

Profilin (Che a 2) and polcalcin (Che a 3) are relevant allergens of *Chenopodium album* pollen: Isolation, amino acid sequences, and immunologic properties

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Background: Little is known about the molecular properties of chenopod allergens. Recently, profilin and 2 EF-hand calcium-binding protein (polcalcin) have been shown to play a role in chenopod pollinosis.

Objective: We sought to analyze these panallergens in chenopod pollen and to evaluate their involvement in the allergy to this biologic source.

Methods: Profilin and polcalcin were purified to homogeneity and characterized by using spectrometric and chemical methods. Immunologic analyses were performed by means of immunoblotting, ELISA, and competitive inhibition assays with olive profilin— and polcalcin-specific rabbit polyclonal antibodies and sera from patients with chenopod allergy. cDNAs encoding these proteins were cloned by means of PCR and sequenced.

Results: Purified Che a 2 (profilin) and Che a 3 (polcalcin) exhibited prevalences of 55% and 46%, respectively, in patients (n = 104) hypersensitive to chenopod pollen. Both purified allergens individually inhibited the IgE binding to the whole pollen extract and showed strong cross-reactivity with the corresponding olive pollen profilin (Ole e 2) and polcalcin (Ole e 3). Chenopod profilin consists of a 131-amino-acid chain that displays identities of approximately 75% and 82% with pollen and food profilins, respectively. Che a 3 (86 amino acids) displays similarity (65% to 82% identity) with polcalcins from pollens of olive, birch, alder, rapeseed, and timothy.

Conclusion: Profilin and polcalcin are relevant panallergens in chenopod pollen and good candidates to be involved in IgE cross-reactivity with other pollen sources, thus explaining the highly frequent polysensitization of patients allergic to chenopod. (J Allergy Clin Immunol 2004;113:1192-8.)

Key words: *Chenopod* allergy, pollinosis, profilin, polcalcin, 2 EF-hand calcium-binding allergen, cross-reactivity, amino acid sequence, panallergen, *Chenopodium album*, *Chenopodiaceae*

Abbreviation used

pI: Isoelectric point

Chenopod (*Chenopodium album*, commonly lamb's quarter) is a perennial plant of the Chenopodiaceae family that has been reported as responsible for hay fever in dried areas.^{1,2} Many members of this family, as well as from the closely related Amarantaceae family, have been involved in inducing allergic diseases.³ Both Chenopodiaceae and Amarantaceae weeds shed locally large amounts of pollen, being common in large areas of the United States, southern Canada, and central and southern Europe. The clinical incidence of chenopod pollinosis has been increasing mainly in desert areas and arid environments, where this weed is well adapted.⁴ Pollution derived from oil industries, sandstorms, or greening programs are among factors contributing to the rapidly increased prevalence of pollinosis in arid and salt-rich soils.⁵

The characterization of sensitizing allergens from *C. album* has received little attention, probably because of its concomitance with other pollinosis. Allergens of 14 and 35 kd were detected by Lombardero et al,⁶ and protein bands of 15, 25, and 55 kd were detected by Würtzen et al.⁷ In addition, our group reported the isolation, cloning, and sequencing of Che a 1 (17 kd), which has been characterized as a major allergen within this pollen.⁸ Most of the studies on chenopod pollen allergy have shown a high degree of cross-reactivity with amaranth and other less taxonomically related pollens,^{6,7,9,10} as well as some foods, such as garlic, onion, and asparagus.¹¹ IgE cross-reactivity mainly arises from the presence of panallergens, with the role of profilin and polcalcin in pollen-pollen cross-sensitization being relevant.¹²⁻¹⁴ These families of proteins possess highly conserved amino acid sequences, thus explaining their involvement in cross-reactivity.¹³⁻¹⁶ Recently, we have detected a role for profilin and polcalcin in allergy to chenopod pollen.¹⁷

Accurate diagnosis for emerging allergenic pollen sources that share allergens with taxonomically related or nonrelated (and better known) pollens might help to simplify specific immunotherapy procedures. Therefore we have isolated and sequenced the profilin and the polcalcin from chenopod pollen and evaluated their clinical importance. Cross-reactivity with olive pan-allergens is also studied.

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METHODS

Human sera and polyclonal antisera

Sera from 104 untreated donors (all displaying a well-documented clinical history of allergy to *C. album* pollen and without immunotherapy) with rhinitis, rhinoconjunctivitis, or both and serum specific IgE levels (CAP class 3-6) and positive skin prick test responses to chenopod pollen extract were used. A pool of 3 sera from nonatopic individuals was used as a negative control.

Polyclonal antisera against profilin (Ole e 2) and polcalcin (Ole e 3) from olive pollen were prepared by immunizing New Zealand rabbits with the olive allergens.^{18,19}

Pollen extract and protein purification

Pollen from chenopod was purchased from ALK-Abelló (Madrid, Spain). Pollen extract was prepared by means of saline extraction.⁸ Protein concentration was determined according to the method of Lowry et al.²⁰

Lyophilized extract from chenopod pollen was dissolved in 0.2 mol/L ammonium bicarbonate, pH 8.0, and fractionated into a gel filtration Sephadex G-75 (Pharmacia, Uppsala, Sweden) column equilibrated in the same buffer. Fractions containing proteins of approximately 15 and 10 kd were separately pooled. The pool of 15 kd, in which profilin elutes, was chromatographed in an affinity column of poly-L-proline, as previously described,¹⁸ and eluted with 20 mmol/L Tris-HCl, pH 7.6, containing 150 mmol/L NaCl, 0.5 mmol/L ATP, and 6 mol/L urea. The eluted material was dialyzed against 20 mmol/L ammonium bicarbonate at 4°C and lyophilized. Afterward, the sample was chromatographed in a Superdex 75 column equilibrated and eluted with 20 mmol/L ammonium bicarbonate, pH 8.0. The pool of 10 kd from the Sephadex G-75 column was purified in a reverse-phase HPLC C₁₈ μ -bondapak column eluted with an acetonitrile gradient of 0% to 60% in 0.1% (vol/vol) trifluoroacetic acid. Different fractions of the chromatographic steps were analyzed by means of SDS-PAGE and immunoblotting by using specific polyclonal antibodies obtained against profilin and polcalcin from olive pollen.

Molecular characterization

Protein concentration and amino acid composition of purified proteins were determined by means of acid hydrolysis.⁸ SDS-PAGE was performed with 15% (wt/vol) polyacrylamide gels.²¹ Proteins were visualized with Coomassie Brilliant blue R-250 or AgNO₃. Molecular mass determinations were done with protein markers (MW-SDS-70L; Sigma-Aldrich, Química, Spain). N-terminal amino acid sequence was performed by means of Edman degradation on a 494 Applied Biosystems sequencer (Applied Biosystems, Foster City, Calif). Mass spectrometry analyses of proteins (1 μ g) were performed in a Bruker Reflex II MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany).⁸

Immunologic assays

Proteins separated by means of SDS-PAGE were transferred to nitrocellulose membranes and immunodetected with a pool of sera (n = 5) from patients allergic to chenopod pollen (diluted 1:10) or olive profilin-specific polyclonal antiserum (1:10,000 diluted) or olive polcalcin-specific polyclonal antiserum (1:5000 diluted).⁸ A mouse monoclonal anti-human IgE antibody (1:5000 diluted), kindly donated by ALK-Abelló (Madrid, Spain), and a horseradish peroxidase-labeled goat anti-mouse IgG (1:5000 diluted; Pierce Chemical Co, Rockford, Ill) were used to detect bound IgE antibodies. Alternatively, for the detection of chenopod protein-specific IgG binding, a horseradish peroxidase-labeled goat anti-rabbit IgG (1:3000 diluted; BioRad, Richmond, Calif) was

used as a second antibody. The signal was developed by the ECL Western blotting reagent (Amersham Biosciences, Barcelona, Spain).

Immunoblotting inhibition experiments were performed as previously described¹⁷ by using a pool of 5 allergic sera (1:10 diluted), which were previously incubated with 2 μ g of profilin or polcalcin from olive pollen, the same proteins from chenopod pollen, as a positive control of inhibition or BSA as a negative control of inhibition. Percentage of inhibition was determined by means of densitometric analysis.

Quantification of IgE levels in the sera from allergic patients was performed by means of ELISA.⁸ Plates were coated with purified protein and incubated with individual allergic sera (1:10 diluted) for 2 hours. Binding of IgE was detected by means of the antibodies described above. Peroxidase reaction was developed with *o*-phenylenediamine reagent.⁸ OD was read at 492 nm. All the determinations were carried out as duplicates. OD values of less than 0.1 were considered negative responses.

ELISA inhibition experiments were performed as previously described.⁸ Briefly, plate wells coated with 20 μ g of chenopod crude extract were incubated with a pool of 12 sera of patients allergic to chenopod (1:10 diluted) preabsorbed for 2 hours with different amounts of the purified proteins as inhibitors. Bound IgE antibodies were detected as mentioned above. The percentage of inhibition was determined according to the following formula:

$$\text{Percentage inhibition of IgE binding} = \\ (1 - \text{OD}_{492\text{nm}} \text{ with inhibitor} / \text{OD}_{492\text{nm}} \\ \text{without inhibitor}) \times 100.$$

Each value was obtained as mean of 2 determinations.

PCR-based cloning strategy and nucleotide sequence determination

Total RNA from chenopod pollen was obtained as previously described.²² First-strand cDNA was synthesized from RNA by using the SMART RACE cDNA amplification kit (BD Biosciences-Clontech, Madrid, Spain).

Profilin from chenopod was cloned by using 2 degenerate oligonucleotides designed with the sequences of homologues: 5'-TCNTGGCARACNTAYGT, corresponding to the N-terminal of the polypeptide chain, and the antisense primer, 5'-TTAGAGNCCYTGYTCHATNAG, to the C-terminal end.

Polcalcin from chenopod was cloned by using the internal amino acid sequence MMAEIDTD. The PCR product with this degenerate antisense oligonucleotide OL3-1 (5'-TACTACCGRCTCTARTRTGGCT) and a nonspecific primer UPM included in the kit were the extreme 5' end of the encoding sequence. The use of the primer corresponding to this extreme NCH3.1 (5'-ATGGCTGCTGAGG-ATACACCT) and the same nonspecific primer allowed us to obtain the complete sequence of the allergen. Finally, the use of the primers corresponding to both ends of the protein, NCH3.1 and NCH3.2 (5'-CTAGAAGATCTTGGAACATC), rendered the full-length encoding sequence. The obtained cDNA fragments were purified and cloned into the pCR2.1 vector included in the TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). The nucleotide sequences were obtained as previously described.²² Homology searches were performed by using the BLAST network. Multiple sequence alignment was performed with the CLUSTAL W program.

RESULTS

Detection of profilin and polcalcin in *C. album* pollen extract

Chenopod-specific IgE reactivity was measured for 104 sera from patients with positive skin prick test responses to

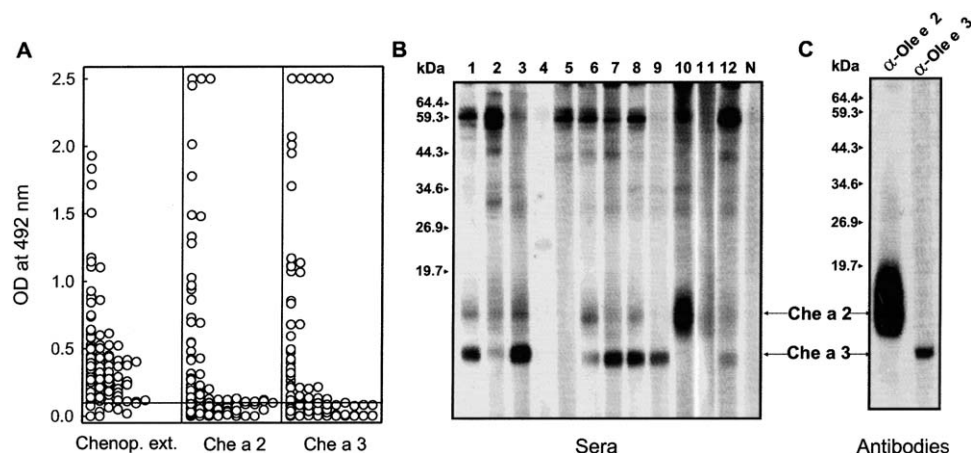


FIG 1. Antigenic reactivity of chenopod pollen. **A**, Chenopod-specific IgE levels and antigen-specific IgE values for 104 sera from patients with chenopod allergy. **B**, Analysis of IgE reactivity for 12 of these sera and for a pool of nonallergic sera (lane N). **C**, Analysis of IgG reactivity against Ole e 2 (olive profilin)— and Ole e 3 (olive polcalcin)—specific polyclonal antisera (lanes α -Ole e 2 and α -Ole e 3, respectively).

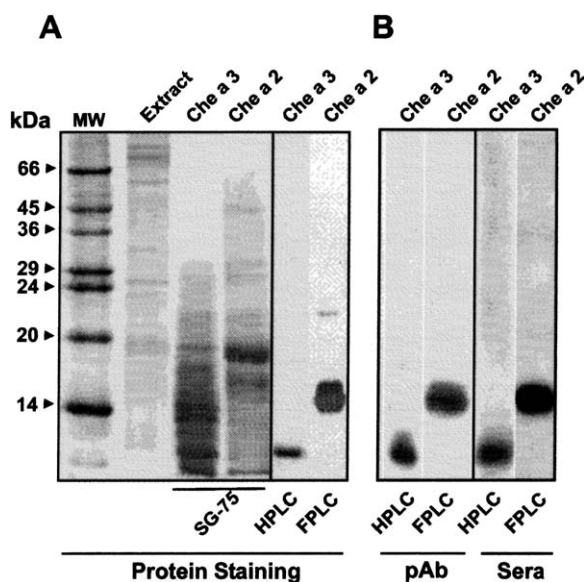


FIG 2. SDS-PAGE analysis of purification steps of Che a 2 and Che a 3. **A**, Protein staining with AgNO_3 . **B**, Immunoblotting of proteins assayed with polcalcin- and profilin-specific polyclonal antisera (lanes pAb) or with a pool of sera from patients with chenopod allergy (lanes Sera).

chenopod pollen extract (Fig 1, A); range and mean were 0.00 to 1.97 and 0.36, respectively. Twelve of these sera were assayed for their IgE-binding capability to chenopod pollen in immunoblotting after separation in SDS-PAGE (Fig 1, B). Most of the sera recognized bands of 10 to 15 kd, thus suggesting a possible role for profilin and polcalcin in the sensitization of these patients. The presence of these proteins was confirmed by using 2 specific antisera obtained in rabbit against olive profilin (Ole e 2) or olive polcalcin (Ole e 3; Fig 1, C).

Isolation and characterization of chenopod profilin

Three chromatographic steps were carried out to obtain pure profilin: a gel filtration chromatography in a Sephadex G-75 column, a poly-L-proline phenyl-Sepharose column, and finally a Superdex 75 column. The yield was about 30 μg of purified protein per gram of dried pollen. Fig 2 shows the silver staining of the protein fraction obtained from the Sephadex G-75 column and after the Superdex 75 column in comparison with the whole extract. The immune responses of purified chenopod profilin to olive profilin-specific antiserum and to sera from patients with chenopod allergy are also given in Fig 2. The purified allergen was called Che a 2 and displayed an apparent molecular mass of 12.6 to 15.2 kd in SDS-PAGE (Fig 2), which suggests the existence of molecular polymorphism. Mass spectrometry analysis of Che a 2 was unsuccessful, and Edman degradation rendered no amino acid, indicating the blockage of the N-terminal end of the protein.

Isolation and characterization of chenopod polcalcin

Polcalcin from chenopod was purified through 2 chromatographic steps: the former was common to obtain profilin and polcalcin (Sephadex G-75), but the pool of eluted fractions selected for the next step was different for isolating each protein. Afterward, a single HPLC step rendered each protein. Afterward, a single HPLC step rendered polcalcin with a yield of 60 μg per gram of dried pollen. SDS-PAGE analyses of the samples are given in Fig 2, in which IgG- and IgE-binding capabilities of the purified protein against anti-olive polcalcin rabbit antiserum and human sera from patients with chenopod allergy are also shown. The positive response allowed us to consider the isolated allergen as polcalcin, which was called Che a 3. Chenopod polcalcin showed a 9.8-kd molecular mass by means of SDS-PAGE (Fig 2) and 2 peaks of 9383 d and 9467 d by means of mass spectrometry (data not shown). Edman degradation was

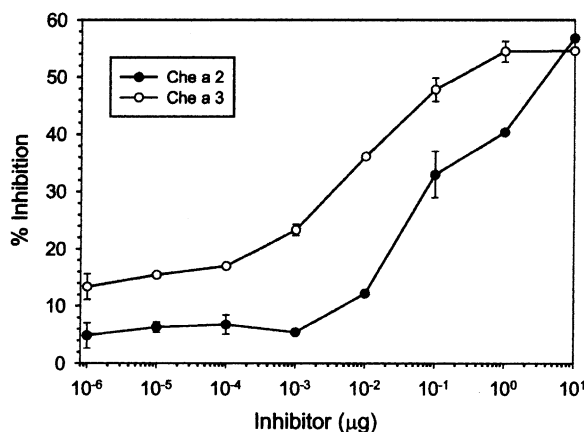


FIG 3. Inhibition of the IgE binding of sera from patients with chenopod allergy to the whole pollen extract by the purified Che a 2 and Che a 3.

unsuccessful, indicating the blockage of the N-terminal end of the protein.

Significance of Che a 2 and Che a 3 in the IgE response to the chenopod pollen extract

Quantification of the IgE response of the 104 sera from patients with chenopod allergy was performed by using ELISA against purified Che a 2 and Che a 3. A pool of sera from nonatopic individuals was used as a negative control, and chenopod pollen extract-coated wells were used as positive controls. Fifty-seven (55%) sera showed positive response to Che a 2, and 48 (46%) sera showed positive responses to Che a 3 (Fig. 1, A); range and mean values for Che a 2-specific IgE-binding values were 0.11 to 2.5 and 0.72, respectively, and those for Che a 3-specific IgE levels were 0.12 to 2.5 and 0.84, respectively. Therefore Che a 2 can be considered as a major allergen of chenopod pollen.

ELISA inhibition experiments with purified Che a 2 and Che a 3 as inhibitors of the IgE binding to the whole extract were performed with an equivolumetric pool of the 12 sera from patients with chenopod allergy. Fig 3 shows that both allergens, Che a 2 and Che a 3, were able to inhibit approximately 50% of the IgE binding to the protein extract, indicating that they are relevant allergens from chenopod pollen.

Immunoblotting inhibition assays of the IgE binding to Che a 2 and Che a 3 were performed with an equivolumetric pool of 5 sera and with Ole e 2 (olive profilin) and Ole e 3 (olive polcalcin) as inhibitors, respectively (Fig 4). BSA was used as a negative control of inhibition. Ole e 2 was able to inhibit 81% (determined by means of densitometric analysis) of the IgE binding to Che a 2, and Ole e 3 reached 100% inhibition of the binding to Che a 3.

cDNA cloning and nucleotide sequencing of Che a 2 and Che a 3

Total RNA was obtained from chenopod pollen, and the single-stranded cDNA was synthesized. This cDNA was used as a template to amplify, by means of PCR, a DNA

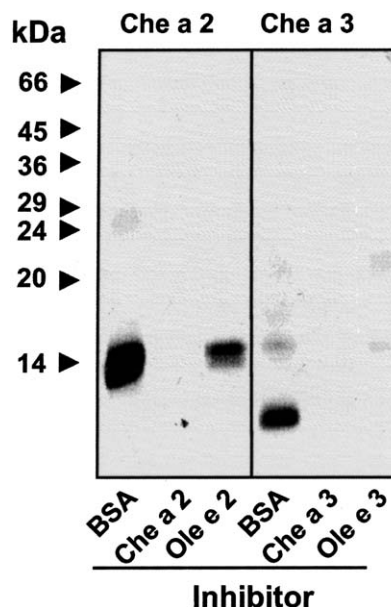


FIG 4. Cross-reactivity between chenopod and olive profilins and chenopod and olive polcalcins analyzed by means of immunoblotting. Strips with purified allergens (left panel, Che a 2; right panel, Che a 3) were incubated with a pool of allergic sera preabsorbed with BSA or the corresponding panallergen from chenopod and olive as inhibitors.

fragment encoding Che a 2, which resulted in 417 bp. Fig 5 shows the nucleotide and deduced amino acid sequences obtained for Che a 2. The polypeptide sequence displays an open-reading frame of 131 residues, a calculated molecular mass of 14,049.1 d, and a theoretic isoelectric point (pI) of 5.19. Some degree of polymorphism was detected in Che a 2; 3 different isoforms are shown in Fig 5. The comparison of Che a 2 amino acid sequence with those from profilins present in database banks confirmed that Che a 2 belongs to this family of proteins (Fig 5). Identities between Che a 2 and other profilins range between 75% for Bet v 2 from birch and 83% for Mal d 4 from apple.

The single-stranded cDNA preparation obtained from chenopod pollen was used to amplify, by means of PCR, a gene encoding Che a 3. In this case the band obtained was 275 bp in size. The nucleotide sequence of this fragment (Fig 6) showed an open-reading frame for 86 amino acid residues with a calculated molecular mass of 9551.5 d (pI of 4.43). Very low polymorphism was detected in Che a 3 because only position 61 was found to be shared by Gly and Asp in different clones. The polypeptide sequence comprises 2 segments that display the consensus 12-amino-acid residues of calcium-binding sites of EF-hand type. Comparison with amino acid sequences of polcalcins from the protein databases resulted in similarities ranging from 65% for the protein of *Brassica napus* to 82% for Aln g 4 (Fig 6).

DISCUSSION

Profilin and polcalcin were hypothesized to have significant roles in allergy to chenopod pollen in a recent

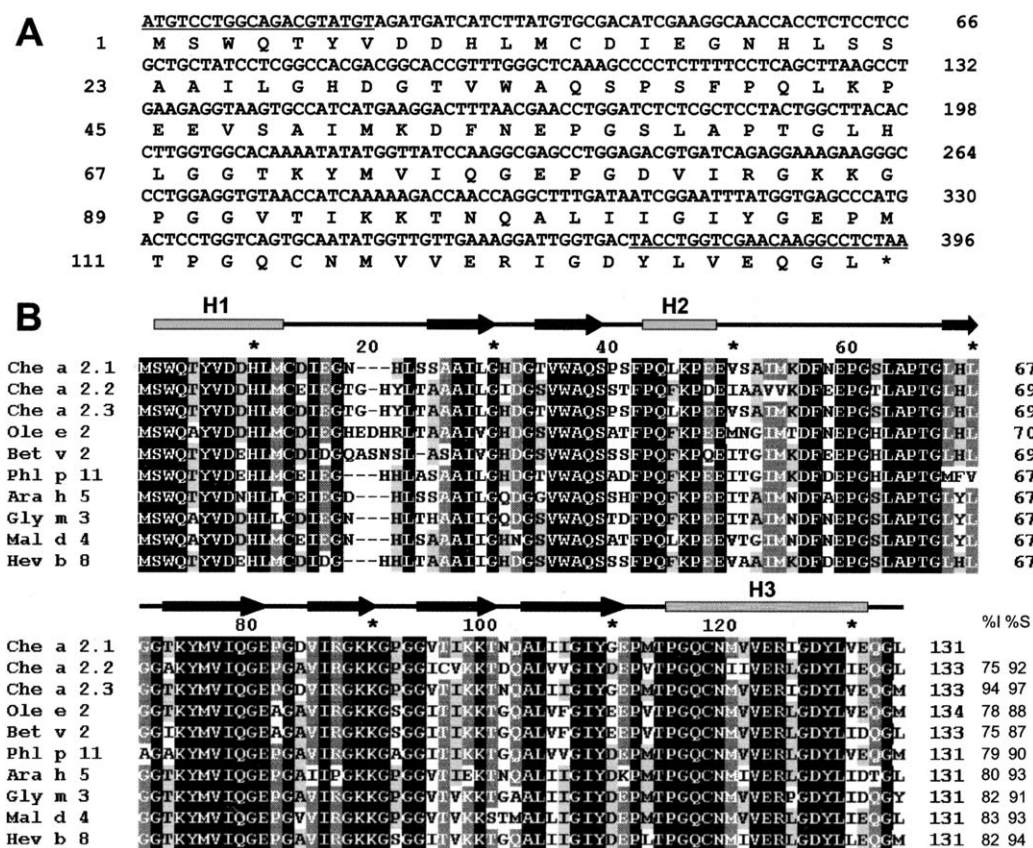


FIG 5. Nucleotide sequence of a cDNA encoding Che a 2 and its deduced amino acid sequence (A): the primers used in the cloning are underlined. Comparison of amino acid sequence of Che a 2 with allergenic profilins from other plant sources (B): percentage of identity (%I) and similarity (%S) are referred to the Che a 2.1 clone, and secondary structure of birch profilin²³ is indicated. H, α -Helix; black arrow, β -sheet.

report from our group.¹⁷ We have now isolated 2 significant allergens from chenopod pollen extract, which have been called Che a 2 and Che a 3. These 2 proteins display structural and functional properties similar to those of profilins and polcalcins, respectively. Profilins consist of a single polypeptide chain of 125 to 153 amino acid residues. Interestingly, Che a 2 shows higher identity to profilin from latex and foods than to profilins from pollens. The alignment of Che a 2 with other profilins involved one gap at position 19 (Fig 5). This polypeptide region is the most variable in length and sequence of vegetable profilins; it corresponds to a significant loop in the 3-dimensional structure and is comprised within the IgE epitope reported.²³ The amino acid sequence of Che a 2 also shows 4 residues at positions 51, 84, 110, and 115 that are not present in other profilins, the 2 latter located in the IgE epitope of the C-terminal portion of birch profilin.²³ These differences could have a role in the degree of cross-reactivity of Che a 2 with other profilins.

Che a 2 displays capability to bind to poly-L-proline affinity columns, a property attributed to actin-binding proteins, and was recognized by IgG antibodies raised in rabbit against olive profilin. Taking into account the

apparent molecular mass of Che a 2, it probably corresponds to allergens previously found,^{6,7} thus supporting its clinical importance in different chenopod-hypersensitive populations. Che a 2 showed high prevalence and cross-reactivity with olive profilin. Since the first studies of pollen profilins, prevalence values of approximately 20% were found in patients allergic to birch, timothy, and artemisia.¹² This result was further confirmed in populations from Central Europe allergic to Gramineae or Betulaceae.^{24,25} Higher values have been reported for profilin from olive (24% to 27%),¹⁸ sunflower (30%),²⁶ and mugwort (36%).²⁷ However, profilin has been found as the major allergen (ie, affecting >50% of allergic patients) in pollen from *Mercurialis annua*²⁸ and saffron.²⁹ Especially interesting is the great relevance of profilin in pollen-food cross-sensitization.¹³ Patients hypersensitive to pollen from Gramineae or Oleaceae and fruits, mainly from the Rosaceae family, showed notably increased positive prevalence (75%)³⁰ to profilin. On the other hand, reactivity to this allergen seems dependent on geographic location because the northern European population is less sensitive than that from warmer areas.³¹ Thus profilin would be a feasible allergen in

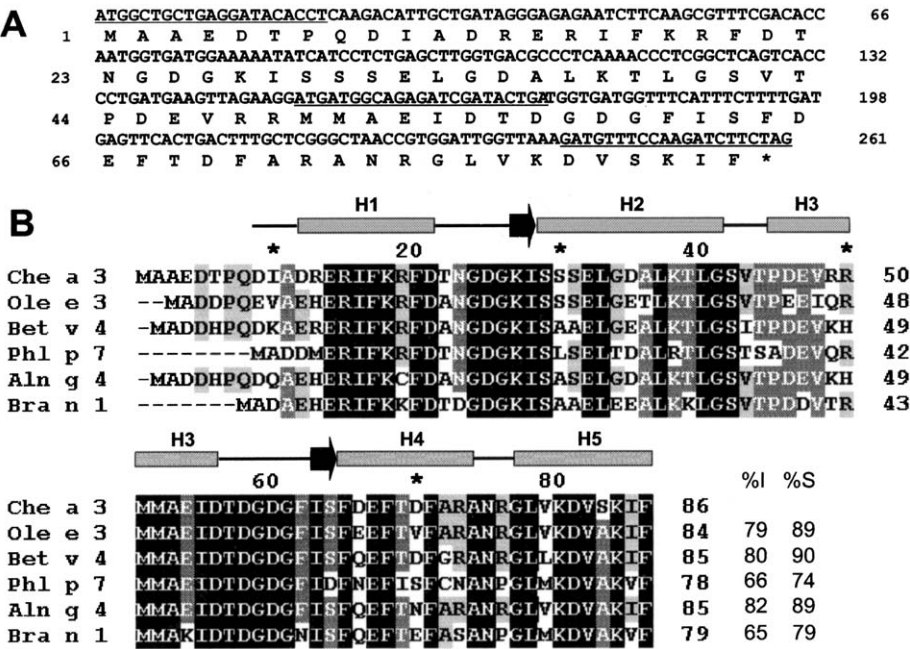


FIG 6. Nucleotide sequence of a cDNA encoding Che a 3 and its deduced amino acid sequence (**A**): 3 primers used are underlined. Comparison of amino acid sequence of Che a 3 with other allergenic polcalcins (**B**): percentage of identity (%I) and similarity (%S) are referred to Che a 3, and secondary structure of timothy polcalcin is indicated.³² H, α -Helix; black arrow, β -sheet.

chenopod because it is a common weed in dry areas. Both the high IgE-binding prevalence of Che a 2 (55%) and the strong IgE cross-reactivity shown with olive profilin suggest that chenopod profilin can be an important mediator of cross-reactivity between pollens.

We also report the isolation and sequence of Che a 3, which belongs to the pollen protein family of polcalcins.¹⁴ Che a 3 displays molecular and immunologic properties similar to those of other members of the family: approximately 9.5 kd of molecular mass and acidic pI and very high content in the α -helical secondary structure (data not shown). IgE- and IgG-binding properties were similar to those of its homologue from olive pollen because (1) olive polcalcin-specific antibody was used to detect chenopod polcalcin and (2) high IgE cross-reactivity was found between olive and chenopod polcalcins. Although the polymorphism found in Che a 3 was very low, it could be responsible for the microheterogeneity observed by means of mass spectrometry. The 3-dimensional structure of Phl p 7 has been recently determined by means of x-ray crystallography analysis, revealing that its polypeptide chain adopts an extended conformation and an intertwined dimer organization.³² The studies on the 3-dimensional structure of Che a 3, which are in progress, could confirm such a polymeric assembly as a general disposition for the monomers of this family of calcium-binding proteins.

The members of this protein family have been identified as potent allergens. However, their prevalence is approximately 20% to 35% in patients with pollinosis. Aln g 4 reacted with serum from 18% of patients with pollen allergy (n = 122).³³ Ole e 3 was assayed with sera from

different populations of patients with olive allergy: the allergen reacted with 17% (n = 195)³⁴ and 18.5% (n = 270) of patients.¹⁹ In addition, a population of 119 sera was assayed in ELISAs and prick tests, and prevalences of 31.9% and 37.8% were found, respectively.³⁵ In this context, Che a 3 is an atypical polcalcin because it showed reactivity to 46% of sera from individuals with chenopod allergy. The high prevalence of this panallergen in these patients might have a relationship with their characteristic polysensitization.

High cross-reactivity has been demonstrated for the polcalcin from alder, Aln g 4, with a notable number of pollen extracts.³³ In that study *C album* was shown as one of the most potent inhibitors of the IgE binding to Aln g 4. Ole e 3 was also shown as a highly cross-reactive allergen to different pollen extracts.^{19,36} In the present work we obtained the complete abolition of the IgE binding of allergic sera to Che a 3 with Ole e 3 as an inhibitor. These data indicate that Che a 3 shares most of the IgE epitopes with the olive polcalcin and suggest an important role for this allergen in the cross-reactivity among pollens.

Recently, the isolation and characterization of Che a 1 was reported.⁸ Although this allergen showed high prevalence, Che a 1-specific IgE levels of individual patients were low. The present work suggests that Che a 2- and Che a 3-specific IgEs are the major contributor antibodies to the whole allergenicity of chenopod proteins.

The fact that panallergens share structural and immunologic properties has allowed us to suggest the use of representative members of such families that carry most of the significant cross-reactive epitopes as marker allergens

of polysensitization for diagnosis and therapy purposes.³⁷ Profilin and polcalcin from chenopod can be considered as representative members of these panallergenic families and therefore are suitable candidates to be included in clinical protocols.

Accession numbers of the nucleotide sequences here reported are AY082337 for Che a 2 and AY082338 for Che a 3. The new allergens have been submitted to the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee.

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