Expression and purification of a major allergen, Pla a 1, from *Platanus acerifolia* pollen and the preparation of its monoclonal antibody

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Abstract. *Platanus acerifolia* pollen is considered an important source of airborne allergens in numerous cities. Pla a 1 is a major allergen from *P. acerifolia* pollen. The present study aimed to express and purify Pla a 1, and to prepare its monoclonal antibody. In the present study, the Pla a 1 gene was subcloned into a pET-28a vector and transformed into the ArcticExpress™ (DE3) RP *Escherichia coli* host strain. The purified Pla a 1 was then used to immunize BALB/c mice. When serum detection was positive, spleen cells were isolated from the mice and fused with SP2/0 myeloma cells at a ratio of 10:1. Hybridoma cells were screened by indirect ELISA and limiting dilution. Positive cells were used to induce the formation of antibody-containing ascites fluid, and the antibodies were purified using protein A-agarose. The results of the present study demonstrated that recombinant Pla a 1 was successfully expressed and purified, and exhibited positive immunoglobulin E-binding to serum from patients allergic to *P. acerifolia*. A total of 11 hybridomas that steadily secreted anti-Pla a 1 antibody were obtained and an immunoblotting analysis indicated that all of these monoclonal antibodies specifically recognized the Pla a 1 protein. These results suggested that specific anti-Pla a 1 antibodies may be obtained, which can be used for the rapid detection of Pla a 1 allergens and in the preparation of vaccines against *P. acerifolia* pollen.

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Introduction

Pollen-induced allergic diseases, including rhinitis (1,2), asthma (3) and atopic dermatitis (4), are significant health problems that are often season-dependent. Numerous plants can release pollen, including poplar, cypress and *Platanus* trees, and grasses, which may cause serious allergic diseases.

Due to its high resistance to diseases, *Platanus acerifolia* is widely planted worldwide (5); however, high concentrations of its pollen are detected during the flowering season (6), and *P. acerifolia* is considered an important source of allergenic pollen in numerous cities (7). The reported prevalence of sensitization to *P. acerifolia* pollen in Mediterranean Europe ranges between 3 and 52% (8). *P. acerifolia* is also a major cause of pollen-induced allergy in Spain; the prevalence ranges between 52 and 56% in central Spain, between 8 and 9% in north-western Spain, and 17% of the population is sensitive to *P. acerifolia* pollen in south-western Spain (9).

Three major allergens have been identified in *P. acerifolia* pollen. Pla a 3 is a non-specific lipid transfer protein (10) and 45% of Spanish patients with *P. acerifolia* pollen allergies were reported to be sensitive to natural Pla a 3 (11). Pla a 2 is a 43-kDa glycoprotein that displays polygalacturonase activity, and is associated with the allergic responses of 84% of patients worldwide with planetree-induced pollinosis (11,12). Pla a 1 is an 18-kDa non-glycosylated protein that has sequence homology to invertase inhibitory proteins (13-15) and pectin methylesterase inhibitor proteins (16). In addition, 84% of patients with *Platanus* allergies in Western European cities are sensitive to Pla a 1 (8).

Previous studies have reported the expression and purification of Pla a 1 (16,17). Monoclonal antibodies (mAbs) that specifically target Pla a 1 may be used in its quantification,

as well as for the further improvement of pollen allergy immunotherapy (18-20). At present, to the best of our knowledge, there is no commercial mAb against the Pla a 1 allergen. Therefore, the present study produced and purified mAbs that specifically bound Pla a 1, which may be used in the quantification of this allergen. In addition, an indirect ELISA was developed with mAbs, which were produced against recombinant Pla a 1.

Materials and methods

Patients and samples. A total of 6 patients (age, 14-34; 3 males and 3 females; recruited between January and May 2015) with allergic rhinitis, with positive skin prick test (allergens supplied by ALK-Abelló, A/S, Hørsholm, Denmark) and positive serum immunoglobulin (Ig)E test to *P. acerifolia* pollen extract (ImmunoCAP assay; Phadia AB; Thermo Fisher Scientific, Inc., Waltham, MA, USA)], and 6 healthy controls (age, 19-45; 3 males and 3 females; recruited in May 2015) were recruited in the present study. The study protocol was approved by the ethical committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). Written informed consent for the use of blood samples was obtained from all participants prior to study entry, according to the declaration of Helsinki. Serum was extracted from whole blood samples (2 ml, collected three times from each individual) by centrifugation at 6,000 x g and 4˚C for 20 min.

Expression and purification of Pla a 1 in E. coli. The nucleotide acid and amino acid sequences of Pla a 1 were obtained from the GenBank database (AJ427413.2; https://www.ncbi.nlm.nih.gov/nuccore/) The open reading frame (ORF) of Pla a 1 comprises 540 bases pairs, encoding 180 amino acids. This ORF contains a 24 amino acid signal peptide. Mature Pla a 1 comprises 468 bases pairs, encoding 156 amino acids. The nucleic acid sequence of mature Pla a 1 was synthesized by GenScript (Nanjing) Co., Ltd. (Nanjing, China) and was subcloned into a pET-28a vector (Novagen; Merck KGaA, Darmstadt, Germany) using *Eco*RI and *Xho*I sites, and the clone was verified by Sanger DNA sequencing, as described previously (21). The nucleotide acid sequence of Pla a 1 is as follows: Gccgatattgttcagggcacatgcaagaaagttgctcagagaagccca aacgtgaactacgatttctgcgtgaaatctcttggagcagatcctaagagccacactgg atcttcaaggacttggggtcatctcagcgaatttagccatacagcatggatctaaaatcc aaacatttattggtcgcatcttgaaaagtaaagtggacccagctcttaagaaatacttgaat gattgtgtggggctttacgctgatgcgaagtcttcagttcaagaggccatagctgacttc aagtccaaggactacgcatcagctaatgtgaaaatgagtgcggctttggacgactcagtg acttgtgaagatgggtttaaggagaagaaaggtatagtatcaccggtgacgaaggag aacaaggattatgtacaactgactgcaatatctcttgcaattaccaaactgcttggtgcttga. The recombinant pET28a-Pla a 1 plasmid was transformed into the ArcticExpress™ (DE3) RP *Escherichia coli* host strain (Sigma-Aldrich; Merck KGaA). A total of 6 colonies were selected and placed separately in 3 ml luria broth (LB)-kanamycin broth induced by 0.5 mM Isopropyl-β-D-thio galactopyranoside (IPTG) for 4 h at 37˚C. After induction, 12% SDS-PAGE was performed and the colony with an obvious band at 23 kDa was selected for inoculation in 3 ml LB-kanamycin broth, and was incubated at 37˚C overnight. Subsequently, 0.5 ml of the culture was inoculated into 50 ml fresh LB-kanamycin broth, and incubated at 37˚C with agitation at 250 rpm, until the optical density (OD) at A600 nm reached 0.6-0.8. IPTG was added to the final concentration of 0.5 mM and the culture was incubated for a further 4 h at 37˚C. The bacterial cells were harvested by centrifugation at 6,000 x g for 10 min at 4˚C, and were lysed in lysis buffer containing 20 mM Tris-HCl and 100 mM NaH_2PO_4 by sonication at 40 kHz (4 sec pulse-on, 8 sec pulse-off). After sonication, proteins from non-induced recombinant Pla a 1 whole cell lysate, IPTG-induced recombinant Pla a 1 whole cell lysate, and supernatant and precipitation (inclusion bodies) fractions after ultrasonication, were boiled for 10 min, visualized with loading buffer [250 mM Tris-Hcl; 10% SDS (w/v); 0.5% bromophenol blue (w/v); 50% glycerin (v/v); and 5% 2-mercaptoethanol (v/v)] and were analyzed by 12% SDS-PAGE (10 μg protein per lane). The BCA method was used to quantify protein. The results demonstrated that the recombinant Pla a 1 was mainly contained within inclusion bodies. So the inclusion bodies were collected by centrifugation at 10,000 x g for 20 min at 4˚C. Following solubilization of the inclusion bodies using 8 M urea, the supernatant was loaded onto a Nickel column [GenScript (Nanjing) Co., Ltd.], washed with running buffer containing 20 mM Tris-HCl, 100 mM NaH_2PO_4, 10 mM imidazole and 8 M urea (pH 8.0), and eluted with elution buffer containing 20 mM Tris-HCl, 100 mM NaH_2PO_4, 250 mM imidazole and 8 M urea (pH 8.0). The eluted fractions were collected and dialyzed with 6, 4, 2, 1 and 0 M urea at 4˚C, each for 3 h. Eluates were subsequently subjected to 12% SDS-PAGE (10 μg protein per lane).

SDS-PAGE analysis of the expression and purification of Pla a 1 in Escherichia coli. These proteins (10 μg per lane), including whole cell lysate, fractions after ultrasonication and eluates were analyzed by 12% SDS-PAGE. The gel was incubated in 100 ml solution with 1.5 mM Coomassie Brilliant Blue at room temperature for 1 h. Subsequently, the gel was washed in 100 ml solution containing 30 ml methyl alcohol (analytic reagent, >99.5%), 10 ml acetic acid (analytic reagent, >99.5%) and 60 ml distilled water for 3 h.

Immunoreactivity of human sera with recombinant Pla a 1. Immunoblotting for the detection of serum specific IgE was performed with recombinant Pla a 1, as described previously (14). Recombinant Pla a 1 (5 μg) was separated by 12% SDS-PAGE under reducing conditions and was then transferred to polyvinylidene difluoride (PVDF) membranes (22). The PVDF membranes were blocked in 5% skim milk at room temperature for 2 h and were then incubated with a mixed serum sample from 6 patients with *P. acerifolia* pollen allergies [diluted 1:40 in phosphate-buffered saline (PBS)] as the primary antibody overnight at 4˚C. Following rinsing with PBS, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-human IgE mAb (cat. no. AHI0504; Thermo Fisher Scientific, Inc.; diluted 1:3,000 in secondary antibody diluent) at room temperature for 1 h and then detected by a ImageQuant LAS 4000 Mini Detection System (GE Healthcare Life Sciences, Little Chalfont, UK) using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA). A mixed serum sample from 6 healthy individuals diluted

in PBS (1:20) was used as negative serum control in this experiment.

Generation, purification and characterization of mAbs against recombinant Pla a 1 immunization. The purified recombinant Pla a 1 was used as an antigen, which was diluted with PBS to a concentration of 1 mg/ml. For the initial immunization, 5 female BALB/c mice (age, 6-8 weeks; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were immunized subcutaneously with 100 μg Pla a 1 emulsified with an equal volume of complete Freund's adjuvant (23-25). Mice were kept at a temperature of 18-22˚C, humidity of 40-70%, a 12-h light/dark cycle and food and water was freely available. A total of 2 and 4 weeks after the initial injection, booster injections were administered subcutaneously, with the same quantity of Pla a 1 emulsified with an equal volume of incomplete Freund's adjuvant (18,26). Subsequently, the serum of each mouse was collected 2 weeks after each immunization by centrifugation of 100 μl blood at 6,000 x g and 4˚C for 20 min. Each serum titer was determined by indirect ELISA, as previously described (27). Finally, the mice with the highest serum titers were administered intraperitoneal injections of Pla a 1 without adjuvant 2 days prior to fusion (26,28). The mouse with the highest serum titer was selected for hybridoma production (29,30).

Cultivation of mouse myeloma cells. Mouse myeloma cells (SP2/0) were cultivated in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 at 37˚C. Cells in the exponential growth phase were grown to concentrations of $4x10^5$ cells/ml prior to cell fusion (20,31).

Fusion and selection of hybridoma cells. Following sacrifice, spleen cells harvested from the immunized mouse with the highest serum titer were fused with SP2/0 cells at a ratio of 10:1 using 50% polyethylene glycol (PEG). The hybridoma cells were selected using hypoxanthine-aminopterin-thymidine (HAT) medium, as previously described (32,33). After 10-14 days of fusion, the supernatants of the harvested spleen cells were screened by indirect ELISA for antibodies against Pla a 1. Hybridoma cells from positive wells were cloned by limiting dilution and were repeatedly subcloned to obtain stable cell lines secreting antibodies (31).

Large-scale preparation of mAbs. Pla a 1-specific mAbs were prepared as previously described (34). Briefly, following intraperitoneal injection of hybridoma cells ($5x10^5$; stable cell lines secreting antibodies) in three mice, ascites fluid was produced in all three BALB/c mice within 7-14 days. Purified mAbs were obtained from the ascites fluid by affinity chromatography using protein A-agarose (Bio-Rad Laboratories GmbH, München, Germany), as previously described (35), and were analyzed by SDS-PAGE.

SDS-PAGE of purified mAbs. The BCA method was used to quantify purified mAbs and the purified mAbs (10 μg per lane) were analyzed by SDS-PAGE (gel concentration of 12%). The gel was incubated in 100 ml solution with 1.5 mM Coomassie Brilliant Blue at room temperature for 1 h. The gel was washed in 100 ml solution containing 30 ml methyl alcohol (analytic reagent, >99.5%), 10 ml acetic acid (analytic reagent, >99.5%) and 60 ml distilled water for 3 h.

ELISA. ELISA for the determination of serum titer was conducted, as previously described (13,36). Briefly, microwell plates were coated with 100 μl 5 μg/ml Pla a 1 and incubated at 4˚C for 24 h. Subsequently, coated wells were blocked with 200 μl PBS containing 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA) and were incubated with 100 μl diluted serum (1:500) from the mouse with the highest serum titer at 4˚C for 1 h. Following incubation with 100 μl HRP-conjugated goat anti-mouse IgG antibody (1:4,000; cat. no. M6898; Sigma-Aldrich; Merck KGaA) at 4˚C for 1 h. Peroxidase activity was measured by adding 100 μl 3,3',5,5'-O-tetramethylbenzidine solution as a substrate and the reaction was terminated by adding 50 μl 3 M H_2SO_4. Subsequently, the optical density was measured at 450 nm (14).

Results

Expression and purification of Pla a 1 in E. coli. The *P. acerifolia* pollen Pla a 1 was subcloned into a pET-28a vector and transformed into the Arctic Express™ (DE3) RP *E. coli* host strain. The results demonstrated that Pla a 1 was predominantly expressed within inclusion bodies (Fig. 1A). The Pla a 1-containing inclusion bodies were purified using Ni columns. Following the successful renaturation of purified Pla a 1, ~1.4 mg recombinant Pla a 1 was obtained from 500 ml cell culture. The purity of the purified Pla a 1 was identified by SDS-PAGE as a single band with an apparent molecular weight of 20 kDa (Fig. 1B).

Immunoreactivity to IgE of Pla a 1. In order to determine the allergenicity of Pla a 1, the ability of Pla a 1 to bind IgE in the serum of patients with *P. acerifolia* pollen allergies was determined by western blotting. As presented in Fig. 2, mixed serum from patients with *P. acerifolia* pollen allergies exhibited positive IgE reactivity to Pla a 1, whereas mixed serum from healthy controls failed to do so.

Generation, purification and characterization of mAbs against recombinant Pla a 1. In the present study, BALB/c mice were immunized four times with the purified Pla a 1 together with an adjuvant, after which splenocytes were collected and fused with SP2/0 using 50% PEG. The fused cells were selected in HAT medium. Positive cells were screened with ELISA and subcloned by limiting dilution at least three times, in order to obtain stable cell lines secreting mAbs. The titers of the hybridoma culture supernatants were determined with indirect ELISA based on purified Pla a 1 (18). In this assay, the supernatant of SP2/0 myeloma cells was used as the negative control. A total of 11 hybridoma cell lines stably secreting mAbs were screened; the cell lines were named as follows: 6D12, 6E1, 6F10, 6F12, 6H2, 10C9, 10D9, 10E9, 10F9, 11D5 and 11F5. An OD analysis of the supernatant from each of the 11 hybridoma cell lines revealed that the optimal hybridoma was 6D12 (Table I). When the ratio of the sample OD/blank OD is >2.1, the highest

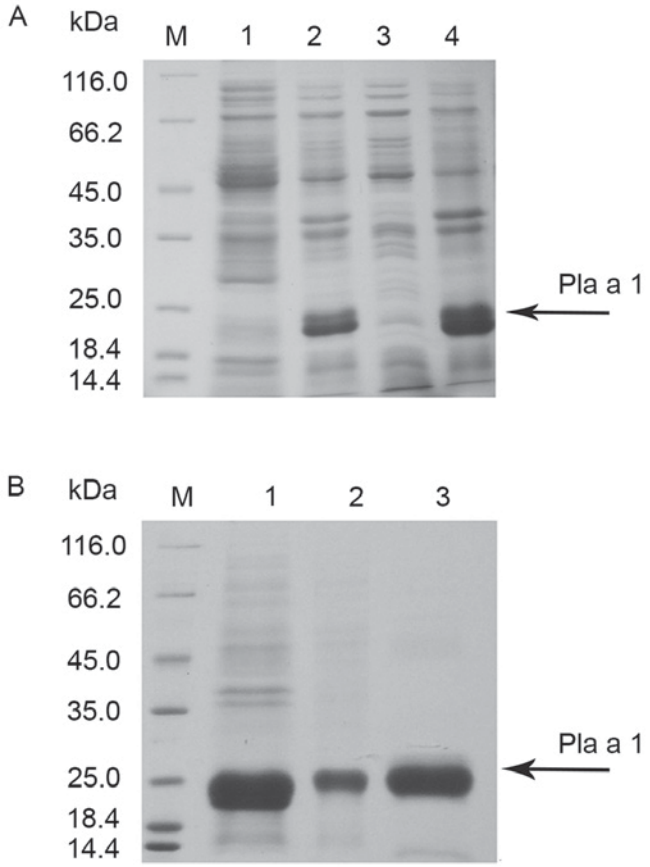

Figure 1. Expression and purification of Pla a 1 in *Escherichia coli*. (A) SDS-PAGE analysis of the expression of Pla a 1 in *Escherichia coli*. Lane M, protein molecular weight standard; lane 1, non-induced recombinant Pla a 1 whole cell lysate; lane 2, isopropyl-β-D-thiogalactopyranosidε-induced recombinant Pla a 1 whole cell lysate; lane 3, supernatant fraction after ultrasonication; lane 4, precipitation fraction (inclusion bodies) after ultrasonication. Pla a 1 was denoted with an arrow. (B) SDS-PAGE following affinity chromatography of Pla a 1 from inclusion bodies. Lane M, protein molecular weight standard; lane 1, unpurified protein; lane 2, eluate of all protein excluding Pla a 1 following purification; lane 3, eluate of target protein. Pla a 1 was denoted with an arrow.

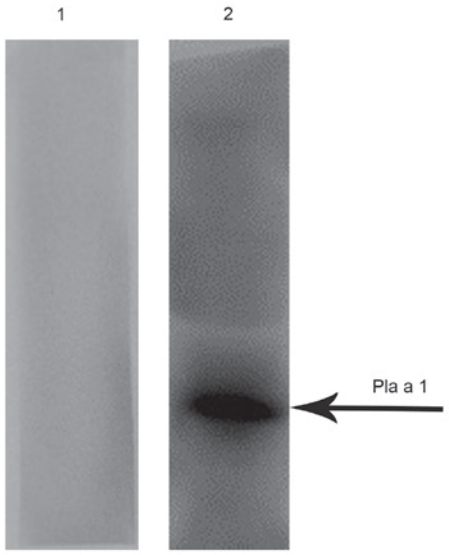

Figure 2. Immunoreactivity to IgE of Pla a 1 was determined by western blot analysis. Lane 1, Pla a 1 was incubated with mixed serum from healthy individuals as a negative serum control; lane 2, Pla a 1 was incubated with mixed serum from patients with *Platanus acerifolia* pollen allergies.

Table I. Optical density of 11 hybridomas.

Hybridoma	Optical density
6D12	3.353
6E1	3.239
6F10	3.312
6F12	3.274
6H2	3.353
10C9	3.353
10D9	3.197
10E9	3.201
10F9	3.099
11D5	3.154
11F5	3.128

dilution degree used (that has a sample OD/blank OD >2.1) is considered to indicate the titer of a mAb. The results of the indirect ELISA indicated that the titer of mAbs purified from the 6D12 hybridoma was >512,000 (Table II). Therefore, 6D12 was used for further cloning using the limiting dilution method. Resurgent cells, cells that exhibited normal activity and good condition after being thawed, were used following liquid nitrogen frozen storage. After three repeats of the limiting dilution method, the positive rate was determined, which is presented in Table III. The monoclonal cell positive rate following the second repeat was 95%, whereas after the third repeat the monoclonal cell positive rate was 100%. These data indicated that, following frozen storage and recovery, hybridoma cells can secrete specific antibodies against Pla a 1, the cell line was named Pla a 1-mAb-6D12.

Finally, the purified mAbs were obtained by protein A-agarose affinity chromatography. The purified antibody was analyzed by SDS-PAGE. It contained a heavy chain of 50 kDa and a light chain of 25 kDa (Fig. 3).

Discussion

The development of high purity and hypoallergenic preparations has become a particular focus of allergic research worldwide in recent years. In addition, screening the main allergens of pollen is a key step for the standardized preparation of pollen allergen vaccines (37). Therefore, the determination of Pla a 1 content is crucial for the development of a *P. acerifolia* pollen allergen vaccine (38,39). In the present study, Pla a 1 was predominantly expressed in the inclusion bodies of *E. coli*. Subsequently, purified Pla a 1 underwent western blot analysis and the results revealed the Pla a 1 exerts immunological activities by binding IgE in the sera from patients with *P. acerifolia* pollen allergies. Furthermore, purified Pla a 1 was used as an immunizing antigen to generate mAbs in mice; a total of 11 hybridoma cell lines stably secreting mAbs against the Pla a 1 protein were screened in the present study. The results of an indirect ELISA confirmed that all 11 mAbs could specifically recognize the recombinant Pla a 1 protein.

mAb-based immunoassays have been used to measure allergen contents in the indoor environment (40). For example,

Table II. Titer of monoclonal antibodies purified from hybridoma 6D12.

Serum dilution times	Optical density
500	2.979
1,000	2.941
2,000	2.483
4,000	2.015
8,000	1.840
16,000	1.632
32,000	1.337
64,000	1.196
128,000	0.845
256,000	0.725
512,000	0.426
Blank	0.074
Titer	>512,000

Table III. Establishment of the monoclonal cell line Pla a 1-mAb-6D12.

Dilution number	Total number of wells	Positive well	Positive rate (%)
1	59	31	52.54
2	40	38	95
3	70	70	100

mAb, monoclonal antibody.

mAbs against Der f 1 (a major allergen of the house dust mite *Dermatophagoides farina*) can be used for the detection of this allergen (41). In addition, Der f 2 is a major allergen from *D. farina*, and mAbs against Der f 2 can be used to create a precise quantitative method to identify allergen components in dust samples (35,42). Der f 7 is another major allergen of house dust mites, and mAbs against Der f 7 may be useful for environmental studies and for the standardization of mite allergen extracts (43,44). Standardization of allergenic extracts is essential to improve their diagnostic and therapeutic quality.

It is important to develop an effective tool to monitor the concentration of allergen components in the outdoor environment. The high titer, highly specific antibodies that have been produced in the present study may be used to reduce the potential for pollen allergens to cause allergy symptoms in individuals that are treated with the antibodies. The antibodies produced may be used as an immunotherapy for humans, which will allow the body to identify antigens associated with the administered antibodies and prevent allergic responses to these antigens, thereby reducing the potential number of allergens in the environment that an individual may be allergic to. In the present study, 11 mAbs were identified that can specifically recognize the Pla a 1 protein. These mAbs may be valuable for the rapid and accurate detection of the Pla a 1 allergen.

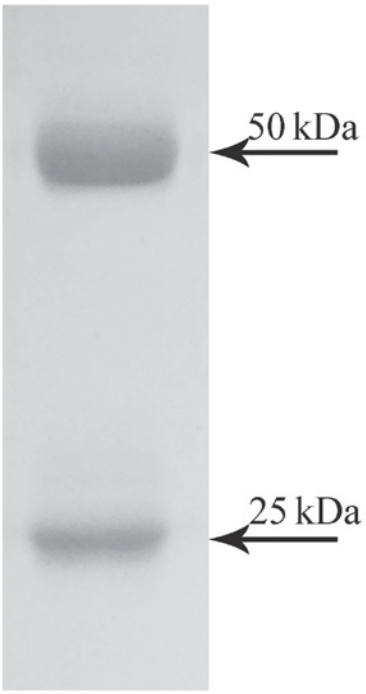

Figure 3. SDS-PAGE analysis of the anti-Pla a 1 antibody purified by affinity chromatography on protein A-agarose. The 50 kDa band indicates the heavy chain of the antibody; the 25 kDa band indicates the light chain of the antibody.

Although a breakthrough has been made regarding the use of mAbs in the accurate quantification of allergen levels (45), there remain some disadvantages to their use. A limitation is that mAbs are specific to only one type of antigen. It is important that more mAbs against *P. acerifolia* pollen allergens are prepared, which may contribute toward the generation of specific immunotherapies, such as a *P. acerifolia* pollen vaccine.

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