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Expression of the major olive pollen allergen Ole e 10 in the yeast *Pichia pastoris*: Evidence of post-translational modifications

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Abstract

Olive pollen allergy is a clinical disorder that affects around 20% of the population in Mediterranean areas. The major olive pollen allergen, Ole e 10, is involved in cross-reactivity phenomena and asthma induction in allergic patients, and, besides its clinical interest, Ole e 10 is the first member of a new family of plant proteins. Ole e 10-specific cDNA has been cloned in the plasmid pPICZαA and expressed in the methylotrophic yeast *Pichia pastoris*. The recombinant protein has been purified in a two chromatographic-step procedure. N-Terminal sequencing, mass spectrometry, IgG, and IgE binding assays were employed to characterize the recombinant allergen. These analyses revealed that the product undergoes a proteolytic cleavage in the N-terminal end with the loss of the first six residues. Different strategies were used to solve this problem, such as changes in the fermentation conditions and the employment of protease-deficient yeast strains. Proteolytic cleavage was minimized and about 51% of rOle e 10 was obtained as a full-length protein. Moreover, a covalent modification was found in the N-terminal end of the full-length rOle e 10. Peptide mapping and mass spectrometry analyses pointed to the existence of a phosphorylation located in a serine residue of the N-terminal segment of rOle e 10 and it was confirmed after treatment of the sample with alkaline phosphatase. Finally, both full-length and truncated rOle e 10 retained most of the IgG- and IgE-binding capabilities of the natural protein isolated from the pollen.

Keywords: *Pichia pastoris*; Allergen; Olive pollen; Proteolysis; Phosphorylation; Recombinant protein

Type-I allergy is an increasing disease that affects more than 20% of the population in industrialized countries. This immunological disorder starts with the recognition of allergens by IgE antibodies setting off a cascade of cellular events and the consequent clinical symptoms of allergy [1]. Pollen from trees, grasses, and weeds are major sources of inhalant allergens. Allergy to olive tree (*Olea europaea*) pollen affects more than 20% of the population in Mediterranean areas, where this tree is widely cultivated, and constitutes an important allergenic source in North America, Australia, and South Africa [2,3]. The olive pollen allergogram is very complex, since a large number of proteins are recognized by human IgE. At date, 10 allergens from this pollen (Ole e 1–Ole e 10) have been isolated and characterized [4–6]. Ole e 10 has been described as a major allergen and shares IgE B-cell epitopes with proteins from different pollens, fruits, and latex [6]. The implication of Ole e 10 in the development and exacerbation of asthma processes has been recently proposed [7]. From a taxonomic point of view, Ole e 10 represents the first member of a new family of plant proteins [6].

Because of the low yield of the Ole e 10 isolation from the pollen (80 μg/g pollen) and the limited availability of this biological material, the expression of the allergen as a recombinant protein could facilitate its accessibility for clinical and scientific purposes. Due to the presence of six cysteine residues in Ole e 10, the yeast *Pichia pastoris* has been selected for its recombinant production. This

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methylotrophic yeast has proved to be a useful system for proteins containing cysteine residues, allowing the obtaining of soluble proteins with a high yield and correct post-translational maturation [8].

In this work, we report the extracellular production in the yeast *P. pastoris* of the major olive pollen allergen, Ole e 10. Isolation, molecular and immunological characterization, as well as the detection and control of post-translational modifications introduced in the recombinant allergen are also described.

Materials and methods

Materials

The *Escherichia coli* strains TOP10 and DH5α were used, respectively, as hosts for cloning the PCR fragments in pCR2.1 (Invitrogen) and pPICZαA (Invitrogen) vectors. *P. pastoris* KM71 and SMD1168 (protease deficient) strains (Invitrogen) were used as hosts for transformations with the plasmid pPICZαA.

Natural Ole e 10 (nOle e 10) was isolated from olive pollen as previously described [6].

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

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

Cloning of Ole e 10 in the expression vector

The coding region of mature Ole e 10 (GenBank/EMBL databank Accession No. AY082335) was amplified by PCR using the plasmid pCR2.1/Olee10 as a template, and a sense (5′ cgtctcgagaaaagaTCTTCGTCGCCCGTCCCA 3′) and an antisense (5′ cgtccgcggTCAAGAGAGGAATGAGCATGA 3′) primers which hybridized, respectively, with the 5′ and 3′ ends of the protein encoding region (codifying sequence in upper-case). The sense primer included the *Xho*I restriction site (underlined) and a sequence that allows fusion of the Ole e 10 encoding region in-frame with the preprosequence of the α-mating factor present in the pPICZαA vector. The antisense primer contains a stop codon and a *Sac*II restriction site (underlined). The PCR product was purified using the Wizard PCR preps kit (Promega) and directly cloned into a linearized pCR2.1 vector and was used to transform Top10 *E. coli* cells. The construction was digested with the restriction enzymes *Xho*I and *Sac*II, subcloned into the same sites of the pPICZαA and used to transform DH5α competent *E. coli* cells to obtain the construction pPICZαA/Olee10.

Transformation of P. pastoris and production of rOle e 10

pPICZαA/Olee10 recombinant plasmid (~10 μg) was linearized with *Sac*I, and the purified fragment was used to transform KM71 or SMD1168 competent yeast cells by electroporation. Transformed cells were incubated on YPDS plates (yeast extract/peptone/dextrose/1 M sorbitol) with 100, 750 or 1500 μg/ml of zeocin at 30 °C for 3–4 days until colonies appeared. For the production of rOle e 10, selected transformed strains were cultured for 48 h at 30 °C in buffered glycerol complex medium (BMGY)[1] (alternatively supplemented with casamino acids 1%), buffered glycerol minimum medium (BMGH) or non-buffered glycerol minimum medium (MGYH). Cells were then collected by centrifugation and resuspended in one-fifth of the original volume of buffered methanol complex medium (BMMY), buffered methanol minimum medium (BMMH) or methanol minimum medium (MMH) for induction of the *AOX*1 promoter. This culture was maintained for 3 days and supplemented daily with methanol (0.5% (v/v)). The culture medium of yeast-induced cells was cleared by centrifugation at 3000*g* at 4 °C. The presence of rOle e 10 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 identical conditions using the colony that rendered the best yield in the small scale experiments.

Purification of rOle e 10

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. Anion-exchange chromatography on a DEAE–cellulose column equilibrated in 20 mM ammonium bicarbonate, pH 8.0, was used to fractionate the sample under a gradient (0.02–0.5 M) of ammonium bicarbonate. Fractions containing rOle e 10, detected by SDS–PAGE and Coomassie blue staining, were pooled and subjected to a reverse-phase HPLC step (Nucleosil C-18 column). The elution was performed with an acetonitrile gradient of 30–50% in 0.1% (v/v) trifluoroacetic acid and registered at 214 nm.

[1] *Abbreviations used:* BMGY, buffered glycerol complex medium; BMGH, buffered glycerol minimum medium; MGYH, non-buffered glycerol minimum medium; BMMH, buffered methanol minimum medium; MMH, methanol minimum medium; MS, mass spectrometry; OD, optical density.