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Introduction

Several cypresses, junipers and cedars belonging to the Cupressaceae family represent important and increasing causes of respiratory allergies in Mediterranean areas, in the southern states of USA and in Japan [1,2].

The common cypress, *Cupressus sempervirens* (*Cups*), also known as the Italian cypress, originates from Eastern Mediterranean regions [3]. During the last three decades, *Cups* and *Cupressus arizonica* (*Cupa*) have been widely cultivated in both private and public green spaces as ornaments or functional edges. *Cupressus* pollen grains currently constitute the major source of late winter/early spring respiratory allergies, reaching to 40% of the annual pollen spectrum in some cities surrounding the Mediterranean basin [4,5].

The characterization and standardization of *Cups* pollen extracts have been the subject of several studies and have led to the identification of two *Cups* pollen allergens: (a) Cup s 1, a 43–45 kDa protein among the pectate-lyase family, currently recognized as the major cypress pollen allergen [6], and (b) Cup s 3, a 34-kDa thaumatin-like protein, reported as a pathogenesis-related (PR-5) protein [7]. Some biochemical and immunological studies also demonstrated the potential role of proteins belonging to the β -galactosidase [8] and profilin [9] families in *Cups* pollen allergy.

The characterization and standardization of *Cupa* as well as *Cryptomeria japonica* (*Cryj*) pollen extracts have already been reported. For both species, four allergens have been described (Cup a 1, 2, 3, 4 and Cry j 1, 2, 3, 4 respectively) (reviewed in [1]).

We recently showed that the use of detergents and chaotropic agents able to solubilize water-insoluble components considerably increased the diversity and quantity of extracted *Cups* pollen proteins [10]. IgE immunoblotting experiments after one-dimensional gel electrophoresis (1-DE), two-dimensional gel electrophoresis (2-DE) and double one-dimensional gel electrophoresis (D1-DE) [11] revealed differential sensitization patterns in allergic patients and evidenced new allergens in *Cups* pollen.

To optimize our sample preparation using procedures that could mimic physiological conditions [12], we compared in the present study the 2-DE patterns of Italian cypress pollen allergenic proteins obtained with various extraction protocols. IgE immunoreactive profiles of cypress sensitized patients were also investigated in two other Cupressaceae pollen extracts, *Cupa* and *Cryj*, as well as in several taxonomically unrelated pollens (grasses and angiosperm trees) in order to evaluate putative IgE cross-reactivities. Finally, a series of

biochemical and immunochemical experiments was performed, with special emphasis on a basic allergen of 14 kDa (called herein BP14) to study its distribution, its kinetics of release, its isoforms, its frequency of IgE reactivity, the nature of its IgE epitopes and the potential presence of cross-reactive carbohydrate determinants (CCDs).

Results

Salts are required to extract basic cypress pollen allergens

The capacity of water, ammonium bicarbonate buffer (AB) and NaCl/P_i to extract proteins from cypress pollen was compared. Silver staining of 2-DE gels showed that water-extractable and salt-extractable proteins gave different 2-DE map patterns (Fig. 1A–C). Additional basic spots were clearly visualized in AB and NaCl/P_i pollen extracts with pI ranging from 8.5 to 10 and *M_r* of about 14, 43 kDa (Fig. 1B,C, boxes 3 and 2, respectively). Moreover, a basic protein with an *M_r* of 60 kDa was visible on 2-DE separation obtained with NaCl/P_i extract (Fig. 1C, box 2). The 2-DE immunoblot using sera of allergic patients showed that the basic spots of 14, 43–45 and 60 kDa present in the NaCl/P_i extract were partially or totally recognized by specific IgE of sensitized patients (Fig. 1F, boxes 3 and 2, respectively). In AB extract the binding of IgE was weak (Fig. 1E).

BP14 was quickly extracted by salts immediately after adding NaCl/P_i as shown in Fig. 2A. The qualitative content of protein did not vary according to extraction duration (Fig. 2A). After 3 h of incubation in NaCl/P_i, the total protein concentration in supernatants finally reached a plateau around 94 $\mu\text{g}\cdot\text{mL}^{-1}$.

BP14 is a marker of the Cupressaceae family highly expressed in *Cups* species

Since no – or very few – proteins were observed at low *M_r* with the exception of BP14 (Fig. 1C), we used 1-DE to screen various sera (*n* = 31) from cypress-allergic patients and to compare the pollen protein patterns as well as IgE reactivities for the two taxonomically closely related trees: *Cups* and *Cupa* that cover overlapping areas in different Mediterranean environments.

As shown in Fig. 3 and as previously reported [10], two IgE binding patterns may be distinguished on the basis of the discriminative IgE reactivity to BP14. Twenty-three patients' sera (74.8%) showed IgE

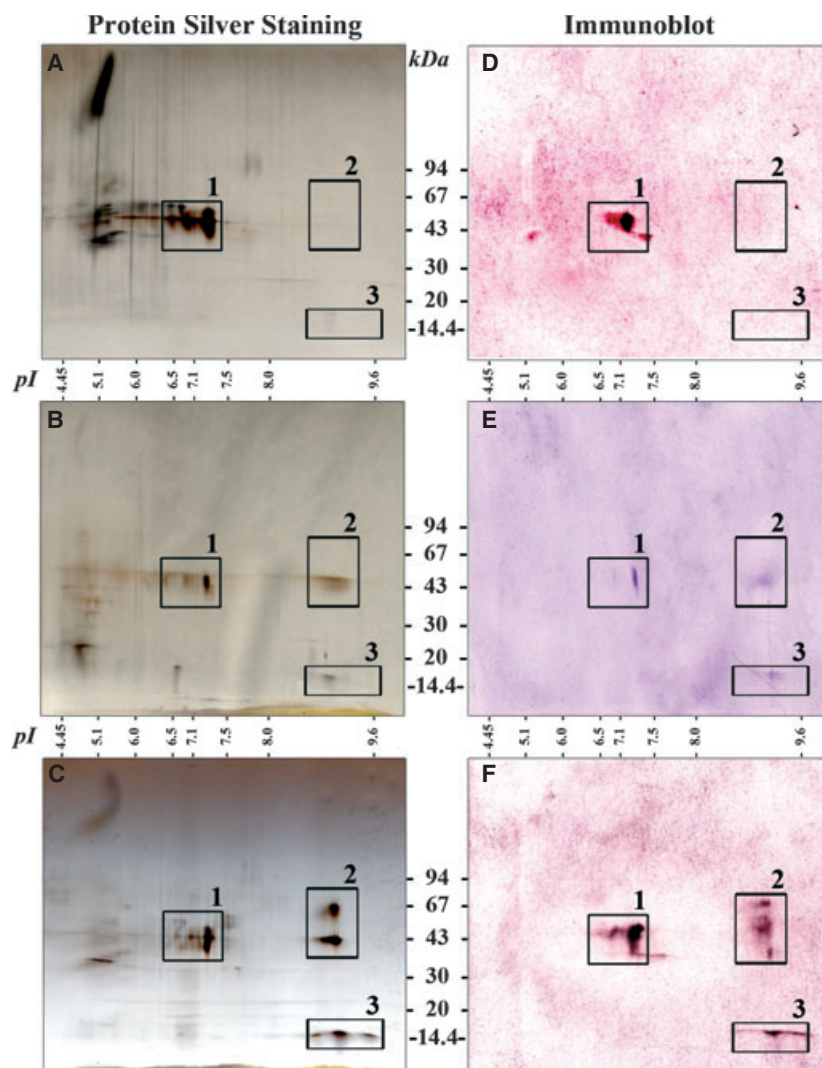


Fig. 1. 2-DE analysis of water- and salt-extractable cypress pollen proteins. The first dimension (IEF) was carried out in a pH 2–11 gradient and the second dimension in an 8–18% acrylamide gradient SDS-gel. M_r markers are expressed in kilodaltons; pI , isoelectric point. (A), (B), (C) 2-DE silver stained separations of *Cups* pollen proteins: (A) water extract; (B) AB extract; (C) NaCl/ P_i extract. (D), (E), (F) 2-DE IgE immunoblots: (D) water extract; (E) AB extract; (F) NaCl/ P_i extract. The same serum is used for the three immunoblots.

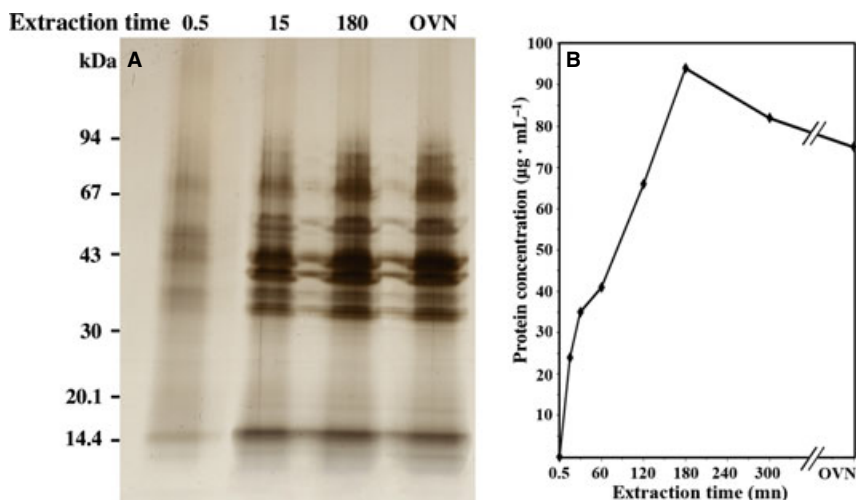


Fig. 2. Kinetics of extraction of *Cups* pollen proteins using NaCl/ P_i . First, 100 mg of *Cups* pollen was incubated for different times [0.5, 15, 30, 60, 120, 180, 300 min and overnight (OVN)] under rotation (22 °C) in 1 mL (1/10 w/v) of NaCl/ P_i . The suspension was then immediately centrifuged and supernatant was collected for (A) 1-DE SDS/PAGE analysis (0.4 μg loaded per lane except for the 0.5 min time point) and (B) the estimation of protein concentration. M_r markers are expressed in kilodaltons.

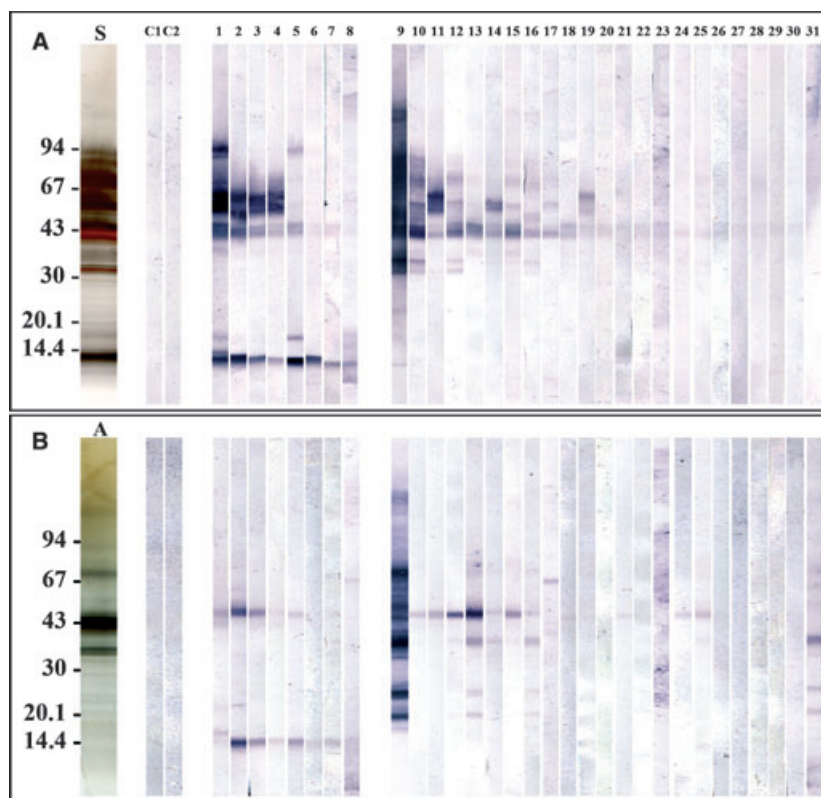


Fig. 3. IgE reactivity to *Cups* and *Cupa* NaCl/P_i pollen extracts. *Cups* (A) and *Cupa* (B) pollen extracts were separated in an 8–18% acrylamide gradient SDS-gel and blotted onto NCa. Individual sera were then incubated on strips and IgE binding was revealed. The 31 sera were provided by the same center and included 14 females and 17 males with a mean age of 31.55 (range 3–77, see Table 1). *Cups*, silver stained protein profile of NaCl/P_i *Cups* extract; *Cupa*, silver stained protein profile of NaCl/P_i *Cupa* extract; strips 1–31, screening of 31 sera from cypress-allergic patients ordered by the intensity and frequency of allergen recognition; strips 1–8, sera showing IgE reactivities against BP14; strips 9–31, sera showing IgE reactivities against HMW and no reactivity to BP14; C1, negative control with no serum; C2, negative control using the serum of a healthy non-atopic individual. *M_r* markers are expressed in kilodaltons.

reactivity only to high molecular weight (HMW) allergens (> 30 kDa) (lanes 9–31) while eight of them (25.2%) had IgE also or mainly against BP14 (lanes 1–8). BP14⁺ and HMW⁺ patients included both mono-sensitized and polysensitized profiles (Table 1).

The comparison between *Cupa* and *Cups* pollen extracts clearly showed that the level of expression of BP14 may differ among cypress species. However, despite the lack of a visible 14 kDa band in the silver staining of *Cupa* pollen extract (Fig. 3B, lane A), all sera highly IgE positive to BP14 in *Cups* pollen extracts also showed some IgE reactivity to a 14 kDa component in *Cupa* pollen grains (Fig. 3A,B, lanes 1–8).

To evaluate the presence of BP14-like proteins in other plants, NaCl/P_i pollen extracts from orchard, timothy, maize, wheat, ash, birch, pine, Italian cypress, Arizona cypress and Japanese cedar were run in paral-

lel in SDS/PAGE and immunoblotted using representative sera recognizing or not the BP14. Results are depicted in Fig. 4. Using a representative serum recognizing BP14 from *Cups*, a weak doublet reactivity around 14 kDa was also observed in *Cryj* which belongs to the Cupressaceae family and induces severe pollinosis in Asia (Fig. 4B, lane 9). The weak reactivity observed with *Cupa* and *Cryj* is in agreement with the very low BP14 content in these two pollen extracts (Fig. 4A, lanes 8 and 9). In contrast, no cross-reactivities were found with the four grass pollens (maize, wheat, orchard and timothy, Fig. 4B, lanes 1–4) or with the two angiosperm tree pollens (ash and birch, Fig. 4B, lanes 5 and 6) or with the pollen of the gymnosperm tree *Pinus sylvestris* (lane 10), a member of the Pinales order to which Cupressaceae belong. Thus, BP14 seems to be specific to the Cupressaceae family and is expressed more highly in *Cups* species than in

Table 1. Sensitizations, age and sex of the 31 patients corresponding to the 31 sera analysed in immunoblots (Fig. 3). Sensitizations were assessed using Phadia ImmunoCAP® assay.

	Serum no.	Sex	Age ^a	Sensitizations other than cypress pollen
BP14+	1	F	39	House dust mite
	2	F	26	Peach
	3	F	44	Cat, weed pollen
	4	M	7	Weed pollen
	5	M	10	
	6	F	8	
	7	F	41	Peach
	8	M	12	Grass pollen
HMW+	9	M	42	Grass, mimosa pollen, moulds
	10	F	50	Weed, grass pollen
	11	M	45	
	12	M	54	Ash, pine pollen
	13	M	3	Grass pollen, milk, house dust mite, dog, bromelain
	14	F	33	Seafood, celery, grass, weed, olive pollen
	15	M	24	House dust mite
	16	M	36	Hazelnut, birch, olive pollen, moulds
	17	F	30	Olive, pollen, house dust mite, dog
	18	F	25	Latex, weed, mimosa pollen
	19	M	53	Cat, grass, weed pollen
	20	F	61	
	21	M	17	Grass, weed, plane tree pollen
	22	M	9	Grass, weed pollen
	23	F	42	House dust mite, cockroach
	24	M	12	Grass, birch pollen
	25	F	51	Grass pollen, wheat
	26	F	77	
	27	M	64	
	28	M	12	House dust mite
	29	M	16	Weed pollen
	30	F	11	House dust mite, horse, grass pollen, moulds
	31	M	24	

^a Mean age 31.55 years.

Cupa and *Cryj*. Interestingly, by using a patient's serum recognizing HMW allergens in *Cups*, numerous cross-reactivities were observed with all other tested pollens (Fig. 4C), likely to be associated to the existence of anti-CCD IgE in this serum. Bromelain inhibition experiments confirmed that the IgE reactivities of this patient are mainly directed against carbohydrates since a quasi total inhibition was observed on immunoblots while, for the anti-BP14 serum (Fig. 4B), the IgE reactivities were not associated with CCD [13]. Furthermore clinical data indicate that besides *Cups* pollen this patient is also sensitized

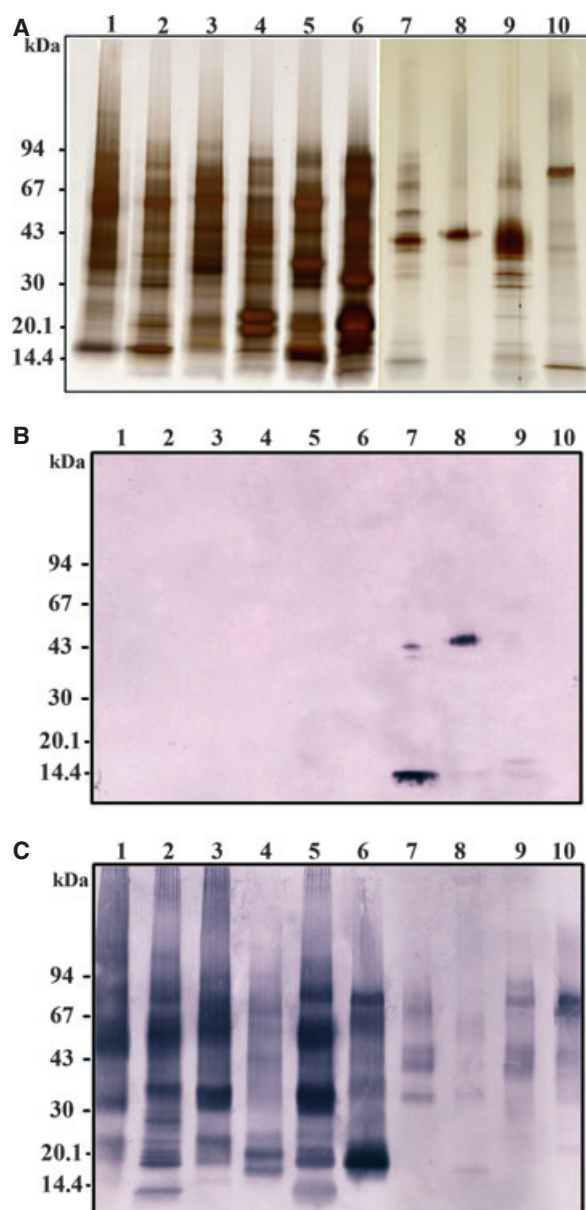


Fig. 4. IgE reactivity to Cupressaceae and non-Cupressaceae pollen grains. Pollen NaCl/P_i extracts from seven non-Cupressaceae species [1, maize (grass); 2, orchard (grass); 3, wheat (grass); 4, timothy (grass); 5, ash (angiosperm tree); 6, birch (angiosperm tree); 10, pine (gymnosperm tree)] and three Cupressaceae species [7, Italian cypress; 8, Arizona cypress; 9, Japanese cedar] were resolved using 1-DE SDS/PAGE. Separated proteins were then either silver stained (A) or blotted onto NCA sheets for subsequent incubation with either a representative serum containing anti-BP14 IgE (B) or a representative serum containing anti-HMW IgE and no anti-BP14 IgE (C). *M_r* markers are expressed in kilodaltons.

to molds, grass and mimosa tree pollen, likely to involve specific IgE to major relevant allergens from these sources.

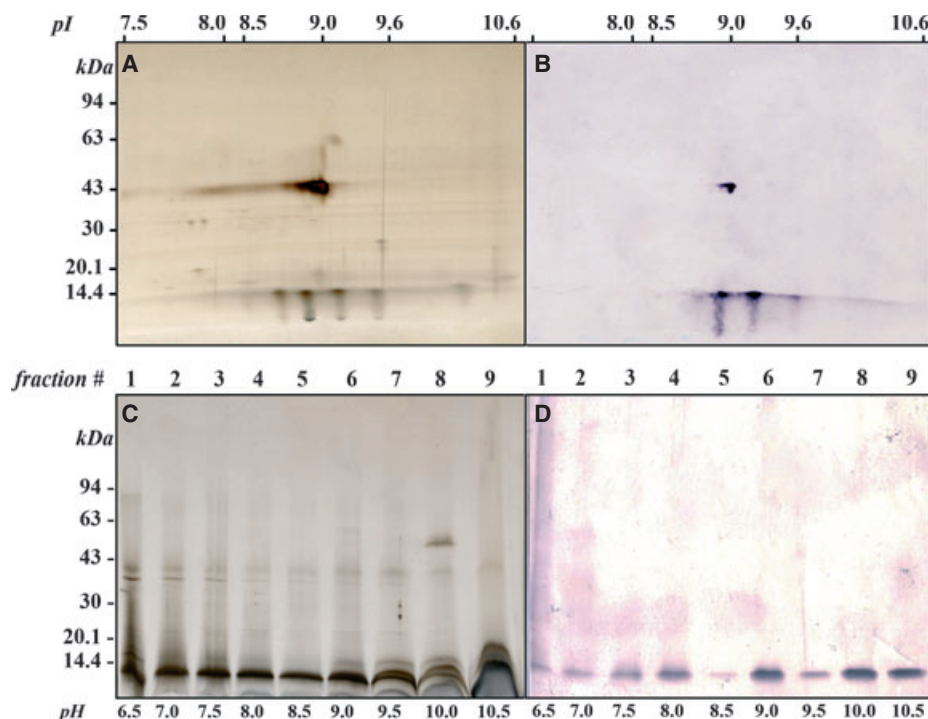


Fig. 5. IgE binding to allergen isoforms isolated by using a short gradient 2-DE and a liquid phase IEF. (A) Silver staining of 2-DE of NaCl/P_i *Cups* pollen extracts made in a short basic gradient (pH 8–11). (B) 2-DE IgE immunoblotting of the pattern illustrated in (A) using a representative serum selected from previous screenings for its anti-BP14 IgE reactivity. *pI*, isoelectric point. *M_r* markers are expressed in kilodaltons. (C) Silver staining of a 1-DE SDS/PAGE separation of the nine consecutive basic fractions (lanes 1–9, *pI* 6.5–10.5) obtained from the Rotofor IEF with a PUN *Cups* pollen extract. (D) 1-DE IgE immunoblotting of the SDS/PAGE separation depicted in (C) using a representative serum selected from previous screenings for its anti-BP14 IgE reactivity. *M_r* markers are expressed in kilodaltons. The measured pH values of each of the nine fractions are indicated.

BP14 exhibits several neutral to basic isoforms

A resolving 2-DE immunoblotting of NaCl/P_i *Cups* pollen extracts made in a short basic gradient from pH 8 to 11 (Fig. 5A,B) and the liquid-phase preparative isoelectric focusing (IEF) (Rotofor[®]) separation of the phosphate urea Nonidet P40 buffer (PUN) pollen extract (Fig. 5C,D) revealed that BP14 is composed of several allergenic isoforms covering a broad range of *pI* from 6.5 to 10.5 (Fig. 5). The amount in protein as well as the IgE reactivity are higher, however, for basic isoforms (pH 8.5–10) than for neutral ones. IgE reactivities observed in some fractions of Rotofor experiments (Fig. 5D) were strong because they were 10 times concentrated (see Materials and methods).

IgE epitopes of BP14 are heat stable and conformational

Heat treatment of NaCl/P_i pollen extract before electrophoresis did not induce any alteration of migration profile and IgE immunoreactivity of BP14 (Fig. 6A,B).

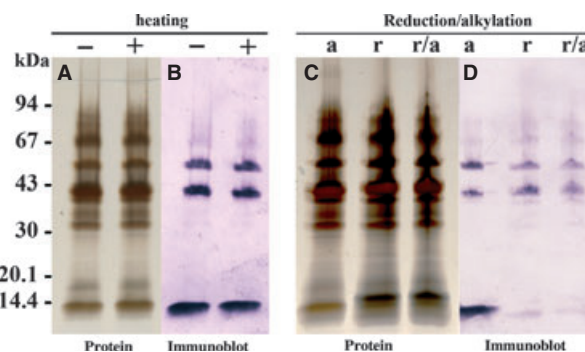


Fig. 6. Effect of heating, 2-Me reduction and alkylation on the IgE binding capacity of cypress pollen allergens. *Cups* pollen extracts, treated or not, were separated in an 8–18% acrylamide gradient SDS/PAGE and either silver stained (A, C) or blotted onto NCA membrane for subsequent incubation with a representative serum selected from previous screenings for its anti-BP14 IgE reactivity (B, D). *M_r* markers are expressed in kilodaltons. (A), (B) Before running, *Cups* pollen extract was heated for 3 min at 100 °C (+) or not (–). (C), (D) Before running, *Cups* pollen extract was reduced (r) with 3% 2-Me and/or alkylated (a) with 0.25 M iodoacetamide.

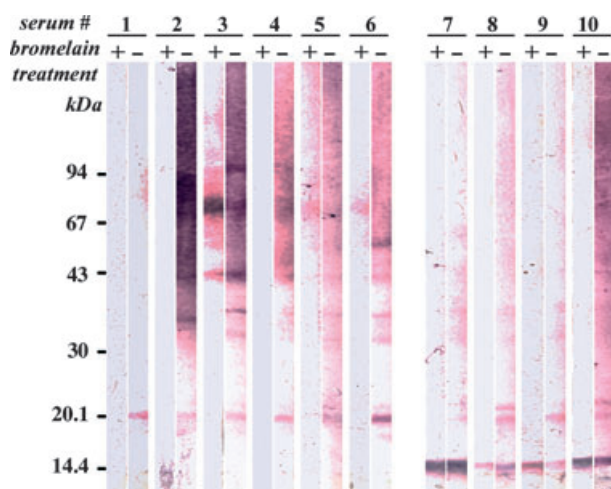


Fig. 7. Detection of cross-reactive IgE directed against carbohydrate determinants. The IgE reactivity to the NaCl/P_i pollen extract separated by 1-DE SDS/PAGE and blotted to NCa was tested with (+) or without (–) preincubation of patients' sera (lanes 1–10) with 0.2% bromelain. Lanes 1–6 correspond to patients' sera showing a specific IgE reactivity to HMW allergens without any BP14 IgE reactivity and lanes 7–10 correspond to patients' sera showing IgE reactivity to BP14. M_r markers are expressed in kilodaltons.

In contrast, BP14 IgE epitopes were shown to be sensitive to a reducing treatment with 2-mercaptoethanol (2-Me) with or without alkylation (Fig. 6C,D). Therefore, BP14 exhibits conformational IgE epitopes abrogated by the rupture of cysteine disulfide bridges maintaining its tertiary structure. The reduced BP14 has an M_r slightly higher (15–17 kDa) than the non-reduced one (14 kDa).

Absence of CCD on BP14

The presence of CCD on cypress pollen allergens was explored by binding inhibition with bromelain, commonly used as a glycoprotein bearing prototypical plant carbohydrate moieties [14]. As shown in Fig. 7, a preincubation of patients' sera with bromelain inhibited partially or totally the IgE reactivities to HMW and also to a 20-kDa protein (Fig. 7, lanes 1–6) but had no effect on the IgE reactivity to BP14 (Fig. 7, lanes 7–11). Furthermore we observed that the lectin concanavalin A did not bind to blotted BP14 in contrast to HMW allergens (data not shown). These results rule out any implication of bromelain-type CCD and manose-containing glycans in the IgE binding to BP14.

Discussion

Biochemical characterization of novel allergens, besides giving fundamental knowledge on protein features, is

an important step towards the development of specific allergen based diagnosis and therapy of allergic disorders. Although several IgE binding proteins of *Cups* pollen extracts were previously isolated and visualized by using 1-DE and 2-DE analysis, few pollen allergens have been unequivocally identified and characterized in this species [10,11,15]. This fact can be explained by the inherent characteristics of water/salt cypress pollen extracts known to be very rich in carbohydrate and poor in protein [16]. These complex matrix properties are directly related to the cypress pollen structure accumulating high amounts of pectine and β -glucan in its thick intine as well as many callose-containing vesicles in its cytoplasm [17]. Our results demonstrated that the low protein concentration of cypress pollen protein extracts is not due to the low protein content of the pollen *per se* but rather to the accessibility of the intrapollenic proteins stuck in a thick gel-like matrix of polysaccharides composing parts of the sporoderm [10].

In the present study, we showed that a phosphate buffer containing 0.15 M NaCl is able to extract basic proteins that could not be extracted with water and are poorly extracted with the classical extraction buffer AB. This confirms the structural peculiarities of cypress pollen since various pollens such as orchard, timothy, birch or ash gave high amounts of proteins, in diversity as well as in quantity, upon water, AB or NaCl/P_i extraction (see Fig. 4 for NaCl/P_i extraction). Furthermore, the basic proteins described were shown to be IgE reactive indicating that these proteins were actually exposed to the immune system. In fact, we previously reported that hydrophobic proteins extracted with detergent and chaotropic agents can be IgE reactive in orchard pollen [18] as well as *Cups* pollen [10] indicating that the 'in vivo natural extraction' on the patient's mucosa is highly efficient while largely unknown. The use of NaCl/P_i, a buffer with a physiological pH and ionic strength, allowed a fast release of BP14. Several studies have reported differences in time release of allergens from various pollen grains [19–21]. The allergenicity of pollen proteins, in terms of both challenge and sensitization, has been partly suggested to be related to the speed of release from the pollen grain [22–24].

The choice of the optimal extraction buffer is thus crucial in sample preparation to ensure the detection and diagnosis of all relevant major and minor sensitizations in allergic patients. The present study demonstrated that, since the level of exposure to different sensitizing cypress pollen allergens may differ, both common allergenic components as well as species-specific allergens have to be considered to obtain an optimal allergen preparation [25].

Results reported herein focused on the description of BP14, a multi-isoform 14-kDa basic protein optimally extracted with NaCl/P_i. It may be hypothesized that this protein, because of its alkaline properties, is a member of a hydrophobic complex which is dissociated under 0.15 M salty conditions. Depending on the cypress-allergic patient cohort this protein is allergenic in 15%–25% of tested individuals. Up to now the reactivity patterns were confirmed in more than 120 European patients' sera (published [10,11] and unpublished results). BP14 seems to induce very high affinity IgE since, even in the absence of detectable protein upon silver staining (as in *Cupa* pollen), an IgE reactivity was observed on immunoblots, making anti-BP14 IgE a very sensitive probe. Cross-reactive proteins were found at 14 kDa only in Cupressaceae pollen *Cupa* and *Cryj* and should correspond to homologous proteins of BP14 since *Cups* belongs to the same tree family. It is much more expressed in *Cups* than in *Cupa* and *Crij*, however. In contrast, no cross-reactivity was detected in other pollen species tested (four grasses, two angiosperm and one gymnosperm tree). The epitopes recognized by specific IgE on BP14 are polypeptidic, not sensitive to heat but to reduction indicating the presence of disulfide bridges and therefore cysteines in the sequence of the protein. These physicochemical characteristics as well as the low molecular weight and the basic isoelectric point are shared by lipid transfer proteins (LTPs), a diversified family of allergens widely distributed in plants [26]. The potential role of LTPs in cypress pollinosis was recently suggested by two studies, one on *Cryj* [27] and the other on *Cupa* [28]. However, according to our results, biochemical (anion exchange purification) and immunochemical (immunoblot with anti-LTP antibodies) experiments were unable to show that BP14 belongs to the family of LTPs. As well, more than 25 MS analyses (MALDI or ESI-MS and MS/MS) performed either with 1-DE or 2-DE of detergent or non-detergent extracts, using various alternative stainings (Coomassie Blue, silver or Sypro Ruby), on four different and independent MS platforms, did not allow BP14 to be identified because, until now, the protein is absent from databases (data not shown). Furthermore, whether this reactivity is associated with a peculiar exposition/sensitization process and/or correlated with symptoms deserves further clinical investigations (to be published).

In contrast to the peptidic nature of IgE epitopes on BP14, IgE reactivity to HMW proteins was shown to be mainly directed against the CCD moiety of glycoproteins although the extent of the bromelain inhibition varied substantially according to the allergic

individuals. These results are in accordance with the studies of Afferni *et al.* [29] and Iacovacci *et al.* [30], showing that a large portion of the IgE reactivity of Cupressaceae-allergic subjects is associated with sugar moieties of *Cupa* glycoproteins. Carbohydrate epitopes of *Cupa* pollen allergens were shown to be shared by taxonomically unrelated allergenic pollen species [31–34]. The N-linked oligosaccharide structures containing $\alpha(1-3)$ fucose and $\beta(1-2)$ xylose are common features of many plant-derived glycoproteins. They are able to induce cross-reactive IgE responses suspected to be poorly clinically relevant and which may affect the *in vitro* diagnosis of allergic diseases [35,36].

In conclusion, we showed here that up to 25% of cypress-allergic patients have a specific IgE reactivity to non-glycosylated epitopes of an as yet undescribed 14-kDa basic allergen that seems to be exclusively expressed in Cupressaceae pollen grains. Since the IgE reactivity to BP14 allows a clear-cut distinction between two groups of cypress-allergic patients, it might be a discriminative factor in the cypress pollinosis otherwise considered as severe by clinicians.

Materials and methods

Patients' sera

Sera were drawn after obtaining informed consent from individuals suffering from cypress pollen allergy. Sera from cypress-allergic patients were selected according to their symptoms and to a positive ImmununoCAP® (Phadia, Uppsala, Sweden) to cypress pollen extracts (with serum-specific IgE higher than 0.71 kU_A·L⁻¹; CAP class 2). They were kindly provided by three different medical biology centers in Lyon, Marseille and Rome after the evaluation of total and specific IgE titer. For each analysis, the serum from a healthy individual with a normal total IgE concentration (< 0.10 kU_A·L⁻¹) was chosen as control.

Cypress pollen protein extractions

All the pollen studied were supplied by Allergon AB (Angelholm, Sweden). For the preparation of *Cups* salt pollen extracts, 100 mg was incubated for different times (30 s, 15 min, 30 min, 1 h, 2 h, 3 h, 5 h and overnight) under rotation (22 °C) in 1 mL (1/10 w/v) of H₂O, or a solution of 125 mmol·L⁻¹ AB (pH 8) [37], or NaCl/P_i (150 mmol·L⁻¹ NaCl, pH 7.4). In preliminary validation experiments commercially supplied *Cups* pollen was shown to be similar in protein content and IgE reactivities to freshly collected pollen originating from the south of France (kindly provided by M. Calleja, SupAgro, Montpellier). Arizona cypress (*Cupa*), Japanese cedar (*Cryj*), pine (*Pinus sylvestris*), ash (*Fraxinus excelsior*), birch (*Betula verrucosa*), orchard

(*Dactylis glomerata*), timothy (*Phleum pratense*), maize (*Zea mays*) and wheat (*Triticum aestivum*) pollen extracts were performed overnight in NaCl/P_i as described above. The pollen suspensions were centrifuged at 18 000 *g* for 20 min at 4 °C. The supernatants were collected and stored as aliquots at –20 °C until use.

For the protein separation by liquid phase IEF (Rotofor®; Biorad, Hercules, CA, USA) pollen grains were suspended in PUN buffer [10 mmol·L^{–1} phosphate buffer pH 7.1 containing 3 mol·L^{–1} urea (GE Healthcare, Uppsala, Sweden) and 0.2% Nonidet P-40 (Sigma-Aldrich, St Louis, MO, USA)]. The suspension was then gently shaken overnight at room temperature and the supernatant separated by centrifugation (18 000 *g* for 20 min). Phenylmethylsulfonyl fluoride and EDTA (Sigma-Aldrich) were added up to 1 mmol·L^{–1} concentration for both, as protease inhibitors. Before loading the extract into the Rotofor chamber, 1 mol·L^{–1} non-detergent sulfobetaine-256 (Calbiochem, Merck Chemicals, Nottingham, UK) was added in order to avoid potential protein aggregation [38].

Protein concentration of the samples was measured using the Bradford protein assay (Pierce, Thermo Scientific, Rockford, IL, USA) with BSA as standard.

Reduction and alkylation

Proteins of *Cups* NaCl/P_i pollen extract were reduced by 2-Me (Sigma-Aldrich) and alkylated by iodoacetamide (Sigma-Aldrich). The reduction was performed for 3 min at 100 °C in NaCl/P_i with 3% 2-Me. After cooling the reaction mixture to 4 °C, 250 mmol·L^{–1} iodoacetamide was added to final concentration and alkylation was performed for 15 min at 4 °C. Assays without 2-Me and iodoacetamide, either non-boiled or boiled, were performed as control.

One- and two-dimensional gel electrophoresis

For 1-DE SDS/PAGE separation, extracted proteins in a 38 mmol·L^{–1} Tris buffer pH 6.8 containing 4% (w/v) SDS were applied to a thin 8–18% gradient polyacrylamide gel (ExcelGel; GE Healthcare) and run on a flat-bed electrophoretic chamber (Multiphor II; GE Healthcare) at 12 °C. The gel was then either transferred to a nitrocellulose membrane for western blotting assays or silver stained for detection of the separated proteins. Molecular mass (*M_r*) markers (GE Healthcare) ranging from 14.4 to 94 kDa were used as comparative references.

For 2-DE, *Cups* pollen extracts were first separated by IEF performed using a polyacrylamide gel 4%T, 3%C (Clean gel; GE Healthcare) hydrated in a solution containing 5% v/v ampholytes (Servalyt®; Serva, Heidelberg, Germany) on a flat-bed electrophoretic chamber (Multiphor II; GE Healthcare) cooled at 15 °C. The ampholytes used covered either a wide range of pH 2–11 or, for a better

resolving separation of basic proteins, a short basic pH from 7 to 11. Samples (55–60 µg of proteins from the total pollen protein extract) were loaded on strips 10 cm long and 0.7 cm wide of a dry Whatman® 3 MM paper (GE Healthcare) placed directly on top of the gel (anode side). Isoelectric point (pI) (Biorad) markers ranging from 4.45 to 9.6 or 7.5 to 10.6 were used in wide or short range IEF, respectively, as comparative references.

After IEF separation, 5-mm strips of the gel were cut, incubated in the equilibration buffer [114 mmol·L^{–1} Tris pH 6.8 containing 12% (w/v) SDS] for 30 min and submitted to the second dimension SDS/PAGE in the same conditions as described above.

One-DE and 2-DE gels were either silver stained according to Blum *et al.* [39], Coomassie Blue stained or blotted onto a cyanogen bromide activated nitrocellulose (NCA) sheet [40] (Optitran®BA-S 83; Schleicher and Schuell, Dassel, Germany).

Immunoblotting

Electroblotting of separated proteins was performed onto NCA sheets with a semidry Novablot apparatus (LKB, Uppsala, Sweden) following the manufacturer's instructions (1 h, 1 mA·cm^{–2}). The membranes were then dried and blocked with NaCl/P_i containing 0.3% (v/v) Tween 20® (Sigma-Aldrich) (NaCl/P_i-Tw) for 1 h at ambient temperature. For 1-DE screening, each NCA membrane was then cut into 2.5-mm-wide strips that were individually incubated with 1 : 10 diluted patient sera in NaCl/P_i-Tw 0.1% (overnight at 20 °C). For 2-DE analysis, the whole NCA membranes were incubated with individual serum. Each membrane was washed three times for 10 min in NaCl/P_i-Tw 0.1% (v/v) and incubated for 2 h at 20 °C with 1 : 700 dilution of alkaline phosphatase conjugated goat anti-human IgE (Sigma-Aldrich). The alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitro blue tetrazolium (Sigma) in 0.1 mol·L^{–1} Tris acetate buffer pH 9.5.

IgE binding inhibition using bromelain

Binding inhibition was tested on immunoblotting strips, obtained after SDS/PAGE of *Cups* pollen extract, by preincubation of the individual serum with 0.2% bromelain (from pineapple stem; Sigma) as previously described [14].

Liquid-phase preparative IEF using the Rotofor®

The pH gradient was obtained by using a solution containing 2% v/v of ampholyte mixture with a wide range of pH 2–11 (Serva). IEF was performed by the method of Egen *et al.* [41] with some modifications. Ten milliliters of ampholyte solution was added to 40 mL of PUN pollen extract. The solution was then injected into the Rotofor

chamber. The extract was focused at 4 °C under constant power (15 W) for 270 min. Twenty fractions (2.2 mL each) were then harvested and pH evaluated. Each fraction was dialyzed against 20 mmol·L⁻¹ phosphate buffer pH 7.2, lyophilized and then solubilized in distilled water (10% of the initial volume). The fractions were analyzed by SDS/PAGE followed by IgE immunoblotting (as described above).

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