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Molecular Cloning and Expression of a New Allergen of *Acacia farnesiana* (Aca f 2)

Najmeh Sepahi, Ali Khodadadi, Mohammad-Ali Assarehzadegan, Akram Amini, Farnoosh Zarinhadideh, and Hosein Ali-Sadeghi

Department of Immunology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

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ABSTRACT

Inhalation of pollens from different species of Acacia is a common cause of respiratory allergy in tropical areas of the world. *Acacia farnesiana* is commonly used as street trees in towns and ornamental shade trees in parks and gardens throughout arid and semi-arid regions of Asia. This study aimed to produce and purify the *A. farnesiana* pollen profilin (Aca f 2) and evaluate its nucleotide sequence homology with profilins of common allergenic plants to predict allergenic cross-reactivity.

Thirty-nine patients who were allergic to Acacia pollens were included in the study. Cloning of Acacia profilin-coding sequence was performed by polymerase chain reaction using primers from Acacia pollen RNA. The cDNA of Acacia pollen profilin was then expressed in *Escherichia coli* using pET-21b(+) vector and purified by metal affinity chromatography. Immunoreactivity of the recombinant Acacia profilin (rAca f 2) was evaluated by specific ELISA, immunoblotting, and inhibition assays.

The coding sequence of the Acacia profilin cDNA was recognized as a 399-bp open reading frame encoding 133 amino acid residues. Eighteen patients (18/39, 46.15%) had significant specific IgE levels against Aca f 2. Immunodetection and inhibition assays indicated that purified Aca f 2 might be the same as that in the crude extract.

Aca f2, the first allergen from *A. farnesiana* pollen, was identified as belonging to the family of profilins. The amino acid sequence homology analysis showed high cross-reactivity between Aca f 2 and other profilins from botanically unrelated common allergenic plants.

Keywords: Acacia; Cloning; Cross Reaction; Expression; Profilin

INTRODUCTION

Acacia farnesiana (*Vachellia farnesiana*), a species of the Fabaceae family, is one of the main causes of allergic rhinitis in subtropical countries such as Iran and other countries along Persian Gulf, where the frequency of sensitization ranges from 25% to 48%.[1-6] The main flowering season of this plant is from February to May.[7] In Iran and throughout arid and sub-arid regions, it is planted as a shade and/or ornamental tree, or for binding sand.[8, 9] Pollens from_Acacia and

Corresponding Author: Mohammad-Ali Assarehzadegan, PhD Department of Immunology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Tel: (+98 611) 3367 54350; Fax: (+98 611) 3332 036, E-mail: assarehma@gmail.com

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mesquite (*P. juliflora*), the common member of Fabaceae family, have been reported as a main source of pollinosis.[1, 2, 4, 10-12]

Protein analysis of Acacia pollen revealed several components ranging from 12 to 105 kDa. Furthermore, up to now there are several different allergic components with molecular weights at 45, 50, 66 and 85 kDa that are reported as dominant IgE-binding components of Acacia pollen.[5, 13] Earlier studies have shown *A. farnesiana* tree pollens to be an important allergen source in tropical and subtropical countries.[1, 2, 5, 13] The proteins of 45, 66 and 85 kDa are recognized as shared allergens among *A. farnesiana* and other allergenic tree pollens.[5, 11, 14] In spite of a high rate of sensitization to Acacia pollen in Iran and neighboring countries, based on our knowledge, there are few studies about the molecular characterization of *A. farnesiana* pollen allergens.

One approach to gain deeper insight into the pathogenesis of allergic diseases is the characterization of allergens. The use of molecular biology techniques during the last decades has significantly improved our knowledge about the structure and biological function of aeroallergens.[15] The combination of data from molecular and structural evaluation of IgE-binding components from pollen extracts is critical for component-resolved diagnosis, specific immunotherapy, and the elucidation of mechanisms underlying sensitization to various allergens.[16, 17] In this study, we produced *A. farnesiana* pollen profilin using *Escherichia coli* and then evaluated the cross-reactivity of its nucleotide sequence with the most common allergenic profilins in tropical regions. Identification and production of the recombinant forms of the common allergens of these pollens may lead to the exploration of new guidelines for diagnostic, therapeutic and preventive purposes.

MATERIALS AND METHODS

Pollen Extract Preparation

Flowers of *A. farnesiana* were gathered from February to May in Ahvaz city, southwest of Iran. Collection of pollen materials and processing was performed as previous studies.[5, 18] Pollens were defatted using repeated changes of diethyl ether. Extraction of pollen proteins was then carried out as described previously,[18] and the protein content of the extract was measured by Bradford's method.[19] Finally, the extract was freeze-dried and stored at -20°C for later use in the current study.

Patient and Skin Prick Tests (SPTs)

The thirty nine respiratory allergic patients participated in this study were referred to Immunology department from outpatient clinics in random. Data about demographic variables and allergy history of patients were obtained by a questionnaire. They were included in the study if they reported at least one ocular, nasal, or respiratory symptom to common indoor or outdoor allergens. Eight healthy subjects who presented with negative SPTs and no specific IgE to the *A. farnesiana* pollen extract were assigned as negative controls.

The patients were also evaluated by a clinical examination and a SPT with common aeroallergens. The human ethics committee of the institute approved the study protocol with informed written consent from each patient. SPTs were performed by an experienced nurse under physician's supervision and patients with a positive SPT donated a serum sample. Afterwards, serum samples of patients were stored at -20°C before use.

Total and Specific IgE by Enzyme-Linked Immunosorbent Assay (ELISA)

Total serum IgE levels were measured using a commercially available ELISA kit according to the manufacturer's instructions (Euroimmun, Lübeck, Germany).

To evaluate levels of specific IgE to *A. farnesiana* pollen proteins in allergic patients, an indirect ELISA was developed. In brief, wells of an ELISA microplate (Nunc A/S, Roskilde, Denmark) were coated with 110µl *A. farnesiana* pollen extract [2µg/well in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6)] at 4°C for overnight. After blocking with 150ml of PBS-2% Bovine serum albumin (BSA) solution for 1 hour at 37°C, the plates were incubated with 100µl patients' sera for 3 hours at room temperature. Each well was then incubated for 2 hours at room temperature with 1:500 dilution of biotinylated goat anti-human IgE antibody (Nordic- Mubio, Susteren, Netherlands) in 1% BSA. The wells were then gently washed 5 times with T-PBS (PBS containing 0.05% Tween 20) to remove unbound anti-IgE and then each received 100μl of horseradish peroxidase-conjugated streptavidin (Bio-Rad, Hercules, CA, USA) (diluted 1:8,000 in PBS- 1%

BSA) and incubated for 1 hour at room temperature. After five washes with T-PBS, each well received 100µl tetramethylbenzidine (TMB-H2O2; Sigma-Aldrich, St. Louis, MO) substrate solution and the plate was incubated at room temperature for 20 min before the reaction was stopped by addition of 100µl of 3 M HCl. Subsequently, the absorbance in each well was assessed at 450nm using an ELISA reader. All results were expressed as optical density (OD) units. OD four times greater than the mean values of three determinations of a pooled sera from negative controls (i.e. >0.15 OD units) was considered to be positive.

Amplification of *A. farnesiana Profilin cDNA* and Determination of Nucleotide Sequence

Total RNA was isolated from 80mg of *A. farnesiana* pollen by Chomczynski and Sacchi methods.[20] First Strand of cDNA was synthesized using RevertAid ™ First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Degenerate primers used for cDNA amplification were designed according to conserved sequence for plant pollen profilins. These primers include the sense 5´-ATGTCSTGGCAGACGTAYGTHGATGA-3´ and the antisense 5´-CATGCCYTGTTCGACCAGRTARTCACC-3´. The amplified product was ligated into the PTZ57R/T TA cloning vector from InsTAclone™ PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instruction. *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA) were transformed with the ligation products using the manufacturer's protocol. Recombinant plasmid was then purified from the gel using a Plasmid Extraction Kit (GeNet Bio, Chungnam, Korea) and sequenced by the dideoxy method and analyzed at the SeqLab Sequence laboratories (Gttingen, Germany).

Construction of Prokaryotic Expression Plasmid Carrying *A. farnesiana Profilin* Gene

The coding sequence from *A. farnesiana* pollen profilin was amplified with *Pfu* DNA polymerase (Thermo Scientific, Waltham, MA, USA), using two specific primers. The obtained sequence (GenBank accession number: KM266374) was implemented in the designation of specific primers for *A. farnesiana* profilin cDNA. These contained overhangs with *Not*I and *Xho*I restriction sites for direct cloning into expression plasmid pET-21b(+) (Novagen, Gibbstown, NJ, USA) as follows: The sense primer (5´-TCCGCGGCCGCAATGTCCTGGCAGACGTATGT AGA-3´, *Not*I restriction site underlined) and the antisense primer (5´-CCCTCGAGCATGCCTTGTTCGACCAGATAGT-3´, *Xho*I restriction site underlined). After PCR amplification, the resulting product was digested with *NotI* and *Xho*I restriction enzymes according to the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA). The purified digested PCR product was ligated into the digested pET-21b(+) plasmid with the same enzymes. Correct constructs were transformed into competent *E. coli* BL21 (DE3) cells (Novagen, Gibbstown, NJ, USA).

Expression and Purification of Recombinant *A. farnesiana Profilin* (rAca f 2)

A fresh clone of recombinant plasmid pET-21b(+)/*Aca f 2* was inoculated into 2ml of LB medium containing 100 µg/ml of ampicillin and incubated at 37˚C. Expression of the recombinant protein was induced by adding isopropyl β-D thiogalactopyranoside (IPTG) to a concentration of 0.2mM. [21] After induction, in order to improve the solubility of rAca f 2, the cultures were incubated at 20˚C and the cells were allowed to grow for a period of 12 hr. Consequently, the cells were harvested by centrifugation (3,000 g, 15 min, 4˚C), resuspended in lysis buffer (50mM Tris–HCl pH 6.8, 15mM imidazole, 100mM NaCl, 10% Glycerol, and 0.5% Triton X-100), and then subjected to three freeze-thaw cycles in liquid nitrogen. Purification of rAca f 2 was performed with Ni-NTA agarose (Invitrogen, Carlsbad, CA, USA) from the soluble phase of lysate, following the manufacturer's instructions.

Specific ELISA for rAca f 2

In order to measure the serum specific IgE levels against the purified rAca f 2, an indirect ELISA was developed as described above, except for the wells of the ELISA microplate which were coated with 15µg/ml of the purified recombinant rAca f 2 per well.

ELISA Inhibition Assays for rAca f 2

ELISA inhibition were performed as described above, except for a pooled serum (1:2 v/v) from hypersensitive *A. farnesiana* allergic patients (Nos. 9,10,14 and 18) which was pre-incubated for overnight

at 4˚C with either 1000,100, 10, 1, 0.1 or 0.01 µg of rAca f 2 as inhibitors or with BSA as a negative control. Inhibition percentage was calculated using the following formula: (OD of sample without inhibitor- OD of sample with inhibitor / OD of sample without inhibitor) ×100.

IgE-Immunoblotting and IgE-Immunoblotting Inhibition for rAca f 2

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of rAca f 2 pollen extract was performed according to Laemmli [22] using 12.5% acrylamide separation gels under reducing conditions. The molecular masses of protein bands were estimated with Image Lab Analysis Software (Bio-Rad, Hercules, CA, USA) in comparison with protein markers of known molecular weights (Amersham Low molecular weight Calibration Kit for SDS electrophoresis, GE Healthcare, Little Chalfont, UK). Separated protein bands from the electrophoresis of rAca f 2 were electro-transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Little Chalfont, UK), as described earlier.[18] Briefly, after blocking and washing, membranes were incubated with a serum pool or individual sera from patients with *A. farnesiana* allergy or with control sera (1:5 dilutions) for 3 hours. Biotinylated goat anti-human IgE (Nordic-Mubio, Susteren, Netherlands) (1:1000 v/v in PBS) was added to the blotted membrane strips and incubated for 2 hours at room temperature. The unbound antibodies were removed from blots by washing with TPBS (PBS containing 0.05% Tween 20) and incubated with 1:10,000 v/v in TPBS-HRP- linked streptavidin (Sigma-Aldrich, St. Louis, Mo, USA) for 1 hour at room temperature. After several washes with TPBS, strips were incubated with Supersignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Waltham, MA, USA) for 5 minutes, and proteins were then visualized by chemiluminescence using ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

To study cross-inhibition between natural and recombinant profilin of *A. farnesiana*, a mixture of 100 µl of pooled serum (1:5 v/v) was incubated with natural *A. farnesiana* pollen extract (20 µg/ml, as inhibitor), rAca f 2 (10 µg/ml, as inhibitor), or BSA (as negative control) overnight at 4˚C and shaking. Preincubated sera were used to assess the reactivity of a PVDF membrane blotted with natural *A. farnesiana* pollen extract and rAca s 2.

RESULTS

Patients and SPTs

Thirty nine patients, 21 males and 18 females (mean age 31.03 ± 8.60 years, age range, 18-49 years), were participated in the study. The patients had seasonal rhinitis without asthma. All subjects had positive SPTs to *A. farnesiana* pollen extract (mean diameter of weal 7.28 ± 1.21 mm; diameter range, 5-10mm). A serum pool from 8 healthy subjects who showed negative SPT responses and no specific IgE against *A. farnesiana* pollen extract were considered as negative control.

Total and Specific IgE Levels

The mean total IgE serum was determined as 278.90 IU/ml (range 145-551 IU/ml). Serum from 39 allergic patients were assessed for specific IgE binding to proteins from *A. farnesiana* pollen extract. All of these patients had significantly elevated specific IgE levels to the extract of Acacia pollen (mean OD_{450}, 1.39±0.41; range, 0.78-2.42) (Table 1).

Cloning and Sequence Analysis of Aca f 2

The sequence analysis of *A. farnesiana* profilin showed an open reading frame of 399-bp coding for 133 amino acid residues with a predicted molecular mass of 14.247 kDa and a calculated isoelectric point (pI) of 4.94. The obtained nucleotide sequence was submitted to NCBI GenBank (Accession Number: KM266374).

The comparison of the deduced amino acid sequence of Aca f 2 with other allergenic plant-derived profilins in the protein database was performed. A high level of sequence identity (95%) was detected between Acacia profilin and Pro j 2 (*P. juliflora* pollen profilin) (Figure 1).

Expression and Purification of Aca f 2

A pET-21b(+)/*Aca f 2* clone was constructed and confirmed by digestion with *Not*I and *Xho*I restriction enzymes. This recombinant plasmid was expressed in *E. coli* strain BL21 (DE3) pLysS as a fusion protein with His_6-tag in the C-terminus.

The rAca f 2 was present in a soluble form in the supernatant, where it was further purified by Ni^{2+} affinity chromatography to yield purified protein. The purified rAca f 2 was quantified by Bradford's protein

Table 1. Clinical characteristics, SPT responses and specific IgE values of patients reactive to recombinant acacia pollen profilin (rAca f 2).

Patients	Age (years)/sex	Clinical history[1]	Acacia pollen extract		rAca f 2
			Skin test[2]	Specific IgE[3]	Specific IgE
1.	28/M	AR,RC	7	1.00	0.85
2.	32/M	AR	7	1.80	0.98
3.	30/F	AR,RC	9	1.75	1.20
4.	21/M	AR,RC	6	0.98	0.80
5.	23/M	AR,RC	7	1.30	0.94
6.	22/F	AR	7	1.50	0.88
7.	38/F	AR	5	1.50	1.00
8.	34/F	AR,RC	10	1.80	0.98
9.	30/M	AR	8	2.40	1.80
10.	32/M	AR,RC	9	1.92	1.20
11.	21/F	AR, RC	7	1.60	0.88
12.	30/F	AR	6	1.54	0.85
13.	42/F	AR,RC	8	1.60	1.10
14.	27/M	AR,RC	10	1.90	1.10
15.	36/F	AR	8	1.50	0.90
16.	34/M	AR	9	1.70	1.10
17.	24/M	AR	6	1.50	0.89
18.	29/M	AR,RC	10	2.10	1.30

[1] AR, Allergic rhinitis; RC, rhinoconjunctivitis. [2] The mean wheal areas are displayed in mm^2. Histamine diphosphate (10 mg/ml)-positive control; Glycerin-negative control. [3] Determined in specific ELISA as OD (optical density) at 450 nm.

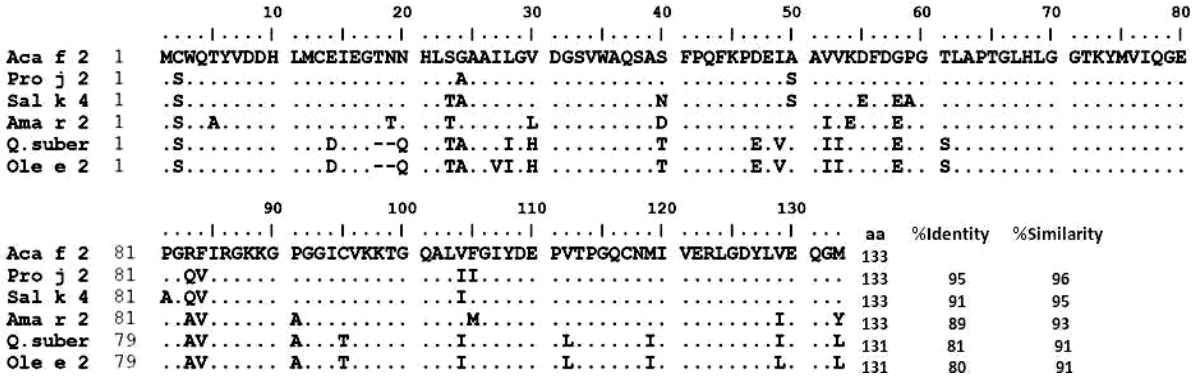

Figure 1. Comparison of the *A. farnesiana* profilin (Aca f 2) amino acid sequence with allergenic profilins from other plants. The amino acid sequence identity and the similarity of Aca f 2 (KM266374) to other members of the profilin family are indicated at the ends of the amino acid sequences. *Prosopis juliflora* (Pro j 2, AHY24177.1); *Salsola kali* (Sal k 4, ACS34771.1); *Amaranthus retroflexus* (Ama r 2, ACP43298.1); *Quercus suber* (AFG16923.1) and *Olea europaea* (Ole e 2, A4GFC2.1).

assay, which showed that approximately 2.5 mg of recombinant protein had been purified from 1 liter of the bacterial expression medium. SDS-PAGE revealed that the apparent molecular weight of the fusion protein was about 18 kDa (Figure 2). The *A. farnesiana* pollen profilin, as a new allergen, was designated Aca f 2 by the WHO/IUIS Allergen Nomenclature Subcommittee.

IgE-binding Analysis of rAca f 2

The levels of specific IgE to the purified rAca f 2 were determined using 39 individual patients' sera. Eighteen patients (18/39, 46.15%) had significant specific IgE levels to rAca f 2 (Table 1). Serum samples from the patients allergic to *A. farnesiana* pollen were further tested for IgE reactivity to rAca f 2 by immunoblotting assays. The results showed that the recombinant form of *A. farnesiana* profilin was reactive with 18 individuals' sera. These results were consistent with those obtained from specific IgE ELISA (Figure 2).

In vitro Inhibition Assays

ELISA inhibition experiments were performed to evaluate the IgE-binding capacity of the purified rAca f 2 compared to its natural counterpart in *A. farnesiana* pollen extract. ELISA inhibition results showed a dose-dependent inhibition of the IgE directed towards rAca f 2 in patients' sera positive to *A. farnesiana*. Pre-incubation of pooled serum with 100 µg/ml of rAca f 2 and *A. farnesiana* pollen extract revealed significant inhibition (90% and 82%, respectively) of IgE binding to rAca f 2 in microplate wells (Figure 3).

Immunoblot inhibition assays showed that pre-incubation of serum samples with rAca f 2 almost completely inhibited the IgE binding to a protein band with an apparent molecular weight of 15 kDa (Figure 4, line 3). Altogether, in vitro inhibition assays revealed a similar IgE reactivity for rAca f 2 and its natural counterpart in *A. farnesiana* pollen extract. In addition, the results indicated that pre-incubation of serum samples with native crude extract of *A. farnesiana* pollen completely inhibited the IgE binding to natural profilin counterparts in *A. farnesiana* pollen extract and other reactive proteins (Figure 4, line 2). However, pre-incubation of the pooled serum with BSA did not affect the IgE-reactivity to rAca f 2 (Figure 4, line 1).

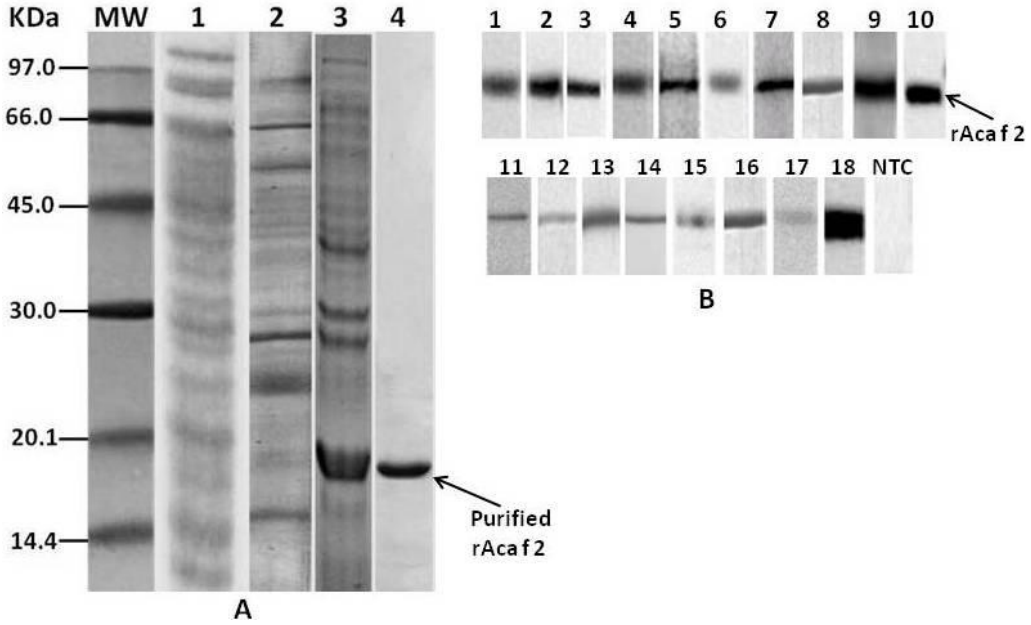

Figure 2. SDS–PAGE and immunoreactivity of recombinant *A. farnesiana* profilin (rAca f 2). A. lane MW, molecular weight marker (GE Healthcare, Little Chalfont, UK); lane 1: Coomassie Brilliant Blue stained SDS–PAGE of the crude extract of *A. farnesiana* pollen in reducing condition; lane 2: Immunoblotting of acacia pollen extract. The strip was first blotted with acacia pollen extract. The strip was then incubated with pooled sera from Acacia allergic patients (Nos. 9,10,14 and 18) and detected for IgE reactive protein bands. Lane 3: rAca f 2 in soluble fraction; lane 4: purified rAca f 2 (as an approximately 18-kDa recombinant protein) on 12.5% acrylamide gel. B. IgE immunoblot of purified rAca f 2 using allergic patients' sera. lanes 1–18, probed with sera from patients with positive for rAca f 2; lane NTC, negative control.

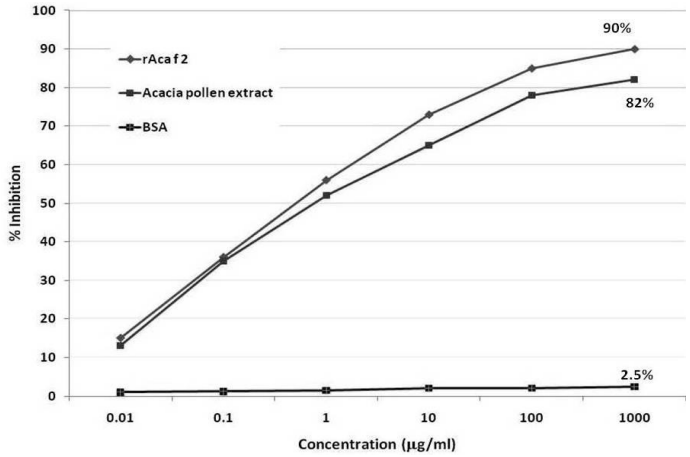

Figure 3. ELISA inhibition with *A. farnesiana* pollen extract and rAca f 2. Inhibition of IgE-binding to rAca f 2 by ELISA using Acacia pollen extract and rAca f 2. Control experiments were performed with BSA.

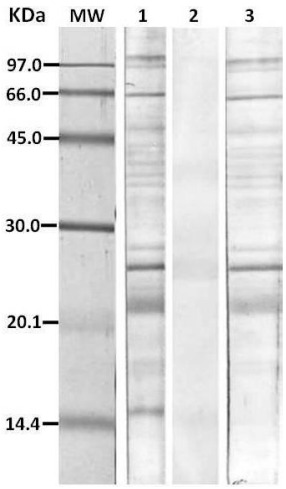

Figure 4: Immunoblotting inhibition assays. lane MW, molecular weight marker (GE Healthcare, UK); lane 1, *A. farnesiana* protein strip incubated with pooled serum without inhibitor (negative control); lane 2, *A. farnesiana* protein strip incubated with pooled serum containing 100µg of *A. farnesiana* pollen extract as inhibitor (positive control); lane 3, *A. farnesiana* protein strip incubated with pooled serum containing 100µg purified rAca f 2, as inhibitor.

DISCUSSION

A. farnesiana is a flowering tree that is widely distributed throughout the tropic and subtropic areas of Iran and other countries in Western Asia.[5, 13] Aca f 2, as the first allergen from *A. farnesiana* pollen, is an allergenic member of the profilin family. The purpose of this study was to clone and produce the recombinant profilin from the *A. farnesiana* pollen in *E. coli* in order to obtain the purified rAca f 2.

Isolated profilins from various sources have indicated high sequence identities and may play roles in IgE-cross reactivity in allergic patients. In previous studies, several allergens from this family, such as Sal k 4, Ama r 2, che a 2 and Ole e 2, have already been identified. [23-26] In this study, profilin from *A. farnesiana* pollen was cloned by a PCR strategy using degenerated primers based on the codons of conserved amino acid sequences from various plant profilins. The open reading frame of *A. farnesiana* profilin contained 399 bases encoding a 14.2 kDa protein that correlates with the molecular characteristics of known plant profilins. The results of immunoblotting assay of *A. farnesiana* pollen extract using pooled sera from the patients was also indicated an IgE-binding protein band with an estimated MW of 15 kDa (figure 1A). So far, different MWs from profilins of varies plant sources have been reported, such as 14.2 -14.6 kDa in three members of the Amaranthaceae family (Sal k 4, Che a 2, Ama r 2), 15 kDa in *Olea europaea* pollen (Ole e 2), and 14.0 kDa in *Artemisia vulgaris* (Art v 4).[23-27] These inconsistencies may be elucidated by diversities in only some amino acid residues, levels of glycosylation or variations in the methods of measuring MWs.[26]

The IgE-binding ability of the purified rAca f 2 to sera from *A. farnesiana* allergic patients was evaluated by specific ELISA and immunoblotting assays in order to confirm that rAca f 2 was correctly folded and bound to IgE as the natural counterpart in Acacia extract. The results of immunoblotting assays for natural profilin

were consistent with those obtained from rAca f 2. A nearly complete inhibition of IgE-binding to natural *A. farnesiana* profilin was also obtained after pre-incubation of pooled serum with purified rAca f 2. Taken together, it seems that rAca f 2 is comprised of IgE-epitopes similar to those of its natural counterpart.

Recently, cross-reactivity of Acacia pollen allergens with other allergenic plants has been described. [5, 28] In tropical areas, the importance of Acacia and the most allergenic members of the Amaranthaceae family (*S. kali*, *A. retroflexus*, *C. album*) and Quercus genus pollens have been described as causing respiratory allergy.[1, 2, 29, 30]

The results of amino acid sequence identity analysis revealed that Aca f 2 have a high degree of identity with the selected profilins from the most common allergenic regional plants, particularly Pro j 2 (*P. juliflora* profilin) (95%). This was expected because Acacia and *P. juliflora* both belong to the Fabaceae family. Generally, this high degree of homology may assume the same tertiary structure.

The successful recombinant bacterial expression of proteins requires denaturation and subsequent refolding in order to obtain the correct conformation, which is especially important for the IgE-binding reactivity of recombinant allergens. The rAca f 2 was successfully expressed in *E. coli* as a soluble molecule. During the process of expression and after induction with IPTG, the temperature of the culture medium was lowered to 18˚C to obtain a high amount of the soluble form of the protein. This low culture temperature strategy was also suggested by previous studies.[24, 26]

In conclusion, *A. farnesiana* pollen is a potent allergenic source with several IgE binding components. The first allergen from the *A. farnesiana* pollen with detectable specific IgE in about 46.15% of *A. farnesiana* allergic patients was identified as the family of profilins. Analysis of the amino acid sequences of *A. farnesiana* profilin and several profilin molecules from other plants also showed cross-reactivity among plant-derived profilins from unrelated families, which may be predicted by the degree of amino acid sequence identity of potential conformational epitopes.

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