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Complementary DNA Cloning and Immunologic Characterization of a New *Platanus orientalis* Pollen Allergen, Pla or 1.0101

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Abstract: Oriental Plane trees, as *Platanus orientalis*, are an important source of airborne allergens in cities of the southwest Asia and southeast Europe. Diagnosis of type I allergy essentially depends on the availability of defined allergens, which can be provided by recombinant technology. This study was aimed to investigate molecular properties of a *Platanus orientalis* pollen allergen, designated as Pla or 1.0101 (accession number EU296476), to produce its immunoreactive recombinant counterpart in *Escherichia coli*. Molecular characterization of the *Platanus orientalis* pollen allergen was performed using, cDNA cloning, expression of the recombinant allergen in *Escherichia coli* and IgE immunoblotting of recombinant allergen. The 18 kDa allergen (Pla or 1.0101) was identified as an important IgE-binding component of *Platanus orientalis* pollen. The Pla or 1.0101-specific cDNA sequences were amplified, using specific primers based on the N and C-terminal sequence of a Pla or 1.0101 homologue in *Platanus acerifolia*, Pla a 1. Sequencing corresponding Pla or 1.0101 cDNA revealed an open reading frame of 513 bp coding for 170 amino acid residues. The recombinant Pla or 1.0101 was produced by pET102/D-TOPO *E. coli* expression system. IgE-binding to the recombinant form of Pla or 1.0101 was proven by immunoblot and specific ELISA.

Key words: Allergen characterization, cDNA cloning, cyclophilin, invertase inhibitors, MALDI-TOF-MS, *Platanus orientalis*, recombinant allergen, SDS -PAGE immunoblotting

INTRODUCTION

Platanus orientalis (Oriental Plane) is the dominant tree species in parks and streets in most cities of Iran (Poorkhabbaz, 2007). The other important plane trees are American sycamore *(Platanus occidentalis)* and London plane tree (*Platanus acerifolia* or *platanus hybrida*) (Hsiao, 1973). Research has shown that *Platanus* pollen is a major contributor to pollinosis symptoms during March and April (Subiza *et al*., 1994; Varela *et al*., 1997). A high prevalence of positive skin prick tests (43.6%) has been recorded for *P. orientalis* in Mashhad, Iran. The highest concentrations of its pollen have been detected during the flowering season, reaching up to15% of the total pollen in Mashhad (Unpublished results). During the past decade, the researches of many groups have been focused on the identification and characterization of proteins responsible for IgE-mediated allergies, since type I allergy is becoming a major world health problem (Johansson and Haahtela, 2004). Currently, allergy diagnosis and specific immunotherapy are carried out with allergenic extracts which contain a variety of allergenic and non-allergenic components that make them too complex to be standardized (Vanree, 1997). Diagnosis of type I allergy essentially depends on the availability of defined allergens, which can be provided by recombinant technology (Valenta *et al*., 1991). Despite the abundance of airborne *P. orientalis* pollens and its proven implication in pollinosis, few studies have actually addressed the molecular characteristics of *P. orientalis* pollen-derived allergens (Pazouki *et al*., 2008).

The purpose of this study was to characterize the molecular properties of the most important allergen of *P. orientalis* pollen in order to produce its immunoreactive recombinant counterpart in *E. coli*.

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MATERIALS AND METHODS

Subjects, skin test and sera collection: Seven patients, who showed adverse clinical reactions to *P. orientalis* pollen and IgE reactivity to the 18 kDa component of *P. orientalis* were included in this study. They comprised 04 male and 3 female, with a mean age of 25±14.5 years. These patients referred to Department of Immunobiochemistry, Mashhad University of Medical Sciences, Mashhad, Iran. All subjects underwent skin prick test (SPT) using the crude extract of *P. orientalis* pollen (0.5 mg mL^{-1}). The SPT was performed according to guidelines from the European Academy of Allergology and Clinical Immunology (Dreborg and Frew, 1993). Histamine 10 mg mL^{-1} and saline were used as positive and negative controls, respectively. After obtaining informed consent, sera were taken from all patients and stored at -20°C until used in immunoblot assays. A serum pool from 5 patients with no clinical history of allergy and with negative SPT to *P. orientalis* pollen extract was used in IgE immunoblotting assays. The *P. orientalis* pollen allergen-specific IgE and Pla or 1.0101-specific IgE of selected sera were measured by indirect ELISA as described previously (Sankian *et al*., 2005).

Preparation of *P. orientalis* total pollen extract: Pollens from *P. orientalis* was purchased from Greer Laboratories, Inc. (Lenoir, North Carolina, USA). Diffusates of the pollens were prepared by thoroughly mixing dry pollen (1 g) with 10 mL phosphate buffer saline (PBS) by continuous stirring for 16 h at 4°C. The mixture was centrifuged at 16,000 g for 10 min. The supernatant was dialyzed against phosphate-buffered 5 mM (pH 8) (cutoff point of 3.5 kDa) and filtered through a 0.22 μm membrane (Millipore Corp., Bedford, MA, USA). The filtrate was then lyophilized and stored -20°C in sterile vials (Chakraborty *et al*., 2005). Determination of the protein concentration was performed according to the Bradford (1976) method with bovine serum albumin (BSA) as standard. To use this extract for skin prick test, glycerol was added to a final concentration of 50% (v v^{-1}).

One dimensional electrophoresis, proteins transfer and immunoblot: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) in 12.5% polyacrylamide gel using a BioRad Mini Protean II system (BioRad, Hercules, CA, USA). Protein bands were visualized by Coomassie Brilliant Blue (CBB) staining. Separated protein bands were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA), essentially by the method of Towbin *et al*. (1979). Immunodetection was carried out on PVDF after treatment with methanol for 15 sec and blocking with Superblock (Pierce, Rockford, IL, USA) at 4°C for 16 h. Membranes were probed with individual sera from *P. orientalis*-allergic patients (diluted 1/5 in PBS containing 1:10 v v^{-1} blocking buffer) or with a pool of sera from non-allergic subjects, diluted as above, for 4 h at room temperature. PBS buffer with 1% BSA was used as a negative control. Membranes are then washed 4 times for 5 min with 0.05% Tween-20 in PBS and incubated for 2 h with a goat biotinylated anti-human IgE (Kirkeggard and Perry Laboratories, Gaithersburg, MD, USA) diluted 1/1000 in PBS containing blocking buffer (1:10 v v^{-1}). After washing, blots were incubated for 1 h with streptavidin horseradish peroxidase-labeled diluted 1/40000 (Sigma Chemical Co., St Louis, MO, USA). The peroxidase reaction was developed with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) for 5 min and IgE-binding proteins were detected by ECL Hyperfilm (Amersham Pharmacia Biotech, Uppsala, Sweden) after exposure for 5 min. The molecular mass of protein bands was estimated with Kodak digital science 1 D Image Analysis Software (Eastman Kodak, Rochester, NY, USA) by comparison with protein markers of known molecular weight (LMW electrophoresis calibration kit, Amersham Pharmacia Biotech, Uppsala, Sweden) (Sankian *et al*., 2007).

Amplification of Pla or 1.0101 cDNA by RT-PCR and nucleotide sequence determination: Total RNA was extracted from fine powder of *P. orientalis* pollen grounded under liquid nitrogen by using a Qiagen RNeasy Plant Total RNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg total RNA using first-strand cDNA synthesis Kit (Fermentas, Lithuania) with a Oligo (dT) 18 as primer. For cDNA amplification, two oligo nucleotide primers: Pla or 1f, 5'-ATGAAGCTTTCCTTCTCTCTCTGTATC-3'andPlaor1r, 5'-AGCACCAAGCAGTTTGGTAATTG-3' were designed on the basis of reported nucleotide and amino acid sequences of *P. acerifolia*, Pla a 1, major allergen (Asturias *et al*., 2003). After amplification of Pla or 1.0101 cDNA, PCR products were cleaned using Gene Clean II kit (Qbiogene, Illkirch, France) and the PCR product was first sub-cloned into the vector pTZ57R/T with the TA cloning kit (Fermentas, Lithuania) and then clones carrying inserts were characterized by restriction analysis and non-radio-active sequencing (MWG Biotech AG, Ebersberg, Germany). Homology search was done using BLASTN for nucleotide sequence alignment.

Analysis of predicted protein sequence: The predicted molecular mass and isoelectric point were determined by the Gene Runner program v 3.05 (Hastings Software). The deduced protein sequence of Pla or 1.0101 was next subjected to a BLAST similarity search (Altschul *et al*., 1997). Multiple sequence alignment was performed by BioEdit software (Hall, 1999). The deduced amino acid sequences of invertase inhibitors were obtained from the NCBI Protein Database with the following accession numbers: invertase inhibitors from *P. acerifolia* (Pla a 1), AJ427413; *Vitis* sp., AX214342; *Arabidopsis thaliana*, AB013394; pistil-specific protein from *Solanum tuberosum*, X80472; *Triticum aestivum*, AX214381; *Nicotiana tabacum*, Y12806 and *Lycopersicon esculentun*, AJ010943. Percentages of identity (% Id) and similarity (% Sim) with respect to Pla or 1.0101 were also indicated.

Expression and purification of recombinant Pla or 1.0101 as a fusion protein: The Pla or 1.0101 coding region was amplified with Pfu DNA polymerase (Fermentas, Lithuania). The 5' primer (5'- CACCATGAAGCTTT CCTTCTCTCTCTGTATC -3') mimics the first nine codons and introduces a pET102D/TOPO expression vector overhang (underlined). The 3' primer (5'-AGCACCAAGCAGTTTGGTAATTG -3') mimics the last 7codons. After PCR amplification, the 513 bp product was ligated into the pET102D/TOPO expression vector according to the instructions in the manufacturer's manual (Invitrogen, San Diego, Calif, USA). The resulting pET102D/TOPO-Pla or 1.0101 construct was transformed into chemically competent Top10 strain of *E. coli* (Invitrogen) and recombinant plasmids were isolated using Qiagen purification kits (Qiagen GmbH, Hilden, Germany). The fidelity of the cloned product was verified by sequencing. Plasmids containing the of Pla or 1.0101 inserts with the appropriate sequence were transformed into competent BL21(DE3) strain of *E. coli* (Invitrogen) for protein expression. BL21(DE3) *E. coli* carrying pET102/D-TOPO-Pla or 1.0101 construct was cultured in 5 mL of LB medium containing 100 μg mL^{-1} ampicillin with shaking at 37°C overnight. The preculture (1 mL) was inoculated into 100 mL of LB medium containing 100 μg mL^{-1} ampicllin in a 250 mL Erlenmeyer flask and was grown with shaking at 37°C. After reaching to an absorbance of 0.5 -0.6 at OD600, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM. Then, after 6 h of growth at 30°C, the cell pellet was collected by centrifugation (4000 rpm, 20 min) and dissolved in 30 mL binding buffer (10 mM imidazole, 0.4 M NaCl, 0.1 M KCl, 10% glycerol, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.6). Suspended bacteria were disrupted with 3 times freeze thaw in liquid nitrogen. Insoluble material of the cell lysate was removed by centrifugation (10,000 rpm, 30 min). The supernatant of the disrupted cells was bound to 5 mL Ni-NTA superflow resin (invitrogen) in a falcon tube for 1 h at RT with gentle shaking. The resin was loaded into a column for purification. Then, the column washed with 10 volume of binding buffer and 5 vol. washing buffer (20 mM imidazole, 0.4 M NaCl, 10% glycerol, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.6). The (His) 6-tagged recombinant Pla or 1.0101 was eluted with 5 vol. elution buffer (200 mM imidazole, 0.4 M NaCl, 0.1 KCl, 10% glycerol, pH 7.6). The purified protein was extensively dialyzed against phosphate buffer pH 7.4 (4°C, 72 h) (Sankian *et al*., 2007).

IgE reactivity of the recombinant allergen: The reactivity of specific IgE antibodies to the recombinant Pla or 1.0101 allergen was examined by immunoblotting and specific ELISA as described previously (Sankian *et al*., 2005). The purified protein and non-induce transformed *E. coli* total extract were subjected to reducing SDS-PAGE and electro-blotted on PVDF membrane. Immuno-detection was carried out as described above, using 7 sera of *P. orientalis* allergic patients, who were reactive to 18 kDa allergen and a pool serum from non allergic individuals.

RESULTS AND DISCUSSION

Amplification of Pla o1 cDNA and sequence analysis: Amplification of *P. orientalis* cDNA using the 5' primer (Pla or 1f) and the 3'-primer (Pla or 1r) resulted in a single 315 bp fragment that was cloned into pTZ57R/T. Four of these clones were sequenced. Sequencing analysis revealed that the PCR products correspond to a 513 bp open reading frame (Accession number EU296476) which encodes Pla or 1.0101, a 170-amino-acid polypeptide with an average molecular mass of 18.2 kDa and a theoretical isoelectric point value of 8.69. There is no potential N-glycosylation (N-X-S/T) site in the predicted amino acid sequence (Fig. 1). The deduced polypeptide shares substantial sequence similarity with invertase inhibitors from other plants. Pair wise nucleotide alignments indicated 92% identity with *P. acerifolia* invertase inhibitor, Pla a 1, 40% with *Vitis* sp. invertase inhibitor, 36% with *Arabidopsis thaliana* invertase inhibitor, 33% with pistil-specific protein from *Solanum tuberosum*, 28% with *Triticum aestivum* invertase inhibitor, 21% with *Nicotiana tabacum* invertase inhibitor and 24% with *Lycopersicon esculentun* invertase inhibitor (Fig. 2). *Platanus orientalis* invertase inhibitor accepted

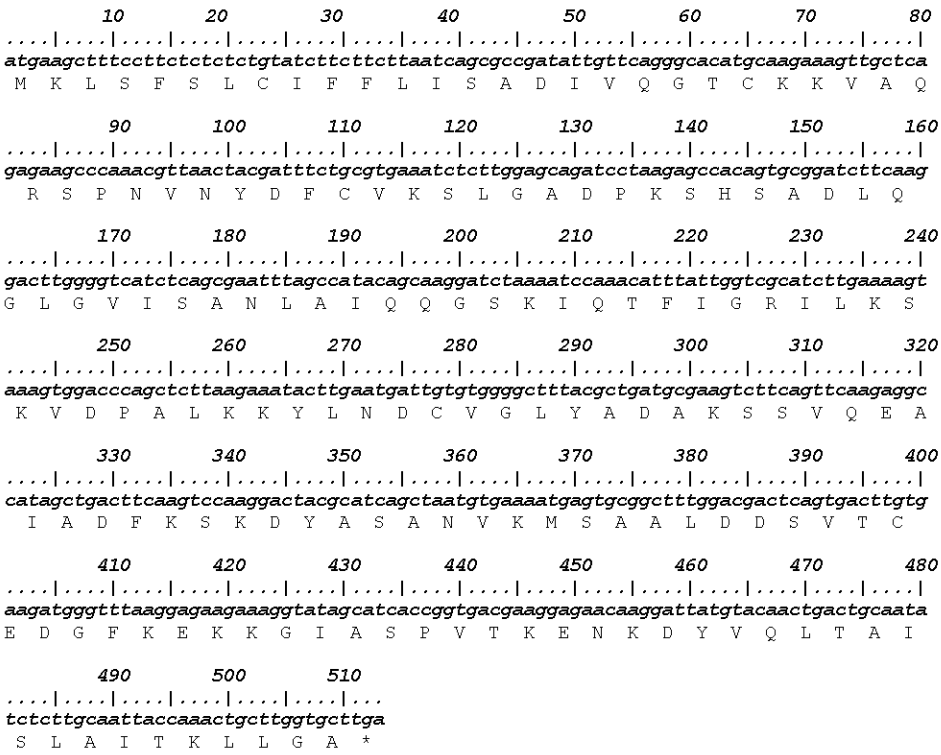

Fig. 1: Sequence analysis of cDNA encoding Pla or 1.0101. Nucleotide and deduced amino acid sequences. The nucleotide sequence for Pla or1.0101 has been deposited in the GenBank database under Accession No. EU296476

(July 2008) as Pla or 1.0101 by the International Union of Immunological Society (IUIS) allergen nomenclature subcommittee.

Expression and purification of Pla or 1.0101 protein: Expression of recombinant Pla or 1.0101 allergen in *E. coli* was performed with the pET102/D-TOPO expression vector. A fusion Protein was obtained with an estimated molecular mass of 36 kDa, consist of Pla or 1.0101 protein, a His-Patch thioredoxin, V5 epitope and a 6xHis tag. Recombinant proteins were found in insoluble inclusion bodies which made soluble by changing incubation temperature from 37-28°C. The yield of Pla or 1.0101 produced using the described conditions (cell density of 0.6, 0.4 mM IPTG and overnight expression) was 30-40% of the total protein (Fig. 3a). The soluble material was purified Ni-NTA affinity chromatography. After elution with a lysis buffer containing 200 mM imidazole, Pla or 1.0101 showed a single band with an apparent molecular mass of 36 kDa. The yield of purified Pla or 1.0101 was about 50 mg L^{-1} of bacterial culture. A purity of more than 95% was achieved as documented using the SDS-PAGE gels (Fig. 3a).

Immunological characterization of recombinant Pla or 1.0101: All the selected patients showed positive skin challenge responses to *P. orientalis* total pollen extract, significantly high levels of IgE to *P. orientalis* extract in ELISA and IgE reactivity to 18 kDa component of *P. orientalis* pollen extract in immunoblot analysis.

The IgE reactivity of purified rPla or 1.0101 was analysed by immunological assay and specific ELISA using seven sera from the selected patients. A pool of sera from 5 healthy control subjects was included as controls. As shown in Fig. 3b, 4 out of 7 patients' sera had specific IgE antibodies to rPla or 1.0101 allergen in immunoblot analysis as well as in specific ELISA.

About 20% of the population in developed countries suffers from IgE mediated atopic diseases, such as allergic rhinitis, rhinoconjunctivitis and bronchial asthma (Wutrich, 1989). The cloning and expression in heterologus systems of the genes for the major allergens is expected to produce important advancements in the study, diagnosis and therapy of allergy (Baldo and Donovan, 1989). In the present study, recombinant Pla or 1.0101 was characterized and recombinantly produced as

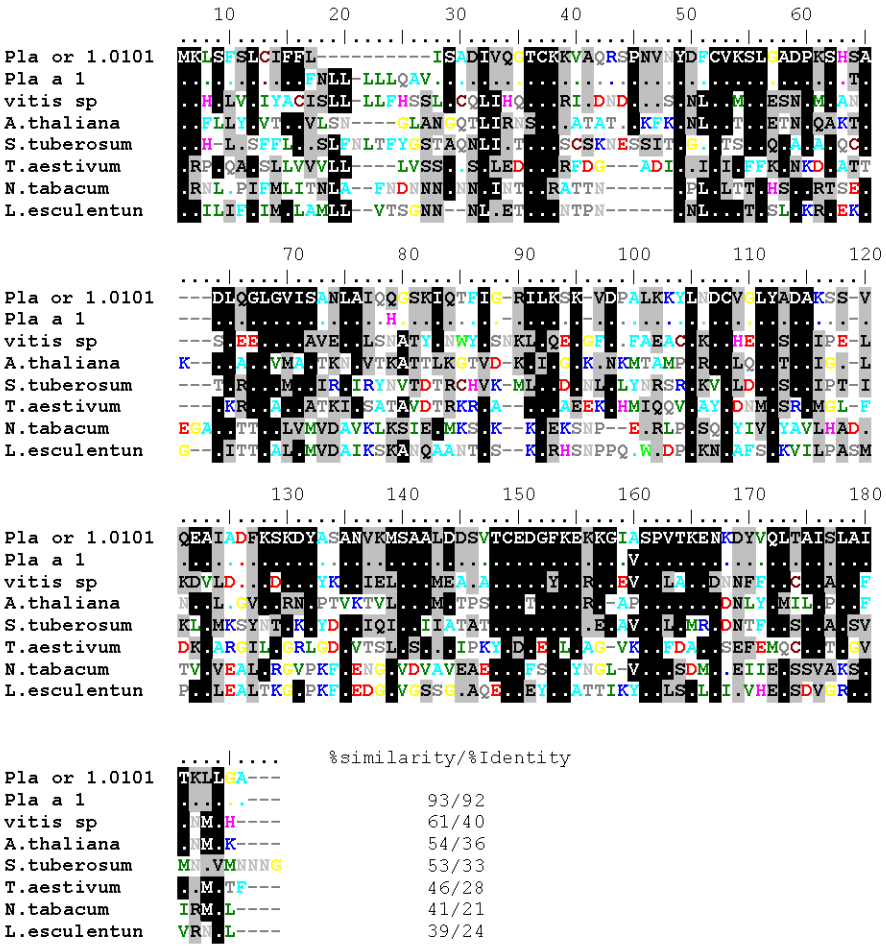

Fig. 2: Comparison of amino acid sequence of plant invertase inhibitors and related proteins. Pla or 1.0101 from *P. orientalis* pollen, invertase inhibitors from *P. acerifolia* (Pla a 1), *Vitis* sp., *Arabidopsis thaliana*, pistil-specific protein from *Solanum tuberosum*, *Triticum aestivum*, *Nicotiana tabacum* and *Lycopersicon esculentun* (GenBank Accession Numbers EU296476, AJ427413, AX214342, AB013394, X80472, AX214381, Y12806 and AJ010943, respectively). Percentages of identity (% Id) and similarity (% Sim) with respect to Pla or 1.0101 were also indicated

an important allergen of *Platanus orientalis* pollen, belongs to a new class of allergens related to proteinaceous invertase inhibitors. Immunreactivity analysis confirmed that IgE-binding capacity of recombinant Pla or 1.0101 was comparable to its natural counterpart.

cDNA encoding Pla or 1.0101 showed sequence homology with invertase proteinaceous inhibitors protein family with similar molecular mass and amino acid sequence to those of *P. acerifolia*, tomato, tobacco, potato and other plants. This family of protein has been previously described as allergen (Asturias *et al*., 2002, 2003). An allergens with the same molecular weight have previously described in *P. acerifolia* pollen extract as Pla a 1. This 18 kDa nonglycosylated protein belongs to an invertase inhibitors family (Asturias *et al*., 2002, 2003)

In immunoblotting 4 out of 7 patients'sera showed IgE reactivity against recombinant invertase inhibitors of *P. orientalis* pollen (Fig. 3b) but three sera showed no reactivity with rPla or 1.0101, in spite of their reactivity with 18 kDa component of *P. orientalis* pollen extract. This may be explained by the presence of other IgE-binding component that have been detected by immunoblotting analysis of *P. orientalis* extract. In another experiment, we analyzed a protein band at 18-kDa corresponding to the IgE reactive protein of *P. orientalis* pollen. This protein band was excised from the gel and analyzed by MALDI-TOF-MS. In peptide mass fingerprint analysis, the masses obtained from excised protein band showed the highest correlation with cyclophilin from *Glycine max*, *Phaseolus vulgaris* and *Populus tremuloides* (unpublished).

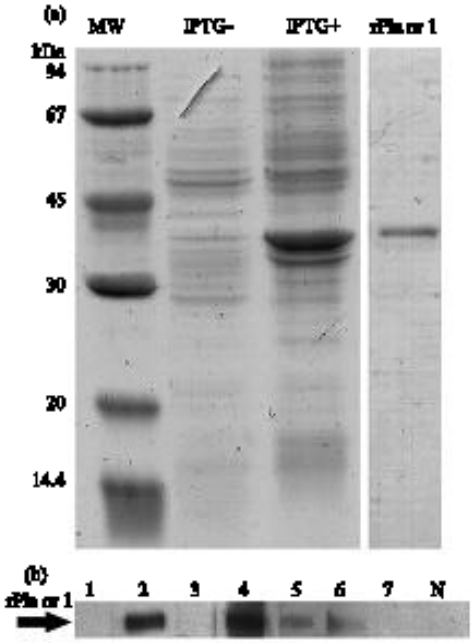

Fig. 3: (A) SDS-PAGE of Pla or 1.0101 expressed in *E. coli*. Total protein extracts of the non-induced bacteria (IPTG⁻), transformed culture induced with 0.4 mM IPTG (IPTG⁺) and SDS/PAGE Coomassi Brilliant blue staining of metal affinity purified recombinant Pla or 1.0101 allergen (rPla or 1) (B) Immuno-blotting of recombinant Pla or 1.0101 with 7 sera of *P. orientalis* allergic patients who were reactive to 18 kDa camponent on pollen total extract (Lane 1-7) and with a pool sera from non allergic individuals (lane 8). MW, Molecular weight

CONCLUSION

The recombinant Pla or 1.0101 was produced as an important allergen of *Platanus orientalis* pollen, belongs to a new class of allergens related to proteinaceous invertase inhibitors. Immunreactivity analysis confirmed that IgE-binding capacity of recombinant Pla or 1.0101 was comparable to its natural counterpart. Immunoblot analysis of recombinant rPla or 1.0101 using patient`ssera, who showed IgE reactivitiy against a 18 kDa protein of *Platanus orientalis* pollen, supports the presence of an other allergenic protein at the molecular weight of 18 kDa. Purified recombinant Pla or 1.0101 could be used allergen-specific diagnosis and immunotherapy instead of natural counterpart.

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