



Molecular cloning and characterization of Cup a 4, a new allergen from *Cupressus arizonica*

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ABSTRACT

Sensitization to *Cupressaceae* pollen has become one of the most important causes of pollinosis in Western countries during winter and early spring. However, the characterization of the extracts, the allergens involved and the cross-reactivity with other pollen sources still remain poorly studied; in the case of *Cupressus arizonica* only two allergens have been described so far. A new allergen from *C. arizonica* pollen, Cup a 4, was cloned and expressed in *Escherichia coli* as an N-terminally His-tag recombinant protein that was characterized biochemically, immunologically and by circular dichroism spectroscopy. The new allergen has high sequence identity with Prickly Juniper allergen Jun o 4 and contains four EF-hand domains. The recombinant protein has structural similarities with other calcium binding allergens such as Ole e 3, Ole e 8 and Phl p 7. Cup a 4 is expressed in mature pollen grains and shares antigenic properties with the recombinant form. Sera from 9.6% *C. arizonica* allergic patients contain specific IgE antibodies against recombinant Cup a 4.

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1. Introduction

Species of the *Cupressaceae* family are an important cause of allergic pollinosis in Mediterranean countries, Japan and South Western United States [1]. In the general population the prevalence fluctuates from 0.6% to 9.6% (reviewed by Charpin [1]) and from 9% to 35% among allergic patients [1]. The explanation for the differences found between different studies may rely in the high variability of the potency and protein content which generally characterize cypress pollen extracts. In any case, there is a general consensus that the prevalence of this pollinosis has constantly been rising in the last 10–15 years [1]. Such an increase seems to be correlated with an increased exposure to cypress pollen due to the frequent use of cypress trees in landscaping, the period of pollinization of cypress trees (January–March) which may mask allergic symptoms as a common winter cold [2], and a possible underestimation of the existing prevalence related to the use of low potency extracts for diagnosis.

Diagnosis of cypress pollen allergy is strongly dependent on the effectiveness of pollen extracts. Standardization of these extracts is complicated due to the characteristics of cypress pollen (low pro-

tein content, less than 3%, and high carbohydrate concentrations [3]). Ideally, in order to obtain a well characterized extract all the allergenic components should be identified and immunologically and biochemically described. Although six protein components from *Cupressus arizonica* pollen extracts have been identified as having IgE reactivity [4] only two allergens have been cloned, Cup a 1 [5] and Cup a 3 [6]. Cloning and expression of the allergenic proteins present in *C. arizonica* pollen may provide a useful tool with applications in both diagnostic and therapeutic fields.

The objective of this study is the identification, molecular cloning, expression and characterization of Cup a 4. The results presented in this paper reveal that recombinant Cup a 4 is an IgE binding protein with a predominant α -helix conformation.

2. Materials and methods

2.1. *C. arizonica* pollen

Pollen was generously donated by Dr. Raddi from the Institute for the Pathology of Forest Plants (Florence, Italy). Pollen contamination was excluded by controlled collecting conditions and by microscopic analysis (purity >99%). Pollen was dried and stored at –70 °C until use.

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2.2. *C. arizonica* pollen extracts

Two types of extracts were used in this paper. Pollen extracts were prepared in native conditions [3] and denaturing conditions by the phenol/SDS method for high polysaccharide-containing recalcitrant tissues [7]. Protein content of both extracts was determined using the commercial BioRad Protein Assay Dye Reagent (BioRad, Italy).

2.3. Molecular cloning of *Cup a 4*

RNA from *C. arizonica* pollen was obtained as previously described [8]. Forward and reverse primers were designed based on known *Juniperus oxycedrus* sequences of allergen Jun o 4 (previously Jun o 2) (accession number AF031471) [9]: forward primer 5'-AATAAATAGACATACGCGCACCAAG-3'; reverse primer 5'-TGTC TTCCAGTTAGGGTTTGCA-3'. The amplified product was cloned into pCR®II-TOPO-TA vector (Invitrogen, USA) and transformed into *Escherichia coli* XL-1 Blue competent cells. Positive clones were selected by PCR and sequenced.

2.4. Expression and purification of recombinant *Cup a 4* (rCup a 4)

Forward and reverse primers targeting the 498 bp open reading frame were designed based on the obtained sequence: forward primer 5'-GGGGATCCATGGACGAAGTCCGTCAAG-3'; reverse primer 5'-GGAAGCTTCATTGCTTATCAGTCA-3' (BamHI and HindIII restriction sites are underlined). The amplified product was cloned into pQE-30 expression vector (Qiagen, UK) and transformed into *E. coli* M15 competent cells. Correct insertion sense and reading frame were confirmed by sequence analysis. Expression and purification in native conditions were performed according to the manufacturer's instructions (Qiagen). Protein purity was analyzed by SDS-PAGE.

2.5. *Cup a 4* sequence analysis

Sequence analysis was performed using ProtParam (physicochemical analysis) [10], ClustalW algorithm (multiple alignments) [11], SignalP 3.0 (signal peptide prediction) [12] and NetPhos 2.0 (phosphorylation site prediction) [13].

2.6. MALDI-TOF molecular weight determination and peptidic signature identification of SDS-PAGE protein bands

All proteomic analysis was carried out in the 'CBMSO Protein Chemistry Facility', a member of ProteoRed network. Bands of interest were cut manually and digested *in situ* with trypsin as previously described [14]. The purified protein and an aliquot of the trypsin digestion supernatant were analyzed directly by MALDI-TOF mass spectrometry as previously described [14]. Obtained mass spectra from the digested band were used as peptidic signatures for the identification of proteins using Mascot and Profound search engines.

2.7. CD spectroscopy

FAR-UV CD spectra were recorded using a JASCO spectropolarimeter, model J-600 (JASCO Europe SLR), equipped with a NESLAB RTE-100 water bath interfaced to a computer. Samples were prepared in 25 mM phosphate buffer, pH 7.4, at a protein concentration of 20 µM. Wavelength scans from 190 to 250 nm were performed at 25 °C as previously described [15]. Calcium induced changes in secondary structure were observed after CaCl₂ addition to a final concentration of 230 µM.

To test the thermal stability of the protein, CD spectra were recorded at 20 °C, 50 °C and 95 °C and at 20 °C after heating to 95 °C. The CD spectrum of the protein recorded in the presence of 6 M guanidinium hydrochloride at 20 °C was taken as a control of complete denaturation.

Protein CD spectra were analyzed for percentages of secondary structure using the DICHROWEB [16] server to apply the analysis program CONTINLL [17] in combination with the reference set SP175 [18].

2.8. Generation of rCup a 4 antiserum

Two New Zealand White Rabbits were used to generate a polyclonal antibody against rCup a 4. One hundred micrograms of purified protein were mixed with 500 µg CpG motif containing oligonucleotide 5'-TCGTCGTTAACGTTGTCGCCTT-3' and adsorbed onto 0.3% aluminum hydroxide. Four doses were administered subcutaneously on days 0, 14, 21 and 40. Five days after the fourth dose, serum was collected and the animals were euthanized. Prior to the first immunization, serum was collected from each rabbit to serve as negative control.

2.9. Human sera

Patients were selected in the context of the EU CRAFT Cyprall project QLK-CT-2002-71661. Sera from 177 patients sensitized to cypress pollen were obtained after written consent. All patients were positive by skin prick test to *C. arizonica* extract prepared in native conditions.

2.10. Western blot

Recombinant protein (1.5 µg) and pollen extracts (30 µg total protein) were separated by SDS-PAGE and transferred onto a PVDF transfer membrane (Millipore, USA) using a tank transfer system. Primary antibodies were diluted as follows: rabbit sera, 1:20,000; human sera 1:50 for IgG detection and 1:2 for IgE detection. Detection was performed with goat anti-rabbit IgG-AP, goat anti-human IgG-AP or goat anti-human IgE-AP (Invitrogen) developing the results with BCIP/NBT Kit (Invitrogen).

2.11. Reverse enzyme allergosorbent test (REAST) procedure

C. arizonica extract prepared in native conditions and rCup a 4 were conjugated to biotin and used in REAST procedures as previously described [19]. Results are determined by interpolating optical density values on an IgE standard curve (International standard (WHO IgE IRP) titrated in terms of total IgE). Positivities are grouped in five classes according to IgE concentration: Class 1 (0.5–1 kU/L), Class 2 (1–2.5 kU/L), Class 3 (2.5–10 kU/L), Class 4 (10–25 kU/L) and Class 5 (>25 kU/L).

3. Results

3.1. Cloning and sequencing of *Cup a 4*

Synthesized cDNA from *C. arizonica* pollen was used in PCR reactions that yielded a 714 bp amplified product. This product was cloned into pCR®II-TOPO TA vector and sequenced (Accession number GU015025). The sequenced region comprised a 498 bp open reading frame coding for a protein of 165 amino acids (Fig. 1A). The calculated molecular mass of the protein is 18037.9 Da and has a theoretical isoelectric point of 4.36. The deduced amino acid sequence shows four EF-hand calcium binding motifs in positions 35–47, 71–83, 104–116 and 140–152. After sequence analysis, no

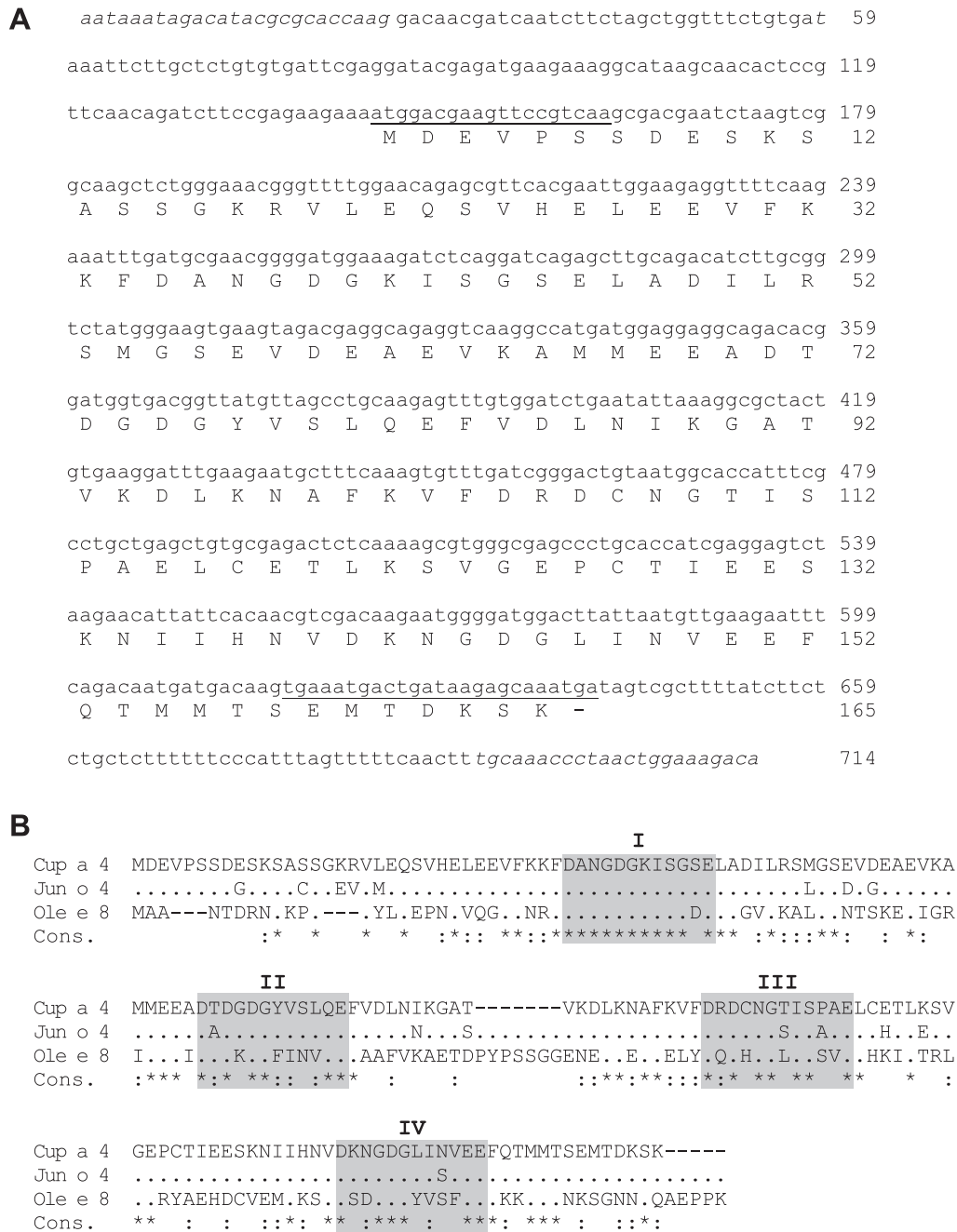


Fig. 1. Molecular cloning of Cup a 4. (A) Nucleotide and deduced amino acid sequence of Cup a 4. Primer binding sites for cDNA amplification are in italics, primer binding sites for rCup a 4 cloning are underlined. The stop codon is marked as a dash (-). (B) ClustalW multiple sequence alignment of Cup a 4 with Jun o 4 and Ole e 8. Shaded areas indicate the EF-hand domains. Identical residues are marked as (.) and gaps are marked as a dash (-). In the consensus sequence: = identical residues and : = conserved substitutions.

phosphorylation sites were found, and signal peptide analysis yielded no cleavage sites. Alignment of the deduced amino acid sequence with previously described Jun o 4 [9] resulted in a 90.30% identity and a 95.15% similarity (Fig. 1B). When the sequence was aligned with Ole e 8, an olive allergen that also contains four EF-hand calcium binding motifs [20], 37.08% identity and 61.24% similarity were observed (Fig. 1B).

3.2. Expression and purification of rCup a 4

After the correct sense and reading frame of the pQE30-Cup a 4 construct was confirmed (Accession number EF523472), rCup a 4

was expressed in *E. coli* as an N-terminally 6 × His-tagged protein. Bacterial culture induction gave rise to a prominent protein band of an apparent molecular weight of ~24 kDa in SDS-PAGE (Fig. 2A). Rupture and fractionation of the cells showed that the expressed protein resided almost entirely in the soluble phase (Fig. 2A). The purified recombinant protein showed a band of the same weight than after culture induction when analyzed by SDS-PAGE (Fig. 2A), yielding 21 mg of purified protein per liter of bacterial culture. After MALDI-TOF analysis of the purified rCup a 4, two species were detected, a predominant one with a molecular weight of 19,380 Da and a less abundant species of 38,670 Da. These molecular weights are in agreement with those calculated

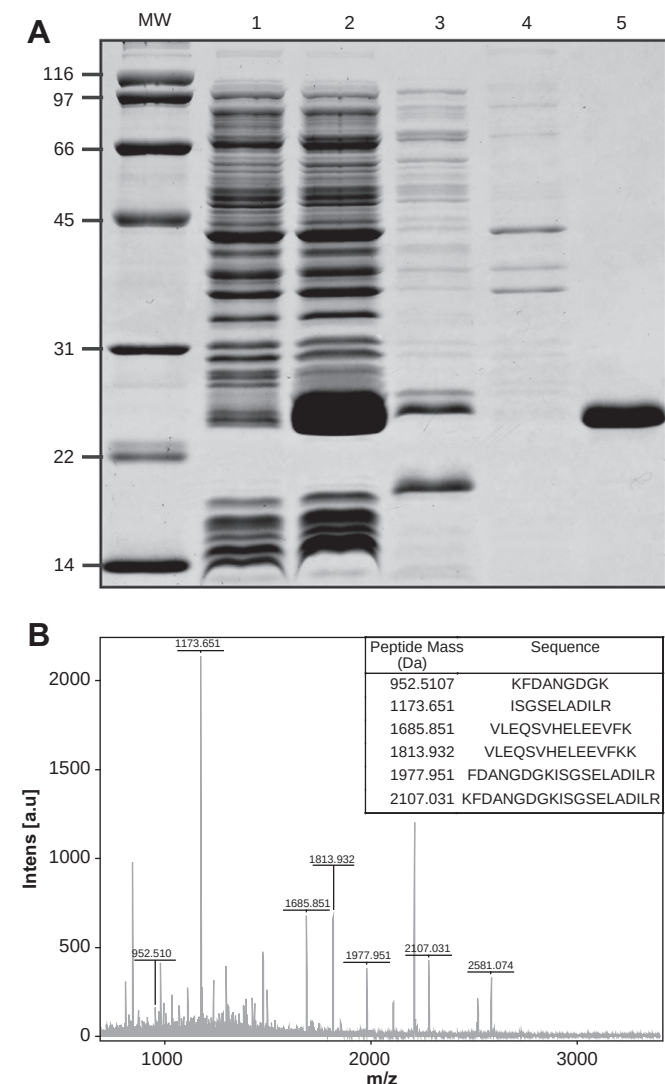


Fig. 2. Expression and purification of rCup a 4. (A) Coomassie staining of 12% SDS-PAGE. (1) Non induced control; (2) IPTG induced cell lysate; (3) soluble and (4) insoluble fractions of the cell lysate; (5) purified rCup a 4. (B) Peptidic map of rCup a 4 obtained after tryptic digestion. Identified peptides and their size in Da are underlined.

for the monomer and dimer forms of the recombinant protein (19,368 Da and 38,736 Da, respectively). The identity of the purified protein was confirmed after tryptic digestion followed by MALDI-TOF analysis; seven of the obtained peptidic signatures matched with peptides present in the rCup a 4 deduced sequence (Fig. 2 B).

3.3. Secondary structure analysis

The far-UV CD spectrum of rCup a 4 at 20 μ M and 20 $^{\circ}$ C displays extreme ellipticity values at $\sim$ 208 and $\sim$ 222 nm, indicating that it is a folded protein that contains a significant amount of α -helical structure and low β -strand content (Fig. 3). The structural conformation of rCup a 4 in phosphate buffer is as follows: 35.6% α -helix, 8.8% β -strand, 12.3% turn and 43.3% random coil (NMRSD = 0.063). When secondary structure was calculated for the recombinant protein in the presence of 230 μ M CaCl_2 , the results were 47.3% α -helix, 4.1% β -strand, 10.3% turn and 39.2% random coil (NMRSD = 0.061). rCup a 4 showed high stability to heating, retaining 40% of its molar ellipticity at 222 nm at 95 $^{\circ}$ C. After cooling, the

recombinant protein returned to its original state, recovering 91% of its molar ellipticity at 222 nm. rCup a 4 lost its secondary structure in the presence of 6 M guanidinium hydrochloride (Fig. 3B).

3.4. Presence of Cup a 4 in *C. arizonica* extracts

Sera from preimmune rabbits did not recognize any bands when tested against the pollen extract or the recombinant protein (data not shown). The sera of rabbits immunized with rCup a 4 reacted against a $\sim$ 24 kDa band corresponding to rCup a 4 (Fig. 4A, lane 1), and weakly recognized two higher molecular weight bands of 45 and 60 kDa.

Since no reactivity could be detected when the hyperimmune serum was tested against pollen extracts prepared in native conditions (not shown), an alternative protein extraction procedure was performed. This procedure is carried out in denaturing conditions and removes most of the polysaccharides in the pollen. When tested against this protein extract, the hyperimmune serum recognized a band (Fig. 4B, lane 1) of similar molecular weight than that of rCup a 4 (Fig. 4A, lane 1).

3.5. Reactivity of patients' sera against Cup a 4

Sera from 177 *C. arizonica* allergic patients were analyzed by REAST for IgE binding to *C. arizonica* native protein extract and rCup a 4. All these sera reacted with the pollen extract with positivity levels ranging from classes 1 to 5 (data not shown). IgE binding was detected in 17 out of 177 samples (9.6%), 8 of them Class 1, 3 Class 2, 4 Class 3 and 2 Class 4.

A pool of the rCup a 4 positive sera was used to analyze IgG and IgE reactivity to the recombinant protein by Western blot (Fig. 4A). The pool of sera showed IgE reactivity to the recombinant protein, recognizing rCup a 4 as well as two weak recognition bands in the 45–60 kDa range. IgG from the same pool of sera also recognized rCup a 4 and two bands in the 66–80 kDa range. A pool of sera from non allergic patients did not show IgG or IgE reactivity to rCup a 4, although it recognized the same two high molecular weight bands that were observed when the positive pool was tested.

When the pool of rCup a 4 positive sera was tested for reactivity against the protein extract prepared in denaturing conditions (Fig. 4B, lane 2) a band of similar size (24 kDa) than the one recognized by the rabbit hyperimmune serum was observed. The pool of sera also showed high IgE reactivity against six protein components ($\sim$ 45, 43, 21, 20, 18, 14 kDa). Weak reactivity was observed against proteins in the 31–38 kDa range and a group of high molecular weight proteins.

4. Discussion

C. arizonica is a representative member of the *Cupressaceae* family, native to the Southwest of North America [21]. It is being widely used as a reforestation plant in the Mediterranean Basin [5]. In this paper we describe a new protein from the Arizona cypress. The high sequence identity of this protein with Jun o 4, along with the IgE binding properties of the recombinant protein, allowed us to classify it as a new allergen from the Arizona cypress. According to the WHO/IUIS nomenclature we have named this new allergen Cup a 4. Previous studies have shown the cloning and expression of two major allergens from *C. arizonica*, Cup a 1 and Cup a 3 [5,6]. These allergens have high sequence identity with group 1 and 3 allergens from *Cupressus* and *Juniperus* genera. Based on these facts, primers were designed using the group 4 allergen from *J. oxycedrus* Jun o 4 as a template, to clone the homologous *C. arizonica* allergen. The cloned cDNA encodes an 18 kDa protein with a high sequence identity to Jun o 4. Both proteins contain four

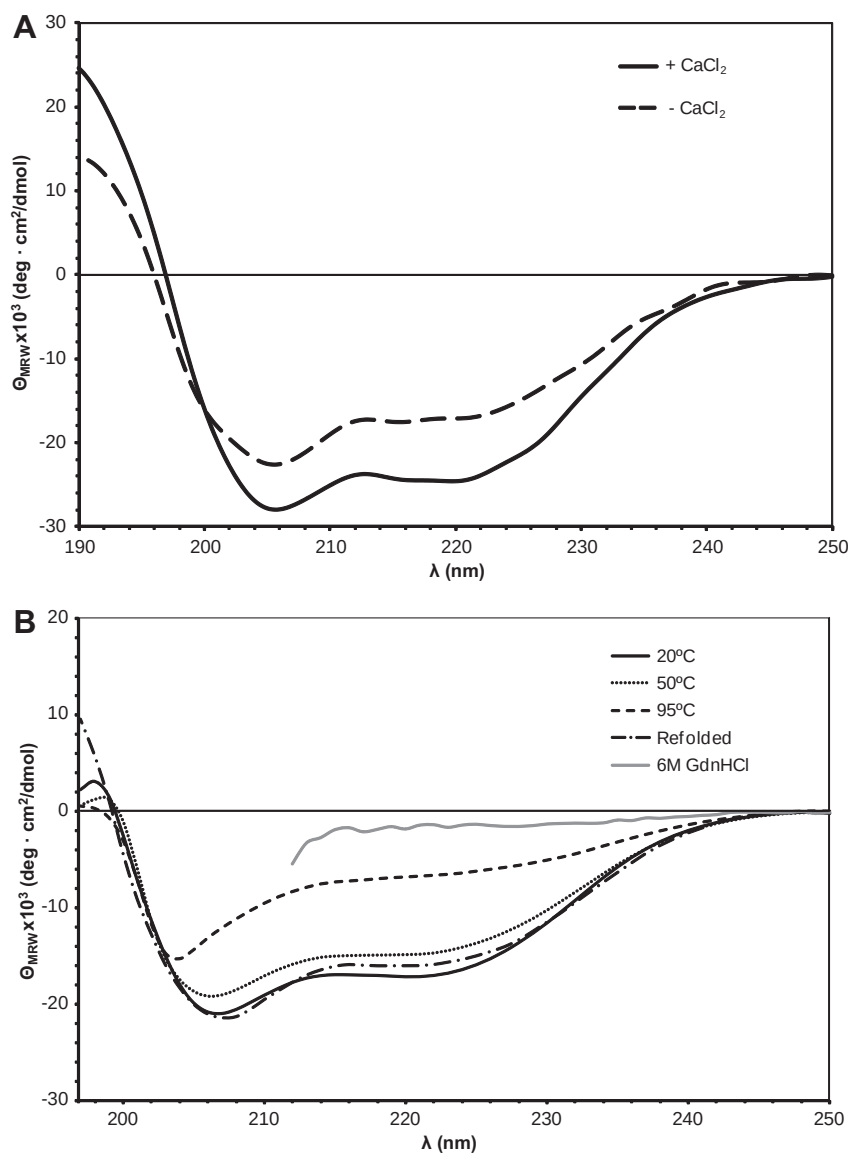


Fig. 3. Far UV CD spectra of 20 μ M rCup a 4. (A) In the presence or absence of CaCl_2 . (B) At 20 $^{\circ}\text{C}$, 50 $^{\circ}\text{C}$, 95 $^{\circ}\text{C}$, at 20 $^{\circ}\text{C}$ in the presence of 6 M guanidinium hydrochloride (GdnHCl) and at 20 $^{\circ}\text{C}$ after cooling (refolded).

canonical EF-hand domains. The olive allergen Ole e 8 has also four EF-hand domains even though the sequence identity with the cloned cDNA is lower. The data presented showed that the highest similarity between these three proteins was found in the first of the four EF-hand domains, where it reached 92%.

Expression of Cup a 4 as a recombinant protein in *E. coli* yielded high quantities of soluble protein. When analyzed by SDS–PAGE, the purified protein had an apparent molecular mass 5 kDa higher than expected, a difference that may be associated with irregular migration patterns of histidine-tagged proteins. The exact molecular weight was determined by mass spectrometry and the identity of the recombinant protein was further confirmed after tryptic digestion and peptide signature analysis.

CD data revealed that the protein has a significant α -helical content and low β -strand structure. Cup a 4 possesses 4 EF-hand domains, and the addition of CaCl_2 to the recombinant protein resulted in an increase in α -helical secondary structure. These data suggest that rCup a 4 has Ca^{2+} binding properties, an indication that it could be included in the polcalcins family. Several studies have established that the proteins belonging to this family share

a high level of cross-reactivity. The polcalcins family contains 35 pollen allergens having two, three or four EF-hand domains [22]. Previous studies regarding thermal stability of three allergenic polcalcins, Phl p 7, Ole e 3 (two EF-hands) and Ole e 8 (four EF-hands) [23,24] agree with the results shown in this paper: rCup a 4 is stable at high temperatures and the small changes in secondary structure induced by heat are largely reversible.

The apparent molecular weight of the band detected when rabbit anti Cup a 4 hyperimmune serum was tested against the protein extract obtained in denaturing conditions does not agree with the calculated weight of native Cup a 4. This fact can be explained by protein aggregation and/or post translational modifications. The high molecular weight bands observed when testing sera for IgG binding to rCup a 4 are most likely due to reactivity against *E. coli* contaminant proteins that must have been carried over during rCup a 4 purification. The obtained results suggest that Cup a 4 is expressed in mature pollen and that it shares antigenic properties with the recombinant form of the protein. Since Cup a 4 was only detected in the protein extract prepared in denaturing conditions it is most likely that this protein is present in very low

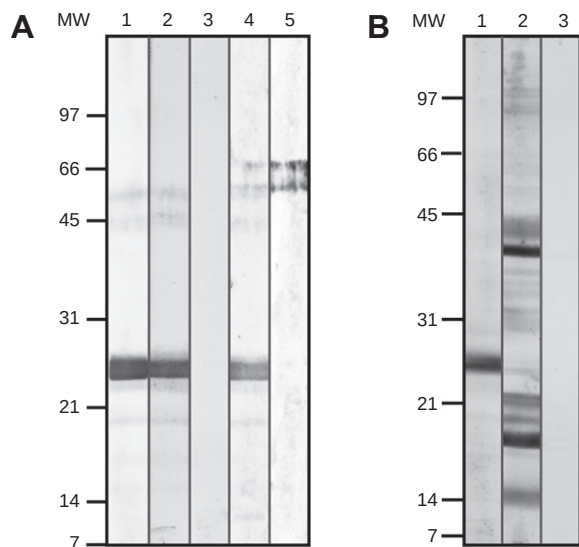


Fig. 4. Western blot analysis. (A) (1) rCup a 4 recognition by IgG in hyperimmune rabbit anti-rCup a 4 serum; (2) rCup a 4 recognition by human IgE from a pool of *C. arizonica* allergic patients' sera; (3) rCup a 4 recognition by human IgE from a pool of non allergic sera; (4) rCup a 4 recognition by human IgG from a pool of *C. arizonica* allergic patients' sera; (5) rCup a 4 recognition by human IgG from a pool of non allergic sera. (B) (1) Recognition of Cup a 4 in *C. arizonica* pollen denatured protein extract by IgG in hyperimmune rabbit anti-rCup a 4 serum; (2) denatured protein extract recognition by human IgE from a pool of *C. arizonica* allergic patients' sera; (3) denatured protein pollen extract recognition by human IgE from a pool of non allergic sera.

quantities in the extract prepared in native conditions or even not present. In this context, the role of Cup a 4 in *Cupressaceae* pollinosis diagnosis should be further studied.

The pool of patients' sera showed IgE binding to a 24 kDa band in the extract prepared in denaturing conditions, the same size as the band recognized by the hyperimmune rabbit serum. These data, along with rCup a 4's IgG and IgE binding activities suggest that the detected protein present in this extract is Cup a 4.

The IgE binding profile of the denatured protein extract is different from previously described native protein extract profiles. In extracts prepared in native conditions the predominant IgE binding component has been identified as the 43 kDa major allergen Cup a 1 [2–5,25]. A recently reported study [26] has described a new 35 kDa predominant IgE recognition band. In the denatured protein extract used in this paper the highest reactivity was observed against two bands of 43 and 18 kDa, both of them described by Di Felice et al. [4]. In addition to these bands, several components showed IgE reactivity in the denatured protein extract, including 24 kDa Cup a 4, a 21 kDa component (possibly Cup a 3 [6]) and two new low molecular weight protein components of 20 and 14 kDa. These findings may indicate that in whole pollen, allergenic proteins may be present that are not detectable in the extracts commonly used for diagnosis or specific immunotherapy.

Due to the fact that large quantities of recombinant Cup a 4 can be obtained, further structural characterization of this allergen may be carried out together with a fine analysis of the potential Ca^{2+} binding sites of the protein and its allergenic properties. Since Cup a 4 is an allergen that may be especially relevant for those patients living in the Mediterranean basin, the cloning and characterization of this allergen from *C. arizonica* could be considered as a useful tool for extract standardization. Cup a 4 may be also considered as an additional potential new diagnostic marker and candidate for allergen specific immunotherapy.

5. Conclusions

We have cloned a new allergen from *C. arizonica* pollen, Cup a 4. Expression and characterization of the recombinant form may provide a useful tool for *C. arizonica* extract standardization and diagnosis of *Cupressaceae* pollinosis.

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