLA-DQA10301-DQB10302, HLA-DQA10401-DQB10402, and HLA-DQA10102-DQB10602 are also shown in Table II and the ultimate results of these 4 methods finally predicted 2 peptides, 46-54 and 65-80. As a result, Per a 9 was predicted to have 7 T cell epitope sequences, 46-54, 65-80, 119-127, 194-202, 210-218, 239-250 and 279-290 as shown in Fig. 7.

Discussion

To better understand the Per a 9-mediated CR allergies and with an aim to improve the diagnosis and treatment of CR allergies, we prepared biologically active and highly pure American CR allergen Per a 9 in relatively large amount in the present study. Per a 9, an arginine kinase, has been reported to be purified from American CR extract by monoclonal antibody based-affinity chromatography (12), but the quantity obtained was limited. Since monoclonal antibody based-affinity chromatography is very costly and tedious, it is not suitable for the production of large amounts of target protein. We therefore prepared recombinant Per a 9 using *E. coli* expression systems in the present study. We found that as little as 1 liter of *E. coli* was able to produce 8.69 mg of highly pure recombinant Per a 9, which is sufficient for the functional analysis of Per a 9.

It is well known that the advantages of the prokaryotic (*E. coli*) system are easy handling and the expression of high-quantity of target proteins (45); however, one of the disadvantages is that insoluble, inactive inclusion bodies are frequently formed, which means that protein needs to be reconstituted *in vitro* following solubilization under denaturing conditions to achieve biological activity. Moreover, the application of non-glycosylated recombinant allergens in allergy diagnosis frequently yields false results, as the recombinant allergens lack glycan structures of their natural counterparts, which IgE supposedly react with (46). It has been discovered that Per a 9 contains 356 amino acids with a calculated molecular weight of 39.74 kDa, and Per a 9 purified from American CR extract by monoclonal antibody based-affinity chromatography exhibits a molecular weight of 40.57 kDa (12), indicating that few glycan structures exist in Per a 9 protein. In the present study, Per a 9 expressed in the ArcticExpress™ (*DE3*) RP host strain was soluble with a molecular weight of 40 kDa, without any reconstitution process, indicating that Per a 9 obtained herein should have its immunological and biological functions.

Our results showed that 81.3% of the sera from patients with CR allergies reacted to Per a 9, proving that Per a 9 is a major allergen of the American CR. A similar finding has been previously reported in the Thai population (12). The

basophil activation test we employed herein is a more advanced technique for the determination of the allerginicity of a given compound. We confirmed that Per a 9 is an active allergen of CR as it was able to activate basophils which were sensitized by CR allergy sera. The availability of recombinant allergens has increased our understanding of IgE-mediated allergies and may improve the diagnosis and treatment of these diseases (47). In our case, recombinant Per a 9 should be a useful tool for the functional and clinical analysis of this allergen.

Allergen-specific IgE is the key molecule for the development of allergic symptoms. The synthesis of IgE requires a B cell to undergo class switch recombination in close contact with allergen-specific T helper 2 cells (Th2) (48). In a previous study, overlapping synthetic peptides were frequently used to validate the IgE-binding capacity. Although this method decreases the possibility of missed epitopes, it needs to synthesize lots of peptides and is very costly and time-consuming (49). *In silico* prediction has already become a familiar and useful tool for selecting epitopes from immunologically relevant proteins, which can save the expense of synthetic peptides and the working time (50). A previous study demonstrated that the use of the bioinformatics approach to predict B cell epitopes correlated well with the experimental approach (51). Many algorithms have been developed to predict B cell epitopes on a protein sequence based on the propensity values of amino acid properties of hydrophilicity, antigenicity, segmental mobility, flexibility and accessibility (48). In the present study, we predicted the B cell linear epitopes of Per a 9 allergens by 3 sequence based tools (the DNASTAR protean system, BPAP and the BepiPred 1.0 server) and predicted 11 peptides (23-28, 39-46, 58-64, 91-118, 131-136, 145-154, 159-165, 176-183, 290-299, 309-320 and 338-344) as potential B cell linear epitopes. Over the past several years, some algorithms have substantially improved their accuracy to predict T cell epitopes. However, most algorithms have targeted HLA-DR molecules, but not HLA-DP and HLA-DQ molecules, even though they are important for antigen presentation. NetMHCpan-2.0 has recently been evaluated to have a per-allele mean accuracy of 0.854 (1.0 being 100% accurate, and 0.5 of no significance) (52). In another study, the per-allele mean accuracy of NN-align was 0.882 (53). In this study, Net-MHCIIpan-2.0 and NetMHCII-2.2 were used to predict the core 9-mer T cell epitopes in the Per a 9 allergens and predicted 5 potential T cell epitope sequences, 119-127, 194-202, 210-218, 239-250 and 279-290.

Allergen-specific immunotherapy (SIT) is the only treatment able to cure allergic diseases. Numerous studies have shown that crude allergen extracts currently used in SIT are clinically effective (54-56); a high allergen dose is more effective, although the potential risk of severe acute side-effects is a limiting factor. Attenuated allergenic molecules, i.e., hypoallergens or synthetic peptide fragments have been used as high-dose and safer alternatives to conventional extract-based SIT (57). Vaccination with a combination of small peptides that together extend across the entire native allergenic protein theoretically could preserve T cell activation, while avoiding IgE-based immune responses. IgE recognizes conformational epitopes of larger peptides (B cell epitopes) and proteins, while T cell receptors recognize small linear peptides of 8 to 10 amino acids (T cell epitope). By immunizing with small peptides, T cell activation could occur, while IgE binding would be lost (58-60).

In conclusion, in the present study, we prepared recombinant Per a 9 allergens using a prokaryotic expression system. We confirm that Per a 9 is a major allergen of the American CR, which can activate basophils *in vitro*. Recombinant Per a 9 may prove to be a useful tool for studying and understanding the role of Per a 9 in CR allergies. We also predicted B and T cell epitopes of the Per a 9 allergen, the major allergen in the American CR, using the *in silico* method, which can be used to benefit allergen immunotherapies and reduce the frequency of allergic reactions. However, their accuracies need to be confirmed in the further experiments.

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