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Cloning, expression and characterization of a novel four EF-hand Ca^{2+}-binding protein from olive pollen with allergenic activity

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Abstract A novel allergenic member of the family of Ca^{2+}-binding proteins has been cloned from olive tree pollen. The isolated DNA codes for a protein of 171 amino acid residues, which displays four EF-hand sequence motifs. The encoded protein was overproduced in *Escherichia coli* and purified. The protein (18 795 Da), which binds Ca^{2+} and IgE antibodies from patients allergic to olive pollen, undergoes Ca^{2+}-dependent conformational changes. It is retained on a phenyl-Sepharose column, which indicates the existence of regulatory EF-hand domains. This fact suggests its involvement in Ca^{2+}-dependent signal transduction events of the pollen grain. This allergen could be considered as a member of a new subfamily of EF-hand Ca^{2+}-binding proteins since it displays a low amino acid sequence similarity with the so far known proteins.

Key words: Ca^{2+}-binding protein; Olive pollen allergen; Ole e 8; Amino acid sequence; Recombinant production

1. Introduction

It is well known the role of Ca^{2+} in a wide variety of cellular processes mediated by Ca^{2+}-binding proteins (CaBPs). In plant tissues, a number of different subfamilies of EF-hand CaBPs have been found. Among them, calmodulin (CaM), CaM-like proteins, CaM-dependent protein kinases, and centrins are the best known CaBPs. As in animal cells, CaBPs from vegetables are involved in transduction of environmental stimuli into Ca^{2+} signals [1], including responses to mechanical stress and cold-shock [2,3]. A crucial biological role has been attributed to Ca^{2+} and CaBPs from pollen tissue in the germination and pollen tube growth since they seem to be strongly dependent on the levels and distribution of this ion [4,5]. Recently, it has been shown that CaM is not responsible for the tube orientation during its growth [6], therefore other CaBPs should be involved in such a process which is dependent on Ca^{2+}. The pollen tissue has been shown to express at least three types of EF-hand CaBPs, all of them able to induce allergy: Bet v 3 [7] from birch, the ubiquitous family of polcalcins [8–13], and Jun o 2 from juniper [14].

The olive pollen extract contains a number of proteins with allergenic activity. One of these allergens is the polcalcin Ole e 3 [9], which has been cloned and expressed in *Escherichia coli* [12] and possesses two EF-hand sites. We herein report the cloning, overproduction and characterization of an allergenic CaBP protein of olive pollen with four EF-hand motifs, which seems likely to belong to a new EF-hand CaBP subfamily.

2. Materials and methods

Olive pollen extract was prepared as previously described [9]. Double stranded cDNA was synthesized from 50 μg of total olive pollen RNA as described [15], using a modified lock-docking oligo-dT primer for the first strand synthesis. After treatment with T4 DNA polymerase, the blunt-ended cDNA was ligated to an adaptor sequence following the Marathon cDNA amplification kit protocol (Clontech). PCR amplifications were performed throughout a two-step method. At first, a gene-specific antisense oligonucleotide (OL3-1: 5′-TCGGTRTCRATCTCRGCCATCAT-3′) was designed based on the internal peptide MMAEIDTD of the olive allergen Ole e 3 [12]. An oligonucleotide (AP1: 5′-CCATCCTAATACGACTCACTATAGGGC-3′) included into the Marathon kit, which hybridizes with the adaptor sequence, was used as a sense primer. PCR conditions and cycle number were done as the Advantage KlenTaq Polymerase Mix Kit (Clontech) describes but adapting the hybridizing conditions to the specific primers. The purified fragment was phosphorylated with T4 polynucleotide kinase and inserted into the *Sma*I site of a blunt-ended dephosphorylated pUC18 plasmid. The transformation of DH5αF′-competent *E. coli* cells and recombinant selection were achieved by standard procedures. The DNA nucleotide sequencing of several clones was automatically carried out according to [16] adapted for PCR. In the second step, the AP1 primer and a non-degenerate oligonucleotide (OL8-1: 5′-ATACATATGGCAGCAAACACGGACAG-3′, based on the putative NH_2-terminal end of the protein obtained in the first step), which contained a *Nde*I restriction site (underlined), were used. The obtained fragment was included into the pUC18 vector and further sequenced. Afterwards, a new primer was designed based on the COOH-terminal end of the protein (OL8-2: 5′-ATAAGCTTCTATTTCGGTGGCTCCGCTTG-3′), in which a *Hin*dIII restriction site (underlined) was included. OL8-1 and OL8-2 were used to amplify the cDNA coding for the whole protein. This cDNA was cloned into the *Nde*I/*Hin*dIII site of a pET-11b expression vector (pET/Ole8). After transformation of HB101 cells, the clone was sequenced to confirm the in-frame introduction of the fragment. Production of the recombinant protein as a non-fusion molecule was performed after transforming BL21(DE3)-competent *E. coli* cells with this pET/Ole8 construction, according to [9]. The soluble fraction, which contained the protein, was chromatographed on a Sephadex G-150 column, in 0.2 M ammonium bicarbonate. Recombinant protein was finally purified using a phenyl-Sepharose CL-4B column equilibrated in 50 mM Tris–HCl, pH 7.4, containing 0.5 mM $CaCl_2$. Retained protein was further eluted with 50 mM Tris–HCl containing 1 mM EGTA, and dialyzed against distilled water containing 2 mM $CaCl_2$.

Protein samples were hydrolyzed with 5.7 M HCl at 105°C for 24 h, and analyzed on a Beckman System 6300 amino acid analyzer. NH_2-terminal Edman degradation was performed on an Applied Biosystems model 477A sequencer. Molecular mass determination was achieved in a Bruker Reflex II matrix-assisted laser-desorption ionization mass spectrometer as described [17]. Amino acid sequence alignments and homology searches were performed according to [18]. Sequence-based secondary structure was predicted according to [19].

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Abbreviations: CaBP, Ca^{2+}-binding protein; CaM, calmodulin; CD, circular dichroism

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SDS–PAGE was performed in 15% polyacrylamide gels [20] and stained with Coomassie blue.

Ca^{2+}-binding probes were carried out according to [21]. Proteins were transferred onto nitrocellulose membranes after SDS–PAGE, and washed with 2 mM EGTA and with calcium buffer (10 mM PIPES, pH 6.9, 50 mM NaCl, 0.1 mM $MgCl_2$). Afterwards, the membranes were incubated with 6 μM $^{45}CaCl_2$ (3000 mCi/mmol) in calcium buffer, and washed with distilled water. Sheets were exposed to Agfa X-ray film for 4 h. Mobility shift experiments were performed according to [22] in 15% SDS–PAGE in the presence of 10 mM EGTA, and in 10 mM $CaCl_2$ after washing with 2 mM EGTA. Samples were stained with Coomassie blue.

Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter as described [12]. Mean residue mass ellipticity was calculated [23] based on 111, obtained from the amino acid composition of this protein. Fluorescence emission spectra were obtained on an SLM Aminco 8000 spectrofluorimeter, using 4 nm slits for both excitation and emission beams. Far-UV CD and fluorescence analyses were performed in the same conditions, 0.15–0.20 mg/ml (near-UV CD analysis at 1.1 mg/ml) of protein concentration, in 20 mM ammonium bicarbonate, pH 8.0, at 20°C, and containing alternatively 2 mM $CaCl_2$, or 2 mM EGTA, or 10 mM $CaCl_2$.

ELISA titration was performed in microtiter plates coated with 100 μl/well of protein (1 μg/ml) in PBS and blocked with PBS-3% defatted milk-0.1% Tween 20 [12]. Afterwards, the plates were incubated with different dilutions of a pool of olive-allergic sera. Binding of human IgE was detected by mouse anti-human IgE antibodies followed by horseradish-peroxidase-labeled goat anti-mouse IgG. Sera from untreated donors with positive skin test and radioallergosorbent test (RAST) class 3–6 to olive pollen extract, and non-allergic sera were used for immunoassays.

3. Results

The cDNA synthesized from olive pollen RNA was the template for a PCR reaction using a primer sequence codifying an EF-hand Ca^{2+}-binding motif. The 750 bp band obtained was cloned into a pUC18 plasmid and further sequenced. Fig. 1 shows the nucleotide and deduced amino acid sequences. The polypeptide chain is 171 amino acids in length and contains four 12-residue segments with consensus sequence of EF-hand type Ca^{2+}-binding sites. A search of homology in the GenBank/EMBL allowed to detect similarities with calmodulin-related proteins (Fig. 2). Greatest identity, 43% (35% outside the EF-hand sites), was obtained with TCH2, which is a stress-induced protein from *Arabidopsis thaliana* containing four EF-hand Ca^{2+}-binding sites [2]. It also exhibited 38% (27% out of the Ca^{2+}-binding sites) with Jun o 2, a pollen allergen from juniper [14], and 33% with the consensus amino acid sequence of calmodulins. Theoretical analysis of the secondary structure of this olive protein, based on its amino acid sequence, is also given in Fig. 2. The four putative Ca^{2+}-binding sites are predicted to be flanked by α-helical segments, which is a structural feature of EF-hand domains [24].

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CGAAAAGCACAAGAATATCAATGGCAGCAAACACGGACAGGAATTCCAAGCCTTCA   56
                    m  A  A  N  T  D  R  N  S  K  P  S     11
GTGTACCTCCAAGAACCGAATGAGGTCCAAGGTGTATTCAACCGCTTCGACGCCAAC  113
 V  Y  L  Q  E  P  N  E  V  Q  G  V  F  N  R  F  D  A  N   30
GGCGACGGCAAGATATCCGGGGATGAGCTAGCCGGCGTGCTTAAGGCGCTGGGATCC  170
 G  D  G  K  I  S  G  D  E  L  A  G  V  L  K  A  L  G  S   49
AACACTTCTAAAGAGGAGATCGGCCGCATAATGGAGGAGATCGACACCGACAAGGAC  227
 N  T  S  K  E  E  I  G  R  I  M  E  E  I  D  T  D  K  D   68
GGTTTCATCAATGTCCAGGAGTTCGCCGCCTTCGTCAAGGCCGAGACAGACCCCTAC  284
 G  F  I  N  V  Q  E  F  A  A  F  V  K  A  E  T  D  P  Y   87
CCCTCCTCCGGCGGCGAGAACGAGCTTAAGGAGGCGTTCGAGCTCTACGACCAGGAT  341
 P  S  S  G  G  E  N  E  L  K  E  A  F  E  L  Y  D  Q  D   106
CATAATGGATTGATCTCATCCGTCGAGCTCCACAAGATCCTAACGCGCCTCGGTGAG  398
 H  N  G  L  I  S  S  V  E  L  H  K  I  L  T  R  L  G  E   125
CGGTACGCCGAACATGACTGCGTTGAGATGATTAAATCTGTTGATTCTGATGGAGAT  455
 R  Y  A  E  H  D  C  V  E  M  I  K  S  V  D  S  D  G  D   144
GGTTACGTTAGCTTTGAGGAATTCAAGAAAATGATGACGAACAAAAGCGGCAATAAC  512
 G  Y  V  S  F  E  E  F  K  K  M  M  T  N  K  S  G  N  N   163
AGCCAAGCGGAGCCACCGAAATAGACAGCGGTGTGGCGCACTGGGATTTTGTTGTGT  569
 S  Q  A  E  P  P  K                                       170
GCTTCTTCCTGGTATATCGCCTTTTCCGATTTTAGGGCAAAATTCCATGATATTGAT  626
GATTTAGGGTTAATTTTGATTTATGTTTTTAAATTTTACTCTAATGTAATGACAGTG  683
CCATTTTATTGACTT(A)16                                       714
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Fig. 1. Nucleotide sequence of the cDNA clone encoding an olive CaBP and its deduced amino acid sequence. The primers used are underlined. The nucleotide sequence reported has been submitted to the GenBank/EMBL Data Bank with accession no. AF078679.

The complete coding sequence of the olive CaBP was subcloned into *Nde*I/*Hin*dIII-digested pET11b expression vector. A non-fusion and intracellular protein of 20 kDa was obtained in the soluble fraction of the transformed and disrupted *E. coli* cells after 4 h induction (Fig. 3A). The recombinant protein was isolated by two chromatographic steps. First, the sample was chromatographed on Sephadex G-150, in which two major molecular species of 20 and 40 kDa were obtained (Fig. 3B). They were separately applied onto a phenyl-Sepharose column. Both proteins were retained on the affinity matrix

```
          hhhhtttcc ct      ee ecccthheee eeettttttc cccehhhhhh hhhhccccch hhhhhhhhhh hhhhttceeh hhhhhhhhht
Ole e 8   mAANTDRNSK PS------VY LQEPNEVQGV FNRFDANGDG KISGDELAGV LKALGSNTSK EEIGRIMEEI DTDKDGFINV QEFAAFVKAE
TCH2      ·---SSK·GV VR------SC ·GSMDDIKK· ·Q···K···· ···V···KE· IR··SPTA·P ··TVTM·KQF ·L·GN···DL D··V·LFQIG
Jun o 2   ·DEVPSSDGS K·ACSGEV·M E·SVH·LEE· ·KK··· ···· ····S···DI ·RS···DVGE A·VKAM···A ·A·G··YVSL ···V------
CaM       -·----·QLTE EQI------- ----A·FKEA ·SL··KD··· T·TTK··GT· MRS··Q·PTE A·LQDMIN·V ·A·GN·T·DF P··LTLMARK
                                          *  **  ***  *   **       *         *         * *  *      **
                                             ——Site I——                                ——Site II——

          tccccccccc h     hhhh hhhhhhhttc ccchhhhhhh hhhhhccthh hhhhhhhhhe ettttttceh hhhhhhhhhc tccccccccc tt
Ole e 8   TDPYPSSGGE N-----ELKE AFELYDQDHN GLISSVELHK ILTRLGERYA EHDCVEMIKS VDSDGDGYVS FEEFKKMMTN KSGNNSQAEP PK
TCH2      I----GG··N ·RNDVSD··· ·····L·G· ·R··AK···S VMKN···KCS VQ··KK··SK ··I····C·N ·D·····S· GG·A------ --
Jun o 2   -·LNNKGASV K-----D··N ··KVF·R·C· ·S··AA··CH T·KSV··PCT IEESKNI·HN ··KN···LI· V···QT···S EMTDK·---- -·
CaM       ---MKDTDS· E------IR· ··RVF·K·G· ·F··AA··RH VM·N···KLT DEEVD···RE A·I····QIN Y···V···MA ·---------- --
                             **   * * * * **  **        **           *    *  ***      **  **
                                  ——Site III——                              ——Site IV——
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Fig. 2. Secondary structure prediction of olive CaBP and alignment of its amino acid sequence with EF-hand CaBPs. Amino acid residues predicted as components of α-helix (*h*), β-sheet (*e*), β-turn (*t*) and random coil (*c*) are indicated in the upper lane. The amino acid sequences of TCH2, Jun o 2 and a consensus sequence for calmodulins were obtained from the Swiss-Prot database. Identities with the olive protein are indicated by points, and gaps by hyphens. The four Ca^{2+}-binding sites are underlined. Residues conserved in all sequences are indicated by asterisks.

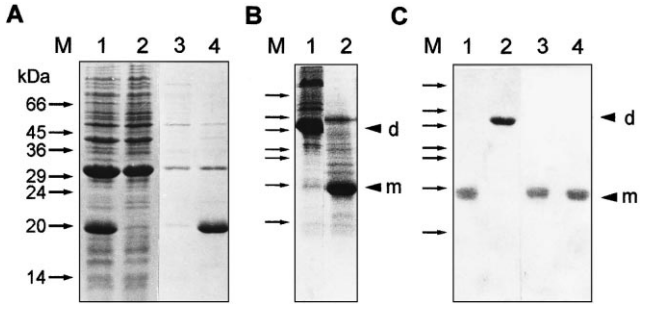

Fig. 3. Expression and isolation of recombinant olive CaBP. Coomassie blue staining of 15% SDS–PAGE of: (A) *E. coli* extracts with (1) and without (2) the specific insert after 4 h of induction; insoluble (3) and soluble (4) fractions of the cell lysate; (B) fractions eluted corresponding to 40 kDa (1) and 20 kDa (2) from Sephadex G-150 column; (C) fractions from lanes B1 (2) and B2 (1) eluted from phenyl-Sepharose column; (3, 4) samples as lanes 1, 2 respectively, under reducing conditions. M, molecular-mass markers; d, dimer and m, monomer positions.

and then eluted by addition of 1 mM EGTA. SDS–PAGE analysis of the isolated fractions is shown in Fig. 3C. The high molecular weight form shifted its mobility from 40 to 20 kDa after treatment with 2-mercaptoethanol (Fig. 3C) suggesting that it is the disulfide-bound dimer form of the low molecular weight protein. To verify this possibility, molecular characterization of these two proteins was independently achieved.

Amino acid compositions of both samples were nearly identical (data not shown), and agree with that of the protein deduced from the nucleotide sequence. NH_2-terminal Edman degradation gave the amino acid sequence AANTD for both proteins, revealing the identity of these polypeptide chains as well as the absence of methionine at the first position of the NH_2-terminal end of the recombinant protein. Mass spectrometry analysis of the high and low molecular mass forms gave 18 795 Da and 37 470 Da, respectively, which agree with those calculated for the polypeptide sequence (18 775.7 Da for the monomer, and 37 549.4 Da for the dimer).

Proteins transferred to nitrocellulose membranes, after SDS–PAGE, were incubated with $^{45}Ca^{2+}$. Fig. 4A shows the positive signal for monomer and dimer forms of the protein. CaM and parvalbumin were used as positive controls, and lysozyme and Ole e 1, a major allergen from olive pollen, as negative controls. Since the binding of Ca^{2+} to proteins in-

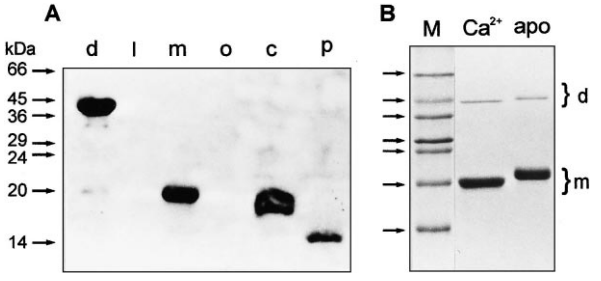

Fig. 4. Ca^{2+}-binding (A) and Ca^{2+}-dependent apparent molecular mass (B) of olive CaBP. Samples were electrophoresed, transferred to membranes and then incubated with $^{45}Ca^{2+}$. A: Dimer (d) and monomer (m), CaM (c), parvalbumin (p), lysozyme (l) and Ole e 1 (o) were also analyzed. B: The protein was incubated in the presence of 10 mM $CaCl_2$ and 2 mM EGTA (lane Ca^{2+}), and in 10 mM EGTA (lane apo), separated by SDS–PAGE and stained with Coomassie blue.

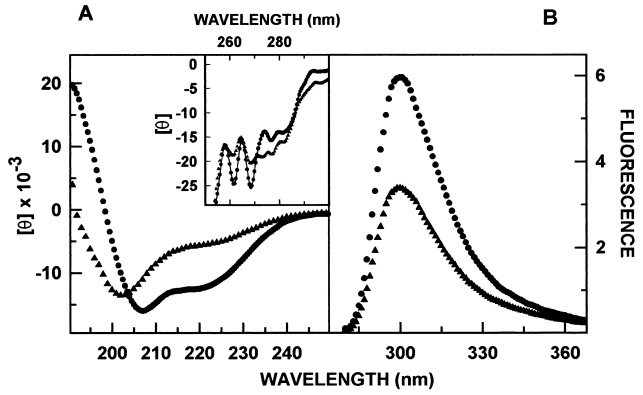

Fig. 5. Spectroscopic analyses of the Ca^{2+}-dependent conformational changes. A: Far-UV (inset, near-UV) CD spectra of olive CaBP; molar ellipticity Θ is represented. B: Fluorescence emission spectra of the protein for excitation at 275 nm. Spectra were carried out in the presence of 2 mM $CaCl_2$ (●), 2 mM EGTA (▲).

duces conformational rearrangements which can be detected by SDS–PAGE as a change in the mass/charge ratio, a Ca^{2+}-dependent electrophoretic mobility assay was performed. The apparent molecular mass of the olive CaBP shifted from 19.5 kDa to 21.9 kDa (for the monomer) and from 41.5 kDa to 44.1 kDa (for the dimer) upon addition of EGTA (Fig. 4B).

The effect of Ca^{2+} on the conformation of olive CaBP was analyzed by CD and fluorescence emission spectroscopy. Far-UV CD spectrum of the native protein (Fig. 5A) displays extreme ellipticity values at 208 and 222 nm, characteristic features of α-helical conformation. Convex constrain analysis of this spectrum gave 38% α-helix, 3% β-strand and 41% non-regular conformation. Addition of 2 mM EGTA results in a remarkable loss of regular secondary structure (Fig. 5A). Only about 3% α-helical content was deduced from the corresponding far-UV spectrum. Near-UV spectra confirmed conformational differences between Ca^{2+}-bound and Ca^{2+}-depleted forms of the protein (Fig. 5A, inset). The fluorescence emission spectrum of the native protein shows a maximum at around 300 nm, characteristic of proteins lacking Trp residues (Fig. 5B). In the presence of 2 mM EGTA, the fluorescence quantum yield of the protein is largely decreased with no shift in the wavelength of the emission maximum (Fig. 5B). Far- and near-UV CD and fluorescence spectra of the native protein were recovered after addition of 10 mM $CaCl_2$, and no significative differences were detected in the behavior of the dimer and monomer forms.

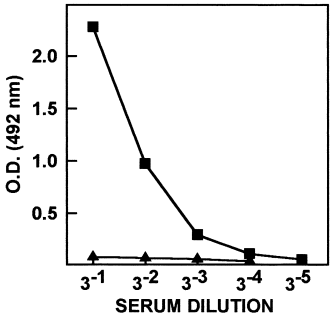

Fig. 6. IgE-binding to Ole e 8 (■) was assayed by ELISA titration using a pool of sera of patients allergic to olive pollen. Binding to a non-allergic sera pool (▲) was used as negative control.

Direct ELISA titration of the recombinant protein was performed with a pool of sera of patients allergic to olive pollen (Fig. 6), in comparison with the binding to non-allergic sera. From a population of olive-allergic patients, 11 out of them were found positive to this olive protein. We propose the name of Ole e 8 for this allergen, according to the IUIS allergen nomenclature committee [25].

4. Discussion

A cDNA encoding a novel CaBP from olive tree pollen has been cloned and sequenced. The deduced polypeptide sequence has four 12-residue segments with the EF-hand type Ca^{2+}-binding motif, such as those described for members of the CaBP family [26]. The cDNA has been expressed in *E. coli* and the recombinant protein is able to bind Ca^{2+} as well as to IgE from sera of patients allergic to olive pollen. Because of this latter property, it has been named Ole e 8. Sequence similarities between Ole e 8 and CaM or CaM-related proteins are mainly restricted to the Ca^{2+}-binding sites, identities not reaching more than 35% outside these domains. Ole e 8 shows a low identity with THC2 and Jun o 2, and the length of their NH_2- and COOH-terminal sequences, as well as the peptide connecting Ca^{2+}-binding sites II and III, are different from those of Ole e 8. In addition, the number of Cys residues is different in Ole e 8 (only one) and TCH2 or Jun o 2 (four Cys). Other pollen allergens have been demonstrated to contain Ca^{2+}-binding sites, but their polypeptide lengths and low sequence similarity discard any reasonable possibility of homology with Ole e 8. In fact, the family of polcalcins, whose members have been described in *Brassica napus* and *B. rapa*, olive tree, Bermuda grass, birch and alder [8–13], contains two EF-hand sites in their short molecules (around 9 kDa), and Bet v 3 from birch has three Ca^{2+}-binding sites within its 203 amino acid sequence [7]. Taking into account these data, Ole e 8 could be the first member described of a new subfamily of EF-hand CaBPs.

Ole e 8 has been expressed in *E. coli* as a single polypeptide chain, but with strong tendency to form a covalent dimer structure. The presence of a free cysteine in the polypeptide chain can explain the formation of the disulfide bond that links the protomers. The capability to form covalent or non-covalent bound dimers was described for S100 proteins, a wide family of 10–12 kDa EF-hand CaBPs [27]. The formation of the Ole e 8 dimer seems not to compromise its capacity to bind Ca^{2+}, IgE antibodies, or phenyl-Sepharose matrix, indicating that the three-dimensional structure of the protein is not significantly affected by the association process.

Ole e 8 is able to bind Ca^{2+}, exhibiting a strong conformational change between the apo- and Ca^{2+}-bound forms, which can be electrophoretically and spectroscopically detected. The apparent molecular mass of Ca^{2+}-depleted Ole e 8 is 2.5 kDa higher than that of the Ca^{2+}-bound protein, a shift that is in the range of those obtained for CaM-related proteins and CaM (between 1.5 kDa and 6.0 kDa [28,29]). Analysis of the secondary structure of Ole e 8 in the presence of EGTA shows a dramatic loss on the α-helix content. Only small changes have been shown on the secondary structure of many CaM-related proteins when Ca^{2+} is removed [30]. These changes have been localized at the α-helix ends flanking the Ca^{2+}-binding loops, when the tertiary structure rearranges from a compact lobe in the free state to an open structure in the Ca^{2+}-bound form [30]. The remarkable changes observed in Ole e 8 can not be explained in these terms, and thus we ought to consider this protein as an unusual member of the CaBP family. However, this feature of Ole e 8 does not seem to disturb the reversibility of the conformational equilibrium between its apo- and Ca^{2+}-bound forms. The olive pollen protein displays capability to establish interactions with a hydrophobic matrix in a Ca^{2+}-dependent manner. Although the biological function of most of the CaBPs is unknown, it is often possible to discriminate between buffer activators and enzyme regulators, since the latter undergo a conformational change in response to Ca^{2+}. The Ca^{2+}-binding promotes the formation of a solvent-accessible hydrophobic surface, which is involved in the interaction of the protein with their targets [29,30]. Thus, Ole e 8 should possess the ability to regulate biochemical processes by means of its regulatory EF-hand domains. Interestingly, from the 26 hydrophobic residues exposed in the Ca^{2+}-bound state of CaM [30], 24 are conserved in Ole e 8, as well as seven hydrophobic residues among the nine exposed in the Ca^{2+}-depleted state.

The role of CaBP in plants is poorly understood. A number of external stimuli, such as light and darkness, gravity, temperature, touch and wind, or phytohormones, induce Ca^{2+} influx into cytosol, which is supposed to trigger specific physiological responses via a signal transduction cascade. Therefore, sensor proteins such as CaM and CaM-related proteins would play a role in the cellular response of plants to environmental stimuli [31]. A limited number of CaM-related proteins have been reported in plants, and their subclasses are very different in sequence except at their Ca^{2+}-binding segments [32]. Interestingly, *TCH* genes were shown to be induced in *A. thaliana* as a response to mechanical and temperature stimuli, their encoded proteins TCH1, TCH2 and TCH3 being CaM and two CaM-related proteins [2]. The pollen grain, as other plant tissues, is exposed to environmental changes and should respond to external stimuli. Pollen germination and tube growth are dependent of Ca^{2+}, and the formation of a steep gradient of intracellular Ca^{2+} was found focused in the tip of the pollen tube during elongation [33]. As mentioned above, several Ca^{2+}-binding proteins have been found in pollen tissue, all involved in type I allergenic activity [7–14], and some of them being specific of pollen tissue [11,12]. Although these allergens have EF-hand loops, Ole e 8 does not show structural homology with them, thus it could constitute a new decoder of Ca^{2+} information in pollen signaling.

IgE antibody recognition was demonstrated for Ole e 8. Taking into account the similarity between EF-hand domains of different proteins, and that the involving of the Ca^{2+}-binding sites of polcalcins in IgE interactions have been proposed [11–13], Ole e 8 could be also involved in allergic cross-sensitization. The availability of Ole e 8 protein and its gene will enable us further studies on its allergenic activity and biological role in plant cell metabolism, in which there are still scarce data [34].

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