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A recombinant functional variant of the olive pollen allergen Ole e 10 expressed in baculovirus system

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Abstract

Pollens have been reported as important sources of antigens causing type-I allergy and, among them, olive pollen has high clinical relevance in Mediterranean countries. The most recently described olive allergen, Ole e 10, is involved in cross-reactivity phenomena and related to asthma induction in allergic patients. These immunologic features make this allergen a good candidate to be included in diagnosis and therapy of protocols of allergic diseases. Since the availability of Ole e 10 from the olive pollen is limited, the allergen has been efficiently expressed in the baculovirus/insect cell system. The Ole e 10-cDNA inserted into the transfer vector pBacPAK8 allowed the expression of the recombinant protein in cultured Sf21 cells. Recombinant Ole e 10 (rOle e 10) was purified from the culture after dialysis and three chromatographic steps. Mass spectrometry, Edman degradation, IgE- and IgG-binding analyses were employed to characterize the recombinant allergen, which showed molecular and immunological equivalence with the natural protein. Affinity gel electrophoresis in presence of laminarin (1,3- β -glucan) revealed that rOle e 10 retains identical carbohydrate-binding capacity than the natural allergen. In conclusion, the recombinant expression of Ole e 10 in baculovirus/insect cell system produces a homogeneous and biologically active allergen that could be useful for clinical and scientific purposes.

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1. Introduction

Type-I allergy is an increasing disease caused by the recognition of normally innocuous antigens (called allergens) by IgE antibodies thus inducing a cascade of cellular activation and the consequent symptoms, rhinoconjunctivitis, asthma, dermatitis, etc. (Kay, 1997). Tree pollens are major causes of pollinosis

Abbreviations: AGE, affinity gel electrophoresis; 2-ME, 2-mercaptoethanol; TFA, trifluoroacetic acid

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and, among them, olive pollen has high clinical relevance in Mediterranean areas and constitutes also an important allergenic source in America, Australia and South Africa (Bousquet et al., 1985; Macchia et al., 1991; Manousis and Moore, 1988). To date 10 allergens (Ole e 1 to Ole e 10) have been isolated and characterized from olive pollen (Rodríguez et al., 2001; Huecas et al., 2001; Barral et al., 2004). The most recently isolated allergen, Ole e 10, is a small (10.8 kDa) and acidic (pI 5.8) protein that has been described as the first member of a new family of plant proteins, with homology to modules from glucanases and glucanotransferases implicated in carbohydrate metabolism (Barral et al., 2004). Ole e 10 is a major allergen recognized by 55% of patients allergic to olive pollen and exhibits IgE cross-reactivity with protein extracts from different pollens (belonging to either *Oleaceae* or non-taxonomically related families), fruits and latex (Barral et al., 2004). Furthermore, the clinical relevance of Ole e 10 has been recently enhanced by the finding of correlation between asthma and Ole e 10 sensitivity in olive pollen allergic patients that remarks the special importance of this allergen in the aetiology of the disease (Quiralte et al., 2005). These clinical features make Ole e 10 a good candidate to be included in diagnosis and immunotherapy protocols for allergy.

Along the last decade, recombinant DNA technology has allowed the availability of a large number of allergens as homogeneous recombinant molecules in high yield (Schmidt and Hoffman, 2002). It is starting to be considered the usefulness of these recombinant forms in diagnosis and immunotherapy of allergy, overcoming the problems associated to the employment of natural extracts that usually are poorly characterized and contain undesirable components (Valenta et al., 1999). Since the yield of the isolation of Ole e 10 from olive pollen is low (~80 µg/g of dried pollen) (Barral et al., 2004), its recombinant production as a homogeneous protein with high yield could be a useful tool in order to complete clinical, immunological and biochemical studies. Over the last few years baculovirus/insect cells expression system has been widely employed for the production of biologically active recombinant proteins (Vialard et al., 1995). The ability to perform many of the post-translational modifications found in eukaryotic cells is the main advantage over prokaryotic expression systems that sometimes produce insoluble or incorrectly folded materials.

In this work, we report the recombinant expression in baculovirus/insect cell expression system of Ole e 10 as a soluble, stable and functional protein. Characterization of its biochemical and immunological properties is also included.

2. Materials and methods

2.1. Materials

pBacPAK8 transfer vector and AcMNPV DNA were included in the BacPAK Baculovirus Expression System from Clontech (Palo Alto, CA). *Spodoptera frugiperda* (IPLB-Sf21) cells and Grace's insect medium were purchased from Clontech and Gibco BRL (Gaithersburg, MD), respectively. *E. coli* strain Top 10 and plasmid pCR2.1 were included into the TOPO TA cloning kit (Invitrogen BV, Groningen, The Netherlands).

Human sera were collected from olive pollen allergic individuals without previous immunotherapy. Patients were characterized by a case history of hypersensitivity to olive pollen with symptoms of rhinitis and/or bronchial asthma, positive skin prick test response to olive pollen extract, positive IgE response to Ole e 10 and exhibiting RAST classes (specific IgE for whole olive pollen extract) of 3–6 by the CAP System (Pharmacia Biotech, Uppsala, Sweden).

Natural Ole e 10 (nOle e 10) was isolated from olive pollen as previously described (Barral et al., 2004).

A polyclonal serum against nOle e 10 was obtained by immunizing New Zealand rabbits over 6-week period by weekly injection of the protein (100 µg) in complete Freund's adjuvant.

2.2. Cloning of Ole e 10 in the transfer vector

The coding region of mature Ole e 10 (GenBank/EMBL databank accession number AY082335) was amplified from total olive pollen cDNA with Taq Advantage polymerase (Clontech) by PCR using a sense (5'-ataggatccATGTCTTCGTCGCCCGTT-3') and an anti-sense (5'-atagcgccGCTCAAGAGAGGAATGAGCA-3') primers which hybridized, respectively, with the 5' and 3' ends of the protein encoding region (codifying sequence in upper-case). The sense

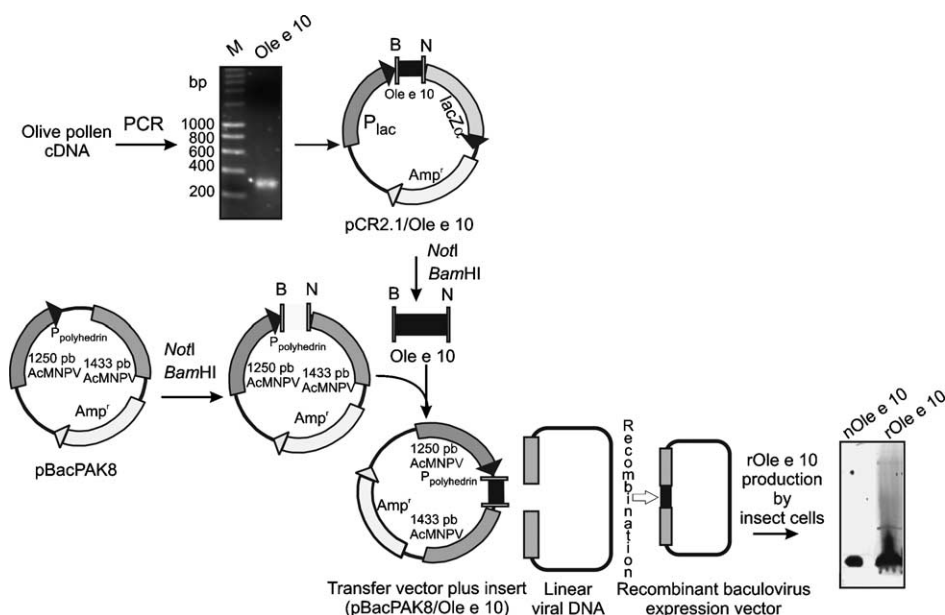


Fig. 1. Construct of the rOle e 10 expression vector. cDNA codifying Ole e 10 was amplified by PCR and cloned into the pCR2.1 vector (pCR2.1/Ole e 10). The construct was digested with the restriction enzymes *Bam*HI (B) and *Not*I (N) and inserted into the transfer vector pBacPAK8 digested with the same enzymes, giving the construction pBacPAK8/Ole e 10. Recombination between the transfer vector and the linearized viral cDNA gives the recombinant baculovirus expression vector with Ole e 10-specific cDNA included in the viral genome. rOle e 10 expression was detected in the cell lysate by immunoblotting with an Ole e 10-specific polyclonal antibody. Amp^r, ampicillin resistance; P_{polyhedrin}, polyhedrin promoter; bp, size markers in base pairs.

primer included the *Bam*HI restriction site (underlined) and the anti-sense one contains a stop codon and a *Not*I restriction site (underlined). The PCR product was cloned into pCR2.1 vector and used to transform Top10 *E. coli* cells. The construction was digested with the restriction enzymes *Bam*HI and *Not*I, subcloned into same sites of the baculovirus transfer vector pBacPAK8 (pBacPAK8/Ole e 10) and used to transform DH5 α competent *E. coli* cells (Fig. 1). Recombinant plasmids were isolated and purified with Chroma Spin columns (Clontech).

2.3. Expression of Ole e 10 in baculovirus-infected insect cells

Recombinant baculovirus were generated by cotransfecting 500 ng of pBacPAK8/Ole e 10 plasmid with linearized AcMNPV DNA into Sf21 cells according to the manufacturer's protocol. Recombinant baculovirus expressing Ole e 10 under transcriptional control of the *Autographa californica* polyhedrin promoter were obtained. Cells were cultured in

Grace's basic insect medium supplemented with 10% of bovine fetal sera at 27 °C during 3 days. Supernatant of the infected cells was harvested and recombinant viruses were isolated by a plaque assay and tested for Ole e 10 expression. Several positive clones were selected and employed to infect Sf21 cells and, after 72 h, both lysate and culture media were tested for rOle e 10 expression by immunoblotting with a specific anti-Ole e 10 antibody.

2.4. Purification of recombinant Ole e 10 (rOle e 10)

Insect cell cultures were harvested by centrifugation at 500 g. Cells were then resuspended in lysis buffer containing 0.6 mM NaCl, 20 mM Tris pH 8.0, 1% (w/v) Triton X-100 and a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin). Cells were lysed by sonication at 4 °C, and soluble material was collected after centrifugation at 12,000 $\times$ g and dialyzed against 20 mM ammonium bicarbonate pH 8.0, with a mem-

brane cut-off of 3.5 kDa. This material was loaded onto a DEAE-cellulose column previously equilibrated in the same buffer and eluted in an ammonium bicarbonate gradient, 20–500 mM. Fractions containing rOle e 10 (detected by immunoblotting with an Ole e 10-specific polyclonal antibody) were pooled, lyophilized and fractionated into a Sephadex G-50 superfine column in 0.2 M ammonium bicarbonate. A RP-HPLC (Nucleosil C-18 column) was used as the final step for rOle e 10 purification. The elution was performed with an acetonitrile gradient of 30–40% in 0.1% (v/v) trifluoroacetic acid (TFA).

2.5. Analytical procedures

The N-terminal amino acid sequence was determined using an Applied Biosystems Procise model 494 sequencer (Applied Biosystems, Foster City, CA). For mass spectrometry analyses, samples (1 µg) were mixed with a matrix solution comprised of saturated α -cyano-4-hydroxycinnamic acid in 30% aqueous acetonitrile and 0.1% TFA. Measurements were performed in a Bruker Reflex II MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with an ion source with visualization optics and a nitrogen laser (337 nm). The equipment was externally calibrated employing singly, doubly, and triply charged signals from either cytochrome *c* (12,360 Da) or bovine serum albumin (66,430 Da).

2.6. Standard and affinity gel (AGE) electrophoresis experiments

SDS-PAGE was performed by the method of Laemmli (1970) in 15% (w/v) polyacrylamide gels, and eventually using 2-mercaptoethanol (2-ME) treatment of the sample. Proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 or blotted onto nitrocellulose membranes (Amersham).

The capability of rOle e 10 to bind soluble polysaccharides was evaluated by affinity gel electrophoresis as previously described (Mimura et al., 1992). Briefly, native polyacrylamide gels were prepared consisting of 15% (w/v) acrylamide in 25 mM Tris, 192 mM glycine buffer, pH 8.3. Laminarin (1,3- β -glucan, 0.1%, w/v) was added to the gels prior to polymerization. Native gels without carbohydrate were run simultaneously

under the same conditions. BSA (Sigma) was used as a negative control. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

2.7. Immunological characterization

Immunodetection of proteins transferred to nitrocellulose membranes was performed as previously described (Villalba et al., 1994), using a specific anti-Ole e 10 antibody (diluted 1:2500) or a pool of sera from patients allergic to olive pollen (diluted 1:10). A mouse anti-human IgE (diluted 1:5000), kindly donated by ALK-Abelló (Madrid, Spain), and a horseradish peroxidase-labeled goat anti-mouse IgG (Pierce) (diluted 1:5000) were employed to detect IgE-binding proteins. A horseradish peroxidase-labeled goat anti-rabbit IgG allowed detecting the binding of polyclonal anti-Ole e 10 antibody. The signal was developed using the ECL Western blotting reagent (Amersham Pharmacia Biotech) as previously described (Villalba et al., 1994).

IgE titration was performed by ELISA indirect according to (Batanero et al., 1996). Briefly, microtiter 96-well ELISA plates were coated with 100 µl of natural or recombinant protein (1 µg/ml in PBS). Plates were incubated with different dilutions of a pool of sera from allergic patients or a polyclonal anti-Ole e 10 antibody. Bound IgG and IgE were detected as described above. Peroxidase reaction was developed with *o*-phenylenediamine (0.63 mg/ml; Merck) and H₂O₂ 0.04% (v/v), in 0.1 M sodium citrate buffer pH 5.0, containing methanol 4% (v/v). Optical density (OD) was read at 492 nm and determinations were carried out as duplicates.

3. Results

3.1. Expression and isolation of rOle e 10

The coding region of Ole e 10 was amplified by PCR using total olive pollen cDNA as template and the hybridized oligonucleotides described in Section 2. Ole e 10-specific cDNA was then inserted in the baculovirus transfer vector (pBacPAK8) and the construct (pBacPAK8/Ole e 10) was cotransfected together with AcMNPV-linearized DNA into Sf21 insect cells. Wild-type virus infected and uninfected Sf21 cells were

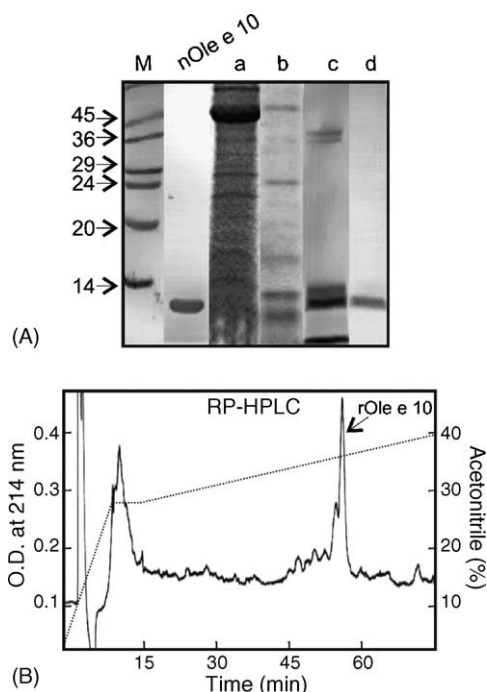


Fig. 2. rOle e 10 purification. (A) SDS-PAGE analyses of the purification steps: cell lysate (lane a), DEAE-cellulose (lane b), Sephadex G-50 (lane c) and RP-HPLC (lane d) eluates; natural Ole e 10 (lane nOle e 10); molecular mass is done in kDa (lane M). (B) Elution profile of rOle e 10 on a RP-HPLC nucleosil C-18 column using an acetonitrile gradient (30–40%) in 0.1% TFA.

employed as negative controls. The production of rOle e 10 was detected by immunoblotting of the insect cell lysate under reducing conditions, with a specific anti-Ole e 10 antibody at 72 h post-infection (Fig. 1). The expression rendered about 1 mg of rOle e 10 per litre of insect cell culture.

The dialyzed medium from the cell lysate was loaded on a DEAE-cellulose column and eluted by using an ammonium bicarbonate gradient from 20 to 500 mM. Fractions containing rOle e 10 were pooled and subjected to a Sephadex G-50 chromatography to eliminate high molecular mass contaminants. A RP-HPLC chromatography was used as a final step to obtain a $\geq 95\%$ pure rOle e 10, with a yield of approximately 420 μg of protein per litre of cell culture. Aliquots from different steps of the purification procedure were analysed by SDS-PAGE and Coomassie-blue staining (Fig. 2).

3.2. Molecular and immunological characterization of rOle e 10

Recombinant Ole e 10 was characterized by different analytical methods to evaluate its purity as well as its similarity to the natural allergen isolated from the pollen. Absence of contaminants in the purified protein as well as the molecular mass of rOle e 10 were determined by mass spectrometry analysis. The mass spectrum of rOle e 10 rendered a single peak of 10,773 Da that fits with the molecular mass described for the natural allergen (10,789 Da) and with that of the amino acid sequence deduced from Ole e 10-cDNA (10,791 Da). These results also confirm the absence of glycosylation in the recombinant allergen in spite of the presence of a potential *N*-glycosylation consensus site in the sequence of Ole e 10. Attempts to obtain the N-terminal sequence of the recombinant protein by Edman degradation were unsuccessful, indicating the existence of a modification of the N-terminal first residue as it happens for nOle e 10.

Like the natural protein, the purified rOle e 10 exhibited an electrophoretic pattern with two bands of 10.4 and 11.6 kDa that were attributed to a disulphide-bond isomerism (Barral et al., 2004) (Fig. 3A). The contribution of both conformations in rOle e 10, estimated by densitometric analysis, was 89 and 11%, respectively, and fits well with the percentage previously reported for nOle e 10 (90 and 10% of 10.4 and 11.6 kDa bands, respectively) (Barral et al., 2004).

Immunological characterization of purified rOle e 10 was performed using a specific anti-Ole e 10 polyclonal antibody and a pool of sera from patients allergic to olive pollen. As shown in Fig. 3B and C, IgG and IgE antibodies exhibited similar reactivity for natural and recombinant proteins in immunoblotting assays, recognizing in both cases the two protein bands present in SDS-PAGE. In addition, SDS-PAGE pattern of both nOle e 10 and rOle e 10 under reducing conditions showed a single band of 14.0 kDa molecular mass, and the reduced proteins transferred to membranes were also recognized by the same pool of allergic sera that bound to the untreated samples (Fig. 3D). The change of electrophoretic mobility of both protein bands to a single one of higher molecular mass in response to 2-ME indicates the loss of tertiary structure as a consequence of the disruption of potential cystine-bridges.

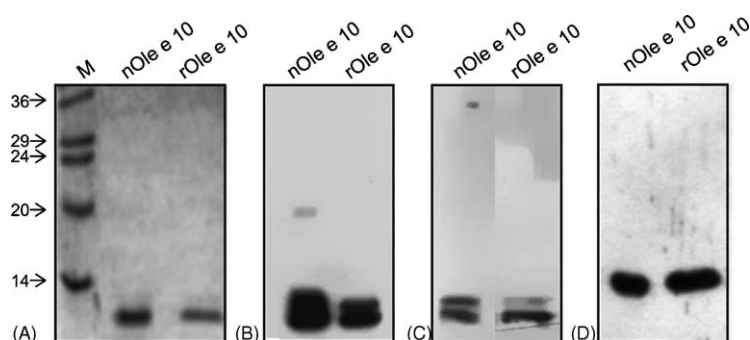


Fig. 3. SDS-PAGE analysis of purified rOle e 10 in comparison with nOle e 10. (A) Coomassie blue staining. (B) IgG-binding analysis with a polyclonal anti-Ole e 10 antibody after SDS-PAGE and transference to membranes. (C) IgE-binding detection with a pool of sera from allergic patients. (D) IgE-binding after reduction of the samples with 2-ME. Samples in B–D panels were immunostained after transference to membranes. M, molecular mass markers in kDa.

The quantification of the IgE and IgG-binding capabilities of nOle e 10 and rOle e 10 was performed by ELISA titrations employing antigen-coated wells and serial dilutions of a specific anti-Ole e 10 antibody as well as a pool of sera from allergic patients (Fig. 4). nOle e 10 and rOle e 10 showed similar IgE- and IgG-binding capabilities, indicating that rOle e 10 retains allergenic and antigenic epitopes that are present in nOle e 10.

3.3. Biochemical function of rOle e 10

nOle e 10 and rOle e 10 were subjected to AGE experiment in native polyacrylamide gels in the presence or absence of laminarin as has been described in Section 2. Both natural and recombinant proteins

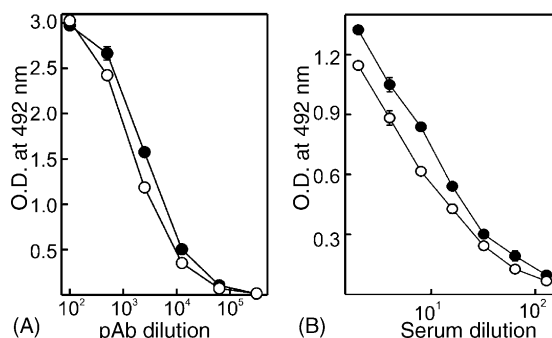


Fig. 4. ELISA analysis of rOle e 10. (A) IgG-binding and (B) IgE-binding to nOle e 10-coated (●) and rOle e 10-coated (○) wells were assayed.

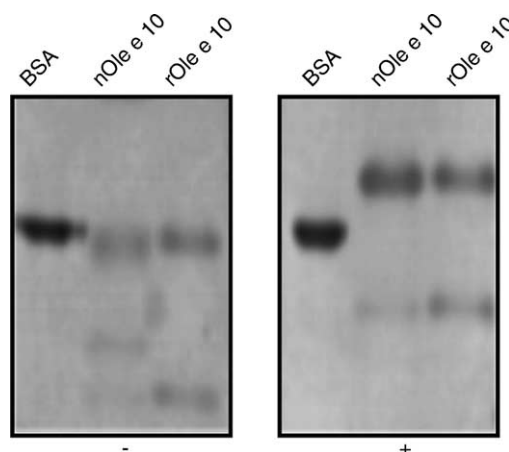


Fig. 5. Carbohydrate-binding assay. AGE experiments for nOle e 10 and rOle e 10 in gels with (+) or without (−) laminarin (0.1%, w/v). BSA was employed as negative, non-interacting control.

showed an indistinguishable behaviour in this assay with an identical retardation in the non-denaturing electrophoretic conditions in presence of the 1,3- β -glucan (Fig. 5), supporting the existence of a proper three-dimensional folding for rOle e10.

4. Discussion

Olive pollen is one of the main causes of allergy in Mediterranean areas, where this tree is extensively cultivated. The characterization of Ole e 10 has acquired special importance due to its high clinical

relevance. However, because of the low amount of Ole e 10 present in the pollen and the limited accessibility of this biological material, the availability of Ole e 10 should be solved by molecular biology techniques. In fact, in the last few years, the expression of allergens as recombinant proteins has facilitated their study (Chapman et al., 2000; Valenta and Kraft, 2002). *E. coli*, the most utilized host system, is not able to perform suitable post-translational processing of many recombinant proteins and often led to biologically inactive products. Thus, eukaryotic expression systems, such as yeast or insect cells, should resolve these problems. While yeast expression could produce hyperglycosylation in recombinant proteins, insect cells usually carry out correct post-translational processing and thus they make biologically active recombinant proteins (Schmidt and Hoffman, 2002). Although most allergens described to date are derived from eukaryotic sources (Schmidt and Hoffman, 2002), few allergens have been expressed in these systems: Cte f 1 the major cat flea salivary allergen (McDermott et al., 2000), Sol i 2 from fire ant venom (Schmidt et al., 1996), Fel d 1 from cat (Vailles et al., 2002; Seppala et al., 2005) and Aed a 1 from the mosquito *Aedes aegypti* (Xu et al., 1998; Peng et al., 2001).

Ole e 10 has been expressed in baculovirus system as a soluble and stable protein, and at significant yield (approximately, 420 µg/l of cell culture) in comparison with that obtained from the pollen (50–65 µg/g of pollen). It was purified to homogeneity from the cell lysate. Despite the presence of a single peak in mass spectrometry analysis, SDS-PAGE experiments revealed the existence of two forms in the purified rOle e 10, in a similar way to that previously described for the natural allergen (Barral et al., 2004). These two conformations were attributed to a cysteine-bridge isomerism and were recognized by allergic sera suggesting their presence in the pollen cell. The coexistence of both immunological reactive conformations in natural and recombinant Ole e 10 suggests a correct folding for the recombinant allergen, and indicates the capability of insect cells to induce the formation of the correct disulphide-bridge arrangement. The molecular characterization of rOle e 10 confirmed the absence of glycosylation that will facilitate the comparison with the natural protein, since the potential *N*-glycosylation site is also empty in the allergen isolated from olive pollen.

To analyze the immunological integrity of rOle e 10, ELISA response to specific antibodies (IgG and IgE) was assayed. Titration experiments demonstrated that the recombinant allergen retains most antigenic and allergenic epitopes of the natural form. The clinical characteristics of Ole e 10 (high prevalence, implication in cross-reactivity among pollens, latex and fruits, and participation in the development and/or exacerbation of asthma in allergic patients) (Barral et al., 2004; Quiralte et al., 2005) make the allergen a good candidate to be included in diagnosis and therapy protocols for allergy. Thus, the recombinant form of Ole e 10 will be a useful tool to guarantee the availability of this molecule that is present in the pollen in very low amount. Several authors have proposed the use of cocktails of recombinant allergens for diagnosis and therapy of allergic disorders instead of poorly standardized natural extracts which are currently used (Valenta and Kraft, 1995; van Ree et al., 1999; Chapman et al., 2000). The use of allergens involved in cross-reactivity may predict future allergic reactions to allergenic sources containing immunologically related molecules. This strategy would permit for diagnosis and immunotherapy the reduction to a limited number of cross-reactive recombinant allergens containing most IgE epitopes present in the corresponding natural extracts (Valenta et al., 1996; Niederberger et al., 1998).

Finally, the correct folding of Ole e 10 was also assessed by AGE experiments in presence of laminarin. In fact, rOle e 10 retains the carbohydrate-binding capability of nOle e 10. In the absence of proteins homologous to Ole e 10 with known structure, the characterization of the three-dimensional folding of the recombinant allergen can help to understand the protein–carbohydrate interaction, as well as the antigenic and allergenic epitopes, eventually allowing the design of hypoallergenic forms to be used in immunotherapy (Valenta and Kraft, 2002; Niederberger et al., 2004).

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