

Molecular cloning of major allergen from *Cupressus arizonica* pollen: Cup a 1

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Summary

Background The family Cupressaceae is a relevant source of allergens that causes winter respiratory allergies. Cloning and sequencing the major antigen of *Cupressus arizonica* is important for a better diagnosis and treatment of sensitized patients.

Objective To obtain a full-length complementary DNA for Cup a 1, the major allergen of *Cupressus arizonica* pollen. It was cloned and sequenced and the recombinant protein was expressed.

Methods Messenger RNA from *Cupressus arizonica* pollen was obtained and the Cup a 1 sequence was established using a 3'-RACE system and primers based on the N-terminal amino acid sequence. Recombinant Cup a 1 was cloned in pBluescript and expressed in a glycosylated form in rabbit reticulocytes. The cDNA was subcloned in pGEX-5X-1 and expressed in *Escherichia coli* as a fusion protein with GST.

Results Recombinant Cup a 1 is highly homologous with the major allergens of mountain cedar (Jun a 1), Japanese cypress (Cha o 1) and Japanese cedar (Cry j 1). Cup a 1 contains three potential N-glycosylation sites that are different from those found in Jun a 1 and Cry j 1. The cloned protein contains a pectate lyase active site identical to those of Cry j 1 and Jun a 1. The IgE from patients' sera recognizes recombinant Cup a 1, and this reactivity is higher with the glycosylated protein.

Conclusion Cup a 1 has been cloned and sequenced. As expected, the high degree of homology with Cha o 1, Jun a 1 and Cry j 1 explains the cross-reactivity of conifer pollens. Different IgE reactivity with the glycosylated and non-glycosylated protein suggests the importance of carbohydrate moieties in the IgE binding site.

Keywords: *Cupressus arizonica*, Cup a 1, cDNA cloning, *Cupressus arizonica* major allergen, pollen RNA extraction

Clinical and Experimental Allergy, Vol. 30, pp. 1750–1758. Submitted 31 January 2000; revised 31 March 2000; accepted 9 May 2000.

Introduction

Allergic diseases are the most prevalent of human disorders, affecting almost 15% of the population in developed countries. Pollen from the Cupressaceae family is an important cause of winter respiratory allergies. *Juniperus ashei*, or mountain cedar, is the main allergen source in Texas (USA) [1], and *Cupressus sempervirens* and *Cupressus arizonica* produce pollinosis in Mediterranean areas such as France, Italy, Israel and Spain [2–5].

Cupressus arizonica pollinates in January and February and partially overlaps with the pollination of *Cupressus sempervirens* (from February to the end of March), making a long season for the sensitized patients who cross-react to both pollens [6]. The related species *Cryptomeria japonica* (Japanese cedar) is a major allergen source in Japan [7].

Increasing attention has been paid to cypress pollen allergy due to the widespread diffusion of *Cupressus* species. In fact, *C. arizonica* is used in many Mediterranean countries for ornamental purposes in gardens and parks, and also as wind and noise barriers.

Subiza *et al.* [5] reported that the prevalence of positive

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skin prick tests for *C. arizonica* and *C. sempervirens* may fluctuate from 0 to 23% in areas with identical climatic conditions and similar pollen counts. One of the reasons for this discrepancy is the low quality of the extracts because of the special characteristics of the grain wall and the high degree of carbohydrates which hamper the production of a soluble extract to be used in *in vitro* and *in vivo* tests [8].

Di Felice *et al.* [9] have identified the IgE-reactive components from arizonica cypress pollen, including a major allergen with a molecular weight of 43 kDa.

The diagnosis and treatment of IgE-mediated allergy requires the production of large amounts of pure, well-defined proteins from pollen extracts. In recent years, this drawback has been overcome by using recombinant DNA technology, which is a convenient tool for obtaining homogeneous protein preparations.

In this study, the cloning, sequencing and expression of the major allergen of *Cupressus arizonica* is reported. In accordance with the WHO guidelines on allergen nomenclature [10], the allergen was named Cup a 1. The IgE-binding properties of the recombinant protein were tested in several *in vitro* assays and compared with its natural homologues.

Materials and methods

Human sera

Human sera were obtained from subjects from several allergy departments in Madrid (Spain). These were diagnosed on the basis of clinical history, a positive skin prick test to *Cupressus arizonica* pollen extract (Prick test, ALK Abelló, Madrid, Spain) and the presence of specific IgE antibodies (determined by the UNICAP System Test, Pharmacia, Uppsala, Sweden). Serum samples were aliquoted and stored at -20°C until use.

Subjects were free from allergen-specific immunotherapy and their informed consent to participate in the study was obtained.

Control sera were obtained from non-allergic subjects with high total serum IgE levels and from healthy controls.

Cupressus arizonica pollen extract preparation

Pollen from *Cupressus arizonica* was purchased from Allergon (Angelholm, Sweden). Extract was prepared as previously described [9]. Protein concentration was measured according to the method of Bradford [11].

Isolation of total RNA

Extraction of RNA from *Cupressus arizonica* pollen was performed by different methods. Acid guanidinium

thiocyanate–phenol–chloroform extraction from crushed pollen [12], centrifugation of homogenized pollen in a CsCl cushion [13], and different commercial kits were used. None were effective as they were unable to break the pollen grain.

For this reason, a different lysis solution [8 M guanidine hydrochloride, 20 mM MES and 20 mM EDTA (Sigma, St Louis, MO, USA)] was used. Pollen (0.5 g) was mixed with the lysis solution and the mixture was frozen in liquid nitrogen and immediately thawed. The resulting slurry was homogenized (Virtishear Cyclon, Virtis, NY, USA) and passed through a QIAshredder column (QIAGEN, Izasa, Spain) to improve the sample homogenization. Total RNA was extracted with the Rneasy Kit (QIAGEN) according to the manufacturer's instructions.

The use of this lysis solution allows breakage of the pollen so that its content is released and total RNA can be isolated (Fig. 1).

N-terminal amino acid sequences

Proteins from *Cupressus arizonica* pollen extract were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Applied Biosystems, CA, USA) and 43 kDa protein, corresponding to the major antigen [9], was cut out for N-terminal sequencing. Microsequencing was performed by Edman degradation using a LF3000 sequencer with an on-line phenylthiohydantoin (PTH) amino acid analyser (System Gold, Beckman, USA). The N-terminal sequence was (D N P I D S C W R G D S N W D Q N R M K L A D C (A/V) V (G)); the analyser could not distinguish between Ala or Val in position 25. Back translation from the peptide sequence to the corresponding DNA was used in order to design two overlapping degenerated 5' primers: ARI4 [5'-GGG TCG ACG ATA ATC CCA T(A/C/T)G A(T/C)A GC] and ARI6 [5'-GGG TCG ACG A(T/C)T C(A/G)T C(A/G)A ACT GGG ATC AA]. The *Sal* I restriction site is underlined. The difference between the two primers is that ARI 6 is designed from nucleotide 13 to nucleotide 33 of the protein sequence, and ARI 4 is designed from nucleotide 1 to nucleotide 18.

cDNA synthesis and 3'-RACE

cDNA synthesis and polymerase chain reaction were performed using the 3'-RACE system (Rapid Amplification of cDNA ends method, 3'-RACE System, GIBCO BRL, USA) according to the manufacturer's instructions.

PCR was performed with sense (ARI 4) and antisense (UAP, Universal Amplification Primer, enclosed in the 3'-RACE system) primers. PCR amplification was

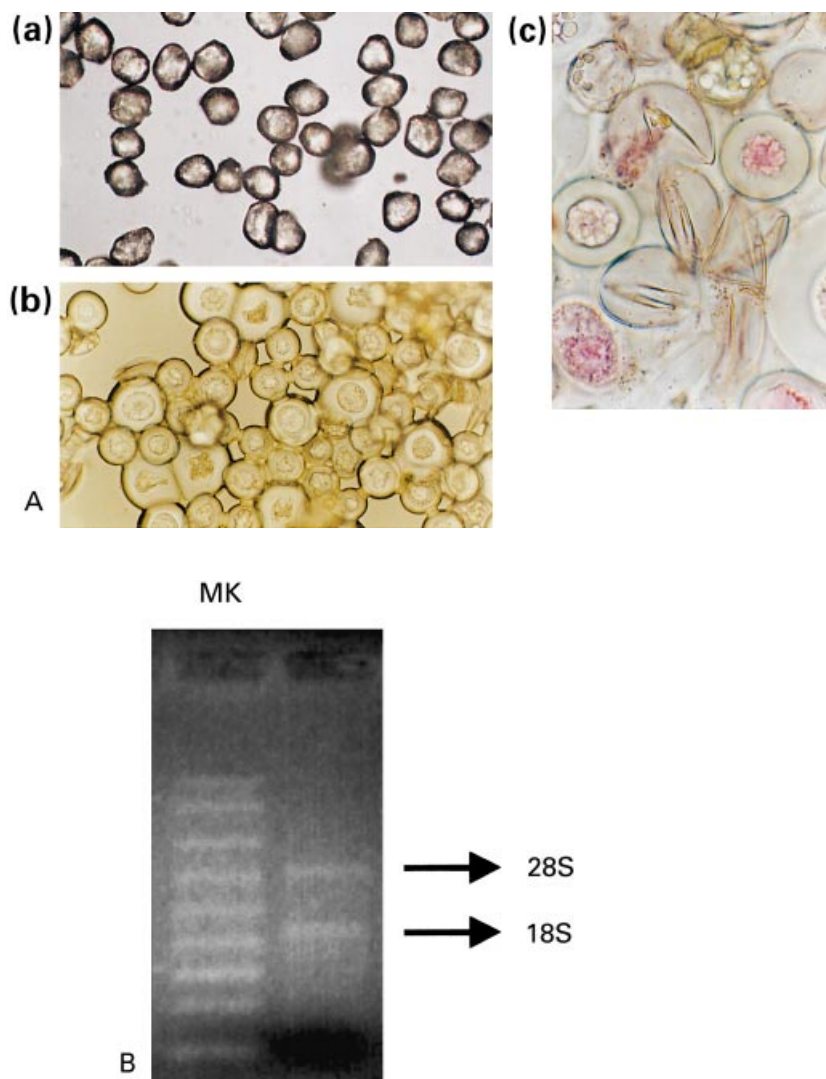


Fig. 1. (A) Photomicrograph of *Cupressus arizonica* pollen grains in different phases of the lysis reaction. Native (a), swollen (b) and broken (c) pollen grains are represented. (B) Total RNA obtained from broken pollen grains. Three different bands were observed corresponding to 28 S and 18 S (the 5S band is not well defined due to its small size).

performed for 35 cycles at 45 °C as annealing temperature. Since the 3'-RACE system used the poly(A) tail region as an initial priming site and degenerated primers were used, multiple amplification products might be synthesized. To generate a specific amplification product, the RACE product was re-amplified using a second nested 5'-primer (ARI 6) and the 3'-primer (UAP). PCR amplification was performed for 35 cycles at 50 °C as annealing temperature.

Cloning and sequencing of *Cup a 1* cDNA

PCR fragments were purified from a 1.5% low melting point agarose gel (Bio-Rad, Richmond, Ca, USA) using the WizardTM PCR Preps DNA Purification System (Promega, Madison, WI, USA), phosphorylated with T4 polynucleotide kinase (Promega) and inserted into the Eco RV site of a blunt-ended dephosphorylated pBluescript (Stratagene, La

Jolla, CA, USA). Epicurean coli XL-1 Blue MR Super-competent cells (Stratagene) were transformed according to the manufacturer's instructions.

Positive clones were selected by PCR screening with the primers T3 (5'-AAT AAC CCT CAC TAA AGG GA-3') and T7 (5'-TAA TAC GAC TCA CTA TAG G-3') (Amersham Pharmacia Biotech, Uppsala, Sweden).

DNA sequence analyses of the subsequently isolated plasmids were performed with an AbiPrism sequencer (Becton Dickinson, San José, USA).

Expression in rabbit reticulocytes

Transcription and translation reactions were performed with the TNT Coupled Reticulocyte Lysate System (Promega) in order to express the cloned protein in the pBluescript plasmid. Small amounts of protein were obtained by this method, although it enables the protein

to be glycosylated. The reactions were performed according to the manufacturer's instructions. Protein was analysed in a 12% SDS-PAGE under reducing conditions and electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad); this was exposed on a Kodak X-ray (X-OMAT UV, France) film for 24 h at -70°C in order to detect the radioactive protein which contains ^{35}S -methionine.

Immunoblotting was performed with the obtained protein from eight TNT Lysate reactions. Protein was analysed in a 12% SDS-PAGE under reducing conditions, and electrophoretically transferred onto nitrocellulose membranes (Bio-Rad) for immunostaining. The membranes were incubated for 2 h at room temperature with the patient sera diluted 1:3 in NET (0.005 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.005 M EDTA, 0.05% Triton X-100, 0.25% gelatin), and incubated with rabbit anti-human ^{125}I -IgE antibodies (RAST RIA, Pharmacia-Upjohn, Uppsala, Sweden) for 2 h at room temperature. Membranes were exposed on a Kodak X-ray (X-OMAT UV) film for 5 days at -70°C .

Expression of recombinant Cup a 1 in Escherichia coli

To obtain full-length coding DNA, the Bam HI and Sal I restriction sites were introduced at the 5' and 3' ends of the Cup a 1 encoding cDNA by PCR using as primers: sense (5'-TTT GGA TCC ACG ATA ATC CCA TCG ACA GC) and antisense (5'-TTT GTC GAC TTA TGC TAC AAC TCC AGC).

The cDNA was inserted into the Bam HI and Sal I sites of the pGEX-5X-1 plasmid vector (Amersham Pharmacia Biotech) and transformed into *E. coli* strain XL1 Blue Supercompetent cell (Stratagene). The pGEX-5X-1/ARI plasmid encodes a fusion protein (GST-Cup a 1), consisting of glutathione S-transferase and a four amino acid peptide (Ile Glu Gly Arg, a Factor Xa cleavage site), followed by the Cup a 1 polypeptide chain.

Production and purification of recombinant fusion protein and Factor Xa treatment

Overnight cultures of *E. coli* XL1-Blue containing the recombinant plasmid pGEX-5X-1/ARI were diluted 10-fold with Luria broth (LB) medium containing 0.1 mg/mL ampicillin. Cells were grown to an absorbance value of 0.7 at 600 nm. After induction with 0.1 mM isopropyl- β -D-thiogalactopyranosid (Boehringer Mannheim, Germany), cultures were maintained for 1 h and harvested by centrifugation at 7000 g for 10 min at 4°C . The pellet was resuspended in a lysis solution, containing 20 mM Tris-HCl pH 8, 5 mM EDTA, 1 mM DTT, 2 mM PMSF, 0.1 $\mu\text{g}/\text{mL}$ lysozyme, 1 $\mu\text{g}/\text{mL}$ leupeptin and 1 $\mu\text{g}/\text{mL}$ pepstatin A, and mildly sonicated. The sonicate was centrifuged at

1200 g for 10 min at 4°C and the supernatant fluid was incubated with glutathione Sepharose 4B (Amersham Pharmacia Biotech) for 30 min at room temperature. The mixture was pelleted and washed with cold PBS. The GST-Cup a 1 protein was eluted with a reduced glutathione buffer (2 M Tris-HCl pH 8, 15 mM reduced glutathione (Sigma)) and treated with Factor X_a (Promega) (10 U/100 mg protein) overnight at 22°C to yield a non-fused Cup a 1. Incubation of the GST and Cup a 1 mixture with Glutathione Sepharose 4B allows separation of the GST from the cloned protein.

Electrophoresis and immunoblotting

Recombinant Cup a 1 was analysed in a 12% SDS-polyacrylamide gel electrophoresis under reducing conditions [14]. Protein was visualized by Coomassie Brilliant Blue staining, or electrophoretically-transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature in NET buffer and incubated overnight with the patients' sera diluted 1:3 in NET; they were then incubated with horseradish peroxidase-labelled goat anti-human IgE antibodies (The Binding Site, Birmingham, UK) diluted 1:1000 in NET. Alternatively, the membranes were incubated with mouse polyclonal serum raised against the 43 kDa protein from the *Cupressus arizonica* extract (diluted 1:500). Horseradish peroxidase-labelled goat anti-mouse IgG (Caltag, CA, USA) was used as the secondary antibody. The peroxidase reaction was developed for 1 min with the ECL Western Blotting reagent (Amersham Pharmacia Biotech).

Results

Cloning of Cup a 1 cDNA

Total RNA from *Cupressus arizonica* pollen was used to synthesize and amplify the Cup a 1 cDNA using a degenerate oligonucleotide primer. The primer was designed according to the N-terminal sequence of the allergen and the adapter primer that hybridized with the sequence linked to the oligodT primer used in the cDNA synthesis.

A PCR fragment of about 1000 bp was consistently obtained. This fragment was cloned in pBluescript and different clones were obtained. Two of these clones, ARI-1 and ARI-5, were positive and their sequences completely identical. Figure 2 shows the nucleotide and amino acid sequences obtained. The nucleotide sequence was submitted to the NCBI Nucleotide Database (AJ243570, Cup a 1).

A TAA stop codon was found at position 1039, which resulted in a mature protein of 346 amino acids. The 3' untranslated region is not shown.

1	gat	aat	ccc	atc	gac	agc	tgc	tgg	aga	gga	gat	tgc	aac	tgg	gat	caa	aac	aga	atg	aag	ctc	gca	gac	tgt	gtt
	D	N	P	I	D	S	C	W	R	G	D	S	N	W	D	Q	N	R	M	K	L	A	D	C	V
26	gtg	gga	ttt	gga	agc	tcg	acc	atg	gga	ggc	aaa	gga	gga	gaa	att	tac	acc	gtc	aca	agc	tca	gaa	gat	aat	cct
	V	G	F	G	S	S	T	M	G	G	K	G	G	E	I	Y	T	V	T	S	S	E	D	N	P
51	gtg	aat	cct	aca	cca	gga	act	ttg	cgc	tat	gga	gca	aca	aga	gaa	aaa	gca	ctt	tgg	ata	att	ttc	tct	cag	aaa
	V	N	P	T	P	G	T	L	R	Y	G	A	T	R	E	K	A	L	W	I	I	F	S	Q	N
76	atg	aat	ata	aag	ctc	cag	atg	cct	ttg	tat	gtt	gct	gga	tat	aag	act	att	gac	ggc	agg	gga	gca	gtt	gtt	cat
	M	N	I	K	L	Q	M	P	L	Y	V	A	G	Y	K	T	I	D	G	R	G	A	V	V	H
101	ctt	ggc	aat	ggc	ggg	ccc	tgt	ctg	ttt	atg	agg	aaa	gcg	agc	cat	ggt	att	ctc	cat	ggg	ttg	cat	ata	cac	ggg
	L	G	N	G	G	P	C	L	F	M	R	K	A	S	H	V	I	L	H	G	L	H	I	H	G
126	tgt	aat	acg	agt	gtt	ttg	ggg	gat	gtt	ttg	gta	agt	gag	tcc	att	ggg	gtg	gag	cct	gtt	cat	gct	cag	gat	ggg
	C	N	T	S	V	L	G	D	V	L	V	S	E	S	I	G	V	E	P	V	H	A	Q	D	G
151	gac	gcc	att	act	atg	cgg	aat	ggt	aca	aat	gct	tgg	att	gat	cat	aat	tct	ctc	tcc	gat	tgt	tct	gat	ggg	ctt
	D	A	I	T	M	R	N	V	T	N	A	W	I	D	H	N	S	L	S	D	C	S	D	G	L
176	atc	gat	gtt	aca	ctt	ggg	tcc	act	gga	att	act	atc	tcc	aac	aat	cac	ttc	ttc	aac	cat	cat	aaa	gtg	atg	ttg
	I	D	V	T	L	G	S	T	G	I	T	I	S	N	N	H	F	F	N	H	H	K	V	M	L
201	tta	gga	cat	gat	gat	aca	tat	gat	gat	gac	aaa	tct	atg	aaa	gtg	aca	gtg	gcc	ttc	aat	caa	ttt	gga	ccc	aat
	L	G	H	D	D	T	Y	D	D	D	K	S	M	K	V	T	V	A	F	N	Q	F	G	P	N
226	gct	ggg	caa	aga	atg	cca	agg	gca	cga	tat	gga	ctt	gta	cat	gtt	gca	aac	aat	aat	tat	gat	caa	tgg	aat	ata
	A	G	Q	R	M	P	R	A	R	Y	G	L	V	H	V	A	N	N	N	Y	D	Q	W	N	I
251	tat	gct	att	ggg	ggg	agt	tca	aat	cca	acc	att	cta	agt	gaa	ggg	aat	agt	ttc	act	gct	cca	aat	gag	agg	tac
	Y	A	I	G	G	S	S	N	P	T	I	L	S	E	G	N	S	F	T	A	P	N	E	S	Y
276	aaa	aag	gaa	gta	aca	aag	cgt	ata	ggg	tgt	gaa	aca	aca	tca	gct	tgt	gcg	aac	tgg	gtg	tgg	aga	tcc	aca	cga
	K	K	E	V	T	K	R	I	G	C	E	T	T	S	A	C	S	N	W	V	W	R	S	T	R
301	gat	gct	ttt	act	aat	gga	gct	tat	ttt	gta	tca	tgc	ggg	aaa	gct	gaa	gac	acc	aat	ata	tac	aat	agt	aat	gaa
	D	A	F	T	N	G	A	Y	F	V	S	S	G	K	A	E	D	T	N	I	Y	N	S	N	E
326	gct	ttc	aaa	gtt	gag	aat	ggg	aat	gca	gct	cct	caa	tta	aca	caa	aat	gct	gga	gtt	gta	gca	taa			
	A	F	K	V	E	N	G	N	A	A	P	Q	L	T	Q	N	A	G	V	V	A	*			

Fig. 2. Nucleotide sequence and deduced amino acid sequence of Cup a 1. A stop codon (TAA) is indicated by an asterisk. The pectate lyase-active site is represented in red and three possible N-linked glycosylation sites are in green. A putative T-cell epitope is shown in blue.

The N-terminal amino acid sequence was identical to that inferred by the nucleotide sequence, which also allowed us to determine that the amino acid in position 25 was Val. The theoretical molecular weight for the cloned protein was 37.5 kDa. This molecular weight is below that described for native major allergen as the carbohydrate content has not been taken into account. The calculated pI was 5.93.

The amino acid sequence of Cup a 1 was compared with those of Jun a 1, Cha o 1 and Cry j 1 in Table 1. Cup a 1 had a high degree of sequence identity to Jun a 1 (91%), Cha o

1 (81%) and Cry j 1 (75%), but also had a significant degree of sequence identity to pectate lyase-like proteins. The cloned protein contained a sequence (Arg²²⁹ Met Pro Arg Ala Arg Tyr Gly Leu Val His Val Ala Asn Asn Tyr²⁴⁵) that was identical to the pectate lyase active site of Cry j 1 and Jun a 1. Amino acid positions 127–129 (NTS), 157–159 (NVT) and 272–274 (NES) agree with the typical amino acid triplet Asn-X-(Ser/Thr) for a possible N-glycosylation site [15]. Several phosphorylation sites are described in the amino acid sequence: 111–114 (RKAS), a cAMP phospho-site; 12–15 (SNWD), 44–47 (TSSE),

Protein	Plants	AC*	Identity (%)
Jun a 1	(<i>Juniperus ashei</i>)	AF106662	91
Cha o 1	(<i>Chamaecyparis obtusa</i>)	D45404	81
Cry j 1	(<i>Cryptomeria japonica</i>)	JC2123	75
Pectate lyase like protein	(<i>Arabidopsis thaliana</i>)	AL022140	51
Pectate lyase	(<i>Zinnia elegans</i>)	Y09541	50
Pectate lyase	(<i>Fragaria ananassa</i>)	U63550	46
Pectate lyase homologue	(<i>Medicago sativa</i>)	U41472	46
Amb a 1	(common ragweed)	C53240	45

*Identification number of the NCBI Nucleotide Database.

Table 1 Degree of amino acid sequence identity of the allergen Cup a 1 with related pollen allergens (Jun a 1, Cha o 1, Cry j 1, Amb a 1) and pectate lyase proteins

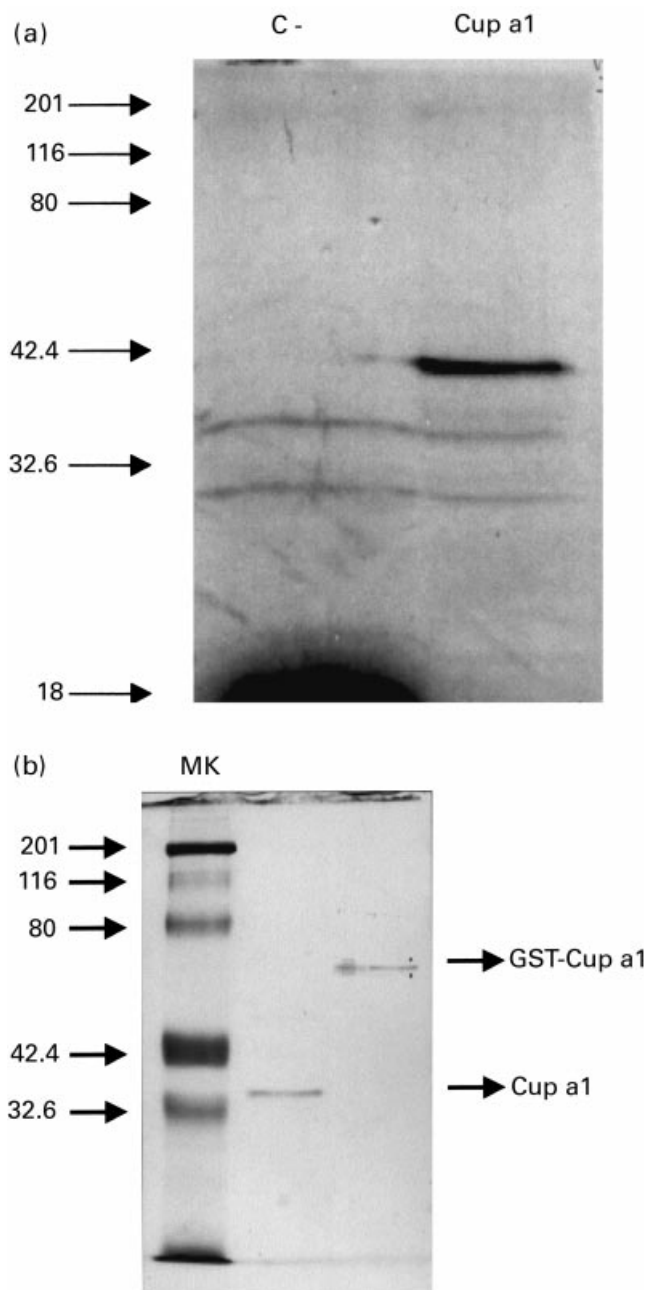


Fig. 3. (a) SDS-PAGE analysis of *Cupressus arizonica* expression performed by rabbit reticulocyte reaction (lane 2). The labelled protein bands were visualized by autoradiography. Negative control (C-) is the pBluescript plasmid without cDNA inserted (lane 1). (b) Coomassie Brilliant Blue-stained SDS-PAGE of rCup a 1 protein. Molecular weight markers (lane 1), rCup a 1 protein (lane 2) and rCup a 1-GST fusion protein (lane 3).

45–48 (SSED), 167–170 (SLSD), 206–209 (TYDD) and 298–301 (STRD), CK2-phospho-sites; 57–59 (TLR), 154–156 (TMR), 212–214 (SMK), 274–276 (SYK), 280–282 (TKR), 298–300 (STR) and 312–314 (SGK),

PKC-phospho-sites; 314–321 (KAEDTNIY), a Tyr-phospho-site. Five myristylation motifs were also found in the amino acid sequence 34–39 (GGKGGG), 125–130 (GCNTSV), 184–189 (GITISN), 265–270 (GNSFTA) and 284–289 (GCETTS).

Expression of recombinant Cup a 1 (rCup a 1)

Expression of Cup a 1 in rabbit reticulocytes was performed with the pBluescript/ARI construct. This expression yields a glycosylated protein of 43 kDa (Fig. 3a).

Expression of recombinant Cup a 1 in *E. coli* was performed with the pGEX-5X-1/ARI plasmid. A fusion protein (GST-Cup a 1) was obtained with an estimated molecular mass of 63 kDa, which agrees with an estimated molecular mass of 26 kDa for glutathione S-transferase [16] and 37 kDa for the non-glycosylated Cup a 1 (Fig. 3b), obtained after Factor Xa cleavage.

Immunological characterization of recombinant Cup a 1

Serum samples from seven allergic patients sensitized to *Cupressus arizonica* pollen and two control subjects, and a mouse antiserum obtained against the 43 kDa protein from *Cupressus arizonica* extract, were selected for IgE-immunoblot analysis of *Cupressus arizonica* extract and rCup a 1.

Serum IgE from the seven allergic patients sensitized to *C. arizonica* reacted with rCup a 1 expressed in rabbit reticulocytes (Fig. 4a). When rCup a 1 was expressed as a fusion protein in *E. coli*, only five of the same sera recognized the recombinant protein (Fig. 4b). A better recognition of rCup a 1 was observed when the protein was expressed in reticulocytes than when expressed as a fusion protein in *E. coli*. This is probably because, in the case of rabbit reticulocyte, the protein is glycosylated, indicating the role of carbohydrate determinants as antigenic structures. All the tested sera reacted with the 43 kDa protein from the *Cupressus arizonica* extract (data not shown).

Discussion

Cypress is one of the major pollen-producing plants in the Mediterranean area. From a clinical point of view, some differences have been described between patients mono-sensitized to cypress pollen and those with added polysensitization to other pollens or inhalant allergens and arizonica [5,17]. These patients have mainly oculo-nasal symptoms during winter; all had positive skin tests to both *C. arizonica* and *C. sempervirens* extracts, and also to *J. sabinoides* extract. Curiously, specific IgE for *C. arizonica* is usually lower in the monosensitized group than in those

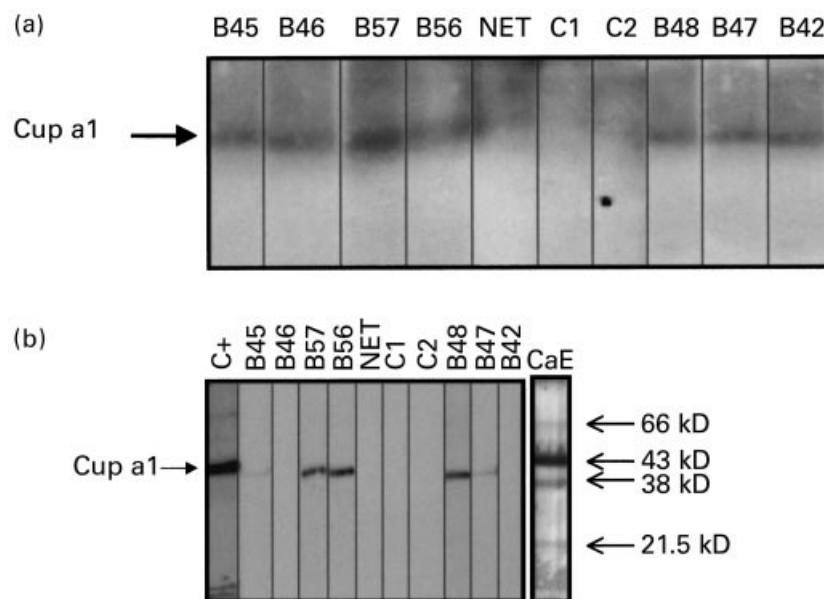


Fig. 4. (a) Immunoblotting analysis of transferred rCup a 1 expressed in rabbit reticulocytes, revealed with anti¹²⁵I-IgE antibody and visualized by autoradiography. Sera from allergic patients B45, B46, B57, B56, B48, B47 and B42 were reactive. C1 and C2 represent control sera from non-allergic patients. NET is a control using buffer instead of sera. (b) Immunoblotting pattern of IgE binding to rCup a 1 expressed in *E. coli*. Protein bands were visualized using HRPO-labelled IgE antibody and developed by ECL system. Allergic sera and controls were the same as in Fig. 4(a). C + : mouse polyclonal serum against 43 kDa protein of *Cupressus arizonica* extract was used as positive control. The last lane (CaE) represents a *C. arizonica* pollen extract after separation by SDS-PAGE in reducing conditions and silver-stained. The major allergen, a 43 KDa protein, differs in its molecular weight from rCup a 1 because of the glycosylation of the molecule.

patients with additional sensitizations. Even the values for specific IgE to *C. arizonica* in polysensitized allergic patients are lower than expected when compared with the values obtained with *J. sabinoides* and *C. sempervirens*. This is indicative of the low quality of the *in vivo* and *in vitro* diagnostic tests prepared from *Cupressus arizonica* pollen, which have always hampered the clinical diagnosis of cypress allergy. This underestimation is augmented by the coincidence of the Cupressaceae pollination period with the winter season when upper respiratory tract diseases are common.

For these reasons, it is obvious that molecular cloning of the *Cupressus arizonica* major allergen, identified as a molecule with a molecular weight of about 43 kDa, is a very useful tool for the production of large amounts of pure protein.

One of the first problems to be overcome in order to obtain non-degraded RNA from *Cupressus arizonica* pollen grains is to find an adequate way of breaking the pollen walls. Several methods have been used, the most suitable being deep-freezing of the grains in a lysis solution and, after thawing, homogenization. Figure 1A shows the different stages of the breaking process of the pollen grains and the corresponding RNA extracted (Fig. 1B).

A simple and efficient method has been used to clone the *Cupressus arizonica* major allergen that avoids the tedious screening of a cDNA library. A PCR strategy based on the known N-terminal sequence, in combination with the 3'-RACE, provided the complete cDNA. The major allergen of *Cupressus arizonica* pollen, Cup a 1, which is homologous to the major allergens of mountain cedar, Japanese cypress and Japanese cedar, was cloned and sequenced. The amino acid sequence of the mature Cup a 1 protein contained 346 residues, making it similar in length to Jun a 1, Cry j 1 and Cha o 1 [18–20].

The cDNA sequence of Cup a 1 has three N-glycosylation motifs (Asn¹²⁷, Asn¹⁵⁷ and Asn²⁷²). Two of the potential N-glycosylation sites (residues 127 and 157) were found in Jun a 1, two in Cha o 1 (127, 157) and one in Cry j 1 (residue 272). Therefore, much of the cross-reactivity among Jun a 1, Cha o 1, Cry j 1 and Cup a 1 may be due to carbohydrate moieties rather than to shared glycoprotein epitopes. Data reported by Afferni *et al.* [21] suggest that epitopes on the major allergen of *Cupressus arizonica* extract are glycosylated and lose their reactivity for IgE after deglycosylation with periodate. When an immunoblotting with patients' sera was performed, IgE reactivity was higher for the glycosylated recombinant protein, indicating that IgE

specificity for carbohydrates plays an important role in the reactivity against pollen (Fig. 4a,b).

T-cell epitopes from Japanese cedar have been described [22]. At least two major epitopes from Cry j 1 were obtained using epitope mapping with overlapping peptides and measuring the proliferation produced in specific T-cell lines. One of these epitopes (between amino acids 108–120) coincides 100% with Cup a 1, indicating the presence of a possible T-cell reactive epitope in this and other recombinant allergens from the same group [18].

The amino acid sequence corresponding to the pectate lyase-active site, also found in Cry j 1 [23] and Jun a 1, suggests that Cup a 1 may have an enzymatic activity similar to pectate lyase. The substrates of pectate lyase are pectic substances found in the cell walls of plant tissues. The physiological role of Cup a 1 in pollen may be to facilitate pollen tube growth during pollination by degrading the pistil of female flowers, as speculated for Cry j 1 [24]. The allergenic cross-reactivity among different families of plant allergens might be due to a conserved pectate lyase motif.

In conclusion, a major allergen from *Cupressus arizonica* pollen has been cloned. The high degree of sequence identity between this allergen and those of other conifers may explain some common IgE epitopes among plants. The recombinant Cup a 1 molecule may contribute to the standardization of the allergens used for diagnosis and immunotherapy. Moreover, the data corresponding to the complete amino acid sequence of Cup a 1 may facilitate experimental determination of T- and B-cell-reactive epitopes.

Acknowledgements

The authors are grateful to Dr Javier Subiza (Madrid, Spain), Dr Martín-Esteban (Madrid, Spain) and Dr Sastre (Madrid, Spain) for providing sera from allergic patients. The authors would like to thank M. del Barrio, C. Pastor and L. Vidarte for their technical support. Also, they appreciate the support of Fundación Lair (Madrid) to Ignacio Arrieta; Sociedad Española de Alergia e Inmunología Clínica to Ascensión Minguez and Fundación Conchita Rábago to Marta Rojo.

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