

***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**

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Abstract The inhalation of *Chenopodium album* (*C. album*) pollen has been reported as an important cause of allergic respiratory symptoms. The aim of this study was to produce the recombinant profilin of *C. album* (rChe a 2) pollen and to investigate its cross-reactivity with other plant-derived profilins based on potential conformational epitopes and IgE reactivity analysis. Che a 2-coding sequence was cloned, expressed, and purified using one step metal affinity chromatography to recover high-purity target protein. We assessed cross-reactivity and predicted IgE potential epitopes among rChe a 2 and other plant-derived profilins. Immunodetection and inhibition assays using sixteen individual sera from *C. album* allergic patients demonstrated that purified rChe a 2 could be the same as that in the crude extract. The results of inhibition

assays among rChe a 2 and other plant-derived profilins were in accordance with those of the homology of predicted conserved conformational regions. In this study, amino acid sequence homology analysis showed that a high degree of IgE cross-reactivity among plant-derived profilins may depend on predicted potential IgE epitopes.

Keywords *Chenopodium album* · Che a 2 · Profilin · Cross-reaction

Introduction

Chenopodium album (*C. album*) or Common lamb's-quarters is a perennial plant of the goosefoot family, Chenopodiaceae [1]. This weed is found in all parts of the world and grows on all types of soils even salty soil, preferring fertile ones [2]. Pollinosis and allergic sensitization to *C. album* has been reported in European countries and North America [3, 4], as well as, is a severe problem in semidesertic countries such as Iran [5, 6], Saudi Arabia [7], and Kuwait [8].

The previously reported chenopod allergen, Che a 1, is a glycoprotein of molecular mass about 17 kDa and 143 amino acid residues, whose sequence exhibits 27–45% identity with known members of the Ole e 1-like protein family [9]. Two other allergens, Che a 2 (profilin), an about 14.4 kDa protein, and Che a 3 (polcalcin), an about 9.5 kDa protein, were cloned and purified and characterized by using Immunochemical methods [10, 11].

Profilins are 12- to 15-kDa monomeric proteins belonging to a ubiquitous family of actin binding proteins. They are prominent allergens in the pollen of trees, grasses, and weeds, along with many fruits and vegetables [12–14]. However, these allergens are usually under-represented in

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the natural extracts used for diagnostic, therapeutic and experimental purposes.

Since chenopod pollen allergens play an important role in sensitization of allergic patients, in this study, we described the production of recombinant profilin of the *C. album* pollen in *Escherichia coli* (*E.coli*) at a relatively high level of expression. Moreover, after purification, the allergenic reactivity of rChe a 2 and its cross-reactivity with other plant-derived profilins was assessed based on predicted conserved conformational regions and employing the experimental method with the sera from *C. album* allergic patients.

Materials and methods

Allergic patients and skin prick test (SPT)

This study included sixteen patients suffering from allergy to *C. album* pollen, nine women and seven men who had seasonal rhinitis without asthma. All subjects had positive SPTs to *C. album* pollen extract. The four other healthy

subjects who showed negative SPT responses and no specific IgE to *C. album* pollen extract were considered as negative controls. Case histories in respect to the *C. album* allergy are summarized in Table 1. The Human Ethics Committee of the Mashhad University of Medical Sciences (MUMS), Mashhad, Iran approved the study protocol with informed written consent from each patient.

Pollen extracts preparation and recombinant profilins

After the process of *C. album* species identification, polleniferous materials were collected from *C. album* flowers as described previously [6]. In brief, pollen grains were separated by passing the dried materials through different sieves. Pollen materials with more than 90% pollen and less than 10% floral parts of the same plant were taken for protein extraction.

Pollen materials were defatted using repeated changes of diethyl ether. Two grams of the *C. album* pollen were extracted as described previously [6]. The protein content of the extract was then measured by the Bradford's method [15].

Table 1 Clinical characteristics, SPT responses and IgE-reactivity of patients allergic to *C. album* pollen

Patients	Age (years)/sex	Clinical history	<i>C. album</i> extract		Recombinant Che a 2	
			Skin test ^a	Specific IgE ^b	Skin test	Specific IgE
1.	24/F	AR, RC	28	0.82	19	0.66
2.	18/M	AR, RC	24	0.75	14	0.59
3.	30/F	AR, RC	25	0.93	15	0.72
4.	30/M	AR	12	0.67	0	0.06
5.	27/M	AR	13	0.74	0	0.07
6.	28/F	AR, RC	27	0.88	16	0.60
7.	17/F	AR	10	0.89	0	0.08
8.	36/F	AR, RC	18	0.68	12	0.60
9.	20/F	AR, RC	27	0.90	16	0.71
10.	26/M	AR, RC	11	0.89	0	0.06
11.	22/M	AR	18	0.98	10	0.78
12.	13/F	AR, RC	15	0.69	0	0.05
13.	14/F	AR	28	1.02	18	0.78
14.	23/M	AR	11	0.72	0	0.07
15.	39/M	AR	13	0.68	0	0.07
16.	38/F	AR	12	0.70	0	0.06
Controls						
17.	34/F	—	0	0.08	0	0.06
18.	24/F	—	0	0.08	0	0.08
19.	29/M	—	0	0.09	0	0.07
20.	35/F	—	0	0.10	0	0.06

AR Allergic rhinitis; RC Rhiniconjunctivitis

^a The mean wheal areas are displayed in mm². Histamine diphosphate (10 mg/ml)-positive control; Glycerin-negative control

^b Determined in specific ELISA as OD (optical density) at 450 nm

Recombinant profilins from *Crocus sativus* (rCro s 2) was expressed in *E. coli* and purified by metal precipitation [16]. Moreover, recombinant profilins from *Cucumis melo* (rCuc m 2) [17], and *Salsola kali* (rSal k 4) [18], *Amaranthus retroflexus* (rAma r 2) (unpublished data), were expressed in *E. coli* and purified using metal affinity chromatography (Ni-NTA).

Amplification of Che a 2 cDNA by PCR and determination of nucleotide sequence

The Che a 2 coding region was amplified using degenerate primers that are designated on the basis of the amino acid sequence comparisons from different plant profilins. The primers used were the sense primer: 5'-ATGTCSTGG CAGACGTAYGTHGATGA-3' and the antisense primer: 5'-CATGCCYTGTTCGACCAGRTARTCACC-3'. A PCR product of 399 bp length was purified from gel (QIAGEN, Hilden, Germany) and cloned into the pTZ57R/T vector from the Ins TAclone™ PCR cloning kit (Fermentas, Vilnius, Lithuania). The resulting plasmids were employed to transform the TOP10 *E. coli* cells and recombinant plasmids were then subjected to sequencing (SeqLab, Gottingen, Germany).

Construction of prokaryotic expression plasmid carrying Che a 2 gene

Based on the obtained sequence, specific primers of Che a 2 contained overhangs with *Not* I and *Xho* I restriction sites for direct cloning into the expression plasmid pET-32b(+) (Novagen, NJ, USA) were developed as following: sense primer (5'-ACCGCGGCCGCAATGTCGTGGCAGACGT ACGTAGA-3', *Not* I restriction site is underlined) and anti-sense primer (5'-TCCCTCGAGCATGCCCTGTTTCG ACCAGGTAGT-3', *Xho* I restriction site is underlined).

The resulting 399 bp amplified product was digested by the *Not* I and the *Xho* I restriction enzymes according to the manufacturer's protocol (Fermentas, Vilnius, Lithuania). Purified digested PCR product was ligated into the digested pET-32b(+) plasmid with the same enzymes before. The pET-32b(+)/Che a 2 expression vector provided a sequence coding for a thioredoxin (TrxA) partner and two hexahistidyl (6 × His) affinity tags at the 5' and 3' ends of the Che a 2 coding sequence. Correct constructs were transformed into competent *E. coli* BL21 (DE3) cells (Novagen, NJ, USA).

Expression and purification of recombinant Che a 2 protein

Recombinant Che a 2 was expressed in the *E. coli* expression strain BL21 (DE3)pLysS (Invitrogen, USA) as

described previously [17, 19]. In brief, A fresh clone of recombinant plasmid pET-32b(+)/Che a 2 was inoculated into 2 ml LB containing 50 µg/ml ampicillin and incubated. The culture was incubated at 37°C with shaking at 300 rpm until the absorbance at 600 nm reached 0.4. Afterwards, 250 µl of this culture was inoculated into 5 ml of LB medium in a 50 ml- flask at 25°C with shaking at 280 rpm until the absorbance at 600 nm reached 0.4. Expression of the recombinant protein was induced by inactivation of the *lac* repressor via addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM and to improve solubility of recombinant Che a 2 (rChe a2), the cultures were incubated at 18°C, overnight. Subsequently, the cells were harvested by centrifugation (3000 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, 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 and 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 fresh tubes on ice. Sample aliquots from both supernatant and pellet were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels by Laemmli method to check for expression of rChe a 2 [20].

The purification of rChe a 2 was performed by metal-affinity chromatography using Ni-NTA agarose (Invitrogen, CA, USA) from the soluble phase of lysate, according to the manufacturer's instructions.

IgE-Immunoblotting and IgE-immunoblotting inhibition

Natural extract of *C. album* pollen and purified rChe a 2 were electrophoresed on 15% SDS-PAGE and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) as described previously [6]. In brief, after washing and blocking with 2% BSA for 16 h at 4°C, membranes were incubated with a serum pool or individual sera of the allergic patients or with control sera, 1:5 dilutions in PBS, for 3 h at room temperature. Biotinylated anti-human IgE (KPL, Inc., MD, USA) (1:1000 v/v in 1% BSA) was added to the blotted membrane strips and incubated for 2 h at room temperature. The unbound antibodies were removed from blots by washing with PBS and followed by incubation with 1:2000 v/v in bovine serum albumin (BSA) 1% HRP-linked ExtraAvidin (SIGMA) for 2 h at room temperature. The bound enzymatic activity of horseradish peroxidase was detected by enhanced chemiluminescence reagents (PIERCE, IL, USA) and documented with G-BOX Chemi-Doc (Syngene, Cambridge, UK).

For cross-inhibition experiments between the natural *C. album* pollen extract and the rChe a 2 protein allergen, a mix of 100 µl of pooled sera (1:5 v/v) were incubated with the equal volume of extract of natural *C. album* pollen extract (50 µg/ml), rChe a 2 and BSA (as negative control), separately, for 3 h at 37°C under shaking. Pre-incubated sera were used to assess the reactivity on PVDF membrane blotted with rChe a 2 or *C. album* pollen extract as described above.

Specific enzyme- linked immunosorbent (ELISA) and inhibition assays

To evaluate the specific IgE reactivity of *C. album* pollen extract, all sixteen sera were assessed by ELISA as previously described [17]. Four control subjects who showed negative SPT responses to *C. album* pollen extract (Table 1, Sample Numbers 17–20) were also used as negative controls. Optical density (OD₄₅₀) greater than four times the median values of the negative controls were considered to be positive.

To study the dose-dependence of IgE reactivity to Che a 2 by other plant profilins, including rSal k 4, rAma r 2, rCuc m 2 and rCro s 2, ELISA inhibition assays were performed as described, except that a pooled serum (1:2 v/v) from *C. album* allergic patients (Nos. 1, 2, 3, 6, and 9) was preincubated for 1 h at room temperature with either 100, 10, 1, 0.1 or 0.01 µg of the purified specific recombinant proteins as inhibitors or with BSA as a negative control. Serum without rChe a 2 served as a sample without an inhibitor. Assays were performed in duplicate. The percentage of inhibition was calculated using the following

formula: (OD of sample without inhibitor -OD of sample with inhibitor/OD of sample without inhibitor) × 100.

Homology modeling and common predicted conformational epitopes

The homology model of *C. album* profilin was generated using the coordinates from the X-ray structure of profilin of *Arabidopsis thaliana* (PDB Number: 3nuaA) by the Internet server Swiss Model (<http://swissmodel.expasy.org/SWISS-MODEL.html>), as previously described [17]. In brief, the program of ZMM software was used with the above constraints to minimize the conformational energy of the protein. Low-energy conformations were obtained by Monte Carlo minimization method. Analysis of Che a 2 molecular model was performed using POCHECK program [21]. The profilin of *Arabidopsis thaliana* has 76% sequence identity with the *C. album* profilin (Fig. 2a).

Amino acid sequences of the profilins (Ama r 2, Sal k 4, Cuc m 2, Hev b 2, and Cro s 2) were evaluated in eleven predicted conformational epitopes which has been recently reported (Table 2) [22]. These predicted epitopes were introduced with a binding potential to specific IgE from profilin allergic patients.

Result

Cloning and sequence analysis of Che a 2

The sequence analysis of Che a 2 showed an open-reading frame of 399 bp coding for 133 amino acid residues with a

Table 2 Comparison of plant-derived profilins with Che a 2 based on predicted conformational epitopes

IgE-binding epitope sequencer	Plant-derived profilins (% identity) Cue in 2					Mean of identity (%) in each epitopes
	A ma r 2	Sal k 4	Cuc m 2	Hev b 8	Cro s 2	
1 2SWQTYVDDH ₁₀ Q ₃₇ Y ₁₂₇ Q ₁₃₁ GM ₁₃₃	86	93	79	100	86	88.8
2 8DDH ₁₀ 13uFPIEENHLT ₂₃ 39SN40 ₁₀₉ DEPVT ₁₁₃	68	68	58	53	37	56.8
3 Q ₄ D ₈ 21HLT ₂₃ V ₃₄ 37QSSTFPQ ₄₃ G ₇₁ N ₁₀₉	80	80	80	80	60	76
4 32GSV ₃₄ 37QSSTFPQLKQE ₄₇ F ₆₈ 71GE ₇₂	59	59	80	65	59	64.4
5 30LDGSV ₃₄ 42PQLKQE ₄₇ 50KAICN ₅₄ T ₆₅ F ₆₈ G ₇₁	63	53	63	53	63	59
6 E ₄₇ 50KA ₅₁ N ₅₄ 60NTLAPT ₆₅ F ₆₈ 71GEKYMV ₇₆	65	65	65	59	65	63.8
7 59PN ₆₀ A ₆₃ 75MVIQGE ₈₃ Q ₈₃ 86RGK ₈₈ P ₁₁₄ N ₁₁₈ E ₁₂₂	94	83	94	94	89	90.8
8 53CN ₅₄ 57DVPNS ₆₁ 76VIQGE ₈₃ Q ₈₃ 86R ₈₆ K ₉₈ E ₁₂₂	78	67	72	67	67	70.2
9 17ETG ^a NHLT ₂₃ 88KKGP ₉₁ 109NEPVT ₁₁₄	80	87	80	60	60	73.4
10 H ₁₀ 13FPIEETG ^a N ₂₀ R ₈₆ 113TPG ₁₁₆₋₁₁₈ NM ₁₁₉ 112EK ₁₂₃ 126DY ₁₂₇	76	76	76	59	47	66.8
11 Y ₆ 98KTNQ ₁₀₁ 122EK ₁₂₃ 126DYLVEQGM ₁₃₃	73	87	60	67	47	66.8
Mean of identity (%) in each plant-derived profilins	75	74	73	69	62	

Adapted from [22].Residue numbering corresponds to a position in *C. album*

^a The residues (in italic) were not found in Hev b 8, Cuc m 2 and Cro s 2

predicted molecular mass of 14.4 kDa and a calculated pI of 4.68. The obtained nucleotide sequence was submitted to the NCBI GenBank (Accession Number: FJ985985).

The comparison of the deduced amino acid sequence of the *C. album* pollen profilin with other allergenic plant-derived profilins in the protein database was performed (Fig. 1). A high level of sequence similarity (90%) was detected with the Che a 2 previously reported (AAL92870). Moreover, the most conserved residues showed involvement in specific biological functions and structural roles (Fig. 1).

Homology modeling and predicted conformational epitopes

Three-dimensional structure of the Che a 2 is shown in Fig. 2a. The model was evaluated in terms of stereochemical and geometric parameters such as bond lengths, bond angles, torsion angles, G-factor and packing environment, and it was found to satisfy all stereochemical and geometric criteria. No residue was located in the disallowed regions of the Ramachandran map. After energy minimization of the models, the overall conformational energy of comparative model of the Che a 2 was -808 kcal/mol. Main-chain C α atoms of 3nula and the Che a 2 superimpose with an RMS deviation of 0.01Å.

Based on eleven predicted conformational epitopes, the results revealed that the highest level of identity (75%) was found between Che a 2 and Ama r 2. Moreover, in accordance with a previous study [22], epitopes 1 and 7 contained more conserved residue in selected profilins (88.8 and 90.8%, respectively) (Fig. 2b).

Expression and purification of rChe a 2

The pET-32b (+)/Che a 2 clone was successfully constructed and confirmed by digestion with *Not* I and *Xho* I restriction enzymes. This recombinant plasmid containing TrxA, to increase the solubility, and his-tags, for metal-affinity chromatography purification, was expressed in *E. coli* strain BL21 (DE3)pLysS as an about 34-kDa recombinant protein (TrxA-rChe a 2).

In this study, high level expression of Che a 2 was obtained using 0.2 mM concentration of IPTG as inducer and optimum temperature at 18°C (Fig. 3). SDS-PAGE analysis of both the soluble and the insoluble protein fractions revealed that most of rChe a 2 was presented in the soluble phase in the supernatant fraction. rChe a 2 was expressed in *E. coli* as a fusion 34 kDa- protein with two his-tagged proteins in two ends and was purified from the expression medium supernatant by Ni-chelate affinity chromatography. The maximum elution was achieved with 250 mM imidazole. The purified fractions were subjected to analysis by SDS-PAGE method and Coomassie brilliant blue R-250 staining (Fig. 3).

The purified rChe a 2 protein was quantified using the Bradford protein assay and the yield of purification was determined 2.1 mg/ml.

SDS-PAGE, IgE-immunoblot and immunoblot inhibition

SDS-PAGE of *C. album* pollen extract indicated several proteins with MWs approximately from 14 to 97 kDa (Fig. 4a). Moreover, the analysis of both natural form of

	10	20	30	40	50	60	70	80	
Che a 2-1	MSWQTVVD	DHLMFPIETG	NHLTAAIV	GLDGSVWA	QSSTFPOLKQ	EEVKAICNEFD	VNTLAPTGL	FLGGER	YIMVIOGE
Che a 2-2		CD..G--..	SS..L.H..T.	..PS....P.	..S..MKD..	NE.GS....	..H...T.		
Sal k 4		CE..G.N...	..L.V....	..AN...F.PD	..IS.VVK...	..EAG....	..H...T.		
Ama r 2		CE..G.T..G	..L.....	..AD...F.PD	..IA..VED..	..E.G....	..H...T.		
Hev b 8	..H....	ER..CE..G--	..S...I.Q...	..N...F.S..	..IT..MSD..	..E.G....	..H...T.		
Cuc m 2		VE..CE..G--	..S...I.Q...	..QN...P...	..AG.VGD..	..AD.G....	..YI...T.		
Cro s 2		EDMG--HV..	..S...L.H...	..AG...E..PA	..IT..L.D..	NE.GS....	..MYIN..A		
	90	100	110	120	130	aa	%identity	%similarity	
Che a 2-1	PGAVIRGK	FKGPGVCIKK	INQALVFGIY	NEPVTFGQ	CNMVVEKLG	GYLVEQGM	133		
Che a 2-2	..D.....	..T.....	..II...G..M.	RI....		131	80	90	
Sal k 4	A.Q.....	..I.V....	..G...I...D.	I..R...		133	77	86	
Ama r 2		..A..I.V..	..G...M...D.	I..R...	..I...Y	133	77	85	
Hev b 8	A.....	..TVR....	..II...D..M.	I..R...	..L....	131	74	86	
Cuc m 2		..ATV....	..GM...I...D	..M.....	..I..R...	131	73	84	
Cro s 2	..V.....	..S...T...	..S.M...I...L	..D..M....	..L...R...	131	68	85	

Fig. 1 Comparison of the Che a 2 amino acid sequence with allergenic profilins from other plants. The amino acid sequence identity and the similarity of Che a 2 (Che a 2-1, ACR77509) with previously reported Che a 2 (Che a 2-2, AAL92870) and other members of the profilin family are indicated at the end of each amino acid sequence. *Salsola kali* (Sal k 4, GQ145223), *Amaranthus*

retroflexus (Ama r 2, ACP43298), *Hevea brasiliensis* (Hev b 8, CAA75312), *Cucumis melo* (Cuc m 2, AAW69549) and *Crocus sativus* (Cro s 2, AAW81034). The highly conserved residues in plant-derived profilins involved in biological and structural functions (Poly-L-proline binding, fold conservation, actin binding site and stabilized turn) were boxed

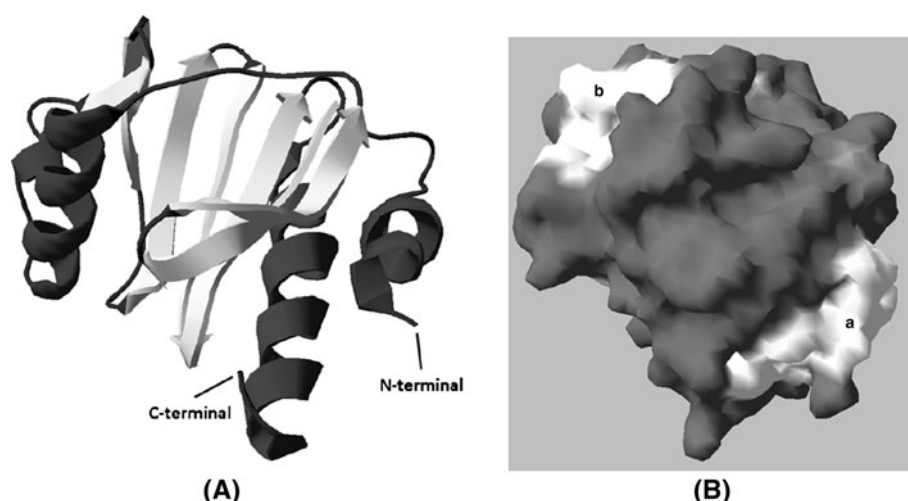


Fig. 2 Three-dimensional view of *C. album* profilin. **a** Ribbon representation of the structure of the Che a 2 modeled according to the three-dimensional structure of the profilin of *Arabidopsis thaliana* (PDB number: 3nulA). **b** Space-filling model of Che a 2 with two predicted conformational epitopes. These epitopes contained more

conserved residue in selected profilins. (epitope a: ^{2} SWQTYVDDH ${}_{10}$ -Q ${}_{37}$ Y ${}_{127}$ Q ${}_{131}$ G ${}_{132}$ M ${}_{133}$ and epitope b: ^{59} PN ${}_{60}$ A ${}_{63}$ ^{75} MVIQGEPGA ${}_{83}$ ^{86} RGK ${}_{88}$ P ${}_{114}$ N ${}_{118}$ E ${}_{122}$, residue numbering corresponds to a position in *C. album*)

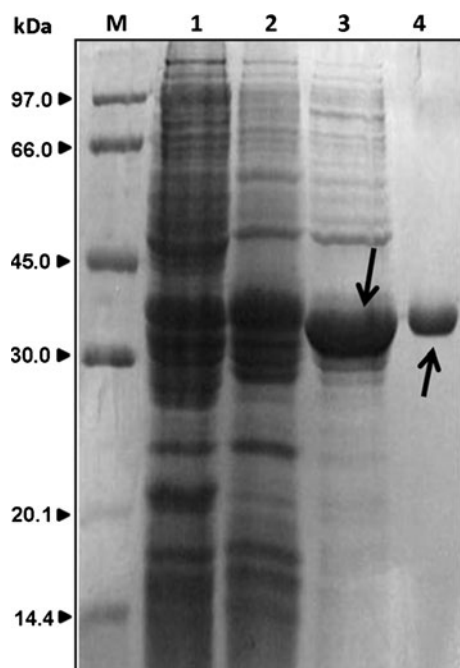


Fig. 3 SDS-PAGE (12%) for analyzing for analysis of expression and purification of rChe a 2. Lane M, Low molecular weight marker (Amersham, UK); Lane 1, IPTG-induced pET-32b(+) vector without insert; lane 2, IPTG -induced of insoluble fraction selected clone with insert; lane 3, IPTG -induced of soluble fraction selected with insert; lane 4, purified rChe a 2 with Ni-NTA affinity chromatography

profilin (Che a 2) in *C. album* pollen extract and purified recombinant protein revealed that they were reactive with eight (50%) individual sera (Fig. 4b, c), Lanes 1, 2, 3, 6, 8, 9, 11, and 13.

Inhibition experiments demonstrated similar IgE reactivity for both purified rChe a 2 and its natural counterpart

in *C. album* pollen extract using a pooled serum, including five *C. album* allergic patients (Nos. 1, 2, 3, 6, and 9). The results indicated that pre-incubation of serum samples with rChe a 2 (Fig. 5a, b, lane 2) or the crude extract of *C. album* pollen (Fig. 5a, b, lane 3) completely inhibited IgE binding to rChe a 2 and natural Che a 2, respectively. However, preincubation of the pooled serum with PBS-BSA had no influence on its IgE-reactivity to Che a 2 (Fig. 5a, b, Lane 1).

Specific IgE ELISA and inhibition assays

The specific IgE values against *C. album* pollen extract and purified rChe a 2 were determined with sixteen individual patients' sera (Table 1). All of these patients had significantly elevated specific IgE levels to *C. album* pollen extract. Eight sera (8/16) gave OD values more than 0.3 (four times greater than the median values of the negative controls), and thus were considered capable to bind to purified rChe a 2 (Table 1). The results were consistent with those obtained from the IgE immunoblotting assays using the recombinant protein.

To investigate cross-reactivity among rChe a 2 and other weed and fruit profilins, an ELISA inhibition with recombinant profilins (rAma r 2, rSal k 4, rCuc m 2, and rCro s 2) was performed (Fig. 6). Almost complete inhibition was attained with 100 μ g/ml rChe a 2 as a positive control. Pre-incubation of pooled serum with a high concentration (100 μ g/ml) of rAma r 2, rSal k 4 and rCuc m 2 revealed significant inhibition of IgE binding to rChe a 2 (72, 66, and 56%, respectively) (Fig. 6).

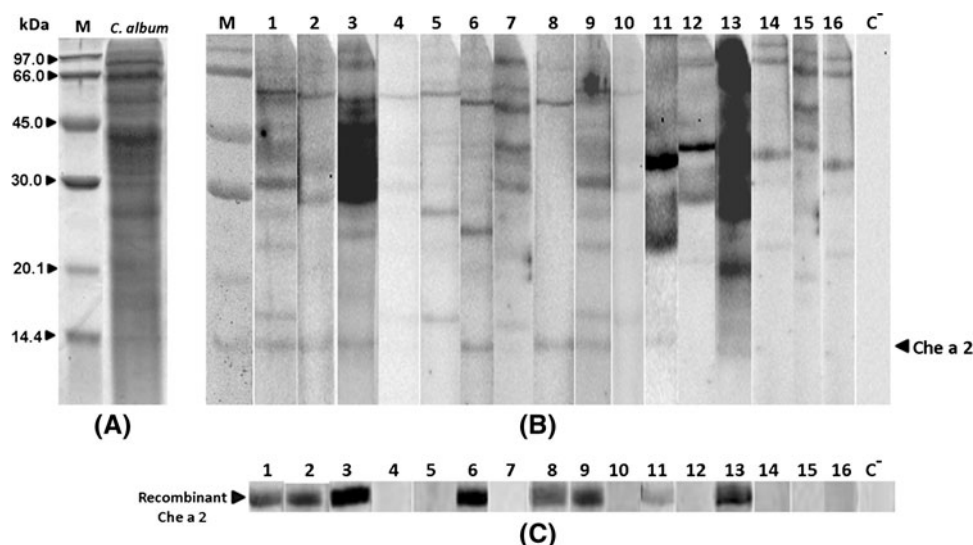


Fig. 4 SDS-PAGE and Immunoreactivity of *C. album* pollen extract and recombinant Che a 2. **a** Coomassie brilliant blue stained SDS-PAGE of the crude extract of the *C. album* pollen on 15% acrylamide gel. Lane M, Low molecular weight marker (Amersham, UK); **b** IgE Immunoblot of *C. album* pollen extract using allergic patients' sera;

lane M, molecular weight marker (Amersham, UK); Lanes 1–16, probed with individual patients' sera; Lane C⁻, Negative control; **c** IgE-immunoblotting analysis of rChe a 2. Lane 1–16, blotted rChe a 2 was probed with individual sera of *C. album* allergic patients; Lane C⁻, negative control

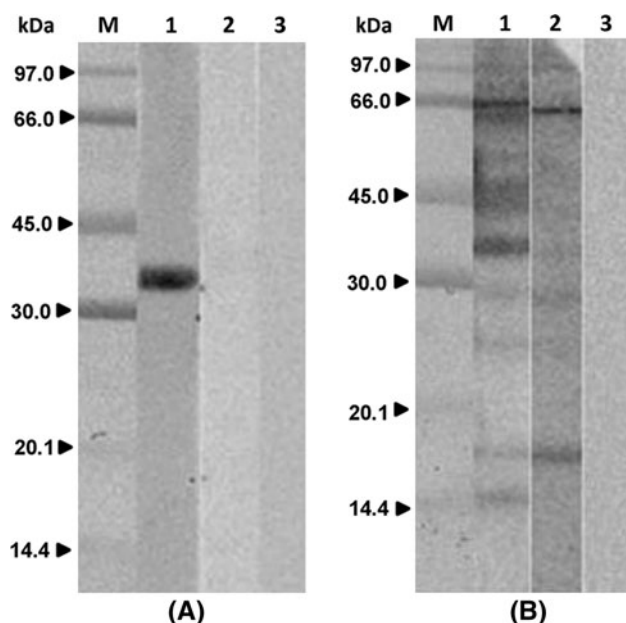


Fig. 5 Inhibition immunoblotting. Purified rChe a 2 **a** and Protein extract from *C. album* pollen **b** were separated by SDS-PAGE and blotted onto PVDF. IgE-binding of a pooled serum, including five *C. album* allergic patients (Nos. 1, 2, 3, 6, and 9) IgE-binding of a *C. album* allergic pooled serum studied after pre incubation with PBS (Lane 1), purified rChe a 2 (10 µg/ml) (lane 2), and *C. album* extract (20 µg/ml) (lane 3). Lane M, Low molecular weight marker (Amersham, UK)

Capacity of rChe a 2 to elicit immediate cutaneous reactions

The capacity of rChe a 2 to induce immediate skin reactions were investigated in volunteer *C. album* allergic

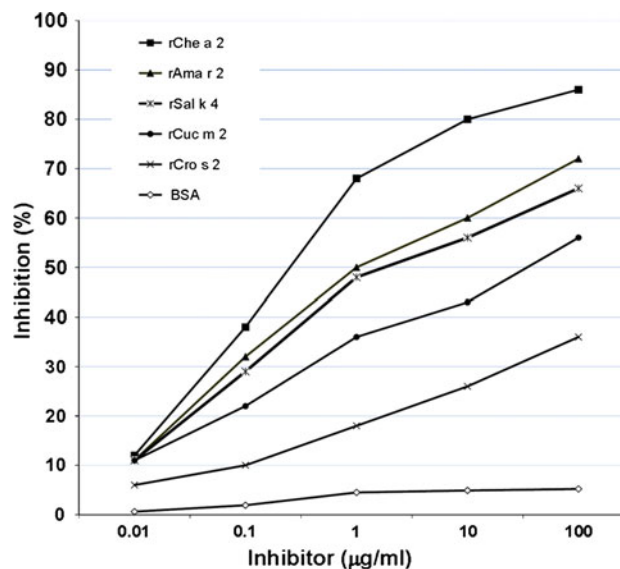


Fig. 6 Inhibition of IgE-binding to rChe a 2 by ELISA using recombinant profilins from other sources: *Amaranthus retroflexus* (rAma r 2), *Salsola kali* (rSal k 4), *Cucumis melo* (rCuc m 2), and *Crocus sativus* (rCro s 2). Control experiments were performed with BSA

patients by in vivo SPT (Table 1). Eight patients exhibited a positive reaction for rChe a 2. In this way, in vivo results confirmed in vitro immunoreactivity assays. In control patients, there were no skin reactions elicited with the application of purified rChe a 2.

Discussion

Chenopodium album is a weedy plant of the Chenopodiaceae family growing even in salty soil [8, 9]. Pollinosis to *C. album* has been reported previously and three proteins, Che a 1, Che a 2, and Che a 3 proteins have defined as allergenic components of *C. album* pollen [9, 11]. Che a 2 is a member of profilin family that has significant role in allergy [4, 11]. The purpose of this research was to produce the recombinant profilin from the *C. album* pollen in *E. coli* and evaluation of its cross-reactivity with other regional allergenic profilins, based on potential cross-reactive IgE binding epitopes and IgE reactivity analysis.

In this study, profilin of *C. album* pollen was cloned by a PCR strategy using degenerated primers based on the codons of conserved amino acid sequences from different plant profilins. The open reading frame of Che a 2 contains 133 bases encoding a 14.4 kDa protein, which correlates with the molecular characteristics of the known plant profilins.

The comparison of the obtained amino acid sequence of Che a 2 (ACR77509) with deposited sequences showed high degree similarity to profilins from other sources. The similarity was found to be 90% with previously reported Che a 2 (AAL92870). Regarding to this difference, it is possible that these are two isoforms of Che a 2 protein of the *C. album* pollen. Nevertheless, additional studies are required to answer to this question.

In this experiment the temperature of culture was lowered to 18°C following IPTG induction. A significant increase in the intensity of soluble expressed rChe a 2 band was detected by SDS-PAGE, confirming that aggregation was reduced at lower growth temperature that is explained by chaperone-assisted folding pathways in *E. coli* [23–25].

The IgE-binding ability of the purified rChe a 2 to sera from *C. album* allergic patients was evaluated in order to confirm that the recombinant protein was equivalent to its natural counterpart in crude extract. The results of immunoblotting assays for natural Che a 2 were consistent with those obtained from recombinant Che a 2. A complete inhibition of IgE-binding to natural Che a 2 was also obtained after preincubation of pooled serum with purified recombinant Che a 2. This study's findings also revealed that, similar to the results obtained from immunoblot assays with natural Che a 2 in *C. album* pollen extract and rChe a 2, eight patients did not show positive SPTs with rChe a 2. Taken together, it seems that rChe a 2 is comprised of IgE-epitopes similar to those of its natural counterpart.

In this study, amino acid identity analysis revealed that there was a high degree of identity among Che a 2 and other allergenic profilins from regional plants, particularly

Sal k 4 (*S. kali*), Ama r 2 (*A. retroflexus*), Cuc m 2 (*C. melo*) and Cro s 2 (*C. sativus*).

The results of the ELISA inhibition assays with pooled sera from patients (Nos. 1, 2, 3, 6, and 9), showed that inhibition by rAma r 2 and rSal k 4 was more intensive than other selected recombinant profilins. This was expected, because all these plants belong to the Amaranthaceae family.

The amino acid sequence analysis of the eleven predicted conformational epitopes from the selected profilins showed a high level of identity between Che a 2 and Ama r 2 conformational epitope regions (75%) and was concurrent with the results of ELISA inhibition assays. Radauer et al. [22] reported several conserved residues with an IgE-binding potential in two predicted conformational epitopes (epitopes 1 and 7, table 2) in plant-derived profilins. The amino acid sequence analysis of the two epitopes from the selected profilins indicated a significant identity among unrelated plant-derived profilins. The present study demonstrated that compared to other epitopes, potential conformational epitopes 1 and 7 contained conserved residues (88.8 and 90.8%, respectively), thus suggesting the essential role of epitopes 1 and 7 in cross-reactivity among profilins.

In addition to conserved residues with an IgE-binding potential, there are several other residues which are different in profilins from various sources. However, residues playing a crucial role in the structure and biological function of the molecule are significantly conserved among unrelated phylogenic profilins (Fig. 1) [26, 27].

In conclusion, this study demonstrates that purified rChe a 2 is comprised of IgE-epitopes similar to that of its natural counterpart. Moreover, according to their phylogenetic relationships, the cross-reactivity among plant-derived profilins from unrelated families may be predicted by the degree of amino acid sequence identity of potential conformational epitopes. As an appropriate expression system, *E. coli* BL21 along with the Ni-NTA agarose column, was showed as a tool for high level production of a pure recombinant allergen suitable for the characterization of allergenic epitopes recognized by T and B cells and finally for possible diagnostic and specific therapeutic purposes.

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