



Constructing a hybrid molecule with low capacity of IgE binding from *Chenopodium album* pollen allergens

Hamid Reza Nouri^a, Abdolreza Varasteh^b, Fatemeh Vahedi^c, Jamshidkhan Chamani^d,
Danial Afsharzadeh^a, Mojtaba Sankian^{a,*}

^a Immunobiochemistry Lab, Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^b Allergy Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^c Biotechnology Department, Razi Vaccine and Serum Research Institute, Mashhad, Iran

^d Department of Biology, Faculty of Sciences, Islamic Azad University-Mashhad Branch, Mashhad, Iran

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ABSTRACT

Allergen specific immunotherapy is the only remedy to prevent the progression of allergic diseases. Nowadays, using of recombinant allergens with reduced IgE-binding capacity is an ideal tool for allergen immunotherapy. Therefore, in this study we focused on a hybrid molecule (HM) production with reduced IgE reactivity from *Chenopodium album* pollen allergens. By means of genetic engineering, a head to tail structure of the three allergens of the *C. album* pollen was designed. The resulting DNA construct coding for a 46 kDa HM was inserted into an expression vector and expressed as hexahistidine tagged fusion protein in *Escherichia coli*. IgE reactivity of the HM was evaluated by western blotting, inhibition ELISA and *in vivo* skin prick test and its immunogenic property was tested by proliferation assay. The recombinant HM was expressed and purified by nickel-affinity chromatography. Comparison of the recombinant HM with a mixture of three recombinant allergens, as well as natural allergens in the whole *C. album* pollen extract via immunological experiments revealed that it has a much lower potential of IgE reactivity. Furthermore, *in vivo* skin prick tests showed that it has a significantly lower potency to induce cutaneous reactions than the mixture of recombinant wild type allergens and whole extract while, it had been preserved immunogenic properties. Our results have demonstrated that assembling three allergens of *C. album* in a hybrid molecule can reduce its IgE reactivity.

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1. Introduction

Type I allergy, a genetically determined hypersensitivity disease which has affected more than a quarter of the population in industrialized areas of the world [1,2]. To date, the only curative treatment strategy for Type I allergy is allergen-specific immunotherapy (SIT), which currently has been performed through subcutaneous or mucosal administration of allergens with the natural sources to the patients in order to switch to an allergen-specific unresponsiveness condition [3,4]. However, this strategy has some disadvantages. Currently, many pollen extracts, which were used in SIT were associated with some side effects in clinical settings due to their complicated composition. Therapeutic

allergen extracts may contain no important allergens; the ratio of the individual components in the extract may be greatly valid and possibly induce a new IgE specificity [5,6]. The use of recombinant allergens can resolve many of these problems. In addition, a few reports on SIT with recombinant wild type allergens have recently been published demonstrating the efficacy of these molecules [7,8]. However, recombinant wild type allergens may lead to IgE mediated side effects such as urticaria, allergic asthma and anaphylactic shock [9]. Therefore, production of recombinant allergens that display reduced allergenic activity is an approach that diminishes IgE mediated side effects.

According to clinical data, one of the most important problems in allergic patients is that they are sensitized to more than a few allergens from one or several sources. In addition, many mono-sensitized patients acquire additional allergies gradually with increasing of the age [10]. It has recently been demonstrated that molecules which consist of several copies of one or more homologous allergens, or even consist of immunologically unrelated allergens has shown the capability to be used for the allergy treatment in the patients who are sensitive to the several allergen components [11,12]. It seems that the use of hybrid molecules can

Abbreviations: SPT, skin prick test; HM, hybrid molecule; Mixture of rAllergens, A mixture with equimolqr of rChe a 1, rChe a 2 and rChe a 3.

* Corresponding author. Tel.: +98 511 7112611; fax: +98 511 7112596.

E-mail addresses: nourihr851@mums.ac.ir (H.R. Nouri), varasteha@mums.ac.ir (A. Varasteh), vahedif@yahoo.com (F. Vahedi), chamani@ibb.ut.ac.ir (J. Chamani), dm.lotus@yahoo.com (D. Afsharzadeh), sankianm@mums.ac.ir (M. Sankian).

overcome many existing problems in immunotherapy with allergenic extract or recombinant wild type allergens.

In *Chenopodiaceae* family, *Chenopodium album* is one of the most recognized allergenic members. *Chenopodiaceae* pollens are the main cause of sensitization in desert and semi-desert areas like Kuwait, Saudi Arabia and Iran [13]. *C. album* pollens were found in atmosphere of our country, Iran, especially from June to early October. Previous studies in Mashhad, northeast of Iran, have demonstrated the susceptibility to *C. album* pollen allergens up to 63.4% of population [14]. Che a 1, Che a 2 and Che a 3 are known allergens in *C. album* pollen, of which the first two have been identified as the major allergens whereas Che a 3 is a minor allergen in the patients who are sensitive to *C. album* pollen. Che a 1 is a single N-glycosylated polypeptide with 168-amino acids length and 18 kDa molecular mass. Che a 2 consists of a 131-amino acids chain and displays up to 75% and 82% identity with pollen and food profilins, respectively. Che a 3, a polypeptide chain with 86-amino acids length, belongs to the polcalcin family. It displays acidic pI and an apparent molecular mass 9.5 kDa [15–18].

Since, *C. album* pollen is considered as an important cause of respiratory allergy in Iranian population and most patients are frequently co-sensitized to immunologically unrelated allergens of this pollen, we were thus encouraged to design a HM from three allergens of *C. album* for therapeutic purposes. Here, we also present an approach that allows the combination of allergen molecules. The chimeric DNA for these allergens expressed as a HM in *Escherichia coli*, and its immunological characteristics were studied by independent techniques.

2. Material and methods

2.1. Serum samples and Skin prick test

Initially 17 patients, eight women and nine men (mean age of 31.76 ± 12.94 years; age range of 19–54 years), presenting to Immunology Research Center of Avicenna Research Institute of Mashhad were included in this study. The selected patients had histories of allergic conjunctivitis, allergic rhinitis, and/or bronchial asthma and positive skin prick test (SPT) to *C. album* pollen extract. Serum samples were collected from these patients and stored at -70°C until usage. None of the patients received glucocorticosteroid, anti-histamine treatment or SIT. Sera were also obtained from 4 non-atopic volunteers who were negative on SPT with *C. album* pollen extract (Table 1). SPTs were performed on the ventral side of the subjects' forearms according to Dreborg method [19]. Histamine phosphate at 10 mg/ml and glycerin were used as positive and negative controls, respectively. Reactions with a wheal of 3 mm greater than the negative control, surrounded by an erythema, were considered positive. SPTs were performed with HM and a mixture of rChe a 1, rChe a 2 and rChe a 3 with several concentration. The Human Ethics Committee of the Mashhad University of Medical Sciences (Mashhad, Iran) approved the study, and written informed consent was obtained from all study participants before the study commenced.

2.2. Production of pollen extract

C. album pollen extract was prepared as previously described [13]. Briefly, pollens were collected from *C. album* plants from Bu-Ali garden of Mashhad, Northeast of Iran. In the first step of pollen extract production, pollens mixed with the cold acetone (1/10 w/v) and incubated at 4°C with agitation. Acetone was removed by suction and then, phosphate-buffered saline (PBS, 0.15 M, pH 7.4) containing 20 mM EDTA (1:20 w/v) was added to the dried pollens followed by overnight agitation at 4°C . After that, it was clarified

by centrifugation at $7150 \times g$ for 20 min at 4°C , and the clear supernatant containing the solubilized proteins was dialyzed for 16 h at 4°C against Tris–HCl 0.02 M, pH 8. The filtrate was then lyophilized and stored at -70°C . The protein content was determined by Bradford's method [20].

2.3. Amplification of the sequence encoding the hybrid molecule by polymerase chain reaction

Total RNA was extracted from 0.8 g of mature non-defatted *C. album* pollen, according to the method of Chomczynski and Sacchi [21]. The first cDNA strand was synthesized from 2.5 μg of total RNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentase, Life Sciences, Lithuanian).

The cDNA coding for the hybrid molecule consist of three *C. album* identified allergens, Che a 1, Che a 2 and Che a 3 were constructed by using overlapping extension polymerase chain reaction (PCR). A chimeric DNA was designed as Che a3–Che a1–Che a2 containing Che a 3 at the 5' end, Che a 1 at the central region and Che a 2 at the 3' end of the HM (Fig. 1). The first step to make this chimeric DNA was amplification of the Che a 1 and Che a 2 coding region from first strand cDNA by conventional PCR using a pair of primers a₁-5' and a₁-3' for Che a 1 and a₂-5' and a₂-3' for Che a 2. The nucleotide sequences of the sense and antisense primers were described in Table 2. The PCRs were performed separately in 20 μl reaction volume containing 5 μg *C. album* cDNA, 12.5 pmol of each primer, 3 μl MgSO₄ (25 mM), 1.2 μl dNTPs (10 mM), 2 μl 10 $\times$ reaction buffer, and 2.0 units of *pfu* polymerase (Fermentase, Life Sciences, Lithuanian). RT-PCR cycling parameters were as follow: initial denaturation at 95°C for 3 min, then 35 continuous cycles of denaturation at 95°C for 30 s, annealing temperatures for Che a 1 and Che a 2 were at 55°C and 57°C , respectively, for 1 min, extension for 2 min at 72°C and the final extension for 3 min at 72°C . Agarose Gel DNA Purification Kit (Bioneer, Korea) was used to obtain purified PCR products. Subsequently an overlapping extension PCR was designed to combine Che a 1 and Che a 2 genes in which exploited previous purified PCR products as template. The annealing temperature was optimized according to the melting temperature (T_m) of overhang's region of a₂-5' primer. The PCR reaction contained 15 μl of each purified Che a 1 and Che a 2 PCR products, 3 μl MgSO₄ (25 mM), 1.2 μl dNTPs (10 mM), 2 μl 10 $\times$ reaction buffer and 2.0 units of *pfu* polymerase. The PCR thermocycling parameters were as follows: initial denaturation at 95°C for 3 min, 10 continuous cycles of denaturation at 95°C for 30 s, annealing temperature for 1 min at 50°C , extension for 2 min at 72°C and final extension for 10 min at 72°C . The desired linked DNA fragment for Che a 1–Che a 2 was isolated from agarose gel and then was amplified by a₁-5' and a₂-3' primers. The cDNA coding for Che a 3 allergen was amplified by a₃-5' and a₃-3' primers. The *Not I* restriction enzyme site was added to a₃-3' primer. The PCR cycling conditions for Che a 3 were very similar to Che a 2 with slightly modification. The annealing temperature was optimized at 60°C . For construction of a chimeric DNA from Che a 3–Che a 1–Che a 2, secondary overlapping extension PCR was performed with Che a 3 PCR products and the linked DNA fragment for Che a 1–Che a 2 as templates. The annealing temperature was adapted to overhang at a₃-3' primer (1 min at 50°C). The other thermocycling parameters for this reaction were as same as the first overlapping extension PCR. After this reaction, the chimeric DNA fragment containing Che a 3, 1 and 2 was amplified by a₃-5' and a₂-3' primers. It should be noted that three allergens of a *C. album* were amplified separately with specific primers via conventional PCRs. All of the PCRs were performed by a Corbett Research Thermocycler (Corbett, Australia).

Table 1
Demographic and clinical features of *C. album* allergic patients.

Allergic patient	Age/sex	Clinical history ^a	Positive SPT to other extracts	Total IgE (IU/ml)	Skin prick test ^b	
					Histamine	<i>C. album</i> pollen extract
1.	20/M	RC	A, S	148	9	14
2.	21/M	AR	A, S, P	362	10	13
3.	54/F	RC	A, S, K	439	12	10
4.	19/F	RC, AR	A, S, K, M	706	15	15
5.	40/M	AR	A, S, K	720	12	12
6.	20/M	RC	A, S, P	76	14	10
7.	41/F	RC, AR	A, S	279	12	12
8.	34/F	RC, AR	A, S, K, P	194	8	10
9.	43/M	AR	A, S, K	830	10	16
10.	19/M	RC	A, S	1120	13	12
11.	23/F	AR	A, S	710	9	10
12.	24/F	RC	A, S, K, P	237.9	11	10
13.	47/F	AR	A, S, K, P, Ar	153.6	11	8
14.	20/F	RC	A, S	1215	8	10
15.	22/M	RC, AR	A, S	275	14	15
16.	40/M	AR	A, S, K	433.4	10	10
17.	54/M	RC, AR	A, S	91	8	15
None allergic patient						
18.	21/M	Negative	–	69	10	0
19.	21/F	Negative	–	19	5	0
20.	21/F	Negative	–	30	9	0
21.	21/M	Negative	–	39	7	0

^a Clinical history including AR, allergic rhinitis and RC, rhinoconjunctivitis

^b Skin test was performed on the *C. album* pollen allergic patients (1–17) and non-atopic individuals (18–21) with 1 mg/ml of *C. album* pollen extract, *Amaranthus retroflexus* (A), *Salsola kali* (S), *Kochia scoparia* (K), *Platanus orientalis* (P) and *Artemisia* (Ar) as well as with 10 mg/ml of the histamine diphosphate and glycerin as positive and negative control, respectively. The mean wheal diameter is displayed in millimeters.

2.4. Constructing expression vector

The chimeric DNA for the HM was isolated from 2% agarose gel (Bioneer, Korea) and cut with *Not I* and *Xho I* restriction enzymes according to the manufactures' protocol (Fermentase, Life Sciences, Lithuanian). The chimeric DNA was purified and cloned into the expression vector pET21b(+) (Novagen, USA) which had been digested by the same restriction enzymes,

using the DNA Ligation Kit (TaKaRa Bio Inc., Japan). Ligation products were transformed into competent *E. coli* cells strain TOP10 (Invitrogen, San Diego, USA) and inoculated onto Luria Bertani (LB) plates containing 50 µg/ml of ampicillin at 37 °C for overnight. The purified plasmids from resistant clones were sequenced by the dideoxy method using fluorescence-labeled primers and analyzed at the SEQLAB Sequence Laboratories (Göttingen, Germany).

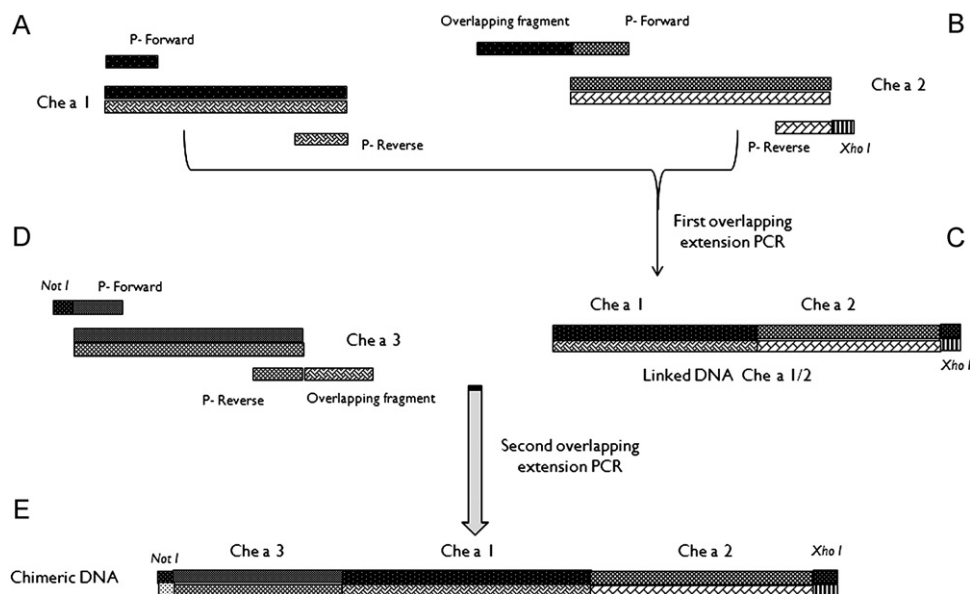


Fig. 1. Schematic view of the gene fusion protocol for construction of the chimeric DNAD. (A) In this part by application of the suitable primers Che a 1 gene has amplified. (B) To amplify Che a 2 gene, a forward primer that linked an overlapping fragment to its 5' end was used. *Xho I* restriction site was added to the 5' end of the reverse primer. (C) The first overlapping extension PCR, Che a 1 gene linked to Che a 2. (D) Che a 3 was amplified with the use of a forward primer that was carrying *Not I* restriction site in 5' end and a reverse primer with overlapping fragment in 5' end. (E) The chimeric DNA was obtained by second overlapping extension PCR with the use of Che a 3 amplified gene and linked DNA Che a 1–Che a 2 as template. P in this figure is tabloid of primer.

Table 2

Nucleotide sequences of the primers which are used for the construction of the hybrid molecule.

Che a 1: F	a ₁ -5'	5'-ATGGCGAAGTGTCAAGCTG-3'
Che a 1: R	a ₁ -3'	5'-ATTAGCTTTAACATCATAAAGATCCA-3'
Che a 2: F ^a	a ₂ -5'	5'-TCTTTATGATGTTAAAGCTAATCTCTATGTCGTGGCAGACGTACGT-3'
Che a 2: R ^b	a ₂ -3'	5'-TATCTCGAGCATGCCCTGTTCCGACCAG-3'
Che a 3: F ^c	a ₃ -5'	5'-ATTGCGGCCGCATGGCTGCTGAGGATACACCTC-3'
Che a 3: R ^a	a ₃ -3'	5'-GCTTGACACTTCGCCATAGGAGGGAAGATCTTGGAAACATCTTTAACCA-3'

The primers designed according to the deposited nucleotide sequences in NCBI for Che a 1 (GenBank accession no. AY049012) Che a 2 (GenBank accession no. FJ985985) and Che a 3 (GenBank accession no. AY082338).

^a Overhang fragments (Italicized) were added to the forward primer of Che a 2 and reverse primer of Che a 3 to combine the amplified DNA with overlapping extension PCR.

^b To reverse primer of Che a2 (a₂-3') added a *Xho* I restriction site (underlined) for cloning process.

^c *Not* I restriction site (underlined) introduced to the forward primer of Che a 3 (a₃-5'). In addition, three nucleotides added to 5' end of the primers to enhance the efficiency of *Not* I and *Xho* I restriction enzyme.

2.5. Expression and purification of the recombinant hybrid molecule

The purified pET21b(+) which carried the chimeric DNA was sub-cloned to 50 µl of *E. coli* BL21 Codon Plus(DE3) RIL (Stratagene, USA). Moreover, the empty vectors pET21b(+) were transformed to the *E. coli* cells as negative control. A single colony of successfully transformed *E. coli* was picked up and inoculated into 2 ml LB medium at 37 °C in the aeration condition to reach an OD₆₀₀ of 0.4–0.6. The fresh media (100 ml) was inoculated with aliquots (1.5 ml) of the earlier culture, and samples were incubated at 18 °C with aeration to reach an OD₆₀₀ of 0.4. After that, 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to the cultures and they were returned to the 18 °C for additional 10 h. Following to recombinant protein production, the cells were collected by centrifugation at 1000 × *g* for 5 min at 4 °C, and suspended in the cell lysis buffer containing 50 mM Tris–HCl (pH 8.0) and 500 mM NaCl, and then were lysed using 50 mg/ml lysozyme (Qiagen, USA). To enhance the cell lysis, the cells were frozen in the liquid N₂ and thawed at 37 °C for three times. During purification process of the HM from bacterial cell culture, 10 units of DNase I was added to 1 ml protein extract and incubated for 20 min at 37 °C to destroy the bacterial DNA and plasmid. The suspension was centrifuged at 14,480 × *g* for 30 min at 4 °C to remove the insoluble debris. The supernatant was added to a Ni-NTA spin column (Qiagen, USA) pre-equilibrated with the lysis buffer. The column was then washed by the native washing buffer (300 mM NaCl, 50 mM Tris–HCl pH 8.0) with different concentrations of imidazole (20, 50 and 100 mM). Finally, the bound recombinant HM was eluted with the native elution buffer containing 200 mM imidazole. 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 10 mM Tris–HCl. The concentration of the recombinant proteins was determined by Bradford's method [20]. Contamination of the purified recombinant HM solution to bacterial endotoxin was determined using the Limulus Amoebocyte Lysate test (GenScript, USA) according to the manufacturer's instructions. Endotoxin level in the hybrid molecule solution was estimated to be lower than the authorized limit of 5 EU/kg, which is described in European Pharmacopeia.

2.6. Characterization of the recombinant hybrid molecule

The aggregation state of the purified HM was investigated by size-exclusion chromatography (SEC). SEC experiment was performed by ZORBAX Bio Series GF-250 column according to the manufacture's structures (Agilent technologies, California, USA). The mobile phase pH was 7.0 and was composed of 130 mM NaCl and 20 mM Na₂HPO₄. The flow rate was set to 1.0 ml/min and eluent was monitored with UV detector at 210 nm.

Circular dichroism (CD) spectroscopy was used to determine secondary structures and monitoring structural changes of the hybrid molecule and an equimolar mixture of rChe a 1, rChe a 2 and rChe a 3. Far-UV CD experiments were performed on a Jasco-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Jasco 2-syringe titrator. Spectra were recorded on samples that prepared in 10 mM Tris–HCl buffer, pH 7.4 and 1-mm path length quartz cuvette. A bandwidth of 1 nm and a response of 2 s were used, with a scanning rate at 50 nm min^{−1} to obtain final spectra as an average of five scans. For exploring changes in secondary structure, far-UV CD spectra were obtained over a wavelength range of 190–240 nm [22].

2.7. Measurement of the total and specific IgE by ELISA

Total serum IgE levels were measured by means of a commercially available ELISA kit according to the manufacturer's instructions (Radim, Pomezia Terme, Italy). Quantification of specific IgE levels in the sera samples of 17 allergic patients to *C. album* pollen extract was performed by means of ELISA. The optical density (OD_{450nm}) values were considered as the positive when they exceeded from the mean OD_{450nm} of the negative controls plus three SDs. To detect the specific IgE levels to the hybrid molecule and all of the wild-type recombinant allergens rChe a 1, rChe a 2 and rChe a 3 in patients' sera, an indirect ELISA was performed as follows: The wells of the ELISA microplate (Nunc MaxiSorp™, Fisher Scientific, Pittsburg, PA) were saturated overnight at 4 °C in 15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6, and 2 µg of each of purified recombinant allergens. Plates were washed with PBS, pH 7.4, and 0.05% Tween20 (T-PBS). After blocking of wells with 2% BSA in PBS, sera diluted 1:5 in 1% BSA/PBS were incubated in coated wells at 37 °C for 3 h in duplicate. Then all of the wells were incubated for 2 h at the same temperature with 1:1000 diluted of biotinylated goat anti-human IgE antibody (KPL, USA). After that, 100 µl of 1:20,000 diluted of horseradish peroxidase (HRP)-conjugated streptavidin (Bio-Rad, USA) were added to the wells and then incubated for 2 h. Each incubation step was followed by five times washing with T-PBS. Subsequently, colorimetric reaction was performed by addition of chromogenic substrate (tetramethyl benzidine (TMB)/H₂O₂) to the each well. The reaction was stopped by 3 M HCl after 15 min in the darkness at room temperature, then the optical density at 450 nm was measured using an ELISA reader (Stat Fax® 2100 Microplate Reader, Awareness Technology, USA).

2.8. SDS-PAGE and human IgE western blotting

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *C. album* extract (50 µg/well), purified HM (5 µg/well) and a mixture of rChe a 1, rChe a 2 and rChe a3 (5 µg/well) were performed using 15% acrylamide separation gels

(Bio-Rad Miniprotean II System, USA) under reducing conditions according to Laemmli [23] and protein bands electro-transferred to the polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, USA). After performance of blocking stage with BSA 2% in PBS for 16 h at 4 °C with mild shaking, the membrane were probed with either individual ($n = 17$) or pooled human sera as the primary antibody solution. Typically, sera were diluted 1:5 in BSA 1% in PBS (v/w) and incubated with membranes overnight at 4 °C on a rocker platform. IgE complexes were detected using a biotinylated goat anti human IgE antibody (KPL, USA). Membranes were incubated in the secondary antibody (diluted 1:1000 in BSA 1%) for 1 h at room temperature on a rocker platform, then membranes were washed 5× with T-PBS and the final incubation was performed with 1:20,000 diluted HRP-conjugated streptavidin (Bio-Rad, USA). Finally the signals were developed by the chemiluminescent method as recommended by the manufacturer (Chemiluminescence Detection kit, Pars Tous, Iran) and documented with G-Box imaging system (Syngene, UK).

2.9. Inhibition assays

For inhibition ELISA assays, the microtiter plates were coated with 2 µg/well of a mixture of the three recombinant allergens which was available in the coating buffer (sodium carbonate, pH 9.5) and saturated with the blocking buffer (2% BSA in PBS). The pooled serum (1:2 diluted in 1% BSA), of the subjects simultaneously shown high levels of specific IgE against all of the three rChe a 1, rChe a 2 and rChe a 3, had been pre-absorbed for 2 h with different concentrations of *C. album* extract, the purified recombinant HM and a mixture of recombinant allergens as inhibitor. The pooled serum was pre-absorbed with an immunologically unrelated protein (BSA) as negative control. This pooled serum with inhibitor were then added to the wells of the ELISA microplate and incubated for 1 h at 37 °C. Bounded IgE complexes were detected as described earlier for indirect ELISA. Each experiment was performed in duplicate. In comparable experiments, inhibition of IgE reactivity with microtitre plates coated with *C. album* extract was tested by pre-incubation of the same serum pool with allergen preparations as described above. The percentage of inhibition was calculated according to the formula: inhibition (%) = $(OD_A - OD_B) / OD_A \times 100$, where OD_A and OD_B represent the optical densities with the non-inhibited and inhibited pooled serum, respectively. For immunoblotting inhibition experiments, 50 µg of *C. album* extract and a mixture of wild type allergens were separated by SDS-PAGE on Bio-Rad Miniprotean II System gels and then blotted onto PVDF membrane. Other steps of the experiment were followed as earlier described for IgE western blotting except for addition of pooled serum (1:5 diluted), which had been individually pre-incubated with equal volume of inhibitors (40 µg/ml, as inhibitor) for 3 h at 37 °C with shaking. Inhibitors included the hybrid molecule, a mixture of recombinant allergens and natural allergens in *C. album* pollen extract. For control purposes, the pooled serum was mixed with PBS.

2.10. Proliferation assay

Female 6-week-old BALB/c mice were purchased from Razi Vaccine and Serum Research Institute (Mashhad, Iran). The study protocols including handling, care and immunization were approved by Animal Ethics Committee of Mashhad University of Medical Sciences. Mice were immunized according to the previous schedule [24] with 5 µg of a mixture of recombinant allergens already adsorbed on 2 mg alum gel suspension (13 mg/ml, Sigma–Aldrich, Germany). In parallel, control mice ($n = 5$) received alum plus PBS. After immunization, splenocytes of each group were seeded 2.5×10^5 cells per well and stimulated with either 20 µg/ml

of the HM or a mix of recombinant allergens in RPMI 1640 contained 10% heat-inactivated fetal calf serum (FCS) and 100 U/ml of penicillin and 100 µg/ml of streptomycin for 3 days. Some wells kept un-stimulated as negative control or stimulated with PHA (1 µg/ml) as positive control. After stimulation, the proliferation of the cultured cells was determined using Cell Proliferation ELISA, BrdU (Colorimetric) kit (Roche Diagnostics, Germany) according to the manufacture structure.

2.11. Statistical analysis

The data were analyzed by GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as means ± SD and statistical analysis was performed using the Mann–Whitney *U* test for IgE levels and skin prick test results. *p* Values < 0.05 was considered as significant.

3. Results

3.1. Patients and skin prick test results

The demographic (age and sex) and clinical data were recorded for each of the patients in Table 1. All of the 17 patients had positive SPTs (mean weal; 11.9 ± 2.4 mm) to *C. album* pollen extract. 88.2% (15/17) of the allergic patients showed total IgE concentrations more than 100 IU/ml while, the mean level of IgE in non-allergic patients was 39.25 IU/ml.

3.2. PCR amplification and cloning of the hybrid molecule

The size of the PCR products for Che a 1 and Che a 2 and Che a 3 were approximately 504 bp, 430 bp and 287 bp, respectively. In the first overlapping extension PCR using a mixture of Che a 1 and Che a 2 PCR products as template, the desired fragment (912 bp) as well as other products with different sizes were generated. In the second reaction of overlapping extension PCR, an intended chimeric DNA of three allergens of *C. album* with 1182 bp was created using Che a 3 and Che a 1–Che a 2 fragments. The chimeric DNA was digested with *Not I* and *Xho I* restriction enzymes, and then inserted to pET21b(+) expression vector by *T4* DNA ligase successfully. Initial transformation of *E. coli* TOP10 was performed for evaluation of correct cloning of chimeric DNA into the pET21b(+) vector. Plasmids were extracted from some colonies grown in LB medium, and then sequenced. Sequence analysis of this plasmid revealed that the chimeric DNA corresponds without mutations, to a 1182 bp open reading frame which encodes *C. album* pollen three allergens (GenBank accession no. AY082338, AY049012 and FJ985985) with 339 amino acids, average molecular mass of 46 kDa, a calculated theoretical isoelectric point value of 4.86 and a potential N-glycosylation sites at Asn-64 in Che a 1 structure. Fig. 2 shows that the amino acids sequence of the HM deduced from the nucleotide sequence of the chimeric DNA is identical to Che a 3, Che a 1 and Che a 2 as previously deposited in the National Center for Biotechnology Information (NCBI).

3.3. Expression and purification of the recombinant hybrid molecule

Following the extraction and identification of the plasmids carrying the chimeric DNA from *E. coli* cells strain TOP10, they were successfully transformed into *E. coli* BL21-CodonPlus(DE3)-RIL for protein expression. A fused form of the recombinant HM with six histidine tail at C-terminus were expressed in the *E. coli* BL21-CodonPlus (DE3)-RIL. The highest level of the recombinant protein production was obtained 12 h after induction with IPTG. The expressed HM was detected by SDS-PAGE, and results showed

HM	MASMTGGQQM	GRDPNSSVD	KLAAMAAED	TPQDIADRER	IFKRFDTNGD	GKISSSELGD	ALKTLGSVTP	70
Q84V36.1	-----	-----	-----	-----	-----	-----	-----	45
Q8LGRO.1	-----	-----	-----	-----	-----	-----	-----	1
ACR77509.1	-----	-----	-----	-----	-----	-----	-----	1
AAL92870.1	-----	-----	-----	-----	-----	-----	-----	1
HM	DEVRRMAEI	DTDGDGFISF	DEFTDFARAN	RGLVKDVSKI	FFEMAKCQAV	FLLVGALCVL	SLAGVANAAE	140
Q84V36.1	-----	-----	-----	-----	-----	-----	-----	86
Q8LGRO.1	-----	-----	-----	-----	-----	-----	-----	27
ACR77509.1	-----	-----	-----	-----	-----	-----	-----	1
AAL92870.1	-----	-----	-----	-----	-----	-----	-----	1
HM	NHFKVQGMVY	CDTCRIQFMT	RISTIMEGAT	VKLECRNITA	GTQTFKAEAV	TDKVGQYSIP	VNGDFEDDIC	210
Q84V36.1	-----	-----	-----	-----	-----	-----	-----	86
Q8LGRO.1	-----	-----	-----	-----	-----	-----	-----	97
ACR77509.1	-----	-----	-----	-----	-----	-----	-----	1
AAL92870.1	-----	-----	-----	-----	-----	-----	-----	1
HM	EIELVKSPNS	ECSEVSHDVY	AKQSAKVSIT	SNNGEASDIR	SANALGFMRK	EPLKECPEVL	KELDLVDVKA	280
Q84V36.1	-----	-----	-----	-----	-----	-----	-----	86
Q8LGRO.1	-----	-----	-----	-----	-----	-----	-----	167
ACR77509.1	-----	-----	-----	-----	-----	-----	-----	1
AAL92870.1	-----	-----	-----	-----	-----	-----	-----	1
HM	NEPMNQTYV	DDHLMFFIEE	TGNHLTAAAI	VGLDGSVWAQ	SSTFFPQLKQE	EVKAICNEFD	VPNTLAPTGL	350
Q84V36.1	-----	-----	-----	-----	-----	-----	-----	86
Q8LGRO.1	-----	-----	-----	-----	-----	-----	-----	168
ACR77509.1	-----	-----	-----	-----	-----	-----	-----	67
AAL92870.1	-----	-----	CD..SS..	L.H.T...	PS....P.	S..MKD.N	E.GS....	65
HM	FLGGEKYMVI	QGEFGAVIRG	KKGPGGVCIK	KTNQALVFGI	YNEPVTGQC	NMVVEKLGDY	LVEQGMLEHH	420
Q84V36.1	-----	-----	-----	-----	-----	-----	-----	86
Q8LGRO.1	-----	-----	-----	-----	-----	-----	-----	168
ACR77509.1	-----	-----	-----	-----	-----	-----	-----	133
AAL92870.1	H...T...	...D....	...T....	...II...	...G..M...	...RI...	-----	131
HM	HHHHH	425						
Q84V36.1	-----	86						
Q8LGRO.1	-----	168						
ACR77509.1	-----	133						
AAL92870.1	-----	131						

Fig. 2. Homology analysis of the hybrid molecule sequence confirmed that it belongs to the allergenic proteins of *C. album* pollen family. The comparison of the respective amino acids sequence of the HM with allergens of *C. album* which has previously deposited in database banks (AAL92871.1 [Che a 3], AAL07319.1 [Che a 1], ACR77509.1 and AAL92870.1 [Che a 2 isoforms]) revealed that, N-terminal end of the hybrid molecule have complete identity with Che a 3, the middle region of hybrid molecule was absolutely similar to Che a1 and finally, C-terminal of the HM had 100 and 73% identity to the ACR77509.1 and AAL92870.1 respectively, which submitted for Che a 2 allergen. Numbers in right side have shown the length of the each sequence. The linkers are shaded in gray. Gaps (–) are inserted into sequences to optimize the sequence alignment.

that the HM was expressed mainly as the soluble protein with an apparent molecular mass of 46 kDa (Fig. 3). This molecular weight is consistent with the sum total of the three rChe a 1, rChe a 2 and rChe a 3 with molecular mass of 22, 18 and 13 kDa, respectively that subtracted the fusion peptide of 7 kDa. This protein was purified by Ni²⁺-chelate affinity chromatography (Fig. 3, lane3). Recombinant wild-type allergens rChe a 1, rChe a 2 and rChe a 3 were previously expressed and purified in our laboratory and a mixture of these allergens shown in Fig. 3, lane 4. Approximately 50 milligrams of the recombinant hybrid molecule was obtained from 1 l of the expression medium.

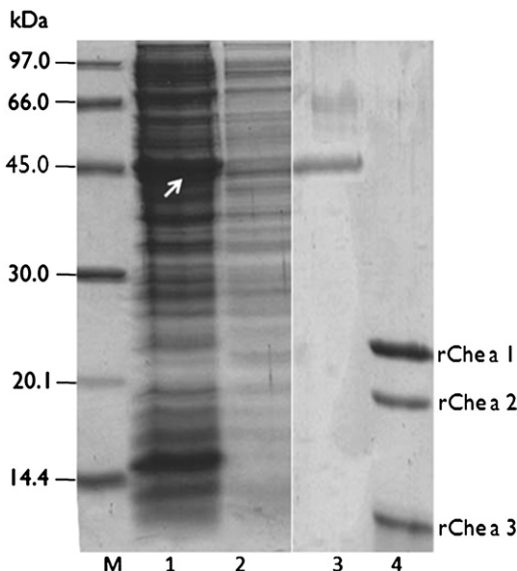


Fig. 3. Electrophoretic analysis of the recombinant HM expressed in *E. coli* BL21-CodonPlus(DE3)-RIL by SDS-PAGE. The 15% polyacrylamide gel stained by Coomassie brilliant blue R-250 revealed that the expressed HM was present in the soluble phase. Lane 1 shows supernatant of cells containing pET21b(+)/HM and lane 2 shows inclusion bodies of above cells. Lanes 3 and 4 show the Ni-NTA purified recombinant HM and a mixture of rChe a 3, rChe a 2, rChe a 1, respectively.

3.4. Characteristics of the recombinant hybrid molecule

As regards most of heterologous proteins are expressed in the soluble phase are less likely to form aggregated structures, so we can state the hybrid molecule expressed and purified from soluble phase have a low chance of aggregation. The aggregation state of the purified hybrid molecule was investigated by size-exclusion chromatography (SEC). One sharp peak eluted between 9 and 10 min (Fig. 4A). According to the calibrated ZORBAX Bio Series GF-250 column, proteins with 45–67 kDa were eluted at these time points. The elution time of the peak observed for the hybrid molecule corresponds with the predicted 46 kDa molecular weight of the HM monomer. Since, there were no peaks prior the mentioned peak; we can conclude that there were no aggregated structures with high molecular mass in the purified recombinant hybrid molecule solution.

The CD spectra of the hybrid molecule and a mixture of recombinant allergens have been shown in Fig. 4B. They exhibit double minima at 208 and 222 nm, respectively, which is characteristic of high α -helical content. Changes in the secondary structural contents of the HM and a mixture of recombinant allergens were calculated. The α -helical content decreased from 50.11% in a mixture of recombinant allergens to 47.21% in the HM. The β -sheet content increased in the HM to 19.96%, while β -sheet content in a mixture of recombinant allergens was 18.72%. The turns were 13.3% and 13.55% for the HM and a mixture of recombinant allergens, respectively. The unordered coils were also 19.33% and 17.62% for the HM and a mixture of recombinant allergens, respectively. This data indicate that the secondary structure content of HM is lower than mixture of recombinant allergens that may cause slight difference in these preparations.

3.5. Serum IgE reactivity with purified recombinant allergens

The binding capacity of natural allergens in the *C. album* pollen extract and the isolated recombinant allergens in a mixture form to the specific IgE was evaluated by ELISA. We performed ELISA experiments with sera of the 17 patients, which had allergic

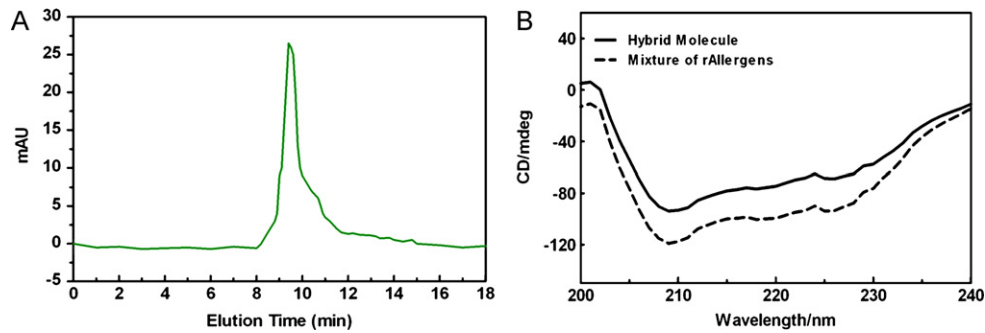


Fig. 4. Characteristics of the recombinant HM by size exclusion chromatography (SEC) and circular dichroism (CD) spectroscopy. (A) SEC of the hybrid molecule. The UV absorption traces at 210 nm (mAU: milli absorption units) are shown at y-axis for a given elution time (x-axis). (B) Far UV CD spectra of the HM (solid line) and an equimolar mixture of recombinant allergens (dashed line). The CD spectrum of these proteins has shown minima at 208 and 222 nm.

symptoms to *C. album* as well as positive skin prick test to its extract. Sera of four non-atopic individuals were used as a negative control. All patient sera (17/17) exhibited positive response to the whole *C. album* pollen extract (OD values 450 nm > 0.4). Table 3 shows that, 94% (16/17) of the patient's sera manifested high OD_{450 nm} values against a mixture of the three recombinant allergens. Therefore the recombinant allergens may have comparable IgE-binding epitopes as well as the natural allergens. In all of the tested individuals, the serum specific IgE significantly showed ($p < 0.05$) less binding affinity to the hybrid molecule than to the mixture of recombinant allergens and the *C. album* pollen extract in ELISA (Table 3). The ELISA results were used to confirm that natural allergens and produced recombinant allergens share IgE epitopes and display an equivalent recognition. It is so notable that IgE reactivity to a mixture of allergens was significantly more than IgE reactivity to the HM (Fig. 5A). The purified HM was analyzed by immunoblotting to detect IgEs binding capacity of pooled sera from *C. album*

pollen-sensitized individuals (patient's no. 3, 5, 9, 13 and 14). Moreover, this experiment involved a mixture of rChe a 1, rChe a 2, rChe a 3 and the pollen extract simultaneously. The patient serum pool showed high IgE reactivity with both the natural allergens in the pollen extract and each of the purified recombinant allergens in the mixture. It is so notable that IgE reactivity to a mixture of allergens was significantly more than IgE reactivity to the HM (Fig. 5B). This is in consistent to the direct ELISA data (Table 3) where IgE reactivity with the HM was at low level in all of the 17 sera.

3.6. Reduced allergenic activity of the hybrid molecule

Inhibition of IgE-binding to the natural allergens in *C. album* pollen extract and a mixture of the recombinant allergens was accomplished by means of ELISA after pre-incubation of the pooled serum from allergic patients with several concentrations of the HM (0.1, 1, 10, 100, 1000 $\mu\text{g/ml}$) or with the similar concentrations of a

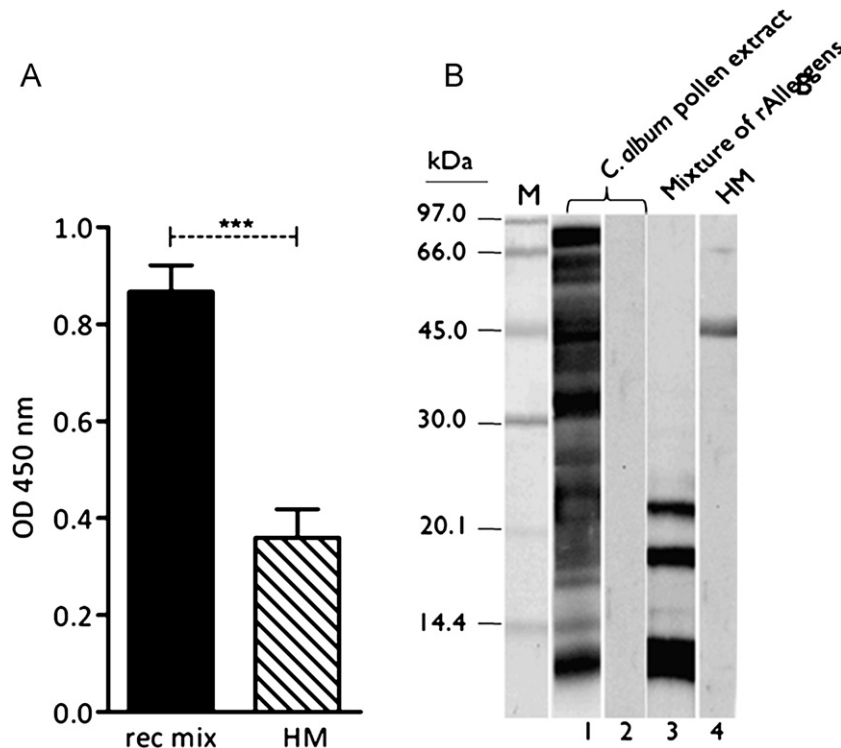


Fig. 5. Analysis of the IgE-binding to pollen extract and purified recombinant allergens. (A) The specific IgE antibodies had a significant tendency to bind to the a mixture allergen compare with the recombinant HM. (B) In this figure, immunoreactivity of *C. album* pollen extract and purified recombinant allergens has been visualized. In the lane 1 the reactivity of IgE, which is available in a pooled sera from five allergic patients (No. 4, 5, 9, 13 and 14) to the *C. album* extract bands are manifested. The negative control serum (lane 2) did not show any reactivity to the *C. album* pollen extract. Lane 3 exhibits interaction of the pooled serum with a mixture of three recombinant allergens. The HM (lane 4) had low reactivity with the pooled serum. Lane M: low molecular weight (Amersham, Buckinghamshire, UK), *** $p < 0.001$ with Mann–Whitney *U* test.

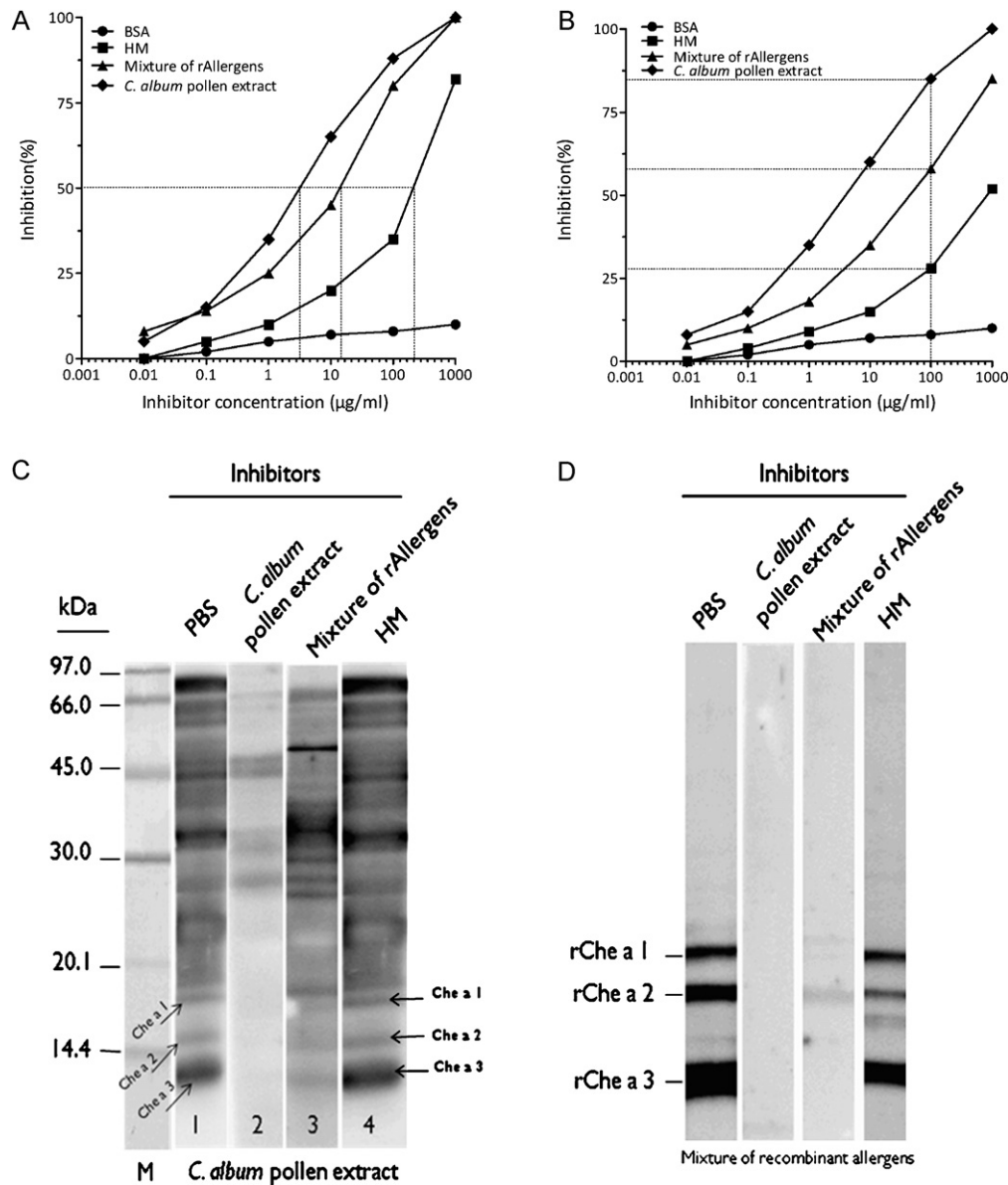


Fig. 6. IgE-binding inhibition assays of the natural and recombinant allergens. (A) ELISA plates were coated with a mixture of rChe a 1, rChe a 2 and rChe a 3 or (B) with *C. album* pollen extract. Percentage of inhibition calculated against pooled sera which pre-adsorbed with one of the following proteins as inhibitor: *C. album* crude extract, a mixture of rChe a 1, rChe a 2 and rChe a 3, the HM and BSA. Evaluation of the IgE binding inhibition shows by immunoblotting assay in part C and D. Strips with (C) 50 μg of crude extract and (D) 5 μg of a mixture of recombinant allergens were incubated with a pooled serum pre-adsorbed with either PBS or *C. album* pollen extract or mixed recombinant allergens or the HM as inhibitors. Lane M: low molecular weight (Amersham, Buckinghamshire, UK).

mixture of recombinant allergens. Fig. 6A has shown *C. album* pollen extract and a mixture of allergens were able to inhibit IgE-binding to recombinant wild-type allergens on a dose-dependent manner. Approximately, 5.5 μg/ml *C. album* and 25 μg/ml of a mixture of recombinant allergens were required to induce 50% IgE binding inhibition (IC₅₀) to a mixture of recombinant allergens. Moreover, similar inhibition level was observed with at least 16 fold more of the HM in comparison of a mixture of recombinant allergens. Furthermore, using the natural allergens in *C. album* pollen extract as the solid phase, it was demonstrated that IgE-binding weakly inhibits with 100 μg/ml of the HM (28% inhibition), while similar concentration of pollen extract and a mixture of recombinant allergens significantly inhibited IgE binding to the natural allergens with high percentage, 85% and 58% inhibition, respectively (Fig. 6B). These results confirmed that natural allergens (Che a 1, Che a 2 and Che a 3) in *C. album* pollen extract and rChe a 1, rChe a 2 and rChe a 3 share many epitopes, while they have the lowest

similarity to the HM. However, previously incubated pooled serum with PBS–BSA did not raised inhibition level to both natural and recombinant allergens (Fig. 6A and B, lane BSA).

Immunoblotting-inhibition experiments showed that three recombinant allergens in a mixture were able to inhibit IgE binding to the natural allergens in pollen extract. Densitometry analysis revealed that a mixture of recombinant allergens is able to inhibit 87.1%, 93.2% and 80% of IgE-binding to the natural allergens of *C. album* pollen extract, Che a 1, Che a 2 and Che a 3, respectively (Fig. 6C, lane 3), while the HM is unable to inhibit the natural allergens (Fig. 6C, lane 4). Also, crude extract as an inhibitor, completely inhibited the IgE binding to natural allergens, while PBS as an inhibitor or a negative control was not able to inhibit IgE reactivity to the natural allergens (Fig. 6C, lane 1 and 2). As it has shown in Fig. 6D, the responses of the protein bands corresponding to rChe a 1, rChe a 2 and rChe a 3 were completely inhibited by pollen extract as inhibitor. In addition, when a mixture of recombinant allergens

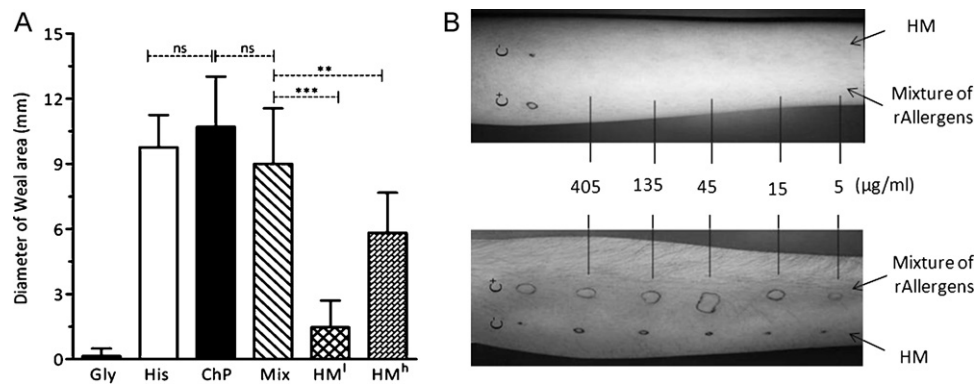


Fig. 7. The ability and potency of the purified recombinant HM, a mixture of wild-type allergens and the pollen extract to cause immediate skin reactions. (A) This bar chart shows the skin reactivity in allergic individuals. Mean diameter of wheal areas are displayed in y-axis versus millimeters and the glycerin (Gly), histamine diphosphate (His), 1 mg/ml of *C. album* pollen extract (ChP), 15 µg/ml of a mixture of wild-type allergens (Mix) and 15 and 405 µg/ml of HM^l and HM^h, respectively on x-axis. The *C. album* pollen extract and a mixture of wild type allergens induced a significantly larger skin reaction in contrast to the 15 and 405 µg/ml of the HM. (B) The results of the skin prick test on a *C. album* allergic patient (down) and a non allergic patient (up) are showed. Indeed 10 mg/ml of histamine diphosphate and glycerin consider as positive (C⁺) and negative (C⁻) controls, respectively. Cutaneous reaction to the various concentrations of the HM and a mixture of the wild-type allergens was defined on the forearm. Wheal areas are encircled. ***p* < 0.01, ****p* < 0.001 and ns, non-significant with Mann–Whitney *U* test.

was used as an inhibitor, a complete inhibition in IgE binding to rChe a 1 and Che a 3 and 96% inhibition in IgE binding to rChe a 2 was observed. The HM had low capability to inhibit IgE binding to recombinant wild-type allergens in the mixture. Inhibition experiments demonstrated that there is a high degree of immunological identity between the purified wild-type allergens and its natural counterparts (Che a 1, Che a 2 and Che a 3) in *C. album* pollen extract, while HM have the lowest IgE reactivity to the natural and recombinant wild-type allergens.

3.7. Low capacity of the HM to elicit immediate cutaneous reactions

The ability of HM and a mixture of the recombinant wild-type allergens of *C. album* in induction of the immediate skin reactions

Table 3
Specific IgE ELISA with *C. album* pollen extract and purified recombinant allergens.^a

Patient no.	<i>C. album</i> extract	Mixture of rAllergens	Hybrid molecule
1.	0.85	0.82	0.17
2.	0.89	0.78	0.11
3.	0.88	1.09	0.17
4.	0.91	0.89	0.59
5.	0.98	0.91	0.68
6.	0.87	0.99	0.11
7.	0.71	0.76	0.21
8.	0.62	0.21	0.04
9.	1.10	0.99	0.36
10.	0.85	0.61	0.11
11.	0.97	0.98	0.54
12.	0.88	0.85	0.72
13.	1.04	1.10	0.71
14.	1.01	1.20	0.49
15.	0.87	0.89	0.16
16.	0.70	0.73	0.39
17.	0.83	0.94	0.56
Mean ± SD	0.88 ± 0.12	0.85 ± 0.21	0.37 ± 0.25
Non allergic patient			
18.	0.10	0.14	0.10
19.	0.14	0.08	0.08
20.	0.13	0.06	0.10
21.	0.15	0.11	0.11
Mean ± SD	0.13 ± 0.02	0.10 ± 0.03	0.10 ± 0.01

^a The result of specific ELISA showed in OD (Optical density) at 450 nm. The mean of OD value of the hybrid molecule is statistically lower than both the natural allergens and a mixture of recombinant wild type allergens (*p* < 0.05).

were investigated in *C. album* allergic patients by *in vivo* SPT. The mean wheal diameter (mm) of SPT with *C. album* extract was compared with the results of SPT with a mixture of allergens and the HM (Fig. 7A). Almost all of the allergic patients significantly showed positive cutaneous reaction to a mixture of allergens (15 µg/ml) in comparison with the obtained results from SPTs of the natural allergens in *C. album* extract. Following the application of the HM on the forearm of allergic patients, 29.4% of patients showed negative SPTs at 15 µg/ml. But in the other patients, the HM showed a significantly (*p* < 0.05) reduced wheal area in comparison with the obtained data of pollen extract and a mixture of recombinant allergens. No skin reactions observed in none allergic individuals to *C. album* pollen extract, a mixture of allergens and the HM. It should be noted that different concentrations of the HM (5, 15, 45, 135 and 405 µg/ml) along with the similar concentrations of a mixture of recombinant allergens were examined on three allergic patient according to Dreborg method [19]. The SPT results from these patients revealed 405 µg/ml of the HM is able to induce cutaneous reactions which were similar to the SPT results of 15 µg/ml of a mixture of recombinant allergens. In fact, in comparison with a mixture of allergens, only very large amounts of the HM (27-fold) were able to induce relative positive skin pick test. However, in control patients who were challenged with the similar concentrations of the HM and a mixture of recombinant allergens no skin reaction was observed.

3.8. Stimulation with the HM induces *in vitro* proliferation in splenocytes

For assessment of the HM immunogenicity, splenocytes proliferative responses to mentioned antigens and a mixture of recombinant allergens were determined by colorimetric BrdU immunoassay and expressed as stimulation index. The highest proliferative responses were observed in response to PHA as a T-cell mitogen (data not shown). It is shown in Fig. 8; the HM was able to induce proliferative response as same as the allergenic mix in the both immunized mice and control group. In fact, induced proliferative responses by these antigens were not statistically different (*p* > 0.05).

4. Discussion

In recent years the production of the effective recombinant allergens for the specific immunotherapy has been developed as one of the main aims in the field of allergy researches, while specific

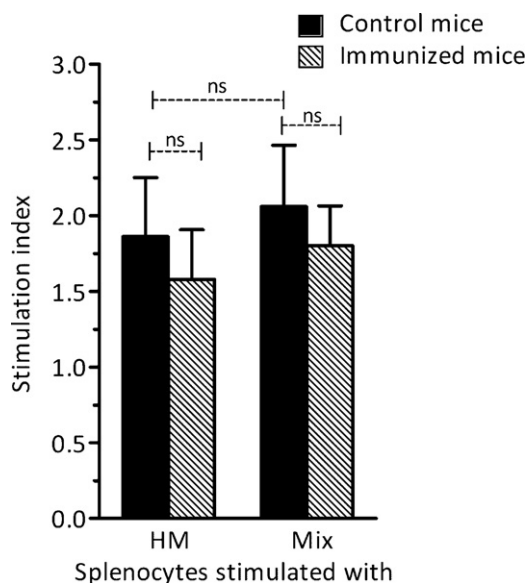


Fig. 8. Splenocytes proliferation assay. Splenocytes were *in vitro* stimulated with the HM or an allergenic mix. Results are represented as mean stimulation index $\pm$ SD that obtained from five individual mice. ns, non-significant with Mann-Whitney U test.

immunotherapy uses allergen extracts that consist of a variety of different allergens and non-allergenic components [25]. Although it is possible to determine the quantity of certain allergens in therapeutic allergen extract, but it is not achievable to eliminate non-allergenic components [26]. It has been reported that extract-based immunotherapy may induce IgE antibodies toward the new allergens and lead to clinically relevant new sensitizations [6]. Fortunately when applying the recombinant allergen molecules for allergen immunotherapy, the treatment is adaptable according to the patient's sensitization profile and thus it is possible to avoid the induction of unrelated immune responses [27]. Treatment of allergy for complex sources induces heterogeneous immune responses, even when these allergens are administered in equimolar mixtures. These immune responses may be inhibited by the individual allergen with different immunogenicity [28]. However, application of well defined and pure allergens in recombinant forms will overcome some of the relevant problems in diagnosis and treatment of sensitized patients. In the current study, we successfully expressed and purified wild type recombinant allergens of *C. album* along with the HM in *E. coli* BL21-CodonPlus(DE3)-RIL as a His-tag fusion protein by use of the pET21b(+). Since, the HM was so sensitive to the existence of rare codons especially to the leucine and isoleucine codons, CUA and AUA respectively, so selection of the *E. coli* BL21-CodonPlus(DE3)-RIL was as an easy approach to avoid of this problem [29]. The high yield of the recombinant proteins (50 mg/l of the bacterial culture) is sufficient for practical uses. Furthermore, combination of three identified allergens of *C. album* pollen extract (Che a 1, Che a 2 and Che a 3) for the purpose of the HM production, may serve as a genetically engineering method to reduce its allergenic activity considerably.

Che a 1, Che a 2 and Che a 3 were selected as identified allergens of *C. album* pollen, for the following reasons: *C. album* pollen allergens are one of the most important pollen allergens in our country and semi dried areas, and they share B cells and T cells epitopes with the related allergens in chenopod family such as *Salsola kali* and *Amarantus retroflexus* [30,31]. Furthermore, almost all of allergic patients to *C. album* pollen are sensitized at least to one of the identified allergens in this plant. Finally, lower molecular weight of Che a 1, Che a 2 and Che a 3 had (17.1, 14.4 and 9.5 kDa, respectively) make them as suitable molecules for assembling in the HM. It should be

noted that up to now only these three allergens are identified in *C. album* pollen. Take together these reasons, we selected Che a 1, Che a 2 and Che a 3 for this study. These allergens were able to combine together and compose a HM with 46 kDa. It also easily express in *E. coli* as a prokaryotic expression system.

The ELISA data revealed that each of the rChe a 1, rChe a 2 and rChe a 3 that produced separately in *E. coli* were immunochemically equivalent to their natural counterpart and had the same IgE-binding capacity as the natural forms. The results of inhibition ELISA and immunoblotting experiments suggested that the produced recombinant wild-type allergens of *C. album* contain most of the IgE-binding epitopes of the natural allergens and are correctly folded. These recombinant allergens with satisfactory properties would be able to use as an appropriate tool in diagnosis of *C. album* allergy [32]. A similar experiment with the HM did not establish a similar condition and showed that it has low IgE reactivity. It assumed that reduction in IgE binding is due to folding differences between the recombinant HM and recombinant wild-type allergens. The obtained results of CD spectra manifested that due to the assembling of three allergens in the HM, a conformational change is appeared in the final product. This inappropriate folding may causes in disruption of some B-cell epitopes at the surface of the HM. Since, IgE epitopes were masked in the aggregated proteins and resulted in reduced IgE binding; we evaluated aggregation state in the purified HM solution by means of size-exclusion chromatography (SEC). SEC data showed that there is no aggregated structure in the purified HM solution. Therefore, we are able to undoubtedly state that reduced IgE binding potential of the hybrid molecule had no relationship to presence of aggregated structure in the purified solution. Moreover, in some other surveys, it has been exposed that covalent head-to-tail linking of monomeric allergens for generation of hybrid molecule causes local relative reorientations of epitopes. Valenta and Kraft showed that IgE epitopes in a hybrid allergen from grass pollens may bind to the IgE antibodies, but they less efficiently crosslink Fc ϵ -RI to IgE [33]. Additionally, the skin test of *C. album* allergic patients to a mixture of allergens and the HM confirmed that allergenic activity of the HM was reduced. As we have shown in Fig. 7A; a mixture of three allergens at 15 μ g/ml elicited a wheal reaction which was comparable to the wheal reaction to natural allergens in *C. album* pollen extract at 1 mg/ml. The hybrid molecule (15 μ g/ml) failed to induce significant skin reactions. For a moderate positive cutaneous reaction in SPTs, about 27-folds (405 μ g/ml) of the HM more than a mixture of recombinant allergens was required. Also, Reese et al. demonstrated that destruction of the native conformation of carrot major allergen, Dau c 1, by hybrid technology acts as a superior plan for reducing its allergenicity [34]. Furthermore, splenocytes from the control group and immunized mice were equally proliferated in response to either the HM or allergenic mix as stimuli. This equal proliferative response indicating that specific T cell epitopes were preserved in the HM while, some of IgE epitopes were altered. Therefore, we proposed to consider the hybrid molecule as a hypoallergenic protein for specific immunotherapy against *C. album* allergy.

In different studies, various side-effects of immunotherapy due to administration of the natural allergens even at very low doses have been manifested. So, to avoid of this side-effects in *C. album* mediated allergy immunotherapy, administration of the hybrid molecules with low reactivity recommended especially in the areas like Iran, where *C. album* has been frequently one of the responsible allergens for type I allergy. In addition, the hypoallergenic nature of HM would permit high doses injection of the protein to allergic patients in specific immunotherapy. It may make the specific immunotherapy possible to be with few injections and decrease the risk of anaphylactic reactions. On the other hand, synthesis of a hybrid molecule comprises the major allergens of different sources cause to save time and production costs that may result in

highly using of this technology in the future. The obtained results of the current study may have clinical applications in the future, but definitely additional studies are required to evaluate the immunogenicity of the hybrid molecule, which are under progress in our lab. Finally, we hope to generalize our approach and results for the treatment of other allergenic sources.

5. Conclusion

In summary, in the present study for the first time the hybrid molecule from three identified allergens of *C. album* pollen was constructed and its allergenicity was evaluated. Immunological experiments confirmed that this molecule had low potential of IgE reactivity and can consider as a candidate for immunotherapy of *C. album* pollen allergic patients in the future.

Conflict of interest

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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