

BRIEF COMMUNICATION

Identification of a polygalacturonase (Cup s 2) as the major CCD-bearing allergen in *Cupressus sempervirens* pollen

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Abstract

As IgE glyco-epitopes, also referred to as cross-reactive carbohydrate determinants (CCDs), can share significant structural homologies between different plants, they are prone to extensive cross-reactivity among allergen pollen extracts. Here, cypress pollen allergens, especially a polygalacturonase (PG), were further characterized using double one-dimensional electrophoresis (D1-DE). The presence of specific IgE directed against CCDs was investigated by bromelain IgE inhibition and concanavalin A binding assays using sera of cypress pollen-sensitized patients. Our results showed that IgE reactivity to CCDs in *Cupressus sempervirens* pollen extracts is mainly related to bromelain-type epitopes of a newly identified cypress PG. This glycoprotein has been further characterized through an immunoproteomic approach and officially indexed as Cup s 2 by the WHO/IUIS allergen nomenclature. Cup s 2 could thus be associated with the increased prevalence of IgE reactivity to cypress pollen extracts because of CCD interference.

KEYWORDS

bromelain, cross-reactive carbohydrate determinant, *Cupressus sempervirens*, cypress pollen allergens, polygalacturonase

1 | INTRODUCTION

Cupressaceae pollen is frequently associated with seasonal patterns of allergic diseases in southern Europe (1), the Middle East, the southern states of North America and Japan (2). However, the prevalence of sensitization to cypress pollen allergens is still difficult to estimate due to the uncertain efficiency of current commercial pollen extracts in the diagnosis of cypress pollen allergy (3,4). It has recently been shown that skin sensitization to cypress correlated with seasonal symptoms for only 64% of tested children suffering from seasonal pollen allergy (5). Inversely, 15% of children with clinically relevant sensitization to cypress pollen had no specific IgE to Cup a 1, the *Hesperocyparis arizonica* pollen major allergen (pectate

lyase, 43 kDa) sharing 97% identity with Cup s 1 from *Cupressus sempervirens* pollen (5). Depending on the region, 60 to more than 95% of cypress pollen-sensitized patients are reported to be polysensitized with co-sensitization to taxonomically unrelated pollen species (6). Therefore, it was claimed that in patients positive to cypress pollen allergen extracts with polysensitization, symptoms were not always clearly related to the cypress pollen, but rather were associated with cross-reactive allergenic plants (3). However, it has not been clarified whether this potential cross-reactivity is due to protein/domain sequence homologies and/or cross-reactive carbohydrate determinants (CCDs) present on glycoproteins of cypress pollen extracts.

In the present study, cypress pollen allergen extracts were further characterized and the presence of specific IgE directed against CCDs was investigated.

Hélène Sénéchal and Pascal Poncet equally contributed to this study.

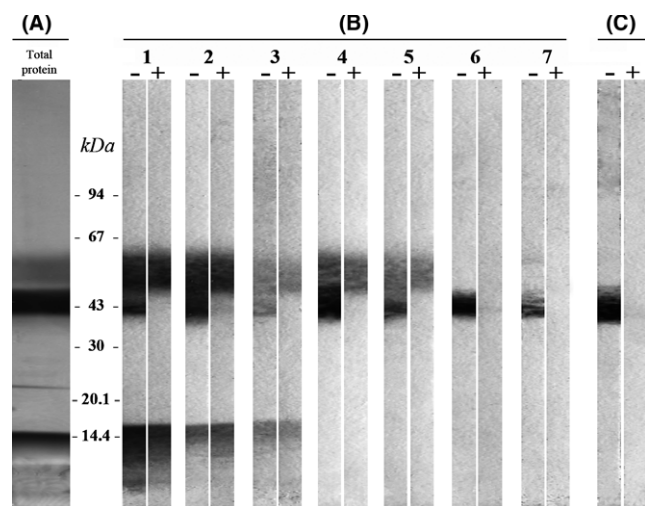


FIGURE 1 IgE immunoreactivity of six cypress pollen-allergic patients (lanes 1 to 6) and one grass pollen-allergic patient (lane 7) in the absence (-) or presence (+) of bromelain (5 U/mL). (A) Silver staining of total cypress basic protein separated in D1-DE. (B) IgE reactivity of patient sera. (C) Concanavalin A binding to cypress pollen basic proteins separated by D1-DE with (+) or without (-) addition of 10 mmol L⁻¹ methyl αD-mannopyranoside and 300 mmol L⁻¹ methyl αD-glucopyranoside. Molecular masses (kDa) are indicated

2 | METHODS

2.1 | Specific IgE screening of patient sera

Common cypress (*Cupressus sempervirens*) pollen phosphate-buffered saline extracts were characterized using double one-dimensional electrophoresis (D1-DE), as previously described (7). Briefly, after an isoelectric focusing separation (pH 2-11), the neutral and basic fractions at pH 7-7.5 and 8.5-9.5, respectively, were cut out and submitted to SDS-PAGE (8%-18%). The D1-DE separated proteins were then subjected to specific IgE immunoblotting using sera of cypress pollen-allergic patients (CPAP) (7) selected according to symptoms (rhinoconjunctivitis or asthma) and positive fluorescence enzyme immunoassay (ImmunoCAP®, Thermo Fisher Scientific Villebon, France and Phadia, Uppsala, Sweden, Phadia) of Cupressaceae pollen extracts. After obtaining informed consent from CPAP when they visited the allergy clinic, serum samples were drawn in agreement with the institutional board review of the pneumology-allergology department.

2.2 | Bromelain IgE inhibition and Con A binding assays

IgE reactivity to carbohydrate- vs peptide-specific determinants was investigated using bromelain IgE inhibition (4,8). The lectin concanavalin A (Con A) binding assay was performed using biotinylated Con A (Amersham, UK) followed by alkaline phosphatase-conjugated streptavidin and appropriate substrate (NBT/BCIP). Bromelain IgE inhibition was carried out with seven sera (Table S1) comprised of six sera positive to Cupressaceae extracts and a single serum

highly reactive to high molecular weight (>30 kDa) allergens from grass pollen extract, previously determined as CCD reactive.

2.3 | MS/MS analyses and N-terminal Edman sequencing

The CCD-bearing protein (glycoprotein) at 43 kDa and the non-CCD-bearing protein at 60 kDa were identified using 10 and 2 MS/MS analyses, respectively. All IgE-binding proteins detected by immunoblotting were manually excised from their matching gel proteins presenting the same Mr and pI. Proteins were then treated as previously described (8-10). Each gel spot was analyzed in duplicate (or sometimes triplicate) by MALDI MS/MS. For each spot, the top seven most intense precursors were automatically selected for MS/MS fragmentation. Tandem mass spectral data were then submitted to database searching (NCBI nr Viridiplantae database) using MASCOT. Homology searches and multiple amino acid sequence alignments were carried out using the Blastp (<http://expasy.org/tools/blast/>) and Clustal Omega (version 1.2.4) (www.uniprot.org/align/) programs, respectively. N-terminal Edman sequencing was carried out using a PPSQ-33A system (Shimadzu, Kyoto, Japan), in two steps of five cycles. A total of 10 residues were sequenced and identified, one at a time.

3 | RESULTS

D1-DE analysis of the basic fraction of cypress pollen extract revealed three protein bands, 60 kDa, 43 kDa, and 14 kDa (Figure 1A). IgE reactivity to the 43-kDa protein was totally inhibited after pre-incubation of patient sera with bromelain, while the intensity of IgE binding to other basic allergens of 14 and 60 kDa remained unchanged (Figure 1B). Moreover, the exclusive binding of Con A to the 43-kDa protein outlined the presence of mannose-containing oligosaccharides in its structure (Figure 1C). Incubation of Con A with two usual ligands, methyl αD-mannopyranoside (10 mmol L⁻¹) and methyl αD-glucopyranoside (300 mmol L⁻¹), inhibited Con A binding to the 43-kDa glycoprotein of (Figure 1C), thereby assessing the specificity of the binding. The same analysis performed at 43-45 kDa on the neutral fraction pI 7-7.5, containing Cup s 1 the major glyco-allergen (7), demonstrated that, in all studied patients, IgE reactivity to Cup s 1 was only partially affected by the pre-incubation with bromelain (data not shown).

MS/MS analyses showed that both IgE-binding basic proteins, the CCD-bearing 43 kDa and the non-CCD-bearing 60 kDa, belong to the polygalacturonase (PG) family. Thirty-two identified peptides from the 43-kDa protein presented a sequence coverage of 75.1/74.7% with Cup a 2 (A0T2M5/M4), 65.9% with Jun a 2, and 54.9% with Cha o 2, the allergenic PGs of *Hesperocyparis arizonica*, *Juniperus ashei*, and *Chamaecyparis obtusa*, respectively (Figure 2). The ten N-terminal amino acid residues were 100% homologous with Cup a 2 and Jun a 2 (Figure 2, shaded underlined sequence). This newly identified *C. sempervirens* PG has recently been approved as Cup s 2

[illegible]

FIGURE 2 Protein sequence alignments of various Cupressaceae PGs: Cup s 2 (PG43, C0HKB1: accession number in UniProt database), Cup a 2, a putative allergen (A0T2M5/M4), Jun a 2 (Q9FY19), and Cha o 2 (Q7M1E7). The sequence coverage was obtained from 10 MS/MS analyses and N-terminal Edman sequencing (underlined shaded sequence) of Cup s 2 (PG43, 32 peptides) and 2 MS/MS analyses for PG60 (11 peptides). The two potential N-glycosylation sites are bold shaded. Asterisks indicate amino acids of the respective PG sequences identical to Cup s 2. Variations in four amino acid positions (87, 88, 124, and 132 according to Jun a 2 amino acid numbering) are indicated in the line variant

by the WHO/IUIS Allergen Nomenclature Sub-Committee. Cup s 2 shares 96.5% sequence identity with Jun a 2, 87.2% with Cup a 2, and 81.5% with Cha o 2. Of the eleven peptides identified in the 60 kDa, nine were redundant with those identified in the 43 kDa. The two remaining peptides were only found in the 60 kDa PG and included the N-glycosylation sites at position 267 or 266 in Jun a 2 or Cha o 2 (KCTIGTGDDCVAVG TGSSNITKD) and 397 in Cha o 2 (KLSNVFLKLTSGKV).

4 | DISCUSSION

Cupressus sempervirens is widely distributed over the regions surrounding the Mediterranean basin and the importance of its pollen as a major aeroallergen has been repeatedly emphasized (1,2). Several sensitization patterns and IgE-binding proteins have already been detected (11) in cypress pollen extracts, from which only two allergens, Cup s 1 (a pectate lyase) and Cup s 3 (a pathogenesis-

related group 5 protein), are officially classified in IUIS (www.allergen.org). In the present work, an immunoproteomic approach was used to study CCDs in cypress allergy.

In a previous study, we showed that among 31 allergic patients sensitized to cypress pollen, 19 were positive to a 43-kDa basic protein (7). This protein is characterized in-depth herein as the PG of *Cupressus sempervirens* pollen and registered as Cup s 2 in the WHO/IUIS allergen nomenclature (<http://allergen.org/viewallergen.php?aid=889>). Moreover, our results revealed that the contribution of bromelain-type CCD to the specific IgE recognition of Cup s 2 is substantial.

PGs are widely present throughout the plant kingdom and comprise the second group of major allergens in Cupressaceae pollen (1). Interestingly, the strongest IgE reactivities to Cup s 2 were found among sera from polysensitized patients (Table S1). All these strongly reactive sera displayed CCD-sIgE to this 43-kDa glycoprotein (Figure 1, lanes 1-6). Furthermore, the serum of a grass pollen-allergic patient exhibiting anti-CCD IgE appeared to be reactive only to Cup s 2 in cypress pollen extracts (Figure 1, lane 7).

Of the two different forms of PG reported, at 43 kDa (PG43) and at 60 kDa (PG60) (7), PG60 appeared to be exclusively recognized for its peptide-specific IgE determinants. Furthermore, the specific binding of Con A lectin to Cup s 2, and not to the PG60 isoenzyme, demonstrated the presence of exposed mannose-containing oligosaccharides only on the 43-kDa protein. This fact reflects fundamental differences between specific IgE-binding epitopes involved in the recognition of the 43-kDa and 60-kDa proteins making these two cypress pollen PGs immunologically distinguishable despite their structural similarity. By comparing the whole set of identified peptides of PG43 to the sequence of Cup a 2, Jun a 2 or Cha o 2, peptides where N-glycosylation is located were not detected. These two peptides were readily detected in the PG60, but not in the PG43, because the grafted carbohydrate moiety would likely prevent the effective proteolysis by trypsin in MS analysis (12). Thus, the presence of oligosaccharide structures containing a mannosyl group on Cup s 2 may explain the extensive cross-reactivity reported between Cupressaceae and non-Cupressaceae pollen PGs (13), as well as between PGs of fruits and Cupressaceae pollen grains (14), despite the poor homology of their respective amino acid sequences. Pollen PGs share sequence identity of up to 97% within Cupressaceae, but only 33%-36% with other angiosperm pollen grains and 40%-47% with fruit PGs, rendering the risk of cross-reactivity between specific peptide determinants very low (15).

To conclude, the present study demonstrates that sensitization to the cypress pollen 43-kDa PG (Cup s 2) in our patients is mainly due to bromelain-type epitopes and could be linked to an increased prevalence of IgE reactivity to cypress pollen extracts because of CCD interference (16). About 31% of pollen-allergic patients (17) and 20% of patients with food allergy have CCD-specific IgE in their sera (18). The clinical relevance of IgE reactivity to CCD epitopes is still a matter of debate, and specific carbohydrate epitopes are often considered clinically irrelevant (15,17-20). Thus, the sometimes confounding role of these glyco-epitopes in serum-based IgE antibody

assays and skin prick tests should be considered, especially when the results do not match the clinical picture (20,21).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors have contributed to the acquisition of data and/or interpretation of data, drafting of the article and revising it critically for important intellectual content and have provided approval for this version to be published.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Table 1: Biological characteristics of studied patients

Lane #	Sex	age	Cup s pollen*	Other pollen*	Other*				
1	F	44	13.1	Par o 0.17	Der p <0.1	Fel d 13. 0			
2	F	26	20.6	Dac g <0.1	Pru p 1.57	Der p <0.1	Fel d <0.1	Hev b<0.1	
3	F	10	ND	Jun a 20.8					
4	M	54	4.03	Jun a 34.6	Gal d <0.1	Bos d <0.1			
5	F	35	ND	Jun a 26.8					
6	M	3	17	Dac g 14.6	Cap h 18.8	Der p 1.5	Can f 34.5	Bromelin 3.0	
7	M	<i>unknown</i>	ND	Dac g >100					

* IgE: kIU/L

ND: Not done