
Isolation of the main allergen Fra e 1 from ash (*Fraxinus excelsior*) pollen: comparison of the natural and recombinant forms

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Background: Fra e 1 is a major allergen for ash pollen-sensitized individuals in northern and central Europe. It belongs to the Ole e 1-like family and displays high cross-reactivity with taxonomically related members.

Objectives: To isolate and characterize natural Fra e 1 (nFra e 1) from ash pollen and to compare its structural, antigenic, and allergenic properties with those of its recombinant form (rFra e 1).

Methods: The allergen was isolated by means of gel permeation chromatography and reverse-phase high-performance liquid chromatography columns. Molecular characterization was performed by means of Edman degradation, mass spectrometry, circular dichroism, concanavalin A lectin reaction, and anti-horseradish peroxidase polyclonal antibody. Immunologic characterization was performed using immunoblotting and enzyme-linked immunosorbent assay, inhibition experiments, and histamine release assays with serum samples from allergic patients with well-known reactivity to Fra e 1 or Ole e 1 and with polyclonal antiserum and monoclonal antibodies against Ole e 1. The protein used as a reference was rFra e 1, which was produced in the yeast *Pichia pastoris*.

Results: Purified nFra e 1 appeared as 5 variants with different glycosylation degrees. Both nFra e 1 and rFra e 1 were equivalently folded as deduced from the spectroscopic analysis using circular dichroism. Both molecules share the antigenic and allergenic epitopes after the purification process, and the glycan group of nFra e 1 is a potential epitope. Natural Fra e 1 displayed strong cross-reactivity with Ole e 1.

Conclusions: Natural Fra e 1 is a heterogeneously glycosylated protein with high allergenic relevance. It displays structural, antigenic, and allergenic similarity with rFra e 1. Both proteins could be used for clinical purposes.

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INTRODUCTION

Ash (*Fraxinus excelsior*) tree is widely distributed across Europe, and its pollen constitutes an important cause of allergy in temperate zones in Europe, including northern Spain, the British Islands, central Europe, Scandinavia, and Russia.^{1–3} The concomitant presence of ash pollen in the atmosphere together with other allergenic pollens, such as birch, has originated the absence of a deep knowledge about its relevance and allergenic profile.

The presence of the major allergen Fra e 1 and panallergens such as profilin and polcalcin has been determined.^{4,5} The recombinant form of Fra e 1 (rFra e 1) has recently been cloned, expressed in the methylotrophic yeast *Pichia pastoris* and purified.⁶ The amino acid sequence of Fra e 1 was found to be highly similar to that of the major allergen Ole e 1 from olive pollen, and, therefore, it has been included in the Ole e

1-like family of proteins, to which Lig v 1 from privet and Syr v 1 from lilac belong. The immunologic response of rFra e 1 using in vitro and in vivo methods has reasonably confirmed its correct folding and potential usefulness in clinical studies.

Glycosylation and polymorphism are 2 common characteristics of natural allergens. Frequently, many variants are represented in the natural allergens in contrast to the recombinant ones, in which only 1 homogeneous form of the allergen is obtained. Moreover, the purification of natural allergens involves important problems, such as the scarce availability of the biologic source and the low yield during the isolation process. The use of recombinant allergens, after their validation, could solve these difficulties. However, the practical availability of recombinant allergens tolerated a main inconvenience, such as the incorrect protein folding, perhaps owing to the lack of posttranslational modifications or to the instability of the allergen during the isolation process. These problems would produce a decrease in the recognition of the recombinant allergen by the patients' sera compared with the natural forms. Independent of the choice of the most representative form of the allergen, the natural form is essential for the validation of the recombinant form.

The aims of this work are to isolate natural Fra e 1 (nFra e 1), to detect the glycan moiety, and to study its equivalence

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with rFra e 1 by means of spectroscopic analysis, enzyme-linked immunosorbent assay (ELISA), immunoblotting, and inhibition experiments using IgG and IgE antibodies. These experiments evaluate the suitability of both forms of the allergen in testing and treating ash-allergic patients.

MATERIALS AND METHODS

Ash Pollen Extract, rFra e 1, and nOle e 1

Ash pollen extract was prepared using *F excelsior* pollen (ALK-Abelló, Madrid, Spain) as described previously.⁷ Recombinant Fra e 1 was expressed in *P pastoris* and was isolated according to the methods of Barderas et al.⁶ Natural Ole e 1 was obtained from *Olea europaea* pollen (ALK-Abelló) as described previously.⁸

Serum Samples and Antibodies

Seventy-two patients from Strasbourg (France) with pollinosis to ash, a class radioallergosorbent test result range of 1 to 6 as determined using the CAP-RAST method (Amersham Pharmacia Biotech, Uppsala, Sweden), positive skin prick test reactions to ash pollen extract,⁶ and well-known reactivity to rFra e 1 were included in this trial. Furthermore, 1 nonatopic patient was used as a negative control. All the patients provided informed consent. A pool of serum samples from Jaén (Spain) with known reactivity to Ole e 1 was selected according to the criteria described by Huecas et al.⁹ Polyclonal antiserum against nOle e 1 was prepared by immunizing a New Zealand white rabbit in a 6-week period by weekly injection of the protein (100 µg) in complete Freund adjuvant. Five different families of monoclonal antibodies, F1, F2, F4, F6, and F7, against Ole e 1^{10–12} were used in the analysis of the structural integrity of this protein.

nFra e 1 Isolation

Two chromatographic steps, gel permeation chromatography and reverse-phase high-performance liquid chromatography, were necessary for the purification of nFra e 1. Ash pollen extract was loaded into a Sephadex G-75 (Amersham Biosciences, Barcelona, Spain) column in 0.2M ammonium bicarbonate (pH 8.0). The presence of nFra e 1 was analyzed by reaction with the nOle e 1-specific polyclonal antiserum. Fractions containing nFra e 1 were loaded onto a Nucleosil C₁₈ (Scharlab S.L., Barcelona, Spain) column with an acetonitrile gradient (0%–60%) in 0.1% trifluoroacetic acid. The elution profile was continuously monitored at 214 nm.

Analytical Methods and Amino Acid Sequencing

Protein concentration of purified samples was determined by amino acid analysis after hydrolysis with 5.7 N hydrochloride, in sealed tubes under vacuum. Samples were analyzed on a 6,300 amino acid analyzer (Beckman Coulter, Fullerton, CA).¹² N-terminal Edman degradation of the protein was performed using a 477A sequencer (PE Applied Biosystems, Foster City, CA).¹³

Electrophoresis and Immunologic Characterization

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli¹⁴ in 15% polyacrylamide gels. Proteins were stained with Coomassie blue or electrophoretically transferred to nitrocellulose membranes. Immunodetection was achieved by using 5 monoclonal antibodies, a pool of serum samples from ash pollen-sensitized patients (diluted 1:10), a polyclonal antiserum against nOle e 1 (diluted 1:5,000), or the anti-horse-radish peroxidase (HRP) polyclonal antiserum (diluted 1:5,000) as described previously.¹⁵ The signal was developed using a Western blotting reagent (ECL; Amersham Biosciences, Barcelona, Spain).

The IgE quantification was performed by ELISA in microtiter plates coated with 100 µg of protein per well.¹⁶ Plates were incubated with allergic serum samples (diluted 1:10). The binding of human IgE was detected by mouse anti-human IgE antibodies (diluted 1:5,000) donated by ALK-Abelló followed by HRP-labeled goat anti-mouse IgG (diluted 1:5,000) (Pierce Chemical Co, Rockford, IL). The monoclonal antibodies and polyclonal antiserum were detected by HRP-labeled goat anti-mouse IgG (diluted 1:5,000) and HRP-labeled goat anti-rabbit IgG (diluted 1:3,000) (Bio-Rad, Richmond, CA), respectively. The reaction was developed with *o*-phenyldiamine, and the optical density was measured at 492 nm.¹⁶ Each value was calculated as the mean of 2 determinations.

The ELISA inhibition assays were performed as described previously.¹² The wells coated with 100 µg of purified protein were incubated with the serum samples (diluted 1:10) or the monoclonal (F7) or polyclonal antibodies, all of them previously incubated with different concentrations of rFra e 1, nFra e 1, or nOle e 1 as inhibitors.

Spectroscopic Characterization

Circular dichroism spectra were obtained in the far-UV using a spectropolarimeter (Jasco J-715; Japan Spectroscopic Co, Tokyo, Japan) with minor modifications.⁷ The protein concentration was 0.20 to 0.25 mg/mL in 50-mmol/L ammonium bicarbonate (pH 8.0). Mean residue mass ellipticities were calculated based on 113.26 as the average molecular mass or residue, obtained from the amino acid composition of rFra e 1, and expressed in terms of θ (degree $\times$ cm² $\times$ dmol⁻¹).

Carbohydrate Detection and Deglycosylation Treatment

Carbohydrate detection of protein transferred to nitrocellulose membranes was performed by using a biotinylated concanavalin A (ConA) lectin solution.¹⁵ The staining was developed by HRP reaction with 0.05% diaminobenzidine 0.03% of 30% hydrogen peroxide in Tris–sodium chloride (pH 7.5) reaction buffer. Enzymatic deglycosylation was performed with peptide-N-glycosidase F endoglycosidase (Boehringer, Mannheim, Germany) as described previously.¹⁷

Mass Spectrometry Analysis

Samples were measured using a matrix-assisted laser-desorption/ionization time-of-flight mass spectrometer (Bruker Re-

flex II; Bruker-Franzen Analytik, Bremen, Germany).¹⁸ The equipment was externally calibrated using singly, doubly, and triply charged signals from either cytochrome C (12,360 Da) or bovine serum albumin (66,430 Da).

Basophile Histamine Release Test

The challenge of whole blood with rFra e 1 and anti-IgE as a positive control was performed in a dose-response manner.¹⁹ Recombinant Fra e 1 and nFra e 1 (from 10^{-8} to $1 \mu\text{g/mL}$) or anti-IgE (from 10^{-4} to $10^{-3} \mu\text{g/mL}$) (e-specific; Dako, Glostrup, Denmark) were used as previously described.²⁰ Histamine values are the mean of 2 determinations. Results are expressed in percentage of histamine release. The patient was selected because of a positive response to nFra e 1 and rFra e 1 in ELISA.

RESULTS

nFra e 1 Isolation and Molecular Properties

A 2-step procedure consisting of size-exclusion chromatography and reverse-phase high-performance liquid chromatography was followed to purify nFra e 1. Five different batches, all of them containing purified nFra e 1, were obtained (Fig 1). Homogeneity, apparent molecular mass, IgG and IgE responses, and glycan composition were analyzed by means of Coomassie blue staining (Fig 1A), Ole e 1-specific polyclonal antiserum (Fig 1B), serum samples from patients with ash pollen sensitization (Fig 1C), ConA lectin (Fig 1D), and anti-HRP polyclonal antiserum (Fig 1E), respectively.

The molecular mass of nFra e 1 variants ranges in SDS-PAGE from 18 to 24 kDa. No differences were found in the

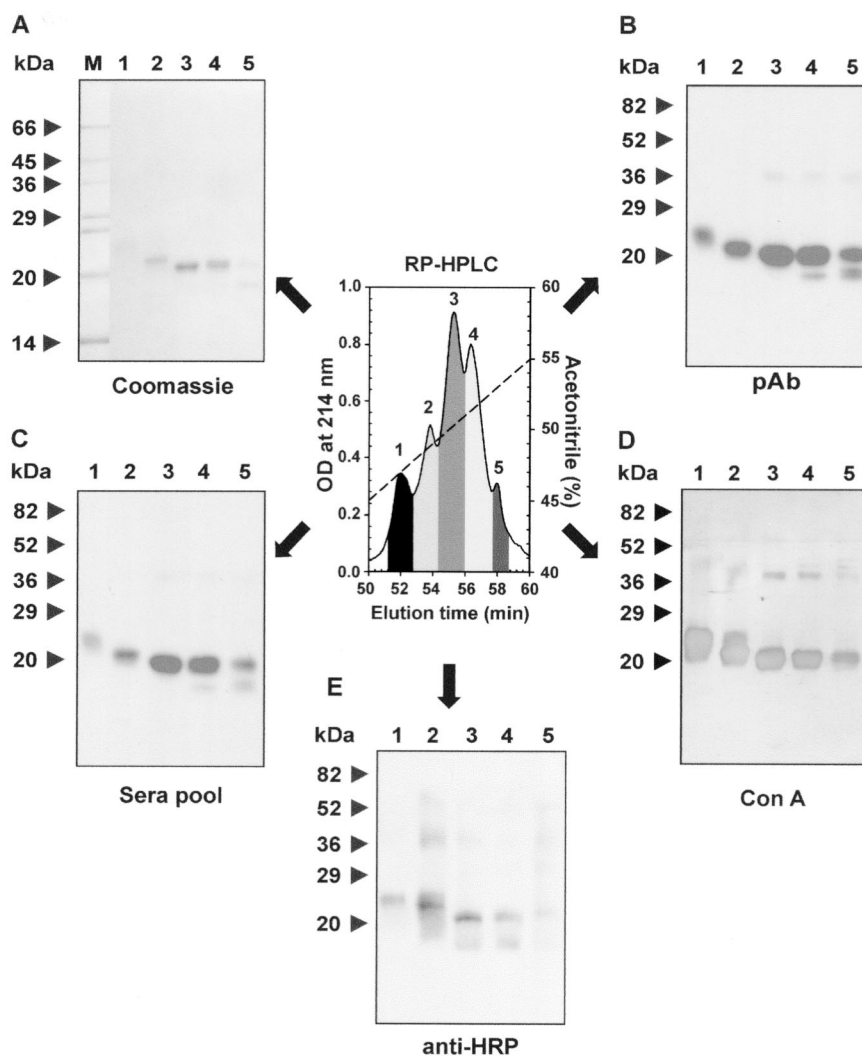


Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analyses of 5 batches (lanes 1 to 5) of natural Fra e 1 obtained after reverse-phase high-performance liquid chromatography (RP-HPLC). A, Coomassie blue staining. Staining after blotting to membranes with polyclonal antiserum (pAb) (B), serum samples (C), concanavalin A (ConA) lectin (D), and anti-horseradish peroxidase (HRP) pAb (E). M indicates molecular weight markers; OD, optical density.

N-terminal amino acid sequence (EDVPQ) of all batches, which is in agreement with the sequence previously obtained by Edman degradation of Fra e 1.⁶ Furthermore, no significant differences were observed in the IgG and IgE responses among the different batches (Fig 1B and C), which suggests the same antigenic and allergenic properties for all variants. Related to the ConA recognition (Fig 1D), bands of 20 and 24 kDa were positive, whereas the 18-kDa band of batches 4 and 5 was not recognized. Furthermore, all the batches of nFra e 1 were recognized by the anti-HRP polyclonal antiserum (Fig 1E), indicating that their glycans contain xylose or fucose residues. To evaluate the glycan size in the different batches, a deglycosylation reaction with peptide-N-glycosidase F was performed using rFra e 1 as control (Fig 2). All the fractions changed their electrophoretic mobility to the 18 kDa size as a result of their sensitivity to the treatment and the consequent loss of the glycan component. Reactions to the polyclonal antiserum, human sera, and ConA were assayed for the deglycosylated forms of nFra e 1. The recognition with the antiserum and the serum samples after SDS-PAGE and transference to membranes was similar, and the ConA reaction was negative (data not shown).

Mass spectrometry of nFra e 1 gave a heterogeneous peak for the glycosylated variant ranging from 17,913 to 19,510 Da. When the deglycosylation was achieved, the molecular mass decreased to approximately 16.5 kDa, being 16,385 Da for nFra e 1 and 16,402 Da for rFra e 1, which is close to the value deduced from the amino acid sequence of rFra e 1, 16,423 Da (Fig 3A).⁶ Therefore, the molecular mass of the main glycan group is approximately 1.5 kDa, and approximately 3.1 kDa that of batch 1, the latter constituting a hyperglycosylated variant.

Evidence of the accurate folding of Fra e 1 was obtained by means of spectroscopic studies comparing both forms of the allergen. The far-UV circular dichroism spectra of nFra e 1 (batch 4) and rFra e 1 showed no significant differences (Fig

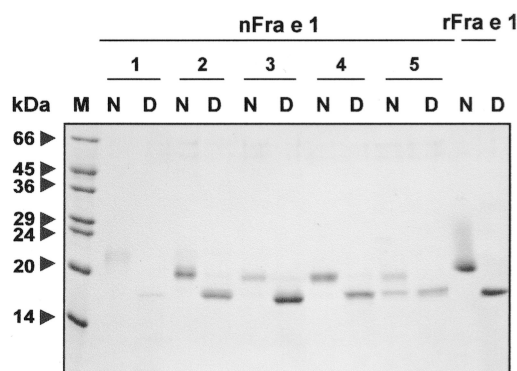


Figure 2. Deglycosylation analysis of Fra e 1. Coomassie blue staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the 5 batches of natural Fra e 1 (nFra e 1) and recombinant Fra e 1 (rFra e 1). D indicates deglycosylated with peptide-N-glycosidase F; N, untreated samples; and M, molecular weight markers.

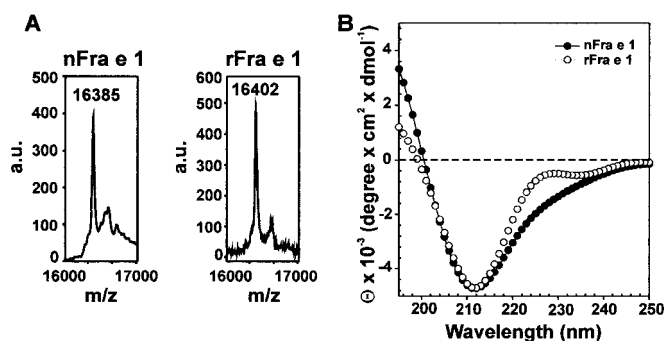


Figure 3. Mass spectrometry (A) and far-UV circular dichroism (B) structural analyses of deglycosylated natural Fra e 1 (nFra e 1) (batch 4) and recombinant Fra e 1 (rFra e 1). a.u. indicates arbitrary units; m/z, mass/charge.

3B). A composition of 15% α -helix, 38% β -sheet, 11% β -turn, and 36% random coil was obtained for rFra e 1 and 15%, 42%, 12%, and 31%, respectively, for nFra e 1.

Immunologic Properties

On the basis of the homogeneity of the polypeptide chain, the high similarity in the SDS-PAGE pattern to Ole e 1 isolated from olive pollen, and the yield obtained, nFra e 1 batch 4 was selected for immunologic comparison with rFra e 1 and the homologous nOle e 1 from olive pollen. The IgG and IgE reactivities of nFra e 1, rFra e 1, and the ash pollen extract were evaluated by means of immunoblotting and ELISA. Five Ole e 1–specific monoclonal antibodies, 1 polyclonal antiserum, and a pool of serum samples from ash pollen–allergic patients were used in immunoblotting analyses (Fig 4). F4 exhibited decreased recognition for rFra e 1 with respect to the ash extract and nFra e 1. Similar recognition was found to both proteins with the serum samples of patients allergic to ash pollen (Fig 4).

The IgG and IgE binding to rFra e 1 and nFra e 1 were quantitatively evaluated using ELISA inhibition experiments with the monoclonal antibody F7, the polyclonal antiserum, and the pool of serum samples used for other analyses (Fig 5A). The 2 proteins exhibited similar inhibition of binding to IgG and IgE antibodies, although rFra e 1 did not completely inhibit the IgG binding of the polyclonal antiserum and the IgE binding to nFra e 1. This could be due to the different glycan composition and polymorphism between the 2 forms of the allergen. Complete inhibition was obtained when rFra e 1 was used for coating ELISA plates and nFra e 1 as inhibitor. The nFra e 1 and nOle e 1 were also compared (Fig 5B). Using 2 pools of serum samples from different populations (Jaén and Strasbourg) with very different pollen exposure, similar behavior was found between both molecules, although total inhibition of the IgE binding to nOle e 1 was not reached for the Jaén serum samples with nFra e 1 as inhibitor. Identical behavior was shown when nFra e 1 was substituted with rFra e 1.

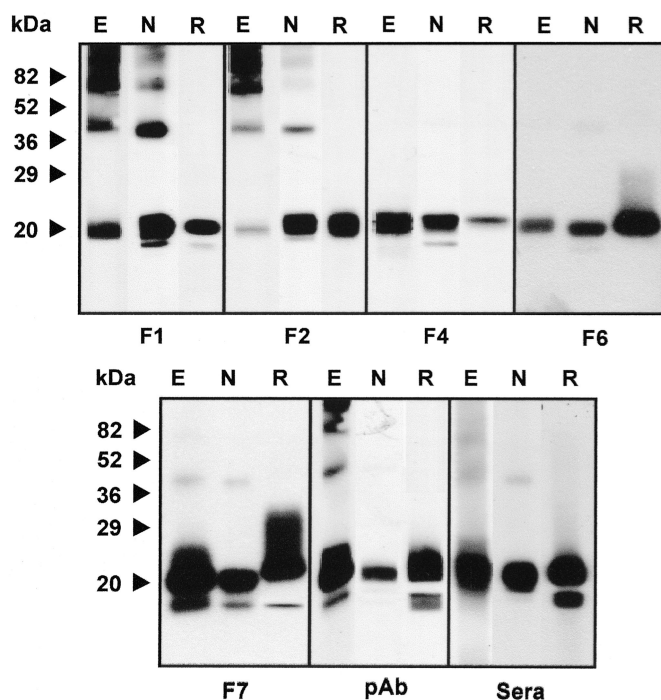


Figure 4. Immunostaining analysis after sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transference to nitrocellulose membranes. Responses to monoclonal antibodies (F1, F2, F4, F6, and F7), polyclonal antiserum (pAb), and serum samples from patients from Strasbourg (France) allergic to ash are shown. E indicates ash pollen extract; N, natural Fra e 1 (batch 4); and R, recombinant Fra e 1.

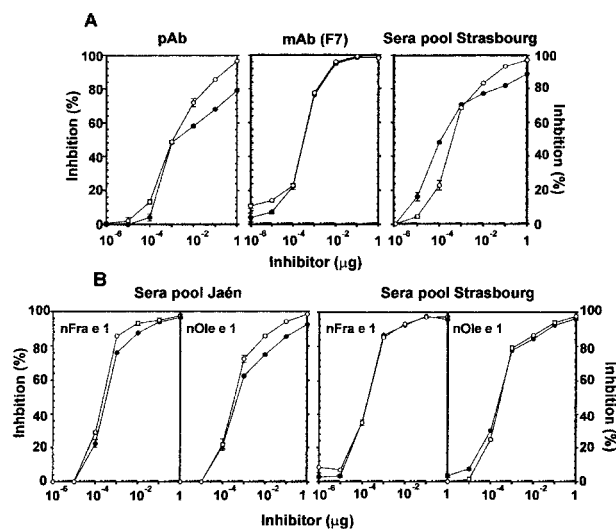


Figure 5. Enzyme-linked immunosorbent assay inhibition experiments. A, Natural Fra e 1 (nFra e 1) (batch 4) was coated to wells and the antisera (monoclonal antibody [mAb], polyclonal antiserum [pAb], and serum samples) was preincubated with recombinant Fra e 1 (●) or nFra e 1 (○) as inhibitors. B, Natural Fra e 1 and Ole e 1 were coated to wells and assayed using IgE from serum samples from Jaén (Spain) or Strasbourg (France) and nFra e 1 (●) or nOle e 1 (○) as inhibitors.

To compare the allergenic correlation between rFra e 1 and nFra e 1, the IgE binding to both proteins was assayed for 72 individual serum samples by ELISA. Quantification of IgE levels, given as optical density at 492 nm, showed that both proteins exhibited similar recognition by the serum samples (slope = 0.88, $r = .98$; $P < .01$) (Fig 6A).

Histamine Release

A positive histamine release test result was obtained with both forms of Fra e 1 (Fig 6B). The basophiles of the patient were good responders. Degranulation with anti-IgE was more than 40% at 10^{-3} $\mu\text{g/mL}$. With rFra e 1, maximum histamine release was obtained with 10^{-3} $\mu\text{g/mL}$, and 30% of total histamine release was obtained with 10^{-6} $\mu\text{g/mL}$. For nFra e 1, the concentrations inducing maximum and 30% of total histamine release were nearly similar.

DISCUSSION

Ash pollen allergy is recognized as an important cause of allergy in middle and northern Europe.^{1–3} Although a previous study⁶ about the relevance of Fra e 1 in ash pollen allergy has been reported using the recombinant allergen, a comparative analysis with the natural allergen is mandatory. The

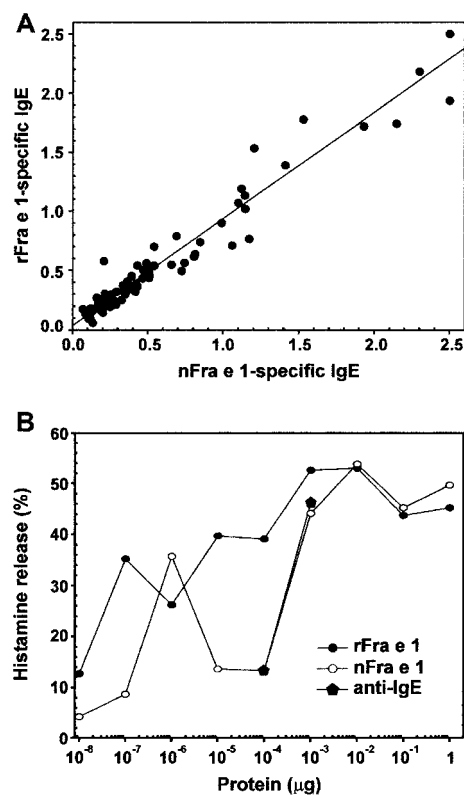


Figure 6. A, Analysis of the IgE binding response to recombinant Fra e 1 (rFra e 1) and natural Fra e 1 (nFra e 1) (batch 4) by using 72 serum samples from patients sensitized to ash pollen; their responses are shown in optical density at 492 nm. B, Histamine release assay for 1 ash pollen-allergic patient; the response to anti-IgE was used as a positive control.

present work evaluated the equivalence between nFra e 1 and rFra e 1 in terms of structure, antigenicity, and allergenicity after isolation and characterization of the former with an atopic population from Strasbourg (northwestern France), where ash pollen is one of the main causes of pollinosis.⁴

These analyses required isolation of the natural allergen from the pollen. The purified protein exhibited several forms with different molecular weights in SDS-PAGE. The glycosylated variants of 5 different batches of nFra e 1 exhibited molecular masses ranging from 18 to 24 kDa, these differences being mainly due to the heterogeneity in the glycan composition of the protein because identical length and a low polymorphism degree had been found in the primary structure of the polypeptide chain. In fact, only 2 different isoforms were identified in previous studies.⁶ Fra e 1 and Ole e 1, Lig v 1, and Syr v 1, all of them members of the Ole e 1-like protein family and belonging to the Oleaceae family, have 1 potential N-glycosylation site at Asn-111,^{13,21,22} which has been demonstrated to be linked to a heterogeneous glycan group in the case of Ole e 1.^{15,23} Ole e 1 carried high-mannose and complex N-glycans as major components and a substitution of the proximal N-acetylglucosamine with an α -1,3-fucose on a minor fraction of the allergen.^{15,24} Other members of the Ole e 1-like family, such as Lol p 11, also show heterogeneity in the carbohydrate moiety²⁴; therefore, the existence of several variants of the glycan component in other members of this family of proteins cannot be discarded.

Despite this polymorphism, the antigenic behavior using IgG and IgE antibodies was near equivalent in all 5 batches obtained. The N-terminal amino acid sequence was identical in all analyzed batches and was in agreement with the sequence deduced from the Fra e 1-specific DNA.⁶ These first residues EDVPQ are conserved in all Oleaceae members of the Ole e 1-like family.^{13,21,22,25} Natural Fra e 1 and rFra e 1 displayed an equivalent secondary structure, as deduced from the spectroscopic analysis, and it is similar to that of other members of the protein family, such as Ole e 1, Syr v 1, and Lig v 1.^{13,26}

The more significant difference between nFra e 1 and rFra e 1 concerned the glycan group because of the differential glycosylation machinery of the cell performing the biosynthesis. Whereas *P. pastoris* yeast mainly produces high-mannose N-glycans, the pollen tissue also originates complex and hybrid glycans containing fucose and xylose residues. This difference can be important for the IgE-binding capability of glycoproteins. In fact, the oligosaccharide of Ole e 1 has been reported to contain a relevant allergenic epitope.^{15,17,23} However, the glycan moieties of Pla l 1 and Che a 1, also members of Ole e 1-like family belonging to the Plantaginaceae and Chenopodiaceae families, respectively, do not seem to have a role in the binding of these allergens to IgE antibodies.^{27,28} The allergenic relevance of the carbohydrate moiety in Fra e 1 could be evaluated by inhibition assays performed with nFra e 1 and rFra e 1. In these analyses, rFra e 1 did not completely inhibit the recognition of nFra e 1 by using a polyclonal antiserum or serum samples from patients allergic

to ash pollen. Otherwise, the behavior of rFra e 1 is equivalent to that of nFra e 1 when a monoclonal antibody directed toward the polypeptide chain is used. These results would agree with the existence of IgE antibodies directed to the glycan group of Fra e 1.

Furthermore, nFra e 1 and nOle e 1 share most allergenic epitopes, as deduced from the inhibition analysis performed with these 2 allergens. The slight difference observed when the serum samples from Jaén were used could be attributed to the significant contribution of the high number of Ole e 1 isoforms that sensitize to the population of this Spanish region.²⁹ The high level of olive pollen counts in Jaén emphasizes the relevance of the existence of several isoforms. The pollen levels are lower in Strasbourg, and with these serum samples the inhibition is identical with both molecules. The biologic activities of nFra e 1 and rFra e 1 are supported by findings from histamine release analysis, which were positive to both proteins in the blood of the patient tested.

In conclusion, because of the similar reactivity found for rFra e 1 and nFra e 1, we suggest the potential use of both proteins in testing and treating ash-allergic patients.

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