

Quantification of profilins by a monoclonal antibody-based sandwich ELISA

J.A. Asturias ^{a,*}, M.C. Arilla ^a, M. Aguirre ^a, N. Gómez-Bayón ^a, A. Martínez ^a,
R. Palacios ^a, F. Sánchez-Gascón ^b, J. Martínez ^a

^a R&D Department, Bial-Aristegui, Alameda Urquijo, 27, 48008, Bilbao, Spain

^b Facultad de Medicina, Hospital Universitario, Universidad de Murcia, Murcia, Spain

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Abstract

Profilins are plant allergens responsible for cross-reactivities in pollen and fruit-allergic patients. A two-site enzyme-linked immunosorbent assay has been developed for the quantification of profilins and its suitability for quantifying profilin in different plant extracts has been evaluated. The assay is based on two profilin-specific monoclonal antibodies (mAbs) with different epitope specificities. These antibodies were immobilized on ELISA plates and incubated with samples containing profilin. Bound profilin was detected by a combination of biotinylated profilin-specific antiserum and peroxidase–streptavidin conjugate. The optimized ELISA measured profilin concentrations ranging from 4 to 250 ng/ml and could quantify profilins from plant species of a variety of different botanical families. No reactivity to mites, molds, or crustaceans was detected, suggesting that the immunoassay is plant-specific. The results indicate that this sensitive profilin-assay will be helpful both for quantifying the profilin content of allergenic extracts intended for clinical use and for studying cross-reactivities between pollen extracts. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Profilin is considered to be a minor allergen present in many plant allergenic extracts. The only exceptions are *Mercurialis annua* (Vallverdú et al., 1997) and saffron (Feo et al., 1997), where the incidence of profilin sensitivity among pollen-al-

lergic patients is more than 50%. Profilins are widely distributed among plants, and have been described as cross-reacting allergenic components responsible for some of the type I allergic reactions caused by pollen and food from distantly related plants (Valenta et al., 1992a,b; Martínez et al., 1995). Plant profilins exhibit an average of 75% identity in amino acid sequence, but this homology decreases to 46% when plant profilins are compared to non-plant profilins (Staiger et al., 1993; Mittermann et al., 1995; Huang et al., 1996; Vallverdú et al., 1998; Asturias et al., 1998).

Crude extracts of naturally occurring allergenic materials used for diagnosis and immunotherapy may

Abbreviations: HRP, horseradish peroxidase; mAbs, monoclonal antibodies; PBS, phosphate-buffered saline; PVDF, polyvinylene difluoride; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis

* Corresponding author. Tel.: +34-94-443-8000; fax: +34-94-443-8016; e-mail: la.bial@sarenet.es

contain complex mixtures of proteins of which only a few have allergenic activity. One way to characterize allergenic extracts is to quantify allergen levels by specific immunoassays with monoclonal antibodies (mAbs). Allergen-specific mAbs have many advantages for allergen characterization and identification, and can be used for quantitative measurement in mass units (μg protein/ml), which is very useful in the standardization of allergen extracts. Such antibodies show specificity for a single epitope and can be produced in unlimited amounts. In this study, a solid phase ELISA based on sunflower profilin-specific mAbs was developed in order to provide a specific and sensitive estimate of the content of this panallergen in pollen extracts.

2. Material and methods

2.1. Profilin purification

Defatted pollen was extracted for 24 h at 4°C in 0.1 M phosphate buffer, pH 8. The extract was clarified by centrifugation at $5600 \times g$ for 30 min, filtered through 0.45 μm pore diameter membranes and dialyzed by ultrafiltration in a Pellicon System (Millipore Milford, MA) with a 5000 Da cut-off. Profilin purification was achieved by poly-L-proline (PLP)-Sepharose 4B (Pharmacia, Uppsala, Sweden) chromatography as previously described (Lindberg et al., 1988). Determination of the protein concentration was performed according to Bradford (1976). Recombinant profilins from *Helianthus annuus* (Hel a 2), *Cynodon dactylon*, *M. annua* and *Olea europaea* were obtained as previously described (Asturias et al., 1997a,b, 1998; Vallverdú et al., 1998). Truncated Hel a 2 allergens fused to glutathione S-transferase (GST) were constructed and produced as previously described (Asturias et al., 1998).

2.2. Immunoblotting

Proteins were separated by sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970) using 12.5% polyacrylamide gels under reducing conditions and electrophoretically transferred to polyvinylene diflu-

oride (PVDF) (Immobilon-P-membranes, Millipore) by the method of Towbin et al. (1979). After blocking with 8.8% skimmed milk, the blot was incubated for 1 h at 37°C with hybridoma culture supernatants. After washing, blots were incubated for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated anti-mouse polyvalent immunoglobulins (Sigma, St. Louis, MO) (1/1000 diluted), and developed with 4-chloro-1-naphthol solution (Bio-Rad, Richmond, CA).

2.3. Production of profilin-specific mAbs

The production of profilin-specific mAbs has been described previously (Arilla et al., 1997). Briefly, 8- to 10-week old BALB/c mice were immunized intraperitoneally with 50 μg of sunflower profilin emulsified in complete Freund's adjuvant. The same amount of antigen and incomplete Freund's adjuvant were given every 15 days during 2 months with a final 50 μg intraperitoneal dose without adjuvant. Three days later the spleen cells were mixed with SP₂O myeloma cells using the method described by Köhler and Milstein (1976). Supernatants were screened by ELISA on day 11 and positive hybridomas containing antibodies of the desired specificity cloned by limiting dilution. Isotype detection was performed using a commercial kit (Boehringer Mannheim, Mannheim, Germany).

2.4. Purification and labeling of antibodies

For antibody purification, hybridoma culture supernatants or rabbit anti-profilin serum were purified on a HiTrap Protein G (Pharmacia) equilibrated in 20 mM phosphate buffer pH 7.0. The adsorbed IgG was eluted from the column using 0.1 M glycine–HCl buffer pH 2.7, and 1 ml fractions were collected in tubes containing 60 μl of 1 M Tris–HCl pH 9.0. The presence of IgG-rich fractions was monitored by measuring absorbance at 280 nm. Peak fractions were pooled and dialyzed against phosphate-buffered saline (PBS), for use as a detector antibody, or against bicarbonate buffer for biotinylation. The purified rabbit IgG was labeled with biotin using the ECL protein biotinylation kit (Amersham, Buckinghamshire, UK).

2.5. Direct ELISA and ELISA-inhibition

Microtiter plates (Maxisorp, NUNC, Denmark) were coated with 200 ng/ml of sunflower profilin in 0.1 M bicarbonate buffer pH 9.6, and after blocking with 1% BSA, 0.05% Tween 20 in PBS (PBS-B-T), mAbs were added and incubated for 1 h at 37°C. Detection was carried out as previously described (Martínez et al., 1985). For ELISA-inhibition experiments different concentrations of competitor hybridoma culture supernatants were added to the wells and incubated for 1 h. Purified labeled mAbs (1/50 diluted) were then added and the ELISA developed as described previously (Jiménez et al., 1994).

2.6. Optimization of detector and capture antibodies concentration

The biotinylated rabbit anti-profilin antiserum used as a detector antibody was titrated for optimum binding to sunflower profilin. A two-fold dilution series of sunflower profilin (19 to 300 ng/ml) was

coated on the solid phase, followed by serial dilutions of biotinylated antiserum (0.5 to 16 µg/ml) and streptavidin–HRP conjugate (diluted 1/1500) (Amersham).

Concentrations of capture mAbs 5F2 and 3H8 ranging from 1.1 to 9 µg/ml were used to coat microtiter plates and incubated with a two-fold dilution series of sunflower profilin (3 to 200 ng/ml). Bound profilin was detected with biotinylated rabbit anti-profilin antiserum (optimum concentration: 16 µg/ml) and streptavidin–HRP conjugate (diluted 1/1500) (Amersham).

2.7. Quantification of profilin using a mAb-based sandwich ELISA

Microtiter plates (Maxisorp, NUNC) were coated overnight at 4°C with 100 µl of a mixture of mAbs 5F2 and 3H8, at 10 µg/ml each, in PBS buffer. Plates were saturated with PBS-B-T for 1 h at 37°C, and 100 µl of serially diluted profilins (0.5–250 ng/ml) or extracts (1 to 2000 µg/ml) were added

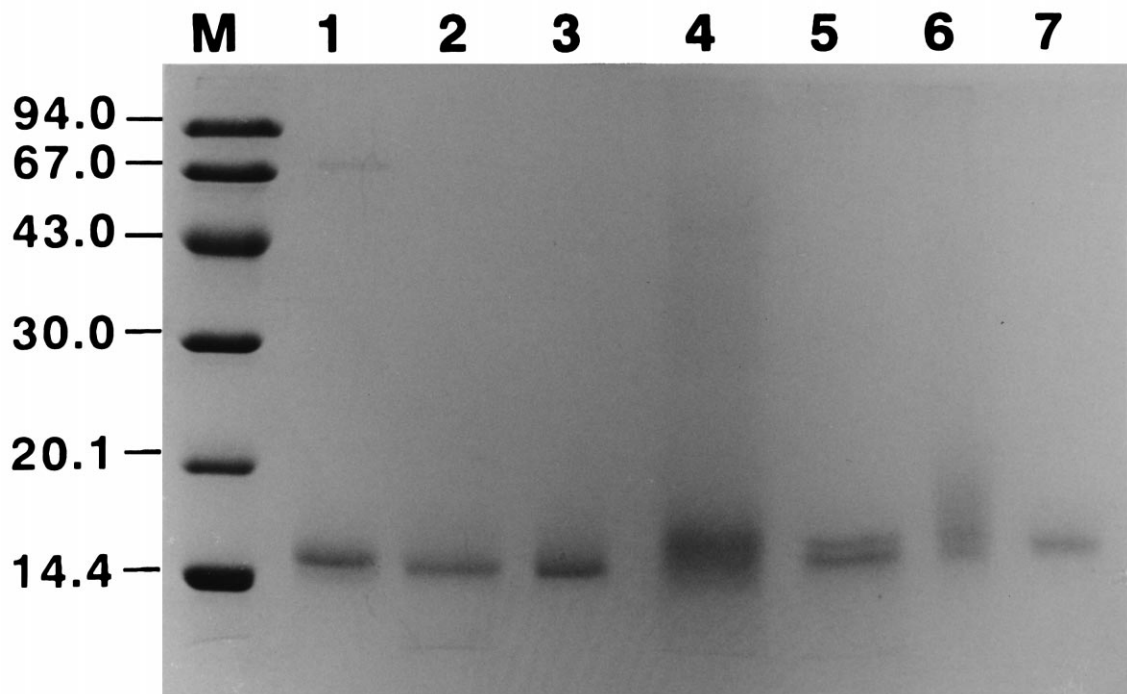


Fig. 1. SDS-PAGE analysis of purified profilins (5 µg per lane) from *H. annuus* (lane 1), *C. dactylon* (lane 2), *P. pratense* (lane 3), *O. europaea* (lane 4), *M. annua* (lane 5), *R. communis* (lane 6) and *Z. fabago* (lane 7).

Table 1

Profilin-binding activity of selected mAbs tested against various plant pollen extracts

MAb	ELISA activity ($A_{600\text{ nm}}$)						
	<i>Helianthus</i>	<i>Cynodon</i>	<i>Mercurialis</i>	<i>Ricinus</i>	<i>Olea</i>	<i>Phleum</i>	<i>Platanus</i>
7B12	0.636	0.119	0.165	0.501	0.148	0.122	0.328
6D2	0.632	0.125	0.170	0.424	0.151	0.135	0.322
5F2	1.788	1.769	1.529	2.140	0.611	0.894	0.712
3G4	1.744	1.706	1.451	1.972	0.551	0.796	0.686
3H8	1.384	1.358	1.006	1.307	0.516	0.790	0.739

and incubated for 1 h at 37°C. After washes with PBS containing 0.05% Tween 20 (PBS-T), 100 μ l of biotinylated rabbit anti-profilin serum (16 μ g/ml) were added to each well and the plate incubated for

1 h at 37°C. Then 100 μ l streptavidin–HRP conjugate (diluted 1:1500 in PBS–B–T) were added for 1 h at 37°C. The plates were washed again and developed by incubating with 200 μ l/well of a solution

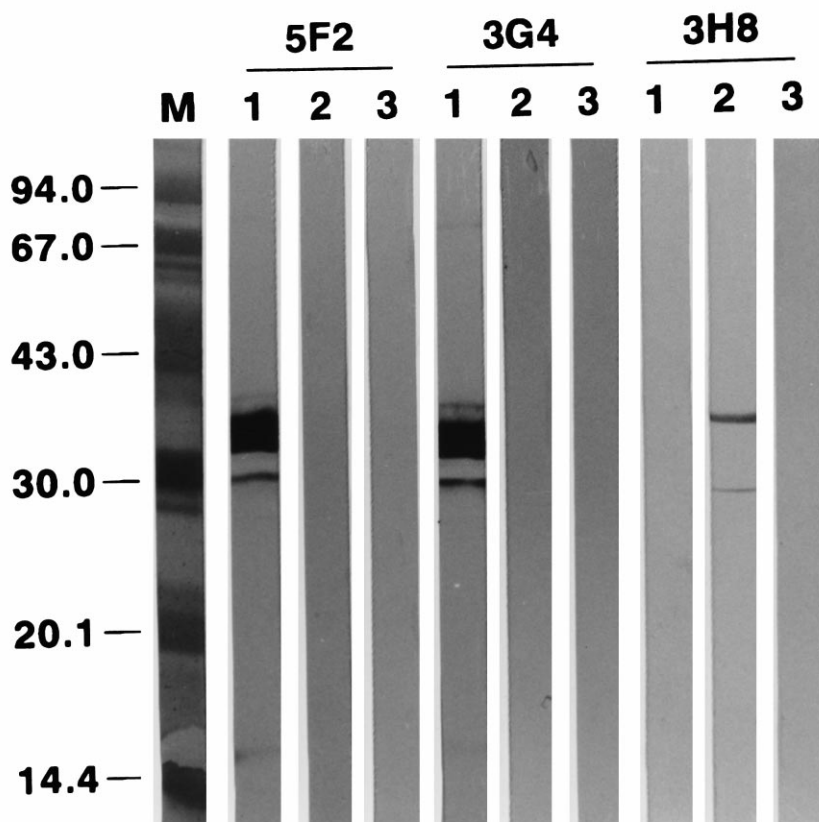


Fig. 2. Characterization of mAbs by immunoblotting with truncated Hel a 2 proteins incubated with 5F2 (A), 3G4 (B) and 3H8 (C). Lane M, molecular mass markers; lane 1, N-terminal half of Hel a 2 fused to GST; lane 2, C-terminal half of Hel a 2 fused to GST; lane 3, control GST.

Table 2

Comparison of the different combinations of profilin-specific mAbs in the solid phase ELISA. Absorbance values at 600 nm were determined after incubation with HRP-detector antibody

MAb bound to the solid phase	Detector antibody			
	5F2	3G4	3H8	Serum
5F2	–	0.454	1.103	1.408
3G4	0.317	–	0.675	0.912
3H8	1.096	1.109	–	0.880
Serum	0.712	0.724	0.843	–
5F2 + 3H8	–	–	–	1.605

of *o*-phenyldiamine. The reaction was stopped by adding 50 μ l of 3 M H_2SO_4 and the absorbance was measured at 492 nm. A standard curve was generated with serial two-fold dilutions (1 to 250 ng/ml) of purified Hel a 2. The concentrations of the sample

were interpolated from the linear portion of the standard curve.

2.8. Evaluation of assay variation

The detection limit was calculated as the amount of profilin which corresponded to the median plus 2.33 times the standard deviation obtained after 30 measurements of the zero standard. In order to evaluate intraassay variation, 20 microtiter wells were incubated with the same concentration of profilin at profilin concentrations of 1, 16, and 250 ng/ml and a coefficient of variation (CV) was calculated. The CV was defined as the standard deviation divided by the mean and multiplied by 100. CVs smaller than 20% were considered not to be significant. Recovery experiments were performed using sunflower extracts (16 and 4 μ g/ml) spiked with serial two-fold dilutions of purified Hel a 2.

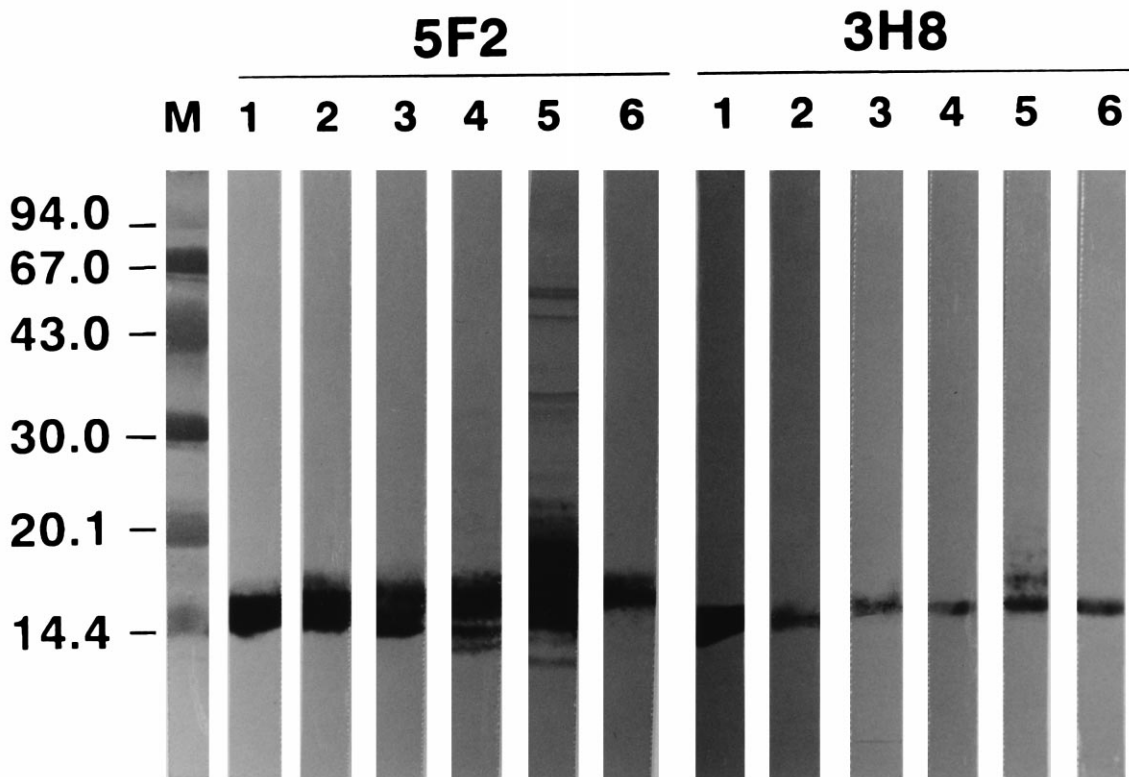


Fig. 3. Immunoblotting of plant extracts (40 μ g per lane) using mAbs 5F2 and 3H8 after SDS–PAGE. Lane 1: *H. annuus*; lane 2: *P. pratense*; lane 3: *O. europaea*; lane 4: *M. annua*; lane 5: *R. communis*; lane 6: *Z. fabago*.

3. Results

3.1. Purification of profilins

Profilins were isolated from pollen extracts of *H. annuus*, *Phleum pratense*, *C. dactylon*, *M. annua*, *O. europaea*, *Zygophyllum fabago* and *Ricinus communis* by means of PLP-Sepahrose chromatography. Proteins with molecular masses of 14–17 kDa were isolated (Fig. 1). Profilins from *O. europaea* and *M. annua* appeared as multiple bands consistent with their isoform composition. These purified profilins were used for the studies of the specificity of the mAbs and as the primary standard preparation in the sandwich ELISA.

3.2. Selection of mAbs

Screening by ELISA against sunflower profilin resulted in 20 profilin-reactive mAbs, which were also tested against different plant extracts. Five mAbs were chosen on the basis of their wide profilin reactivity pattern (Arilla et al., 1997). All of them were typed as IgG1 subclass proteins, with κ light chains. Their reactivity with profilin was measured

by ELISA (Table 1), and the three mAbs (namely 5F2, 3G4 and 3H8), showing the highest absorbance values for all the extracts tested, were selected for further studies. Immunoblotting of N- and C-terminal truncated recombinant Hel a 2, incubated with these three mAbs, revealed that 5F2 and 3G4 bound to the N-terminal half of Hel a 2, and 3H8 to the C-terminal half (Fig. 2). Table 2 shows a comparison of the combinations of the anti-profilin mAbs and rabbit anti-profilin serum in the sandwich ELISA. When the same mAb was employed as both the capture mAb (bound to the solid phase) and as the (HRP-conjugated) detector, no binding was observed. The results also suggest that 3G4 and 5F2 probably recognized overlapping (or very similar) epitopes of Hel a 2 since very low absorbance values were observed when one mAb was used to detect the profilin captured by the other one. This finding was further corroborated by cross-inhibition studies (data not shown). The combination of mAbs 5F2 or 3H8 with anti-profilin antiserum as detector resulted in the highest absorbance values. Given that the use of more than one mAb is advisable, a mixture of 5F2 and 3H8 was selected as the coating mAb, while the rabbit anti-profilin serum, linked to HRP, was cho-

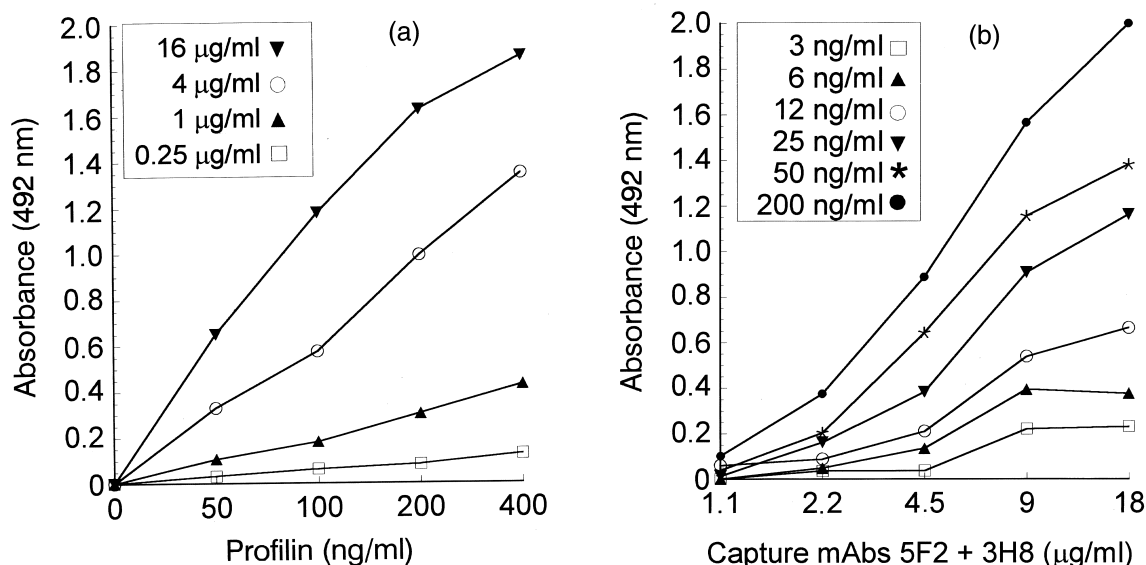


Fig. 4. (a) Reactivity of serial dilution series of rabbit anti-profilin serum using increasing concentrations of purified Hel a 2. (b) Optimization of capture mAbs 5F2 + 3H8 and reactivity of serial dilutions of purified Hel a 2 using serial dilution of capture mAbs. The optimal concentration of detector antiserum (16 $\mu\text{g/ml}$) was used for detection.

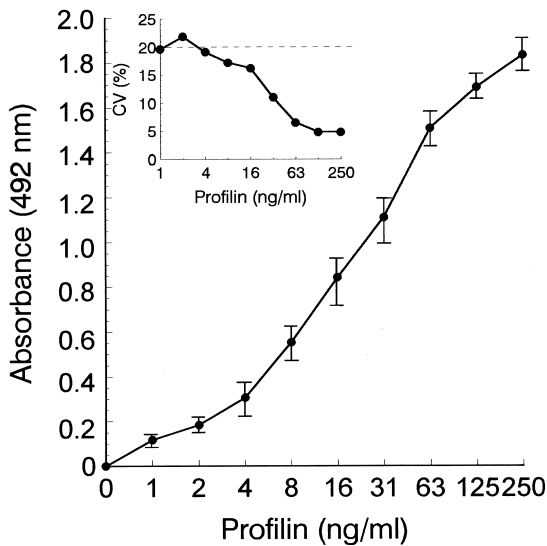


Fig. 5. Dose–response curves in ELISA of Hel a 2 showing day-to-day variation. The error bars indicate standard deviation. The inset presents the corresponding precision profiles.

sen as the detector. The reactivity of mAbs 3H8 and 5F2 to profilin in pollen extracts was tested by immunoblotting (Fig. 3). Both mAbs had a similar

affinity for sunflower profilin but the affinity of 5F2 for profilin from other plants was stronger than that of 3H8.

3.3. Determination of optimal concentrations of detector profilin-specific antiserum and capture mAbs 3H8 and 5F2

Serial dilutions of detector anti-profilin rabbit anti-serum were assayed for the ability to bind increasing concentrations of profilin, as shown in Fig. 4A. The highest concentration of serum tested, 16 $\mu\text{g/ml}$, was chosen as optimal because it yielded high yet usable absorbance values over a wide range of Hel a 2 concentrations. The use of antiserum labeled with biotin or HRP gave similar patterns, but higher absorbances were measured with the former. Fig. 4B shows the reactivity of different coating dilutions of the mAbs 5F2 and 3H8 with increasing amounts of Hel a 2, using the optimal concentration of anti-profilin serum. A 9 $\mu\text{g/ml}$ concentration for each capture mAb (5F2 and 3H8) was deemed optimal since higher concentrations did not yield any significant increase of antibody binding in the middle range of Hel a 2 concentrations.

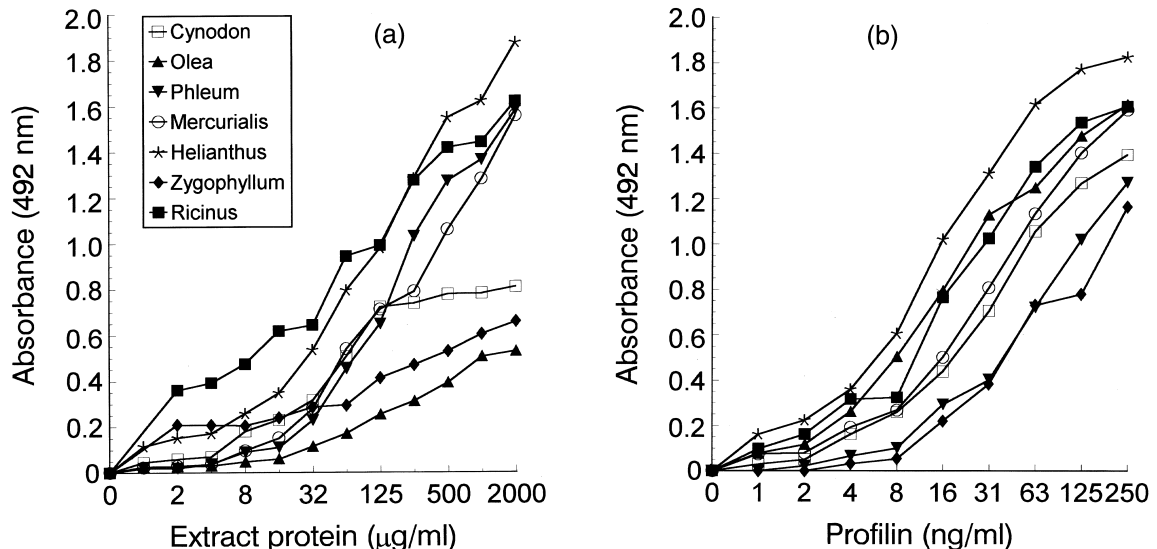


Fig. 6. Dose–response curves in ELISA of (a) pollen extracts and (b) the corresponding purified profilins. The values shown are the mean of four independent experiments.

Table 3
Specificity of the assay and estimation of profilin content

Extract	$A_{492\text{ nm}}$	% Profilin/total protein using as reference	
		Hel a 2	Own profilin
<i>H. annuus</i>	0.979	0.021	0.021
<i>C. dactylon</i>	0.722	0.015	0.025
<i>P. pratense</i>	0.709	0.015	0.050
<i>O. europaea</i>	0.253	0.004	0.003
<i>M. annua</i>	0.707	0.015	0.019
<i>R. communis</i>	0.992	0.021	0.020
<i>Z. fabago</i>	0.413	0.007	0.025
<i>Dermatophagoides farinae</i>	0.035	< 0.00001	na
<i>Aspergillus fumigatus</i>	0.039	< 0.00001	na
Shrimp	0.091	< 0.00001	na

na: not applicable.

3.4. Assay variations and standard curve

The intraassay CV was acceptable at Hel a 2 concentrations as low as 1 ng/ml. Interassay variation values decreased as the Hel a 2 concentrations increased. The average interassay variation was 13.4% for the whole concentration range (1 to 250 ng/ml), but it fell to 11.2% over the range from 4 to 250 ng/ml. In this range, all interassay CVs were lower than 20%. The standard dose–response curves obtained with the purified Hel a 2 are shown in Fig. 5. The sensitivity or detection limit, defined as the antigen concentration that produced a significant positive signal, was determined to be 0.5 ng/ml. According to the intra- and interassay CVs, the practical working range for the ELISA was from 4 to 250 ng/ml of Hel a 2. These results and the $99 \pm 7\%$ of recovery obtained supported the validity of the assay.

3.5. Estimation of profilin content in other pollen extracts

Fig. 6A shows the dose–response curves obtained with the whole *H. annuus*, *C. dactylon*, *P. pratense*, *O. europaea*, *M. annua*, *R. communis* and *Z. fabago* extracts. In order to estimate the profilin content in various pollen extracts, standard dose–response curves were calculated using purified profilin from each extract (Fig. 6B). Similar curves were obtained for profilins from distantly related botanical families, such as the Gramineae (*Cynodon*, *Phleum*), the

Oleaceae (*Olea*), the Euphorbiaceae (*Mercurialis*, *Ricinus*) and the Zygophyllaceae (*Zygophyllum*). Nevertheless, the curve slopes differed, particularly those for the *Phleum* and *Zygophyllum* profilins. The specificity of the ELISA was ascertained by testing and assay of allergenic plant and non-plant extracts (Table 3). The profilin content was negligible in extracts of shrimp, mites and molds. For plant extracts, the profilin content was calculated using both the Hel a 2 and their own profilin as standard. The results showed that the profilin estimation could differ significantly according to the profilin used as reference. The greatest divergences corresponded to those extracts whose dose–response curves differed the most. For instance, the *Zygophyllum* extract would contain 0.007% profilin if Hel a 2 was used as the reference, but 0.025% if *Zygophyllum* profilin was used instead. A similar situation was found for the *Cynodon* and *Phleum* extracts. Dose–response curves were obtained comparing natural and recombinant profilins from *Helianthus*, *Olea*, *Cynodon* and *Mercurialis* (data not shown). When using recombinant allergens, the absorbance values were 35% to 60% lower than those obtained with their natural counterparts.

4. Discussion

Crude allergen extracts are currently used for allergy diagnosis and immunotherapy although their allergen composition and allergenic potency may

differ from extract to extract. This probably reflects the variability in the natural source materials, which in turn may depend on environmental conditions in the case of pollens, the phase of maturation in the case of molds, and the species of mites, types of pets, etc. In addition, some extracts may contain important allergens at insufficient concentrations. This occurs with profilins which are underrepresented in some pollen extracts (Susani et al., 1995; Asturias et al., 1997b).

In this study, a solid phase ELISA method was set up to measure plant profilin with mAbs. This powerful tool has been applied to the characterization of extracts from etiologically important pollen (Lombardero et al., 1992; Ayuso et al., 1995; Ramírez et al., 1997; Fahlbusch et al., 1998) and indoor (Chapman et al., 1987; Chapman et al., 1988; Pollart et al., 1991) allergens. Sandwich immunoassays present several advantages over direct or inhibition ELISA procedures since non-relevant proteins do not interfere with target detection and the antigens are detected as native proteins in the liquid phase, thus avoiding any conformational changes after absorption to the solid phase. The possible occurrence of conformational changes produced by mAb binding has been reported for the Bet v 1 specific mAb Bip 1 (Laffer et al., 1997). The use of a specific polyclonal serum would avoid the loss of detection produced by conformational changes affecting one or more epitopes. Hence, a specific anti-profilin polyclonal serum has been used as the secondary antibody in our assay.

The main criteria of a good immunoassay are high sensitivity, reproducibility and specificity. The present sandwich ELISA has a sensitivity threshold of about 0.5 ng/ml of Hel a 2 sunflower profilin, comparable in magnitude to the Par j 1, Bet v 1 and Pen a 1 specific assays, which detect these allergens at concentrations as low as 0.2, 0.4 and 1 ng/ml, respectively (Ayuso et al., 1995; Jeoung et al., 1997; Ramírez et al., 1997). The assessment of day-to-day, inter- and intraassay variability reveal that this ELISA is reproducible using the linear portion of the standard curve, i.e., over the 4 to 250 ng/ml range. Moreover, when different pollen extracts as well as non-plant extracts, from mites, molds and seafood, were tested, only the plant profilins were found to be reactive, confirming the specificity of the assay.

Given that profilins display a considerable heterogeneity among plants, and that isoforms have been described in several species (Staiger et al., 1993; Huang et al., 1996; Asturias et al., 1997a,b), the use of more than one mAb is advisable. Consequently, the immunoassay described here is based on the use of two capture mAbs (5F2 and 3H8), which recognize different epitopes at both termini of the profilin molecule. The highest homology regions within plant profilins are located towards the N- and C-terminus of the molecule (Valenta et al., 1991). Both regions are involved in the interaction with actin, the principal physiological ligand of profilin (Fedorov et al., 1997), and consequently, the mAbs that bind to those regions would be expected to show considerable cross-reactivity. This could be the case with mAbs 5F2 and 3H8 since preliminary results indicate that 5F2 binds to a region located between Ala³⁶–Met⁴⁹, whereas 3H8 binds to the polypeptide stretch containing the last 20 amino acids of the C-terminus.

Although the ELISA has been developed using sunflower profilin, it also permits the detection of other plant profilins that share common epitopes with the former. In order to design an exact quantification assay of profilin in plant extracts, separate control curves should be established for each plant species using the corresponding purified profilin. In a two-site ELISA method for quantification of tropomyosin, purified shrimp, crab and lobster tropomyosins should be used for the construction of standard curves in order to quantify the tropomyosin content in each extract (Jeoung et al., 1997). For the detection of group 4 allergens in grass species, parallel curves were observed with extracts of different grass pollens, but the percentages of group 4 allergens relative to the total protein content were very different (Fahlbusch et al., 1998). As profilin is not considered a glycosylated protein (Vallverdú et al., 1998), the different reactivities of the profilins tested should be due to the existence of similar but not identical epitopes in all of them. Therefore, the estimation of profilin content in a sample may vary according to the profilin used as standard. The variability of the profilin content in pollen of different extracts has also been reported previously (Van Ree et al., 1992).

Recombinant profilins are sometimes less reactive than their natural counterparts in ELISA (Asturias et

al., 1997b; Vallverdú et al., 1997; Asturias et al., 1998). It is likely that the recombinant allergens represent only one of the various isoforms occurring naturally and hence possess only some of the IgG epitopes present in natural profilin. Since the serum was produced against epitopes present on all the natural sunflower profilin isoforms, the use of a unique recombinant isoform as standard could overestimate the profilin content of some samples.

The ELISA procedure described here could be very useful for the evaluation of extracts as possible sources of this panallergen, as well as for the quality control of preparations intended for clinical use. The assay is highly sensitive and specific for plant profilins from the most important pollen allergenic extracts.

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