

Are Ca^{2+} -binding motifs involved in the immunoglobulin E-binding of allergens? Olive pollen allergens as model of study

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Summary

Background Several Ca^{2+} -binding proteins, which possess EF-hand sites with a high sequence similarity, have been found to be able to induce Type-I allergy.

Objective To study whether the common EF-hand sequential motifs can be involved in the IgE-reactivity of these proteins, thus being responsible of a degree of cross-reactivity among different Ca^{2+} -binding proteins.

Methods Two olive pollen allergens, Ole e 3 and Ole e 8, have been used in the study. Parvalbumin and calmodulin were included in immunological analyses. Sera from patients allergic to olive pollen, as well as Ole e 3- and Ole e 8-specific rabbit antisera were used in indirect enzyme-linked immunosorbent assay (ELISA), ELISA inhibition assays and immunoblotting. Conformational analyses (circular dichroism spectra and thermal stability) and specific immunodetection assays were performed in the presence and the absence of Ca^{2+} . Chemical breakdown and high-performance liquid chromatography (HPLC) was used to obtain fragments from Ole e 3 containing a single EF-hand motif.

Results Thirty-four (17%) and 16 (8.2%) out of 195 sera from patients allergic to olive pollen contained specific IgE against Ole e 3 and Ole e 8, respectively. The IgE-binding of 12 allergic sera diminished up to 22% for Ole e 3 and to 82% for Ole e 8, when depleted Ca^{2+} . A pool of these sera recognized the two olive allergens and parvalbumin, but at very different extent. Inhibition of the IgE-binding was only achieved between two olive allergens. No structural relationships between Ole e 3 and Ole e 8 were established when specific polyclonal antisera against both proteins were used.

Conclusion EF-hand Ca^{2+} -binding sites can not be considered as general allergenic motifs responsible for the cross-reactivity between Ca^{2+} -binding allergens. Different families of Ca^{2+} -binding allergens have specific epitopes that could be involved in the cross-reactivity among members of the same family.

Keywords Ca^{2+} -binding protein, cross-reactivity, olive pollen allergy

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Introduction

It has been found that the pollen tissue contains several types of allergens which belong to the widespread family of Ca^{2+} -binding proteins (CaBPs) [1]. All of them possess in their polypeptide sequence a variable number (2–4) of structural motifs called 'EF-hand' sites, which are composed of 12 consecutive amino acid residues [2]. These sequential motifs are directly implicated in the binding of the protein to Ca^{2+} ions and their amino acid sequences have been found to be very conserved. Three different types of CaBPs from pollen tissue, according to the number of EF-hand sites, have been demonstrated to be inducers of Type I allergy: (i) Bet v 3 from birch with three EF-hand motifs [3]; (ii) an ubiquitous family of small

CaBPs with two EF-hand sites (polcalcins) that have been detected in rapeseed (Bra n 1, Bra n 2, Bra r 1 and Bra r 2), olive tree (Ole e 3), birch (Bet v 4), Bermuda grass (Cyn d 7), alder (Aln g 4) and timothy grass (Phl p 7) [4–9]; and (iii) Jun o 2 from juniper and Ole e 8 from olive tree, which are four EF-hand CaBPs [10, 11]. These three types of CaBPs do not show sequence similarity out of the EF-hand sites. Although there is not direct evidence to support the idea that biological activity of an allergen is related to its ability to induce allergy [12], the common structural organization of the EF-hand motifs in the CaBPs could be responsible of the IgE interaction. In fact, these polypeptide segments are excellent candidates to constitute immunodominant areas because they are salient regions of the protein surface, as well as to be involved in cross-reactivities due to their high sequence conservativeness.

The allergy to olive tree (*Olea europaea*) pollen is an important health problem in Mediterranean countries and some areas of North America [13]. Olive pollen extract contains a complex mixture of allergenic proteins, from which many components

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(Ole e 1 to Ole e 9) have been so far characterized [14, 15]. Among the olive pollen allergens, two CaBPs have been identified: Ole e 3 and Ole e 8 [5, 11, 16]. Ole e 3 is a two EF-hand CaBP that belongs to the family of polcalcins [5, 16]. Ole e 3 shares a very conserved amino acid sequence with other members of this CaBPs family and thus shows a high degree of cross-reactivity. Recently, a novel four EF-hand CaBP with IgE-binding activity, Ole e 8, has been found in olive pollen [11]. This allergen seems not to have counterparts with conserved amino acid sequences in other allergenic pollens, as significant cross-reactivity was observed only with Oleaceae pollens.

We herein studied the possible involving of the EF-hand sites in the recognition of IgG and IgE antibodies using these two CaBPs allergens from olive pollen, as well as calmodulin and parvalbumin as exogenous EF-hand type CaBPs. Analyses of the influence of Ca^{2+} ions on their IgE binding ability and stability are also reported.

Materials and methods

Preparation of pollen extracts

Pollen (Allergon AB) proteins from *Olea europaea* were extracted (1:10, w/v) for 1 h at room temperature with 50 mM ammonium bicarbonate (pH 8.0) containing 1 mM phenylmethylsulphonyl fluoride and then centrifuged at 12 000 g for 20 min at 4 °C [5]. The pellet was re-extracted twice in the same solution. The supernatants were lyophilized and stored at -20 °C.

Obtaining of Ole e 3 and Ole e 8 olive allergens, and protein sources

Recombinant form of Ole e 3 was prepared as described [16]. Ole e 8 was produced in *Escherichia coli* by expression of its cDNA synthesized from total RNA of olive pollen [11]. The protein was purified by size exclusion chromatography in Sephadex G-150 followed by an affinity chromatography in phenyl-Sepharose CL-4B [11]. Frog parvalbumin and bovine calmodulin were from Sigma-Aldrich (Sigma-Aldrich Quimica S.A., Madrid, Spain).

Human sera and rabbit antibodies

One hundred and ninety-five sera from untreated donors with seasonal allergic symptoms from April to June, a positive skin prick test to olive pollen extract and CAP-FEIA System classes 3–6 to olive pollen extract were used for immunoassays.

Two polyclonal antisera were prepared by immunizing adult male White New Zealand rabbits over an 8-week period with 50 µg of Ole e 3, and over a 6-week period with 100 µg of Ole e 8.

Protein concentration determination

Protein concentration was determined by both a colourimetric assay [17] and quantitative amino acid analysis [5].

Spectroscopic analysis

Circular dichroism spectra were obtained on a Jasco J-715 spectropolarimeter connected to a Weslab RTE-111 thermostabilizer bath as described [5], with minor modifications. The protein concentration was 0.10–0.25 mg/mL in 20 mM ammonium bicarbonate. Mean residue ellipticities were calculated based on 110

and 111, respectively, to Ole e 3 and Ole e 8, as the average molecular mass/residue, obtained from the amino acid compositions, and expressed in terms of θ (degree $\times \text{cm}^2 \times \text{d/mol}$).

The thermal stability of the allergens was analysed by circular dichroism between 20 °C and 80 °C, with an increasing of temperature of 30 °C/h. The samples contained, alternatively, 2 mM CaCl_2 or 2 mM EGTA. The alteration suffered in protein structures of Ole e 3 and Ole e 8 during the thermal denaturing process was measured as changes in the molar ellipticity at 222 nm.

CNBr treatment of Ole e 3 and peptide separation

Ole e 3 (10 mg/mL) was cleaved in 70% formic acid at 100 molar excess of the reagent over the methionine content. The treatment was performed during 20 h in nitrogen atmosphere, at room temperature (25 °C) in the dark. Afterwards, the lyophilized sample was chromatographed in a C-18 reverse-phase high-performance liquid chromatography (HPLC) column and eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Molecular characterization of the fragments was achieved by mass spectrometry (according to [5]) to analyse molecular masses, Edman degradation to determine NH_2 -terminal amino acid sequences [5], and acid hydrolysis and amino acid analysis [5] to obtain the amino acid compositions.

ELISA experiments

Enzyme-linked immunosorbent assay (ELISA) titrations were performed in microtiter plates coated with 100 µL/well of protein (1 µg/mL) in phosphate-buffered saline (PBS) and blocked with PBS–3% (w/v) defatted milk–0.1% (v/v) Tween-20 [16]. Afterwards, different dilutions of a pool of olive-allergic sera were added and incubated for 2 h. Binding of human IgE was detected by mouse anti-human IgE antibodies (kindly donated by Dr M. Lombardero from ALK-Abelló, Madrid, Spain) followed by horseradish peroxidase (HRP)-labelled goat anti-mouse IgG (1:5000 diluted) (Pierce, Rockford, IL, USA). Alternatively, proteins were titrated with different dilutions of rOle e 8-specific rabbit antiserum or Ole e 3-specific antiserum, followed by HRP-labelled anti-rabbit IgG (1:3000 diluted; Pierce).

Specific IgE-binding to Ole e 3 and Ole e 8 was determined as described above in ELISA titration, but in this case using individual allergic sera 1:10 diluted. The threshold value to consider a positive response was 0.1 optical density (O.D) at 492 nm.

ELISA inhibition assays were performed as described in [5]. After being coated with 100 µL of the antigen Ole e 8 (1 µg/mL) plates were incubated with a pool of olive-allergic patient sera (diluted 1:10), with a rabbit antiserum specific for Ole e 8 (1:200 000 diluted) or specific for Ole e 3 (1:50 000 diluted), these sera were previously incubated with different concentrations of Ole e 3, Ole e 8, calmodulin or parvalbumin as inhibitors. Binding of IgE or IgG was detected as described above.

IgE-binding dependence on Ca^{2+}

The influence of the Ca^{2+} ion in the IgE binding to the antigen was analysed by ELISA with individual sera from olive-allergic patients (1:10 diluted) in the presence and the absence of 2 mM EGTA. Defatted milk of blocking buffer was substituted by 2%

(v/v) polyvinylpyrrolidone in the standard ELISA above described. Ole e 1 allergen was used as negative control.

Results

Clinical data and frequency of sensitization

One hundred and ninety-five sera from patients allergic to olive pollen were tested in ELISA for their IgE binding to Ole e 3 and Ole e 8. Thirty-four (17%) were found positive to Ole e 3 and 16 (8.2%) to Ole e 8 (Table 1). Complementary clinical data of these patients are shown in Table 1. All of the sera that bound to Ole e 8 were among those positive to Ole e 3. Twenty-eight out of the sera positive to these CaBPs were 5–6 CAP values, determined by CAP. Fourteen sera out of the 16 positive to Ole e 8 displayed Ole e 8-specific IgE values significant lower than to Ole e 3.

Role of Ca^{2+} ions in the IgE binding to Ole e 3 and Ole e 8

The influence of the protein-bound Ca^{2+} on the IgE recognition of Ole e 3 and Ole e 8 was examined by ELISA, comparing the response of individual sera in the presence and the absence of EGTA, which chelates Ca^{2+} ions (Fig. 1). A decrease of the IgE-binding capability was observed for both allergens when depleted Ca^{2+} , being notably higher for Ole e 8. The reduction of the IgE reactivity ranged from 17 to 82% for Ole e 8 and from 0 to 22% for Ole e 3, although the reactivity against these allergens was never abolished.

Thermal stability of olive CaBP in the presence and the absence of Ca^{2+}

Thermal denaturation of the allergens was measured as the changes in the molar ellipticity values at 222 nm when temperature was rising from 20 °C up to 80 °C. The analyses were performed in the presence of 2 mM CaCl_2 or in the presence of 2 mM EGTA. In addition, the spectra at initial 20 °C, at 80 °C,

Table 1. Clinical data, total and specific IgE values of 34 patients allergic to olive pollen

Serum	Sex	Age (years)	Symptoms*	Other pollinosis†	Total IgE (kU/L)	Olive-specific IgE‡ (RAST)	Ole e 3-specific IgE	Ole e 8-specific IgE
1	Male	28	RC,BA	G,P,Q,A,PL	399	50.0 (4)	> 2.5	0.51
2	Female	18	RC	G,P,Q,A,PL,PA	475	22.4 (4)	1.35	(-)
3	Female	20	RC,BA	G,P,Q,A,PL,PA	141	10.8 (3)	0.26	(-)
4	Male	26	RC,BA	G,P,Q,A,PL	581	> 100 (6)	0.57	0.16
5	Female	33	RC,BA	G,P,Q,A,PA	213	33.0 (4)	1.72	(-)
6	Male	19	RC,BA	G,P,Q,A,PA,PL	194	56.9 (5)	0.11	(-)
7	Female	25	RC,BA	G,P,A	172	60.6 (5)	0.13	(-)
8	Male	40	RC,BA	G,P,Q,A,PA,PL	341	95.6 (5)	> 2.5	0.57
9	Female	24	RC	G,P,Q,PL	107	20.0 (4)	0.11	(-)
10	Female	15	RC,BA	G,C,P,A,PA,PL	166	55.4 (5)	0.58	0.23
11	Female	37	RC,BA	G,P,Q,A,PA,PL	938	> 100 (6)	> 2.5	(-)
12	Male	22	RC,BA	G,P,Q,A,PL	2255	> 100 (6)	1.51	0.16
13	Male	19	RC,BA	G,P,Q,A,PA,PL	2057	> 100 (6)	> 2.5	(-)
14	Male	18	RC,BA	Q	387	10.0 (3)	0.65	(-)
15	Male	62	RC	G,C,P,Q,A,PL	nd	68.9 (5)	1.92	0.12
16	Female	16	BA	G,C,P,Q,PA,PL	1490	72.8 (5)	> 2.5	0.18
17	Female	33	R, BA	G,C,P,Q,PA,PL	1375	88.7 (5)	1.85	0.19
18	Male	13	RC	G	866	73.8 (5)	1.31	0.14
19	Male	15	RC, BA	G,C,PA	1400	69.0 (5)	0.14	(-)
20	Female	28	RC, BA	G,A,PA	3233	> 100 (6)	> 2.5	> 2.5
21	Female	13	RC, BA	G,P,Q,A,PA,PL	253	95.6 (5)	1.09	(-)
22	Female	31	RC, BA	G,C	128	75.1 (5)	0.98	(-)
23	Female	8	BA	G	1525	90.2 (5)	0.22	(-)
24	Female	4	RC	G	909	83.7 (5)	> 2.5	1.98
25	Male	12	BA	G,PA	192	71.4 (5)	2.27	0.53
26	Female	18	RC, BA	G,C,Q,A	586	51.9 (5)	0.57	0.15
27	Male	19	BA	G,C,Q,A	562	60.7 (5)	0.73	(-)
28	Male	31	RC	G,Q,A,PA	1103	75.7 (5)	1.66	0.29
29	Male	6	BA	G,C,A,PA	9549	> 100 (6)	0.18	(-)
30	Male	26	RC, BA	G,PA	670	90.5 (5)	1.82	1.10
31	Male	8	RC	G,C,P,Q,A,PA,PL	2139	64.5 (5)	0.18	(-)
32	Female	9	RC, BA	G,C,P,Q,A,PA,PL	1524	61.4 (5)	0.77	(-)
33	Male	4	RC, BA	G	858	74.3 (5)	1.92	(-)
34	Male	42	BA	G,Q,A,PA	754	62.6 (5)	0.63	0.72

*R, rhinitis; RC, rhinoconjunctivitis; AB, bronchial asthma. †G, *Gramineae*; C, *Cupresaceae*; P, *Parietaria*; Q, *Chenopodium album*; A, *Artemisia vulgaris*; PL, *Plantago lanceolata*; PA, *Platanus acerifolia*. ‡Olive-specific IgE is done in kU/L. RAST values are given in parenthesis. Ole e 3- and Ole e 8-specific IgE values are the mean of two determinations and were achieved by OD measurements at 492 nm. Values under 0.1 were considered as negative responses (-).

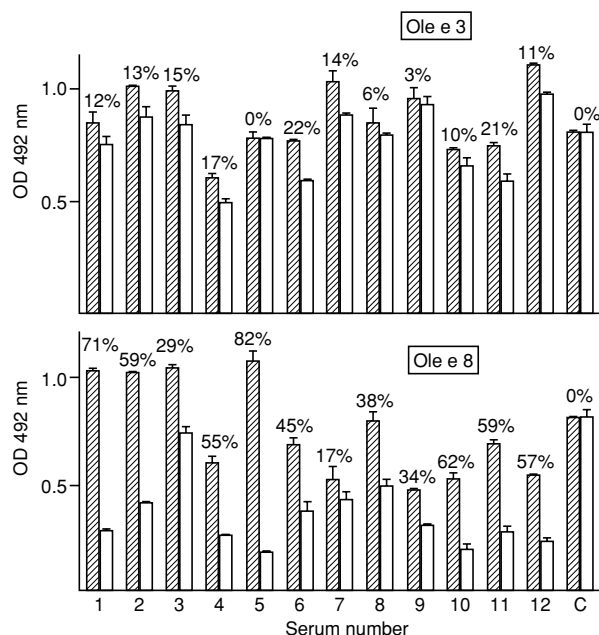


Fig. 1. Effect of Ca^{2+} on the IgE binding to Ole e 3 and Ole e 8. ELISA values (OD, optical density) were obtained in the absence (dashed bars) and in the presence (empty bars) of 2 mM EGTA with 12 sera both for Ole e 3 and for Ole e 8. Values are means of duplicate samples. The decrease of the binding in the presence of EGTA is indicated as percentage of the value in the presence of Ca^{2+} . The binding to Ole e 1 of a pool of sera was used as negative control (C).

and after cooling the samples at 20 °C, were registered (Fig. 2). Ole e 3 showed a high stability to heating in the presence of Ca^{2+} , as it retained most of the α -helix conformation (75% of ellipticity at 222 nm). After a return to the initial conditions, the protein recovered 90% of its original ellipticity. However, in the presence of EGTA, Ole e 3 exhibited at 80 °C a CD spectrum typical of unordered conformation indicating its denaturation, but the allergen recouped 82% of the periodic structure of the original sample when cooled at 20 °C.

Ole e 8 exhibited in the absence of Ca^{2+} a different behaviour than Ole e 3. The allergen is very sensitive to the presence of EGTA, showing at 20 °C a completely disordered secondary structure, therefore no changes were suffered by the protein in response to heating. In the presence of Ca^{2+} , Ole e 8 showed a similar stability to Ole e 3, as it lost 65% of ellipticity at 222 nm when heated to 80 °C, and recovered 88% when cooled at 20 °C.

Analysis of cross-reactivity among EF-hand CaBPs

In order to know the possible reactivity of Ole e 8-sensitive sera to CaBPs containing different number of EF-hand motifs, ELISA titrations were performed for Ole e 3 (two EF-hand Ca^{2+} -binding sites), parvalbumin (three EF-hand sites) and calmodulin (four EF-hand sites) with a pool of eight sera, which were reactive to both Ole e 3 as Ole e 8, and with Ole e 3-specific antiserum and Ole e 8-specific antiserum. Amino acid sequence identities between EF-hand motifs from these Ca^{2+} -binding proteins are shown in Table 2. The two olive allergens and parvalbumin showed IgE-binding capacity, but with different affinities against the antibodies (Fig. 3a). As can be seen, Ole e 3 exhibited higher binding capability than Ole e 8, at the same serum concentration. On the other hand, anti-Ole e 8 antiserum

bound to the four EF-hand CaBPs assayed, although showing remarkably higher affinity against Ole e 8 (Fig. 3b). Ole e 3-specific antiserum also shows affinity against olive allergens, Ole e 8 and Ole e 3 (Fig. 3c).

The inhibition of the IgE binding to the four EF-hand allergen Ole e 8 by other EF-hand proteins was measured by ELISA, performing the pre-incubation of the allergic pool of human sera alternatively with Ole e 8, Ole e 3 and parvalbumin as inhibitors (Fig. 4a). Interestingly, Ole e 3 allergen exhibits greater inhibition strength than Ole e 8 itself.

The differences observed in the ELISA titrations for IgG-binding were confirmed by inhibition assays with the olive allergen-specific antisera (Fig. 4b). Although the binding of the Ole e 8-specific antiserum to Ole e 8-coated wells can be inhibited at 67% with Ole e 3, the amount of inhibitor to reach 50% of inhibition is $1\text{--}2 \times 10^2$ higher than with Ole e 8. None of Ole e 8, parvalbumin, or calmodulin were able to inhibit at all the binding of Ole e 3-specific rabbit antiserum to Ole e 8-coated wells (Fig. 4c).

Analysis of IgE reactivity of CNBr fragments of Ole e 3

Two peptides of the protein were obtained by chemical treatment with CNBr, separated by HPLC and characterized (Figs 5a and b). Structural characterization by mass spectrometry, Edman degradation and amino acid analysis of these peptides indicated that they corresponded to the NH_2 -terminal (positions 1–48/49) and the COOH -terminal (positions 49/50–83) halves of Ole e 3, and each fragment contained one EF-hand site of the allergen. Quantification of the IgE-binding to these peptides was performed by direct ELISA with 22 sera from patients sensitive to Ole e 3 (Fig. 5c). A remarkable loss of binding capability was observed for the peptides in comparison with the whole protein, but 16 sera were able to recognize the COOH -terminal domain and six to recognize the NH_2 -terminal peptide.

Inhibition analysis of the IgE-binding to Ole e 3-coated wells was performed by using individual peptides and an equimolecular mixture of both (Fig. 5d). Although the NH_2 -terminal peptide was not able to inhibit the binding to the antibodies, the COOH -terminal fragment of Ole e 3 reached up to 50% of inhibition, but at $10\times$ concentration of the inhibitor higher than the whole protein. The mixture of peptides did not improve this result. These assays indicate the different epitopic behaviour of the two EF-hand-containing segments of this allergen.

Discussion

Since the discovery of parvalbumin from codfish as a type-I allergy inducer [18, 19], a number of allergenic CaBPs from different sources have been reported. All of these calcium-binding allergens have in common at least two units of a specific sequential motif called EF-hand [1, 5–11], which are directly involved in the binding to calcium and exhibit very conserved amino acid sequences [2]. Two calcium-binding allergens with a different number of EF-hand sites have been found in olive pollen extracts, Ole e 3 and Ole e 8 [5, 11, 16, 20]. The availability of sera from patients allergic to both proteins, as well as allergen-specific antisera allowed us to analyse putative cross-reactivities between these allergens and with other allergenic/

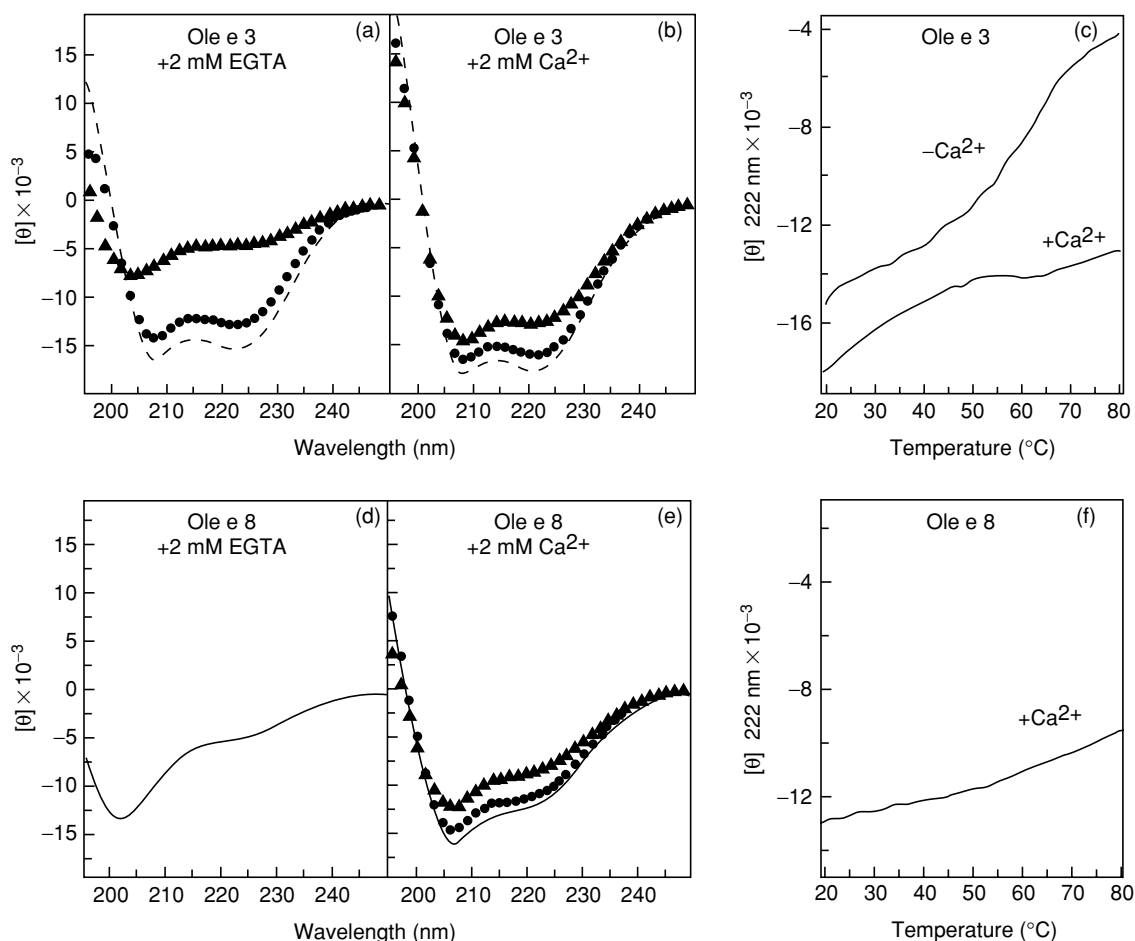


Fig. 2. Thermal stability of Ole e 3 (a,b,c) and Ole e 8 (d,e,f). Circular dichroism spectra were obtained in the presence of 2 mM EGTA (a,d) and the presence of 2 mM CaCl_2 (b,e); the spectra were recovered at 20 °C (dashed line), after heating to 80 °C ($\blacktriangle$), and then cooling to 20 °C ($\bullet$). Changes in the ellipticity at 222 nm were registered in the presence (^+Ca) and the absence of Ca^{2+} (^-Ca ; c and f, respectively, to Ole e 3 and Ole e 8).

Table 2. Amino acid sequence identities between the EF-hand sites of Ole e 3 and Ole e 8, and those of these olive allergens, parvalbumin (Par) and calmodulin (CaM)

Sites	I Ole e 3	II Ole e 3	II Par*	III Par	I CaM	II CaM	III CaM	IV CaM
I Ole e 8	10	7	5	8	6	6	6	6
II Ole e 8	5	8	6	6	6	5	6	7
III Ole e 8	6	6	6	5	5	6	7	5
IV Ole e 8	6	9	4	6	6	6	6	7
I Ole e 3		7	4	7	6	6	6	6
II Ole e 3			6	7	7	7	8	8

*EF-hand site I of parvalbumin has not been included because it is not functional and has deletions.

non-allergenic CaBPs, in order to highlight the possible involvement of the highly conserved EF-hand sites in the antigenic and allergenic activity of these group of allergens.

The allergenic potency of Ole e 3 and Ole e 8 seems to be different, as we did not find allergic sera exhibiting a positive reaction exclusively to Ole e 8, but all of the sera reactive to Ole e 8 were also reactive to Ole e 3. No correlation of the sensitivity to Ole e 3 or Ole e 8 was found with particular clinical symptoms or sensitivities to other pollen sources, except the general

coincidence in the sensitivity to *Gramineae* pollens. The significant difference on the prevalence of these allergens could be explained by intrinsic factors to these molecules, but also in terms of the particular amount of each protein expressed in the pollen. Whereas Ole e 3 is expressed in a relatively high amount ($> 500 \mu\text{g}/10 \text{ g}$ of dried pollen), Ole e 8 is present at a very low level ($15\text{--}40 \mu\text{g}/100 \text{ g}$ of dried pollen). These two allergens have been shown to belong to different subfamilies of CaBPs, Ole e 3 is a calcium buffer protein and Ole e 8 is a calcium sensor protein [11]. Whether the specific functional role is involved or not in the allergenicity of the molecules remains a question, but little evidence supports the suggestion that allergens share structural or functional features [12]. However, the sharing of very conserved amino acid sequences by allergens, such as the EF-hand motifs, may make feasible its link to allergenicity as a hypothesis of work.

Although Ca^{2+} ions are not strictly required for the IgE binding to Ole e 3 and Ole e 8, most of the sera from allergic patients showed a decreased reactivity in the absence of the ion, this being more substantial for Ole e 8. These results indicate that IgE epitopes of both allergens are affected by the conformational change induced by the binding/releasing of Ca^{2+} . This conformational alteration is supported by the spectroscopic data and the thermal stability analysis: the secondary

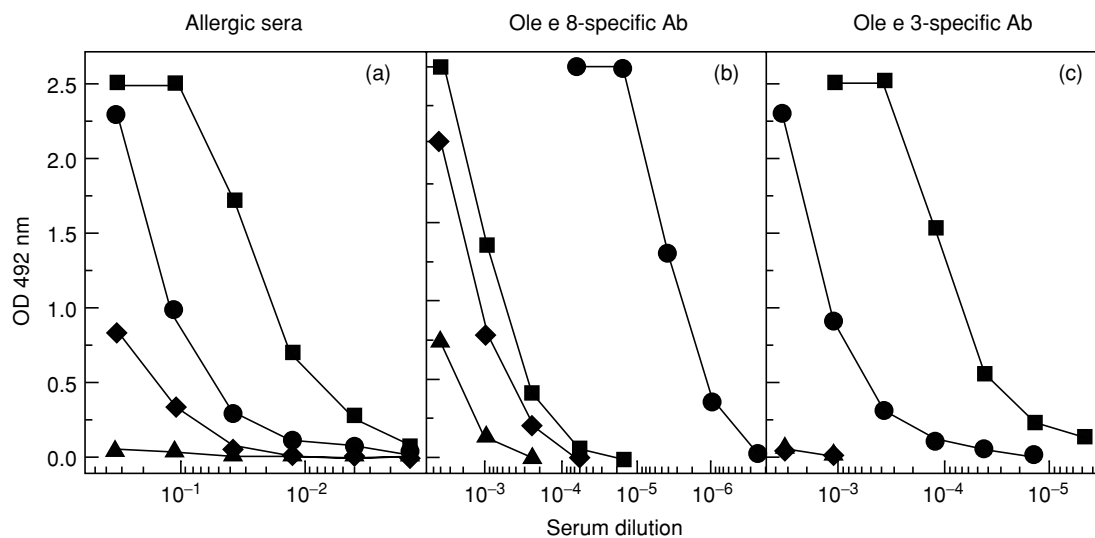


Fig. 3. Analysis of the IgE- and IgG-binding to Ole e 3 (two EF-hand), Ole e 8 (four EF-hand) and other CaBPs. ELISA titration of Ole e 3 (■), Ole e 8 (●), calmodulin (▲) and parvalbumin (◆) with: (a) IgE of a pool of sera from olive-allergic patients who are sensitive to Ole e 3 and Ole e 8; (b) IgG of Ole e 8-specific polyclonal antiserum; (c) IgG of Ole e 3-specific polyclonal antiserum. Values are done in optical density (OD) at 492 nm, and are the mean of two determinations.

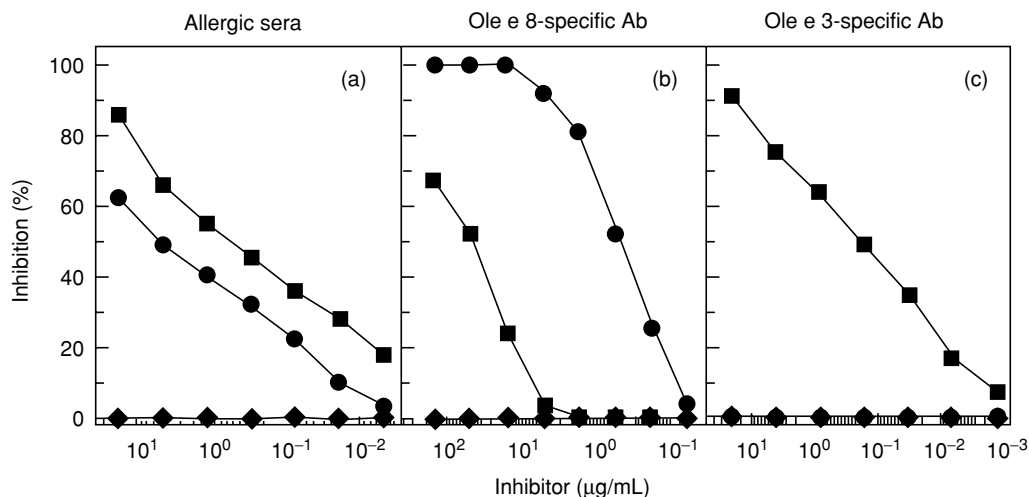


Fig. 4. Inhibition of the binding of antibodies to Ole e 8-coated wells by using Ole e 3 (■), Ole e 8 (●), calmodulin (▲) and parvalbumin (◆) as inhibitors: (a) IgE from a pool of olive-allergic sera, and seven serial fourfold dilutions of inhibitors from 20 $\mu\text{g/mL}$; (b) IgG from an Ole e 8-specific polyclonal antiserum, and eight serial threefold dilutions of inhibitors from 150 $\mu\text{g/mL}$; and (c) IgG from an Ole e 3-specific polyclonal antiserum and seven serial fivefold dilutions of inhibitors from 20 $\mu\text{g/mL}$.

conformation of Ole e 8 is completely lost in the calcium-free form, whereas the stability of Ole e 3 is notably decreased when the protein loses Ca^{2+} . Previous analyses on the fluorescence properties of Ole e 8 in the presence and the absence of Ca^{2+} , which inform about the microenvironment of aromatic amino acids in the three-dimensional structure of the protein, have suggested such a strong conformational change [11]. The results obtained for Ole e 3 fit well to the reported IgE dependence on calcium binding for several members of the ubiquitous family of the two EF-hand polycalcins [6, 8, 9, 21]. However, the strong dependence of the conformation of Ole e 8 on calcium binding suggests a different scaffold for these EF-hand proteins than for the family of the two EF-hand polycalcins. A great influence of conformational changes in dependence on calcium

was also detected for the IgE-binding to parvalbumin, at least for many reactive sera [22]. In all cases, the Ca^{2+} -free proteins are low reactive forms against IgE and this should be considered when designing 'hypoallergenic' derivatives to be used in immunotherapy [1].

Ole e 8 shows a low amino acid sequence identity with Ole e 3 and other allergenic CaBPs out of the EF-hand sites. Ole e 3 belongs to the widespread family of polycalcins, which share very similar polypeptide sequences both out and within the EF-hand sites, and exhibit a high degree of IgG and IgE cross-reactivity among them [5, 8, 9, 16]. By contrast, the absence of IgG cross-reactivity of Ole e 8 with other pollen extracts from taxonomically unrelated plants [20] suggests a low conservation for the amino acid sequence of its counterparts present in

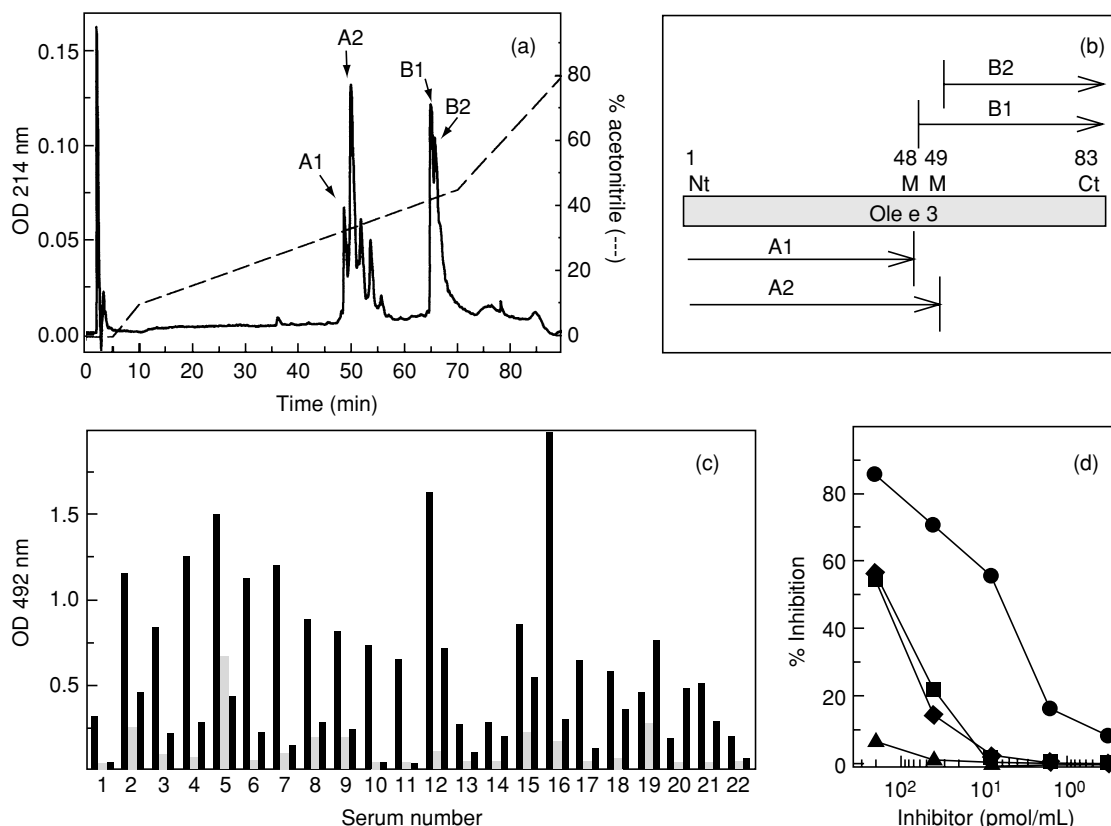


Fig. 5. IgE-binding to CNBr fragments of Ole e 3. (a) HPLC purification of peptides from Ole e 3 treated with CNBr; optical density (OD) of the eluate was registered at 214 nm. (b) Molecular organization of the peptides A1, A2, B1, and B2 obtained from HPLC in the molecule of Ole e 3; Nt, NH₂-terminal end; Ct, COOH-terminal end; M, methionine residues of Ole e 3 located at positions 48 and 49. (c) ELISA quantification of the IgE-binding of 22 sera allergic to Ole e 3 (black lines), NH₂-terminal fragment (A1 + A2, grey lines), and COOH-terminal fragment (B1 + B2, dark grey lines); OD values under 0.1 were considered as negative responses. (d) Inhibition data of the IgE-binding to Ole e 3-coated wells by using as inhibitors: Ole e 3 (●), NH₂-terminal peptide (▲), COOH-terminal peptide (◆), and a mixture of NH₂- and COOH-terminal peptides (■). A pool of sera sensitive to Ole e 3 was used in (d).

other allergenic pollens. This suggests a non-significant role of the EF-hand motifs of these proteins in their immunogenicity. In fact, although it has been proposed that at least one IgE epitope of Cyn d 7, a member of the polcalcin family, resides in the continuous (linear) EF-hand Ca²⁺-binding loop located near the COOH-terminal end [7], most of the experimental data support the absence of major IgE epitopes in the highly conserved Ca²⁺-binding sites [1]. We have found that IgE from patients allergic to Ole e 3 and Ole e 8 were reactive against parvalbumin, although at a 100-fold lesser extent, but not against calmodulin. In addition, Ole e 8-specific rabbit antibodies bind to Ole e 3, parvalbumin and calmodulin, also to a remarkably lesser extent. The very low capacity of recognition to parvalbumin by Ole e 8-specific antibodies may explain the absence of inhibition shown in Fig. 4(b). Binding exhibited by Ole e 3, which is two orders lower than for Ole e 8, may be considered as an unspecific recognition. In addition, the similarity between sequences of EF-hand motifs from Ole e 8 and Ole e 3 is higher than those from Ole e 8 and parvalbumin or calmodulin. IgE-inhibition experiments were also conclusive: only Ole e 3 was able to inhibit the binding of the IgE to Ole e 8-coated wells. Therefore, our data support the idea that the Ca²⁺-binding sites of EF-hand proteins have a very limited role, if possible, in their IgE and IgG epitopes and cross-reactivity.

Finally, the different IgE reactivity of the two half portions of Ole e 3, each containing one EF-hand site, again corroborates the different immunologic behaviour of peptides containing Ca²⁺-binding sites, as it was observed for Cyn d 7 [7].

In conclusion, the IgE epitopes seem not to be located at the EF-hand motifs, but the binding of the IgE antibodies to the EF-hand Ca²⁺-binding allergens is affected by conformational changes occurring in the whole tertiary structure of these proteins, which are induced by the binding/release of Ca²⁺ ions.

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