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Cloning and Expression of Che a 1, the Major Allergen of *Chenopodium album* in *Escherichia coli*

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Abstract *Chenopodium album* is a weedy annual plant in the genus *Chenopodium*. *C. album* pollen represents a predominant allergen source in Iran. The main *C. album* pollen allergens have been described as Che a 1, Che a 2, and Che a 3. The aim of this work was to clone the Che a 1 in *Escherichia coli* to establish a system for overproduction of the recombinant Che a 1 (rChe a 1). In order to clone this allergen, the pollens were subjected to RNA extraction. A full-length fragment encoding Che a 1 was prepared by polymerase chain reaction amplification of the first-strand cDNA synthesized from extracted RNA. Cloning was carried out by inserting the cDNA into the pET21b (+) vector, thereafter the construct was transformed into *E. coli* Top10 cells and expression of the protein was induced by IPTG. The rChe a 1 was purified using histidine tag in recombinant protein by means of Ni–NTA affinity chromatography. IgE immunoblotting, ELISA, and inhibition ELISA were done to evaluate IgE binding of the purified protein. In conclusion, the cDNA for the major allergen of the *C. album* pollen, Che a 1, was successfully cloned and rChe a 1 was purified. Inhibition assays demonstrated allergic subjects sera reacted with rChe a 1 similar to natural Che a 1 in crude extract of *C. album* pollen. This study is the first report of using the *E. coli* as a prokaryotic system for Che a 1 cloning and production of rChe a 1.

Keywords Allergy · Recombinant allergen · *Chenopodium album* · Che a 1 · Cloning

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Introduction

Allergic rhinitis is a worldwide problem, can be seasonal (occurring during specific seasons), or perennial (occurring year round). Seasonal allergic rhinitis, also known as hay fever, is most commonly caused by outdoor allergens, including grasses, weeds, and tree pollens [1, 2]. Symptoms appear most often during the spring, summer, and fall. The symptoms of pollen allergy or pollinosis include sneezing, itchy and watery eyes, scratchy throat, runny nose, and skin rashes [3].

Chenopodium album, or common lamb's quarters, is a perennial weedy plant of the goosefoot family Chenopodiaceae growing everywhere, even in salty soil [4].

Pollinosis and allergic sensitization to *C. album* have been reported in European countries and North America [4, 5]; in addition, it is a severe problem in semidesertic countries such as Iran [6], Saudi Arabia [7], and Kuwait [8].

The inhalation of *C. album* pollen has been known as an important cause of allergic respiratory symptoms in Iran. The prevalence of allergy to *C. album* has been evaluated 56% among allergic rhinitis patients in Mashhad, Iran [6]. High-level sensitivity to *C. album* was detected in 47.1% of patients with airway allergy residing in Riyadh, Saudi Arabia [9].

Che a 1, Che a 2, and Che a 3 proteins have been defined as allergenic components of *C. album* pollen [4, 5].

The molecular properties of chenopod allergens have not been fully known yet. An allergen from *C. album* pollen has been isolated by gel permeation and reverse-phase high-performance liquid chromatography [4, 5]. Molecular characterization with mass spectrometry, Edman degradation, and cDNA sequence have showed that the isolated allergen, Che a 1, is a glycoprotein of molecular mass 17.088 kD and 143 amino acid residues, whose sequence exhibits 27–45% identity with known members of the Ole e 1-like protein family [4, 5].

Antigenic analyses by immunoblotting, ELISA, and ELISA inhibition, using sera of allergic patients, two Ole e 1-specific monoclonal antibodies, and an Ole e 1-specific polyclonal antiserum have shown that 77% of sera from patients allergic to chenopod pollen were reactive to Che a 1. This allergen has been known as a major allergen of *C. album* pollen [4, 5].

The crude extract of *C. album* pollen is a mixture of both allergic and non-allergic compounds. The lack of a standardized *C. album* pollen extract makes difficult the reliable diagnosis and making possible mistake for seasonal illness. On the other hand, the process of purification and standardization of allergens is complicated and expensive [10–12].

It was previously described by Barderas and collaborators that recombinant form of Che a 1 (rChe a 1) was produced in *Pichia pastoris* yeast [5].

The purpose of this research was to clone and produce recombinant Che a 1 in *Escherichia coli* with the aim of establishing an easy system for expression and purification of this allergenic protein, using pET21+ vector. The rChe a 1 could lead to use in diagnosis, treatment approaches, and basic research in allergy field.

Materials and Methods

Subjects

Sera were collected from six patients with documented clinical history of pollinosis and showing positive skin reactions to crude extract of *C. album* with their consent. Sera were collected from six healthy volunteers to act as negative control. The pooled sera of subjects

as allergic positive and negative controls were prepared individually and stored in small aliquots at −70 °C for future use.

Pollen Collection and Extraction

C. album pollens were collected from flowering plants grown in Mashhad, Iran during spring season 2009. The pollens were passed through a wired fine mesh to drop out of dust particles. Refined pollens were kept in a plastic bag in the freezer until further experiment.

To prepare pollen extract, cold acetone (1/20 *w*/*v*) was used in defatting the pollens and the suspension was kept under shaking for 18 h. Acetone was removed by filtration through Whatman. No 1 filter paper and then phosphate-buffered saline (PBS, pH=7.4, 0.01 M) was added and shaken for 20 h. The supernatant was achieved after centrifuging at 5,600×*g* for 30 min and dialyzed in PBS for 24 h. All extraction steps were performed at 4 °C.

RNA Extraction

A modified guanidinium isothiocyanate method [13] was used to extract total RNA from pollens as following. In brief, 3 g of pollen was put in a ceramic mortar, freezed by adding liquid nitrogen on it, and was manually grounded with pestle. About 0.1 g of grounded pollen was transferred to a sterile microtube and 600 μl of lysis buffer (guanidium isothiocyanate 4 M, sodium citrate 25 mM, sarkosyl 0.5%, polyvinyl pyrrolidine 0.5% and 2-mercaptoethanol 0.1%) was added and mixed by pipetting up and down with a micropipette. Then, 80 μl of acetate sodium 2 M, 900 μl distilled water-equilibrated phenol, and 180 μl of chloroform were added, respectively. The tube was vortexed for 30 s and incubated on ice for 15 min followed by centrifugation (1,200× g in 4 °C for 15 min). The upper phase was transferred to a new microtube and three volumes of isopropanol added and mixed. The new tube was subjected to the centrifugation again and after removing the supernatant, pellet was dissolved in 300 μl of lysis buffer and isopropanol was added the same as the total volume. The solution was kept at −20 °C and then centrifuged for 10 min in 4 °C. The obtained pellet was washed with ethanol 70% in diethyl pyrocarbonate (DEPC)-treated water, air-dried, and finally dissolved in 30 μl of DEPC-treated water.

To remove the possible DNA, the extracted sample was subjected to DNAase and purified by phenol–chloroform method. The quality of purified RNA was evaluated by gel electrophoresis using agarose 1%. Total RNA concentration and purity were measured by optical density at 260 and 280 nm (260/280).

cDNA Synthesis and PCR Amplification of cDNA Encoding Che a 1

The cDNA was synthesized from 2 μg total RNA using a first-strand cDNA synthesis Kit (Fermentas, Lithuania) with a random hexamer as primer. The Che a 1 coding region was amplified with *Pfu* DNA polymerase (Fermentas, Lithuania) using specific Che a 1 primers: forward primer, 5′-AAGGCGGCCGCAATGGCGAAGTGTCAAGCTGT-3′ contains a *Not I* site, and reverse primer, 5′-TATCTCGAGATTAGCTTTAACATCATAAAGATCCA-3′ contains a *Xho I* site, (Bioneer, Korea) for directed cloning in pET21b+ vector.

Cloning

After electrophoresis of PCR product on low melting gel agarose 1%, the amplified PCR product was purified using *AccuPrep*™ Plasmid Mini Extraction kit (Bioneer, Korea) and

subjected to two-step digestion with *Xho I* and *Not I* restriction enzymes. After each digestion, the product was electrophoresed on low melting gel agarose 1% and purified. The purified digested PCR product was cloned into the pET21b+ in which the expressed protein contains a six-histidine tag at the C-terminal end for easier purification.

The resulting construct was transformed into competent TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA) with Inoue method [14]. TOP10 *E. coli* transformants were selected on LB agar plates supplemented with Ampicillin. The plasmids were purified and analyzed by restriction enzyme digestion using *Not I* and *Xho I*. After evaluation on gel agarose 1%, the purified plasmids were subjected to sequencing (Seq Lab, Germany).

Expression of rChe a 1

For expression of Che a 1, the purified pET21b+Che a 1 constructs were transformed in *E. coli* (DE-3) competent BL21 Star cells (Invitrogen, Carlsbad, CA) with Inoue method, and rotein synthesis was induced with 0.2 mM isopropyl β–D–thiogalactoside (IPTG) at 37 °C. Subsequently, the cells were harvested by centrifugation (3,000×*g*, 15 min) at 4 °C, and then resuspended by lysis buffer (50 mM Tris–HCl pH 6.8, 15 mM imidazole, 100 mM NaCl, 10% glycerol, and 0.5% Triton X-100). Cell disruption was achieved by three cycles of freezing the suspension in liquid nitrogen followed by thawing at 37 °C. The insoluble materials were removed from cell lysate by centrifugation at 9,000×*g* for 15 min at 4 °C. The supernatant containing soluble materials was transferred to the fresh tubes on ice. Sample aliquots from both supernatant and pellet were evaluated by Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to analyze the expression of rChe a 1.

Purification of rChe a 1

Purification of rChe a 1 was performed by immobilized metal affinity chromatography using Ni–NTA agarose (Invitrogen, Carlsbad, CA) from insoluble phase of lysate using guanidine hydrochloride 6 M to dissolve the inclusion [15].

Two milliliters of Ni–NTA resin was packed into a syringe, washed, and equilibrated in 10 column volumes of deionized water followed by 10 column volumes of binding buffer (50 mM potassium phosphate pH 7.8, NaCl 400 mM, KCl 100 mM, 10 mM imidazole, 10% glycerol, and 0.5% Triton X-100). The filtered supernatant of lysate insoluble phase through a 0.45-μm membrane was loaded onto Ni–NTA column and then washed with 10 column volumes washing buffer containing 20 mM imidazole. Target protein was eluted using an imidazole gradient (100–500 mM) in the binding buffer. Subsequently, the eluted solution containing protein was collected and dialyzed against 50 mM phosphate buffer. The purified rChe a 1 was analyzed by SDS–PAGE gel and Coomassie brilliant blue staining. The concentration of purified protein was assessed by Bradford method.

Western Blotting

Purified rChe a 1 was subjected to 12% SDS–PAGE with the reducing agent 2-mercaptoethanol and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA).

After blocking with 2% BSA, the blots were exposed for 3 h at room temperature to the positive and negative pooled sera (diluted 1:5 in PBS), individually. Using anti-human IgE

biotin-conjugated goat antibody (Sigma, USA; 1:1,000 diluted in BSA 1%) and horseradish peroxidase–streptavidin (1:20,000 diluted), the observed antigen–antibody reactions were documented by G-BOX Chemi-Doc (Syngene, Cambridge, UK) after exposure.

IgE Reactivity of Purified rChe a 1

ELISA was performed to examine the reactivities of specific IgE antibodies to the rChe a 1 as described previously [16]. ELISA plates were coated with 1 μg/well of total *C. album* pollen extract or purified rChe a 1. IgE antibodies were detected using biotinylated goat anti-human IgE (epsilon chain specific; Vector, Burlingame, CA) and streptavidin–peroxidase (Sigma). The signal was developed using 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Optical density (OD=450) more than four times the medium values of the negative control was considered to be positive.

ELISA inhibition was performed with 100 μl of adsorbed pooled positive serum by 100 μl (400 μg/ml) of total extract of *C. album* pollen and purified rChe a 1 for 2 h at room temperature that were done individually. These adsorbed sera were then added to the ELISA plates that had been coated with rChe a 1 or total extract of *C. album* pollen, respectively, in different concentrations. IgE antibodies were detected as described above. The percentage of inhibition was calculated using the following formula: (OD without inhibitor−OD of inhibitor/OD without inhibitor)×100. Inhibition with BSA was used as negative control. This experiment was repeated two times.

Results and Discussion

RNA Extraction

The A260/A280 ratio that estimates the suitable purity of the obtained RNA was found to be 1.95. The gel electrophoresis of RNA showed the good quality of RNA, with visible 25 S and 18 S bands and absence of DNA. The concentration of final obtained RNA was calculated 2,360 μg/ml, totally, 70.8 μg RNA was obtained from 0.1 g of pollens.

RNA isolation is the first step in the study of gene expression and recombinant protein production. However, the isolation of high-quantity and high-quality RNA from pollens containing large amounts of polysaccharides has demonstrated to be a difficult process [17, 18]. Here, we describe an improved technique for RNA isolation from *C. album* pollen grains, including a special disruption and homogenization process followed by RNA isolation technique and removing the DNA residues, which may interfere with RT-PCR and other downstream applications.

The A260/A280 ratio should be 1.8–2. A ratio below 1.8 means that there is protein contamination [19]. A ratio over 2 means that there is degradation of the nucleic acids. Here, using our protocol, we achieved the best ratio for extracted RNA from pollen.

Amplification and Cloning of Che a 1 Coding Region

A 519-bp fragment was obtained after PCR amplification on cDNA of *C. album* pollen (Fig. 1a). After cloning of this PCR product in the pET21b+ vector, analysis of the purified plasmids on gel agarose showed the correct expected size (Fig. 1b). Restriction digestion analyses confirmed the integrity of constructed plasmid (Fig. 1c), and sequencing

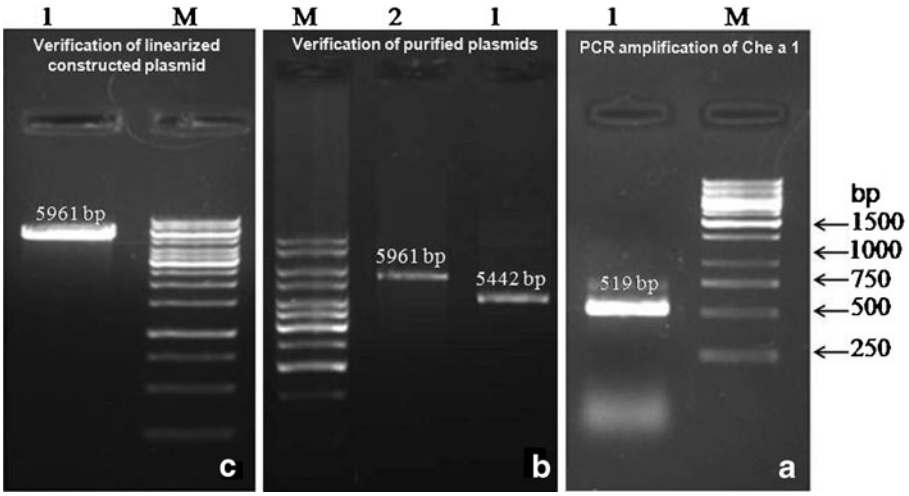

Fig. 1 Analyses of of insert (Che a 1 cDNA) and plasmids (pET21 and pET21+Che a 1) on agarose gels. *Lane M* shows 1 kb GeneRuller (Fermentas), fragment sizes 10,000, 8,000, 6,000, 5,000, 4,000, 3,500, 3,000, 2,000, 1,500, 1,000, 750, 500, and 250. **a** cDNA coding Che a 1 was amplified from total RNA of *C. album* pollen using specific primers in PCR. *Lane 1* shows 519-bp amplicon of Che a 1. **b** After cloning of Che a 1 in the pET21b+ vector, the resultant plasmids were purified and verified on gel agarose. *Lane 1* shows empty plasmid with lower size. *Lane 2* shows the plasmid with insert. **c** Linearized plasmid with *Xho I*

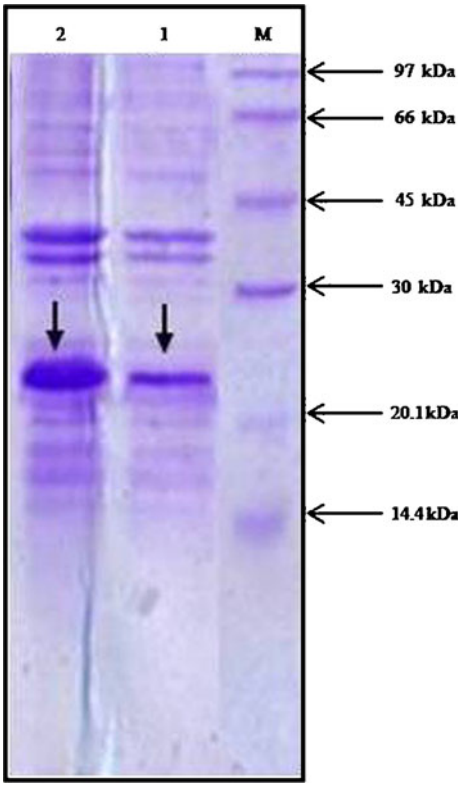

Fig. 2 SDS–PAGE followed by Coomassie blue gel staining showing the result of rChe a 1 expression in insoluble fraction. *Lane M* molecular weight protein marker. After IPTG induction, rChe a 1 was found in insoluble phase. Insoluble phase was treated by guanidine hydrochloride. *Lane 1* 1 h after IPTG induction. *Lane 2* 8 h after IPTG induction. rChe a 1 protein is indicated by *black arrows* in lysate insoluble phase

confirmed accurate cloning (result not shown). The sequence obtained in this study showed 100% identity to reported Che a 1 sequence [5].

The pET21b vector has been designed to express the proteins with a C-terminal-hexahistidine-tagged sequence [20]. The amplified fragments through the PCR were digested with *Xho I*/*Not I*, then ligated into the digested pET21b with the same enzymes. The consequential plasmids were transformed into *E. coli* Top10 cells, which resulted in ampicillin-resistant colonies.

The propagation of vectors containing inserts is recommended in recombination-deficient (recA), endonuclease A-deficient (endA) *E. coli* strains such as Top10 cells, contain *recA* for stable replication of high copy number plasmids, *endA* for enhanced yield and quality of DNA, and *hsdRMS* to eliminate cleavage of recombinant plasmid by the endogenous *EcoR* restriction system [21].

For preparing the competent *E. coli* cells, the Inoue method was used [14]. This method is more reproducible than other chemical methods. The procedure works well with many strains of *E. coli* in widespread use in molecular cloning.

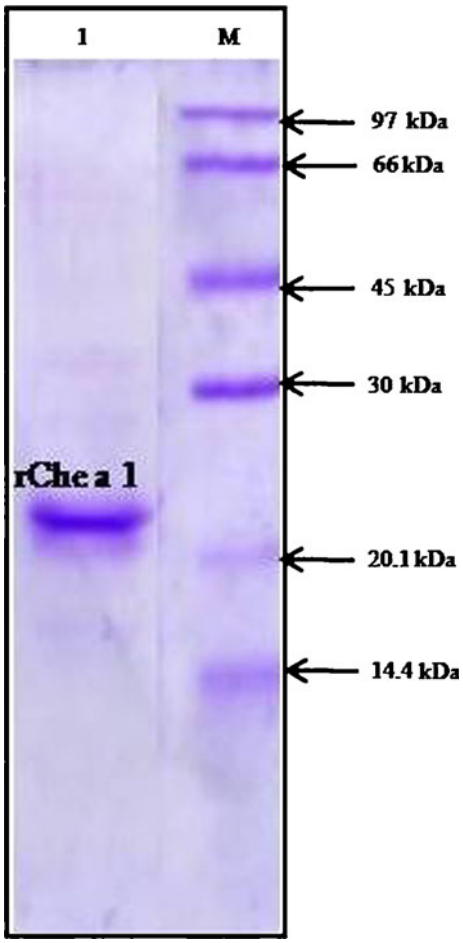

Fig. 3 SDS–PAGE (12%) for analyzing of rChe a 1 purification from insoluble fraction of lysate bacteria with Coomassie blue staining. *Lane M* molecular weight marker. *Lane 1* purified rChe a 1, a single 24-kDa band is visualized

Expression and Purification of rChe a 1 Protein

The selected clones of Top10 cells on ampicillin agar were subjected to plasmid purification. For expression, the pET21b+Che a 1 plasmids were successfully transformed into *E. coli* strain BL21 (DE3). Induction of expression was done using IPTG. The presence of interest expressed protein 24-kDa rChe a 1 was confirmed by SDS–PAGE analyses and Coomassie Brilliant blue staining in insoluble phase (results not shown). After solubilization in guanidine hydrochloride, the supernatant of lysate was verified on SDS–PAGE gel, and the newly expressed rChe a 1 was detected (Fig. 2).

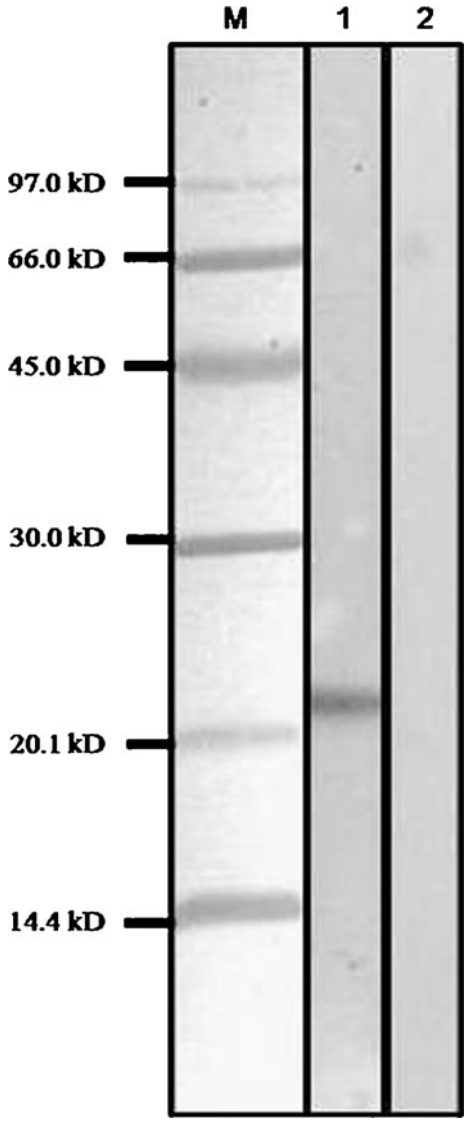

Fig. 4 Western blot analyses of purified rChe a 1. Purified protein separated by SDS–PAGE in 12% acrylamide gel was subjected to western blot analyses with *C. album* pollen allergic and non-allergic pooled sera. *Lane M* protein marker. *Lane 1* a single band is seen using allergic pooled sera. *Lane 2* there was not found any IgE reactivity of rChea 1 with negative pooled serum

Purification of rChe a 1 was done successfully (Fig. 3), and the reactivity of purified rChe a 1 with pooled serum from allergic patients to *C. album* pollen was detected with immunoblotting (Fig. 4).

Barderas et al. used *P. pastoris* yeast as expression system to produce the rChe a 1 [5]. In that study, rChe a 1 was isolated using a two-step procedure consisting of a size exclusion chromatography and an RP-HPLC column from supernatant of cell culture [5].

The expression of proteins in *E. coli* is the easiest, quickest, and cheapest method. There are many available commercial and non-commercial expression vectors with different N- and C-terminal tags and many different strains that are optimized for special applications.

In this study, we used *E. coli* BL21 strain as expression host. This strain is widely known as the strain of choice for expression of target proteins in bacterial systems. It lacks both *lon* and *ompT* proteases, which promote recombinant protein stability. The BL21 (DE3) strain bears a copy of the T7 RNA polymerase gene on their chromosome driven by the lacUV5 promoter. Therefore, when expressing a target gene under a T7 promoter-based system, the BL21 (DE3) strains offer a source of T7 RNA polymerase with simple IPTG induction for high-level expression of recombinant proteins [22].

The rChe a 1 in our system was expressed as a fusion protein with six extra Histidine amino acids in C-terminal. A simple one-step metal affinity purification system, utilizing the Ni–NTA resin, which binds to Histidines, was used.

IgE Reactivity of rChe a 1 Protein

Immunoblotting with sera of allergic patients revealed the reactivity of sera with purified rChe a 1 produced in *E. coli* cells and showed the functional expression in prokaryotic system.

The inhibition studies were carried out using the adsorbed sera of allergic patients to *C. album* pollen with crude extract of *C. album* pollen and the recombinant allergen rChe a 1. Inhibition effect of rChe a 1 was found stronger than total extract (Fig. 5). Using 150 μg/well

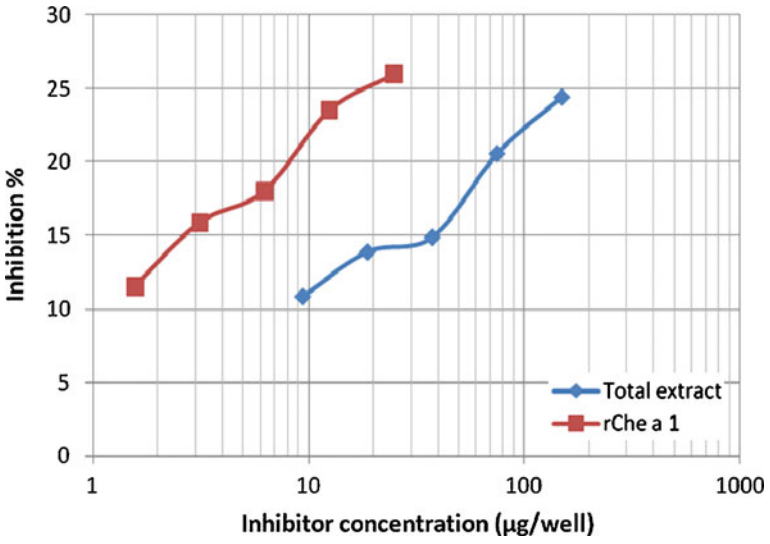

Fig. 5 ELISA inhibition analyses. IgE binding of *C. album* pollen allergic pooled sera was inhibited with rChe a 1 and crude extract of *C. album* pollen. IgE reactivity was inhibited by total extract and rChe a. *Each point* represents the average of two independent experiments

of total extract, inhibition was found to be 24.45%, whereas same inhibition was achieved by using 12.5 μg/well of rChe a 1. These results are average of two independent experiments and may be explained by lowering the relative concentration of the major allergen in the total protein extract than the recombinant allergen.

These inhibition assays suggest cross-reactivity between natural form of Che a 1 allergen in total extract and rChe a 1 that shows presence of tags at the ends recombinant protein that did not have effect on the specific reactivity of rChe a 1 with IgE. The presence of tags in recombinant proteins may not interfere with their activity [23–26].

Conclusion

Recombinant allergens have a wide range of uses, from the diagnosis and development of immunotherapy to the standardization of allergenic proteins to use as tools in molecular allergology.

Most of the existing recombinant allergens have been expressed in *E. coli* and are usually comparable with their natural templates in structural features and immunobiological properties. Recombinant allergens mostly bind to IgE antibody comparable to that of natural allergens and show good reactivity in diagnostic tests [27].

In this research, rChe a 1 was produced and purified successfully. To the best of our knowledge, this is the first report of Che a 1 expression, major allergen of *C. album* pollen in *E. coli* system that is the most frequently used organism for production of recombinant DNA and proteins due to the fact that it is the least expensive, easiest, and quickest system for expression of proteins. The proteins that require post-translational modifications, including disulfide bonds and glycosylations, and higher system such as yeasts, are the systems of choice [28, 29]. In this study, immunoreactivity of rChe a 1 expressed in *E. coli* was shown.

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