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Article

Identification of Potential IgE-Binding Epitopes Contributing to the Cross-Reactivity of the Major Cupressaceae Pectate-Lyase Pollen Allergens (Group 1)

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Abstract: Pectate-lyase allergens, the group 1 of allergens from Cupressaceae pollen, consist of glycoproteins exhibiting an extremely well-conserved three-dimensional structure and sequential IgE-binding epitopes. Up to 10 IgE-binding epitopic regions were identified on the molecular surface, which essentially cluster at both extremities of the long, curved β -prism-shaped allergens. Most of these IgE-binding epitopes possess very similar conformations that provide insight into the IgE-binding cross-reactivity and cross-allergenicity commonly observed among Cupressaceae pollen allergens. Some of these epitopic regions coincide with putative *N*-glycosylation sites that most probably consist of glycotopes or cross-reactive carbohydrate determinants, recognized by the corresponding IgE antibodies from allergic patients. Pectate-lyase allergens of Cupressaceae pollen offer a nice example of structurally conserved allergens that are widely distributed in closely-related plants (*Chamaecyparis*, *Cryptomeria*, *Cupressus*, *Hesperocyparis*, *Juniperus*, *Thuja*) and responsible for frequent cross-allergenicity.

Keywords: pectate-lyase; pollen; cupressaceae; *Cryptomeria japonica*; *Cupressus sempervirens*; *Juniperus ashei*; *Thuja plicata*; IgE-binding epitopes; IgE-binding cross-reactivity; cross-reactive carbohydrate determinant; glycotope



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1. Introduction

Allergy to Cupressaceae pollen has now become a major public health concern with respect to the widespread distribution of Cupressaceae as ornamental trees (*Cupressus sempervirens*, *Hesperocyparis/Cupressus arizonica*, *Cryptomeria japonica*, *Thuja occidentalis*) in European countries, or as forest species (*Cryptomeria japonica*, *Juniperus ashei*) in Japan and USA [1,2]. The severity of symptoms like nasal congestion, itchy eyes, and sleep disturbance is apparently related to the amount of pollen grains in the atmosphere, as deduced from a questionnaire covering a 3-year period in Japan [3]. The major allergens of Cupressaceae pollen have been identified as pectate-lyases (PL): Cup s 1 (*Cupressus sempervirens*) [4], Cup a 1 (*C. arizonica*) [5], Cry j 1 (*Cryptomeria japonica*) [6], Jun a 1 (*Juniperus ashei*) [7], Jun o 1 (*J. oxycedrus*), and Jun v 1 (*J. virginiana*) [8]. PL allergens consist of apparently inactive *N*-glycosylated enzymes built up from three parallel β -sheets organized in a long, curved β -prism [9]. Except for Jun a 1 [10–13], IgE-binding B-cell epitopes of Cupressaceae PL

allergens remain to be identified and characterized. However, the strong IgE-binding cross-reactivity reported among Cupressaceae suggests that Cupressaceae pollen allergens share identical or very similar B-cell epitopes [14–18]. Mapping of sequential IgE-binding epitopes on the molecular surface of Cupressaceae PL allergens was investigated to decipher the molecular basis for the cross-reactivity currently observed among the Cupressaceae pollens.

2. Materials and Methods

2.1. Sera from Allergic Patients

Blood samples were drawn after informed consent patients experiencing anaphylaxis selected according to symptoms as rhino-conjunctivitis and/or asthma to Cupressaceae pollen, and individual sera were used in SPOT experiments. All the used cypress pollen allergic patient (CPAP) sera interacted with Cupressaceae pollen extracts as previously checked by positive fluorescence enzyme immunoassay (ImmununoCAP, Thermo Fisher Scientific, Phadia, 67400 Illkirch, France) (Table 1). The specificity of the used sera was assessed in a previous publication [19].

Table 1. List of sera from patients allergic to cypress pollen, used in SPOT and ELISA inhibition experiments. Specific IgE is against *Cupressus sempervirens* pollen extract.

Subjects	Sex/Age	Allergic History	Specific IgE (kU/L)
1	F/5	* CY, DERE, PAR	0.19
2	M/4	CY, DERP, PAR, DAC, PN, SIN, SHR	0.44
3	F/29	CY, DERP/F, PAR	0.70
4	M/11	CY, DERP/F, CAT, DOG	1.10
5	M/5	CY, OLI, PAR	1.27
6	F/42	CY, DERP/F, CAT	1.39
7	F/43	CY, PAR, DAC	1.98
8	F/16	CY, PAR	4.49
9	F/46	CY, DERP, PAR, DAC	4.92
10	M/33	CY, DERP, PAR, DAC, ALT	6.70

* CY: cypress, DERE: *Dermatophagoides farinae*, DERP: *D. pteronyssinus*, PAR: *Parietaria*, DAC: *Dactylis*, PN: peanut (*Arachis hypogaea*), SIN: *Sinapis*, SHR: shrimp, OLI: olive tree, ALT: *Alternaria*.

2.2. ELISA Inhibition

The 9-mer peptides corresponding to the predicted epitope #1 (10GDSNWDQNR18) and epitope #10 (297RSTRDAFSN305) of Cup s 1 were synthesized using the Fmoc chemistry (Genscript USA Inc., Piscataway, NJ, USA). The capacity of synthetic peptides to inhibit the IgE-Cup s 1 interaction was checked by ELISA. Briefly, the wells were coated with 1 $\mu\text{g mL}^{-1}$ of purified Cup s 1 in phosphate buffer saline (PBS, pH 7.5) [20], and after overnight incubation (4 °C), the wells were washed 3 times with PBS and then incubated (2 h, room temperature) with PBS containing 0.1% Tween 20 (*v/v*) and 1% BSA (*w/v*) (PBSTB). Then, 50 μL of 1:30 diluted CPAP serum in PBSTB, previously incubated with 1 mM or 2 mM of synthetic peptide, was added, and the plates were incubated (1 h 30, room temperature) under constant stirring. Then, mouse monoclonal anti-human IgE coupled to alkaline phosphatase (mAb anti human-IgE-AP) was added (diluted 1:500, Sigma-Aldrich, St. Louis, MO, USA) and 1 h incubation was performed. One hundred microliters of AP substrate, the 5-bromo-4-chloro-3-indolylphosphate (BCIP, Promega Corporation, Madison, WI, USA), was added, and after incubation (45 min in the dark), the reaction was stopped. Between each incubation step, 3 washes with PBS containing 0.1% Tween 20 (*v/v*) were performed. The absorbance at 405 nm was recorded on a Titertek Multiscan spectrophotometer (Labsystems, Thermo Fisher Scientific, Villebon-sur-Yvette, France). Each value is

the mean of three separate experiments, and appropriate controls were performed under the same conditions.

2.3. IgE-Binding Epitope Mapping: SPOT Experiments

Overlapping 15-mer peptides, frameshifted by three residues, corresponding to the entire amino acid sequences of PL Cry j 1 (access number (AN): BAA05542.1), Cup a 1 (AN: CAB62551.1), Cup s 1 (AN: AAF72625.1), Jun o 1 (AN: CAC48400.1), and Jun v 1 (AN: AAF15427.1), were synthesized using the SPOT technique [21]. For these experiments, we used the Multiprep automatic SPOT synthesizer (Intavis Bioanalytical instruments, Cologne, Germany). After different treatments to bind the peptides as described previously [22], the membranes were soaked overnight in tris-buffered saline (TBS) containing 2 mL blocking buffer (Roche-Diagnostics, Meylan, France) and 1 g sucrose. Then, membranes were incubated (2 h) with CPAP serum (1:10 *v/v*) in the presence of an anti-protease cocktail (Roche) in a moist chamber. Membranes were then soaked in a 1:4000 dilution of mAb anti human-IgE-AP for 1 h. After, the interacting peptide spots were stained for 30 min by adding BCIP. Then, the membranes were washed three times with deionized water and dried for scanning. Negative controls were obtained by using either TBS without serum or a serum from non-allergic patients to Cupressaceae pollen. Between each incubation step, 3 washes with TBS containing 0.1% (*v/v*) tween (TBSTw) were performed.

Membranes can be used repeatedly after regeneration steps consisting of 1–3 washes in dimethylformamide for 10 min each, 3 washes in deionized water, 3 washes in 8 M urea containing 1% (*w/v*) SDS and 1% (*w/v*) β -mercaptoethanol, and finally 3 washes in a mixture of ethanol-acetic acid-H₂O (50:10:40, *v/v/v*).

2.4. Bioinformatics

Multiple amino acid sequence alignment of Jun a 1, Jun o 1, Cup s 1, Jun v 1, and Cry j 1 was carried out with CLUSTAL-X [23]. Molecular modeling of pectate-lyase allergens Cha o 1 (*Chamaecyparis obtusa*), Cry j 1 (*Cryptomeria japonica*), Cup s 1 (*Cupressus sempervirens*), Cup a 1 (*C. arizonica*), Jun o 1 (*Juniperus oxicedrus*), Jun v 1 (*J. virginiana*), pectate-lyase homologs from *Metasequoia glyptostroboides* (PL-Mg), and *Taxodium distichum* (PL-Td) was carried out with YASARA Structure [24], using the atomic coordinates of the PL allergen Jun a 1 of mountain cedar (*Juniperus ashei*) (RCSB Protein Data Bank code 1PXZ) [9] as a template. PROCHECK [25], ANOLEA [26], and the calculated QMEAN scores [27,28] were used to assess the geometric and thermodynamic qualities of the three-dimensional models. As an example, none of the residues of the Cup s 1 model occurred in the non-allowed regions in the Ramachandran plot. Using ANOLEA to evaluate the model, only 8 residues (over 345) of the Lol p 1 model exhibited an energy over the threshold value. Both residues are mainly located in the loop regions connecting the β -sheets and α -helices in the model. The calculated QMEAN score of the model gave a value of 0.717. Reliable values of 0.743 (Cha o 1), 0.730 (Cup a 1), 0.686 (Cry j 1), 0.705 (Jun o 1), and 0.718 (Jun v 1) were obtained for the QMEAN score of the different modeled PL allergens, respectively. Assuming that the putative *N*-glycosylation sites Asn-X-Thr/Ser are actually glycosylated, a classic bi-antennary high-mannose glycan chain with a tri-mannoside core (Man)₂-(Man)₃-(GlcNAc)₂ was modeled using the GlyProt server of Glycosciences (<http://www.glycosciences.de/modeling/glyprot/php/main.php>, accessed on 14 March 2021) and represented in CPK on the molecular surface of the Cupressaceae PL allergens. The PL allergens from the less closely related species, PL-Td, PL-Mg, and pectate-lyase homolog from *Prunus persica* (PL-Pp), were similarly modelled using various PL proteins as templates: the endo-xylogalacturonan hydrolase of *Aspergillus tubingensis* (PDB code 4C2L) [29], the endo-polygalacturonases of *Erwinia carotovora* (PDB code 1BHE) [30] and *Colletotrichum lupini* (PDB code 2IQ7) [31], the rhamno-galacturonase A of *Aspergillus aculeatus* (PDB code 1RMG) [32] and the exo-poly- β -D-galacturonidase of *Thermotoga maritima* (PDB code 3JUR) [33] for PL-Td and PL-Mg, respectively, and the PL allergen Jun a 1 (PDB code 1PXZ) [9] for PL-Pp. Rather satisfactory QMEAN scores of 0.500 (PL-Td), 0.5819 (PL-Mg), and 0.595 (PL-Pp) were calculated for the three protein PL models, respectively.

The surface occupied by the SPOT identified sequential IgE-binding epitopic stretches of the modeled allergens that were differently colored and displayed on the molecular surface of the PL models with YASARA. Molecular cartoons were displayed with Chimera [34]. Electrostatic potentials of Cup s 1, Cry j 1, and Jun o 1 were calculated with YASARA and displayed on the molecular surface as red (electro-negatively charged) and blue (electro-positively charged) patches. The root-mean-square deviation of atomic positions (rmsd, in Å) between the superposable C α of pairwise superposed PL was calculated at the SuperPose web server (<http://www.wishart.biology.ualberta.ca>, accessed on 16 March 202) [35].

3. Results

Cupressaceae PL allergens consist of a highly conserved family of pollen proteins with amino acid sequences that share high percentages of both identity ($\geq 75\%$) and similarity ($\geq 95\%$) (Figure 1).

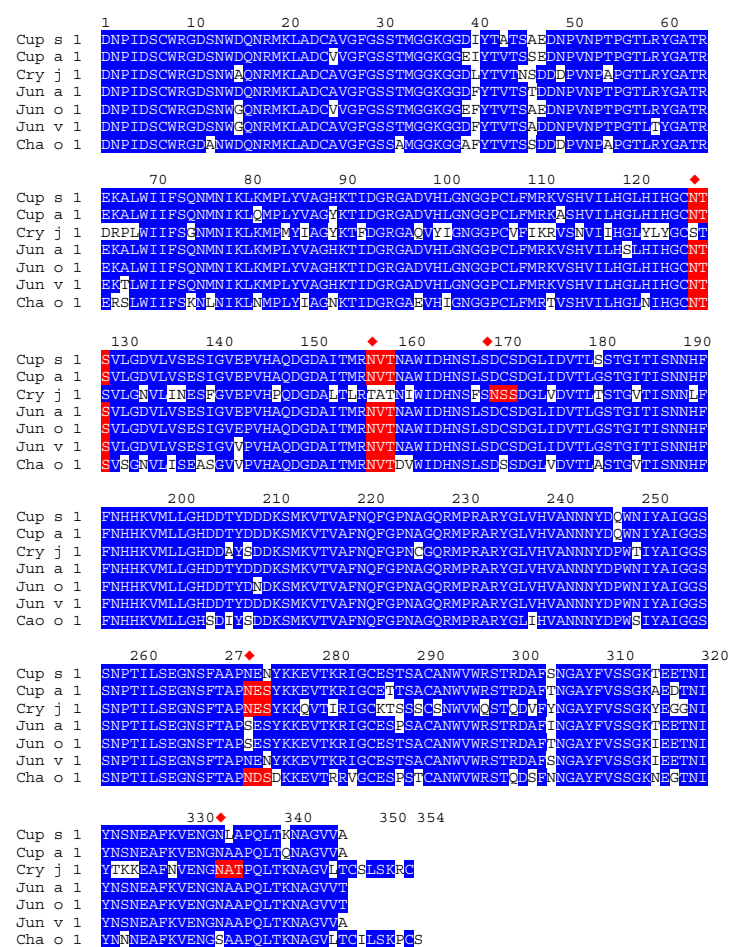


Figure 1. Multiple alignment of Cupressaceae pollen pectate-lyase allergens from *Cupressus sempervirens* (Cup s 1), *Cupressus arizonica* (Cup a 1), *Cryptomeria japonica* (Cry j 1), *Juniperus ashei* (Jun a 1), *Juniperus oxycedrus* (Jun o 1), and *Chamaecyparis obtusa* (Cha o 1). Fully conserved amino acid residues observed among the aligned amino acid sequences are in blue-boxed white letters. Non-conserved residues are in black letters. Putative N-glycosylation sites are in red-boxed white letters, indicated by a red diamond. The amino acid sequence of PL from *Chamaecyparis obtusa* (Cha o 1), which also belongs to the Cupressaceae, was included in the alignment for comparison.

According to their amino acid sequence similarities, the three-dimensional models built for Cup s 1, Cry j 1, Jun o 1, Cha o 1, Cup a 1, Jun v 1, and PL-Td from the atomic coordinates of Jun a 1 used as a template exhibit a closely related structural pattern made of three parallel β -sheets organized in a long, curved helical β -prism. Only Cup s 1, Cry j 1, and Jun o 1 structures are shown in Figure 2.

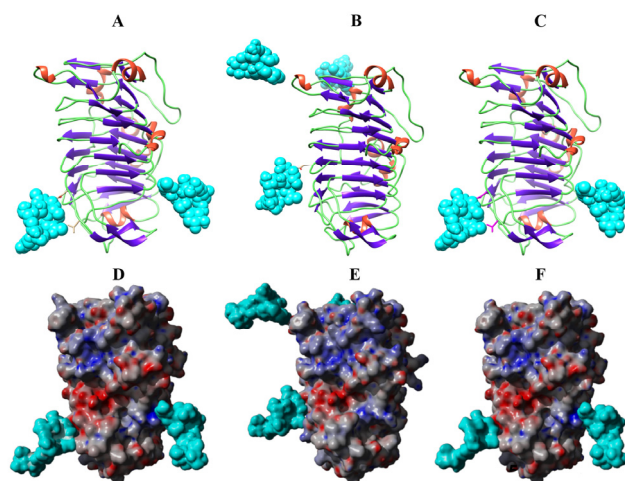


Figure 2. (Ribbon diagram of Cup s 1 (A), Cry j 1 (B), and Jun o 1 (C), showing the overall organization in a long, curved helical β -prism of PL from the Cupressaceae. The *N*-glycan chains associated to the PL allergens are represented in balls colored cyan. Distribution of the electrostatic potentials calculated at the surface of Cup s 1 (D), Cry j 1 (E), and Jun o 1 (F). Electropositive and electronegative potentials are colored blue and red, respectively. Neutral surfaces are colored grey. The *N*-glycan chains decorating the molecular surface of the PL allergens are represented in balls colored cyan.

However, they essentially differ by the degree of *N*-glycosylation due to the occurrence of two (Cup s 1, Jun a 1, Jun o 1, Jun v 1), three (Cup a 1), or four (Cha o 1, Cry j 1) *N*-glycosylation sites along their amino acid sequences (Figure 2A–C). In fact, all of these PL allergens consist of glycoallergens or cross-reactive carbohydrate determinants (CCDs), as previously demonstrated for Jun a 1 [11] and Cup a 1 [20]. The *N*-glycan chains decorating the molecular surface of PL allergens are well exposed and should be readily accessible to the corresponding cross-reacting anti-oligosaccharide IgE antibodies. Up to 10 different IgE-binding epitopic stretches were identified along the amino acid sequences of Jun a 1, Jun o 1, Cup s 1, Jun v 1, and Cry j 1 using the SPOT technique (Figure 3).

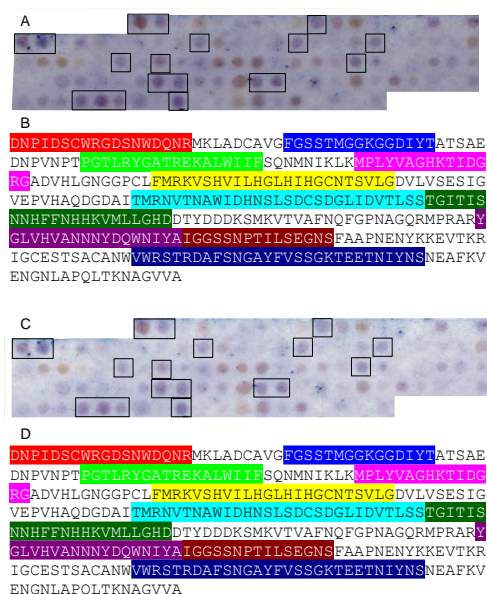


Figure 3. IgE-binding peptides (boxed violet spots) revealed on the Jun v 1 SPOT membrane with two different CPAP (A,C). Mapping of the corresponding continuous IgE-binding epitopic regions (colored boxed white letters) along the amino acid sequence of Jun v 1 (B,D). Epitopes are colored red (#1), blue (#2), green (#3), magenta (#4), yellow (#5), cyan (#6), dark green (#7), purple (#8), brown (#9), and dark blue (#10), respectively.

However, some discrepancies occur between the Cupressaceae PL in the number and the extent of the sequential IgE-binding epitopic stretches along the amino acid sequences. In this respect, epitope #9 only occurs in Cup s 1 and Cry j 1 (Figure 4).

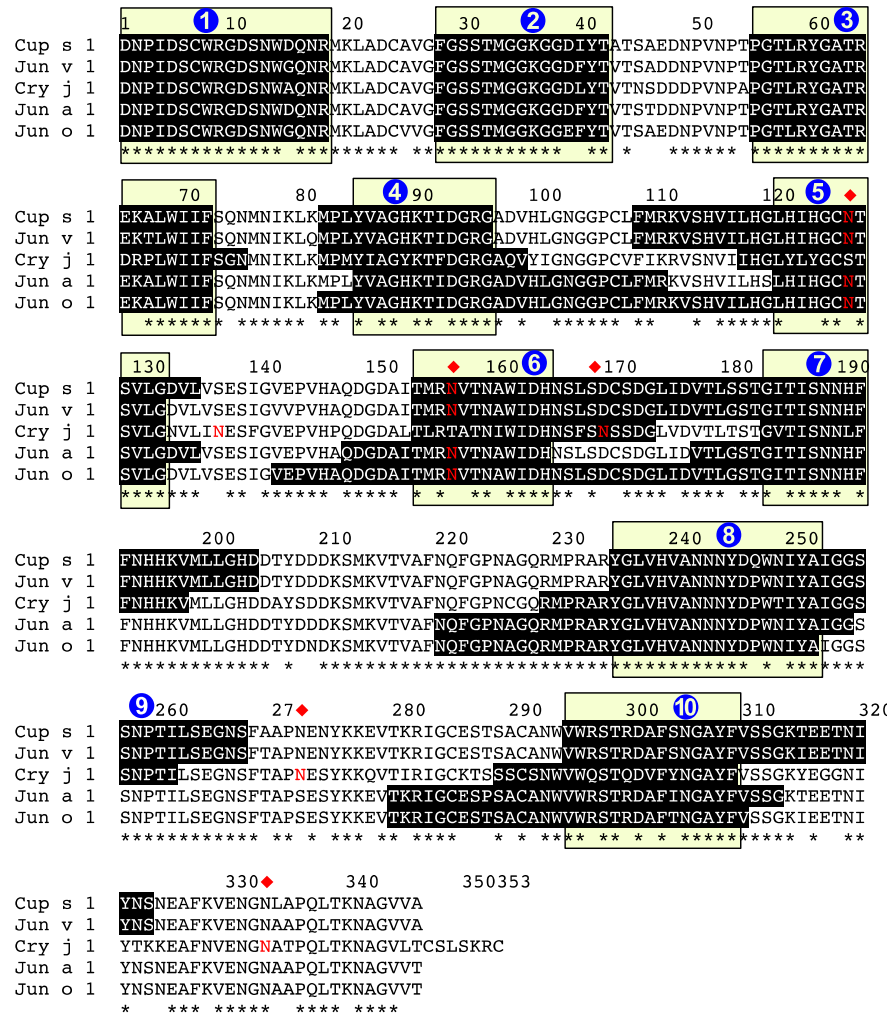


Figure 4. Multiple alignment of Cupressaceae pollen pectate-lyase allergens Cup s 1 (*Cupressus sempervirens*), Jun v 1 (*Juniperus virginiana*), Cry j 1 (*Cryptomeria japonica*), Jun a 1 (*Juniperus ashei*), and Jun o 1 (*Juniperus oxycedrus*), showing the delineation of potential IgE-binding epitopic regions defined in SPOT experiments. IgE-binding epitopic regions are numbered 1–10 and highlighted in black-boxed white letter stretches. Yellow boxes indicate the fully conserved IgE-binding epitopic regions common to all of the pectate-lyase allergens. Fully conserved amino acid residues observed among the aligned amino acid sequences are indicated with asterisks. Asparagine residues corresponding to putative N-glycosylation sites are in red letters and indicated by a red diamond.

Owing to the extremely conserved sequential and structural characteristics observed among PL allergens of the Cupressaceae, all of these molecules share IgE-binding epitopes arrayed on both extremities of the three-dimensional models (Figure 5).

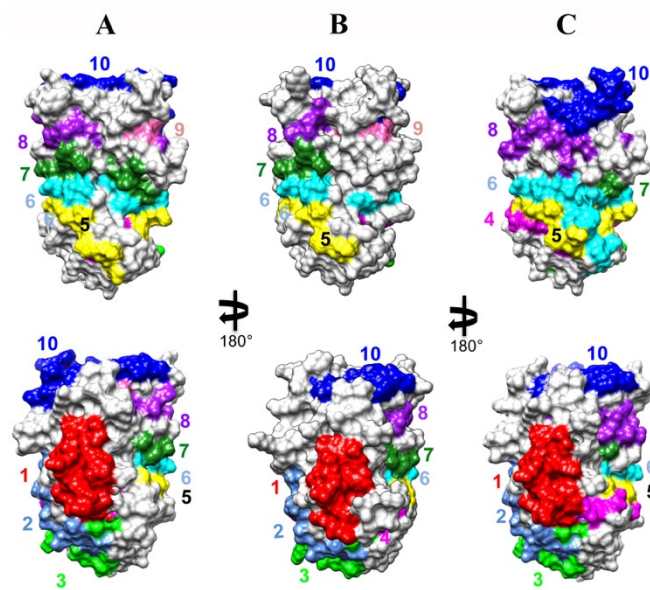


Figure 5. Localization of exposed IgE-binding epitopic regions on both faces of the molecular surface of Cup s 1 (A), Cry j 1 (B), and Jun o 1 (C). Exposed IgE-binding epitopes are numbered and colored red (epitope 1), pale blue (epitope 2), green (epitope 3), magenta (epitope 4), yellow (epitope 5), cyan (epitope 6), dark green (epitope 7), purple (epitope 8), pink-brown (epitope 9), and blue (epitope 10), respectively. Note that epitopes 4 and 9 are rarely exposed at the surface of the PL allergens.

These IgE-binding epitopic regions coincide with exposed areas that mainly consist of hydrophilic and charged residues with negative (epitopes 1 and 6) or positive (epitopes 3, 5, 8, and 10) net charges (Figure 2D–F). In addition, IgE-binding epitopic regions 5 and 6 harbor a putative *N*-glycosylation site at 127NTS (except for Cry j 1) and 157NVT (except for Cry j 1), respectively, which are predicted to be actually glycosylated according to the GlyProt server (Figure 2D–F). Although Cry j 1 exhibits four putative *N*-glycosylation sites, a single site 170NSS, which coincides with IgE-binding epitope 6, is predicted to be actually glycosylated (Figure 5B). Some coalescence occurs between epitopic regions 1–2–3 and 7–8–9, respectively, which creates two patches of more extended IgE-binding epitopic regions at both ends of the allergen structure (Figure 6). However, although closely linked to the exposed epitope 8, the potential epitope 9 is scarcely exposed on the surface of the allergens.

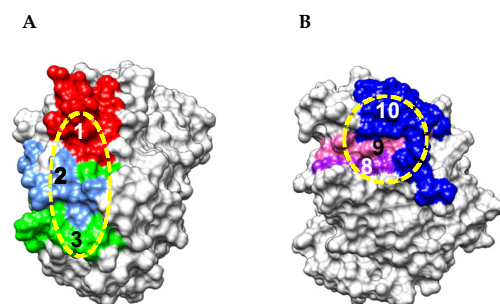


Figure 6. Two different orientations of the Jun v 1 model showing the coalescence of the *N*-terminal epitopic regions 1–2–3 (A) and the *C*-terminal epitopic regions 8–9–10 (B), respectively.

Synthetic 9-mer peptides corresponding to the well-exposed epitopes 1 (10GDSNWDQNR18), 8 (240NNNYDQWNI248), and 10 (311SSGKTEETN319) of Cup s 1 inhibited (23% for epitope 1, 16% for epitope 8, and 36% for epitope 10, respectively) the interaction of Cup s 1 with IgE from allergic patient sera in ELISA inhibition experiments (Figure 7), which confirms some IgE-binding capacity of the epitopic regions identified by the SPOT technique. No inhibition occurred with an unrelated 9-mer peptide RTTADRQTA corresponding to an IgE-binding epitope of the Mal d 3 fruit allergen [36], used as a negative control.

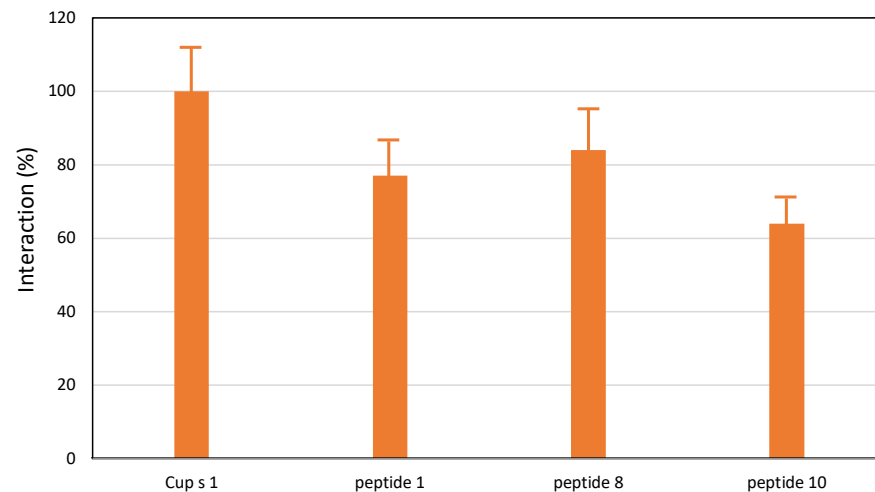


Figure 7. ELISA inhibition of the interaction Cup s 1-IgE from cypress allergic patients, in the presence of peptide 1 (10GDSNWDQNR18), peptide 8 (240NNNYDQWNI248), and peptide 10 (311SSGKTEETN319). All values correspond to the mean values \pm sd from three separate measurements.

All the three-dimensional models built for PL allergens from Cupressaceae including Cup s 1 from *Cupressus sempervirens*, Cup a 1 from *C. arizonica*, Cry j 1 from *Cryptomeria japonica*, Jun o 1 from *Juniperus oxycedrus*, Jun v 1 from *J. virginiana*, Chao 1 from *Chamaecyparis obtusa*, PL-Td from *Taxodium distichum*, and PL-Mg from *Metasequoia glyptostroboides* and PL-Pp from *Prunus persica* (Rosaceae), exhibit a very similar fold and are, therefore, readily superposable (Figure 8A,B).

According to these structural similarities, most of the identified potential continuous IgE-binding epitopes are similarly distributed on the molecular surfaces of the PL allergens (Figure 5A–C). Looking at the conformation of the surface occupied by the conserved IgE-binding epitope 1 in closely related PL allergens, it reveals an overall conformation all the more similar as it corresponds to an identical or very close amino acid sequence (Figure 8C). In this respect, epitope 1 from Cup s 1, Cup a 1, Cry j 1, Jun o 1, Jun v 1, and Cha o 1, which possess an identical or almost identical (Cha o 1) sequence, exhibit a very similar conformation. Conversely, epitope 1 from PL-Td and PL-Mg, which differ from other epitopes by a divergent amino acid sequence, exhibit a quite different overall conformation. Surprisingly, PL-Pp from peach (*Punus persica*), a member of the distantly related Rosaceae family, is more like the PL allergens from cypresses (Cup s 1, Cup a 1) and junipers (Jun o 1, Jun v 1) than the more closely related PL allergens from drawn redwood (PL-Mg) and bald cypress (PL-Td).

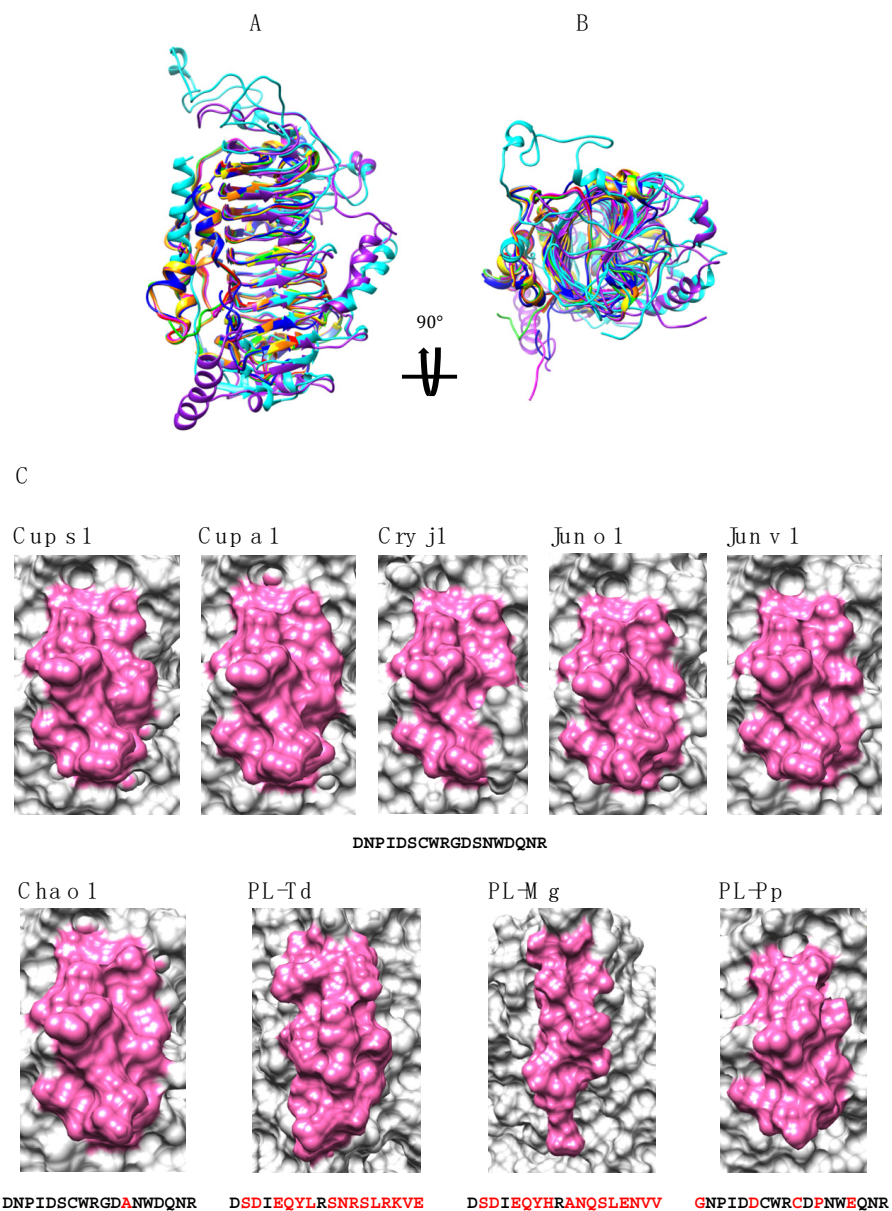


Figure 8. Lateral (A) and upper (B) views of the superposed ribbon diagrams of Cup s 1 (colored red), Cup a 1 (colored pale blue), Cry j 1 (colored green), Jun o 1 (colored yellow), Jun v 1 (colored orange), Cha o 1 (colored magenta), PL-Td (colored cyan), PL-Mg (colored purple), and PL-Pp (colored dark blue). Overall conformation of epitope 1 at the molecular surface of Cup s 1, Cup a 1, Cry j 1, Jun o 1, Jun v 1, Chao 1, PL-Td, PL-Mg, and PL-Pp (C). The corresponding amino acid sequences are indicated with the amino acid changes indicated in red letters, compared to the Cup s 1 sequence.

In addition to the partial conformational similarity of epitope 1 observed between the Cupressaceae PL allergens and PL-Pp, the alignment of amino acid sequences of Cup s 1 and PL-Pp suggests that additional conformational similarities would occur between both allergens (Figure 9). Especially, the amino acid sequence stretches of PL-Pp corresponding to epitopes 3, 4, 7, 9, and 10 of Cup s 1 display more than 50% identity, which suggests additional conformational epitopic similarities between both proteins.

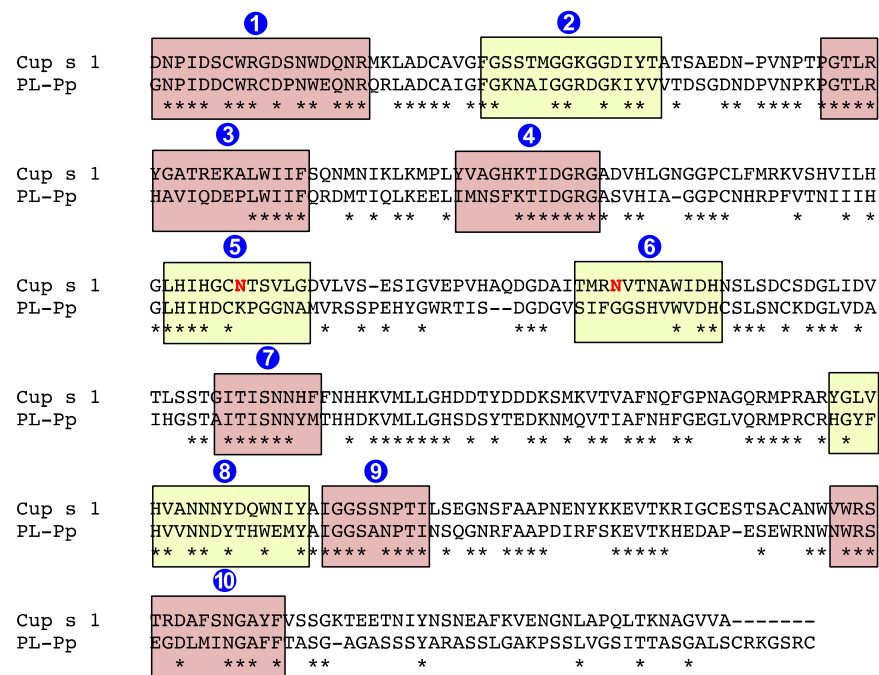


Figure 9. Comparison of Cup s 1 and PL-Pp amino acid sequences, showing the rather conserved (pink-colored boxes) and non-conserved (pale yellow-colored boxes) nature of the IgE-binding epitopic regions identified in Cup s 1, along the amino acid sequence of PL-Pp. The IgE binding epitopic regions of Cup s 1 are numbered 1–10. Fully conserved amino acid residues observed among the aligned amino acid sequences are indicated with asterisks. Asparagine residues corresponding to putative *N*-glycosylation sites are in red letters.

4. Discussion

Pectate-lyase allergens of Cupressaceae pollen consist of a homogenous family of structurally conserved glycoproteins. Their 38 kDa-polypeptide chains exhibit a similar three-dimensional fold made of three parallel β -sheets organized in a long, curved β -prism structure. However, they differ by the degree of glycosylation due to the occurrence of two (Cup s 1, Jun a 1, Jun o 1, Jun v 1), three (Cup a 1), or four (Cha o 1, Cry j 1) putative *N*-glycosylation sites along their polypeptide chains.

Using the SPOT technique, up to 10 sequential IgE-binding epitopic stretches were identified along the amino acid sequences of the PL allergens. In spite of a few discrepancies from one allergen to another, they occur at the same locations in the amino acid sequences of the different allergens. Moreover, the observed localization was remarkably reproducible for each of the assayed PL allergens. Most of these epitopic regions cluster at both ends of the β -prism structure to form more extended epitopic areas that are reminiscent of discontinuous IgE-binding epitopes. They essentially consist of hydrophilic and charged residues susceptible to create hydrogen bonds and electrostatic interactions with the corresponding paratope of IgE antibodies. Along this line, the PL allergen Jun a 1 from the mountain cedar has been reported to contain both linear and conformational IgE-binding epitopes [13]. Two of the IgE-binding epitopes identified in *Cupressus* and *Juniperus* PL allergens, namely epitopes 5 and 6, contain a *N*-glycosylation site, which is predicted to be actually glycosylated according to the GlyProt server and could thus correspond to glycotopes (Figure 2G–I). However, grafted oligosaccharides do not prevent the induction of IgE antibodies against the epitopic region corresponding to the glycosylation site. Carbohydrate moieties of the Cupressaceae allergens were extensively investigated [11,20], and their role as IgE-binding determinants/glycotopes has been clearly demonstrated for Cup a 1 from Arizona cypress pollen [4,37–39] and Jun a 1 from mountain cedar pollen [11].

According to their amino acid sequence conservation, PL allergens from the Cupressaceae and other closely related families of Taxodiaceae (PL-Mg, PL-Td) are readily

superposed with a rms of 0.36-Å, 0.34-Å, and 0.31-Å for the couples Cup s 1/Cry j 1, Cup s 1/Cha o 1, and Cup s 1/Jun o 1, respectively. With other less closely related PL-Td and PL-Mg, rms of 16.59-Å and 19.41-Å were measured for the superposed couples Cup s 1/PL-Td and Cup s 1/PL-Mg, respectively (Figure 3A,B).

The IgE-binding epitopes of the Cupressaceae PL allergens and, especially, the IgE-binding epitopes 1, 2, and 3, are nicely conserved in other closely related PL allergens from Cupressaceae, e.g., in Cup a 1 from *Cupressus arizonica* (*Hesperocyparis arizonica*) and Cha o 1 from *Chamaecyparis obtusa* (Figure 3C). Pectate lyase PL-Td from the western red cedar (*Taxodium distichum*), which also belongs to the Cupressaceae family, readily cross-reacts with Cup s 1 from *Cupressus sempervirens* pollen [37]. Some IgE-binding epitope similarity also occurs with a PL from *Pinus sitchensis* (access. ABK 2501), which belongs to the closely related Pinaceae family, with identity percentages ranging from 15% (epitope 7) up to 60% (epitopes 1 and 6). This IgE-binding epitope relatedness could extend the IgE-binding cross-reactivity of Cupressaceae to other closely related conifers.

A rather frequent cross-allergenicity between Cupressaceae pollen and peach fruit has been reported in patients allergic to cypress from Mediterranean countries defining a pollen food-associated syndrome [40]. Besides allergens from the recently described Gibberellin-regulated protein family shown to be totally or partly responsible for such cross-allergenicity in peach and cypress pollen [19,41], a 45-kDa protein was also reported as a cross-reactive allergen [40]. In this respect, pectate-lyase from peach fruit (access BAF43572, ≥ 42 kDa) exhibits some IgE-binding epitope similarity to Cup s 1 with identity percentages from 25% (epitopes 6 and 9) up to 67–70% (epitopes 1 and 7), which could also participate in the reported cross-allergenicity (Figure 4).

All PL allergens from the Cupressaceae (Cha o 1, Cry j 1, Cup s 1, Jun o 1, Jun v 1) and Taxodiaceae (PL-Mg, PL-Td), contain two (Cup s 1, Jun o 1, Jun v 1, PL-Td), three (Cup a 1, PL-Mg), four (Cry j 1), or five (Cha o 1) putative *N*-glycosylation sites, and thus correspond to often highly glycosylated allergens. The *N*-oligosaccharide linked to one of the three *N*-glycosylation sites of Cup a 1 has been characterized as a mixture of complex glycans containing β 1,2-Xyl and α 1,3-Fuc linked to the core β Man and the first core GlcNAc residues, respectively [20]. A monoclonal antibody mAb 5E6 that specifically recognizes this Cup a 1 glycotope was further characterized [39]. Comparison between the native glycosylated Cup a 1 and a non-glycosylated recombinant Cup a 1 expressed in *E. coli* allowed researchers to discriminate between IgE from cypress allergic patients that recognize the carbohydrate epitopes and those IgE that interact with peptidic epitopes [42]. Moreover, IgE that recognize carbohydrate epitopes are able to induce histamine release from basophils, suggesting they display a functional role in the cypress allergy and should be responsible for some cross-reactivity and cross-allergenicity between closely related Cupressaceae species.

According to their widespread distribution in plant organs, e.g., in pollens and fruits, pectate-lyases appear as structurally conserved pan-allergens responsible for both the sensitization and allergenic reaction in susceptible individuals. Consequently, they display a high degree of clinically relevant IgE-binding cross-reactivity. This cross-allergenicity is of paramount importance for allergic patients, since the pollination periods of the various species of Cupressaceae follow one another, thus extending the exposure to cross-reactive allergens from February to July in Mediterranean countries.

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