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

Yago Pico de Coaña [a,*], Nuria Parody [a,b], Miguel Ángel Fuertes [a], Jerónimo Carnés [b], Daniela Roncarolo [c], Renato Ariano [d], Joaquín Sastre [e], Gianni Mistrello [c], Carlos Alonso [a]

[a] *Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Madrid, Spain*
[b] *R&D Department, Laboratorios LETI S.L., Madrid, Spain*
[c] *Lofarma S.p.A., Milan, Italy*
[d] *Allergy Unit, Bordighera Hospital, Imperia, Italy*
[e] *Fundación Jiménez Díaz, Servicio de Alergia, Madrid, Spain*

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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.

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 protein 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.

* Corresponding author. Fax: +34 911964420.
E-mail addresses: ypico@cbm.uam.es, yagopico@gmail.com (Y. Pico de Coaña).

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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 TTTCCAGTTAGGGTTTGCA-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′-GGGGATCCATGGACGAAGTTCCGTCAAG-3′; reverse primer 5′-GGAAGCTTTCATTTGCTCTTATCAGTCA-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 $CaCl_2$ 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) titered 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