

Quantification of Pla or 3, a *Platanus orientalis* Allergen, Grown under Different Environmental Conditions, by Sandwich ELISA

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Abstract

Background: *Platanus* species are widely cultured around the world and considered an important cause of allergic reactions. In the present study, we developed a sandwich ELISA to quantify Pla or 3 allergen in *P. orientalis* pollen extracts grown near high-traffic roads and compared it to pollen extracts collected from rural areas as control.

Methods: Pollen samples were collected from three polluted and two unpolluted sites in Mashhad, northeast Iran. Recombinant Pla or 3 was expressed and used for polyclonal antibody production in rabbit. A sandwich ELISA was developed and validated to quantify Pla or 3 levels in pollen extracts from the different sites.

Results: The coefficients of variation (CVs) for the intra- and inter- day assays were less than 5 and 18%, respectively. The working range of the standard curve was between 0.1 and 25 ng/ml, with the detection limit being 0.037 ng/ml. The recovery percentage was 88-106.4% at working concentrations from 0.31 to 26.5 ng/ml. Pla or 3 levels were significantly greater in pollens grown near high-traffic roads than in those grown in rural regions ($p < 0.0001$).

Conclusions: A sandwich ELISA was developed and validated to quantify Pla or 3 in pollen extracts. Using this validated ELISA, we showed a substantial difference between the amounts of Pla or 3 in pollens grown in different environments. This finding should be considered in developing public policies to reduce traffic pollution, which leads to reduced allergic reactions in atopic subjects.

Keywords: Pathogenesis-related proteins, Pla or 3, *Platanus orientalis*, Sandwich ELISA

Introduction

Plant-derived pollens are considered as a main cause of asthma and allergic diseases (1, 2). *Platanus orientalis* is cultured as an ornamental tree in southwest Asia, southeast Europe, and the USA (3), and thus, is one of the most important sources of pollen allergens in the environment. Moreover, as reported in recent years, skin reactions to *Platanus* species are increasing (4). The prevalence of allergy to *P. orientalis* pollen has been reported as 43.6% in Mashhad, the second most populated city of Iran (3).

Three major allergens have been identified in *P. orientalis*: Pla or 1, a non-glycosylated protein of 18 kDa and a member of the invertase inhibitors family, Pla or 2, a glycoprotein of 43 kDa and a polygalacturonase, and Pla or 3, a 9-10 kDa protein belonging to non-specific lipid-transfer proteins (ns-LTPs) (3).

Non-specific lipid-transfer proteins are soluble proteins of 7-9 kDa categorized as members of the pathogenesis-related (PR) protein family. Pathogenesis-related proteins are over-expressed

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Received: Mar 9, 2016; Accepted: Apr 20, 2016

in stress situations such as pathogen attacks, drought, freezing temperature, and ozone (5-7). Few studies have compared the expression levels of allergens categorized as PR proteins in plants grown in different environments.

Lu et al. (8) used a laboratory-based experiment to assess the effect of air pollutants on oriental pollens. This research showed increased allergenicity and allergenic protein production in *P. orientalis* after exposure to pollutant gases. In a field-based study, Suarez-Cervera et al. (6) reported greater amounts of Cup a 3 allergen, derived from *Cupressus arizonica* pollen and a PR-5 protein, in pollen grains sampled from polluted sites than from unpolluted sites using immunocytochemical techniques. This study suggested that cypresses up-regulated Cup a 3 expression as a defensive mechanism in polluted conditions. Moreover, the effects of exposure to cadmium (Cd) -contaminated soil on the annual blue grass, *Poa annua* L, pollen was investigated by Aina et al. (9) using proteomic techniques. This group found that pollen allergens exposed to Cd-contaminated soil are more easily released into the environment and also bound more to specific IgE than allergens from uncontaminated soil. A PR-3 class I chitinase-like protein was also detected in this study.

In the present study, we developed a sandwich ELISA, using a specific rabbit polyclonal antibody (pAb) to recombinant Pla or 3 (rPla or 3), to quantify this allergen in *P. orientalis* pollen extracts grown near high-traffic roads. Recombinant Pla or 3 protein was used as a reference standard for the ELISA.

Materials and Methods

Pollen sampling and preparation of extracts

Pollen samples were collected from *P. orientalis* trees during pollination in March and April (10). Three main streets and two vegetated regions (as controls) were sampled in Mashhad, the capital of Razavi Khorasan Province in northeast Iran (11). To prepare extracts from pollen grains, 3 mg of each sample were defatted in acetone and then dried. Proteins were extracted using 0.01 M phosphate-buffered saline (PBS pH 7.4), for 16 hours at 4° C with stirring. The suspensions were then centrifuged at 14000 g for 1 h at 4° C and

the supernatants were dialyzed against PBS. The protein concentrations of the extracts were determined by the Bradford method (12) and stored at -20 °C until use.

Expression and purification of Pla or 3

BL21-CodonPlus cells were transformed with rPla or 3 DNA and cultivated in LB medium containing 100 µg/ml ampicillin at 37 °C. Recombinant Pla or 3 expression was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 14-16 hours of incubation, bacterial cells were harvested by centrifugation at 3000 g for 15 min at 4 °C. To prepare the cell lysate, the cells were treated with lysis buffer and sonicated for 2 min, followed by centrifugation at 10000 g for 10 min at 4°C. The supernatant containing the expressed allergen was then purified by metal affinity chromatography on a Ni-IDA column (Pars Tous Biotechnology, Iran).

Polyclonal antibody production

A 10-16 week old female rabbit (2.5-3.0 kg weight) was purchased from Razi Institute. The animal was housed and fed with standard laboratory methods. To raise polyclonal antibodies against rPla or 3, the rabbit was sensitized by injection of 100 µg of polyacrylamide gel homogenate containing the recombinant allergen. The rabbit was injected four times over six weeks, at two-week intervals. Antibody production was confirmed by immunoblotting against rPla or 3 using pre-immunization rabbit serum as the control. Polyclonal antibodies were purified using Protein A affinity chromatography (Amersham Pharmacia Biotech).

Biotinylation of polyclonal antibody

To increase the sensitivity of the assay, the purified rabbit IgG was conjugated to biotin as described by Edward and Meir (13), and used as the secondary antibody in the sandwich ELISA.

Sandwich ELISA development and validation

An indirect sandwich ELISA was developed and validated to quantify Pla or 3 levels in *P. orientalis*. Polystyrene 96-well microtiter plates (MaxiSorp™, Nunc, Roskilde, Denmark)

were coated with 100 µl of pAb (1:1000 diluted) in 0.05 M carbonate/bicarbonate buffer (pH 9.6) overnight at 4 °C. The plates were washed three times with PBS-Tween (PBST) (300 µl/well) to remove unbound antigens. After blocking with 2% bovine serum albumin (BSA) (Biosera, UK) in PBS for 1 h at 37 °C, rPla or 3 or pollen extracts were assayed diluted in PBS containing 1% BSA (100 µl/well) for 1 h at room temperature. Biotinylated pAb (1:2000 diluted in PBS containing 1% BSA) was used as the secondary antibody for 2 hours at room temperature. Then, streptavidin horseradish peroxidase (HRP) (1:20000 diluted in PBS containing 1% BSA) was added and incubated for 1 h at room temperature. After three washings with PBST, the binding was visualized using 100 µl/well of TMB substrate for 10 min at room temperature. Finally, the reaction was stopped by 1 M HCl solution (50 µl/well), and the optical density (OD) was measured at 450 nm and reference wavelength at 620 nm using a plate reader (Stat Fax 2100 plate reader, Awareness Technology, USA) with Hyper Terminal software (version 5.1). A standard curve was obtained by plotting the OD values against rPla or 3 concentrations.

Negative (assay dilution buffer) and positive (125 ng/ml of rPla or 3 in PBST containing 0.1% (w/v) BSA) controls were included on the ELISA plate. All samples were analyzed in triplicate. The sandwich ELISA was validated by examining the intra- and inter- day precision, accuracy, and limits of detection and quantification (LOD and LOQ).

Intra- and inter- day precision

To assess intra-day variations, five Pla or 3 concentrations from the linear portion of the standard curve were measured in three replicates within a single assay, and to evaluate inter-day variations, a standard curve was plotted each day for five consecutive days. The precision of the method was reported as the coefficient of variation (CV) for each concentration.

Accuracy

A spiking recovery test was used to determine the accuracy of the sandwich ELISA. Two *P. orientalis* pollen extracts at three dilutions (1:1, 1:4,

and 1:64) were spiked with 3.1 ng/ml of recombinant Pla or 3, and the recovery was calculated as the ratio of the measured over the expected Pla or 3 concentration.

Limits of detection and quantification (LOD and LOQ)

The LOD was defined as the average concentration of Pla or 3 that could be calculated from the blank (assay buffer) OD plus three standard deviations. The LOQ was defined as the lowest Pla or 3 concentration that could be quantified with a CV ≤ 20%.

Results

Expression and purification of Pla or 3

Recombinant Pla or 3 was expressed in *E. coli BL21-CodonPlus* cells with a histidine tag to facilitate protein purification (Fig. 1). The purified allergen was used to prepare ELISA standard curves.

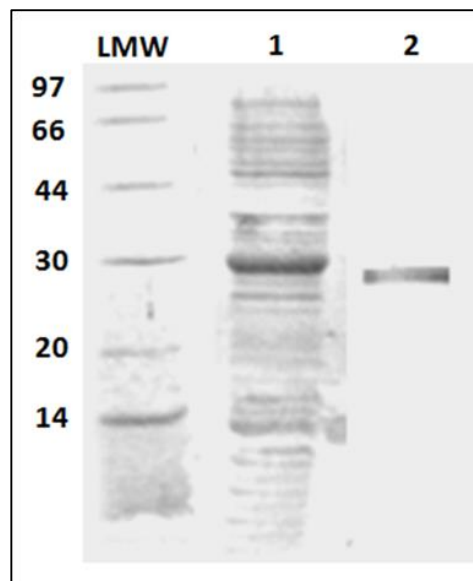


Fig. 1. SDS-PAGE analysis of Pla or 3 expression and purification in *E. coli BL21-CodonPlus* cells. LMW, low molecular weight protein marker; 1, expressed Pla or 3 in *E. coli BL21-Codon Plus* cells; 2, Ni-IDA column-purified Pla or 3.

Sandwich ELISA development and validation

A sandwich ELISA was optimized to use 3 µg/mL of pAb as the capture antibody, 1:2000-diluted biotin labeled pAb as the detection antibody, and 1:20000-diluted streptavidin-HRP. The assay showed the lowest background and highest sensitivity using these antibody concentrations.

Sandwich ELISA for Pla or 3 Quantification in Pollen Samples

Intra- and inter- day precision

To determine the precision of the sandwich ELISA, intra- and inter- day assay variation was determined using five Pla or 3 concentrations from the working range of the standard curve (25, 6.25, 1.62, 0.4, and 0.1 ng/ml) three times per day for five days. Within

this range, the intra-day assay CVs were 2.37, 1.23, 1.66, 1.36, and 4.05% at 25, 6.25, 1.62, 0.4, and 0.1 ng/ml, and the inter-day assay CVs were 7.52, 8.31, 9.53, 11.97, and 17.7%, respectively (Table 1).

Table 1. Determination of intra- and inter- day variation for detection of Pla or 3 allergen in *P. orientalis*

Concentration of standard (ng/ml)	%CV intra-day	%CV inter-day
25	2.37	7.52
6.25	1.23	8.31
1.62	1.66	9.53
0.4	1.36	11.97
0.1	4.05	17.7

Accuracy

The recovery was assessed by the ability of the ELISA to recover a known concentration of recombinant Pla or 3 added to each extract dilution.

The recovery percentage was 88-106.4% at working concentrations from 0.31 to 26.5 ng/ml (Table 2).

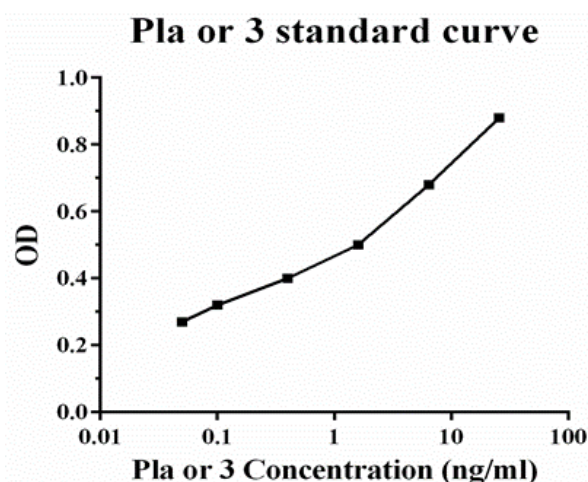
Table 2. Determination of recovery for two different samples at three dilutions (1:1, 1:4, and 1:64).

Sample	Dilution	Pla or 3 level in sample (ng/ml)	Spiking with rPla or 3 (ng/ml)	Observed (O) value (ng/ml)	Expected (E) value (ng/ml)	Recovery (O/E) (%)
Vakilabad Boulevard	-	26.5	3.1	32.2	30.5	105.5
	1:4	6.7	3.1	9.4	9.8	96
	1:64	0.47	3.1	3.2	3.5	91.4
Azghad	-	18.8	3.1	23.3	21.9	106.4
	1:4	5.2	3.1	7.3	8.3	88
	1:64	0.31	3.1	3	3.4	88.2

Limits of detection and quantification (LOD and LOQ)

A linear standard curve was plotted on a semi-logarithmic scale, in the quantification range between 0.1 and 25 ng/ml (Fig. 2). The calculated LOD was 0.037 ng/ml. The LOQ for Pla or 3 with a CV \leq 20% was determined to be 0.1 ng/ml.

Fig. 2. Standard curve of the sandwich ELISA for Pla or 3. Each point represents the optical density (OD) of each Pla or 3 concentration. The quantification range of the standard curve was between 0.1 and 25 ng/ml.



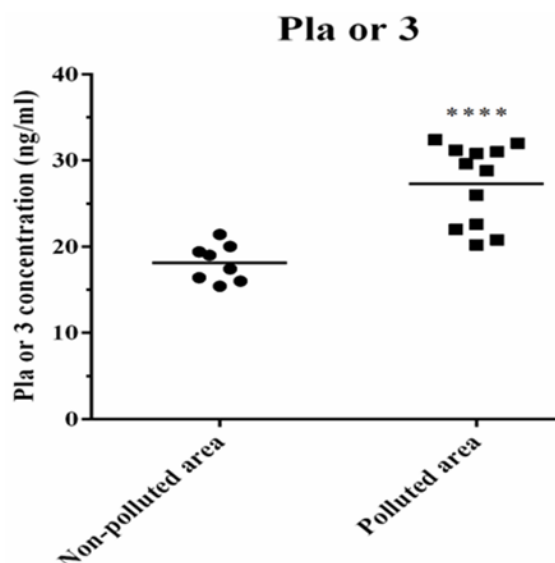


Fig. 3. Pla or 3 concentrations (ng/ml) in *P. orientalis* pollen extracts collected from polluted and non-polluted sites. Pla or 3 levels were significantly greater in pollens from polluted than in pollens from non-polluted sites. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Pla or 3 levels in pollen extracts sampled from different sites

The ELISA was used to measure Pla or 3 in *P. orientalis* pollen extracts grown near high-traffic roads and those from rural regions. As shown in Fig. 3, Pla or 3 levels were significantly greater in pollen extracts from polluted areas than in those collected from unpolluted sites ($p < 0.0001$).

Discussion

Exposure to *Platanus* pollens is considered as a major cause of seasonal allergic reactions leading to high sensitization rates during the pollination period (8). This genus is widely cultivated in parks and streets of Mashhad city (3).

Pla or 3, a major allergen of *P. orientalis*, is a member of the ns-LTP group, categorized as the PR-14 family. Pathogenesis-related proteins (16) are part of the plant defense system, and their expression is up-regulated in response to environmental stressors (5, 7). Several allergens have been identified in the PR protein family (5, 9). Previous studies suggest that air pollution might also stimulate the expression of these proteins (9), leading to greater abundance of allergens categorized as PR proteins in polluted areas.

In this study, we quantified Pla or 3 levels in pollen extracts from different sites, using a sandwich ELISA. For this reason, specific polyclonal antibodies were produced by immunizing a female

rabbit with recombinant Pla or 3. The LOD of this assay was set at 37 pg/ml; similarly, Arilla et al. (4) developed a sandwich ELISA to quantitate two *Parietaria judaica* major allergens, Par j 1 and par j 2, belonging to the lipid transfer protein family. They reported 25 pg/ml as the LOD, similar to that of our study.

With regard to the validation of the sandwich ELISA, the intra- and inter- day assay variabilities were less than 18%, demonstrating the assay's reproducibility.

We found greater Pla or 3 levels in heavy-traffic urban areas than in unpolluted areas. This result agrees with the findings of Suarez-Cervera et al. (6) and Cortegano et al. (7), which reported greater amounts of Cup a 3 allergen, a PR-5 protein, in *Cupressus arizonica* pollen sampled from heavy-traffic roads than in pollens from low-traffic areas. In addition, a study performed by Armentia et al. (5) showed greater protein content in urban areas than in rural districts.

In summary, this report describes an ELISA technique for Pla or 3 measurement in pollen extracts prepared from different environments. The acceptable sensitivity, reproducibility, accuracy, and precision acquired in this study made this method a valuable tool for the immunodetection of Pla or 3 in *P. orientalis* pollen. Using the developed and validated ELISA, we showed that Pla or 3, a

member of the PR protein family, is substantially more abundant in pollens grown along traffic-polluted roads than in those from unpolluted sites. This result should be considered in developing public policies to reduce traffic pollution, which leads to reduced allergic reactions in atopic subjects.

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Acknowledgement

This study was supported by grant number 920506 from the Vice Chancellor of Research of Mashhad University of Medical Sciences, Mashhad, Iran. The results of this article are part of F Sedghy's Ph.D. dissertation. The authors declare no conflict of interest.

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