Expression, purification and epitope analysis of Pla a 3 allergen from *Platanus acerifolia* pollen

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Abstract. *Platanus acerifolia* (*P. acerifolia*) is an important cause of pollinosis in cities. The use of allergen extracts on patients with allergic diseases is the most commonly applied method to attempt to treat pollinosis. Pla a 3, a non-specific lipid transfer protein, is a major allergen present in *P. acerifolia* pollen extracts. In the present study, the Pla a 3 gene was sub-cloned into a *pSUMO-Mut* vector using *Stu I* and *Xho I* sites and transformed into the Arctic Express™ (DE3) RP *E. coli* host strain. The purified Pla a 3 allergen was analyzed by western blotting and the results revealed that the Pla a 3 allergen has the ability to bind IgE in the *P. acerifolia* pollen of allergic patients' sera. Moreover, the authors predicted the potential B cell epitopes of the Pla a 3 allergen using the DNAStar Protean system, the Bioinformatics Predicted Antigenic Peptides system and the BepiPred 1.0 server. In addition, the T cell epitopes were predicted by the SYFPEITHI database and the NetMHCII-2.2 server. As a result, two B cell epitopes (35-45 and 81-86) and four potential T cell epitopes including 2-15, 45-50, 55-61 and 67-73 were predicted in the present study. The current results can be used to contribute to allergen immunotherapies and useful in peptide-based vaccine designs of pollen allergy.

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Introduction

Allergenic pollens that cause allergic diseases, for example, asthma (1) and rhinitis (2,3), are those from trees or plants that pollinate through the air. *P. acerifolia* trees are frequently used as ornamental trees in the urban environment. *P. acerifolia* pollen has been described to cause airway allergies worldwide (4), in particular in the early spring from March to April. The reported prevalence of sensitization to *P. acerifolia* pollen in Mediterranean Europe ranges between 3 and 52% (5).

So far, three major allergens including the invertase inhibitor Pla a 1 (6-8), the polygalacturonase Pla a 2 (9,10) and the non-specific lipid transfer protein (nsLTP) Pla a 3 (9,11), have been identified from *P. acerifolia* pollen. Pla a 1 (18 kDa) and Pla a 2 (43 kDa) have been reported as major allergens with 80-90% sensitization frequencies among Spanish patients with plane pollen allergy (5,12), whereas 45% of plane pollen allergies were sensitized to purified natural Pla a 3 (9).

Allergen-specific immunotherapy (AIT) is an effective treatment for allergic diseases. There is evidence that AIT can relieve symptoms in patients with asthma (13,14), allergic conjunctivitis (15) and allergic rhinitis (16). The administration of increasing doses of allergen extracts to patients is the most commonly applied method. However, the use of crude extracts has several disadvantages. It may increase the risk of allergic side effects or result in the sensitization towards new allergens present in crude extracts (17,18). Compared with allergen extracts, recombinant allergens can be formulated with high quality and precise quantity.

In the present study, the authors expressed and purified the Pla a 3 allergen in the *E. coli* system, which will provide a foundation for further study of this allergen in diagnosis and treatment of plane pollen allergy. Moreover, the identification of B cell and T cell epitopes of allergens will benefit to the diagnosis, therapy and development of effective vaccines for immunotherapy (19,20). Until now, there has been no report of the epitope of the Pla a 3 allergen. In the present study, the authors identified the B and T cell epitopes of Pla a 3 allergen, which contribute to the potential utility in a peptide-based vaccine design for pollen allergy.

Materials and methods

Patients and samples. A total of 12 subjects were included in the study, comprising two patient groups: (A) 6 allergic rhinitis patients (aged 15-34; 3 males and 3 females; recruited between January and June 2015) with positive skin prick test (allergens were supplied by ALK-Abelló, Copenhagen, Denmark) and positive serum IgE test to the *P. acerifolia* pollen extract by using ImmunoCAP assay (Phadia AB, Uppsala, Sweden); (B) 6 healthy controls (aged 20-45; 3 males and 3 females; recruited in June 2015). The study protocol was approved by the ethical committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). Written informed consent for the use of blood samples were obtained from all participants before study entry according to the Declaration of Helsinki.

Sequence retrieval. The complete nucleotide acid and amino acid sequences of Pla a 3 allergen were retrieved from the GenBank database. The open reading frames (ORF) of Pla a 3 allergen had 354 bases pairs, encoding 118 amino acids. Because this ORF contained a 26 amino acid signal peptide, the mature Pla a 3 allergen had 276 bases pairs, encoding 92 amino acids.

Expression and purification of Pla a 3 allergen in E. coli. The nucleotide acid of mature Pla a 3 allergen was synthesized by GenScript (Piscataway, NJ, USA) and sub-cloned into *pSUMO-Mut* vector using *Stu I* and *Xho I* sites and verified by DNA sequencing. The recombinant pSUMO-Mut-Pla a 3 plasmid was transformed into Arctic Express™ (DE3) RP *E. coli* host strain.

A positive colony was selected to inoculate in a 3 ml lysogeny broth (LB)-kanamycin broth (Shanghai Sanger Biotech Technology, Co., Ltd., Shanghai, China), and incubated at 37˚C overnight. A total of 1% of the overnight culture of the transformant was transferred into fresh LB-kanamycin broth and cultured at 37˚C with shaking at 200 x g up to the absorbance 0.6-0.8 at 600 nm. The culture was induced with 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and harvested following an additional 4 h incubation. The culture was disrupted by sonication at 60 kHz, 4 sec pulse-on, 8 sec pulse-off and centrifuged at 10,000 x g for 30 min at 4˚C. The results indicated that recombinant Pla a 3 was mainly in supernatant fraction (Fig. 1) and purified by Nickel affinity chromatography (GenScript). The washing buffer contains 100 mM NaH_2PO_4, 20 mM Tris-HCl, 10 mM imidazole, pH 8.0, and the eluting buffer contains 100 mM NaH_2PO_4, 20 mM Tris-HCl, 250 mM imidazole, pH 8.0.

IgE binding activity of recombinant Pla a 3 allergen. An IgE binding assay was conducted as previously described. Briefly, proteins were analyzed via 12% sodium dodecylsulfatc-polyacrylamide gel electrophoresis (SDS-PAGE) (21). The gel was transferred to polyvinylidene difluoride membranes (22). The blots were blocked in 5% skim milk for 2 h, then incubated with serum of *P. acerifolia* pollen allergic patients (diluted 1:40 in PBS) as the first antibody overnight at 4˚C. Then, IgE that bound to the allergen was detected with a horseradish peroxidase-conjugated goat anti-human IgE mAb (1:3,000, catalog no. A9667, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4˚C for 1 h and then detected using a ImageQuant LAS 4000 mini detection system (GE Healthcare Life Sciences, Chalfont, UK). A total of 6 healthy individuals were used as a negative serum control.

Homology modeling. The Pla a 3 allergen protein sequence was searched for homology in the PDB. As well, the homologous templates suitable for Pla a 3 allergen were selected from SWISS-MODEL server (http://swissmodel.expasy.org/) (23). SWISS-MODEL Repository is a database of 3D protein structure models generated by The Pla a 3 allergen protein sequence *Homology modeling*. The Pla a 3 allergen protein sequence was searched for homology in the Protein Data Bank. In addition, the homologous templates suitable for the Pla a 3 allergen were selected from the SWISS-MODEL server (http://swissmodel.expasy.org/) (23). The SWISS-MODEL Repository is a database of 3D protein structure models generated by the SWISS-MODEL homology-modeling pipeline (24). The best template was retrieved from the results of previous methods and used for homology modeling. Pla a 3 allergen modeled protein structure was built through alignment mode in SWISS-MODEL using the complete amino acid sequence.

Prediction of B cell epitopes. A total of three immunoinformatics tools including the DNAStar protean system, the 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 the Pla a 3 allergen. The ultimate consensus epitope results were obtained by combining the results of the three tools together (25). In the DNAStar protean system, four properties (hydrophilicity, flexibility, accessibility and antigenicity) of the amino acid sequence were chosen as parameters for epitopes prediction (26). The peptide regions with good hydrophilicity, high flexibility, surface accessibility and high antigenic index were chosen as candidate epitopes for further investigation.

Prediction of T cell epitopes. T cell epitopes are principally predicted indirectly by identifying the binding of peptide fragments to the major histocompatibility complexes (MHCs). The binding significance of each peptide to the given MHC molecule was based on the estimated strength of binding exhibited by a predicted nested core peptide at a set threshold level. SYFPEITHI and NetMHCII-2.2 (http://www.cbs.dtu.dk/services/NetMHC II-2.2) (27) were used to predict T cell epitopes of the Pla a 3 allergen. In the current study, HLA-DR10101 and HLADR50101 were used to predict HLA-DR-based T cell epitope prediction. As a result, the ultimate results of T epitopes were obtained by combining the results of the SYFPEITHI and NetMHCII-2.2. B cell and T cell epitopes identified by immunoinformatics tools were mapped onto a linear sequence and presented on the three dimensional model of the Pla a 3 allergen to determine their position.

Results

Expression and purification of the Pla a 3 allergen in E. coli. The Pla a 3 allergen was subcloned into a *pSUMO-Mut* vector and transformed into the *E. coli* host strain. The culture was induced with IPTG and expressed at 37˚C for 4 h and the

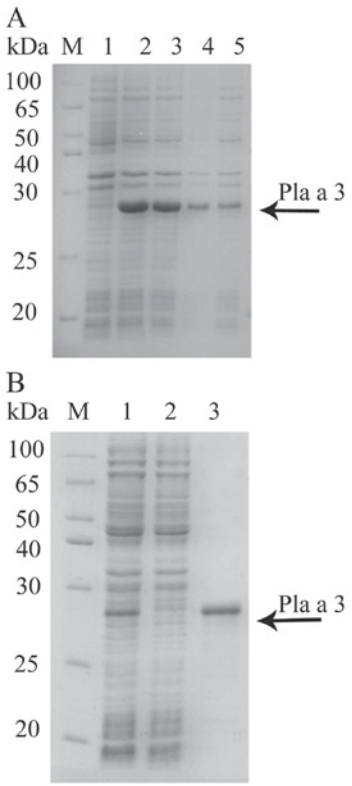

Figure 1. Expression and purification of the Pla a 3 allergen in *E. coli*. (A) Lane M, protein molecular weight standard; lane 1, before induction; lanes 2 and 3, isopropyl-b-D-thiogalactopyranoside-induced recombinant Pla a 3 allergen; lane 4, precipitation fraction following ultrasonication; lane 5, supernatant fraction following ultrasonication. The Pla a 3 allergen is denoted with an arrow. (B) SDS-PAGE following affinity chromatography of the Pla a 3 allergen. Lane M, protein molecular weight standard; lane 1, no-binding protein; lane 2, elute washed with 10 mM imidazole, 100 mM NaH_2PO_4, 20 mM Tris-HCl; lane 3, elute with 250 mM imidazole, 100 mM NaH_2PO_4, 20 mM Tris-HCl. The arrow points to the purified Pla a 3 allergen.

resulting protein presented a band with a apparent molecular weight of 28 kDa (Fig. 1A; lane 2 and lane 3), and no such band was seen in the non-induced cells (Fig. 1A; lane 1). Protein was produced primarily in the supernatant fraction following sonication (Fig. 1A; lane 5) and purified by Ni column. Finally, the purity of the purified Pla a 3 allergen was identified by SDS-PAGE. As presented in Fig. 1B, the recombinant Pla a 3 allergen was successfully purified by elution buffer.

IgE binding activity of the recombinant Pla a 3 allergen. The ability of recombinant Pla a 3 allergen to bind IgE from the *P. acerifolia* pollen allergy patients' serum was determined by western blotting. Fig. 2 demonstrated that mixed *P. acerifolia* pollen allergy patients' serum revealed positive IgE reactivity to the Pla a 3 allergen, but healthy controls failed to.

Homology modeling. Searching for the proteins with known tertiary structure in the Protein Data Bank, 1t12.1.A (PDB accession number) reported the highest sequence identity (62.64%) to the Pla a 3 allergen. Therefore, the best template 1t12.1.A was used for homology modeling. Fig. 3A demonstrated the overall 3D structure of the Pla a 3 allergen, and Fig. 3B demonstrated the predicted B cell and T cell epitopes superimposition on the surface of the Pla a 3 allergen structure.

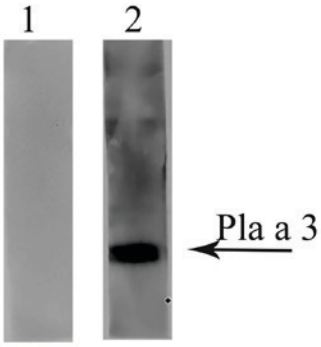

Figure 2. IgE binding activity of Pla a 3 allergen. Allergen was identified by western blotting. Lane 1, Pla a 3 allergen incubated with sera from healthy individual as a negative serum control; lane 2, Pla a 3 allergen incubated with mixed serum of *P. acerifolia* pollen allergic patients as the first antibody.

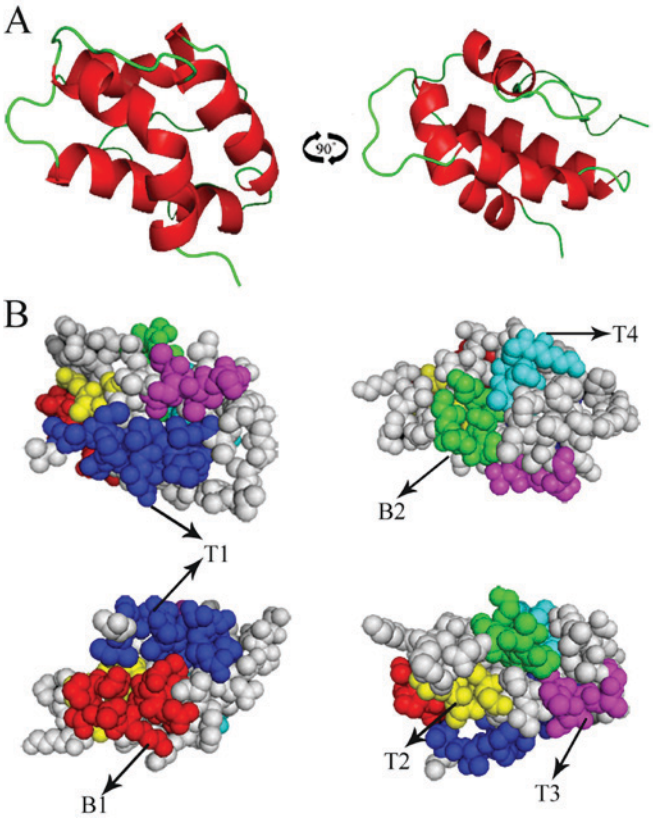

Figure 3. (A) Protein structures of the Pla a 3 allergen homology model. (B) Predicted B cell and T cell epitopes presented on the surface of the Pla a 3 allergen structure.

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 provides strong evidence for epitope identification. Antigenic index directly indicated the epitope forming capacity of the Pla a 3 allergen sequence. Based on these sequence properties, the final predicting regions of the Pla a 3 allergen by DNAstar were obtained as: 35-45 and 81-86 (Table I). The predicted results of the BPAP system were 4-17, 19-34, 45-54 and 63-88. The predicted results of the BepiPred 1.0 server were 22-26, 35-47, 57-59 and 81-87. Furthermore, the final potential B cell epitopes of the Pla a 3 allergen were selected on the basis of

Table I. Location of the B and T cell epitopes of the Pla a 3 allergen predicted by immunoinformatics tools.

	Tools	Location of the prediction results
B cell epitope prediction	DNAStar protean	
	Hydrophilicity	33-47, 83-92
	Flexible regions	10-12, 19-22, 35-45, 53-61, 81-86
	Antigenic index	33-48, 50-57, 86-92
	Surface probability	35-45, 54-55, 81-82, 91-92
	BPAP	4-17, 19-34, 45-54, 63-88
	BepiPred	22-26, 35-47, 57-59, 81-87
T cell epitope prediction	SYFPEITHI	4-18, 29-37, 57-63, 67-73, 77-85
	NetMHCII (HLA-DR)	
	DRB1*01:01	2-16, 45-50, 55-61
	DRB5*01:01	2-15, 47, 56-59

BPAP, bioinformatics predicted antigenic peptides.

Table II. Predicted B and T cell epitopes of the Pla a 3 allergen.

Number	Type of epitope	Position	Sequence
P1	B1	35-45	LNNDAKTTPDR
P2	B2	81-86	KISPTI
P3	T1	2-15	ITCGTVVTRLTPCL
P4	T2	45-50	RQAACG
P5	T3	55-61	ASTSISG
P6	T4	67-73	AASLAGK

the results of these three tools. The ultimate results of the three immunoinformatics tools finally predicted two peptides (35-45 and 81-86) and these peptides are presented in Fig. 3.

T cell epitope prediction. SYFPEITHI and NetMHCII-2.2 were used to identify the T cell epitope of the Pla a 3 allergen. The predicted results of SYFPEITHI were 4-18, 29-37, 57-63, 67-73 and 77-85 (Table I). For HLA-DR-based T cell epitope prediction, the final predicting regions of HLA-DR10101 and HLA-DR 50101 are presented in Table I. As a result, the Pla a 3 allergen was predicted to have four T cell epitope sequences including 2-15, 45-50, 55-61 and 67-73, as presented in Table II.

Discussion

P. acerifolia pollen is the most frequently present in pollen counts (28,29) and the contribution of it in pollen allergies has increased over the past decade. A high percentage of patients with seasonal pollinosis are sensitive to it. The quantification of major allergens has become a crucial goal for the standardization of allergen products intended for clinical use (30,31). Therefore, the determination of Pla a 3 allergen content is very important for the development of a *P. acerifolia* pollen allergen vaccine since it is the major allergen in *P. acerifolia* pollen. In the present study, the Pla a 3 allergen was expressed successfully in the soluble form in *E. coli*. The purified Pla a 3 allergen was analyzed by western blotting and the results revealed that the Pla a 3 allergen has the ability to bind IgE in the *P. acerifolia* pollen allergic patients' sera (5,32,33).

Although used in clinical settings for more than a century, AIT is currently the only known causal treatment of allergic diseases (34). To relieve allergic symptoms, AIT serves a significant role in preventing new allergies and shows long-term effects following termination of treatment. The safety and efficacy of AIT has been demonstrated in a large number of clinical trials. However, AIT is not effective in all allergic individuals, and it carries the risk of adverse effects. For this reason, there is a strong requirement to increase its safety and effectiveness by further research. Establishing the optimal route and dose of administration may benefit to a develop better clinical desensitization effect than before. Growing evidence on the immunological effects of AIT, especially B cell and T cell epitopes related to allergen tolerance, provide novel concepts for safer and more effective vaccination. Therefore, the authors predicted B and T cell epitopes of the Pla a 3 allergen in the present study. Firstly, in order to better understand the structure and function of the Pla a 3 allergen, the basic sequence properties were analyzed and the 3D structures of the Pla a 3 allergen were studied. The 3D structure of the Pla a 3 allergen was performed by homology modeling which is widely used in many areas of structure-based analysis. The Protein Data Bank server was used to search templates of the Pla a 3 allergen and reported that the structure of 1t12.1.A was the best template with the highest identity (62.64%). An earlier study demonstrated that the use of bioinformatics approach to predict B cell epitopes correlated well with the experimental approach (35). In the current study, the authors used three immunoinformatics tools (DNAStar protean system, BPAP and the BepiPred 1.0 server) to predict the B cell epitopes. As a result, two B cell epitopes (35-45 and 81-86) were predicted as the B cell epitopes. However, these B cell epitopes need to be confirmed in further clinical samples. On the other hand, SYFPEITHI and NetMHCII-2.2 were used to predict the T cell epitopes and predicted four potential T cell epitopes including 2-15, 45-50, 55-61 and 67-73. Despite the

high accuracy of these predictions, this approach has not yet been applied to peptide-based vaccine development for allergic diseases. In summary, the successful production of the recombinant Pla a 3 allergen revealed the importance of the Pla a 3 allergen in *P. acerifolia* pollen allergy, and provides a foundation for further study of the allergen in diagnosis and treatment of plane pollen allergy. Moreover, the authors have predicted two B cell epitopes (35-45 and 81-86) and four T cell epitopes including 2-15, 45-50, 55-61 and 67-73 of the Pla a 3 allergen, which can be used to contribute to allergen immunotherapies and is useful in peptide-based vaccine designs of plane pollen allergy.

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