

Identification of a New Allergen from *Amaranthus retroflexus* Pollen, Ama r 2

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ABSTRACT

Background: Pollinosis from *Amaranthus retroflexus* pollen is a common cause of respiratory allergy in Iran with a high positive rate (68.8%) among Iranian allergic patients. The aim of the present study was to evaluate the allergenicity of the *A. retroflexus* pollen profilin.

Methods: Using sera from twelve patients allergic to *A. retroflexus* pollen, IgE-binding proteins from the *A. retroflexus* pollen extract was identified by immunoblotting. The cDNA of *A. retroflexus* pollen profilin was amplified, then cloned into the pET-21b (+) vector, expressed in *Escherichia coli*, and finally purified by metal affinity chromatography. The IgE-binding capacity of the recombinant protein was then analyzed by the ELISA, immunoblotting, and inhibition assays, as well as by the skin prick test (SPT).

Results: Immunoblotting results indicated a 14.6 kDa protein with IgE-reactivity to 33% (4/12) among *A. retroflexus* pollen-allergic patients. Nucleotide sequencing of the cDNA revealed an open reading frame of 399 bp encoding for 133 amino acid residues which was belonged to the profilin family and designated as Ama r 2. A recombinant Ama r 2 (rAma r 2) was then produced in *E. coli* as a soluble protein which showed a strong IgE-reactivity via ELISA confirmed by the SPT. Inhibition experiments revealed high IgE cross-reactivities with the profilins from other plants.

Conclusions: The profilin from the *A. retroflexus* pollen, Ama r 2, was firstly identified as an allergen. Moreover, rAma r 2 was produced in *E. coli* as a soluble immunoreactive protein with an IgE-reactivity similar to that of its natural counterpart.

KEY WORDS

allergen characterization, Ama r 2, *Amaranthus retroflexus*, cloning, profilin

INTRODUCTION

Immunoglobulin E (IgE)-mediated allergy to pollens from the Amaranthaceae is common in semi-desert countries such as Saudi Arabia, Iran, and Kuwait.^{1,4} Particularly among Iranian allergic patients, allergy to *A. retroflexus* (redroot pigweed) pollen is well documented with the high positive rate of 68.8%.⁴

In 1985, Lombardero *et al.* reported that pollens from the Amaranthaceae family, including *A. retroflexus*, Lamb's Quarter (*Chenopodium album*), and Russian Thistle (*Salsola kali*), contained significant allergens with molecular weights (MWs) of 35 and 14 kDa. In addition, it was shown that all of these pollens contained cathodic migrating allergens.⁵ In 1995,

Würtzen *et al.* introduced eleven allergenic proteins from the *A. retroflexus* pollen including three proteins with MWs of 14, 15.5 and 17 kDa.⁶ Recently, we showed an allergenic protein from the *A. retroflexus* pollen with an apparent MW of 14-15 kDa which had not yet been identified.⁷

Profilin is a well-known ubiquitous cytoskeleton protein in eukaryotic cells. It is presumably a link between the microfilament system and signal transduction pathways.⁸ Profilin was first recognized as an allergen in birch pollen and named Bet v 2.⁹ Up to now, profilins have been recognized as the allergens in several pollens, as well as some plant-derived foods.¹⁰

In this study, a new allergen of *A. retroflexus* pollen was identified which was designated as Ama r 2.

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METHODS

PATIENTS

Twelve adult respiratory allergic patients were enrolled from the Outpatient Allergy Clinic of Mashhad University of Medical Sciences. After getting informed consent, patients were asked to complete a detailed questionnaire. They were regarded to have a history of allergy if they stated at least one eye, nasal, or respiratory symptom to common allergens such as house dust, domestic animals, food, or pollen. Patients were also evaluated by clinical examination and skin-prick test with common aeroallergens. Two control subjects with no history of allergy and negative SPTs were also included in the study. The study was approved by the Human Ethics Committee of the university.

SKIN PRICK TEST

Skin prick test (SPT) was performed according to Dreborg's Method.¹¹ Tests were performed with 5 µl of each of *A. Retroflexus*, *C. album*, and *S. kali* pollen extracts (All from Hollister-Stier Laboratories LLC, Spokane, WA, USA) placed on the ventral side of the forearm with standard lancets. After 10 to 15 minutes, the region where the allergen was applied was observed for wheal (raised area) and flare (redness). Histamine as a positive control was also used to make confident of no anti-histamines have been taken which can interfere with the testing results. As a negative control, 50% glycerin solution was applied to make sure the patient was not dermatographic and falsely being identified as sensitive to *A. retroflexus*. A wheal at least 3 mm larger in diameter than the negative control, surrounded by an erythema, was considered as a positive skin test.

TOTAL EXTRACT PREPARATION

Pollen from *A. retroflexus* was purchased from Allergon AB (Välinge, Sweden) and extracted in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) in 1:20 w/v overnight on a shaker, at 4°C. Then it was centrifuged at 7150 x g for 20 min, and the clear supernatant was dialyzed for 16 h at 4°C against Tris-HCl 0.02 M, pH 8.8. The filtrate was then lyophilized and stored at -70°C. Lyophilized samples were reconstituted in distilled water (1/10 w/v) and the protein content of which was determined using the Bradford's method.¹²

ENZYME- LINKED IMMUNOSORBENT ASSAYS (ELISAs)

Total serum IgE levels were measured by means of a commercially available ELISA kit (Radim, Pomezia Terme, Italy) according to the manufacturer's instructions.

To measure the levels of specific IgE to *A. retroflexus* pollen in patients' sera, an indirect ELISA was

developed as described earlier.⁷ Briefly, 2 µg of *A. retroflexus* pollen extract in 100 µl carbonate buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6) per well of a 96-well microtiter plate (Nunc MaxiSorp™, Fisher Scientific, Pittsburg, PA, USA) was incubated overnight at 4°C. Each well was then blocked for 1 h at 37°C with 150 µl of 2% Bovine Serum Albumin (BSA) in PBS following by incubation for 3 h with 100 µl of serum at room temperature with shaking. Each well was then incubated for 2 h at room temperature with 1 : 1000 dilution of biotinylated goat anti human IgE antibody (Kirkegaard & Perry Laboratories, MD, USA) in 1% BSA. Each incubation step was followed by 5 washes with PBS-T (PBS containing 0.05% Tween 20). One hundred microliters of a 1 : 30000 dilution of horseradish peroxidase-conjugated streptavidin (Bio-Rad, MD, USA) were then added to each well. Following 5 washes with PBS-T, 100 µl of chromogenic substrate was added to each well and the plate was incubated for 15 min in the dark. Color development was stopped by the addition of 20 µl of 3 M hydrochloric acid. The plate was read at 450 nm with an ELISA reader. Optical density (OD₄₅₀) greater than three times the median values of negatives control were considered to be positive.

SDS-PAGE, IgE IMMUNOBLOTTING AND INHIBITION ASSAYS

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the *A. retroflexus* pollen extract was performed according to Laemmli¹³ using a 15% acrylamide separation gel under reducing conditions. Separated protein bands from the electrophoresis of *A. retroflexus* pollen extract were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA, US), as described by Assarehzadegan *et al.*³

Inhibition experiments were performed using two recombinant allergens which were previously expressed in our lab and belonged to profilin family including the recombinant Cuc m 2 (from *Cucumis melo* fruit),¹⁴ and the recombinant Che a 2 (from *C. album*, unpublished data). Ten micrograms of the purified rChe a 2, 3.3 µg of the purified rCuc m 2, or 7 µg of BSA (Negative control) were pre-incubated with a pooled serum (from patient 1, 5 and 6 as in Table 1) for 1 h at 37°C. The pre-adsorbed sera were then used for immunoblotting assays. BSA was also used for inhibition as control.

CLONING AND SEQUENCING

Total RNA was extracted from the pollen basically by the method of Chomczynski and Sacchi.¹⁵ First Strand of cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturers' instructions. PCR was performed using two degenerate primers which hybridized with a DNA whose se-

Table 1 Clinical data, specific IgE levels and SPT responses of the selected patients with allergy to *A. retroflexus* pollen

Patients	Age (years)/sex	Clinical history [†]	Total IgE IU/ml	<i>A. retroflexus</i> pollen extract		Recombinant Ama r 2
				Skin test [‡]	Specific IgE [§]	Specific IgE [§]
1	21/M	RC	716	14	1.06	0.98
2	19/M	RC	1120	16	1.05	0.86
3	21/M	R	102	8	0.78	1.02
4	20/M	RC	41	14	0.48	1.04
5	22/M	RC	275	12	1.06	0.6
6	20/F	R	1215	10	1.1	0.6
7	21/F	RC	144.1	8	1.1	0.97
8	24/M	RC	53.1	7	1.04	0.79
9	19/M	RC	514.9	10	0.59	0.91
10	42/M	RC	597	5	1.05	0.64
11	47/F	R	153.6	10	1.09	1.06
12	21/F	R	19	5	0.88	0.54
Controls						
13	21/M	-	69.8	0	0.18	0.18
14	40/M	-	10	0	0.17	0.16

[†] AR, Allergic rhinitis; RC, rhinoconjunctivitis.[‡] The mean weal diameters are displayed in mm². Histamine diphosphate (10 mg/ml)-positive control; Glycerin-negative control.[§] Determined in ELISA as OD (optical density) at 450 nm.

quence derived from that of the conserved sequences of profilin family. The primers used were the sense 5'-ATGTCGTGGCARRCBTAYGT-3' and the antisense 5'-ATRADCCYTGNTCDATNAGRTART-3'. The amplified product was ligated into the PTZ57R/T TA cloning vector from InsTAcloning™ PCR Cloning Kit (Fermentase), according to the manufacturer's instruction. *Escherichia coli* TOP 10 cells (Invitrogen, Life Technologies, Carlsbad, CA, USA) were transformed with the ligation products by using the manufacturer's protocol. Recombinant plasmid was then purified from the gel using a Plasmid Extraction Kit (GeNet Bio, Chungnam, Korea) and sequenced by the dideoxy method and analyzed at the SEQLAB Sequence laboratories (Göttingen, Germany).

EXPRESSION AND PURIFICATION OF rAma r 2

The coding region from Ama r 2 was amplified with *Pfu* DNA polymerase (Fermentase), using two specific primers. According to the nucleotide sequence of Ama r 2 (GenBank accession number: FJ899746), the sense primer (5'-TCCGCGGCCGCAATGTCTGTG GCA AGCGTATGT-3') is complementarily overlapped with the first six codons and introduces a *NofI* restriction site (underlined). The antisense primer (5'-TCCCTCGAGGTAACCCTGCTCGATGAGGT-3') mimics the last six codons, excluding the stop translation codon, and introduces an *XhoI* restriction site (underlined). After PCR amplification, the 399-bp product was ligated into pET-21b (+), (Novagen, WI, USA). The resulting plasmid containing the Ama r 2 gene in pET-21b (+) was used to transform the host cell *E. coli* BL21 (DE3) (Novagen). Cells were grown

at 37°C until to an OD₆₀₀ of 0.4 was reached, when expression was induced by the addition of 0.2 mM IPTG. After induction, in order to improve the solubility of the recombinant protein, the cultures were incubated at 18°C and the cells were allowed to grow for a period of 12 h. The cells were then disrupted by freezing in liquid nitrogen following by thawing at 37°C. The produced rAma r 2 was purified from the soluble phase of lysate by an Ni-NTA agarose (Invitrogen, CA, USA) according to the manufacturer's instructions. The protein concentration was determined according to the Bradford's method. The purified protein was then subjected to reducing SDS-PAGE and immunoblotting.

rAma r 2-SPECIFIC ELISA

In order to determine the serum IgE levels to the purified rAma r 2, an indirect ELISA was developed as described above, except that the wells of the ELISA microplate were coated with 100 µl of the purified rAma r 2 at a concentration of 10 µg/well in coating buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6) overnight at 4°C. Results were expressed as optical density (OD) units. Based on the mean value of two normal sera OD₄₅₀ greater than three times the median values of negatives control were considered to be positive.

rAma r 2-SPECIFIC IMMUNOBLOTTING AND INHIBITION ASSAYS

rAma r 2 was electrophoresed and transferred on a PVDF membrane as described above. Sixty microliters of pooled serum (from patients 1, 5 and 6) was

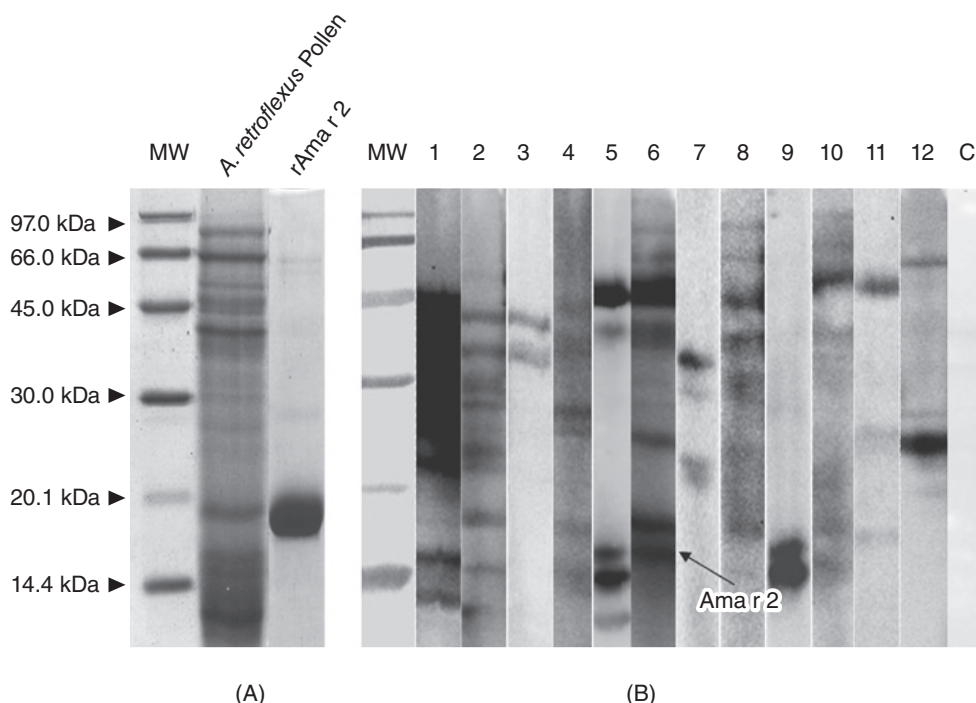


Fig. 1 (A). SDS-PAGE of the crude extract of the *A. retroflexus* pollen on a 15% acrylamide gel. Lane MW, low molecular weight (Amersham, Buckinghamshire, UK); (B). IgE-Immunoblot of *A. retroflexus* pollen extract using allergic patients' sera; Lane MW, low molecular weight (Amersham); Lanes 1-12, probed with individual allergic patients' sera; Lane C-, probed with a pooled serum from control subjects.

mixed with 5 µg of the purified rAma r 2, rChe a 2, rSal k 4 (a recombinant profilin from *S. kali* pollen produced in the same lab), or with BSA. Thereafter, samples were diluted to 600 µl and incubated 2 h at 37°C on a shaker. The pre-adsorbed sera were then used for immunoblotting assays.

SPT WITH rAma r 2

Different concentrations of the purified rAma r 2 were mixed with equal volume of glycerin and used for SPTs using two volunteer allergic individuals. Final concentrations of rAma r 2 used for SPTs were 0.4 and 0.2 µg/ml.

RESULTS

PATIENTS

Twelve patients suffering from respiratory allergy were included in our study. Case histories with respect to respiratory allergy are summarized in Table 1. Rhinitis and rhinoconjunctivitis was the most prominent clinical manifestations among these patients. All patients showed positive SPTs to the *A. retroflexus* pollen extract (Table 1) as well as the *C. album* and the *S. kali* pollen extracts (data not shown). Sera from nine of the twelve (75%) patients showed total IgE concentrations more than 100 IU/ml.

IgE-BINDING PROFILE OF THE *A. retroflexus* POLLEN WITH HUMAN SERA

The protein profile of *A. retroflexus* crude extract on a 15% acrylamide gel showed at least nine bands in the MW range of 10-70 kDa; including 85, 66, 50, 45, 39, 25, 18, 15, and 10 kDa (Fig. 1A). Specific IgE binding fractions probed against sera from all twelve allergic patients along with a pooled serum from the two control subjects are shown in Figure 1B. Six IgE binding protein fractions of 85, 45, 39, 18, 15, and 10 kDa were detected from the blot. Among all twelve samples, sera from four patients (patient number 1, 5, 6 and 10; as in Table 1) recognized a band with the apparent MW of 14-15 kDa (Fig. 1) which were estimated by Kodak1D Image (Eastman Kodak) as 14.6 kD. However, when the pooled sera of normal volunteers were used no IgE binding fractions was observed. According to the results of the specific ELISA, 9/12 of patients showed specific IgE levels of *A. retroflexus* pollen (Table 1).

INHIBITION OF IgE REACTIVITY TO Ama r 2 BY PROFILINS FROM OTHER PLANTS

Immunoblotting-inhibition experiments showed that both rChe a 2 and rCuc m 2 were able to inhibit IgE binding to Ama r 2 in *A. retroflexus* pollen extract (Fig. 2). According to densitometric analysis, rChe a

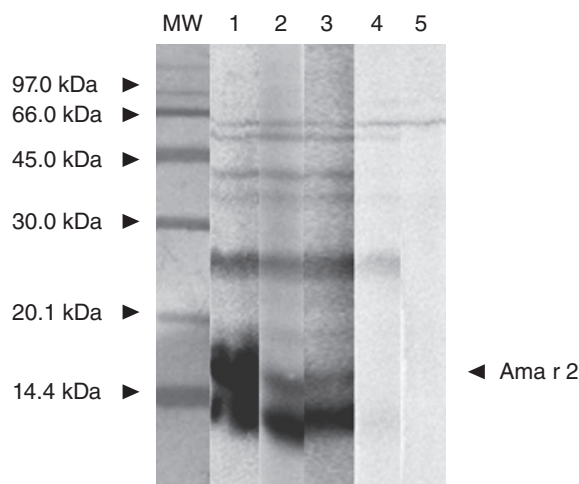


Fig. 2 IgE immunoblot inhibition with *A. retroflexus* pollen extract. Lane MW, low MW (Amersham); Lane 1, *A. retroflexus* protein strip probed with pooled sera from patients' number 1, 5, and 6, with reactivity to 14.6 kDa components, without inhibitor; Lane 2, *A. retroflexus* protein strips incubated with pooled sera containing 10 µg of purified rChe 2 as inhibitor; Lane 3, *A. retroflexus* protein strips incubated with pooled sera containing 3.3 µg of purified rCuc m 2 as inhibitor; Lane 4, *A. retroflexus* protein strips incubated with pooled sera containing 280 µg of *A. retroflexus* pollen extract as inhibitor; Lane 5, *A. retroflexus* protein strip probed with 1%BSA, without inhibitor.

2 and rCuc m 2 were able to inhibit 87.1% and 93.2% of the IgE-binding band (14.6 kDa) detected in *A. retroflexus* pollen extract, respectively.

cDNA AMPLIFICATION AND SEQUENCE ANALYSIS OF Ama r 2

cDNA amplification of Ama r 2 using degenerate primers resulted in a single 399 bp fragment which was cloned into pTZ57R/T. Two of these clones were sequenced. Sequence analysis revealed that the PCR product correspond to a 399 bp open reading frame (GeneBank accession number FJ899746) which encodes Ama r 2, a 133 amino acid peptide belonging to the family of profilins. It had an average MW of 14.6 kDa and a theoretical isoelectric point value of 4.32. It was also submitted to WHO/IUIS Allergen Nomenclature Sub-Committee.

Amino acid sequence of Ama r 2 was compared with those of profilins from other plants. The selected plant-derived profilins were Ama v 1 (*Amaranthus viridis*), Sal k 4 (*S. kali*), Hev b 8 (*Hevea brasiliensis*), Cuc m 2 (*C. melo*), Che a 2 (*C. album*), and Cro s 2 (*C. sativus*). High levels of sequence identity and similarity (87% and 93%, respectively) were detected between the Ama r 2 and the Sal k 4. The most conserved residues showed involvement in specific bio-

logical functions and structural roles (Fig. 3).

EXPRESSION AND PURIFICATION OF rAma r 2

The Ama r 2 was expressed in *E. coli* BL21 (DE3) as a fusion protein with a His₆-tag to permit affinity purification on a Ni²⁺-charged resin. The target protein containing His₆-tag in the C-terminus was present in a soluble form in the supernatant, where it was further purified by Ni²⁺ affinity chromatography to yield purified, homogenous protein.

The purified rAma r 2 was quantified using the Bradford's protein assay which showed that approximately 2 mg of recombinant protein had been purified from one liter of the bacterial expression medium. SDS-PAGE (Fig. 3) showed that the MW of the expressed product was about 18 to 19 kDa, which confirmed that the recombinant protein was expressed as a fusion protein tagged with 6 histidine residues.

IgE REACTIVITY OF rAma r 2

Using an indirect ELISA, the specific IgE levels to the purified rAma r 2 were determined with twelve individual patients' sera (Table 1). All except one patient (No. 12) showed significantly elevated specific IgE levels to *A. retroflexus* pollen extract and to purified rAma r 2 (Table 1).

A pooled serum from three patients allergic to *A. retroflexus* pollen (patients 1, 5 and 6) was tested by immunoblotting assays for IgE reactivity to rAma r 2 and showed a reactive band with the MW of 18 to 19 kDa (Fig. 4, Lane 1).

Inhibition experiments demonstrated that preincubation of serum samples with rAma r 2, rChe a 2 or rSal k 4, completely inhibited IgE binding to rAma r 2 (Fig. 4, Lanes 2-4).

The results of SPTs with rAma r 2 on two volunteer patients confirmed the IgE reactivity of rAma r 2. rAma r 2 was used at concentrations of 0.4 and 0.2 mg/ml which elicited wheals with the mean diameters of 6 and 3 mm, respectively (Fig. 5).

DISCUSSION

In this study, the characterization, cloning and expression of the first allergen of the *A. retroflexus* pollen is reported. This allergen was shown to be a part of the profilin family and was designated as Ama r 2. Previously, there were several reports of the allergenicity of profilins in pollens of the Amaranthaceae/Chenopodiaceae family. In 2003, Che a 2 was isolated as a significant protein allergen from the *C. album* pollen extract.¹⁶ It was also indicated that this protein displayed structural and functional properties similar to those of profilins. In 2007, Ama v 1 was reported as an allergen of the *A. viridis* pollen. It showed a high similarity to the profilin family (unpublished data). In 2010, an allergenic profilin molecule from the *S. kali* pollen was identified and designed Sal k 4.¹⁷ Concur-

	10	20	30	40	50	60	70	80	
<i>Ama r 2</i>	MSWQAYVDDH	LMCEIEGTTN	HLTGAAILGL	DGSVWAQSAD	FPQFKPDEIA	AIVEDFDEPG	TLAPTGLHLG	GTKYMVIQGE	80
<i>Ama v 1</i>		...D.SE.	R.SA...I.V	S	...E...K..ED..	Y..			80
<i>Sal k 4</i>	...T.....	...N.....	...A...V	N	...S.V.KE...A.				80
<i>Hev b 8</i>	...T.....	...D.D.--H	R..A...I.H	SS	...S..V..VMK...S.				78
<i>Cuc m 2</i>	...V...E.	...--	...S...I.Q	...QN	...L..E.V. G..G..AD..	YI.			78
<i>Che a 2</i>	...T.....	...FP..E.G.	...A...V	ST	...L.QE.VK	...CNE..V.N	...F..E.		80
<i>Cro s 2</i>	...T...E.	...DMD.--H	V..S...H	G	...EL..A..T	...LN..N...S....	MYIN	A.....	78
	90	100	110	120	130	%Identity %Similarity			
<i>Ama r 2</i>	PGAVIRGKKG	AGGICVKKTG	QALVMGIYDE	PVTPGQCNCMI	VERLGDYIE	QGY	133		
<i>Ama v 1</i>	P.....	N	I		V..M		133	87	94
<i>Sal k 4</i>	A.Q.....	P.....	...IF.....		V..M		133	87	93
<i>Hev b 8</i>	S..T.....	II	...L.....	LD	...L		131	81	90
<i>Cuc m 2</i>	P..AT.....	M..I.....	...M.....	D	...L		131	81	89
<i>Che a 2</i>	P..V.I...N	...F...N	V	...K...V	...M		133	77	85
<i>Cro s 2</i>	...V.....	S..VTI..SN	M..IF..L...	...M.....LV			131	70	85

Fig. 3 Comparison of the *Ama r 2* amino acid sequence with allergenic profilins from other plants. The amino acid sequence identity and the similarity of *Ama r 2* (ACP43298) with other members of the profilin family are indicated at the end of each amino acid sequence. *A. viridis* (*Ama v 1*, ABW37744), *S. kali* (*Sal k 4*, GQ145223), *H. brasiliensis* (*Hev b 8*, CAA75312), *C. melo* (*Cuc m 2*, AAW69549), *C. album* (*Che a 2*, ACR77509), and *C. 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) are shaded in gray.

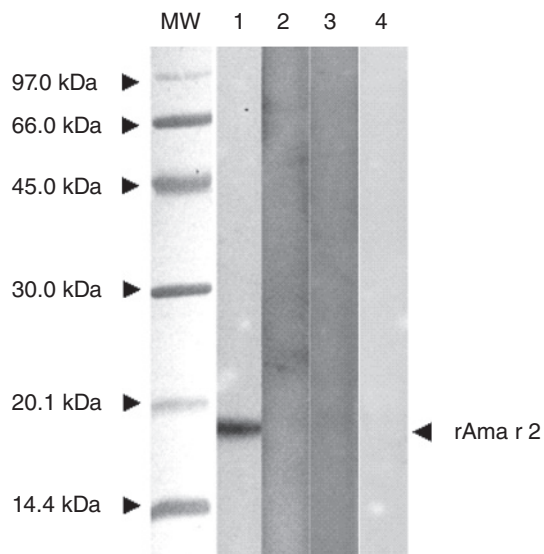


Fig. 4 Immunoblotting inhibition. Lane MW, low MW (Amersham); Lane 1, rAma r 2 protein strip incubated with pooled serum without inhibitor; Lane 2, rAma r 2 protein strip incubated with pooled serum containing 5 µg of rChe a 2 as inhibitor; Lane 3, rAma r 2 protein strip incubated with pooled serum containing 5 µg of rAma r 2 as inhibitor; Lane 4, rAma r 2 protein strip incubated with pooled serum containing 5 µg of rSal k 4 as inhibitor.

rent with these studies, one of our investigations reported an IgE binding protein with an estimated MW of 15 kDa which was detected by immunoblotting of the three related pollen extracts including *A. retroflexus*, *C. album* and *S. kali*. It was also suggested



Fig. 5 The results of SPTs with rAma r 2 on a volunteer allergic patient. 1) Histamine, 2) Glycerin, 3) *A. retroflexus* pollen extract (Hollister-Stier Laboratories LLC, Spokane, WA, USA), 4) rAma r 2 (0.4 mg/ml), 5) rAma r 2 (0.2 mg/ml).

that this protein band is a common minor allergen of the three tested pollens.⁷

In this study, the apparent MW of *Ama r 2* was 14.6 kDa. Via immunoblotting, this protein was recognized in about one third of patients allergic to the *A. retroflexus* pollen extract. So far, different MWs for profilins from different plant sources have been reported, such as 15.0 kDa in celery,¹⁸ 14.4 kDa in date palm pollen,¹⁹ and 14.0 kDa in oranges.²⁰ These discrepancies could be explained by diversities in a few amino acid residues or variations in the methods of measuring MWs.

Immunoblot inhibition studies showed cross-reactivities between *Ama r 2* and other plant profilins, including *Che a 2*, *Sal k 4*, and *Cuc m 2* (Figs. 2, 4). In addition, amino acid homology indicated a close rela-

tionship (over 70% sequence identity) between Ama r 2 and other tested profilins from pollens and fruits (Fig. 3). These results were concomitant with a previous study of ours which suggested profilin as a common minor allergen among members of the Amaranthaceae family.⁷ In addition, several previous studies demonstrated extensive IgE cross-reactivities among profilins from different plants.^{21,22}

According to the results of immunoblotting inhibition assays, both rChe a 2 and rCuc m 2 inhibited the IgE reactivity of the natural Ama r 2 (Fig. 2). Considering that lower amounts of rCuc m 2 produced a more intensive inhibition than higher amounts of rChe a 2, it was suggested that IgE cross-reactivity between Ama r 2 and Cuc m 2 exceeded that between Ama r 2 and Che a 2. These results are in contrast to the phylogeny of these plants, since both *A. retroflexus* and *C. album* are from the Amaranthaceae family while *C. melo* is a fruit which is related to another division of plants. Furthermore, the amino acid sequence homology of profilins from different plants showed higher identities and similarities between Ama r 2 and Cuc m 2 than those of Ama r 2 and Che a 2 (Fig. 3). Therefore, it appears that, although Ama r 2 and Cuc m 2 belong to unrelated plants, their IgE binding epitopes are very similar. Concomitant with these results, other studies have reported IgE cross-reactivities of food allergens and pollens,²³ the so-called pollen-food allergy syndrome, in which there are common IgE epitopes in pollens and foods.²⁴ Especially, profilin molecules were indicated to be resistant to gastric digestion and so producers of IgE cross-reactive responses.²⁵

Finally, rAma r 2 was successfully expressed in *E. coli* as a soluble molecule. During the process of rAma r 2 expression and after induction with IPTG, the temperature of the culture medium was lowered to 18°C to obtain a high amount of the soluble form of the protein. This was also showed by our previous studies.^{17,26}

The recombinant Ama r 2 showed IgE reactivity to patients' sera via ELISA and immunoblotting, which also confirmed the correct folding of the molecule. The results of SPTs with the recombinant protein revealed an *in vivo* IgE reactivity as well. Taken together, it seems that rAma r 2 preserves conformational IgE binding epitopes similar to those of its natural counterpart in *A. retroflexus* pollen extract.

During the last decade, purified native or recombinant allergens have shown promise as a safe and reliable diagnosis and therapy of allergic disorders.²⁷ For this propose, the identification of relevant allergens using appropriate techniques is a crucial step.²⁷

In conclusion, the first allergen from the *A. retroflexus* pollen was identified as belonging to the family of profilins and designated as Ama r 2. This allergen was successfully expressed in *E. coli* as a soluble recombinant protein (rAma r 2). It was also shown to

be IgE cross-reactive with several profilin molecules from other plants. The study of the tertiary structure may be helpful in identifying cross-reactive epitopes.

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