Detection of immunogens from Fraxinus *spp. pollen grains*

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ORIGINAL PAPER

Detection of immunogens from *Fraxinus* spp. pollen grains

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Abstract This work is aimed at characterising immunogens from *Fraxinus uhdei* pollen and to develop ELISA assays for their immunodetection, since it is a relevant aeroallergen. *F. uhdei* pollen extract contained proteins in a wide range of molecular mass from 22 to 112 kDa by SDS-PAGE; moreover, 187 fractions were detected in the 2D electrophoresis. Pollen extract was used to generate rabbit hyperimmune serum. Western blot revealed six main immunogens in a range from 40 to 21 kDa and several protein spots in 2D immunoblot. Tryptic peptides from six spots were analysed by nano-LC-ESI-MS/MS, indicating that most of proteins show homology with proteins involved in metabolism and with Ole e 11 and Fra e 2. The minimum concentration of *Fraxinus* spp. proteins detected by ELISA assays was 0.25 μg/mL. The detection of one 21.5 KDa protein by WB in the dust obtained from air sampling suggests the possibility to be a *Fraxinus* spp. protein suspended in the atmosphere. ELISA assays of the air samples detected the higher concentrations of *Fraxinus* spp. proteins when its airborne pollen concentrations were higher, according to the Mexican Aerobiology Network.

Keywords Aeroallergens · ELISA · *Fraxinus* · Immunoproteomics

1 Introduction

The ash tree (*Fraxinus* spp.) belongs to the family Oleaceae, and the pollen from this family has been considered to act as an allergen (Morfín-Maciel et al. 2009; Hemmer et al. 2000; Barderas et al. 2005; Mas et al. 2014). Several allergens produced by members of the Oleaceae such as *Fraxinus* spp., *Ligustrum* spp., and *Olea* spp. have been described, and the crossreactivity of these allergens has been analysed (Esteve et al. 2012). *Fraxinus* allergens such as Fra e 1 have been studied in natural (Barderas et al. 2005; Hrabina et al. 2007) and recombinant forms (Barderas et al. 2006). It has been concluded from these studies that natural *Fraxinus* allergens are heterogeneously glycosylated and appear to display several structural and allergenic properties. Because they show these properties, it has been suggested that these allergens could be used for clinical purposes.

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Particulate matter in the atmosphere, including pollen grains, mould spores, dust mites, or their components, such as proteins, can trigger respiratory allergies in sensitised individuals (Emberlin 1995; Salvaggio et al. 1971; Reid et al. 2009). Airborne *Fraxinus* pollen grains are considered aeroallergens (Salvaggio et al. 1971; Reid et al. 2009; Metz-Favre et al. 2010) and to act as a sensitising factor for allergies towards Oleaceae pollen grains and towards allergens from other plant families (Hemmer et al. 2000). It has been suggested that in sensitised patients, the duration of allergic reactions to olive pollen grains when these reactions are present longer than the pollination season is a result of polysensitisation (Kimraz et al. 2005) or of the persistence of allergens in pauci-micronic particles (Reid et al. 2009). Hence, it is necessary to assess airborne pollen concentrations as well as the concentration of specific allergens in the atmosphere. *Fraxinus* spp. pollen represents one of the most abundant pollen grains in Mexico City's atmosphere, according to the Mexican Aerobiology Network (REMA). *F. excelsior* pollen is the most common species studied in Europe, whereas *Fraxinus uhdei* is the species present in Mexico (Galindo et al. 2012) and is responsible for certain cases of pollinosis caused by Oleaceae family members in Mexico (Larenas et al. 2009). This study seeks to identify the main immunogenic proteins of *F. uhdei* pollen grains and to develop an ELISA assay for their detection in the atmosphere. With this information, it will be possible to establish the presence of the main allergens as aeroparticles.

2 Methods

2.1 Extract

Pollen grains were harvested from *F. uhdei* flowers collected in Mexico City during the flowering period (January and February) in 2012 and 2013. The trees were identified using a guide to the trees of Mexico City (Rodríguez and Cohen 2003). The pollen was stored at −20 °C until use. *Fraxinus* pollen (150 mg) was suspended in 1.2 mL of phosphate saline buffer (PBS) and 300 µL of protease inhibitor cocktail (Roche, Basel, Switzerland), with agitation overnight at 4 °C. The extract was centrifuged at 9168*g* for 10 min at 4 °C, and the supernatant was stored at −20 °C until use.

2.2 Analytical methods

Protein concentration was determined with the bicinchoninic acid method (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Richmond, CA, USA), using bovine serum albumin as standard.

2.3 Hyperimmune serum

Hyperimmune serum was produced in a 2-kg New Zealand rabbit. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Immunisation was performed by subcutaneous administration of serum at 1 mg/kg plus 200 µL of Freund's complete adjuvant. Immunisations were performed every 2 weeks. The reactivity was tested with ELISA (see ELISA section).

2.4 1D-Electrophoresis and Immunoblotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing and denaturant conditions in 10 % polyacrylamide gels for 3 h at 60 V, 100 mA, as described in a previous publication (Laemmli 1970). The gel, containing proteins, was then transferred onto a nitrocellulose membrane, as previously reported (Towbin et al. 1979). A Western blot (WB) analysis of the *F. uhdei* pollen allergens was performed with the *F. uhdei* pollen extract and air samples, as previously described (Alpuche et al. 2010). The allergens were detected with anti-*Fraxinus* hyperimmune serum (diluted 1:100) and revealed with goat anti-rabbit horseradish peroxidase (HRP) antibody (Jackson Inc., PA, USA). The detection of glycoproteins present in the extract was performed with WB using biotin-labelled concanavalin A (ConA 1:200) specific for mannose/glucose residues or wheat germ agglutinin (WGA 1:75) specific for N-acetylglucosamine. The glycoproteins were revealed with streptavidin–HRP (Sigma–Aldrich, St. Louis, MO).

2.5 2D Electrophoresis and Immunoblotting

Two-dimensional electrophoresis was performed in a Multiphor II system (Amersham Biosciences, Uppsala, Sweden) using ampholytes (pH 3–10) for the first dimension and a 10 % acrylamide gel for the second dimension. The gel was transferred onto a nitrocellulose

membrane and detected with anti-*Fraxinus* hyperimmune serum, as previously described.

2.6 Nano-LC-ESI-MS/MS

Six of the identified spots in the 2D electrophoresis recognised by immune blotting were excised from the 2D electrophoresis gel for trypsin digestion (Promega, Madison, WI), trapped in a LC-Packing PepMap C18 µ-pre-column cartridge (Dionex, Sunnyvale, CA), and loaded into an integrated nano-LC-ESI-MS/MS system through an analytical C18 capillary column connected online to an Ultima API quadrupole acceleration time-of-flight mass spectrometer (Micromass, Manchester, UK). Data acquisition and analysis were performed under the full control of MassLynx 4.0 (Micromass). The 1-s survey scans were run in the m/z mass range of 400–2000. Product ions were analysed with Mascot software (www.matrixscience.com) using both NCBInr and EST databases; only proteins with ion scores >30 were reported (Findlay and Geisow 1989).

2.7 Air sampling

A Cyclon multi-vial sampler (Burkard Co., UK) was used to perform continuous air sampling during 2012, with an air flow of 16.5 L/min. Dust was collected in 1.5-mL Eppendorf tubes weekly, and pooled in a monthly sample, and stored at −20 °C. The samples from January were used for WB detection, as higher concentrations of *Fraxinus* pollen occurred during this month according to REMA. The remaining samples were used for ELISA detection.

2.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA assays were performed in microtitration polystyrene plates (Nunc, Denmark). The detection curve was calculated for *Fraxinus* pollen extract with concentrations from 0.01 to 50 µg/mL in carbonate buffer (pH 9.5). The material to be tested was fixed to the bottom of each well by incubating for 1 h at 37 °C and overnight at 4 °C. The plates were washed four times with PBS/Tween-20 at 0.01 % (PBS-T). The plates were blocked with 5 % non-fat dry milk/PBS incubated for 90 min at 37 °C and washed several times with PBS-T (Agundis et al. 2000). Rabbit anti-*Fraxinus* hyperimmune serum (1:200) was then incubated for 90 min at 30 °C, and the plates were then washed with PBS-T. Peroxidase-labelled mAbs against rabbit IgG (1:400) (Jackson Inc., USA) was then added and incubated for 90 min at 37 °C. The plates were then washed with PBS-T and PBS and revealed by the addition of 50 µL O-phenylenediamine (Sigma–Aldrich) and H_2O_2 in 100 mM citrate buffer, pH 5.6. The reaction was stopped by adding 3 N HCl, and samples were read at 492 nm in an ELISA microplate reader. All assays were performed in triplicate, and the results represent the mean of three determinations, with controls for each conjugate.

3 Results and discussion

3.1 Extract and analytical methods

The concentration of protein solubilised with PBS was 15 mg/mL. This value was equivalent to an amount of protein equal to 12 % of the total weight of the pollen.

3.2 Hyperimmune serum

The recognition of immunogens by the anti-*Fraxinus* hyperimmune serum was determined by ELISA. The titration curve was performed with different protein concentrations of the *Fraxinus* extract (0.01–50 µg/mL) by ELISA. In this analysis, the anti-*Fraxinus* hyperimmune serum at optimal dilution (1:500) recognised a minimum protein concentration in the extract of 0.25 µg/mL (Fig. 1). This value was calculated with the mean of the lowest concentration plus 4 SD.

3.3 1D-Electrophoresis and Immunoblotting

The SDS-PAGE technique showed that *Fraxinus uhdei* extract contained proteins with a molecular mass from 21 to 112 kDa; moreover, four main protein complexes with molecular weights ranging from 22 to 66 kDa were present at relatively high concentrations. The determination of glycoproteins with lectins performed with ConA resulted in the detection of six fractions. This analysis suggested the presence of high-mannose N-glycosidically linked proteins with molecular masses of 86, 76, 70, 54, 47, and 44 kDa. WGA detected the 21.5, 62, and 68 kDa fractions (Fig. 2a); this lectin is specific for GlcNAc, which is a

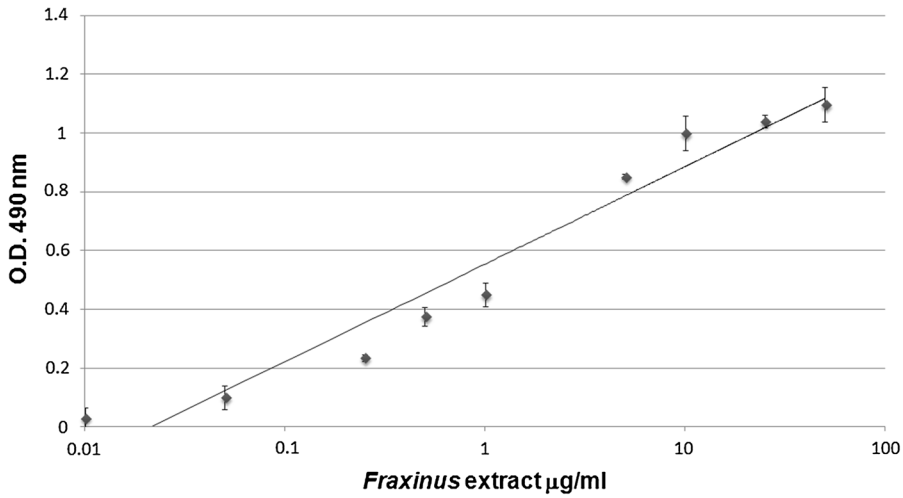

Fig. 1 Detection curve of the *Fraxinus* immunogens in the protein extract by ELISA. The x-axis represents the *Fraxinus* protein concentrations, from 0.01 to 50 µg/mL

part of the N-glycosidically linked glycans, but is specific also for chitobiose and triose, which represent the main structure of polymeric chitin and have been considered to be involved with the cross-reactive carbohydrate determinants (CCDs), which trigger reactions, such as asthma (Salazar et al. 2013). The anti-*Fraxinus* hyperimmune serum detected six main fractions in WB analysis (21.5, 24.6, 28.5, 29.4, 35.2, and 38.5 kDa) (Fig. 2b). The 21.5 kDa band was detected by the anti-*Fraxinus* hyperimmune serum and by WGA, suggesting that this fraction could be the homologue of that reported for *F. excelsior* (Hemmer et al. 2000; Barderas et al. 2005).

3.4 2D Electrophoresis and Immunoblotting

The 2D electrophoresis (Fig. 3a) showed that *F. uhdei* extract contained 187 proteins with a molecular mass

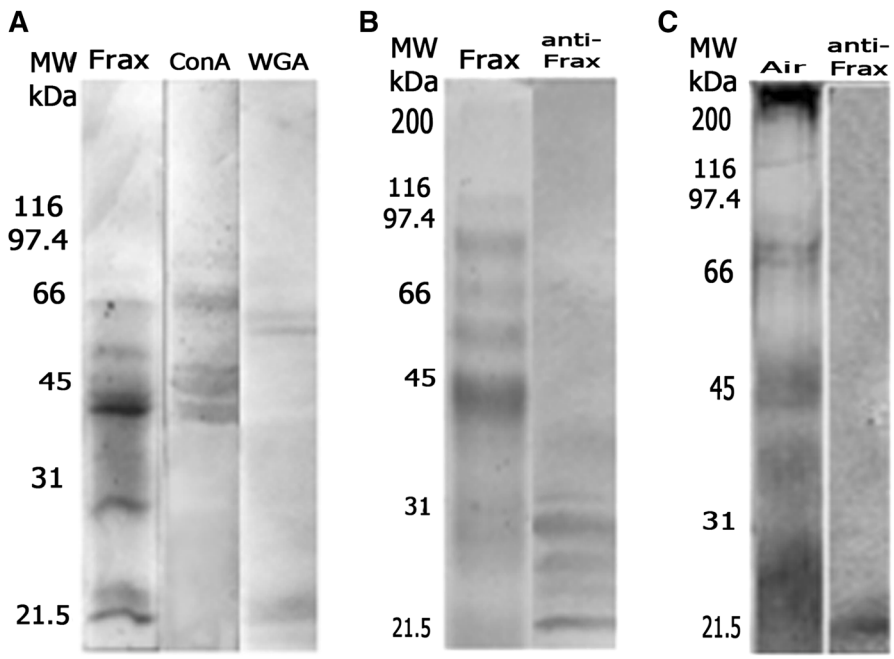

Fig. 2 Electrophoresis and WB of *Fraxinus* proteins in the extract and air sample. **A** *Fraxinus* extract stained with Coomassie blue (Frax) and WB revealed with Con A (Con A) and WGA (WGA) lectins. **B** Immunodetection of *Fraxinus* extract (Frax) revealed with rabbit hyperimmune serum anti-Frax (Anti-Frax). **C** Air sample collected proteins, stained with Coomassie blue (Air) and revealed with anti-Frax serum. MW, molecular weight markers.

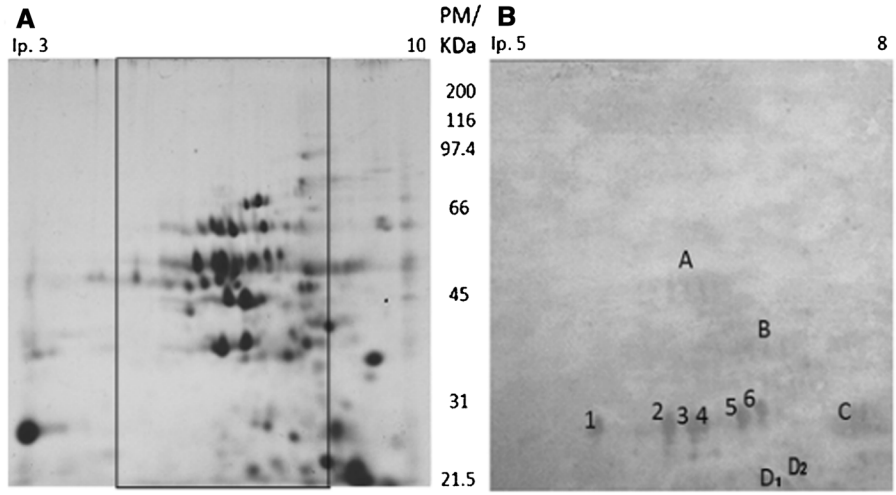

Fig. 3 **a** Two-dimensional electrophoresis of *Fraxinus* extract stained with Coomassie blue. **b** WB of *Fraxinus* immunogens in the *Fraxinus* extract with anti-*Fraxinus* hyperimmune serum

from 21.5 to 100 kDa and a pH between 4 and 10. This result was obtained with PDQuest software (Biorad, Hercules, CA). These findings differ from the results previously reported for *F. excelsior* in the number of spots (200 spots), isoelectric point, and weights of the components (Hemmer et al. 2000; Barderas et al. 2005; Mas et al. 2014; Martín et al. 1994; Poncet et al. 2010). These variations could be due to differences between the studied species and between the conditions present during the production and release of pollen grains (ĎAmato et al. 2007). The anti-*Fraxinus* hyperimmune serum detected a different set of proteins. It identified five groups of proteins with a molecular mass ranging from 30 to 32 kDa (1–6 in Fig. 3b) and four proteins with a molecular mass of 46 (A), 40 (B), 29 (C), and 21.5 (D) kDa (Fig. 3b) and an isoelectric point of 5–8.5.

3.5 Nano-LC-ESI-MS/MS

A mass spectrometry analysis of tryptic peptides from *F. uhdei* immunogens revealed a homology with proteins that participate in grain metabolism (Table 1). Spot 1 was detected as pectinesterase 2 produced by *Olea* spp. pollen grains. It is known as Ole e 11.0102 allergen, with a mass of 39.6 kDa. It has also been reported that this constituent may be secreted (Salamanca et al. 2010). Spot 2 was recognised as probable monodehydroascorbate reductase, a cytoplasmic enzyme of 46.5 kDa produced by *Arabidopsis thaliana* (Salanoubat et al. 2000). One spot from cluster A showed homology with UTP–glucose-1-phosphate uridylyltransferase, which is a 51.8 kDa cytoplasm enzyme produced by potato cells and used for callose deposition. Callose is a necessary constituent of pollen and pollen tubes (Katsube et al. 1991). A spot from cluster D was recognised as adenosine kinase 2, a 37.8 kDa cytosolic and membrane enzyme that has been studied in *Arabidopsis thaliana* (Moffatt et al. 2000).

One of the 21.5 kDa proteins was recognised as the 27 kDa isopentenyl-diphosphate delta-isomerase produced by *Camptotheca acuminata*. This protein is necessary for chloroplast metabolism during photosynthesis (Pan et al. 2008). Two other proteins of 21.5 kDa present in the cluster D were D1, which was identified as Os02g0778400 [*Oryza sativa* Japonica Group], and uncharacterised protein from *Zea mays*. Both of these proteins show homology to Ole e 5 (Carnés and Fernández-Caldas 2002), whereas another fraction (D2) was detected as Fra e 2, which is a *Fraxinus* pollen profilin (known as an allergen), and Bet v 2, a well-known allergen produced by *Betula verrucosa* pollen (Fedorov et al. 1997). The recognition of all these proteins, which are involved in metabolism, could be explained because they are part of pollen development (McConn and Browse 1993). These and other proteins are functionally necessary (Rounds et al. 2011).

3.6 Detection of *Fraxinus* immunogens in air samples

SDS-PAGE of the air sample from the highest pollination period showed that the sample contained

Table 1 LC/MS analysis of the *Fraxinus* immunogens recognised in the 2D electrophoresis

Spot	Name	Access number (UniProt)	Function
1	Pectinesterase 2 (Ole e 11.2) Salamanca et al. (2010)	AL11B_OLEEU	Catalyses demethylesterification of homogalacturonan components of pectin. May be involved in pollen tube development
3	Monodehydroascorbate reductase, cytoplasmic isoform 3 (MADR 3) Salanoubat et al. (2000)	MDAR3_ARATH	Catalyses the conversion of monodehydroascorbate to ascorbate, oxidising NADH in the process
A	UTP–glucose-1-phosphate uridylyltransferase Katsube et al. (1991)	UGPA_SOLTU	Glycosyl donor in cellular metabolic pathways
C	Adenosine kinase 2 Moffatt et al. (2000)	ADK2_ARATH	ATP-dependent phosphorylation of adenosine and other related nucleoside analogues to monophosphate derivatives. Methyl recycling
D1	Os02g0778400 [*Oryza sativa Japonica* Group] and Uncharacterised protein (*Zea maiz*) Carnés and Fernández-Caldas (2002)	OJ1293_A01.3 B4FGF7_MAIZE	ATP binding. Nucleotide kinase activity. Phosphotransferase activity, phosphate group as acceptor
D2	Fra e 2.01 allergen Mas et al. (2014)	W8NXD0_FRAEX	Uncharacterised protein. Profilin family
D2	Pollen allergen Bet v 2 Fedorov et al. (1997)	PROF2_BETPN	Binds to actin and affects the structure of the cytoskeleton. At high concentrations, profilin prevents the polymerisation of actin, whereas it enhances it at low concentrations. Profilin family

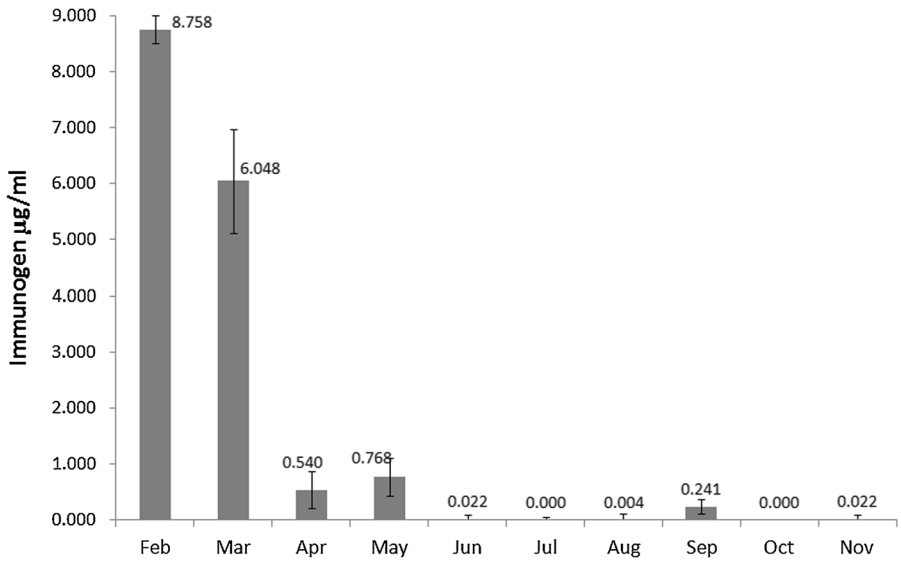

Fig. 4 Detection of *Fraxinus* immunogens in air samples by ELISA. Sampling period: February–November, 2012

various proteins ranging from 21.5 to 116 kDa; the anti-*Fraxinus* hyperimmune serum detected one fraction of 21.5 kDa (Fig. 2c) by WB, suggesting the presence of proteins sharing immunogenic epitopes with the 21.5 kDa protein from *F. uhdei* immunogens. ELISA assays demonstrated that the highest concentration of *Fraxinus* proteins detected was 8.75 and 6.04 µg/mL during February and March, respectively, whereas the concentration in other months, e.g., September, was <1 µg/mL (Fig. 4). These findings are influenced by *Fraxinus* phenology and by the meteorological conditions that were present during pollen development and release (Ziska and Caulfield, 2000; Bartra et al. 2007). According to the Mexican Aerobiology Network, the highest annual concentrations of airborne *Fraxinus* pollen occur from January to March. This result agrees with our detection results obtained with ELISA. As *F. uhdei* provides almost

30 % of the total pollen load, it is an important source of Oleaceae-associated aeroallergens in Mexico City.

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