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Ash pollen immunoproteomics: identification, immunological characterization and sequencing of six new allergens.

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Capsule Summary

Immunoproteomics, IgE-inhibition assays and cDNA-cloning reveals that ash and olive allergenic protein profiles are mostly equivalent, thus explaining their high cross-reactivity. Our data suggest simplifying diagnosis of patients by using indistinctly ash or olive pollen.

Key words

2D-electrophoresis; immunoproteomics; ash pollen; allergen; *Fraxinus excelsior* pollinosis; cross-reactivity

To the Editor:

Ash (*Fraxinus excelsior*) is widely distributed in Northern and Central Europe. Although yearly ash pollen counts are in the range of 1000 grains/m³, its clinical relevance has been undervalued because ash pollination overlaps with that of Betulaceae.¹ Subsequently, ash has not been included in diagnostic assays,² although is responsible for extensive pollinosis in Alsace (France) and Austria where ash pollination occurs immediately after birch,² and Switzerland where it is as frequent as birch pollinosis.³

Allergic patients are commonly diagnosed and treated with protein extracts from natural sources. An alternative approach for allergy diagnosis and personalized treatment of patients consists of the utilization of purified allergens. Thus, it is necessary to know the near complete allergogram of the source responsible for the sensitization.

Fra e 1 is the only ash pollen allergen isolated, produced as recombinant protein and characterized,⁴ despite the complexity of the IgE-reactive protein profile observed by 1D-electrophoresis (1DE) analysis of *F. excelsior* pollen.^{4, 5} We therefore performed an extensive 2D-electrophoresis (2DE) analysis to identify the IgE-reactive proteins content using serum of 25 ash pollen sensitized patients with IgE reactivity to ash and olive pollen extracts by ELISA (see Table E1 in this article's Online Repository at www.jacionline.org). On average, silver-staining gels showed 165 ± 15 spots between isoelectric points (pI) 3.5 to 8.2 and molecular masses (MW) between 4 and 70 kDa (see Figure 1A). To visualize IgE-binding proteins, 2D-immunoblots were performed using two independent pools containing 8 different ash pollen-allergic patient's sera (see Figure 1B and 1C). We found 35 ± 2 IgE-binding proteins with pIs from 3.8 to 8.1 and MW ranging between 8 and 66 kDa. A main group of IgE spots centered at mildly acidic-neutral pI and about 18-22 kDa corresponded to Fra e 1 isoforms.^{4, 5}

We hypothesized that most of the IgE-reactive observed bands might correspond to proteins with similar molecular features (pI and MW) to previously described olive pollen allergens: Ole e 1, Ole e 2, Ole e 3, Ole e 6, Ole e 7, Ole e 9, Ole e 10, Ole e 11 and Ole e 12.⁶ To address this question, we then performed a complete immunoproteomic study of ash pollen extract by 2DE using polyclonal antisera raised against the most relevant olive pollen allergens (see Figure 1D). We identified IgG-reactive proteins and named them as Fra e 1, Fra e 2, Fra e 3, Fra e 6, Fra e 7, Fra e 9, Fra e 11, and Fra e 12, according to the olive homologues recognized by the rabbit antisera. All of them and their isoforms were responsible for most of the identified IgE-reactive proteins (see Figure 1B and 1C). Fra e 10 was only detected by 1DE (data not shown).

These findings suggest high sequence identity between olive and ash pollen allergens. To confirm this hypothesis, cDNA encoding six ash pollen allergens not previously reported (Fra e 2, Fra e 3, Fra e 6, Fra e 10, Fra e 11, and Fra e 12) was amplified by PCR, cloned and sequenced (see Table E2 in this article's Online Repository at www.jacionline.org).⁴ cDNA encoding the complete sequences of 6 of the most IgE-reactive allergens from ash pollen rendered polypeptides ranging from 50 amino acids for Fra e 6 to 341 for Fra e 11 (see Figure 1E). Ash allergens possessed similar physicochemical properties to those from olive pollen: acidic to mildly acidic proteins (4.3 to 6.5) except Fra e 7 with a basic pI of 8.2; low to medium MW (5.8 to 37.4 kDa) and the presence of signal peptides for Fra e 6, Fra e 10 and Fra e 11. The sequence identity between ash and olive pollen allergens ranged between 84 and 97%, and similarity from 94 to 99%, except for Fra e 11 that presented the lowest identity and similarity (79 and 90%, respectively) with its olive counterpart (see Figure 1E).

Consistently with the high amino acid sequence identity and similarity between ash and olive pollen allergens, we hypothesized that purified olive pollen allergens could be used to assess the significance, potency and prevalence of ash pollen allergens. We then performed IgE-inhibition assays of the ash pollen extract with ash

sensitized patients sera and the panel of purified olive pollen allergens as inhibitors. By densitometry of 1DE and 2DE IgE-inhibition assays, we measured a 95% reduction of the IgE-binding obtained with the IgE serum pool without inhibitors (see Figure 2A and 2B). The residual IgE-positive signal might be due to the recognition of specific isoforms not so cross-reactive to olive pollen allergens because of differences in their amino acid sequence or glycosylation patterns. Alternatively, the presence of other allergens different from those present in olive pollen should not be discarded. In a recent publication analyzing ash pollen components by proteomics based on 2D and mass spectrometry,⁷ the authors identified Fra e 1, Fra e 2, and Fra e 3 as relevant allergens, and β -galactosidase, malate dehydrogenase, and a 4-EF hand Ca-binding protein as minor ash pollen allergens; with the vast majority of major allergen spots unidentified.⁷ Then, the residual 5% of the IgE-reactivity observed in the IgE inhibition assays might be associated to these minor allergens.⁷

As IgE-inhibition assays validated the use of purified olive pollen allergens to characterize the IgE immune response of ash pollen sensitized patients, we tested their IgE-binding ability by ELISA. The deduced prevalence for the cross-reactive ash allergens ranged between 64 to 92% for the major allergens Fra e 1, Fra e 2, Fra e 9 and Fra e 11 and between 4 to 32% for the minor allergens Fra e 3, Fra e 6, Fra e 7, Fra e 10 and Fra e 12 (see Figure 2C and 2D). These findings are in accordance with previous reports involving related and non-related allergenic sources, except for Fra e 10 that was previously described as major allergen in olive pollen.^{6, 8}

Collectively, our findings show that the *F. excelsior* allergogram responsible for the allergy symptoms in ash-sensitized patients is highly similar to that obtained from *O. europaea*, which supports the high cross-reactivity of patients to both pollens. Moreover, the high sequence identity of olive and ash pollen allergens and the IgE-inhibition results suggest using indistinctly purified allergens from ash or olive pollen for diagnosis. For treatment, the most prevalent pollen extract in the living area of the sensitized patient should be used. For personalized treatment, a comparative trial with

patients receiving either olive or ash allergens should be made to ensure that their differences do not affect patient's desensitization.

Additional information is available (see this article's Methods section and References in the Online Repository at www.jacionline.org).

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Figure legends

Figure 1. IgE-reactive proteins identified by immunoproteomic profiling of ash-pollen extract. **(A)** 2DE separation of ash-pollen extract followed by silver staining. IgE-immunoblot after 2DE using **(B)** 15% or **(C)** 17% polyacrilamide gels. IgE-immunostaining was performed with a selected equivolumetric pool of 8 sera directed against high MW allergens **(B)** or 8 sera directed to low MW allergens **(C)**.^{4, 5} **(D)** 2DE as in **B** and **C**, and immunostaining with the indicated polyclonal antisera raised against known olive pollen allergens to identify the main IgE-reactive components of ash pollen extract. The IgE-reactive spots named in panels B and C were identified according to the IgG reactivity in panel D. **(E)** Amino acid sequences of the six new allergens from *F. excelsior* pollen and alignment with their *O. europaea* counterparts. Dots, same amino acid than reference. Dash, gaps. Identity and similarity were calculated without the theoretical signal peptide. Accession numbers: Fra e 2 (KC920922), Fra e 3 (KC920923), Fra e 6 (KC920921), Fra e 10 (KC920924), Fra e 11 (KC920915), Fra e 12 (EF626802), Ole e 2 (A4GE44), Ole e 3 (O81092), Ole e 6 (O24172), Ole e 10 (Q84V39), Ole e 11 (D8VPP5), and Ole e 12 (E1U332).

Figure 2. IgE-binding inhibition assays to ash pollen extract and allergenic contribution of ash-pollen allergens. **(A)** Inhibition of the IgE binding to ash pollen extract subjected to 1DE using a pool of 8 randomly selected sera different from those used in Figure 1 to avoid any bias towards any particular allergen and a cocktail of 2.5 µg/mL of each of the olive pollen allergens homologous to Fra e 1, Fra e 2, Fra e 3, Fra e 6, Fra e 7, Fra e 9, Fra 10, Fra e 11 and Fra e 12 as inhibitors. -, no inhibitor. +, with inhibitors. N, non-atopic serum. **(B)** Inhibition of the IgE binding to ash pollen extract subjected to 2DE using the same olive allergen cocktail. Left, no inhibitor. Right, with inhibitors. A non-atopic serum was used as control. **(C-D)** Specific IgE levels for each olive pollen allergen in OD for the 25 ash pollen sensitized sera obtained by ELISA. Median value (red dot) and standard deviation (red error bars) are also represented.

Supplementary Methods

Pollen protein extracts

Pollens from *Fraxinus excelsior* and *Olea europaea* were obtained from ALK-Abelló (Madrid, Spain). Pollen protein extracts were obtained by saline extraction as described previously.^{E1, E2} Protein extract concentration was determined by Lowry method according to established protocols.

Serum samples, allergenic proteins and antibodies

The Ethical Committee of the Complutense University (Madrid, Spain) approved the protocols used for experimental work with mice and all the methodology related to the use of human sera in the study.

Twenty-five patients from Strasbourg (France) with pollinosis to ash (see Table E1 in this article's Online Repository at www.jacionline.org), a class radioallergosorbent test result ranging from 1 to 6 as determined using the CAP-RAST method (Amersham Pharmacia Biotech, Uppsala, Sweden), positive skin prick test reactions to ash pollen extract, and no migratory background to or from the Mediterranean area were included in this study.^{E1, E2} Furthermore, one non-atopic patient was used as a negative control. All patients provided informed consent. Patient's sera were positive to ash and olive pollen extract by ELISA (correlation of 0.93).

Polyclonal antiserum against indicated natural purified olive pollen allergens were prepared by immunizing separately New Zealand white rabbits in a 6-week period by weekly injections of the protein (100 µg) in complete Freund adjuvant. Horseradish peroxidase-labeled goat polyclonal antibody against rabbit IgG was obtained from Bio-Rad (Richmond, Calif). Mouse monoclonal antibody against human IgE was kindly donated by ALK-Abelló (Madrid, Spain). Horseradish peroxidase-labeled rabbit polyclonal antibody against mouse IgG was purchased from Dako (San Just Desvern, Barcelona, Spain).

Allergenic olive proteins were purified according to previously published protocols,^{E3} and concentration determined by micro BCA Protein Assay kit (Pierce).

1D-, 2D-electrophoresis and analytical procedures

SDS-PAGE was alternatively performed in 15% or 17% polyacrylamide gels under reducing conditions in the presence of 5% β -mercaptoethanol according to established protocols. Proteins were stained with Coomassie Blue R-250 (Sigma-Aldrich, Química, Spain). Molecular mass was determined with protein markers MW-SDS-70L (Sigma-Aldrich).

2DE was performed in a PROTEAN IEF Cell using 7 cm length, pH 3-10 linear ReadyStrip IPG (Bio-Rad) under reducing conditions in the presence of tributylphosphine, and/or dithiothreitol, and iodoacetamide, followed by 15% or 17% SDS-PAGE. Proteins were visualized after silver staining or alternatively transferred to nitrocellulose membranes for immunostaining with ash pollen sensitized patient's sera or polyclonal antisera. Experiments were performed in triplicate. Images show a representative image of the experiment.

Immunological assays

Proteins were alternatively transferred to nitrocellulose membranes (Amersham Biosciences) after SDS-PAGE or 2DE under reducing conditions. Membranes were incubated with individual or an equivolumetric pool of human sera (diluted 1:10), mouse anti-human IgE monoclonal antibody (Alk-Abelló) (diluted 1:5000) and rabbit anti-mouse IgG horseradish peroxidase-labeled antibody (Dako) (diluted 1:2500). Alternatively, membranes were incubated with rabbit polyclonal antisera raised against olive pollen allergens (diluted 1:10000) followed by goat anti-rabbit IgG horseradish peroxidase-labeled polyclonal antibody (Bio-Rad) (diluted 1:3000) as described.^{E1, E4} For immunoblotting inhibition assays an equivolumetric pool of 8 sera (diluted 1:10) was preincubated with PBS, or 2.5 μ g/mL of each purified allergen as inhibitors.^{E1, E4}

Chemiluminiscent signal was developed by ECL-Western blotting reagent (Amersham Bioscience). Immunoblotting inhibition assays were quantified by densitometry using the Multi Gauge software (Fujifilm, Barcelona, Spain). Percentage of IgE inhibition was calculated considering the IgE binding signal with the membranes incubated with the IgE serum pool in absence of inhibitors as 100%.

Indirect ELISA was performed in 96 well plates coated with 0.1 µg of purified proteins or 20 µg of pollen extract per well. Assays were performed using individual human serum samples, or alternatively, an equivolumetric pool of human sera (n=5) (diluted 1:10), previously adsorbed with indicated concentrations of inhibitors for the inhibition assays.^{E1, E4} Binding of human IgE or specific polyclonal antisera was detected as above described. Signal was developed with o-phenylenediamine diluted in citrate buffer, stopped with 1 M H₂SO₄, and measured at 492 nm in an iMark Microplate Absorbance Reader (Bio-Rad). ELISA experiments were performed in triplicate and absorbance values above 0.1 were taken as positives.

Cloning strategy

cDNA from *F. excelsior* pollen was synthesized from total RNA using the SMART RACE cDNA amplification kit (BD Biosciences-Clontech, Madrid, Spain) according to the manufacturer instructions and directly used as template in PCR reactions. Amplification of *F. excelsior* pollen allergens by PCR was performed using degenerate and non-degenerate oligonucleotides (see Table E2 in this article's Online Repository at www.jacionline.org) designed from conserved nucleotide sequences from related (olive) and non-related phylogenetically homologues as previously described.^{E5, E6} PCR products were cloned into the pCR2.1 plasmid (Invitrogen, Groningen, The Netherlands), and DNA from 4-6 clones of each construction sequenced using M13 forward and M13 reverse oligonucleotides.

Statistical analysis, amino acid sequences analysis, alignment, and modeling

We used Microsoft Excel 2007 for the analysis of ELISA data sets to calculate mean, and standard error of the mean. The presence and location of signal peptide cleavage site in the amino acid sequences of ash pollen allergens was calculated with SignalP 4.1,^{E7} and the theoretical molecular mass of the mature proteins without the signal peptide with the ProtParam tool.^{E8} Alignment of sequences was made using the freely available GeneDoc software. 3D structure modeling of the allergens was made using SWISS-MODEL, the fully automated protein structure homology-modeling server of the Swiss Institute of Bioinformatics (<http://swissmodel.expasy.org/>).^{E9} Models obtained were represented with VMD.^{E10}

Supplementary References

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1 **Table E1.** Clinical data of the ash pollen sensitized patients included in the study

Patient number ^Δ	Gender*	RAST		Skin test (D+d)/2 (mm)		ELISA (OD at 492 nm)	
		Class	kU/L	Histamine	Ash pollen extract	Ash pollen extract	Olive pollen extract
1	F	3	7.45	4.5	9	0.62	0.92
2	F	2	2.77	5	7	0.17	0.17
3	M	3	7.03	5.5	-	0.11	0.11
4	F	2	2.27	7.5	5	0.23	0.43
5	F	4	17.8	6.5	8.5	0.31	0.37
6	M	3	13	8	8.5	0.14	0.15
7	M	3	11.7	6.5	8.5	0.55	0.69
8	M	3	4.95	5.5	8.5	0.81	1.28
9	M	6	>100	4	5	0.74	1.12
10	F	2	1.42	6.5	7.5	0.24	0.32
11	F	2	3.35	4.5	9	0.33	0.32
12	M	2	1.14	4.5	6.5	0.15	0.12
13	F	3	16.5	4.5	4.5	0.50	0.89
14	F	3	4.95	6	10	0.19	0.24
15	F	2	1.19	6.5	9.5	0.09	0.10
16	M	3	5.26	5.5	8	0.32	0.63
17	F	4	25.4	6.5	10	0.92	1.12
18	F	5	56.9	3.5	7.5	0.43	0.53
19	M	3	7.36	6.5	14	0.24	0.35
20	M	2	2.34	7	-	0.20	0.18
21	F	4	21.7	5	9	0.35	0.62
22	M	2	2.64	6	-	0.41	0.64
23	M	2	0.83	4.5	6.5	0.23	0.18
24	F	3	16.1	6	4.5	0.07	0.06
25	M	4	49.4	6.5	8.5	0.52	0.95

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3 Patients were recruited according to a positive cutaneous test response to ash pollen extract and absence
4 of a migratory background to or from Mediterranean area. Patients age ranges between 18 to 63 years
5 (mean 28.5 years).
6 *F, female; M, male.
7 -, Not done.

8 **Table E2: Oligonucleotides used to clone ash pollen allergens by PCR.**

Allergen	Forward primer 5'->3'	Reverse Primer 5'->3'
Fra e 2	ATG TCN TGG CAR RCN TAY GT	TTA RAA NAT YTT NGC NAC RTC YTT
Fra e 3	ATG GCC GAC GAT CCA CAG GAA GT	CTA GAA GAT TTT TGC AAC ATC CTT GAC
Fra e 6	ATG GAC GAG GCC CAG TTT	TTA RTT NGG YTT RAA RTT NGC
Fra e 10	ATG CGA GGA ACC GCA	TCA AGA GAG GAA TGA GCA
Fra e 11	ATG GAT GAT AGA GTA CCT ATT CC	TTA AGC TTT GGC GGG GGG
Fra e 12	ATG GCT GAG AAA ACC AAG ATT TTG	TTA AAC AAA TTG ATT AAG GGA CTC CTC

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