

Diagnosis of *Chenopodium album* allergy with a cocktail of recombinant allergens as a tool for component-resolved diagnosis

Hamid Reza Nouri · Mojtaba Sankian · Fatemeh Vahedi ·
Danial Afsharzadeh · Leila Rouzbeh · Maliheh Moghadam ·
Abdolreza Varasteh

Received: 11 April 2011 / Accepted: 11 June 2011
© Springer Science+Business Media B.V. 2011

Abstract *Chenopodium album* pollen is one of the main sources of pollen allergy in desert and semi-desert areas and contains three identified allergens, so the aim of this study is comparison of the diagnostic potential of *C. album* recombinant allergens in an allergenic cocktail and *C. album* pollen extract. Diagnostic potential of the allergenic cocktail was investigated in 32 individuals using skin

prick test and obtained results were compared with the acquired results from *C. album* pollen extract. Specific IgE reactivity against the pollen extract and allergenic cocktail was determined by ELISA and western blotting tests. Inhibition assays were performed for the allergenic cocktail characterization. The exact sensitization profile of all patients was identified which showed that 72, 81 and 46% of allergic patients had IgE reactivity to rChe a 1, rChe a 2 and rChe a 3, respectively. Almost all of *C. album* allergic patients (30/32) had specific IgE against the allergenic cocktail. In addition, there was a high correlation between IgE levels against the allergenic cocktail and IgE levels against the pollen extract. The allergenic cocktail was able to completely inhibit IgE binding to natural Che a 1, Che a 2 and Che a 3 in *C. album* extract. In addition, positive skin test reactions were seen in allergic patients that tested by the allergenic cocktail. The reliable results obtained from this study confirmed that the allergenic cocktail with high diagnostic potential could be replaced with natural *C. album* allergen extracts in skin prick test and serologic tests.

Electronic supplementary material The online version of this article (doi:10.1007/s11033-011-1083-9) contains supplementary material, which is available to authorized users.

H. R. Nouri · M. Sankian · D. Afsharzadeh · M. Moghadam ·
A. Varasteh (✉)
Immunobiochemistry Lab, Immunology Research Center,
School of Medicine, Mashhad University of Medical Sciences,
Mashhad, Iran
e-mail: varasteha@mums.ac.ir

H. R. Nouri
e-mail: nourihr851@mums.ac.ir

M. Sankian
e-mail: sankianm@mums.ac.ir

D. Afsharzadeh
e-mail: dm.lotus@yahoo.com

M. Moghadam
e-mail: moghadamm1@mums.ac.ir

F. Vahedi
Biotechnology Department, Razi Vaccine and Serum Research
Institute, Mashhad, Iran
e-mail: vahedif@yahoo.com

L. Rouzbeh
Department of Food Science, Nour Branch, Islamic Azad
University, Nour, Iran
e-mail: Leila_roozbeh@yahoo.com

Keywords Allergenic cocktail · Recombinant allergen ·
Skin prick test · Component resolved diagnosis

Introduction

Pollens from anemophilous plants are the major problem in Type I allergy and they are the most predominant source of allergens in outdoor environment [1]. *Chenopodium album* (Lambs quarter) is a summer annual plant that belongs to *Amaranthaceae/Chenopodiaceae* family, which grows in temperate zones of southern Europe and the western United States [2]. *C. album* pollen is one of the most important

causes of allergic diseases in desert and semi-desert areas, with a prevalence of 62.9, 53 and 70.7% in Iran, Saudi Arabia and Kuwait respectively [3–5]. Over the last decade, *C. album* pollen allergenic molecules have been analyzed and three antigens of 10–18 kDa have been identified that have capacity of binding to human IgE. Che a 1, Che a 2 and Che a 3 are the most important allergens which identified in *C. album* pollen extract [2, 6]. Che a 1 has been characterized in detail, as a single *N*-glycosylated polypeptide chain, and recognized in more than 77% of *C. album* allergic patients [2]. Profilin (Che a 2) and pol-calcin (Che a 3) in *C. album* pollen extract have been distinguished by IgE inhibition assays. *C. album* allergic patients showed 55 and 46% reactivity to Che a 2 and Che a 3, respectively [6].

Type I allergy is one of the most major health problems that affected more than 25% of the population in industrialized countries [7]. In type I allergy IgE antibodies induce allergic rhinitis, conjunctivitis, asthma, and anaphylaxis against the different allergens [8]. Currently diagnosis of type I allergy performs on the basis of case history, skin prick test (SPT) and detection of allergen-specific IgE antibodies in the serum [9]. To date, the allergic diagnostic tests had been based on natural allergen extracts obtained from natural sources that contain unknown concentrations of allergenic components and high amounts of undefined products [10, 11]. Therefore, in the patients who are sensitized to more than one type of allergenic source it would be difficult and often impossible to precisely identify the disease-eliciting allergen. In such cases, it would be important for the clinicians to know whether a patient is co-sensitized to the several allergen sources, or the patient is sensitized to the several sources due to sensitization to cross-reactive components [7]. One of the main goals of allergy research programs is to improve the tools for diagnosis and specific immunotherapy. The recently suggested concept for allergy diagnosis, component-resolved diagnosis (CRD), that identify allergy eliciting proteins by using pure allergen molecules, which are either produced by recombinant technology or by purification from natural allergen sources, for determination of the patients sensitization profile [12]. Using CRD with recombinant allergens, make it possible to determine the disease-causing molecules and to discriminate two clinical conditions of ‘co-sensitization’ and ‘cross-sensitization’ [13, 14]. Therefore, the first aim of this study is determination of allergenic profile in *C. album* allergic patients in Mashhad, northeast of Iran via recombinant allergens. Subsequently, we designed a cocktail of *C. album* recombinant allergens (rChe a 1, rChe a 2, rChe a 3) which may be replaceable with *C. album* pollen extract for diagnostic purposes.

Materials and methods

Chenopodium album pollen extract and purification of recombinant *C. album* allergens

Aqueous extract of *C. album* was prepared as it described previously [15]. In brief, pollens were mixed with 1:20 w/v phosphate-buffered saline (PBS, 0.15 M, pH 7.4) containing 20 mM EDTA and were shaken at 4°C for 16 h. Subsequently, they were clarified by centrifugation at 7,150×*g* for 20 min at 4°C, and the clear supernatants containing the solubilized proteins were dialyzed, lyophilized and stored at –20°C to use. The proteins quality were checked by SDS-PAGE and Coomassie Blue staining according to Laemmli under the reducing condition [16]. The protein concentration of pollen extract was determined by Bradford’s method [17].

Coding regions of Che a 1, Che a 2 and Che a 3 were amplified using the first strand cDNA as template. The sense primer for Che a 1 was 5′ ATTGCGGCCGCATGGCGAAGTGTCAAGCTG 3′, and the antisense primer was 5′ TATCTCGAGATTAGCTTTAACATCATAAAGATCCA 3′. In the case of Che a 2, the sense and antisense primers were 5′ ATTGCGGCCGCATGTCGTGGCAGACGTACGT 3′ and 5′ TATCTCGAGCATGCCCTGTTTCGACCAG 3′ respectively. Finally Che a 3 was amplified by sense primer 5′ ATTGCGGCCGCATGGCTGCTGAGGATACACCTC 3′ and antisense primer 5′ TATCTCGAGGAAGATCTTGGAAACATCTTT AACCCA 3′. The primers designed according to the deposited nucleotide sequences with GeneBank accession numbers AY049012, FJ985985 and AY082338 for Che a 1, Che a 2 and Che a 3, respectively. The *Not* I restriction sites which were incorporated in the sense primers have been underlined, and *Xho* I restriction sites in antisense primers have been written in italics. The PCR products were first cut with *Not* I and *Xho* I restriction enzymes, and purified by preparative agarose gel electrophoresis (Bioneer, Korea), then subcloned into *Not* I and *Xho* I sites of plasmid pET21b(+) (Novagen, USA). Plasmids were transformed into *Escherichia coli* BL21 codon plus (DE3) RIL (Stratagene, USA). Plasmids from positive clones were isolated using Qiagen tips (Qiagen, USA) and were sequenced according to Sanger method. Expression of rChe a 1, rChe a 2 and rChe a 3 was initiated by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then, recombinant allergens produced as histidine-tag fusion proteins. The rChe a 2 and rChe a 3 which had been produced in the soluble phase, were purified by the native condition of Ni-NTA chromatography (Qiagen, USA). In the other hand, rChe a 1 was expressed in the inclusion bodies and purified via a special method. Allergen expressed cells were resuspended in lysis buffer A (50 mM Tris-HCl; pH 8.0,

10 mM NaCl, and 5 mM EDTA) plus 16 µl of 50 mg/ml lysozyme per gram of pellet. After three times freezing and thawing, the suspension was centrifuged at $14,480\times g$ for 40 min at 4°C. The pellet was resuspended in the buffer B (20 mM Na₂HPO₄ pH 7.2; 20 mM NaCl, and 5 mM EDTA), and again centrifuged under the same condition. Inclusion bodies were solubilized after addition of 8 M urea; 100 mM NaH₂PO₄, and 10 mM Tris-HCl, pH 8.0, and then centrifuged at $12,000\times g$ for 20 min. The resultant supernatant was diluted to 6 M urea and then, loaded on a Ni-NTA column. Afterwards the rChe a 1 was purified according to the denaturing condition (Qiagen, USA). The purified proteins were renatured by using a linear 6–1 M urea gradient in 20 mM Tris-HCl (pH 7.4), 500 mM NaCl and 10% glycerol over a period of 8 h. The purity and integrity of the recombinant proteins were checked by 15% SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. All of the recombinant proteins were desalted via dialysis against PBS, 0.15 M. The concentration of the recombinant proteins was determined by Bradford's method [17].

Demographic and clinical characterization of patients

Most of *Chenopodiaceae* allergic patients are sensitized to more than one allergen source in this family, which may be due to the sensitization to *C. album*, *Amarantus retroflexus* or *Salsola kali*. So this study focused on these co-sensitized patients. In brief, thirty-two allergic patients to *chenopodiaceae* pollens (14 female and 18 male patients; mean age, 28.1 years old; 19–54 years old) were involved in this study. The diagnosis of *chenopodiaceae* pollen allergy in the respective patients was based on a clinical history of seasonal allergic conjunctivitis/rhinitis, and SPT. As control group, four non-atopic individuals (2 female and 2 male patients; mean age 27.8 years old; 19–37 years old) took part in the study. The Patient's characteristics including age, sex, clinical symptoms and total IgE level are summarized in supplementary Table 1. The Human Ethics Committee of the Mashhad University of Medical Sciences (Mashhad, Iran) approved this study. After providing the informed patient consent, skin tests were performed on the ventral side of the subjects' forearms according to Dreborg method [18]. Reactions with a wheal of 3 mm greater than negative controls, surrounded by the mild to moderate erythema, were considered as the positive results. Finally, blood was collected for serum sampling.

Determination of total and specific immunoglobulin E levels and inhibition assays

Total serum IgE levels (kU per liter) were measured by means of a commercially available ELISA kit according to

the manufacturer's instructions (Radim, Pomezia Terme, Italy). The serum-specific IgE levels in the *chenopodiaceae* allergic patients were quantified by means of ELISA as previously described [19, 20]. The specific IgE antibodies against the allergenic cocktail of rChe a 1, rChe a 2 and rChe a 3, as well as the single allergens were measured by using an indirect ELISA in patients' sera. The wells of the ELISA microplate (Nunc MaxiSorp™, Fisher Scientific, Pittsburg, PA) were overnight saturated at 4°C in 15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6 with the allergenic cocktail which includes an equimolar concentration of each of the recombinant allergens. Nonspecific binding sites were blocked using 2% Bovine Serum Albumin (BSA) in PBS, and then the wells were incubated with the allergic patient's serum (1:5 diluted in 1% BSA) at 37°C in the agitation condition. In the next step, 1:1,000 diluted of biotinylated goat anti human IgE antibody (KPL, USA) was added to each of the wells and incubated for 2 h at 37°C. Then, for visualization of IgE complex, the wells were incubated with 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated streptavidin (Bio-Rad, USA) for 45 min. Finally, the colorimetric reaction was performed by addition of chromogenic substrate (tetramethyl benzidine (TMB)/H₂O₂) and the optical density was read at 450 nm by an ELISA reader (Stat fax2100). The optical density (OD_{450nm}) values higher than the mean OD_{450nm} of the negative controls plus three SDs were considered as positive. ELISA inhibition assays were carried out in the microtiter plates, which had been coated with *C. album* pollen extract (50 µg/well). The following steps had exactly similar conditions as were explained earlier in the case of direct ELISA. Except that in the current ELISA, we used a pooled serum (diluted 1:2 in 1% BSA) that overnight pre-incubated at 4°C with equivolumetric of each of inhibitors, including different concentrations of *C. album* pollen extract, allergenic cocktail and BSA/PBS. The percentage of inhibition was calculated according to the formula: inhibition (%) = $[\text{OD}_A - \text{OD}_B / \text{OD}_B] \times 100$, where OD_A and OD_B represent optical densities which were obtained from the non-inhibited and inhibited pool sera, respectively.

IgE-binding capacity of recombinant allergenic cocktail and immunoblotting inhibition assays

Natural *C. album* pollen extract and the allergenic cocktail were tested for IgE-binding capacity by Western blotting. For immunoblotting, the pollen extract and the allergenic cocktail were separated via denaturing SDS-PAGE with 15% polyacrylamide gels, according to the Laemmli method [16]. Protein (60 µg of the pollen extract, 5 µg of each recombinant allergens and the allergenic cocktail

which contains an equimolar concentration of rChe a 1, rChe a 2 and rChe a 3) bands were electro-transferred to the polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp, Bedford, USA) and after that, the PVDF membranes were cut into the strips of approximately 0.6 cm in width. The strips were incubated with BSA 2% in PBS for 16 h at 4°C to block non specific sites. Immunodetection on the strips was achieved by using a pooled serum obtained from the allergic patients (diluted 1:10) or from the individual sera. Complexes of IgE were detected using a biotinylated goat anti human IgE antibody (KPL, USA). The strips were incubated in the secondary antibody (diluted 1:1,000 in BSA 1%) for 1 h at the room temperature. Then the last incubation was performed with 1:20,000 diluted HRP-conjugated streptavidin (Bio-Rad, USA). Finally, the signals were developed using the chemiluminescent method as it was recommended by the manufacturer (ECL-Plus™, Amersham Biosciences) and documented with the use of G: box imaging system (Syngene, UK). For immunoblotting inhibition assays, *C. album* pollen extract was electrophorized and blotted to PVDF and was immunostained with the pooled serum. The pooled serum had been previously incubated with 60 µg of *C. album* pollen extract or the allergenic cocktail as inhibitors or alternatively with PBS as a negative control. The percentage of the inhibition was calculated by densitometric analysis of protein bands with the use of Tiny-Quant v1.55 software (Iscove Lab).

Skin prick test with the allergenic cocktail

The ability of allergenic cocktail in induction of the immediate skin reactions was investigated in the allergic patients. SPT was performed on the forearms of the individuals with *C. album* pollen extract and the allergenic cocktail according to the standard procedure. To construct the allergenic cocktail (15 µg/ml), equimolar concentration of rChe a 1, rChe a 2 and rChe a 3 were mixed and diluted in NaCl 0.9% for SPT. Histamine (10 mg/ml) and NaCl 0.9% served as the positive and negative controls. Elicited weal areas were recorded after 20 min and the weal surfaces of greater than 4 mm were considered as positive.

Statistical analysis

Statistical analysis of data were performed using the Pearson *r* correlation test and Student's *t*-test which are included in the statistical GraphPad Prism 5 software. Correlation coefficients were determined by means of linear regression. A *P*-value of less than 0.05 was considered to be significant.

Results

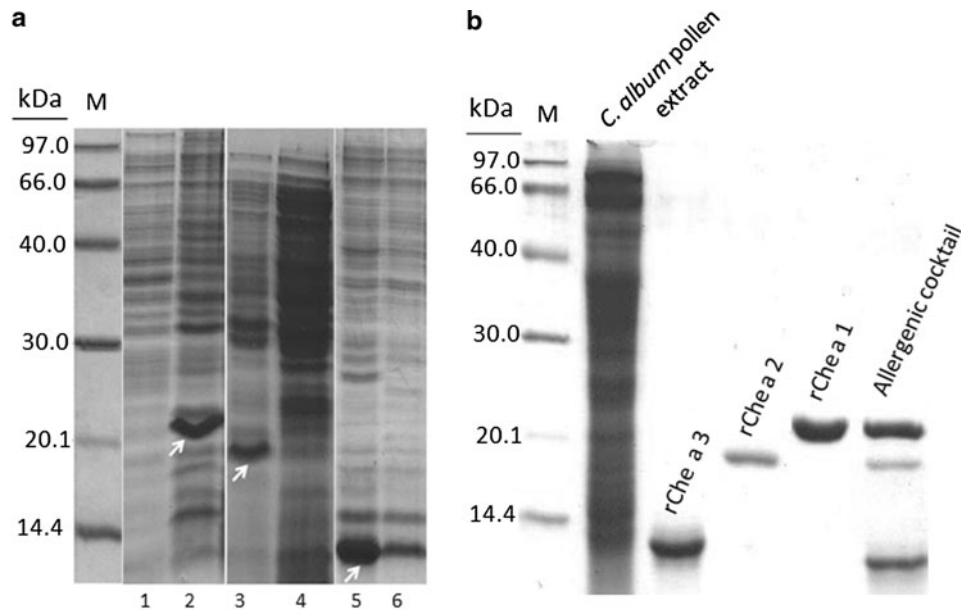
Cloning and expression of an allergenic cocktail from *C. album* pollen

The coding sequence for recombinant Che a 1, Che a 2 and Che a 3 proteins were amplified by RT-PCR of the first strand cDNA which had been prepared from the total RNA. These sequences were successfully inserted into pET21b(+) vector. The sequencing data resulted in a high similarity with deposited sequences for Che a 1, Che a 2 and Che a 3 which reported by AY049012, FJ985985 and AY082338 GenBank accession numbers, respectively. Production of rChe a 2 and rChe a 3 was successfully fulfilled by *E. coli* BL21 codon plus (DE3) RIL and accumulated mainly in the soluble fraction, while rChe a 1 was remained in the insoluble fraction of the cell homogenate under the same conditions (Fig. 1a). After SDS-PAGE and staining of the gels by CBB rChe a 1, rChe a 2 and rChe a 3 were visualized with an apparent molecular mass of 22, 18 and 13 kDa, respectively. These proteins were further purified by Ni²⁺-chelate affinity chromatography (Fig. 1b). The yield of the rChe a 1, rChe a 2 and rChe a 3 were estimated, according to the Bradford's method, which were around 1, 0.4 and 0.6 µg per 1 ml of cell culture, respectively. According to the concentration and molecular weight of recombinant allergens, the molarities of these proteins were calculated about 45.4, 22.2 and 33.3 nmol (nM) respectively. In the same way, 1 nM of rChe a 1 solution was equal to 22 µg of rChe a 1. Also, 1 nM of rChe a 2 and rChe a 3 solutions were equivalent to 18 and 13 µg of rChe a 2 and rChe a 3, respectively. There were 6×10^{14} molecules of each purified recombinant allergens in 1 nM of each recombinant allergen solutions. Therefore, an allergenic cocktail was constructed with an equimolar concentration (0.3 nM) of these recombinant allergens.

Western blot analysis of the purified recombinant allergens

Assessment of the polyacrilamide gel, indicated that *C. album* pollen extracts (Fig. 1b) contained several proteins of approximately 9–85 kDa that occur in variable quantities. These proteins are probably responsible for the allergic sensitization to *C. album* extracts. In details, in staining of *C. album* extract proteins, large quantity of Che a 2 was detected, whereas Che a 1 and Che a 3 were not well represented in the extract. IgE immunostaining of purified rChe a 1, rChe a 2 and rChe a 3 that blotted to PVDF membranes was performed with the sera of 32 patients included in this study. Che a 1 and Che a 2 which were previously reported as the major allergens [2, 6] of

Fig. 1 Coomassie blue stained gel for *C. album* purified recombinant allergens. **a** The polyacrylamide gel stained by coomassie brilliant blue R-250 revealed that rChe a 2 and rChe a 3 express in soluble phase, while rChe a 1 mainly expresses in insoluble phase. Lanes 1, 3 and 5 are supernatants of cells including pET21b(+)/rChe a 1, rChe a 2 and rChe a 3, respectively. Lanes 2, 4 and 6 are inclusion bodies of above cells. **b** SDS-PAGE of crude extracts of *C. album* pollens, Ni-NTA purified recombinant Che a 3, Che a 2, Che a 1, and the allergenic cocktail on a 15% polyacrylamide gel. Lane M low molecular weight (Amersham, Buckinghamshire, UK)



C. album were recognized by 72% ($n = 23$) and 81% ($n = 26$) of the Iranian patient's sera (Fig. 2a). Frequencies of sensitization to Che a 3 (Fig. 2a), an allergenic components from polcalcin family proteins, was 41% ($n = 13$) among 32 patients. IgE immunoblotting with natural pollen extracts indicated that patients have exhibited individual IgE reactivity profiles (Fig. 2b, lane 1). Analysis of data which obtained by immunoblotting was revealed that almost all of *C. album* allergic patients are at least sensitized to one of the rChe a 1 or rChe a 2 or rChe a 3. Two serum samples of allergic patients (no. 7 and 11) had no IgE reactivity with three recombinant allergens. Moreover, 25% ($n = 8$) of allergic patients sera (no. 12, 18, 21, 24, 25, 27, 28, 30 and 32) were recognized which are simultaneously sensitized to all of these allergens. A pooled serum was prepared from these patients sera for inhibition assays. The allergenic cocktail with equimolar concentration of each recombinant allergen showed high potential of IgE reactivity (Fig. 2b, lane 4). To assess the participation of the allergenic cocktail in IgE reactivity, an IgE-binding immunoblotting inhibition assay was designed using a pooled serum, with *C. album* as coating extract, the allergenic cocktail and *C. album* pollen extracts as alternative inhibitors (Fig. 2c). After densitometry analysis of the immunoblotting experiment, a complete inhibition in the responses of IgE reactive bands was obtained when *C. album* extract had been used as an inhibitor. Using the allergenic cocktails as another inhibitor, an inhibition of 95–100% was occurred in the Che a 1, Che a 2 and Che a 3 bands in *C. album* extract. But when the allergenic cocktail had been used as inhibitor no inhibition was accomplished in the other allergenic bands in *C. album* extract.

The patients' IgE reactivity profile: component-resolved in vitro allergy diagnosis with recombinant pollen allergens

Using ELISA test on *C. album* pollen extract, 30 of 32 *C. album* pollen allergic patients were diagnosed. In all of these patients, specific IgE levels (OD values of $450 > 0.4$ nm) were significantly elevated against *C. album* pollen extract. Moreover, there was a significant increase in specific IgE antibodies against the combination of three recombinant allergens (rChe a 1, rChe a 2 and rChe a 3) as the allergenic cocktail in 30 of the 32 allergic patients. Patients no 7 and 11, who had not exposed IgE reactivity to rChe a 1, rChe a 2 and rChe a 3 in immunoblotting, showed OD_{450nm} values of lower than 0.4 with *C. album* extract and the allergenic cocktail (supplementary Table 1). Analysis of serum-specific IgE levels to *C. album* extract and allergenic cocktail showed a highly significant correlation ($r = 0.89$, $P < 0.0001$) (supplementary Fig. 1) between them. Subsequently there was no significant correlation between OD_{450nm} obtained from each recombinant allergen and *C. album* extract or the allergenic cocktail (data not shown). Approximately 72%, 81% and 41% of the sera exhibited IgE reactivity to rChe a 1, rChe a 2 and rChe a 3 respectively. The quantitative analysis of specific IgE levels indicated that rChe a 2 has higher IgE binding capacity in comparison with rChe a 1 and rChe a 3. Although most of allergic patients displayed IgE reactivity to more than one recombinant allergens, but there were a few allergic patients that their sera specifically reacted to rChe a 1 (patient no. 16 and 31) and rChe a 2 (patient no. 6, 19, 20 and 26). In addition, certain patients

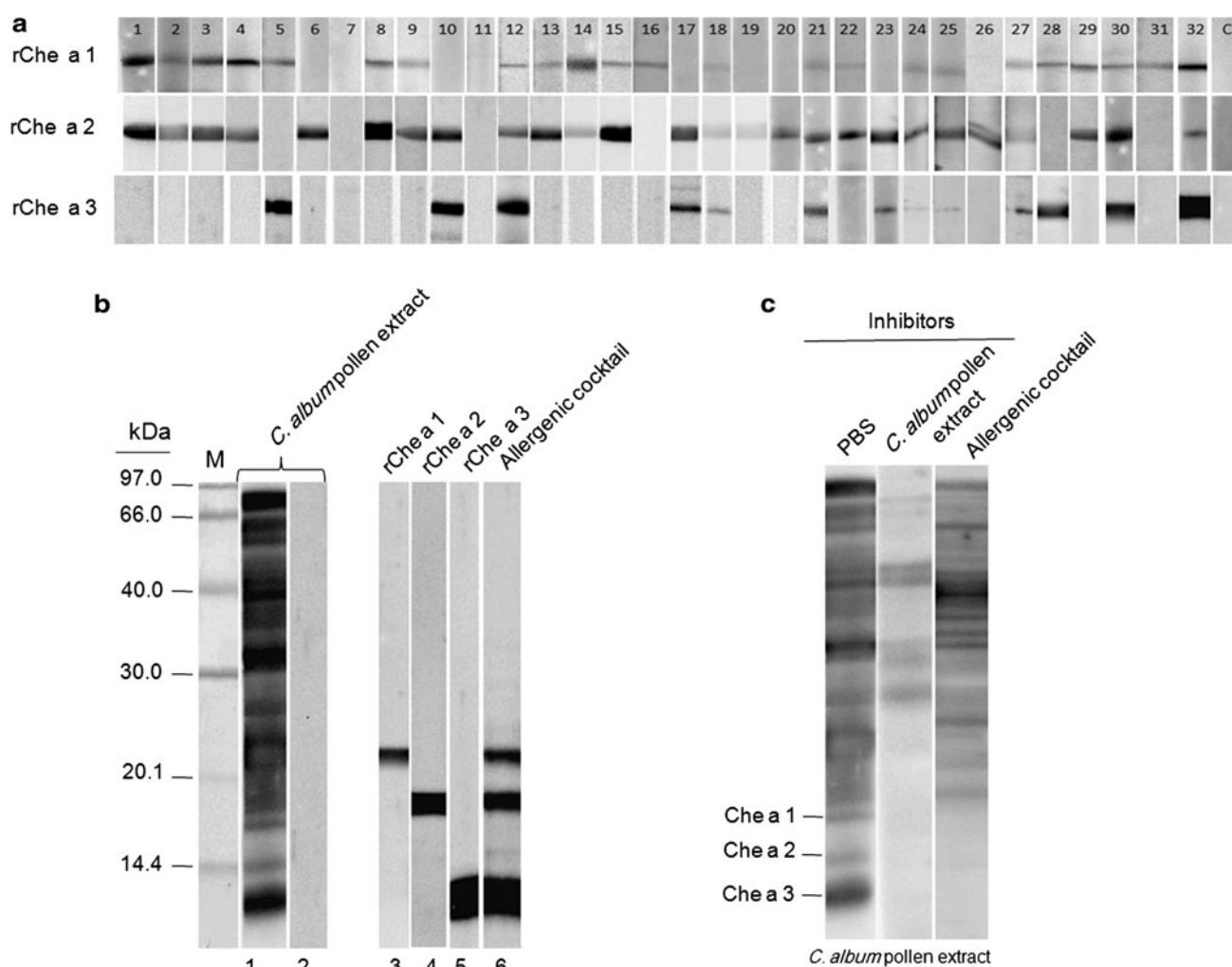


Fig. 2 Immunoreactivity of purified recombinant allergens. **a** IgE binding capacity of purified rChe a 1, rChe a 2 and rChe a 3 checked by sera of allergic and control (C^-) patients. Eight of allergic patients had IgE reactivity to all of rChe a 1, rChe a 2 and rChe a 3. **b** In the lanes 1 and 2, the IgE reactivity of a pooled serum of allergic and non-allergic patients to the *C. album* extract bands are manifested. Lanes 3, 4 and 5 represent interaction of the pooled serum with rChe

a 1, rChe a 2 and rChe a 3, respectively. Lane 6 shows recognition of the allergenic cocktail protein bands by pooled serum. **c** The strips blotted with 60 μ g of crude extract and incubated with a pooled serum pre-adsorbed with PBS, *C. album* pollen extract and the allergenic cocktail as inhibitors. Lane M low molecular weight (Amersham, Buckinghamshire, UK)

(patient no. 12, 18, 21, 24, 25, 27, 28, 30 and 32) had IgE antibodies with varying levels to all three recombinant allergens.

Inhibition of IgE-binding to the natural allergens in *C. album* pollen extract was examined by means of ELISA inhibition. The pooled serum was pre-incubated with (0.1, 1, 10, 100, 1,000 μ g/ml) of the allergenic cocktail or *C. album* pollen extract. About 92% of inhibition was observed in 100 μ g/ml of *C. album* pollen extract as an inhibitor. In contrast, the allergenic cocktail at the same concentration was able to inhibit only 65% of IgE binding to natural allergens in *C. album* pollen extract. When the concentration of allergenic cocktail was increased up to 1,000 μ g/ml, the inhibition percentage moved up to 82%

(Fig. 3). These inhibition data supported that rChe a 1, rChe a 2 and rChe a 3 in the allergenic cocktail had shared many epitopes with their equal natural allergens in *C. album* pollen extract. However, BSA as an unrelated protein did not significantly increase inhibition to the natural allergens.

A cocktail of three recombinant pollen allergens allows in vivo diagnosis of *C. album* pollen allergy

Through use of *C. album* pollen extract (1 mg/ml) in SPT, 31 of 32 allergic patients were diagnosed. In our data, there was only one patient (supplementary Table 1 patient no 26) who showed negative response to SPT with *C. album*

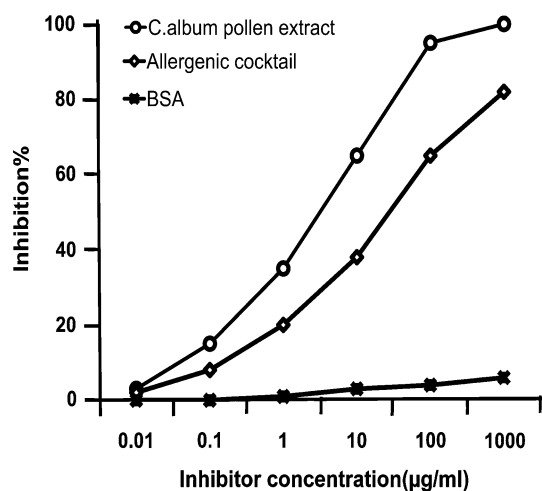


Fig. 3 IgE binding inhibition assay. This experiment was assayed against a pooled serum that pre-adsorbed with allergenic cocktail and crude extract

pollen extract, while he showed positive SPT to *S. kali* and *A. retroflexus* pollen extract beside possessing IgE reactivity to *C. album* extract in ELISA experiment. Moreover, we found two patients with positive skin reactions (supplementary Table 1, patient no 7 and 11) without significant IgE against *C. album* pollen extract in serum. Neither immediate skin type reactions nor adverse side-effects were observed in SPT results of four non-allergic patients. The potency of the allergenic cocktail in induction of in vivo immediate cutaneous reactions was evaluated by applying SPT on 15 volunteer of the respective allergic patients. Almost all of the allergic patients (14 of 15 individuals) exhibited positive skin reactivity to 15 µg/ml of the allergenic cocktail (Fig. 4). Only the patient who had been reported as the non-responding individual to *C. album* extract (patient no 26) did not show any skin reaction to the allergenic cocktail. The mean diameter in millimeters of the wheals that had been induced by the crude extract in *C. alum* sensitized individuals (10.8 ± 0.8) was statistically (*t*-student test, $P > 0.05$) equal to the mean diameter induced to the allergenic cocktail (9.0 ± 0.5).

Comparison between allergen specific IgE antibody levels and SPT results

The level of skin sensitivity that elicited through application of 1 mg/ml *C. album* extract or 15 µg/ml of the allergenic cocktail and determined via the mean wheal diameter, compared to serum specific IgE levels and showed as OD results at 450 nm. According to the results, we found that in almost all of the respective allergic patients, the presence of specific IgE is associated with positive skin reactions to *C. album* extract or/and to the allergenic cocktail.

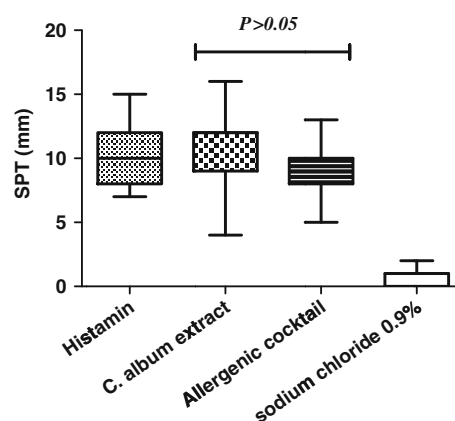


Fig. 4 Skin prick test results from *C. album* allergic patients. This box-plot chart exhibits the skin reactivity in allergic patients. Y-axis is displays mean diameter of wheal areas in mm and X-axis shows the immediate skin reaction inducing factor that include *C. album* pollen extract and the allergenic cocktail; additionally histamine diphosphate (10 mg/ml) and 0.9% sodium chloride are as positive and negative control, respectively

Despite of this association, as it presented in supplementary Table 2, there were weak correlations between the levels of the above mentioned skin sensitivity and serum specific IgE level in respective patients. It means that, there were both patients that exhibited low IgE binding capacity along with the strong skin reactions (supplementary Table 1, patient no. 7 and 11) and patients with high IgE binding capacity to *C. album* and/or to the allergenic cocktail with low or mild skin reactions (supplementary Table 1, patient no. 26).

Discussion

Natural allergen extracts are a poorly defined mixture of allergenic proteins and other unknown macromolecules, which cause some clinical limitations in immunotherapy and diagnosis [21].

The purified natural allergens were introduced as an alternative option in the allergy diagnostic methods and immunotherapy. However, because of low yield and technical difficulties in the purification process of allergens from their natural sources [22], recombinant allergens has been developed in CRD and component resolved immunotherapy (CRIT) [23–25]. During the past decade, with development in molecular biology techniques, many important allergens of different sources such as mites, birch, timothy grass, phleum and parietaria judaica produced and characterized [26–31]. Nowadays, many of researches in the field of allergy focused on construction panels of recombinant allergens and their usage in diagnosis and specific allergen immunotherapy.

In the west of USA and the temperate areas of Europe, *Chenopodiaceae* pollens are one of the most significant pollen allergen sources [2]. Among *Chenopodiaceae* family, *C. album* was selected for this study due to two following reasons. Firstly, *C. album* pollen was one of the most important allergenic sources in desert and semi-desert areas especially in our country, Iran [3]. Secondly, two pan allergens, which belong to profilins and procalcin proteins, were identified in *C. album*. Currently obtained results from this study, has made us able to diagnose the allergic sensitivity to other allergenic members of *Chenopodiaceae* family and additional allergenic sources related to these two pan allergens. The *S. kali* and *A. retroflexuse* pollens have two identified pan allergens belong to profilins protein family which are specified as Sal k 4 and Ama r 2 respectively. Ama r 2 and Sal k 4 showed high cross-reactivity to Che a 2 [32, 33] which caused to propose Che a 2 as a gatekeeper allergen to identify the patients with primary sensitization to *Chenopodiaceae* pollen.

Che a 1, Che a 2 (profilin) and Che a 3 (procalcin) are three identified allergens in *C. album*. So far, Che a 1 has been expressed in *Pichia pastoris*, as Che a 2 and Che a 3 have been expressed in *E. coli* BL21(DE3) in the previous studies [2, 6]. In this study, we have used the *E. coli* BL21 codon plus (DE3) RIL for heterologous expression of these allergens. *E. coli* BL21 codon plus (DE3) RIL as a host contain extra copy of tRNA for leucine, isoleucine and argenine. So all respected allergens with high content of mentioned amino acids were expressed in high yields [34]. Then, rChe a 1, rChe a 2 and rChe a 3 after successfully expression purified by metal affinity chromatography. The purity of each of recombinant allergens was checked by SDS-PAGE which no contamination with other molecules was found. Although, each of recombinant allergens had a defined concentration, we calculated molarity of each allergen according to its molecular weight and concentration. Consequently, the allergenic cocktail with equimolar concentration of each of recombinant allergens was made.

In spite of allergenic extracts which were only applicable in determination of the type of allergenic sources in allergy diagnosis methods, recombinant allergens, make it possible to identify the disease-eliciting allergen molecules [21]. In this study, profile of antibody responses in *C. album* allergic patients was investigated via immunoblotting and ELISA. IgE binding capacity of purified rChe a 1, rChe a 2 and rChe 3 checked via performing western blot test on sera from allergic patients. IgE binding profile of each patient to the recombinant allergens has been shown in Fig. 2a. We resulted that all of the patients have specific sensitization profile to the identified allergens in *C. album* pollen extract. In the other hand, application of immunoblotting inhibition assays confirmed that the allergenic cocktail inhibits IgE-binding to natural Che a 1, Che a 2

and Che a 3 completely (Fig. 2c). The obtained data from ELISA inhibition experiments are in agreement with those obtained from immunoblotting assays. As shown in Fig. 4, lack of complete inhibition of IgE binding by the allergenic cocktail might be explained through the presence of unknown allergens in pollen extract. The results of inhibition assay revealed this possibility that IgE epitopes of rChe a 1, rChe a 2 and rChe a 3 in the allergenic cocktail are similar to their natural counterparts in the *C. album* extract. The above mentioned theory is corroborated by obtaining a high correlation (Supplementary Fig. 1) between measured specific IgE levels of total extract and the allergenic cocktail-specific IgE.

In two separate studies by Rodrigo Barderas and his colleagues, 77, 55 and 46% IgE reactivity with isolated Che a 1, Che a 2 and Che a 3 respectively, has been reported in Spanish *C. album* allergic patients [2, 6]. Up to date, in other studies there is no data about the IgE recognition patterns in sera of different populations. However, in this study through the component-resolved diagnosis concept, we successfully determined the exact sensitization profile of *C. album* allergic patients in Mashhad, northeast of Iran. Approximately 72, 81 and 41% of *C. album* allergic patients showed IgE reactivity to rChe a 1, rChe a 2 and rChe a 3, respectively. It is clear that patients from different geographical areas who exposed to the various species of *C. album*, produce different kinds of IgE profiles. Accordingly, it is uncovered that, in any population there is a variety of IgE recognition patterns. Among three identified allergens of *C. album*, Che a 2 had the maximum reactivity with serum IgE which was determined by western blot and ELISA assays. Therefore, in the future it may use as a gatekeeper allergen for detection of primary sensitization to *Chenopodiaceae* pollen in allergic patients.

Almost all of allergic patients to *C. album* are detectable by applying direct ELISA on the respective allergenic cocktail. Furthermore we found patients who had positive SPT to *C. album* extract (supplementary Table 1 patient no. 7 and 11) with no significant specific IgE against *C. album* extract or the allergenic cocktail. Analysis of sera of two *C. album* allergic patients with the allergenic cocktail and each of the rChe a 1, rChe a 2 and rChe a 3 allergens revealed that they have very low levels of specific IgE ($OD_{450nm} < 0.4$) against each of the mentioned recombinant allergens and the allergenic cocktail. Also, they showed no IgE reactivity to rChe a 1, rChe a 2 and rChe a 3 which blotted to PVDF membrane. These results point out the necessity of provocation assays (SPT) to involve in evaluation of allergenic activity of an allergen molecule. Hence, provocation test was interfered in this study and the capacity of the allergenic cocktail to induce immediate skin reactions was investigated in 15 *C. album* allergic patient.

It found that 15 µg/ml of the allergenic cocktail was able to induce significant immediate skin reactions in almost all of allergic patients. In addition, the skin reactivity of allergic patients to the allergenic cocktail and the natural allergen extract has been compared in Fig. 4. The intensity of induced skin reactivity against the allergenic cocktail was significantly equal to obtained results of SPT against the natural allergens in *C. album* pollen extract in almost all of the enrolled *C. album* allergic patients in this study. No false positive result was found in negative control patients. Additionally, we investigated about the existence of any association between allergen-specific IgE antibody levels in serum and skin sensitivity to the allergenic cocktail, extract or both in *C. album* allergic patients. We found that although, positive skin reactions to the allergenic cocktail were observed in the patients who had specific serum IgE antibodies against *C. album* but, there was a patient (supplementary Table 1, patient no 26) with high level of specific IgE that had negative skin reactivity to the allergenic cocktail and *C. album* pollen extract. So we resulted that allergen specific IgE levels and skin sensitivity were poorly correlated (supplementary Table 2). The recent result and other mentioned consequences corroborate that, it is important to perform SPT and serologic tests simultaneously to recognize the allergenic activity of allergen molecules.

Finally, data analysis from this study points out that reliable in vivo and in vitro diagnosis of *C. album* allergy is now possible with the allergenic cocktail and rChe a 1, rChe a 2 and rChe a 3. Therefore, we propose use of the allergenic cocktail for a new form of component-resolved diagnosis tools in diagnosis of *C. album* allergic patients. In addition, diagnostic tests that establish on the recombinant allergens are able to determine the disease-eliciting allergens. So careful formulation of the allergenic cocktail with the use of responsible allergens improve the CRIT in adjacent future.

Acknowledgments This study was supported by Research administration of Mashhad University of Medical Sciences.

References

- Nicole A, Prem LB, Mohan BS (2002) Aeroallergens and pollinosis: molecular and immunological characteristics of cloned pollen allergens. *Aerobiologia* 18:87–106
- Barderas R, Villalba M, Lombardero M, Rodriguez R (2002) Identification and characterization of Che a 1 allergen from *Chenopodium album* pollen. *Int Arch Allergy Immunol* 127:47–54
- Fereidouni M, Hossini RF, Azad FJ, Assarehzadegan MA, Varasteh A (2009) Skin prick test reactivity to common aeroallergens among allergic rhinitis patients in Iran. *Allergol Immunopathol* 37:73–79
- Suliaman FA, Holmes WF, Kwick S, Khouri F, Ratard R (1997) Pattern of immediate type hypersensitivity reactions in the eastern province, Saudi Arabia. *Ann Allergy Asthma Immunol* 78:415–418
- Ezeamuzie CI, Thomson MS, Al-Ali S, Dowaisan A, Khan M, Hijazi Z (2000) Asthma in the desert: spectrum of the sensitizing aeroallergens. *Allergy* 55:157–162
- Barderas R, Villalba M, Pascual CY, Batanero E, Rodriguez R (2004) Profilin (Che a 2) and polcalcin (Che a 3) are relevant allergens of *Chenopodium album* pollen: isolation, amino acid sequences, and immunologic properties. *J Allergy Clin Immunol* 113:1192–1198
- Kay AB (2001) Allergy and allergic diseases. First of two parts. *N Engl J Med* 344:30–37
- Casolaro V, Georas SN, Song Z, Ono SJ (1996) Biology and genetics of atopic disease. *Curr Opin Immunol* 8:796–803
- Heiss S, Mahler V, Steiner R, Spitzauer S, Schweiger C, Kraft D, Valenta R (1999) Component-resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin testing. *J Invest Dermatol* 113:830–837
- Seppala U, Daulay C, Robinson S, Hornshaw M, Larsen JN, Ipsen H (2011) Absolute quantification of allergens from complex mixtures: a new sensitive tool for standardization of allergen extracts for specific immunotherapy. *J Proteome Res* 10(4):2113–2122. doi:10.1021/pr101150z
- Becker WM, Vogel L, Vieths S (2006) Standardization of allergen extracts for immunotherapy: where do we stand? *Curr Opin Allergy Clin Immunol* 6:470–475
- Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gronlund H (1999) The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 29:896–904
- Barber D, de la Torre F, Feo F et al (2008) Understanding patient sensitization profiles in complex pollen areas: a molecular epidemiological study. *Allergy* 63:1550–1558
- Skamstrup Hansen K, Poulsen LK (2010) Component resolved testing for allergic sensitization. *Curr Allergy Asthma Rep* 10:340–348
- Vahedi F, Sankian M, Moghadam M, Mohaddesfar M, Ghobadi S, Varasteh AR (2010) Cloning and expression of Che a 1, the major allergen of *Chenopodium album* in *Escherichia coli*. *Appl Biochem Biotechnol* 163(7):895–905. doi:10.1007/s12010-010-9093-y
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Dreborg S (1989) The skin prick test in the diagnosis of atopic allergy. *J Am Acad Dermatol* 21:820–821
- Amini A, Sankian M, Assarehzadegan MA, Vahedi F, Varasteh A (2010) *Chenopodium album* pollen profilin (Che a 2): homology modeling and evaluation of cross-reactivity with allergenic profilins based on predicted potential IgE epitopes and IgE reactivity analysis. *Mol Biol Rep* 38(4):2579–2587. doi:10.1007/s11033-010-0398-2
- Assarehzadegan MA, Sankian M, Jabbari F, Tehrani M, Falak R, Varasteh A (2011) Identification of methionine synthase (Sal k 3), as a novel allergen of *Salsola kali* pollen. *Mol Biol Rep* 38:65–73
- Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A (2000) Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 106:409–418

22. Scheurer S (2006) Improvement of the diagnosis of allergy by using purified allergens. *Clin Exp Allergy* 36:1483–1486
23. Salcedo G, Diaz-Perales A (2010) Component-resolved diagnosis of allergy: more is better? *Clin Exp Allergy* 40:836–838
24. Lidholm J (2009) The importance of molecular allergens for in vitro testing: a critical evaluation of component-resolved diagnostics. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 96:157–161
25. Goikoetxea MJ, Cabrera-Freitag P, Sanz ML, Fernandez-Benitez M (2010) The importance of in vitro component-resolved diagnosis in paediatric patients. *Allergol Immunopathol* 38:37–40
26. Pittner G, Vrtala S, Thomas WR et al (2004) Component-resolved diagnosis of house-dust mite allergy with purified natural and recombinant mite allergens. *Clin Exp Allergy* 34:597–603
27. Cudowska B, Kaczmarek M (2004) Diagnostic value of birch recombinant allergens (rBet v1, profilin rBet v2) in children with pollen-related food allergy. *Rocz Akad Med Bialymst* 49:111–115
28. Laffer S, Spitzauer S, Susani M et al (1996) Comparison of recombinant timothy grass pollen allergens with natural extract for diagnosis of grass pollen allergy in different populations. *J Allergy Clin Immunol* 98:652–658
29. De Amici M, Alesina R, Moratti R, Ciprandi G (2010) Component-resolved diagnosis for phlegm allergy: the role of recombinants. *J Asthma* 47:750–753
30. Gonzalez-Rioja R, Ferrer A, Arilla MC et al (2007) Diagnosis of *Parietaria judaica* pollen allergy using natural and recombinant Par j 1 and Par j 2 allergens. *Clin Exp Allergy* 37:243–250
31. Barber D, de la Torre F, Lombardero M et al (2009) Component-resolved diagnosis of pollen allergy based on skin testing with profilin, polcalcin and lipid transfer protein pan-allergens. *Clin Exp Allergy* 39:1764–1773
32. Tehrani M, Sankian M, Assarehzadegan MA, Falak R, Jabbari F, Varasteh A (2010) Immunochemical characterization of *Amaranthus retroflexus* pollen extract: extensive cross-reactive allergenic components among the four species of *Amaranthaceae/Chenopodiaceae*. *Iran J Allergy Asthma Immunol* 9:87–95
33. Assarehzadegan MA, Amini A, Sankian M, Tehrani M, Jabbari F, Varasteh A (2010) Sal k 4, a new allergen of *Salsola kali*, is profilin: a predictive value of conserved conformational regions in cross-reactivity with other plant-derived profilins. *Biosci Biotechnol Biochem* 74:1441–1446
34. Galles C, Gomez RL, Spampinato CP (2011) PMS1 from *Ara-bidopsis thaliana*: optimization of protein overexpression in *Escherichia coli*. *Mol Biol Rep* 38:1063–1070