



Purification and characterization of enolase as a novel allergen in *Platanus acerifolia* pollen

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ABSTRACT

Background: The pollen from *Platanus acerifolia* (*P. acerifolia*) is one of the main causes of allergic disorders. To date, only 4 allergens have been identified from this pollen. But previous studies showed that there still exist under-recognized allergens in it. The aim of this study was to comprehensively investigate the newly identified enolase (Pla a 6) as a novel allergen in the *P. acerifolia* pollen.

Methods: The natural (n) Pla a 6 was purified by combined chromatographic strategies. According to the identified internal peptides, the cDNA sequence encoding this allergen was matched from the mRNA-sequencing results of *P. acerifolia* pollen, which was further amplified and cloned. The recombinant (r) Pla a 6 was expressed and purified from *E. coli*. The allergenicity of this novel allergen was characterized by enzyme linked immunosorbent assay (ELISA), Western blot, inhibition ELISA, and basophil activation test (BAT).

Results: A novel allergen from *P. acerifolia* pollen, named as Pla a 6 was thoroughly studied, which contained an open reading frame of 1338 bp encoding 445 amino acids. The IgE-binding activity of nPla a 6 was initially proved by Western-blot, and a similar IgE-binding pattern to rPla a 6 was also exhibited. Moreover, the positivity for specific IgE against rPla a 6 was tested as 45.95% (17/37) by ELISA, and IgE binding to pollen extract could be inhibited up to 45.77% by 10 µg/ml of rPla a 6. The protein was also confirmed to activate patients' basophils.

Conclusions: In this study, a novel allergen belonging to enolase family was comprehensively investigated and characterized through its natural and recombinant forms in *P. acerifolia* pollen. The study will contribute to the development of novel molecular-based diagnostic and therapeutic approaches for *P. acerifolia* pollen allergy.

1. Introduction

The prevalence of allergic diseases has increased dramatically in recent years [1]. These diseases affect approximately 20% of the population worldwide, and are considered to be one of the most common diseases [2]. Exposure to outdoor allergens is an important cause of

allergic diseases. Among these, pollen grains have been recognized as one of the most extensive and abundant sources of outdoor allergens [3], which have greater impact on human health.

London planetree (*Platanus acerifolia*) is a widely cultivated ornamental tree in urban areas. In recent years, the concentration of *P. acerifolia* pollen has increased significantly, being one of the

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important causes of pollinosis [4]. The European academy of Allergy and Clinical Immunology (EAACI) has identified *P. acerifolia* pollen as an important allergen causing respiratory symptoms and introduced it as a mandatory measure in pollen allergen surveys in all patients with suspected allergic rhinitis in Europe [5]. However, there are few studies concerning on the investigation of actual disease-eliciting allergens in this pollen [6]. So far, only 3 allergens (Pla a 1, Pla a 2, Pla a 3) of *P. acerifolia* pollen have been identified and officially listed in the Allergen Nomenclature database (<http://www.allergen.org/>). Pla a 1 is a specific non-glycosylated protein belonging to the invertase inhibitor family with the high IgE-prevalence of 83%-92% in *P. acerifolia* pollen allergic patients [7]. Pla a 2, a glycoprotein with a weight of 43 kDa, displays polygalacturonase (PG) activity, and also exhibited a high IgE-prevalence of 84% [8]. Pla a 3 is a minor allergen belonging to non-specific lipid-transfer protein (nsLTP) family, with IgE-prevalence of 27.3% [9]. However, in our preliminary 1-dimensional electrophoresis following by immunoblot (data not shown), more than 3 IgE-binding protein bands existed in pollen extract, which was consistent with the previous results of IgE-immunoblot on crude extract of *P. acerifolia* pollen [7]. Recently, our group reported the fourth new allergen from *P. acerifolia* pollen. It is a profilin protein with a molecular weight of 14 kDa, and identified as one of the major allergens in *P. acerifolia* pollen with high IgE-prevalence (76.9%) [10]. Thus, the above hints about understudied allergens prompted us to investigate the potential allergens in *P. acerifolia* pollen in depth.

In the present study, we screened a new IgE-binding band from the crude extract of *P. acerifolia* pollen by immunoblotting assays and identified the band as enolase by mass spectrometry. Subsequently, we isolated and purified the natural form of this protein from the crude extract of *P. acerifolia* pollen. Furthermore, the protein was cloned and expressed in *E. coli* to obtain its recombinant form. The allergenicity of this enolase was comprehensively characterized. It is now named as Pla a 6 by the Allergen Nomenclature database maintained by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. Our findings undoubtedly enriched the allergen components of *P. acerifolia* pollen and promoted the development of component-resolved diagnosis and molecular therapy of the *P. acerifolia* pollen allergy.

2. Material and methods

2.1. Serum samples

Serum samples were obtained from 37 patients with positive serum sIgE to *P. acerifolia* pollen measured by ImmunoCAP (Thermo Fisher Scientific/Phadia, Uppsala, Sweden), sIgE concentration value >0.35 kU/L to t11 in ImmunoCAP system were considered as positive. Sera from 7 healthy subjects were used as negative controls. This study was approved by the First Affiliated Hospital of Nanjing Medical University. (NO.2020-SR-401).

2.2. Preparation of crude extract and IgE immunoblot analysis

The pollen was mixed with 60 mL 10 mM phosphate-buffered saline (PBS) and a final concentration of 1 mM PMSF (Sangon, Shanghai, China). The mixture was incubated overnight at 4 °C with end-over-end mixing. After centrifugation at 12000 rpm, the supernatant was collected and filtered with a 0.22 µm membrane for further use.

For IgE immunoblot analysis, *P. acerifolia* pollen extract was separated on 13% SDS-PAGE and transferred onto 0.22 µm polyvinylidene difluoride (PVDF) membranes. PVDF membranes were then blocked with 5% (w/v) skimmed milk and incubated with 4 serum pools (diluted 1:10 in PBST) of *P. acerifolia* pollen allergic patients and a negative control pool of sera from healthy individuals at 4 °C overnight. After washed with PBST, bound IgE was detected by incubation with HRP-conjugated goat anti-human IgE (KPL, MD, USA) (1:5000 dilution) for

1 h at room temperature. The IgE-binding bands were visualized by Tanon 5200 multi-imaging system (Tanon, Shanghai, China) after incubation with Immobilon™ Western HRP Substrate Luminol Reagent (MerckMillipore, MA, USA).

The protein band at approximate 48 kDa exhibited positive IgE binding activity in the above immunoblot experiment (Figure S1) and contained mainly enolase as identified by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (data not shown).

2.3. Purification and identification of nPla a 6

P. acerifolia pollen extract was separated by sequential chromatography steps consisting of anion-exchange, gel filtration and cation-exchange chromatography on the ÄKTA-go System (GE Healthcare, Uppsala, Sweden). Briefly, the crude pollen extract was first loaded onto HiTrap desalting column equilibrated with 20 mM Tris-HCl (pH 8.4), the collected fraction was injected to HiTrap Q HP column (GE Healthcare, Uppsala, Sweden) equilibrated with the same buffer above. The protein adsorbed on the column was eluted with 20 mM Tris-HCl containing 1 M sodium chloride (pH 8.4) at a linear gradient from 0% to 100% at a flow rate of 5 mL/min and a time of 40 min. Fractions were then analyzed by 13% SDS-PAGE and visualized by Coomassie Blue R250 staining. The fractions with similar protein distribution were pooled and applied to a Superdex G200 increase 10/300 GL column (GE Healthcare, Uppsala, Sweden) which had been pre-balanced with 50 mM sodium phosphate containing 150 mM sodium chloride (pH 7.2), and eluted at a flow rate of 0.5 mL/min. The obtained fractions were analyzed by 13% SDS-PAGE and divided into different parts accordingly. The pooled fractions were further loaded onto 1 mL HiTrap S HP column (GE Healthcare, Uppsala, Sweden), and bound proteins were eluted with a linear salt gradient of 50 mM 2-morpholinoethanesulfonic acid containing 1 M sodium chloride (pH 6.0) from 0% to 50% at a flow rate of 1 mL/min and a time of 30 min, and subjected to 13% SDS-PAGE analysis.

The purified protein was then identified by LC-MS/MS. First, the protein was resolved on 13% SDS-PAGE and stained with Coomassie Bright Blue R250. The single protein band with molecular weight approximate 48 kDa was excised from the gel and digested with trypsin (Promega, WI, USA). The peptides were analyzed by Easy-NanoLC1200 and Q-Exactive HF mass spectrometry (Thermo Fisher Scientific, MA, USA) at Nanjing Helan Biotech Co. Ltd. The internal peptides of this protein were *de novo* analyzed using pNovo software with default settings.

2.4. cDNAs cloning and sequencing of Pla a 6

According to transcriptome sequencing results [10], the internal peptides from LC-MS/MS were matched with the results of mRNA sequencing to obtain the theoretical cDNA sequence encoding Pla a 6. T-A cloning and Sanger sequencing were performed to further confirm the sequence. Briefly, total RNA was isolated from pollen by using MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China) and the quantification was performed by measuring the absorbance ratio at 260/280 nm. Then, total RNA was converted to cDNAs using a commercial cDNA synthesis kit according to the manufacturer's instructions (Takara Biotech Co., Dalian, China). The cDNA encoding Pla a 6 was amplified by PCR using primers based on the matched nucleotide sequence of Pla a 6 gene. The forward and reverse primers were designed as 5'-CGA-GAAAAATCGGAGAGAACTAC-3' and 5'-CTTCTTGCTAACCGTAA-TAAGC-3', respectively. PCR condition was 98 °C/5 s (one cycle), 98 °C/10 s, 55 °C/30 s and 72 °C/1 min (30 cycles), and 72 °C/10 min (one cycle). The PCR amplification products were recovered, purified and cloned into pCE2 TA/Blunt-zero vector (Vazyme, Nanjing, China) for transformation into the *E. coli* JM109. Positive clones were screened by on Luria-Bertani (LB) plates containing 50 µg/mL kanamycin and confirmed by DNA sequencing.

2.5. Expression and purification of the recombinant Pla a 6 in *Escherichia coli* (*E. coli*)

The rPla a 6 protein was expressed as 6 × His-tagged protein. The forward primer 5'-TAAGAAGGAGATATACCATGGCGACGATCAAGG CCGTTAAA-3' and the reverse primer 5'-GTGGTGGTGGTGGTCTCG AGGTAAGGTTCCACTGGTGC-3' were designed and the Nco I and Xho I restriction sites (bolded) were added, respectively. The PCR condition was 98 °C/5 s (one cycle), 98 °C/10 s, 55 °C/30 s and 72 °C/1 min (30 cycles), and 72 °C/10 min (one cycle). Then, the PCR amplification products were subcloned into the pET28a vector by using a *one-step-clone kit* according to the manufacturer's instructions (Vazyme Biotech Co., Ltd, China). The positive clones were verified by DNA sequencing. The recombinant plasmid was transformed into *E. coli* BL21 Star (DE3) competent cells, and the positive recombinant strains were grown up based on kanamycin resistance. The positive colonies were selected and cultured in LB medium containing 50 µg/mL kanamycin at 37 °C with shaking at 230 rpm. When the optical density of the culture reached 0.6 at A_{600nm} , isopropyl-β-D-thio-galactopyranoside (IPTG) was added at a final concentration of 1 mM, followed by incubation for another 6 h at 37 °C with shaking at 160 rpm. After recombinant enolase expression, the cells were collected via centrifugation at 4000 rpm for 20 min. Subsequently, the bacterial cells were re-suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) and then lysed with 60% ultrasonic power (9 s pulse-on, 5 s pulse-off) for 30 min on ice by sonication. After centrifugation at 12000 rpm for 20 min at 4 °C, the soluble supernatant and insoluble particles containing inclusion bodies were collected and analyzed by SDS-PAGE. The inclusion bodies were solubilized for the next purification.

The rPla a 6 was purified by a combination of High Affinity Ni-NTA Resin (GenScript, Nanjing, China) and Superdex G75 increase 10/300 GL column (GE Healthcare, Uppsala, Sweden). In brief, after the inclusion body was dissolved with 8 M urea solution buffer (100 mM NaH₂PO₄, 10 mM Tris and 8 M urea, pH 8.0), the solution was loaded onto the pre-balanced nickel affinity column and washed with low concentration imidazole buffer (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, 10–20 mM imidazole pH 8.0) to remove the low affinity binding protein. Then elute the protein with higher concentration imidazole (250 mM). The eluted recombinant Pla a 6 was characterized and refolded according to the previously reported method [11]. The protein after renaturation was pooled and applied to a Superdex G75 increase 10/300 GL column (GE Healthcare, Uppsala, Sweden) which had been pre-balanced with 50 mM sodium phosphate containing 150 mM sodium chloride (pH 7.2), and eluted at a flow rate of 0.8 mL/min. The obtained recombinant Pla a 6 was analyzed by SDS-PAGE.

2.6. IgE immunoblotting

The IgE binding activity of nPla a 6 was detected by IgE Immunoblotting. Briefly, the purified nPla a 6 protein was electrophoresed on 13% SDS-PAGE and transferred onto 0.22 µm polyvinylidene difluoride (PVDF) membranes. After blocked with 5% skimmed milk in phosphate buffered saline (PBS) for 2 h at room temperature, membranes were cut into strips and incubated with 37 allergic patients' sera (diluted 1:10 in PBST) at 4 °C overnight. After washing three times with PBST, the membranes were incubated with HRP-conjugated goat anti-human IgE (KPL, MD, USA) (1:5000 dilution) followed by incubation with Immobilon™ Western HRP Substrate Luminol Reagent (MerckMillipore, MA, USA). Finally, the IgE-binding bands were visualized by Tanon 5200 multi-imaging system (Tanon, Shanghai, China).

Further, we selected 12 serum samples that were positive in the above experiment to conduct western blot on the recombinant form of Pla a 6 with the same procedure, in order to confirm that it has the similar IgE binding pattern with its corresponding natural form.

2.7. Immunoreactivity of human serum with the rPla a 6 by ELISA

The 96-well plates (Corning Costar, Massachusetts, USA) were coated with 1 µg of rPla a 6 in PBS per well and incubated at 4 °C overnight. Then, each well was blocked with PBST (PBS-0.1% Tween 20) solution containing 1% BSA at 37 °C for 2 h. Serum samples (diluted 1:10 in PBST) were added to the plate (100 µL/well) and incubated at 37 °C for 1 h. After washing three times with PBST, 100 µL of HRP-conjugated goat anti-human IgE (KPL, MD, USA) (1:2500 diluted in PBST) was added and incubated at 37 °C for 1 h. Subsequently, the color was developed using TMB substrate (Beyotime, Shanghai, China) for 13 min. Then 50 µL 2 M H₂SO₄ was added to terminate the reaction, and the absorbance was read at 450 nm using Multiskan GO (Thermo Fisher Scientific, MA, USA). The cut-off value was presented as mean optical density (OD) value + 3 standard deviations (SD) of negative controls [10].

2.8. Inhibition ELISA test

The 96-well plates (Corning Costar, NY, USA) were coated with 100 µL crude extract at 10 µg/mL in PBS overnight at 4 °C. Five serum samples that showed positive both in ELISA and western blot experiments were selected to form a mixed serum pool (1:10 diluted in PBST). The serum pool was pre-incubated overnight with increasing concentrations of the crude extract and rPla a 6 at 4 °C. Then, each well was blocked with PBST solution containing 1% BSA at 37 °C for 2 h. After washing three times, 100 µL of pre-incubated serum was added to each well and incubated at 37 °C for 1 h. The IgE binding activity was detected by 100 µL HRP-conjugated goat anti-human IgE (1:2500 dilution) and reacted with TMB substrate (Beyotime, Shanghai, China) for 13 min. Then 50 µL 2 M H₂SO₄ was added to terminate the reaction, and the absorbance was read at 450 nm using Multiskan GO (Thermo Fisher Scientific, MA, USA). The inhibition rate was calculated as following formula: (OD450 uninhibited-OD450 inhibited) / (OD450 uninhibited-OD450 control) × 100%.

2.9. Basophil activation test

The basophil activation test (BAT) is a functional assay that measures basophil response to allergen cross-linking IgE on cell surface [12]. The expression of CCR3 and CD63 on the surface of basophils is considered to be the indicator of basophil activation. Whole blood donated by 5 volunteers within 24 h was lysed with red blood cell lysis buffer (Femac, Nanjing, China) according to the previously reported method [10]. Basophils were stimulated with nPla a 6 protein (final concentration, 10 µg/mL) at 37 °C for 30 min, anti-IgE antibody was used as a positive control and buffer alone as a negative control. The reactions were terminated with 900 µL pre-cooled PBS solution containing 2.5 mM EDTA, followed by centrifugation at 1000 rpm for 5 min at 4 °C. Subsequently, the supernatant was removed, and the pellets were resuspended with 100 µL of the staining solution containing 5 µL of Alexa647-conjugated anti-CD63 mAb (Biolegend, CA, USA) and 5 µL of PE-conjugated anti-CCR3 mAb (Biolegend, CA, USA) and incubated for 20 min on ice. Basophil expressions of CD63 and CCR3 were evaluated by FACSCanto Plus and analyzed using the build-in Diva software (BD Bioscience, CA, USA).

2.10. Conservation analysis and structure prediction of Pla a 6

MEGA7 was used to generate phylogenetic trees based on the homologous enolase allergen sequences recorded in WHO/IUIS database, and Figtree software was used for visualization (<https://tree.bio.ed.ac.uk/software/figtree/>). The amino acid sequence alignments among Pla a 6, Amb a 12 and Hev b 9 were performed by ClustalX2 [13] and displayed by ESPript 3.0 [14]. Homology modeling was performed by AlphaFold2, the three-dimensional (3D) protein structure was displayed

using Discovery Studio 2021 Client.

3. Results

3.1. Screening and purification of nPla a 6 from *P. acerifolia* pollen extract

In our preliminary screening results, a protein band at approximate 48 kDa exhibited positive IgE binding activity with sera from allergic patients (Figure S1), which was identified as enolase. The enolase was purified from the crude extract of *P. acerifolia* pollen by sequential chromatography (Fig. 1). It migrated as a single protein band with a predicted molecular mass of 48 kDa by SDS-PAGE (Fig. 1C). The identity was confirmed by LC-MS/MS, which was consistent with the IgE binding active band we initially identified in the crude extract. A total of 9 peptides were obtained by *de novo* sequencing of the nPla a 6 based on the LC-MS/MS raw data, which were all, EALELR, DGGSDYLGG, KIPLYK, HIANLSGNK, NGGSHAGNK, MGVEVYHHLK, FKEENNDSQK, RAGWGVMSHR and YNQLLR (Figure S3). The *de novo* analyzed internal peptides were underlined in Fig. 2, which had 17.3% coverage to the full-length amino acid of Pla a 6.

3.2. cDNA cloning of Pla a 6

Based on the identified peptides of nPla a 6, the corresponding cDNA sequence was retrieved from the results of transcriptome sequencing, and amplified by PCR based on the designed primers. The cDNA fragments contained a 1338-bp open reading frame (ORF) encoding 445 amino acids (Fig. 2). The sequence data has been deposited in the GenBank database (Accession No. OK323202). According to the amino acid sequence, the molecular weight of Pla a 6 was calculated as 48 kDa, which was consistent with that in SDS-PAGE (Fig. 1C).

3.3. Expression and purification of the recombinant Pla a 6

The recombinant Pla a 6 was expressed as inclusion bodies (Fig. S2A, lane 4). The targeted recombinant protein was purified by a combination of High Affinity Ni-NTA Resin under denaturing conditions. Gradual removal of denaturant by dialysis along with redox refolding buffer for protein refolding. The refolded protein was further purified and migrated as a single band in SDS-PAGE (Fig. S2B, lane 1).

3.4. IgE reactivity of sera from patients to *P. acerifolia* pollen

In order to determine the allergenicity of Pla a 6, we tested its ability to bind IgEs in the serum of *P. acerifolia* pollen-allergic patients. The IgE-binding activity of nPla a 6 was detected by IgE immunoblots using 37 individual serum samples from *P. acerifolia* pollen-allergic patients. Twelve patients (32.4%) revealed IgE-binding activity to the nature Pla a 6 (Fig. 3A), of which seven serum samples had high specific IgE binding capability, showing relatively strong IgE binding bands, and the remaining five serum showed weak IgE binding bands. Further, these 12 positive sera were selected to confirm the IgE-binding capability of rPla a 6. Immunoblots showed that rPla a 6 had almost the same IgE-binding pattern with nPla a 6 among these 12 positive patients' sera, in which 11 had distinct or slight IgE binding bands to rPla a 6 and only one (No. 9) had negative result (Fig. 3B). The allergenicity of the recombinant Pla a 6 was then determined by ELISA using the same 37 individual serum samples. As shown in Fig. 3C, seventeen (45.95%) out of 37 allergic serum samples showed positive reaction to rPla a 6.

We performed competitive inhibition ELISA with rPla a 6 on sera pool from *P. acerifolia* pollen allergic patients. Five patients with high IgE-binding reactivity to Pla a 6 were collected to form a mixed serum pool for competitive inhibition ELISA test. IgE binding to pollen extract could be inhibited up to 45.77% by 10 µg/mL of rPla a 6 (Fig. 3D).

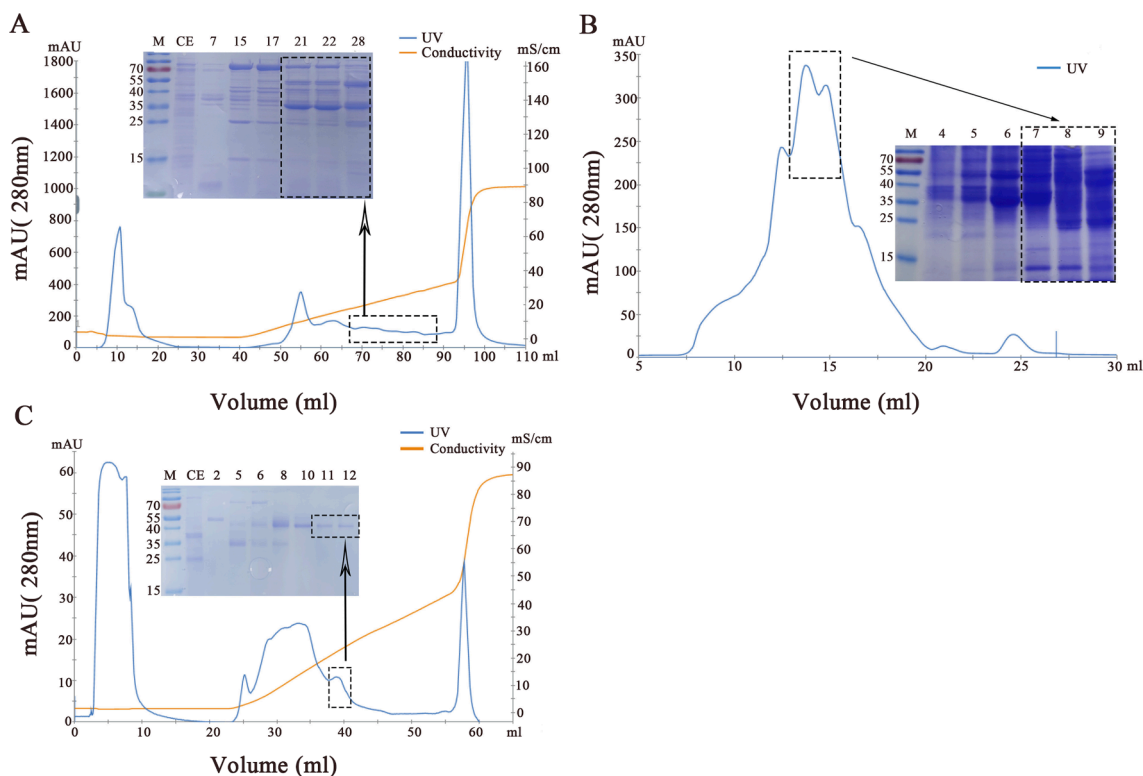


Fig. 1. Purification of nPla a 6 from *P. acerifolia* pollen extract. (A) Elution profile of *P. acerifolia* pollen extract on HiTrap Q HP anionic exchange column, the fractions highlighted with arrow were pooled for further purification. (B) Elution profile of the pooled fractions on Superdex G200 column, the fraction 7–9 were pooled for further purification. (C) The pooled fractions were further purified by loading onto a 1 mL HiTrap SP HP column. The purified Pla a 6 from *P. acerifolia* pollen extract was highlighted with black box.

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1      CGAGAAAAATCGGAGAGAACTACTTCGATCGAACAAAAAATGGCGACGATCAAGGCCGTT
      M A T I K A V
21     AAAGCTAGGCAGATCTTTCGACAGCCGTGGAACCCCTACCGTTGAAGTGGATGTTACTATT
      K A R Q I F D S R G N P T V E V D V T I
41     TCCGATGGAACCTAGCTAGGGCTGCTGTTCCGAGCGGTGCTTCGACTGGTATCTACGAA
      S D G T L A R A A V P S G A S T G I Y E
61     GCTCTGGAATTAAGAGATGGAGGATCGGACTACCTTGGGAAGGGTGTACTTAAGGCTGTA
      A L E L R D G G S D Y L G K G V L K A V
81     GAGAATGTCAATGCAATCATTGGACCCGCTCTGATTGGAAAGGATCCAAGTACGAGACA
      E N V N A I I G P A L I G K D P T E Q T
101    AAGATTGACAACCTTCATGGTACAAGAGCTAGATGGAAACAGTTAATGAGTGGGGTGGTGC
      K I D N F M V Q E L D G T V N E W G W C
121    AAACAGAAGCTTGGTGCAAAATGCTATATTGGCAGTATCACTTGCCATCTGTAAGGCAGGA
      K Q K L G A N A I L A V S L A I C K A G
141    GCTAGTGTGAAAAAGATCCCCCTTTATAAGCATATCGCCAATCTTTCTGGGAACAAAACC
      A S V K K I P L Y K H I A N L S G N K T
161    CTAGTGTTGCCGGTCCCTGCGTTCAATGTCATCAATGGAGGTTACATGCAGGCAATAAA
      L V L P V P A F N V I N G G S H A G N K
181    CTAGCGATGCGAGAGTTTATGATCCTTCTGTTGGGGCGTCTTCTTCAAGGAAGCAATG
      L A M Q E F M I L P V G A T S F K E A M
201    AAGATGGGTGTAGAAGTATATCATCACTTAAAGGCTGTGATTAAGAAAAATATGGCCAA
      K M G V E V T Y H H L K A V I K K K Y G Q
221    GATGCCACTAATGTTGGTGATGAAGGTGGTTTGGCCCTAACATTACAGGAAAACAAAGAG
      D A T N V G D E G G F A P N I Q E N K E
241    GGCTTTGAAGTGTGAAGACGCCATAGCAAAAGCTGGATACACTGGAAAAGTGGTGATT
      G L E L L K T A I A K A G Y T G K N K T
261    GGGATGGATGTGCGAGCGTCAGAGTTCTATGATAACAAGACAAGACTTATGATTGAAT
      G M D V A A S E F Y D N K D K T Y D L N
281    TTTAAGGAGGAGAACAATGATGGGTACAGAAAATATCAGGAGATAGTTGAAGATGTC
      F K E E N N D G S Q K I S G D S L K N V
301    TACAAGTCCTTTGTGACTGATTATCCAATCGTATCCATAGAGGATCCATTTGATCAGGAT
      Y K S F V T D Y P I V S I E D P F D Q D
321    GATTGGGAGCACTATGCCAAGTTGACGGCTGAAATTGGCCAAACAGTACAGATTGTTGGG
      D W E H Y A K L T A E I G Q Q V Q I V G
341    GATGATCTCCTTGTGACGAATCCAAAGCGTGTAGATAAGGCAATCAAGGAGAAGACCTGC
      D D L L V T N P K R V D K A I K E N K T
361    AATGCCCTTTTGTGTAAGGTGAATCAAATTGGCTCTGTCACTGAGAGCATTGAAGCTGTC
      N A L L L K V N Q I G S V T E S I E A V
381    AAAATGTCCAACGTGCTGGCTGGGGTGTGATGGCAAGCCACCGAAGTGGTGAGACCGAG
      K M S K R A G W G V M A S H R S G E T E
401    GATACTTTCATTGCAGACCTTTTCAGTTGGTTTGGCAACGGGTCAGATTAAAGACAGGAGCT
      D T F I A D L S V G L A T G Q I K T G A
421    CCTTGCAGGTCAGAACGTCTTGCAAAATATAACCAGCTTCTTAGGATTGAAGAAGAGCTT
      P C R S E R L A K Y N Q L L R I E E E L
441    GGATCAGCAGCAGTCTATGCGGGAGCAAATTTCCGAGCACCAGTGGAACCTTACTAGATG
      G S A A V Y A G A N F R A P V E P Y *
461    CTTATTACGGTTAGCAAGAAG

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Fig. 2. cDNA sequence encoding Pla a 6 and deduced amino acid sequence. Complete nucleotide sequence and amino acid sequence of Pla a 6. The underlined peptides were identified by LC-MS/MS from the crude extract.

For BAT, 5 different *P. acerifolia* pollen-allergic patients' basophils was used to determine the proportion (%) of CD63-positive out of CCR3-positive cells after stimulation. Anti-IgE was used as a positive control and PBS buffer along as a negative control. In comparison with the negative control, the nPla a 6 at 10 µg/mL induced an approximate 1.8-fold to 12.7-fold increase in the proportion (%) of CCR3 and CD63 double-positive cells (Fig. 4), which further confirmed the allergenicity of Pla a 6.

3.5. Conservation analysis and structure prediction of Pla a 6

The phylogenetic tree and multiple sequence alignment result demonstrated that Pla a 6 was closer with Amb a 12 (91%), the enolase from ragweed and Hev b 9 (90%), the enolase from rubber latex (Fig. 5A, B). A new computational method, AlphaFold 2, was used to predict the structure of Pla a 6. The three-dimensional (3D) protein structure was displayed using Discovery Studio 2021 Client. The secondary structure prediction showed that the single chain of Pla a 6 consists of 14 α-helices

(residues 64–72, 74–78, 86–95, 113–130, 135–143, 183–205, 225–238, 281–294, 309–319, 335–344, 354–357, 360–372, 390–397, 411–427) and 16 β-sheets (residues 4–13, 19–27, 32–36, 101–102, 105–106, 152–159, 172–176, 246–250, 257–258, 263–265, 278–279, 297–299, 324–327, 349–352, 376–380, 401–404) (Fig. 5C). The residues with high solvent accessible score were calculated according to the 3D model of Pla a 6 (Fig. 5D).

4. Discussion

Pollen is one of the most significant outdoor allergens. The number of allergen-sensitized individuals has been increasing in recent years, especially in industrial areas [15]. *P. acerifolia* pollen have been shown to be an important cause of pollinosis in Spring. The trees are widely planted in urban areas of Western Europe, Australia, New Zealand, North America, Iran and also in many cities in China [16]. Three allergens have been identified in *P. acerifolia* pollen, two of which were recognized as major allergens (Pla a 1 and Pla a 2) and the other one (Pla

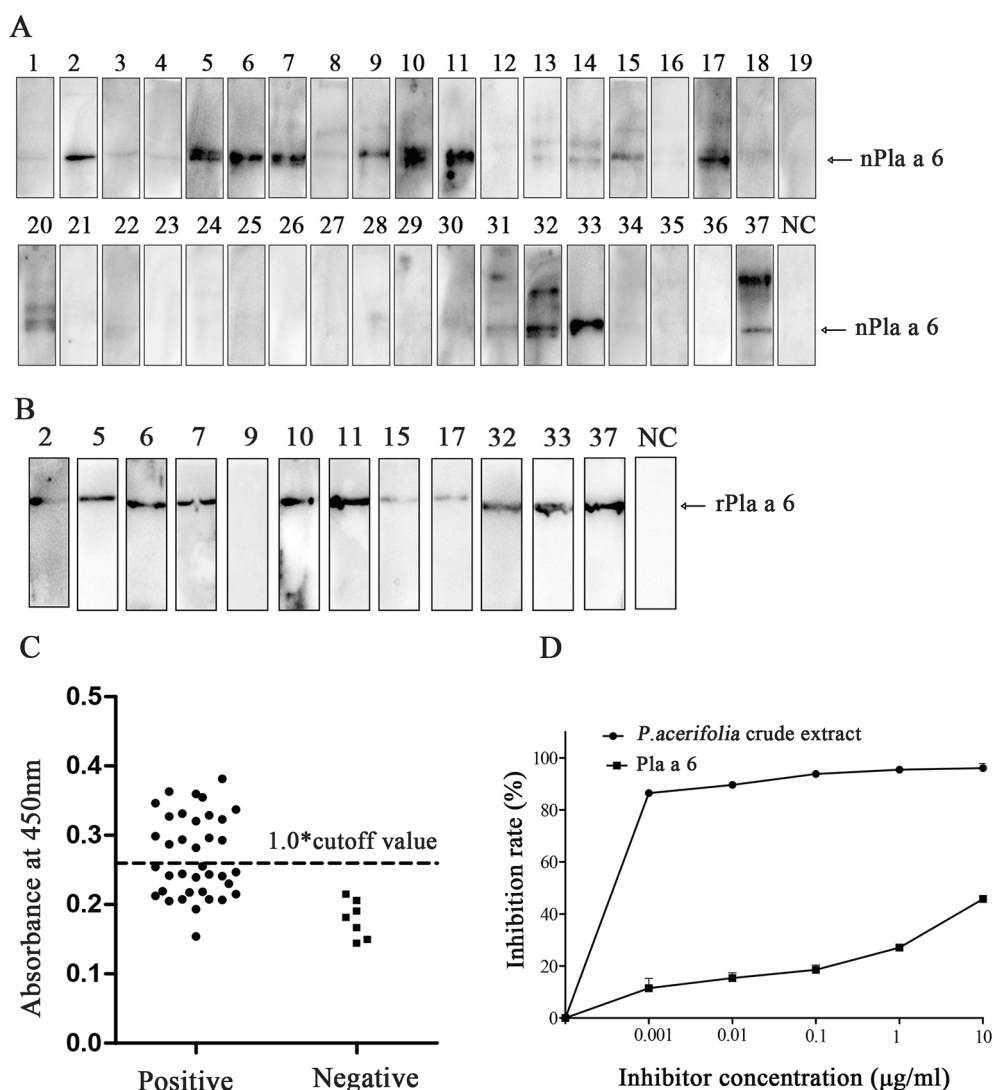


Fig. 3. IgE reactivity of Pla a 6. (A) Western blot analysis of IgE reactivity to nPla a 6 in the sera from the patients. nPla a 6 was incubated with serum samples of 37 (lanes 1–37) *P. acerifolia* pollen-allergic patients and 1 (lane NC) non-allergic individual. (B) Confirmation of the IgE-reactivity by western blot analysis. The rPla a 6 was incubated with 12 sera samples that were positive for nPla a 6 in western blot and 1 non-allergic individual. Numbers referred to that in Fig. 3A; NC, non-allergic individual. (C) Assessment of IgE-reactivity of rPla a 6 by ELISA using sera from 37 *P. acerifolia* pollen allergic patients and 7 healthy subjects. The cutoff value was calculated as the mean absorbance value of the 7 negative samples plus 3 standard deviations. (D) Inhibition ELISA test with the rPla a 6 and *P. acerifolia* pollen extract. Inhibitor concentrations ranged from 0 to 10 μg/mL. The data was presented as mean ± SD (n = 3).

a 3) was reported as a minor allergen. Recently, our group reported the fourth allergen, profilin from *P. acerifolia* pollen as a major allergen in *P. acerifolia* pollen [10]. However, there were still several unknown IgE binding components in this pollen [7]. Therefore, it is necessary to make in-depth research to this pollen allergen components.

In the present study, we conducted a preliminary western blot experiment on the crude extract of *P. acerifolia* pollen, and screened a high positive IgE binding activity band with a molecular weight of 48 kDa. The protein band in the crude extract was mainly identified as enolase by mass spectrometry. Through the traditional chromatographic strategies combined with LC-MS/MS characterization, the new IgE binding protein belonging to enolase family was purified from the extract of *P. acerifolia* pollen, which was identical to the band we initially identified by mass spectrometry in the crude extract. A total of 9 internal peptides were obtained from the MS results. The cDNA sequence encoding Pla a 6 was obtained by matching the internal peptides with the results of high-throughput pollen transcriptome sequencing, and was further confirmed by T-A cloning and Sanger sequencing. The cDNA fragments contained a 1338-bp open reading frame (ORF) encoding 445 amino acids. The sequence information has been submitted to GenBank under accession number of OK323202. This novel allergen belonging to enolase family was named as Pla a 6 by the WHO/IUIS Allergen Nomenclature Sub-committee. Analysis of the secondary structure of Pla a 6 showed that it contained 14 α -helices and 16

β -sheets. Further, the IgE-binding activity of nPla a 6 was detected by IgE immunoblots using 37 individual serum samples from *P. acerifolia* pollen-allergic patients. Twelve patients (32.4%) revealed IgE-binding activity to the nPla a 6. However, a small amount of nPla a 6 western blot results showed the existence of other IgE binding bands, indicating that there may still be a very small number of other components in this purified nPla a 6, which can bind to the serum IgE of some patients. Hence, it is difficult to get very high-purity natural enolase protein from pollen extract, so we obtained the recombinant form of the protein through expression and purification, in order to further study its characteristics more comprehensively. We firstly demonstrated that rPla a 6 and its natural counterpart had the similar IgE binding pattern through western blot. Then, the allergenicity of the recombinant Pla a 6 was determined by ELISA using the same 37 individual serum samples mentioned above and the positive rate was 45.95%. The difference in positive rate between western blot and ELISA might be related to the destruction of the conformational epitopes of enolase during western blot. In ELISA inhibition assays, the maximum inhibition rate of rPla a 6 on the positive serum pool were 45.77%. Furthermore, we demonstrated that Pla a 6 had the ability to induce basophil activation through BAT experiments. CD63 was used as the degranulation marker of basophils, which was the most commonly used activation marker at present. The CD63 molecule is a tetraspan granule protein that is not expressed on resting basophils, but is upregulated in high density after being

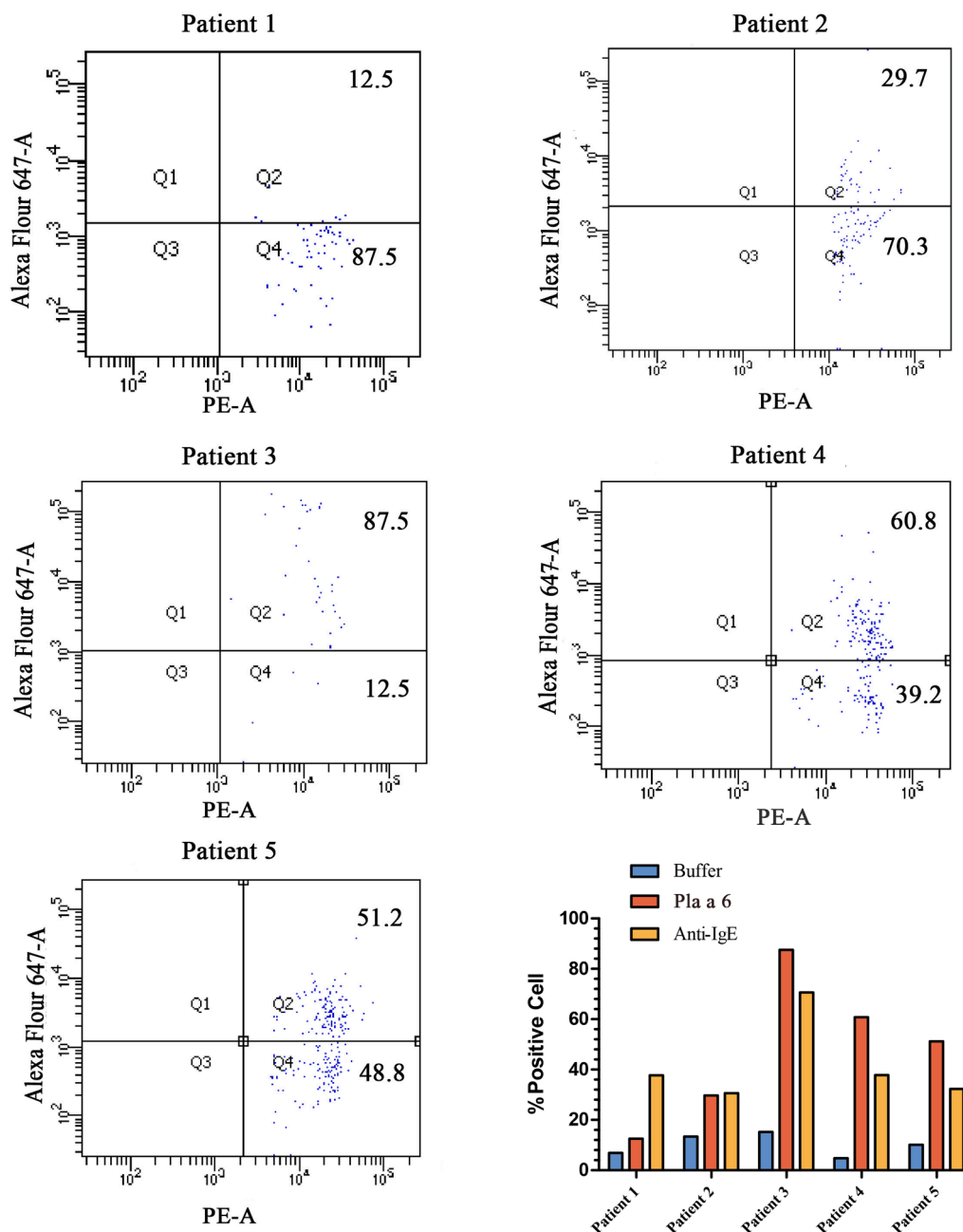


Fig. 4. Basophil activation test. (A) Representative flow cytometry plots of basophils from different *P. acerifolia* allergic patients and stimulated with nPla a 6. Basophil activation was assessed by monitoring the proportion of CCR3 and CD63 positive cells. (B) The calculated proportion (%) of CCR3 and CD63 positive cells after stimulated with PBS, anti-IgE, and Pla a 6.

stimulated [17]. In the present study, Pla a 6 caused an approximate 1.8-fold to 12.7-fold upregulation in the proportion (%) of CCR3 and CD63 double-positive cells, which further indicated that it was an important allergen component in *P. acerifolia* pollen.

Enolase is a key glycolytic enzyme, which plays an important role in plant growth and development. It is required to produce ATP as part of the glycolysis pathway and a highly conserved enzyme in all eukaryotes and most prokaryotic cells [18]. It was reported that enolases from a variety of organisms, including plants [19], yeasts [20], fungi [18], cockroaches [21] and pollens [22,23] can cause IgE induced responses. Unfortunately, only two enolases found in pollen were registered in IUIS database, namely Amb a 12 from ragweed pollen and Cyn d 22 (the amino acid sequence is currently unknown) from Bermuda grass pollen. But there was no biochemical characterization of Amb a 12 and Cyn d 22 has yet been reported. To the best of our knowledge, this is the first

report to purify enolase from tree pollen and to conduct in-depth research on it.

Previous studies on the IgE-binding activity of enolases have been mainly focused on a variety of fish and different fungi. The sensitization rate of enolase in fish species ranges from 17% to 56%, among which 19% of Thu a 2 from *Thunnus albacares*, 56% of Gad m 2 from *Gadus morhua*, 34% of Sal s 2 from *Salmo salar* [24], 17% of Cyp c 2 from *Cyprinus carpio* and 21% of Pan h 2 from *Pangasianodon hypophthalmus* [25]. In fungus, the sensitization rate of enolase ranges from 20% to 100%, such as 22% of Alt a 6 from *Alternaria alternata* [26], 20% of Cla h 6 from *Cladosporium herbarum* [27], 100% of Cur l 2 from *Curvularia lunata* [28], 21% of Rho m 1 from *Rhodotorula mucilaginosa* [20], 30% of Pen c 22 from *Penicillium citrinum* and Asp f 22 from *Aspergillus fumigatus* [29]. Similarly, the sensitization rate of enolase was also varied widely in plant species. For example, Amb a 12 (66%) from

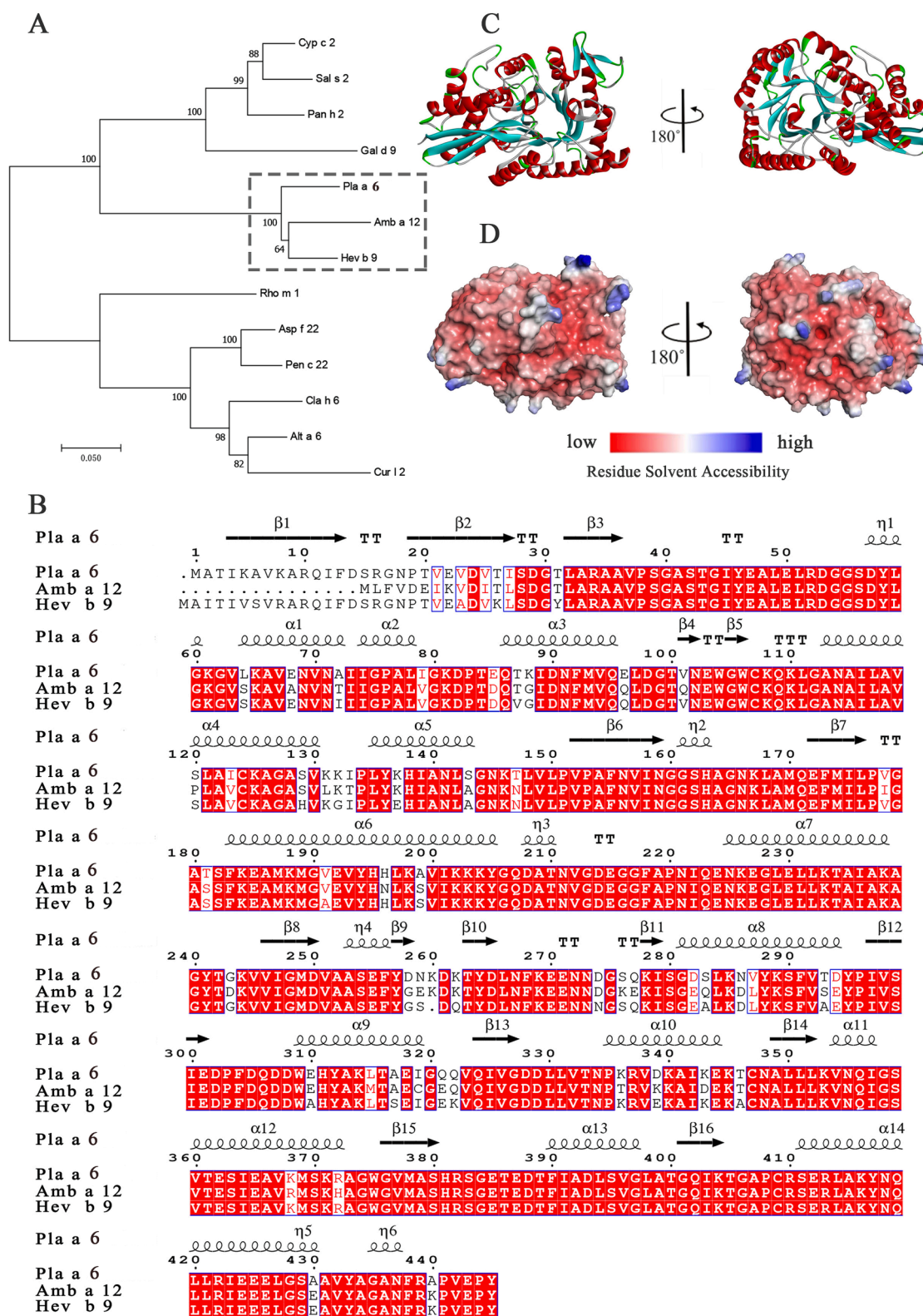


Fig. 5. Conservation analysis and structure prediction of Pla a 6. (A) Phylogenetic analysis of Pla a 6 and other homologous allergens recorded in WHO/IUIS. (B) The positions of typical secondary structures in Pla a 6 and the alignment of the primary sequence with Amb a 12 and Hev b 9. The identical and conserved sequences were highlighted by red block and white block, respectively. The sequence identity was 91% with Amb a 12 and 90% with Hev b 9. (C) A three-dimensional (3D) structure shown in cartoon mode. Monomers were used as an illustration and surface characterization, in which the α -helix, β -strand, and loop are painted red, blue and green, respectively. (D) Construction of the 3D protein structure of Pla a 6. The score of residue solvent accessibility was presented from red to white to blue, which indicated the low to high score. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ragweed have been identified as major allergens while Hev b 9 from *Hevea latex* was only 14.5% [30]. In this study, the IgE-binding rate of rPla a 6 in ELISA was 45.95%, lower than those of Pla a 1 (79%-83%) [7,31], Pla a 2 (37.9%-84%) [8,9] and Pla a profilin (76.9%) [10] but higher than Pla a 3 (27.3%) [9].

Some *P. acerifolia* pollen allergens seem to have a potential for cross-reactivity, including well-conserved proteins such as profilin [10], polygalacturonase (Pla a 2) [8,32], and nsLTP (Pla a 3) [33]. The increasing number of polysensitized patients to several pollens has emphasized the importance of pan-allergens. In some previous studies, enolase has been proved to be a cross-reacting component between fungi and plants [30], and between different food such as fish and chickens [34]. The immunoproteomic studies also found that enolases among the proteins recognized by IgE antibodies present in the serum of patients sensitized to several kinds of pollens [35]. Sequence comparisons and three-dimensional structure determinations can facilitate future studies on the role of enolase in pollens or plants cross-reactivity. The phylogenetic tree showed a high degree of similarity between Pla a 6, Amb a 12 (enolase of ragweed) and Hev b 9 (enolase of rubber latex), which may be evolved originally from the same ancestor gene. On this basis, we conducted homology alignment for these three sequences and found that the amino acid sequence of Pla a 6 was highly homologous with Amb a 12 (91%, accession No. [KU593514.1](#)) and Hev b 9 (90%, accession No. [AJ132580](#)). The important amino acid residues responsible for the active site of *S. cerevisiae* enolases have been described and proved to be located in conserved regions. Stefan *et al.* [30] have demonstrated that this region presents in Hev b 9 sequence. In this study, sequence alignment of enolases revealed that the regions also exist in Pla a 6. The 3D structure of Pla a 6 was modeled by AlphaFold 2 server and contained a total of 14 α -helices and 16 β -sheets. In general, although Pla a 6 was not the main *P. acerifolia* pollen allergen in this study, its cross-reactivities and the possible cross-reactivities between pollens or plants need to be investigated in the future.

In conclusion, the study identified and characterized a new allergen belonging to the enolase family in *P. acerifolia* pollen and this is the first report to in-depth study enolase from tree pollen. Currently, allergy diagnostic tests still mainly based on natural allergen extracts. Our study further enriched the molecular information of allergen component in *P. acerifolia* pollen, and it will pave the way for molecular-based diagnosis and treatment for *P. acerifolia* pollen allergy.

Authors' contributions

Ji-Fu Wei, Jin-Lyu Sun and Man Tian designed the study. Yong-Xin Jiao, Le-Bin Song, Zhi-Qiang Xu, Yong-Shi Yang and Dan-Xuan Zhu contributed to data collection. Yong-Xin Jiao and Zhi-Qiang Xu performed the statistical analysis and interpretation of the results. Yong-Xin Jiao and Le-Bin Song wrote the manuscript and Ji-Fu Wei revised it.

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CRediT authorship contribution statement

Yong-Xin Jiao: Investigation, Formal analysis, Data curation, Writing – original draft. **Le-Bin Song:** Methodology, Validation, Data curation. **Zhi-Qiang Xu:** Project administration, Resources, Investigation. **Dan-Xuan Zhu:** Resources, Investigation. **Yong-Shi Yang:** Investigation. **Man Tian:** Conceptualization, Supervision, Writing – review & editing. **Jin-Lyu Sun:** Conceptualization, Supervision, Writing – review & editing. **Ji-Fu Wei:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2022.109313>.

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