# Identification and characterization of a pan-allergen profilin, a major allergen from *Caryota mitis* pollen

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**Summary**

***Background:*** ***Caryota mitis*** **is a common plant in tropical and subtropical areas. It produces larger amount of pollen, which has great potential for allergenicity in the pollination season.**

***Objective:*** **This study aims to identify the components of *Caryota mitis* pollen contributing to human allergic diseases.**

***Methods:*** **The sera from 20 patients with a positive skin prick test to *Caryota mitis* pollen crude extract (CmPCE) were collected; the allergic components were determined by the forming of immune complexes in the sera and CmPCE. The results were confirmed by the immune competitive inhibitory assay.**

***Results:*** **Sixteen out of 20 collected serum samples reacted to a 14 kDa protein fractioned from CmPCE; this 14 kDa antigen also had positive reactions to CmPCE in an ELISA assay. 12 samples from 20 collected serums positively reacted to recombinant CmProfilin (rCmProfilin), as shown by Western blotting, and also showed positive reactivity in ELISA. Preincubation of sera with rCmProfilin eliminated the reactivity of the patients' sera to this 14 kDa band.**

***Conclusion:*** **A 14 kDa protein from the CmPCE was identified as the major allergic component of CmPCE.** ***(Asian Pac J Allergy Immunol 2014;32:39-45)***

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## Introduction

The prevalence of asthma and allergy has continued to increase in the last few decades. It has been estimated that more than 20% of the world's population suffers from IgE-mediated allergic diseases.[1] Airborne allergens derived from pollens are considered to be one of the most important causes of asthma and rhinitis.[2]

In urbanized environments, the plantating of trees for ornamental purposes is increasing, both in city avenues, street-walks and in public parks, which may impose a great allergenic impact on the health of the residents. A relationship between tree pollen allergy and hospital admission or exacerbation of respiratory allergic symptoms has been recognized and several tree pollen allergens have been identified and characterized.[3] Previous research showed that the Fagales, including the families of the Betulaceae, Corylaceae, and Fagaceae, developed in North and Middle Europe, North-West Africa, East Asia, and from North America to the Andes, predominantly flower in spring. Moreover the Oleaceae family extended southward and was found in the Mediterranean area, where the olive tree is the most potent elicitor of type I allergy.[3] Oriental plane trees, *Platanus orientalis*, are an important source of airborne allergens in cities of southwest Asia and southeast Europe.[4] Platanus pollen is a major contributor to symptoms of pollinosis during March and April.[5] A high prevalence of positive skin prick tests for *P. orientalis* pollen extract (43%) has been recorded in Mashhad, Iran. Platanus, Acer, Salix, Quercus, Betula and Populus pollen were aerobiologically characterized and linked with monthly emergency hospital admissions.[6] The most important pollens producing pollinosis in Spain are those from cypress trees, birch trees, *Platanus hispanica*, olive trees, Parietaria and Chenopodium and/or Salsola.[7]

Currently, the allergenic extracts are used in allergy clinic to diagnose allergic disorders, which are as well as the major vaccine employed in the specific immunotherapy.[8] After reviewing the literature available, we realized that among the allergen directory (about 400-500 species) provided by World Health Organization (WHO) and world-famous allergy companies (e.g., ALK company, Merck & Co. Inc.) for diagnosis and treatment, no tropical airborne allergen from pollen has been described so far.

*Caryota mitis* is commonly planted in parks and streets in most cities of tropical and sub-tropical zones. In the pollination season, it produces larger amounts of pollen. Though there are more studies on tree pollen allergy, few reports on tropical airborne pollen allergens have been published.

Profilins are ubiquitous proteins found in all eukaryotic cells where they function as actin and phosphatidylinositol 4,5-bisphosphate binding proteins that are involved in the regulation of the actin cytoskeleton and signal transduction.[9] These highly conserved proteins were described as the allergens of many grasses, weeds, trees, fruits and vegetables[10] as well as the natural rubber latex.[11] The presence of profiling-specific IgE antibody is a mediator for cross-reactivity to botanically unrelated allergens; thus, profilins are termed as pan allergens from a wide variety of plant sources.[11]

In the present study, the cDNA of *Caryota mitis* profilin was cloned and expressed in *E. coli.* The recombinant protein was tested for its IgE reactivity.

## Methods

### *Pollen samples*

Six batches of *Caryota mitis* inflorescences were collected from public gardens or sidewalks in Hainan province of China in the early morning in May 2010. Some inflorescences were buds, some were just coming into bloom. The inflorescences was inserted into a bottle which was full of water. Under the inflorescences there was a clean paper to collected the pollen. The experimenter tapped the inflorescences with a small wooden stick every day in order to make the mature pollen fall onto the paper. After all the pollen was collectd, the pure pollen was obtained by using the 80 and 160 mesh sample sieves to eliminate impurities. Then five pollen samples were taken randomly and smear stained in preparation for testing of the purity under the microscope. Test results showed that the purity was up to 95%, and all other impurities were only 5%. The pollen samples were stored at -20 °C until use.

### *Patients' serum collection*

Eighty-six patients with pollinosis (manifested by disease history, positive skin prick test (SPT) and allergen-specific IgE in their serum) were recruited into this study. The procedures were approved by the Shenzhen University Medical College Ethical Committee. Written informed consents were obtained from each patient. SPTs against CmPCE (10μg/ml) were conducted in the Municipal People's Hospital of Haikou, Hainan province, China. The SPT results were recorded 15 min later. People who responded with a wheal diameter larger than 5 mm were regarded as positive to the test. Serum from 20 positive patients (10 males, 10 females, average age 24 years old, range 11–48) was collected.

### *Immunology Assay of CmPCE*

Following published procedures,[12] dry *Caryota mitis* pollen was extracted at 4°C and named as CmPCE. In brief, 2 g *Caryota mitis* pollen was frozen in liquid nitrogen and ground in a mill without thawing. The powder was homogenized and defatted by suspending it in 30 ml acetone at 4°C overnight. The extract was then lyophilized and the pollen powder was resuspended in a 1:20 ratio in coca's buffer for 72 h. The supernatant was collected and dialyzed against distilled water, lyophilized and stored at -20°C. The protein concentration was determined by a Bio-Rad protein assay (Bio-Rad, Hercules, Calif., USA).

In the ELISA assay, 20 μg/ml of CmPCE was coated onto a 96-well microtiter plate (100 μL/well) and incubated at 4°C overnight. After being blocked with 5% skimmed milk powder for 1h at room temperature, 100μL of $IgE^+$ sera from patients with CmPCE allergy was added and incubated overnight at 4°C. The bound IgE was then reacted with 100 μL biotin-labeled goat anti-human IgE (1:3,000) followed by 100 μL horseradish peroxidase-conjugated streptavidin (1:2,000). 100 μL TMB was used as the substrate and the reaction was stopped by 25 μL of 2 M $H_2SO_4$. The plate was read with a microplate reader (BioTek, Shanghai, China).

### *Cloning of cDNA of profilin from Caryota mitis pollen*

Nucleotide sequences of profilin from 67 plant foods were downloaded from SWISS-PROT, TrEMBL (http://www.expasy.org) and NCBI (http://www.ncbi.nlm.nih.gov). A homologous comparison was proformed to determine their

conserved regions by using ClustalX (1.81) software. Two specific primers were designed based on the sequence of the conserved region. The sequences of the forward primer (F1) and reverse primer (R1) were as follows:

F1: 5′-ATGTCGTGGCAGGCGTACGT-3′;

R1: 5′- GGACAA( G/A) TGCAACATG( A/G) TTGTTG -3′.

Total RNA was extracted from *Caryota mitis* pollen using an RNeasy mini kit (Qiagen Inc., Germany) and converted to cDNA using reverse transcriptase with specific primers. The cDNA was amplified by PCR. The resulting amplican was cloned into a pMD18-T vector (Takara Inc., Japan) and the resulting sequence was correct as checked by sequencing. The 3'-end of cDNA was rapid amplified using a 3'-RACE kit (Invitrogen Life Tech. Inc., USA). The resulting product was cloned into pMD 18 T-vector and sequenced. The sequence was spliced to obtain the full-length allergen gene sequence after analyzing. In addition, a pair of primers which included Nde I and Xho I restriction sites were synthesized as follows:

F2:5'-GAACATATGATGTCGTGGCAGGCG TAC-3';

R2: 5'-GAACTCGAGCTAAAAACCCTGAT CAAT-3'. The full-length Open reading fram (ORF) for profilin was amplified by PCR. PCR products were purified from the agarose gels using a Gel Extraction Kit (Omega Bio-Tek, USA).

***Expression and purification of profilin from Caryota mitis pollen***

For CmProfilin expression, the full-length gene of CmProfilin was ligated into pET-28a (+) Vector (Novagen Inc., USA) and transformed into *E. coli* BL21. It was grown at 37°C until the OD was 600 of 0.6–0.8 and the profilin expression was induced by adding 1 mM isopropyl-D-thiogalactopyranoside (IPTG) to the culture; the cells were harvested 3 h later. The harvested cells were resuspended in 20 mmol /L Tris-HCl (pH 7.5) and sonicated after freezing and thawing three times to lyse the bacteria. The cell lysate was clarified by centrifuging at 15,000 ×*g* for 15 min at 4°C; the supernatant was loaded into a Ni-NTA column (Amersham Pharmacia Inc., Canada) balanced with a buffer containing 20 mM Tris-HCl (pH 7.5) and 200 mM NaCl. After exhaustive washing with the same buffer, the column was eluted with 2 column volumes (40 mL) of 200 mM imidazole in the balanced buffer. The elution peak was collected, dialyzed against 1×phosphate buffer solution (PBS) and the homogeneity was analyzed by 12% SDS-PAGE.

***Circular dichroism spectroscopy analysis***

The secondary structure of the purified recombinant *Caryota mitis* profilin was assessed by using circular dichroism (CD) spectroscopy in a Jasco J-810 spectropolarimeter (Jasco, Easten, USA) in a 0.1 cm quartz cuvette. Data from measurements were accumulated to calculate the mean spectra.

***Immunoblot and Immunoblot Inhibition assay of rCmProfilin***

For mixture serum blot, CmPCE (70 μg) and purified r CmProfilin (20 μg) were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. A serum pool from 20 patients showing a positive skin prick test reactions to CmPCE was used. For single serum blot, purified r CmProfilin (0.2 mg) was separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The sera from 20 allergic individuals were separately diluted 1:5 in Tris-buffered saline solution (TBST). The membranes were blocked with a blocking buffer (TBSTcontaining 5% BSA and 0.1% Tween 20, TBST, pH 7.6) for 1 h, the blots were incubated with sera from patients (1:5 diluted in TBST), as well as control sera from 3 healthy people. After washing three times, biotin-labeled goat anti-human IgE antibody (Katzenbach Partners LLC; 1:3,000 diluted in TBST) and horseradish peroxidase-conjugated streptavidin (Katzenbach; 1:2,000 diluted in TBST) were sequentially added. Lastly, the bands were visualized by using 3,3',5,5'-tetramethyl-benzidine (TMB; Katzenbach) as a substrate. For inhibition assays, mixed serum from 20 patients was preincubated with 70 μg/mL purified r CmProfilinfor 1 h at room temperature.

***ELISA and ELISA Inhibition assay of rCmProfilin***

In the ELISA assay, 5 μg/well of r CmProfilin was coated onto a 96-well microtiter plate containing 100 μL PBS and incubated overnight at 4 °C. After being blocked with BSA in PBS at 4 °C for overnight and washed for three times, 100 μL of sera from patients 1–20 and 3 healthy controls (1:10 diluted with PBS) were added and incubated overnight at 4 °C. The bound IgE was then reacted with 100 μL biotin-labeled goat anti-human IgE (1:3,000) followed by 100 μL horseradish peroxidase-conjugated streptavidin (1:2,000). TMB was used as the substrate and the reaction was stopped by 100 μL of 2 M $H_2SO_4$ .

For the inhibition assay, pooled sera from patients 1-20 were pre-incubated with inhibitors (CmPCE and rCmprofilin) at different concentrations (0.05, 0.1, 1, 10, 100, 1000, 1200, 1400 and 1,600 μg/mL) for 2 h at 37 °C and then reacted with the coated r CmProfilin(5 μg/well) or CmPCE (5 μg/well) as antigens. After washing, the coated plates were incubated with 100 μL biotin-labeled goat anti-human IgE (1: 3,000) followed by 100 μL horseradish peroxidase-conjugated streptavidin (1:2,000). Color development was performed by using TMB as the substrate and stopped by 100 μL of 2 M $H_2SO_4$. Then the OD at A450 nm was measured. Buffer and a serum pool from three nonallergic subjects was used as negative controls. Data from three measurements were accumulated.

## Results

### *SDS-PAGE and immunogenicity assay of CmPCE*

CmPCE and the sera from 20 patients with positive response to CmPCE in SPT were fractioned by SDS-PAGE respectively. The fractions of CmPCE were14, 28, 40, 55, 85, 100 and 120 kDa respectively (Figure 1a). In the results of immunoblotting assay, the serum fractions showed positive staining at 14, 16, 20, 28, 33 and 100 kDa respectively (Figure 1b) in 16 (80%) out of 20 patients. The sera from healthy subjects did not show any positive reactivity to CmPCE. The results were further confirmed by ELISA (Figure 2).

### *Similarity of gene sequence of CmProfilin with profilin from other sources*

The cDNA of the profilin from *Caryota mitis* (Cm) was obtained by RT-PCR and 3'-Race and the sequence was confirmed by GenBank entry (accession number EF173600). The amino acid sequence (131 amino acids) of CmProfilin was deduced from a 396-bp ORF . The cDNA encoding profilin from Cm was cloned and analyzed. The alignment results of CmProfilin compared to profilins from other plants were presented in Figure 3. CmProfilin had 99% identity similar to *Cocos nucifera* profilin (Coc n), 97% to *Roystonea regia* (Roy r) profilin, 75% to hazelnut profilin, 80% to *Ananas comosus* profilin (Ana c), 78% to *Corylus avellana* Pollen profilin (Cor a 1), 77% to *Phleum pratense* pollen profilin (Phl p 2) and 77% to *Olea europaea* pollen profilin (Ole e).

### *Purification and physicochemical characterization of rCmProfilin*

The CmProfilin cDNA was cloned into the expression vector pET-28a (+) and expressed as a 6-histidine-tagged protein in E. coli BL21 (Figure 4a). Affinity chromatography resulted in a highly pure recombinant protein, migrating at about 14 kDa in SDS-PAGE. The secondary structure of rCmProfilin was evaluated by CD spectroscopy. Circular dichroism spectroscopy showed that the rCmProfilin isoform is folded protein containing alpha helices (Figure 4b).

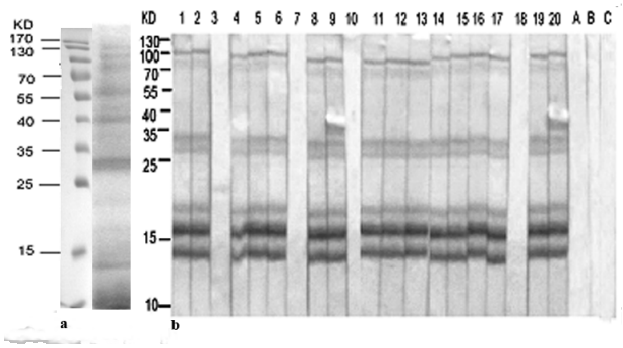


**Figure 1.** Detection of CmPCE in the serum of patient with pollen allergy. a, SDS-PAGE analysis of *Cm*PCE. b, Western blot of CmPCE with sera from 20 patients and 3 healthy controls. Immunoblot with CmPCE to allergic patients (lane 1–20) and healthy control (lane A, and C) by SPT.

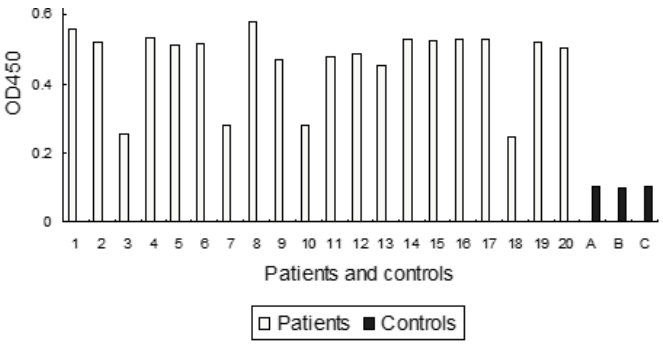


**Figure 2.** ELISA assay of human sera IgE binding to CmPCE. The sera were obtained from patients with positive SPT responses to CmPCE. The bars indicate the OD450 value of ELISA. The open bars indicate the OD value from patients. The closed bars indicate the OD value from healthy controls.

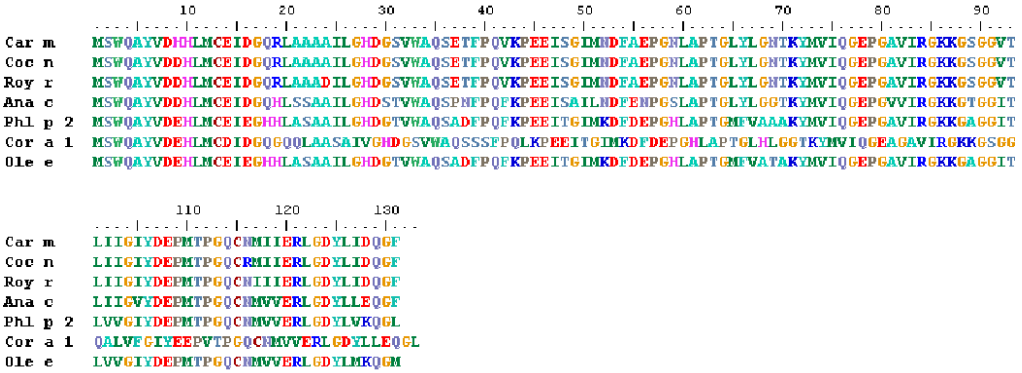


**Figure 3.** Alignment of profilins from different sources. Coc n= *Cocos nucifera* profilin; Roy r= *Roystonea regia* profilin; Ana c= *Ananas comosus* profilin; Phl p 2= *Phleum pratense* pollen profilin variant 2; Cor a 1= *Corylus avellana* pollen profilin variant 1; Ole e= *Olea europaea* pollen profiling.

### *IgE/Inhibition Immunoblot Assay*

The sera were collected from 20 CmPCE SPT-positive patients and 3 healthy controls. Western blot analysis of patient sera was used to identify patterns of IgE reactivity to r CmProfilin (Figure 5a). Based on sequence analysis of Cmprofilin, it was hypothesized that the 14 kDa band detected in Western blot analysis of patient sera represented the panallergen profilin. This hypothesis was tested with an immunoblot inhibition assay by using patient sera preincubated with rCmprofilin. Following preincubation of the pooled sera with rCmprofilin, the IgE-binding ability of the 14 kDa band was inhibited, thereby identifying the 14 kDa band as CmProfilin (Figure 5b).

### *ELISA and ELISA Inhibition Assay*

The reactivity of patient sera to rCmProfilin was also examined by ELISA assay (Figure 6). Of the 20 patient serum samples, 12 (1, 2, 5, 8, 9, 11, 12, 14, 17-20) samples showed a positive reaction with rCmProfilin reflected by increases in the OD value of the ELISA. In contrast, the three healthy controls gave negligible values in ELISA.

An ELISA inhibition assay was performed to investigate the specificity of the serum binding to r CmProfilin. At a concentration of 1,600 μg/mL, CmPCE had up to 88.1% and 83.2% of inhibition rate towards itself and rCmProfilin had an 83.2% inhibitory effect on the binding to the sera.

## Discussion

*Caryota mitis* is a typical tropical plant which in previous research was regarded as an important contributor to pollinosis; a high prevalence (53.2%) of positive skin prick tests for *Caryota mitis* pollen extract has been recorded in Haikou, China.[13] There has been little research about the palm's pollen allergens and the mechanism of the allergic response to the pollen at home and abroad. It is therefore very important to study the structure and allergenic properties of the *Caryota mitis* pollen allergens. Profilin is a protein which is known as a pan-allergen and it is common among different species of plants or different tissues, with 70%-85% homology; but it is less conserved among different species of low eukaryotes or animals, with 25%-40% homology.[14] Profilin is a widely distributed allergen with standard amino acid sequences, which is considered to be one of the main factors causing

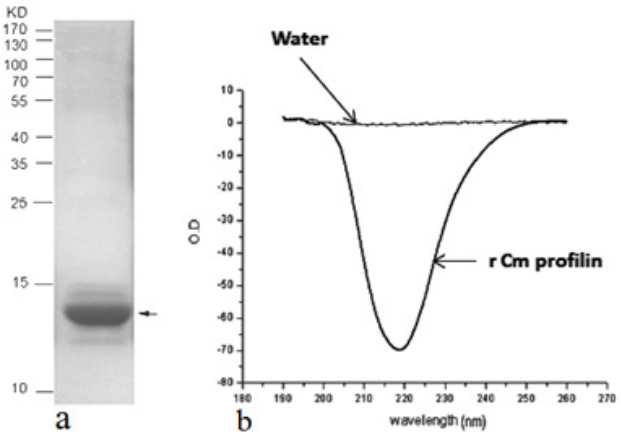


**Figure 4.** Physicochemical characterization of rCmProfilin. a: The immune blot at 14 kDa (indicated by an arrow) shows the rCmProfilin. b: The curve indicates the circular dichroism analysis results of rCmprofilin.

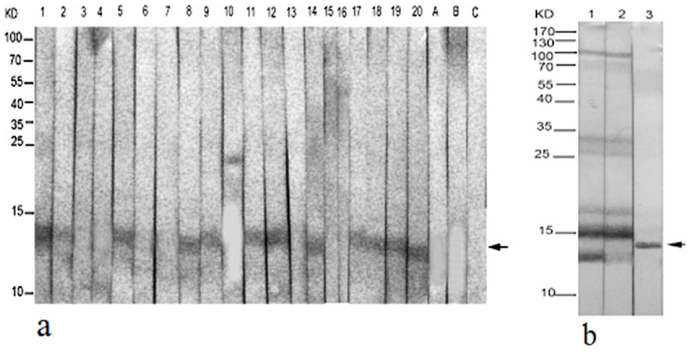


**Figure 5.** Detection of specific antibodies of rCmprofilin from patient sera. a: The immunoblots at 14 kDa (pointed by an arrow) indicate the immune complexes of specific antibodies and rCmProfilin. The serum samples in lanes 1–20 were obtained from patients with positive SPT to CmProfilin. Samples in lanes A, B and C were obtained from healthy controls. b: The serum from the 20 patients was pooled and analyzed by an inhibitory assay with specific antigens. Lane 1 shows CmPCE; lane 2 shows rCmProfilin was inhibited, and lane 3 shows rCmProfilin (pointed by an arrow).

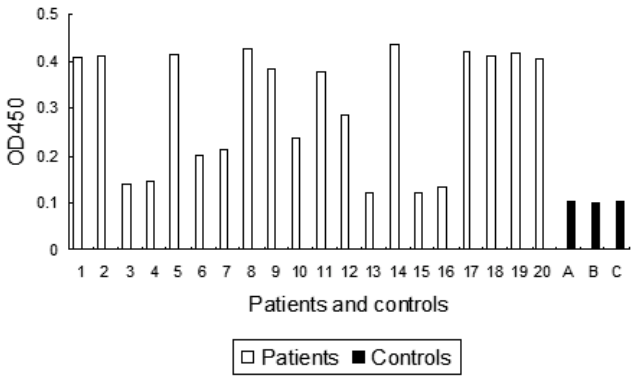


**Figure 6.** ELISA assay of human sera IgE binding to rCmprofilin. The sera were obtained from patients (bars 1-20) with positive SPT to Cmprofilin and healthy individuals (bars A-C). All sera were diluted 1: 10 in PBS and analyzed by ELISA. The bars indicate the OD values of the ELISA.

cross-allergic reactions between pollens and plant foods,[11] and between pollens and rubber latex.[15] CmProfilin has a standard sequence which contains about 131-134 amino acids and it focused between pI 4.6- pI 5.4 and has a molecular weight of 14.0 kDa-14.5 kDa. The protein secondary structure of CmProfilin mainly consists of an alpha-helix, beta-folding random coil.[16] ; the protein tertiary structure of CmProfilin is α/βtypes by CD spectrum analysis. This study analyzed the gene sequence, protein structure and physicochemical characteristics of CmProfilin which is important in determining the mechanism of CmProfilin sensitivity.

In this study, we identified a major allergen from a common plant, the *Caryota mitis*. A14 kDa protein was identified from the extracts of *Caryota mitis* pollen (CmPCE), which reacted with the serum from patients with SPT positive response to the CmPCE. We also recombined the allergen; the rCmProfilin also reacted with the positive serum from the patients. The present data showed that 16 out of 20 (80%) of collected sera samples reacted to the 14 kDa CmProfilin proteins in their natural form; and 12 of 20 (60%) collected sera samples reacted to rCmProfilin. The results imply that this 14 kDa protein is a novel allergen from *Caryota mitis*. The inference has been further supported by the finding that incubation of sera with rCmProfilin eliminated the reactivity of the patients' sera to this 14 kDa protein.

From the CmPCE, we isolated several protein members but the major reactive member is the one at 14 kDa, and the CmProfilin is the major allergen from *Caryota mitis* pollen. We may regard this member as the chief allergen in CmPCE. If we use CmPCE for the specific immunotherapy, we would carry out desensitization therapy with this 14 kDa protein, which may induce specific immune tolerance to this specific allergen.[17,18] Meanwhile, several irrelevant proteins from CmPCE are also introduced to the patient and have the potential to induce new sensitization to those proteins. Therefore, we propose to use purified allergens in specific immunotherapy. This paper is the first to research the pollen allergen of this tropical plant (*Caryota mitis*), to analyze the molecular structure, allergenicity and immunogenicity of the pollen allergen, and has filled a gap in this domain to a certain extent. The results of this study can provide significant in the clinical diagnosis and the understanding of the cause of palmas pollen allergy and the prevention of recurrence.

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