

Short communication

Olive pollen allergen Ole e 8: identification in mature pollen and presence of Ole e 8-like proteins in different pollens

In a first approach, Ole e 8, a novel Ca^{2+} -binding protein from olive pollen, was cloned and produced in *Escherichia coli*. We have obtained the natural form of Ole e 8 (nOle e 8) from the pollen and examined its immunologic equivalence with its recombinant form (rOle e 8). Size exclusion chromatography and a phenyl-Sepharose CL-4B affinity column were used to obtain nOle e 8 from the olive pollen. Inhibition assays by immunoblotting, using rOle e 8-specific rabbit antiserum, were performed to analyze the immunologic equivalence between the natural and the recombinant allergen, as well as to detect its presence in other pollens. Recombinant and natural Ole e 8 resulted immunologically equivalents, since they completely inhibited the IgG binding of the polyclonal antiserum to each other. Ole e 8-like proteins were detected in *Oleaceae* and *Juniperus communis* pollen, and might contribute to cross-reactivity processes between taxonomically related pollens.

**A. Ledesma, M. Villalba¹,
F. Vivanco², R. Rodríguez¹**

¹Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, Madrid; ²Departamento de Inmunología Fundación Jiménez Díaz, Madrid

Key words: Ca^{2+} -binding protein; cross-reactivity; Ole e 8; olive pollen allergy.

Dr Rosalía Rodríguez
Departamento de Bioquímica y Biología Molecular
Facultad de Ciencias Químicas
Universidad Complutense
28040 Madrid
Spain

Accepted for publication 31 August 2001

Allergy to olive tree (*Olea europaea*) pollen is an important health problem in Mediterranean countries and some areas of North America (1). Olive pollen extract contains a complex mixture of allergenic proteins, from which many components with different clinical significance have been characterized (2).

It has been found that pollen tissue contains at least three different types of Ca^{2+} -binding proteins (CaBP), all of them being able to induce IgE-mediated allergy in the terms of the recently revised nomenclature for allergic diseases (3): (i) an ubiquitous family of proteins with two EF-hand sites (4–9), which have been detected in rapeseed (Bra n 1, Bra n 2, Bra r 1 and Bra r 2), olive tree (Ole e 3), Bermuda grass (Cyn d 7), birch (Bet v 4), alder (Aln g 4) and timothy grass (Phl p 7); (ii) Bet v 3 from birch with three EF-hand motifs (10); and (iii) Jun o 2 from juniper which is a four EF-hand CaBP (11). Allergens which share such structural motifs can be responsible for cross-reactivity between different sources (12).

Recently, a new olive CaBP has been produced as a recombinant form in *E. coli* cells, using a cDNA prepared from pollen RNA (13). This protein (20 kDa of apparent molecular mass) contained four EF-hand motifs and was called Ole e 8 as it exhibited binding activity to IgE from the sera of olive allergic patients. The aim of this work is to evaluate the

existence of Ole e 8 in mature olive pollen and to analyze the immunologic equivalence between its natural and recombinant forms. The presence of proteins homologous to Ole e 8 in other pollens is also examined.

Material and methods

Preparation of pollen extracts

Pollen proteins from *Olea europaea*, *Lolium perenne*, *Cynodon dactylon*, *Betula verrucosa*, *Fraxinus excelsior*, and *Syringa vulgaris* were extracted for 3 h, as described (4, 14, 15). *Juniperus communis* pollen was extracted with 100 mM ammonium bicarbonate (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, for 16 h at 4°C. Supernatants were filtered using a 1.2- μm pore size filter, dialyzed and stored at -20°C (16).

Ole e 8 purification and analytical methods

Natural Ole e 8 was purified from extract of olive pollen obtained at 3 h, by using a size exclusion chromatography in Sephadex G-150, in 0.2 M ammonium bicarbonate, followed by affinity chromatography in phenyl-Sepharose CL-4B equilibrated in 50 mM Tris-HCl, pH 7.4 containing 0.5 M CaCl_2 and eluted with 50 mM Tris-HCl containing EGTA. rOle e 8 was produced in *E. coli* by expression of its specific cDNA prepared from total RNA of olive pollen, amplified by PCR, and purified as described (13).

Protein concentration was determined by colorimetric assay (17), or quantitative amino acid analysis (13). SDS-PAGE was performed in 15% polyacrylamide gels (18).

Ole e 8 immunodetection and immunoblot inhibition assays

Polyclonal antiserum against rOle e 8 was prepared by immunizing adult male White New Zealand rabbits over a 6-week period with 100 µg of the purified protein. Samples of pollen extracts and purified protein were electrophoretically transferred onto nitrocellulose membranes. Immunostaining of membranes was performed by incubation with the polyclonal antiserum (diluted 1/25 000) raised against rOle e 8 (13). The IgG-binding was detected with horseradish peroxidase-labeled anti-rabbit IgG (Pierce Chemical Co., Rockford, Ill.) (4). The signal was developed using the ECL Western-blotting reagents (Amersham Corp, Buckinghamshire, England).

Immunoblotting inhibition assays were performed with olive pollen extract (50 µg of total protein), the phenyl-Sepharose eluate (0.2 µg of total protein, and rOle e 8 (0.1 µg), separated by SDS-PAGE and transferred to nitrocellulose membranes. Olive pollen extract (0.1 mg and 1 mg), the phenyl-Sepharose eluate (0.25 µg and 2.5 µg), rOle e 8 (0.5 mg) and Ole e 1 (0.5 µg) were used as inhibitors. Anti-rOle e 8 polyclonal antiserum (diluted 1/25 000) was preincubated with the corresponding inhibitor for 2 h at room temperature. The rOle e 8-specific antiserum preincubated with PBS was used as a control.

Results

Two chromatographic steps were carried out for the purification of nOle e 8 (Sephadex G-150 and phenyl-Sepharose affinity column). An enriched protein preparation was the result of this procedure. The allergen is present in the pollen extract under a monomer form of 20 kDa (Fig. 1A, lanes OP). However, it has strong tendency to dimerize and is eluted from the exclusion column mainly as a dimer (40 kDa), whose form is maintained in the affinity chromatography (Fig. 1A, lanes PS). This property had also been observed for the recombinant form of the allergen (13) and could be due to the presence of a free Cys at position 132 of the polypeptide chain. The estimated amount of Ole e 8 in the pollen was 0.2–0.5 µg per mg of total protein (0.02–0.05%).

Both nOle e 8 and rOle e 8 were able to completely inhibit the IgG binding of anti-rOle e 8 polyclonal to each other. The assay was performed by using rOle e 8 as inhibitor against partially purified nOle e 8 electrophorized and blotted to membranes (Fig. 1A), as well as with nOle e 8 as inhibitor against rOle e 8 (both monomer and dimer forms) attached to the nitrocellulose membranes (Fig. 1B). In the latest, no complete inhibition was reached by using crude pollen extract, probably due to its very low content of Ole e 8; however, 100% inhibition was achieved with 2.5 µg of the fraction eluted from the phenyl-Sepharose column, thus revealing the antigenic equivalence between natural and recombinant forms of Ole e 8.

The presence of proteins either homologous to Ole e 8 or sharing antigenic epitopes with this olive allergen was investigated in the pollen from different allergenic species: ash, lilac, Bermuda grass, ryegrass, birch, and juniper. Immunoblotting of these pollen extracts was achieved with rOle e 8-specific rabbit antiserum in Western blotting. Reactive proteins with

a molecular mass of 20 kDa were recognized in *Oleaceae* species (ash and lilac) and, to lesser extent, in juniper (Fig. 2). No positive bands were found in pollen from *Gramineae* species and birch. This suggests that Ole e 8-like proteins do not display highly conserved amino acid sequences, or they are not detectable because of their lower levels in these pollens. The reactive bands from ash, lilac and juniper pollen did not appear in the immunoblotting when the anti-rOle e 8 antibody was preincubated with rOle e 8 as inhibitor (Fig. 2), indicating the existence of cross-reactivity between the recognized proteins.

Discussion

The recombinant production of protein allergens can overcome the frequent problems caused by the polymorphism of allergens, the variability of the natural extracts, and the limited availability of most of the allergenic sources. Therefore, recombinant forms of allergens should progressively substitute to the natural forms for clinical and scientific purposes, after accurate demonstration of their equivalence. As a part of the study of the allergenic pattern of olive pollen, a cDNA encoding Ole e 8 was recently cloned, sequenced and expressed in *E. coli* (13). However, the natural form of Ole e 8 was not explored in the pollen. In this work, it has been demonstrated that Ole e 8 is biologically expressed in mature pollen, and both forms of the allergen, natural and recombinant, are immunologically equivalents.

The levels of Ole e 8 are very low in the olive pollen, 15–40 µg per 100 g of dried pollen (0.02%–0.05% of total protein). This protein represents a good example of the interest in producing recombinant allergens since only very small amounts of Ole e 8 could be obtained from the pollen, while a pure and fully active rOle e 8 was produced by molecular biology techniques at the level of milligrams in *E. coli* (13). In contrast, the isolation of Ole e 8 from the pollen has turned out to be difficult because of the low concentration of the protein and the presence of abundant Ole e 1, which shares with Ole e 8 molecular mass and acidic pI.

An additional difficulty on the isolation of Ole e 8 was the absence of allergic sera exhibiting only positive reaction to this allergen. Ole e 8 shows a low prevalence in comparison to other allergens from olive pollen as Ole e 1 (>70%) or Ole e 7 (47%). Interestingly, all sera reactive to Ole e 8 also recognized to Ole e 3, which is also a CaBP, but containing only two EF-hand sites (4). Although the amino acid sequences of Ole e 3 and Ole e 8 are very different, and only share similarity within the EF-hand motifs, we can not exclude the possibility that they share antigenic determinants. In

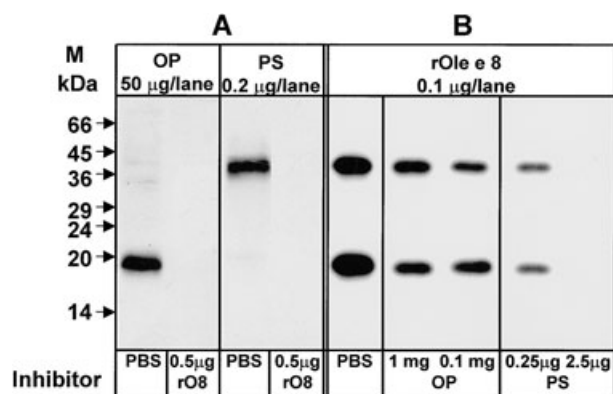


Figure 1. Analysis of the immunologic equivalence of natural and recombinant Ole e 8 by SDS-PAGE and immunoblotting inhibition assays, using anti-rOle e 8 rabbit polyclonal antiserum. (A) Olive pollen extract (lanes OP) and phenyl-Sepharose fraction (lanes PS) were separated by SDS-PAGE and transferred to membranes; inhibition was performed with rOle e 8 (rO8, 0.5 µg). (B) After SDS-PAGE and blotting of rOle e 8 (monomer plus dimer forms), the IgG-binding was inhibited by olive pollen extract (OP, 1 or 0.1 mg total protein) and phenyl-Sepharose fraction (PS, 0.25 or 2.5 µg). Incubation with phosphate saline buffer (PBS) was used as negative control in both assays. M, molecular mass markers.

fact, whether their EF-hand sites are involved in the concomitant sensitivity to both olive allergens is to be determined.

Ole e 8 shows a low amino acid sequence identity with other CaBPs out of the EF-hand sites. The highest sequence similarity was observed for Jun o 2 – a major allergen from juniper (11) – and for the stress-induced protein from *Arabidopsis thaliana* (19) called TCH2 (27% and 35% identity out of the Ca²⁺-binding sites, respectively). These proteins are CaBPs containing four EF-hand sites and could be functionally related to Ole e 8. The rOle e 8-specific rabbit antiserum recognized a 20-kDa protein from *Juniperus communis* extract (Fig. 2), which could correspond to a homologue of Jun o 2 or to a

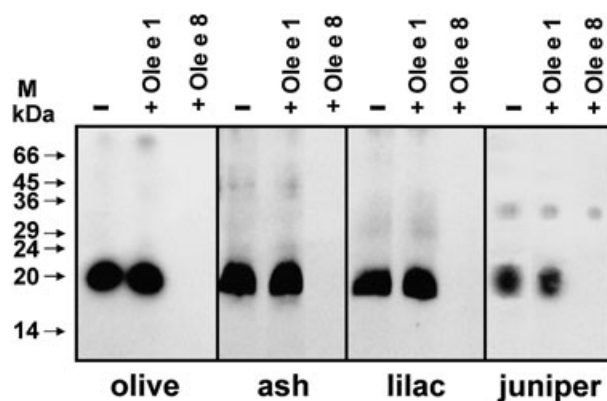


Figure 2. Detection of Ole e 8-related proteins in pollen extracts. Blotting of pollen extracts (50 µg of total protein) from olive, ash, lilac, and juniper were probed with rOle e 8-specific rabbit antiserum (lanes -). Pollen extracts of ryegrass, Bermuda grass and birch were also probed, resulting in a negative response (data not shown). The IgG binding was also analyzed after incubation of the serum with Ole e 1 (0.5 µg) or rOle e 8 (0.5 µg) as inhibitors (lanes +). M, molecular mass.

protein more closely related to Ole e 8. The absence of IgG cross-reactivity of Ole e 8 with other pollen extracts from taxonomically unrelated plants suggests a low conservativeness for the amino acid sequence of Ole e 8-like proteins.

In conclusion, the finding of Ole e 8 enlarges the landscape of the CaBPs as molecules involved in processes of IgE binding that in some extent can be responsible of cross-reactivity. The recombinant production of Ole e 8 and its homologues could overcome the difficulties on the purification procedure derived from their low levels in the pollen.

Acknowledgments

This work was supported by grants PM98/0062 and PM98/0094 from the Dirección General de Enseñanza Superior (MEC, Spain)

References

- BOUSQUET J, COUR P, GUERIN B, MICHEL F-B. Allergy in the Mediterranean area. I. Pollen counts and pollinosis of Montpellier. Clin Allergy 1985;14:249–258.
- RODRIGUEZ R, VILLALBA M, MONSALVE RI, BATANERO E. The spectrum of olive pollen allergens. Int Arch Allergy Immunol 2001;125:185–195.
- JOHANSSON SGO, HOURIHANE J, O'B, et al. A revised nomenclature for allergy. Allergy 2001;56:813–824.
- LEDESMA A, VILLALBA M, BATANERO E, RODRIGUEZ R. Molecular cloning and expression of active Ole e 3, a major allergen from olive-tree pollen and a member of a novel family of Ca²⁺-binding proteins (polcalcins) involved in allergy. Eur J Biochem 1998;258:454–459.
- TORIYAMA K, OKADA T, WATANABE M et al. A cDNA clone encoding an IgE binding protein from *Brassica* anther has significant sequence similarity to Ca²⁺-binding proteins. Plant Mol Biol 1995;29:1157–1165.
- ENGEL E, RICHTER K, OBERMEYER G et al. Immunological and biological properties of Bet v 4, a novel birch pollen allergen with two EF-hand calcium-binding domains. J Biol Chem 1997 272:28630–28637.
- SUPHIOGLU C, FERREIRA F, KNOX RB. Molecular cloning and immunological characterization of Cyn d 7, a novel calcium-binding allergen from Bermuda grass pollen. FEBS Lett 1997;402:167–172.

8. HAYEK B, VANGELISTA L, PASTORE A, SPERR WR, VALENT P, VRTALA S., Molecular and immunologic characterization of a cross-reactive two EF-hand calcium-binding alder pollen allergen, Aln g 4: Structural basis for calcium-modulated IgE recognition. J Immunol 1998;**161**:7031–7039.
9. NIEDERBERGER V, HAYEK B, VRTALA S et al. Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p. 7. Faseb J 1999;**13**:843–, 856.
10. SEIBERLER S, SCHEINER O, KRAFT D, LONSDALE D, VALENTA R. Characterization of a birch pollen allergen, *Bet v*, III, representing a novel class of Ca^{2+} -binding proteins; specific expression in mature pollen and dependence of patients'IgE binding on protein-bound Ca^{2+} . EMBO J 1994;**13**:3481–3486.
11. TINGHINO R, BARLETA B, PALUMBO S et al. Molecular characterization of a cross-reactive *Juniperus oxycedrus* pollen allergen. Jun o 2: a novel calcium-binding allergen. J Allergy Clin Immunol 1998;**101**:772–777.
12. VAN REE R, AALBERSE RC. Pollen-vegetable food cross-reactivity: serological and clinical relevance of cross-reactive IgE. J Clin Immunoassay 1993;**16**:124–130.
13. LEDESMA A, VILLALBA M, RODRIGUEZ R. Cloning, expression and characterization of a novel four EF-hand Ca^{2+} -binding protein from olive pollen with allergenic activity. FEBS Lett 2000;**466**:192–196.
14. BATANERO E, VILLALBA M, LÓPEZ-OTÍN C, RODRIGUEZ R. Isolation and characterization of an olive allergen-like protein from lilac pollen. Sequence analysis of three cDNA encoding protein isoforms. Eur J Biochem 1994;**221**:187–193.
15. BATANERO E., González de la Peña MA, Villalba M, Monsalve RI, Martín-Esteban M, Rodríguez R. Isolation, cDNA cloning and expression of Lig v, 1, the major allergen from privet pollen. Clin Exp Allergy 1996, : 26:1401–1410.
16. REID MJ, NISH W, WHISMANN BA et al. HLA-DR4-associated nonresponsiveness to mountain cedar allergen. J Allergy Clin Immunol 1992;**89**:593–598.
17. LOWRY O, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;**193**:265–275.
18. LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1977;**227**:680–685.
19. BRAAM J, DAVIS RW. Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell 1990;**60**:357–364.