Expression, purification and epitope analysis of Pla a 2 allergen from *Platanus acerifolia* pollen

DE-WANG WANG[1*], WEI-WEI NI[1,2*], YAN-JUN ZHOU[1,2*], WEN HUANG[1,2], MENG-DA CAO[1,2], LING MENG[1,2] and JI-FU WEI[1,2]

[1]Research Division of Clinical Pharmacology, The First Affiliated Hospital of Nanjing Medical University; [2]Department of Clinical Pharmacology, The College of Pharmacy, Nanjing Medical University, Nanjing, Jiangsu 210029, P.R. China

Received February 22, 2017; Accepted September 6, 2017

DOI: 10.3892/mmr.2017.7883

Abstract. *Platanus acerifolia* is one of the major sources of outdoor allergens to humans, and can induce allergic asthma, rhinitis, dermatitis and other allergic diseases. Pla a 2 is a polygalacturonase and represents the major allergen identified in *P. acerifolia* pollen. The aim of the present study was to express and purify Pla a 2, and to predict B and T cell epitopes of Pla a 2. The gene encoding Pla a 2 was cloned into the *pET28a* vector and subsequently transfected into ArcticExpress™ (DE3) *Escherichia coli* cells; purified Pla a 2 was analyzed by western blot analysis. The results of the present study revealed that the Pla a 2 allergen has the ability to bind immunoglobulin E within the sera of patients allergic to *P. acerifolia* pollen. In addition, the B cell epitopes of Pla a 2 were predicted using the DNAStar Protean system, Bioinformatics Predicted Antigenic Peptides and BepiPred 1.0 software; T cell epitopes were predicted using NetMHCIIpan -3.0 and -2.2. In total, eight B cell epitopes (15-24, 60-66, 78-86, 109-124, 232-240, 260-269, 298-306 and 315-322) and five T cell epitopes (62-67, 86-91, 125-132, 217-222 and 343-350) were predicted in the present study. These findings may be used to improve allergen immunotherapies and reduce the frequency of pollen-associated allergic reactions.

Introduction

The morbidity and prevalence associated with allergic diseases has increased markedly over the past few decades (1,2), and allergic diseases are considered the most common type of human disease worldwide (3). A survey investigating the epidemiology of allergic diseases within 30 countries revealed that 22% of individuals had immunoglobulin E (IgE)-mediated allergic diseases, including asthma, rhinitis and conjunctivitis; severe allergic diseases associated with trees or plants have been reported to affect the quality of life of children and adults (4,5). Among 1,500 million patients, ~50% of adults and ≥80% of children who suffer from asthma were reported to be induced by allergic factors; the World Health Organization have estimated that >180,000 annual cases of mortality are associated with asthma (6).

Platanus acerifolia pollen has been described to cause airway-associated allergies worldwide, particularly during early spring (7). Three major allergens have been identified within *P. acerifolia* pollen, including Pla a 1, Pla a 2 and Pla a 3. The nonglycosylated protein Pla a 1 has a prevalence of 84% among *Platanus*-allergic patients (8). Pla a 2 is a glycoprotein associated with the allergic responses of 84% of patients with *Platanus*-induced pollinosis (9,10). Pla a 3 is a non-specific lipid transfer protein (9). Evidence has indicated that 45% of patients allergic to *P. acerifolia* pollen are sensitive to natural Pla a 3 (11). In the present study, the Pla a 2 allergen was expressed and purified from an *Escherichia coli* system, which may provide a foundation for the future study of the diagnosis and treatment of *P. acerifolia* pollen-associated allergies.

Allergen-specific immunotherapy (AIT) has been reported to reduce the severity of symptoms in people with allergic asthma (12,13), allergic rhinitis (14) and conjunctivitis (15). The efficacy and safety of AIT has been demonstrated in numerous clinical trials (16,17). In addition to the reduction of allergy-associated symptoms, AIT serves a key role in preventing the emergence of novel allergies and demonstrates long-term effects following the termination of AIT treatment. Increasing evidence regarding the immunological-associated effects of AIT, in particular the use of allergen-derived B and T cell epitopes (18), has provided novel concepts for the improvement of safe and effective vaccination (19). However, to the best of our knowledge, the epitopes of Pla a 2 have not been investigated. In the present study, B and T cell-associated epitopes of Pla a 2 were identified using numerous immunoinformatics tools; these epitopes may aid the development of potential peptide-based vaccine design for pollen allergy.

Correspondence to: Professor Ling Meng or Professor Ji-Fu Wei, Research Division of Clinical Pharmacology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, Jiangsu 210029, P.R. China
E-mail: mengling-ml@163.com
E-mail: weijifu@hotmail.com

*Contributed equally

Key words: Pla a 2, *Platanus acerifolia, Escherichia coli*, B cell epitopes, T cell epitopes, vaccine

Materials and methods

Sequence retrieval. The complete amino acid sequence of Pla a 2 was acquired from the Nucleotide database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nucleotide/), accession number Q6H9K0.

Patients and samples. A total of 10 adult individuals comprised two groups: i) Five allergic subjects (aged between 20 and 43; 2 males and 3 females; recruited in May 2016; diagnosis was established based on clinical symptoms associated with allergic rhinitis during the pollination season, a positive skin prick test result and a seropositive IgE test to *P. acerifolia* pollen extract; and ii) five healthy controls (age, 19-45; 2 males and 3 females; recruited in May of 2016). Written informed consent for the use of blood samples was obtained from the fingertips of all participants prior to study entry according to The Declaration of Helsinki. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China).

Expression and purification of Pla a 2 allergen in E. coli. The nucleotide sequence corresponding to the mature Pla a 2 allergen (signal peptides were removed, which cannot be excised by cell recognition and they may affect the proper folding of proteins.) was synthesized by GenScript (Nanjing, China) and was sub-cloned into a *pET28a* vector (Novagen, Madison, WI, USA) using *Bam*HI and *Xho*I sites, and was verified by Sanger (20). Specifically, the full-length Pla a 2 gene was amplified by reverse transcription-quantitative polymerase chain reaction with a pair of specific primers and the PCR product was cloned into the *pET28a* vector using Tag enzymes (Takara Biotechnology Co., Ltd., Tokyo, Japan). The recombinant *pET28a-Pla a 2* plasmid was transformed into the ArcticExpress™ (DE3) RP *Escherichia coli* host strain (21,22). Firstly, 1 μl *pET28a-Pla a 2* plasmid was transformed into 100 μl ArcticExpress™ (DE3) *Escherichia coli* cells and placed on ice for 30 min and then heated in a water bath at 42˚C for 45 sec, followed by an ice bath for 2 min. A total of 500 μl LB medium without antibiotics was added to the tube with shaking at 200 rpm for 1 h at 37˚C. Subsequently, 200 μl transformed cells were added to the LB agarose solid state medium and placed at 37˚C for 1 h. Finally, the tube was cultured overnight at 37˚C.

The positive clones were cultivated overnight in 3 ml lysogeny-broth kanamycin (concentration 0.05 g/ml) at 37˚C until an absorbance of 0.6-0.8 at 600 nm was attained. The culture was induced with 1 mM isopropyl-b-D-thiogalactopyranoside and harvested following incubation for 4 h at 37˚C. Uninduced culture (subject to the same conditions without IPTG) was used as a control. Expression of recombinant Pla a 2 was confirmed using 12% SDS-PAGE. The cell mass from 200 ml induced culture was resuspended in 20 mM Tris-HCl buffer (pH 8.0). The cell suspension was sonicated at 40 kHz in 10 cycles of 4 sec pulse on and 8 sec pulse off at 4˚C, and was subsequently centrifuged at 10,000 x g for 30 min. Analysis revealed that recombinant Pla a 2 was mainly located in the supernatant (Fig. 1); purification was conducted via Nickel affinity chromatography (GenScript) (23,24). The washing buffer contained 100 mM NaH_2PO_4, 20 mM Tris-HCl and 100 mM imidazole, pH 8.0; eluting buffer contained 100 mM NaH_2PO_4, 20 mM Tris-HCl and 250 mM imidazole, pH 8.0 (25).

IgE binding activity of recombinant Pla a 2 allergen. Purified Pla a 2 (5 μg) protein was separated by 12% SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes and were blocked with 5% skim milk at room temperature for 2 h. Subsequently, PVDF membranes were incubated with a serum mixture (1:40 in PBS) from six patients with *P. acerifolia* pollen allergy, overnight at 4˚C. The serum mixture served as the primary antibody. Subsequently, IgE-allergen complexes were detected using horseradish peroxidase-conjugated goat anti-human IgE monoclonal antibody (cat no. A9667; diluted 1:3,000; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at room temperature; an ImageQuant LAS 4000 mini detection system (GE Healthcare, Chicago, IL, USA) was employed for the detection of these complexes. An enhanced chemiluminescence substrate including luminol/enhancer solution and peroxide solution was used (1:1 mixed; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Serum mixture from six healthy individuals was applied as a negative control and the experiment was repeated three times.

Homology modeling. SWISS-MODEL Repository is a database of three-dimensional (3D) protein structure models generated by the SWISS-MODEL homology modeling pipeline (26). The homologous templates suitable for Pla a 2 were selected using the SWISS-MODEL server (http://swissmodel.expasy.org/) based on its complete amino acid sequence (27). The most appropriate template was retrieved from results of previous analyses and was utilized for homology modeling.

B cell epitope prediction. Three immunoinformatics tools, including DNAStar protean system (https://www.dnastar.com/t-allservices.aspx), BepiPred 1.0 server (http://www.cbs.dtu.dk/services/BepiPred/) and Bioinformatics Predicted Antigenic Peptides (BPAP) system (http://imed.med.ucm.es/Tools/antigenic.pl) were used to predict the B cell-associated epitopes of Pla a 2 (28). The results obtained from the three immunoinformatics tools were combined to produce the most informative results (29). Four properties (hydrophilicity, flexibility, accessibility and antigenicity) of the amino acid sequence were selected as parameters for epitope prediction in the DNAStar protean system (30). The BepiPred 1.0 server and the BPAP system required the amino acid sequence of Pla a 2.

Prediction of T cell epitopes. T cell epitope prediction was based on the identification of bound peptide fragments on major histocompatibility complex (MHC) complexes. 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. NetMHCII-2.2 (http://www.cbs.dtu.dk/services/NetMHCII/) (31) was employed to investigate human leukocyte antigen (HLA)-DQ alleles. NetMHCIIpan-3.0 (http://www.cbs.dtu.dk/services/NetMHCIIpan/) was applied to predict HLA-DR-based T cell

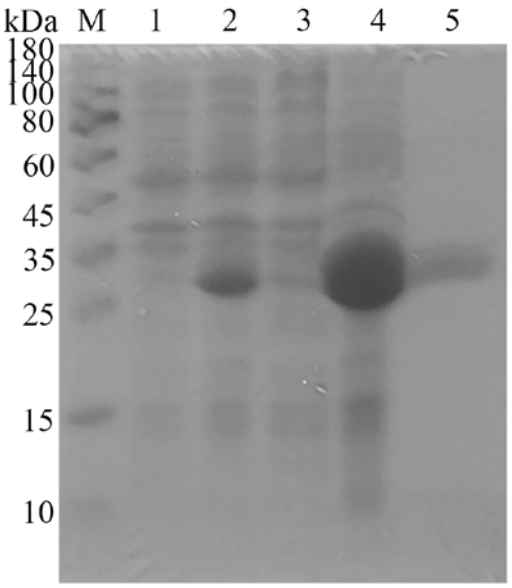

Figure 1. Expression and purification of Pla a 2 allergen in *Escherichia coli*. Lane M, protein molecular weight standard; lane 1, before induction; lane 2, IPTG-induced recombinant Pla a 2 allergen; lane 3, precipitation fraction after ultrasonication; lane 4, supernatant fraction after ultrasonication; lane 5, elute washed with 250 mM imidazole, 100 mM NaH_2PO_4 and 20 mM Tris-HCl.

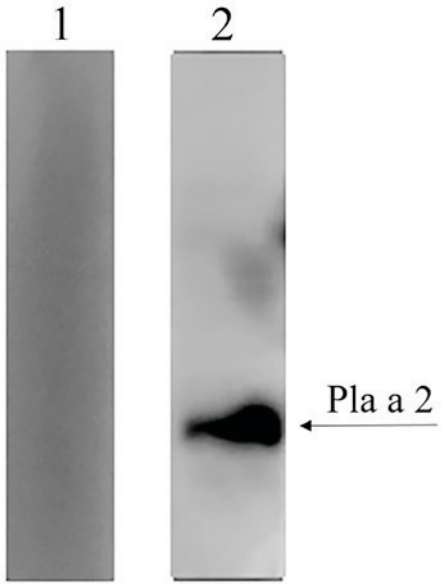

Figure 2. IgE binding activity of Pla a 2 allergen. IgE binding activity of Pla a 2 allergen was identified by western blot analysis. Lane 1, Pla a 2 allergen incubated with a pool of serum from 5 healthy controls as the negative serum control; lane 2, Pla a 2 allergen incubated with mixed sera of patients with *Platanus acerifolia* pollen allergy used as the primary antibody. IgE, immunoglobulin E.

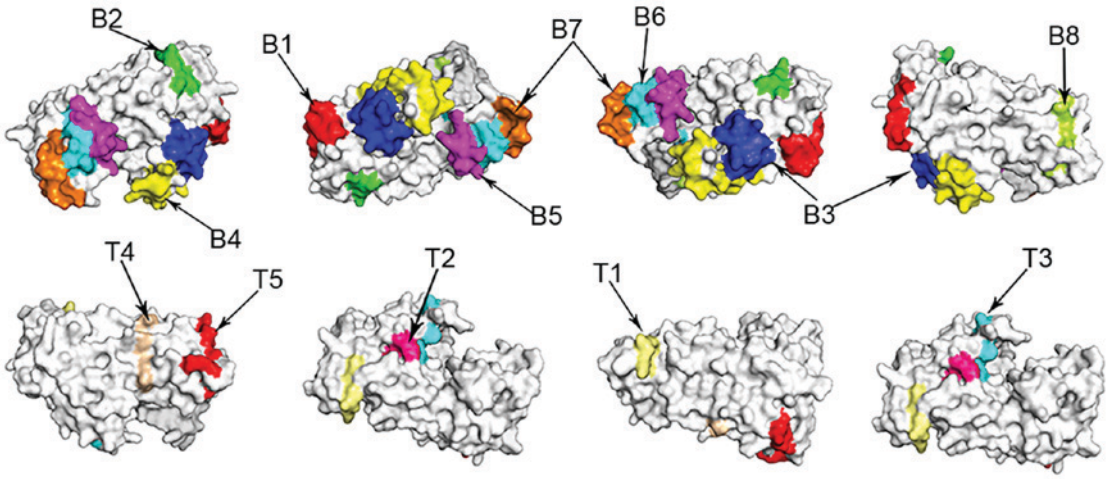

Figure 3. Predicted B and T cell epitopes identified on the surface of the 3-dimensional structure of the Pla a 2 allergen. Specifically, B1 refers to B cell epitope 15-24, B2 refers to B cell epitope 60-66, B3 refers to B cell epitope 78-86, B4 refers to B cell epitope 109-124, B5 refers to B cell epitope 232-240, B6 refers to B cell epitope 260-269, B7 refers to B cell epitope 298-306, B8 refers to B cell epitope 315-322, T1 refers to T cell epitope 62-67, T2 refers to T cell epitope 86-91, T3 refers to T cell epitope 125-132, T4 refers to T cell epitope 217-222 and T5 refers to T cell epitope 343-350.

epitopes (32). In the present study, HLA-DQA10101-DQB10501, HLADQA-10501-DQB10201, HLA-DQA10501-DQB10301 and HLA-DQA10102-DQB10602 were used to predict HLA-DQ-based T cell epitopes. Combining the four results indicated the most informative HLA-DQ-associated T cell epitope result. Providing three epitopes were revealed, the consensus would suggest an epitope. This method was also applied to HLA-DR-based T cell epitope prediction. HLA-DRB-10101, HLA-DRB30101, HLA-DRB40101 and HLA-DRB50101 were used to predict HLA-DR-based T cell epitopes. The most informative results were obtained by combining the results of the HLA-DQ- and HLA-DR-based T cell epitope predictions. B and T cell epitopes identified by computational tools were mapped into a linear sequence and onto a 3D model of Pla a 2 to determine their position (33).

Results

Expression and purification of Pla a 2 in E. coli. *P. acerifolia* pollen Pla a 2 was subcloned into a *pET28a* vector and transformed into BL21 (DE3) *E. coli* host strain. It was demonstrated that Pla a 2 was expressed mainly in the supernatant fraction (Fig. 1). The dissolved inclusion body of Pla a 2 was purified via Nickel affinity chromatography. The purity of the purified

Table I. Predictions of B and T cell epitopes.

Prediction	Tools	Location of the prediction results
B cell epitope	DNAStar protean	15-24, 60-66, 78-86, 105-111, 117-125, 232-240, 260-268, 298-306, 315-322
	BepiPred	2-9, 15-24, 36-46, 58-66, 74-87, 100-126, 170-179, 202-213, 219-226, 233-240, 260-273, 294-306, 317-323, 350-373
	BPAP	5-12, 23-30, 32-38, 40-47, 67-80, 88-100, 133-144, 153-162, 164-172, 180-189, 190-207, 210-220, 224-230, 244-250, 283-301, 303-309, 322-335, 356-365, 368-374
T cell epitope (HLA-DQ)	HLA-DQA10101-DQB10501	84-90, 146-151
	HLA-DQA10102-DQB10602	2-4, 17-29, 46-52, 90-92, 121-125, 131-137, 141-152, 160-163, 182-192, 204-212, 234-240, 248-253, 277-281, 317-324
	HLA-DQA10501-DQB10201	149-154, 279-283
	HLA-DQA10501-DQB10301	2-5, 9-20, 23-35, 45-52, 63-74, 87-108, 161-169, 173-188, 198-206, 212-227, 243-252, 256-265, 291-303, 311-325, 334-353, 360-364
T cell epitope (HLA-DR)	DRB101:01	86-90, 127-133, 309-311
	DRB301:01	62-67, 77-83, 86-91, 125-130, 134-139
	DRB401:01	127-132
	DRB501:01	127-132, 295

BPAP, bioinformatics predicted antigenic peptides; HLA, human leukocyte antigen.

Pla a 2 was identified by SDS-PAGE. A single band with an apparent molecular weight of ~25 kDa was observed (Fig. 1).

Immunoreactivity of Pla a 2 to IgE. The ability of Pla a 2 to bind IgE within the sera of patients associated with *P. acerifolia* pollen allergy was determined by western blotting. Pla a 2 demonstrated positive IgE reactivity to mixed serum of patients associated with *P. acerifolia* pollen allergy; reactivity to healthy controls was not observed, as presented in Fig. 2.

Homology modeling. Protein Data Bank accession no. 4c2I revealed a marked sequence homology (25.22%) to the Pla a 2 allergen and was used for homology modeling. The predicted B and T cell-associated epitopes were superimposed on the surface of the Pla a 2 allergen as presented in Fig. 3.

Prediction of B cell epitopes. The probability of epitope formation within the Pla a 2 sequence is indicated by antigenic index. Regions of high hydrophobicity are also associated with epitope identification. In addition, surface accessibility and fragment flexibility are important features for antigenic epitope prediction. From these sequence properties, the final predicted epitope regions of Pla a 2 were 15-24, 60-66, 78-86, 105-111, 117-125, 232-240, 260-268, 298-306 and 315-322 (Table I) using DNAstar. The predicted results of BepiPred 1.0 server were 2-9, 15-24, 36-46, 58-66, 74-87, 100-126, 170-179, 202-213, 219-226, 233-240, 260-273, 294-306, 317-323 and 350-373. Additionally, the predicted results of BPAP system were 5-12, 23-30, 32-38, 40-47, 67-80, 88-100, 133-144, 153-162, 164-172, 180-189, 190-207, 210-220, 224-230, 244-250, 283-301, 303-309, 322-335, 356-365 and 368-374. Potential B cell epitopes of Pla a 2 were then selected from the results of these three tools. The most informative result of the three immunoinformatics analyses led to the prediction of eight peptides (15-24, 60-66, 78-86, 109-124, 232-240, 260-269, 298-306 and 315-322), which are presented in Table II.

Table II. Predicted B and T cell epitopes of Pla a 2.

Peptide	Type of epitope	Position	Primary sequence
P1	B1	15-24	DYGAKGAGDI
P2	B2	60-66	GPCKGSK
P3	B3	78-86	PADPSKFKS
P4	B4	109-124	QGQTAWAKNNCDKNPN
P5	B5	232-240	GRYNNEKEV
P6	B6	260-269	KTWPNSPPGA
P7	B7	298-306	QCSRQAPSR
P8	B8	315-322	NNIRGTST
P9	T1	62-67	CKGSKI
P10	T2	86-91	SDGWVS
P11	T3	125-132	CKHAAMNL
P12	T4	217-222	QVNCGP
P13	T5	343-350	GEINLSYR

Prediction of T cell epitopes. NetMHCII-2.2 and NetMHCIIpan-3.0 were used to identify the T cell epitopes of Pla a 2. For HLA-DQ alleles, the results of HLADQA10101-DQB10501, HLA-DQA10501-DQB10201, HLADQA10501-DQB10301 and HLA-DQA10102-DQB10602

are presented in Table I. For HLA-DR-based T cell epitopes, the results of HLA- DRB1*01:01, HLA- DRB3*01:01, HLA- DRB4*01:01, and HLA- DRB5*01:01 are also presented in Table I. As a result, Pla a 2 was predicted to have five T cell epitope sequences, 62-67, 86-91, 125-132, 217-222 and 343-350, as presented in Table II.

Discussion

Despite improved understanding of the pathophysiology of allergic diseases and advances in associated pharmacological treatment, the prevalence has continued to increase over the past several decades (34). *P. acerifolia* pollen-associated allergies constitute ~50% of cases of allergy and are frequently associated with severe allergic diseases, including rhinitis and asthma. Characterization and identification of pollen allergens will be beneficial to the diagnosis and treatment of pollen-induced allergic illnesses (35).

P. acerifolia is an important cause of pollinosis in many cities. The quantification of major allergens has become a significant goal for the standardization of allergen products intended for clinical use (36). Therefore, determination of the Pla a 2 allergen is crucial for the development of *P. acerifolia* pollen allergen-associated vaccines. The first objective of the present study was to express and purify the Pla a 2 allergen. The second objective was to predict the B and T cell epitopes of Pla a 2. It was demonstrated that Pla a 2 expression in the soluble form was successful within *E. coli*. In addition, purified Pla a 2 allergen was analyzed by western blot analysis and was demonstrated to possess the ability to bind IgE within the sera of patients with *P. acerifolia* pollen allergy (8,37,38).

To further understand the structure and function of Pla a 2, the sequence properties and 3D structure of Pla a 2 were analyzed. Homology modeling was applied to investigate the 3D structure of Pla a 2; the structure of 4C2I was the most appropriate template with marked identity to Pla a 2. Prediction using informatics tools is a familiar and useful method for selecting epitopes from immunologically relevant proteins. In the present study, three tools were employed (DNAStar protean system, BepiPred 1.0 server and BPAP) to predict B cell epitopes. As a result, eight peptides (15-24, 60-66, 78-86, 109-124, 232-240, 260-269, 298-306 and 315-322) were predicted as B cell epitopes; however, further investigation in clinical samples is required. In addition, NetMHCIIpan-3.0 and NetMHCII-2.2 were used to predict T cell epitopes of Pla a 2-associated allergies; five potential T cell epitope sequences were identified: 62-67, 86-91, 125-132, 217-222 and 343-350.

In conclusion, the predicted B and T cell epitopes of Pla a 2 allergen may be used to benefit allergen immunotherapies and reduce the frequency of allergic reactions. However, the accuracy associated with such immunotherapy must be confirmed in future experiments.

Acknowledgements

The present study was supported by grants from the Special Fund for Forest-scientific Research in the Public Welfare (grant. no. 201304103); the National Natural Science Foundation of China (grant nos. 81571568, 31340073 and 81273274); Jiangsu Province's Key Provincial Talents Program (grant. no. RC201170) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

1. Malmstrom K, Kaila M, Kajosaari M, Syvanen P and Juntunen-Backman K: Fatal asthma in Finnish children and adolescents 1976-1998: validity of death certificates and a clinical description. Pediatr Pulmonol 42: 210-215, 2007.
2. Sullivan PW, Smith KL, Ghushchyan VH, Globe DR, Lin SL and Globe G: Asthma in USA: Its impact on health-related quality of life. J Asthma 50: 891-899, 2013.
3. Moorman JE, Rudd RA, Johnson CA, King M, Minor P, Bailey C, Scalia MR and Akinbami LJ: National surveillance for asthma-United States, 1980-2004. MMWR Surveill Summ 56: 1-54, 2007.
4. Snijders BE, Thijs C, Dagnelie PC, Stelma FF, Mommers M, Kummeling I, Penders J, van Ree R and van den Brandt PA: Breast-feeding duration and infant atopic manifestations, by maternal allergic status, in the first 2 years of life (KOALA study). J Pediatr 151: 347-351, 351.e341-342, 2007.
5. Hendaus MA, Jomha FA and Ehlayel M: Allergic diseases among children: nutritional prevention and intervention. Ther Clin Risk Manag 12: 361-372, 2016.
6. Warner JO, Kaliner MA, Crisci CD, Del Giacco S, Frew AJ, Liu GH, Maspero J, Moon HB, Nakagawa T, Potter PC, *et al*: Allergy practice worldwide: A report by the world allergy organization specialty and training council. Int Arch Allergy Immunol 139: 166-174, 2006.
7. Alcazar P, Garcia-Mozo H, Trigo Mdel M, Ruiz L, Gonzalez-Minero FJ, Hidalgo P, Diaz de la Guardia C and Galan C: *Platanus* pollen season in Andalusia (southern Spain): Trends and modeling. J Environ Monit 13: 2502-2510, 2011.
8. Fernandez-Gonzalez D, Gonzalez-Parrado Z, Vega-Maray AM, Valencia-Barrera RM, Camazon-Izquierdo B, De Nuntiis P and Mandrioli P: *Platanus* pollen allergen, Pla a 1: Quantification in the atmosphere and influence on a sensitizing population. Clinical and experimental allergy: J Br Soc Allergy Clin Immunol 40: 1701-1708, 2010.
9. Lauer I, Miguel-Moncin MS, Abel T, Foetisch K, Hartz C, Fortunato D, Cistero-Bahima A, Vieths S and Scheurer S: Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid-transfer protein, Pru p 3. Clin Exp Allergy 37: 261-269, 2007.
10. Ibarrola I, Arilla MC, Martinez A and Asturias JA: Identification of a polygalacturonase as a major allergen (Pla a 2) from *Platanus acerifolia* pollen. J Allergy Clin Immunol 113: 1185-1191, 2004.
11. Wangorsch A, Larsson H, Messmer M, Garcia-Moral A, Lauer I, Wolfheimer S, Schulke S, Bartra J, Vieths S, Lidholm J, *et al*: Molecular cloning of plane pollen allergen Pla a 3 and its utility as diagnostic marker for peach associated plane pollen allergy. Clin Exp Allergy 46: 764-774, 2016.
12. Abramson MJ, Puy RM and Weiner JM: Allergen immunotherapy for asthma. Cochrane Database Syst Rev Cd001186, 2003.
13. Vadlamudi A and Shaker M: New developments in allergen immunotherapy. Curr Opin Pediatr 27: 649-655, 2015.
14. Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A and Durham S: Allergen injection immunotherapy for seasonal allergic rhinitis. Cochrane Database Syst Rev Cd001936, 2007.
15. Dahl R, Kapp A, Colombo G, de Monchy JG, Rak S, Emminger W, Rivas MF, Ribel M and Durham SR: Efficacy and safety of sublingual immunotherapy with grass allergen tablets for seasonal allergic rhinoconjunctivitis. J Allergy Clin Immunol 118: 434-440, 2006.
16. Madonini E and Musarra A: Allergen immunotherapy in asthma: Current evidence and future requirements. Eur Ann Allergy Clin Immunol 43: 103-110, 2011.
17. Kim JM, Lin SY, Suarez-Cuervo C, Chelladurai Y, Ramanathan M, Segal JB and Erekosima N: Allergen-specific immunotherapy for pediatric asthma and rhinoconjunctivitis: A systematic review. Pediatrics 131: 1155-67, 2013.
18. Thomas WR: The advent of recombinant allergens and allergen cloning. J Allergy Clin Immunol 127: 855-859, 2011.
19. Jutel M, Kosowska A and Smolinska S: Allergen immunotherapy: Past, Present, and Future. Allergy Asthma Immunol Res 8: 191-197, 2016.

20. Shendure JA, Porreca GJ, Church GM, Gardner AF, Hendrickson CL, Kieleczawa J and Slatko BE: Overview of DNA sequencing strategies. Curr Protoc Mol Biol Chapter 7: Unit7.1, 2011.
21. Minato Y, Ueda T, Machiyama A, Shimada I and Iwai H: Segmental isotopic labeling of a 140 kDa dimeric multi-domain protein CheA from Escherichia coli by expressed protein ligation and protein trans-splicing. J Biomol NMR 53: 191-207, 2012.
22. Kumar S, Jain KK, Singh A, Panda AK and Kuhad RC: Characterization of recombinant pectate lyase refolded from inclusion bodies generated in E. coli BL21(DE3). Protein Expr Purif 110: 43-51, 2015.
23. Liu Y, Sun X, Wang G, Tao A, Wu Y, Li M, Shi H and Xie M: Expression, purification and identification of Pla a1 in a codon-optimized *Platanus* pollen allergen. Mol Med Rep 12: 2197-2202, 2015.
24. Zhang Y, Du WX, Fregevu C, Kothary MH, Harden L and McHugh TH: Expression, purification, and characterization of almond (Prunus dulcis) allergen Pru du 4. J Agric Food Chem 62: 12695-12700, 2014.
25. Tong X, Guo M, Jin M, Chen H, Li Y and Wei JF: *In silico* epitope prediction, expression and functional analysis of Per a 10 allergen from the American cockroach. Int J Mol Med 38: 1806-1814, 2016.
26. Kiefer F, Arnold K, Kunzli M, Bordoli L and Schwede T: The SWISS-MODEL Repository and associated resources. Nucleic Acids Res 37: D387-392, 2009.
27. Arnold K, Bordoli L, Kopp J and Schwede T: The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. Bioinformatics 22: 195-201, 2006.
28. Yang H, Chen H, Jin M, Xie H, He S and Wei JF: Molecular cloning, expression, IgE binding activities and *in silico* epitope prediction of Per a 9 allergens of the American cockroach. Int J Mol Med 38: 1795-1805, 2016.
29. Yang X and Yu X: An introduction to epitope prediction methods and software. Rev Med Virol 19: 77-96, 2009.
30. Zheng LN, Lin H, Pawar R, Li ZX and Li MH: Mapping IgE binding epitopes of major shrimp (*Penaeus monodon*) allergen with immunoinformatics tools. Food Chem Toxicol 49: 2954-2960, 2011.
31. Nielsen M and Lund O: NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. BMC Bioinformatics 10: 296, 2009.
32. Karosiene E, Rasmussen M, Blicher T, Lund O, Buus S and Nielsen M: NetMHCIIpan-3.0, a common pan-specific MHC class II prediction method including all three human MHC class II isotypes, HLA-DR, HLA-DP and HLA-DQ. Immunogenetics 65: 711-724, 2013.
33. Chen H, Yang HW, Wei JF and Tao AL: *In silico* prediction of the T-cell and IgE-binding epitopes of Per a 6 and Bla g 6 allergens in cockroaches. Mol Med Rep 10: 2130-2136, 2014.
34. Goldblum RM, Ning B, Judy BM, Holthauzen LM, van Bavel J, Kamijo A and Midoro-Horiuti T: A single mouse monoclonal antibody, E58 modulates multiple IgE epitopes on group 1 cedar pollen allergens. Mol Immunol 74: 106-112, 2016.
35. Li X, Yang HW, Chen H, Wu J, Liu Y and Wei JF: *In silico* prediction of T and B cell epitopes of Der f 25 in Dermatophagoides farinae. Int J Genom 2014: 483905, 2014.
36. van Ree R: The CREATE project: EU support for the improvement of allergen standardization in Europe. Allergy 59: 571-574, 2004.
37. Asero R, Mistrello G, Amato S and Villalta D: Monosensitization to a novel plane pollen allergen. Eur Ann Allergy Clin Immunol 44: 167-169, 2012.
38. Enrique E, Alonso R, Bartolome B, San Miguel-Moncin M, Bartra J, Fernandez-Parra B, Tella R, Asturias JA, Ibarrola I, Martínez A, *et al*: IgE reactivity to profilin in *Platanus acerifolia* pollen-sensitized subjects with plant-derived food allergy. J Investig Allergol Clin Immunol 14: 335-342, 2004.