

Recombinant expression of Ole e 6, a Cys-enriched pollen allergen, in *Pichia pastoris* yeast: detection of partial oxidation of methionine by NMR

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Received 23 March 2004, and in revised form 10 June 2004

Available online 28 July 2004

Abstract

Olive pollen is one of the main causes of allergy in Mediterranean countries. Ole e 6, an olive pollen allergen, is a small (5.8 kDa) and acidic protein (*pI* 4.2) and no homologous proteins have been isolated or characterized so far. Ole e 6 has been efficiently expressed in the methylotrophic yeast *Pichia pastoris*. The cDNA encoding Ole e 6 was inserted into the plasmid vector pPIC9 and overexpressed in GS115 yeast cells. The recombinant product was purified by size-exclusion chromatography followed by reverse-phase HPLC. N-terminal sequencing, amino acid composition analysis, CD, NMR, and IgG-binding experiments were employed to characterize the purified protein. NMR data revealed the oxidation of the methionine at position 28 in approximately 50% of the recombinant protein but, although this alters its electrophoretic behavior, it did not affect folding or IgG-binding properties of rOle e 6. The recombinant form of Ole e 6 expressed in *P. pastoris* can be employed for structural and biochemical studies.

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Keywords: *Pichia pastoris*; Allergen; Olive pollen; NMR

Type-I allergy represents an IgE-mediated hypersensitivity affecting more than 25% of population in industrialized countries. This disease is caused by the recognition of normally innocuous antigens (denominated allergens) by IgE antibodies inducing the symptoms of allergy (rhinoconjunctivitis, asthma, dermatitis, etc.) [1]. Tree, weed, and grass pollens are major sources of airborne antigens causing allergic symptoms. Among them, olive pollen has high clinical relevance in Mediterranean countries, since this pollinosis affects more than 20% of the population in this area where olive tree is widely cultivated [2,3].

At date, 10 allergens from olive pollen have been described and characterized [4–6]. Ole e 6 has been

described as an acidic (*pI* 4.2) and small (5.8 kDa) protein which is recognized by up to 55% of sera from patients allergic to olive pollen [7]. Ole e 6 consists of a single polypeptide chain of 50 amino acids with six Cys residues (12% of the total amino acid composition). Cys are arranged in two sets of a sequential motif Cys-X₃-Cys-X₃-Cys [7], which has been also identified in deduced sequences of cysteine-rich proteins from *Nicotiana tabacum* pollen (74 amino acids length) and *Caenorhabditis elegans* (1513 amino acids). The products of these genes have not been isolated or characterized, and their biological and biochemical functions are unknown. The complete characterization of Ole e 6 (3D structure, functional analysis, epitope mapping, etc.) has not been solved because of the very low yield of the protein during its isolation from olive pollen [7]. The application of molecular cloning techniques for the recombinant production of proteins of difficult accessibility may

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facilitate the availability of these molecules. In the last few years, the expression of allergens as recombinant proteins has facilitated their study because it has provided larger amounts of protein [8,9]. *Escherichia coli*, the most utilized host system, is not able to perform the adequate posttranslational processing of many polypeptides and the products are insoluble or incorrectly folded. Recombinant expression in the methylotrophic yeast *Pichia pastoris* has proved to be a useful system for proteins containing Cys residues, because it can be used to produce soluble proteins with a very high yield and correct posttranslational maturation [10].

In this work, we report the expression of the olive pollen allergen Ole e 6 in *P. pastoris* as a soluble and stable protein. Purification to homogeneity, molecular, spectroscopic, and immunological characterization are also described.

Materials and methods

Materials

Pichia pastoris GS115 his4 strain (Invitrogen) was used as host for transformations with the plasmid pPIC9. The *E. coli* strains INV α F' and TG1 were used, respectively, as hosts for cloning the PCR fragments in pCR2.1 (Invitrogen) and pPIC9 (Invitrogen).

A polyclonal antiserum against natural Ole e 6 was obtained by immunizing New Zealand rabbits over 6-week period by weekly injection of the protein (100 μ g) in complete Freund's adjuvant.

Natural Ole e 6 was isolated from olive pollen as previously described [7].

Cloning of Ole e 6 in the expression vector

The coding region of Ole e 6 was amplified by PCR with a Vent DNA polymerase (New England Biolabs) using the plasmid pUC18/Ole e 6 as a template and two non-degenerate primers, a sense primer 5'-cgtctcgagaaaagaGACGAGGCCAGTTT-3' and an antisense primer 5'-cgggaattcTTAATTTTTTGGCTTGTAGTTTTC-3', which hybridized, respectively, with the 5' and 3' ends of the protein-encoding region (codifying sequence in upper-case). The sense primer allows fusion of the Ole e 6-encoding DNA in-frame with that of the preprosequence of the α -mating factor present in plasmid pPIC9, and also included a *Xho*I restriction site (underlined). The antisense primer contains a stop codon and an *Eco*RI restriction site (underlined). Ole e 6-DNA was purified employing Wizard PCR preps kit (Promega) and ligated into a linearized pCR2.1 vector. DNA from several clones was sequenced as described by Sanger et al. [11]. DNA fragments were digested with *Xho*I/*Eco*RI restriction enzymes, subcloned into the same sites

of the plasmid pPIC9, and used to transform TG1 *E. coli* cells to obtain the construction pPIC9/Ole e 6.

Transformation of *P. pastoris* GS115 and production of rOle e 6

pPIC9/Ole e 6 recombinant plasmid (~20 μ g) was linearized with *Bgl*II restriction enzyme, and the purified fragment was used to transform GS115 cells by a gene replacement using lithium acetate treatment [12]. Transformed cells were incubated on minimal dextrose (MD) plates at 30°C for 4–6 days until colonies appeared. Screening for gene replacement of the construct by homologous recombination at the *AOX1* locus rendering a (His⁺ Mut^s) phenotype was performed by patching the His⁺ colonies in replica-plating on minimal dextrose vs. minimal methanol plates. For the production of rOle e 6, selected (His⁺ Mut^s) transformed strains were cultured for 48 h and 30°C in buffered glycerol minimum medium (BGM). Cells were then collected by centrifugation and resuspended in one-fifth of the original volume of buffered methanol minimum medium (BMM) for induction of the *AOX1* promoter. This culture was maintained for 72 h and supplemented daily with 5 ml of methanol per liter of culture. The culture medium of GS115-induced cells was cleared by centrifugation at 3000g at 4°C. The production of rOle e 6 in the supernatant of the culture medium was analyzed by SDS-PAGE of aliquots taken at different times (0, 24, 48, and 72 h). Large-scale production was performed under similar conditions using the colony that rendered the best yield in the small-scale experiments.

¹⁵N-rOle e 6 for NMR experiments was produced in the same way as rOle e 6 but substituting the NH₄(SO₄)₂ by ¹⁵NH₄(SO₄)₂ (Cambridge Isotope Laboratories) in both BMG and BMM growth media.

Purification of rOle e 6 and analytical procedures

The extracellular medium obtained after the centrifugation was exhaustively dialyzed against 20 mM ammonium bicarbonate, pH 8.0, with a membrane cut-off of 3.5 kDa in order to remove small contaminants. Afterwards, the sample was fractionated in a Sephadex G-50 superfine column eluted with 0.2 M ammonium bicarbonate, pH 8.0. Fractions containing rOle e 6 were detected by Coomassie blue staining and positive reaction to anti-Ole e 6-specific antibody. These fractions were pooled and lyophilized. A reverse-phase HPLC (Nucleosil C-18 column) was used as the final step for the allergen purification. The elution was performed with an acetonitrile gradient of 15–50% in 0.1% (v/v) trifluoroacetic acid.

Composition and protein concentration of purified samples were determined by amino acid analysis. Protein samples (1–2 nmol) were hydrolyzed with 5.7 M HCl and analyzed on a Beckman System 6300 amino acid

analyzer. The N-terminal amino acid sequence was determined by Edman degradation using an Applied Biosystems Procise Model 494 sequencer.

Circular dichroism

Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter (Japan Spectroscopic) fitted with a 150-W xenon lamp and connected to a Weslab RTE-111 thermostabilizer bath. Far-UV spectra were registered in the range of 190–250 nm. Samples (0.1 mg/ml) were analyzed in sodium phosphate 20 mM, pH 7.0, at 25 °C. Mean residue mass ellipticities were calculated based on 116 as the average molecular mass/residue, obtained from the amino acid composition, and expressed in terms of θ (deg cm²/dmol). Secondary structure estimations were performed by computer fit according to the method of Perczel et al. [13].

NMR spectroscopy

¹⁵N-rOle e 6 samples were prepared for NMR experiments at 0.5–1 mM in 95% H₂O/5%D₂O containing sodium-4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at pH 6.0. NMR spectra were carried out at 308 K on a Bruker AV-600 NMR spectrometer operating at 600.13 MHz for the proton, equipped with a triple-resonance cryoprobe and an active shielded z-gradient coil. Proton chemical shifts were referenced to the internal reference DSS and ¹⁵N chemical shifts were referenced indirectly using gyromagnetic ratios of ¹⁵N:¹H [14]. 1D ¹H spectra, COSY, TOCSY, NOESY, ¹H–¹⁵N-HSQC, and 3D NOESY-HSQC were acquired. Conventional pulse sequences and phase-cycling procedures were used for the experiments. Spectra were processed by using the programs XWIN-NMR (Bruker Biospin) and NMR-Pipe [15]. The spectral analysis and assignment were performed with ANSIG 3.3 [16].

Electrophoresis and immunological characterization

SDS–PAGE was performed in 15 or 17% (w/v) polyacrylamide gels [17], and alternatively using 2-mercaptoethanol (2-ME) treatment of the sample. Proteins were stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose membranes. PAGE experiments were also performed in absence of SDS in the same conditions as described above. Immunodetection was achieved as described [18] employing a polyclonal antiserum raised against nOle e 6 (1/10,000) and a secondary antibody horseradish peroxidase-labeled goat anti-rabbit IgG. The peroxidase reaction was developed using the ECL Western blotting reagent (Amersham Pharmacia Biotech) as described [18].

ELISA titration was performed as described [19]. Plates were coated with 0.1 µg per well of antigen (1 µg/

ml in PBS). After blocking, coated wells were incubated with different dilutions of the polyclonal anti-Ole e 6 antiserum, followed by a horseradish peroxidase-labeled goat anti-rabbit IgG. Peroxidase reaction was developed with freshly prepared substrate [19]. The optical density (OD) was read at 492 nm and all determinations were carried out as duplicates.

For ELISA inhibition experiments, anti-Ole e 6 antiserum (1/10,000) was preincubated with different concentrations of pure proteins as inhibitors and added to the nOle e 6-coated wells. Bound IgG was detected as described above.

Results

Expression of Ole e 6

The coding region of Ole e 6 was amplified by PCR using the plasmid pUC18/Ole e 6 as a template and the primers described in “Materials and methods.” Then, the coding sequence for Ole e 6 was inserted in the yeast expression plasmid (pPIC9) in-frame with the rest of the leader sequence. The linearized construct carrying the Ole e 6-specific cDNA inserted downstream of the *AOX1* promoter (pPIC9/Ole e 6) was employed for the transformation of *P. pastoris* GS115 cells obtaining the efficient cellular secretion of rOle e 6. The time course of the expression in the extracellular medium was analyzed by SDS–PAGE and Coomassie blue staining. Major protein bands appeared at 10 and 14.2 kDa. The highest level of production was detected after 72 h of induction (Fig. 1). After small-scale production, the best producer colony was selected for large-scale preparation.

Purification of rOle e 6 and molecular characterization

rOle e 6 was purified from the culture medium after dialysis and two chromatographic steps. The dialyzed extracellular medium of the culture was lyophilized and fractionated on Sephadex G-50 superfine column. The presence of rOle e 6 was detected by staining with a polyclonal Ole e 6-specific antibody. Fractions containing rOle e 6 were subjected to reverse-phase HPLC that rendered two peaks reactive to the antibody (designated rOle e 6-p1 and -p2), which were collected and analyzed (Fig. 2). The remaining peaks were contaminants of the *P. pastoris* culture media. The final yield of the purified allergen (including both peaks) was 25 mg per liter of cell culture.

Both peaks obtained in HPLC showed different mobility in SDS–PAGE (Fig. 3). rOle e 6-p1 migrated with an apparent molecular mass in the range 13.5–14.2 kDa and rOle e 6-p2 gave a major component of 10 kDa and a minor one at 13.5 kDa. The natural protein isolated from olive pollen showed a major band at 10 kDa (Fig. 3A). Both recombinant forms were recog-

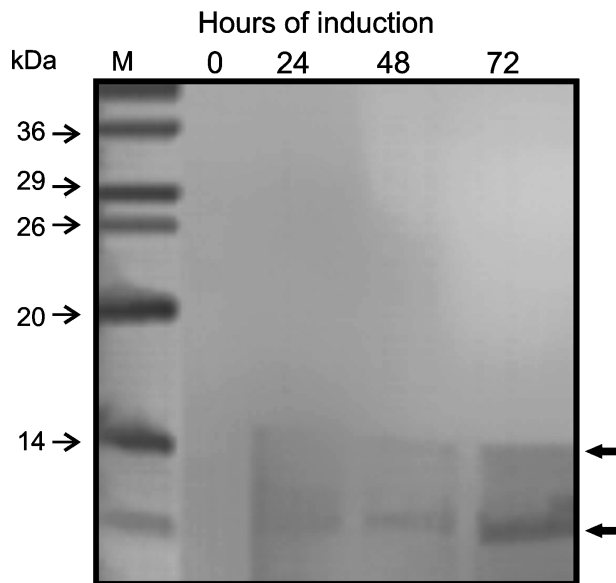


Fig. 1. Time course of rOle e 6-expression in *P. pastoris*. Supernatants of the yeast culture were taken at different time points and analyzed by Coomassie blue staining after SDS-PAGE (17% polyacrylamide). (M) Molecular mass markers.

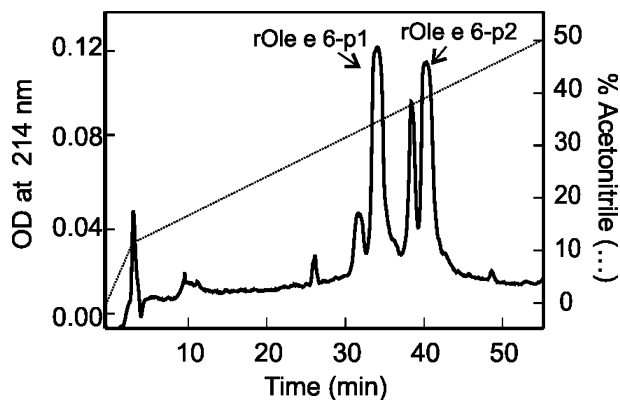


Fig. 2. RP-HPLC elution profile of rOle e 6 in a C-18 column using an acetonitrile gradient (15–50%). Two major peaks (rOle e 6-p1 and rOle e 6-p2) were detected at 214 nm.

nized in immunoblotting by a polyclonal antibody raised against the nOle e 6 (Fig. 3B). The treatment of the samples with 2-ME gave a single band with identical apparent molecular mass for natural and recombinant proteins (10 kDa) that indicated the reduction of disulfide bonds and elimination of the tertiary structure (Fig. 3C). PAGE experiments performed in absence of SDS and in non-reducing conditions (Fig. 3D) showed the same pattern for both recombinant proteins indicating differences between them in the SDS binding but not in the mass/charge relation.

Edman degradation of the N-terminal end for both HPLC peaks gave the sequence DEAQF indicating that both forms correspond to rOle e 6 with a correct processing of the α -mating signal peptide used for the secretion of the protein to the extracellular medium of the yeast culture. The amino acid composition was identical for both rOle e 6 forms and fits well with the composition of the deduced sequence from the cDNA encoding Ole e 6 (Table 1). Evidence for the correct folding of both forms of rOle e 6 was obtained by spectroscopic studies with the determination of far-UV spectra for natural and recombinant allergens (Fig. 4A). No significant differences were observed among the natural allergen and both recombinant forms in terms of the shape of the spectra and molar ellipticity values, indicating a correct folding for both rOle e 6 species in terms of secondary structure. Moreover, similar values of secondary composition were calculated for natural and recombinant proteins, with a predominance of α -helix (~60%) in their structure (Fig. 4B).

NMR experiments indicate different oxidation states between rOle e 6-p1 and rOle e 6-p2

To analyze the observed HPLC and SDS-PAGE behavior, and also to elucidate differences between rOle e 6-p1 and p2 at the molecular level, we have carried out a detailed study by NMR. The NMR spectra of the two species were completely and unequivocally assigned

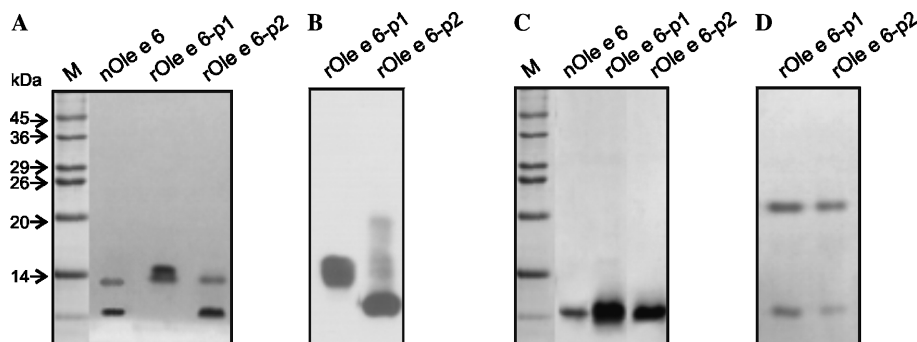


Fig. 3. PAGE analysis (15% polyacrylamide) of purified rOle e 6 (p1 and p2). (A) Coomassie blue staining of nOle e 6 and rOle e 6 after SDS-PAGE. (B) IgG-binding analysis with a polyclonal anti-Ole e 6 antibody to rOle e 6 after SDS-PAGE and transference to membranes. (C) Coomassie blue staining of nOle e 6 and rOle e 6 in SDS-PAGE after reduction of the samples with 2-ME. (D) Coomassie blue staining after PAGE (without SDS) of rOle e 6. (M) Molecular mass markers.

Table 1
Amino acid composition determined for rOle e 6 (p1 and p2)

Amino acid	Ole e 6 ^a	rOle e 6	
		Peak 1	Peak 2
Asx	9	8.7	8.3
Thr	3	2.5	2.6
Ser	2	1.6	1.6
Glx	7	7.3	7.6
Pro	1	0.7	0.6
Gly	2	2.4	2.6
Ala	1	1.2	1.3
Val	2	1.9	1.4
Met	1	1.2	1.1
Ile	0	0	0
Leu	1	1.2	1.1
Tyr	2	2.0	2.2
Phe	3	3.3	3.2
His	1	1.0	1.0
Lys	9	9.1	9.4
Arg	0	0	0
Cys	6	ND	ND
Trp	0	0	0
Total	50	44 + 6 Cys	44 + 6 Cys

ND, Non-determined.

^a Composition deduced from the amino acid sequence.

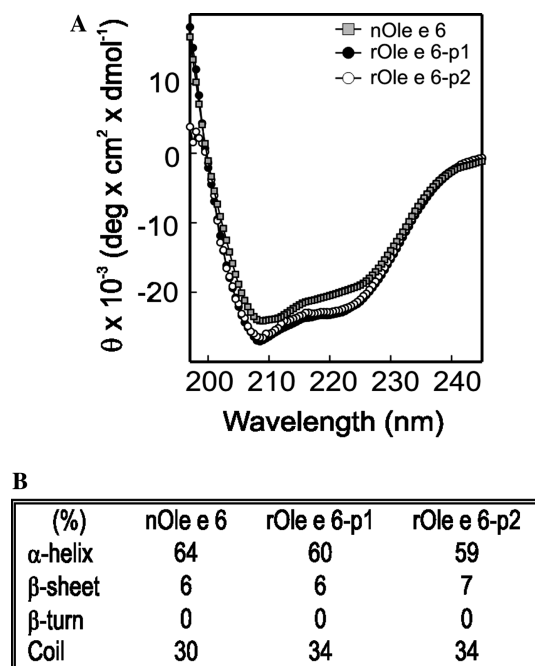


Fig. 4. (A) Far UV-CD spectra of nOle e 6 and rOle e 6. (B) Secondary structure values calculated from the spectra.

following the specific sequential assignment procedure [20]. Both species showed a good chemical shift agreement for all residues except for methionine-28. For rOle e 6-p2, one spin system was identified for each amino acid of the sequence, but two different sets of signals assignable to methionine-28 were identified for rOle e 6-p1. This is clearly observed in the superposition of the

2D-HSQC NMR spectra of rOle e 6-p2 and rOle e 6-p1 (Fig. 5B). For all the amino acids out of methionine-28, the corresponding signals superimpose perfectly. The obtained chemical shifts of methionine-28 are summarized in Table 2, together with the reported values for a methionine amino acid in a random coil structure [21].

First of all, based on the number of protons and their corresponding chemical shift values, the same type of spin system can be attributed to the three sets of signals corresponding to the two species of rOle e 6. No significant differences were observed in the chemical shift values of the backbone atoms ^{15}N , HN, and H_α . However, the signal positions importantly differ for the side chain protons (in bold in Table 2), being the chemical shift values of the two forms of rOle e 6-p1 only slightly different. Additionally, the chemical shifts of side chain protons belonging to rOle e 6-p2 are always smaller when compared with those in rOle e 6-p1. These differences between rOle e 6-p2 and rOle e 6-p1 can be easily

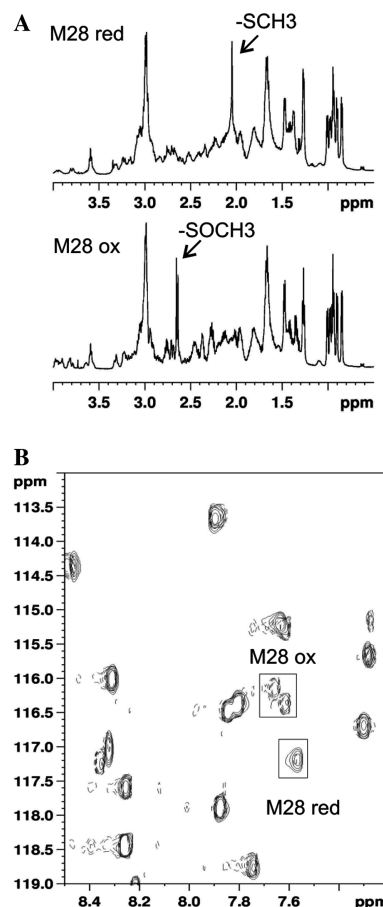


Fig. 5. NMR spectra of Ole e 6-p1 (methionine-28 oxidized form, M28 ox) and Ole e 6-p2 (methionine-28 reduced form, M28 red). (A) 1D spectra of Ole e 6-p2 (above) and Ole e 6-p1 (below). Singlets corresponding to the methyl group of the oxidized and reduced form are labeled. (B) Detail of the superposition of the 2D ^1H - ^{15}N -HSQC spectra of Ole e 6-p1 and Ole e 6-p2. Signals corresponding to the ^1HN - ^{15}N correlation are labeled. Continuous line, Ole e 6-p2. Broken line, Ole e 6-p1.

Table 2

Proton and nitrogen NMR chemical shifts relative to DSS of Ole e 6, pH 6.0, 35 °C

	Chemical shifts							
	¹⁵ N	HN	H _α	H _β	H' _β	H _γ	H' _γ	Me _δ
rOle e 6-p1	116.13	7.65	4.25	2.20	2.20	2.95	3.09	2.65
	116.36	7.61	4.25	2.21	2.21	2.86	3.14	2.64
rOle e 6-p2	117.17	7.56	4.24	2.06	2.06	2.71	2.71	2.05
Methionine RC	119.6	8.28	4.48	2.11	2.01	2.60	2.54	2.10

Chemical shifts corresponding to side chain are in bold.

Methionine RC, reported values for a methionine in random coil structure.

observed in the 1D NMR spectra (Fig. 5A). Thus, both spectra show key differences in some peaks located in the aliphatic region. In the rOle e 6-p2 spectrum (Fig. 5A, top), a singlet signal integrating for three protons emerges at 2.05 ppm. This signal is absent in the corresponding spectrum of rOle e 6-p1 and two new peaks at 2.65 ppm are observed (Fig. 5A, bottom). All the NMR data suggest that there is a chemical modification located in the side chain of one of these protein forms. It is well known that the chemical shift values of the side chain protons are less influenced by the structure conformation than the backbone ones, so they could be used as appropriate tools to determine specific modifications. rOle e 6-p2 values can be compared with those accepted for a methionine in a non-structured environment with good agreement. Taking together all these evidences, we can conclude that rOle e 6-p2 has a methionine at position 28 and corresponds to the native sequence of Ole e 6.

At this point, a reasonable explanation is lacking for the structure of rOle e 6-p1. An additional comparison with an extended chemical shift database [22] allows the identification of a sulfoxide group in its side chain. Additionally, the oxidation of the sulfur atom explains the presence of the two sets of signals for this protein as sulfoxides are chiral atoms due to their pyramidal bounding. Moreover, the presence of the sulfoxide modifies the chemical shift values of neighbor atoms (H_γ, H'_γ, and Me_δ) with respect to the reduced form, and makes non-equivalent both H_γ protons of rOle e 6-p1. In short, rOle e 6-p2 corresponds to the non-modified protein, whereas rOle e 6-p1 is the oxidized S-form.

Immunological properties

The immunological equivalence between the recombinant forms of rOle e 6 (oxidized and non-oxidized) and the natural allergen was demonstrated performing ELISA experiments with the three proteins. IgG-binding analysis of a polyclonal anti-nOle e 6 antibody to nOle e 6-, rOle e 6-p1-, and rOle e 6-p2-coated wells showed identical titration curves for natural and recombinant forms of the allergen (Fig. 6A).

ELISA inhibition experiments were performed for the analysis of the binding to nOle e 6-coated wells of the same polyclonal antibody preincubated with different

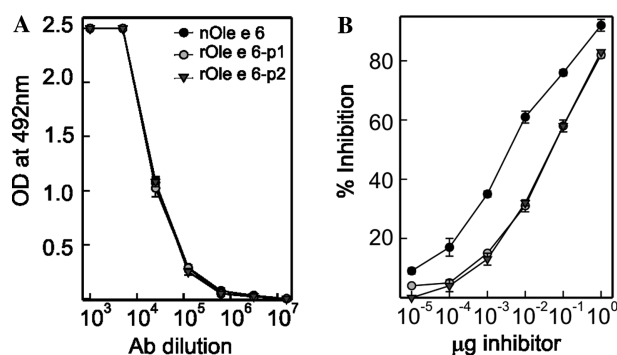


Fig. 6. Immunological analysis of rOle e 6 (p1 and p2). (A) IgG-binding with a polyclonal anti-Ole e 6 antiserum to natural and recombinant Ole e 6-coated wells (0.1 μg protein/well). (B) ELISA inhibition analysis of the IgG binding to nOle e 6-coated wells using nOle e 6, rOle e 6-p1 or rOle e 6-p2 as inhibitors.

amounts of natural or recombinant (oxidized and non-oxidized) forms. Fig. 6B shows the identical inhibition capability obtained for both recombinant forms of the allergen that reach more than 80% of inhibition of IgG binding to nOle e 6, although they did not share the highest capability of inhibition at the same amounts of inhibitor as the natural allergen.

Discussion

Olive pollen is a major cause of rhinoconjunctivitis and asthma in Mediterranean area, where large amounts of this pollen are present as a result of intensive cultivation. Ole e 6 constitutes an interesting protein from the structural, functional, and immunological point of view. It has been described as a significant allergen of the olive pollen, and no homologous proteins have been isolated or characterized so far. Because of the low amount present in the pollen and the limited availability of this biological material, we expressed Ole e 6 using the methylotrophic yeast *P. pastoris*.

Pichia pastoris has been successfully used for the recombinant expression of foreign proteins, and its products are usually obtained as secreted forms in large amounts and with the correct disulfide bonds [10]. One of the main advantages of this expression system is the easy purification of the recombinant products since they

are secreted to the extracellular medium, which contains low levels of endogenous proteins, resulting in enrichment of the overexpressed protein. Ole e 6 has been produced as a soluble monomer, with a very high yield and can be easily purified to homogeneity using a two-chromatographic step procedure.

The production of Ole e 6 by the yeast rendered two recombinant forms that can be separated by RP-HPLC. These forms seemed to be identical to each other and to native Ole e 6 in their structural properties. They showed the same N-terminal sequence, amino acid composition, and secondary structure. Additionally, the close similarity of the NMR data corresponding to the backbone atoms is a clear indication that the backbone conformation is retained in both recombinant forms. However, they differ in their behavior in electrophoresis experiments. Although both peaks, p1 and p2, present a minor component of 13.5 kDa molecular mass that could be attributed to the dimer of the protein, the SDS-PAGE pattern showed different major bands for both recombinant forms, with an estimated molecular mass value of 10 and 14.2 kDa, respectively, to p2 and p1. These values do not correspond with that deduced from the cDNA encoding Ole e 6 (5833 Da). This anomalous correlation between the molecular mass determined in SDS-PAGE and the value calculated for the polypeptide chain has been also described for the native protein nOle e 6 [7]. This could be explained by the strong acidic character of Ole e 6, which can reduce binding of SDS to the protein. Moreover, the apparent molecular mass in SDS-PAGE also depends on the three-dimensional conformation of the protein and non-globular molecules can give anomalous results. The reduction of the samples with 2-ME gave a single band of identical apparent molecular mass for natural and recombinant forms. The change of electrophoretic mobility in response to 2-ME indicates an elimination of the tertiary structure resulting from reduction of disulfide bonds. PAGE experiments in absence of SDS showed an identical migration for both recombinant forms, indicating differences in the SDS binding but not in the mass/charge relation for both rOle e 6 molecules.

The chromatographic and electrophoretic behavior of the recombinant forms of Ole e 6 can be explained on the light of the molecular data obtained from NMR. In fact, the NMR study revealed that the differences between rOle e 6-p1 and rOle e 6-p2 were restricted to the methionine at position 28, and more specifically on the different oxidation states of the sulfur atom of their corresponding side chain. The different covalent structure can explain the different polarity properties between both molecules, as the oxidized form rOle e 6-p1, which contains one more polar bond than the reduced form, elutes from the HPLC column at a higher solvent polarity than the reduced rOle e 6-p2. The electrophoretic properties can be understood assuming a less efficient

incorporation of SDS to the oxidized protein due to steric or polar impediments. As a consequence of the minor charge, the SDS-rOle e 6-p1 complex would show minor mobility (14.2 kDa apparent molecular mass) than SDS-rOle e 6-p2 (10.0 kDa apparent molecular mass), as experimentally observed.

The oxidation of methionine residues is not an exceptional feature and similar modifications have been described for other proteins produced on *P. pastoris*: for instance, the Sonic hedgehog-Fc fusion protein showed the oxidation of three methionine residues as has been demonstrated by mass spectrometry and peptide mapping [23]. In the present work, this oxidation was detected by NMR. The oxidation phenomenon could be due to the presence of hydrogen peroxide in the medium produced during the metabolism of the methanol or to the existence of trace metals in the culture medium, or it could also arise out as a part of a homeostatic mechanism used by *P. pastoris* in order to prevent its oxidation stage [24].

rOle e 6 forms are indistinguishable in their IgG-binding properties, as they are equally recognized for a polyclonal anti-Ole e 6 antiserum and exhibited identical inhibition capability to the antiserum. However, these expressed forms displayed less affinity to the antibody than the natural form in the inhibition experiments, although they were able to reach similar final inhibition values (more than 80%). These data indicated the existence of equivalent IgG epitopes between the natural and recombinant forms of the allergen.

In conclusion, both forms of Ole e 6 expressed in *P. pastoris* can be employed in structural and functional studies, thus allowing the complete characterization of Ole e 6 from two points of view: as a new pollen protein as well as an allergenic component of olive tree. Finally, the presence of the Cys-repeated motifs in non-related proteins suggests a putative structural-functional relationship and, therefore, Ole e 6 could serve as a model of study to elucidate this point.

Acknowledgments

This work was supported by Grants SAF2002-02711 from the Dirección General de Investigación (MCYT, Spain) and 07B/0054/2002 from the Comunidad Autónoma de Madrid (CAM, Spain).

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