The C-terminal segment of the 1,3- β -glucanase Ole e 9 from olive (*Olea europaea*) pollen is an independent domain with allergenic activity: expression in *Pichia pastoris* and characterization

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Several allergenic proteins, such as the 1,3- β -glucanases, have been associated with plant defence responses. Ole e 9 (46 kDa) is a 1,3- β -glucanase and major allergen from olive pollen, which is a principal cause of allergy in Mediterranean countries. Its C-terminal segment (101 amino acid residues) has been produced as a recombinant polypeptide in the yeast *Pichia pastoris*. The cDNA encoding the polypeptide was inserted into the plasmid vector pPICZ α -A and overexpressed in KM71 yeast cells. The recombinant product was purified by size-exclusion chromatography followed by reversed-phase HPLC. Edman degradation, MS and CD were used to determine molecular properties of the recombinant polypeptide, which exhibited 16 % α -helix and 30 % β -sheet as regular elements of secondary structure. Disulphide bridges of the molecule were determined at positions Cys-14–Cys-76, Cys-33–Cys-94 and Cys-39–Cys-48. The high IgE-binding capability of the recombinant C-terminal

segment of Ole e 9 against sera from Ole e 9-sensitive individuals, which was determined by immunoblotting and ELISA inhibition, supported the proper folding of the polypeptide and the maintenance of antigenic properties that it exhibits as a part of the whole allergen. These data indicated that this portion of Ole e 9 constitutes an independent domain, which could be used to study its three-dimensional structure and function, as well as for clinical purposes such as diagnosis and specific immunotherapy. Since it shows sequence similarity with portions of 1,3- β -glucanases from plant tissues and the Gas/Phr/Epd protein families involved in yeast morphogenesis, we suggest that this domain could play an equivalent functional role within these enzymes.

Key words: allergen, glucan hydrolase, *Olea europaea*, recombinant production.

INTRODUCTION

Type I allergy is an increasing disease in industrialized countries, which involves the formation of IgE antibodies in response to substantially innocuous antigens, so-called allergens. Identification and characterization of proteins responsible for IgE-mediated allergies are relevant to developing an accurate diagnosis and therapy of this immunological alteration. Plant pollens have been reported as the main biological source of airborne allergens causing pollen-dependent type I allergies (pollinosis), and therefore they have been a major focus of the research into allergies. Pollinoses are strongly dependent on the geographical distribution of the vegetable source. Allergy to olive tree (*Olea europaea*) pollen affects more than 20 % of the population in countries of the Mediterranean area, where the olive tree is widely cultivated [1,2]. Nine protein allergens have been characterized from olive pollen [3,4]. Among them, Ole e 9 has been reported as a major inducer of IgE-mediated hypersensitivity, affecting more than 65 % of the patients allergic to olive pollen [4]. Ole e 9 constitutes a single polypeptide chain (46 kDa) with 1,3- β -glucanase activity.

1,3- β -Glucanases (glucan endo-1,3- β -D-glucosidases; EC 3.2.1.39) have been reported to be widespread proteins in higher plants [5,6]. They belong to the group 2 of the pathogenesis-related ('PR') protein superfamily and appear as multiple forms that differ in their molecular properties (such as size, isoelectric point and primary structure), cellular localization or expression

pattern. Seeds, roots, fruits and floral tissues are among the producer tissues of 1,3- β -glucanases [6]. Although many of them are constitutively expressed during the natural development of the plant, others have been related to defence responses since they are proteins induced by pathogens [6–10]. Most of the 1,3- β -glucanases so far characterized belong to a family of proteins with a molecular mass of 33–36 kDa, whose three-dimensional structure, either (β/α)₈ conformation or TIM-barrel structure, and enzymic activity have been well established [11,12]. However, a very limited number of larger 1,3- β -glucanases (42–46 kDa) with an extension at the C-terminal end have been reported: (i) a moderately acidic enzyme in wheat A1-intoxicated roots [13]; (ii) two basic 1,3- β -glucanases from *Arabidopsis thaliana* and rapeseed anthers, called A6 proteins [14]; (iii) the gene *SgGN1* product from willow male catkins with a basic deduced amino acid sequence [15] and (iv) Ole e 9, with an acidic pI (5.62), found in pollen tissue [4]. Only A6 proteins and Ole e 9 have been isolated and characterized. Ole e 9 shows 39, 32 and 33 % sequence identities, respectively, with these other 1,3- β -glucanases. The N-terminal portions of larger 1,3- β -glucanases, which comprise 320/350 amino acid residues, contain a tetrad of ionizable amino acids that has been proposed to be involved in the catalytic mechanism [12]. Their three-dimensional modelling fits well to a TIM-barrel structure common to 36 kDa 1,3- β -glucanases [4]. However, no data are available on the structure and function of the remaining C-terminal segment, which consists of around 100 residues and could constitute a defined domain.

Abbreviations used: conA, concanavalin A; CtD, C-terminal domain of Ole e 9; rCtD, recombinant CtD; TFA, trifluoroacetic acid.

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Ole e 9 is an appropriate model to analyse structural and functional properties of this portion of 1,3- β -glucanases. The allergen contains seven cysteine residues, six of which are located at the C-terminal segment. In addition, Ole e 9 possesses two putative N-glycosylation sites, located at positions 355 and 447, and at least one of them should be occupied by a glycan moiety, since the protein is reactive to concanavalin A (conA) lectin [4]. The application of molecular cloning techniques for the recombinant production of proteins of difficult accessibility may facilitate the availability of whole molecules and their domains. Recombinant expression in *Pichia pastoris* has proved to be a successful system for proteins containing cysteine residues involved in disulphide bridges [16,17].

In this work, we report on the expression in the yeast *P. pastoris* of a polypeptide containing the 101 amino acid residues of the C-terminal domain of Ole e 9 (CtD). Characterization of its chemical, spectroscopic and immunological properties, as well as the arrangement of its disulphide bridges, are also reported.

EXPERIMENTAL

Materials

Escherichia coli strains INV α F' and TG1 were used as hosts for plasmids pCR2.1 and pPICZ α -A (both from Invitrogen, Carlsbad, CA, U.S.A.), respectively. *P. pastoris* KM71 yeast cells (Invitrogen) were used as hosts for transformation by electroporation with the plasmid pPICZ α -A. Sera from hypersensitive individuals, which exhibited positive reaction to Ole e 9 in ELISA, were selected from a population of patients with a clinical history of allergy to olive pollen and positive skin-prick and radioallergosorbent tests (classes 3–6) to olive pollen extract.

Cloning of CtD in the expression vector

The cDNA encoding CtD (GenBank™/EBI database accession number AF249675 [4]) containing positions 360–460 was amplified by PCR using Ole e 9-specific DNA as a template, two non-degenerate primers (sense primer OL9TR-NT1, 5'-cgt-ctcgagaaaagaGCAACACCAACCCCAACA-3', and antisense primer OL9TR-CT1, 5'-ctgcgcgcgcTCAGTTACTGCCACTGGAAA-3'; upper-case sequence codes for the N-terminus of CtD) and the *Taq*Gold DNA polymerase (Applied Biosystems-PE, Branchburg, NJ, U.S.A.). The sense primer OL9TR-NT1 contains a *Xho*I restriction site (underlined above), a sequence that allows fusion of the CtD encoding region in-frame with the preprosequence of the α -mating factor present in pPICZ α -A and the sequence corresponding to amino acids at positions 360–365 of Ole e 9 (ATPTPT). The antisense primer contains a *Not*I restriction site (underlined above), a stop codon and the sequence corresponding to the last six residues of Ole e 9 (FPSGSN). This CtD was amplified by PCR using a programme with five cycles of denaturation at 94 °C (60 s), annealing at 47 °C (60 s) and chain extension at 72 °C (90 s) and 25 cycles at 94 °C (60 s), 55 °C (90 s) and 72 °C (90 s). The fragment was purified using the Magic PCR Prep Kit (Promega, Madison, WI, U.S.A.) and directly cloned into a linearized pCR2.1 vector in order to transform INV α F' *E. coli* cells. Several clones were sequenced to confirm the correct sequence. This construction was digested with *Xho*I/*Not*I restriction enzymes and the released fragment was subcloned into the same sites of the pPICZ α -A vector and used to transform TG1 *E. coli* cells to obtain the construction pPICZ α -A/CtD.

Transformation of *P. pastoris* KM71, production and purification of recombinant CtD (rCtD)

pPICZ α -A/CtD recombinant plasmid (10 μ g) was linearized with *Sac*I restriction enzyme and the product was used to electroporate competent KM71 yeast cells. Plates with yeast/peptone/dextrose medium containing 1 M sorbitol and 100 μ g/ml zeocin were plated out for selection of recombinant clones. For the production of rCtD, transformed colonies were processed as reported previously [17]. The extracellular culture medium of induced KM71 cells was cleared by centrifugation at 3000 g at 4 °C. The presence of rCtD in the supernatant of the culture medium was analysed by SDS/PAGE; samples being taken at different times. Large-scale production of rCtD was performed in buffered minimal methanol medium supplemented with histidine using the colony that rendered the best yield in the low-scale experiments. The extracellular medium obtained after centrifugation of the yeast cells was dialysed against 20 mM ammonium bicarbonate, pH 8.0, and lyophilized. The sample was dissolved in 0.2 M ammonium bicarbonate, pH 8.0, and applied to a Sephadex G-50 column. Eluted fractions were tested for protein with Coomassie Blue staining and for IgE reactivity with a pool of allergic sera by Western blotting, and those containing the rCtD were further purified by reversed-phase HPLC on a Nucleosil C₁₈ column with a gradient (0–60 %) of acetonitrile in 0.1 % trifluoroacetic acid (TFA).

SDS/PAGE and analytical procedures

SDS/PAGE was carried out as described by Laemmli [18] in 15 % polyacrylamide gels. Proteins were stained with Coomassie Blue or electrophoretically transferred on to nitrocellulose membranes. Amino acid composition was determined on a Beckman System 6300 amino acid analyser after hydrolysis at 105 °C with 5.7 M HCl for 24 h. Cysteine content was determined as cysteic acid after performic acid oxidation. Tryptophan and tyrosine were detected spectrophotometrically in peptides before acid hydrolysis. The concentration of purified protein samples was determined from absorbance measurement at 280 nm, using a specific absorption coefficient, $A^{0.1\%}$, of 1.72. The N-terminal amino acid sequence was determined by Edman degradation using an Applied Biosystems model 477A sequencer. MS analysis was carried out on a Bruker Reflex II matrix-assisted laser-desorption ionization time-of-flight mass spectrometer as described in [17].

Carbohydrate detection

Carbohydrate detection of protein transferred to nitrocellulose membranes was performed as described previously [19] by using a biotinylated conA solution. The staining was developed by horseradish peroxidase reaction with 0.05 % diaminobenzidine/0.03 % of 30 % H₂O₂ in Tris/NaCl reaction buffer.

Spectroscopic analyses

CD spectra were obtained on a Jasco J-715 spectropolarimeter as described previously [17] with minor modifications. The protein concentrations were 0.15 and 0.6 mg/ml, respectively, for the analyses in the far- and near-UV, in 20 mM sodium phosphate buffer, pH 7.0. Mean residue mass ellipticity was calculated based on 105 as the average molecular mass/residue, obtained from the amino acid composition, and expressed in terms of mean residue weight ellipticity, $[\theta]_{\text{MWR}}$ (degree $\cdot$ cm² $\cdot$ dmol⁻¹).

Proteolytic treatments and peptide separation

Thermolysin digestion of rCtD was performed according to a previous report [20] with minor modifications. Purified rCtD (200 μ g) was dissolved in 0.1 M ammonium acetate buffer, pH 6.5, containing 10 mM CaCl_2 . Thermolysin was added to this solution, at an enzyme/substrate ratio of 1:20 (w/w) and the digestion was carried out for 16 h at 37 °C. The mixture of peptides was separated by reversed-phase HPLC on a Nucleosil C_{18} column with an acetonitrile gradient in 0.1% TFA.

Limited proteolysis of Ole e 9 was performed in 0.1 M ammonium bicarbonate with sequence-grade tosylphenylalanine-chloromethane-treated trypsin at an enzyme/substrate ratio of 1:250 (w/w) at 25 °C. The reaction was stopped by adding SDS/PAGE electrophoresis sample solution and frozen in liquid nitrogen until analysis. Aliquots at different times of digestion (0, 15, 60 and 180 min) were analysed by SDS/PAGE.

Specific IgE quantification, ELISA inhibition and Western blotting

IgE quantification was performed by ELISA in microtitre plates coated with 100 μ l of protein/well (1 μ g/ml), according to Tejera et al. [21]. Plates were incubated with individual olive pollen allergic sera (diluted 1:10). Binding of human IgE was detected by mouse anti-human IgE antibodies (kindly donated by Dr M. Lombardero, ALK-Abelló, Madrid, Spain) diluted 1:5000, followed by horseradish peroxidase-labelled goat anti-mouse IgG (Pierce, Rockford, IL, U.S.A.) diluted 1:5000. Peroxidase reaction was developed using fresh enzyme substrate [21] and measuring absorbance at 492 nm. Each absorbance value was calculated as the mean of two determinations. Absorbance values under 0.1 were considered to be negative responses.

ELISA inhibition assays were performed as described in [17]. After coating with 100 μ l of antigen (1 μ g/ml), the plates were incubated with the pool of allergic sera (diluted 1:10) previously incubated with different concentrations of the inhibitor (0.1–10 000 ng). This step was followed by a treatment with mouse anti-human IgE and horseradish peroxidase-labelled goat anti-mouse IgG.

For immunoblotting, proteins transferred to nitrocellulose membranes were immunostained as described in [22]. Briefly, membranes were incubated with sera from patients allergic to olive pollen (diluted 1:10) followed by reaction with mouse anti-human IgE, diluted 1:5000. The negative control was achieved using sera from patients allergic to the major allergen from mustard seeds. Afterwards, blots were incubated with horseradish peroxidase-labelled goat anti-mouse IgG, diluted 1:5000. The peroxidase reaction was developed using the ECL Western blotting reagent (Amersham Biosciences, Little Chalfont, Bucks., U.K.) as described in [22].

For inhibition Western blotting assay, two identical aliquots of protein sample obtained after 180 min of tryptic digestion of Ole e 9 in limited conditions were submitted to SDS/PAGE and transferred electrophoretically to nitrocellulose membranes. The IgE binding of one of these blotted samples was analysed after being treated with a pool of allergic sera (diluted 1:10) previously incubated with 10 μ g of rCtD as an inhibitor for 2 h at 25 °C. The second transferred sample was used as a positive control for IgE binding, as it was treated with the allergic sera pool without preincubation with the inhibitor. In order to detect the IgE response, the membranes were treated as described for the immunoblotting assay: first with mouse anti-human IgE (diluted 1:5000) and then with horseradish-peroxidase labelled goat anti-mouse IgG (diluted 1:5000). The signal was developed using the ECL Western blotting reagent [22].

RESULTS

Expression in *P. pastoris* and isolation of rCtD

The cDNA encoding CtD was amplified by PCR using two non-degenerate primers that are described above. The resulting DNA fragment was cloned downstream of the AOX1 promoter and in-frame of the α -factor secretion sequence. This construction, pPICZ α -A/CtD, was used for transforming KM71 yeast cells. rCtD was obtained as an extracellular secretion protein.

The time course of the production of rCtD was followed by SDS/PAGE analysis of the extracellular medium of the culture (Figure 1). A major band with an apparent molecular mass of 13 kDa appeared after 24 h of induction and its level was maximum after 96 h. At this time, aggregation or degradation products were not observed by immunostaining of electrophoresed protein transferred to membranes with a pool of sera specific to Ole e 9 (results not shown). After small-scale expression, the best-producing colony was selected for large-scale isolation of the protein. The extracellular medium of the culture was exhaustively dialysed to remove small metabolic contaminants from the yeast and then lyophilized. To purify the recombinant protein, gel-filtration chromatography on a Sephadex G-50 column was performed (Figure 2a). Fractions containing the recombinant product were lyophilized and chromatographed in a reversed-phase HPLC C_{18} column (Figure 2b). The efficiency of this procedure was analysed by SDS/PAGE (Figure 2a, inset). The final yield of the purified protein was approx. 100 mg/l of cell culture, and it displays an apparent molecular mass of 13 kDa, as determined by SDS/PAGE (Figure 2b, inset).

Molecular characterization of rCtD

Edman degradation of the N-terminal end of rCtD gave the sequence ATPTP, which indicates the correct processing of

Figure 1 Time course of the expression of rCtD in *P. pastoris*

Supernatants from cultures were harvested at different times and analysed by Coomassie Blue staining after SDS/PAGE (15% polyacrylamide gel). M, molecular mass markers. The arrow indicates the mobility of rCtD.

Figure 2 Purification of rCtD

(a) Elution profile of gel-filtration chromatography on a Sephadex G-50 column (5 cm × 60 cm) of the extracellular medium after exhaustive dialysis. Inset, SDS/PAGE analysis and Coomassie Blue staining of different fractions. M, molecular mass markers. Fractions containing rCtD (horizontal line) were pooled and subjected to the following step. (b) Elution profile of rCtD on a reversed-phase HPLC Nucleosil C₁₈ column using an acetonitrile gradient in 0.1% TFA. Inset, SDS/PAGE and Coomassie Blue staining of the purified protein (peak underlined). Molecular mass markers are shown.

Figure 3 MS analysis of purified rCtD and glycan detection

Inset, sugar staining of electrophoresed and blotted rCtD (1 µg) with biotinylated conA. Molecular mass markers are shown.

the α -mating signal peptide used for the secretion of the product to the extracellular medium of the yeast. The amino acid composition of rCtD (results not shown) fits well with that deduced from the cDNA clone.

MS analysis of rCtD was carried out, rendering a heterogeneous peak with four maxima around 11.5 kDa (values of 10919, 11250, 11569 and 11727; Figure 3). The molecular mass deduced from the sequence of the clone was 10584 Da (= 10590 Da – 6 Da, removed at the disulphide bridge formation). The difference observed between theoretical and experimental data can be attributed to the presence of heterogeneous carbohydrates in rCtD, which should be inserted during the endogenous maturation of the recombinant protein (the polypeptide possesses a potential N-glycosylation site). This possibility was analysed by reaction of rCtD transferred to nitrocellulose membrane with biotinylated conA lectin. The positive response (Figure 3, inset) showed that rCtD is a glycoprotein. MS spectrum could be explained in terms of a polymorphic glycosylation of rCtD. The difference between pairs of the five major species was approx. 330 or 160 Da, which could correspond to 2 or 1 mannose residues, respectively. Thus MS data for rCtD would agree with the presence of 2, 4 or 5 mannose residues linked to the (GlcNAc)₂ core.

CD in the far-UV range (190–250 nm) tells us about the secondary structure of polypeptide chains, thus giving a measure of the folding of the molecule. The CD spectrum of rCtD (Figure 4a) fits well to that of a theoretical protein containing 16% α -helix, 30% β -sheet, 17% β -turns and 37% non-regular conformation. Figure 4(b) shows the near-UV CD spectrum (245–315 nm) of rCtD with bands around 275 and 290 nm, which proves the asymmetry of the local environment of its aromatic residues, thus supporting the existence of tertiary structure in the recombinant polypeptide.

Figure 4 CD spectra of rCtD

(a) Far-UV (190–250 nm). (b) Near-UV (245–315 nm). The protein was dissolved in 20 mM phosphate buffer, pH 7.0. $[\theta]_{\text{MWR}}$, mean residue weight ellipticities.

Figure 5 Assignment of the disulphide bonds of rCtD

(a) Reversed-phase HPLC elution profile of the thermolytic digestion of rCtD using a gradient of acetonitrile in 0.1% TFA. Numbered peaks were collected and analysed. * indicates cystine-containing peptides. (b) Complete amino acid sequence of rCtD produced in *P. pastoris*. Horizontal lines indicate the locations of the numbered peaks separated in (a), and arrows show the peptides that were sequenced. Cysteine residues are shown in circles. (c) Topological representation of the disulphide bond pattern of rCtD; a solid circle indicates a half cystine residue at that position.

Figure 6 IgE-binding capability of rCtD

Immunodetection with 20 individual sera (1–20) from patients allergic to olive pollen with positive response to Ole e 9 was carried out after SDS/PAGE (15% polyacrylamide gel) and transfer to membranes; M, molecular mass markers. The numbers below indicate the IgE response of each serum (S) to rCtD by ELISA; values are from measurements of absorbance ($^{\circ}$ OD) at 492 nm. C, negative control.

Disulphide bridge arrangement

Thermolytic digestion of rCtD was performed under native conditions to preserve the natural disulphide bridges. A total of 17 major peptides were separated by HPLC in a reversed-phase C_{18} column and analysed further (Figure 5a). The acid hydrolysis and automatic amino acid quantification of these peptides allowed us to assign 95 out of the 101 residues of the molecule (Figure 5b). The four cystine-containing peptides (numbers 13, 15, 16 and 17), determined after oxidizing treatment, were subjected to Edman degradation. Peptides 15 and 16 corresponded to the same region of rCtD; this can be explained because of partial cleavage by thermolysin. These results provide an unambiguous determination of the disulphide arrangement of the cystines, which are bound as follows: Cys-14–Cys-76, Cys-33–Cys-94 and Cys-39–Cys-48 (Figure 5c).

Immunological properties

Ole e 9 is a highly prevalent allergen from olive pollen. Thus, in a hypersensitive population, it induces the synthesis of specific IgE antibodies able to bind the natural allergen. In order to analyse the response of these Ole e 9-specific antibodies from sera of allergic patients against the rCtD, ELISA detection, immunoblotting and ELISA inhibition studies of IgE binding were carried out. All olive allergic sera positive to Ole e 9 ($n = 40$) were found to be reactive to rCtD in ELISA (results not shown). This suggested that rCtD was folded correctly. Out of these sera, 20, whose IgE quantification by ELISA are shown in Figure 6, were also analysed by immunoblotting for their individual response in the presence of SDS. Although several sera seem to lose IgE-binding capability in SDS/PAGE to some extent, mainly those with a low IgE response in the soluble phase (Figure 6), these results point to the existence of a correlation between the IgE reactivities of rCtD in the presence and absence of the denaturing agent.

In order to confirm that the rCtD has a similar conformation to that of natural CtD, limited proteolysis of the whole allergen and immunoblotting inhibition assay of the products was performed. A partially purified preparation of Ole e 9 was digested

Figure 7 SDS/PAGE analysis of the limited proteolysis of Ole e 9 and inhibition assay with rCtD

(a) Ole e 9 (15 μ g) was treated with trypsin under mild conditions; aliquots (3 μ g) were taken at different lengths of time (0, 15, 60 and 180 min) and analysed by SDS/PAGE in 15% polyacrylamide gel; CBS, Coomassie Blue staining is shown; M, molecular mass markers. (b) Samples (1.5 μ g) after 180 min of treatment were transferred to a nitrocellulose membrane and immunostained with a pool of sera containing Ole e 9-specific IgE, which was previously preincubated with (+) or without (–) 10 μ g of rCtD as inhibitor (Inh). Mobilities of Ole e 9, N-terminal domain (NtD) and C-terminal domain (CtD) are indicated with arrows.

Figure 8 ELISA inhibition assays

IgE binding of a pool of Ole e 9-allergic sera to Ole e 9- and rCtD-coated wells (0.1 μ g of protein/well) was assayed using both proteins as inhibitors.

with trypsin (lysines are located in the amino acid sequence of Ole e 9 at positions 8 and –9 from the first alanine of rCtD [4]). Restricted conditions of proteolysis resulted in the appearance of two bands of 36 and 10.5 kDa, which should correspond to the N- and C-terminal domains, respectively. The time course of

the proteolysis was analysed by SDS/PAGE (Figure 7a). This supports the fact that native Ole e 9 consists of two independent domains. We found that 19% of the whole protein (determined by scanning densitometry) remained undigested after 180 min of treatment. Immunostaining of this sample transferred to membranes with a pool of Ole e 9-reactive sera demonstrated IgE-binding capability for the separate domains, as well as the high reactivity of the complete Ole e 9 (Figure 7b). The inhibition assay, which was performed with the pool of allergic sera preincubated with rCtD, resulted in a complete inhibition of IgE binding to the protein band of 10.5 kDa, as well as abolition of the signal at 46 kDa. This demonstrates the presence of IgE epitopes of Ole e 9 within the structure of rCtD, and the similarity of the natural CtD and rCtD conformations.

ELISA inhibition studies were achieved to quantify the contribution of rCtD to the allergenicity of the whole Ole e 9 allergen. When Ole e 9 was fixed to plate wells and rCtD used as an inhibitor, around than 60% of inhibition was reached (Figure 8). When rCtD was fixed to wells and Ole e 9 used as an inhibitor, a 90% of inhibition was reached, indicating that Ole e 9 is able to block almost completely the IgE epitopes present in the rCtD and confirming that they share allergenic epitopes.

DISCUSSION

The olive allergen Ole e 9 (46 kDa) has recently been purified from pollen and its 1,3- β -glucanase activity demonstrated [4]. Alignment of its amino acid sequence with those of other 1,3- β -glucanases contained in databases hinted at the existence of two well-defined domains in the three-dimensional structure of the allergen: an N-terminal portion (around 340 residues), which is common to all known 1,3- β -glucanases, including the shortest ones (33–36 kDa), and a C-terminal domain of around 100 amino acids, which is only present into the larger 1,3- β -glucanases (45–46 kDa). The former contains the catalytic site, but structure and function of the latter is so far unknown. In this work, we proved that Ole e 9 consists of two independent domains, which can be separated by mild proteolysis of the whole allergen, and that both are immunologically active. A precise analysis of the C-terminal domain has been feasible because we have achieved the recombinant expression in *P. pastoris* of the cDNA encoding this segment of Ole e 9, which comprises positions 360–460 of the open reading frame of the whole protein (334–434 of the mature protein).

P. pastoris has been used successfully for the recombinant expression of foreign proteins. The yield of product in this methylotrophic yeast is usually high and the maturation mechanisms involve disulphide-bond formation and N-glycosylation processes. In addition, steps in the purification of recombinant proteins can be reduced to a minimum because they are frequently secreted into the extracellular medium, which is poor in endogenous proteins, and it thus constitutes a solution that is highly enriched with the expression product. CtD has been produced as a completely soluble monomer with a very high yield. This suggests proper folding of its tertiary structure, because otherwise it would aggregate and be insoluble due to its high content of Cys residues.

rCtD was glycosylated, as deduced from its reaction with conA. The heterogeneous profile of the mass spectrum of rCtD is due to the polymorphic nature of the glycan moiety. *P. pastoris* is able to incorporate oligosaccharides at Asn residues involved in consensus sequences of N-glycosylation: Asn-Xaa-Ser/Thr. Thus the Asn-88 of the amino acid sequence of rCtD, which is the single putative site of N-glycosylation, might be occupied by

a conA-reactive glycan. Interestingly, this glycan component is notably shorter than those introduced during the maturation process of recombinant molecules in the yeast *P. pastoris*, which always contain 8–13 mannose residues [23,24]. Glycosylation of rCtD can also explain its higher apparent molecular mass (13 kDa) in comparison with that of the corresponding protein band (10.5 kDa) obtained by limited proteolysis, although the possibility cannot be ignored that natural CtD released by trypsin could be shorter than rCtD if the enzyme is cleaved at Lys-8 of the equivalent recombinant protein (see Figure 5c).

1,3- β -Glucanases are group 2 pathogenesis-related-like proteins, which belong to the superfamily of glycosyl hydrolases (EC 3.2.1), and are found widely in the three major kingdoms (eukaryotes, eubacteria and archaeobacteria). Both types of 1,3- β -glucanase (short and long), including Ole e 9 [4], show significant sequence similarities in the 320 or 360 amino acid residues of their N-terminal portions. An eight-stranded β/α barrel framework has been reported for this segment of 1,3- β -glucanases, on the basis of that described in barley [11], which is also common to many families of glycosyl hydrolases [25,26]. TIM-barrel structures have been found in a wide diversity of proteins, most of which are enzymes with very different functions including isomerization of small sugar molecules and degradation of sugar polymers [11,25,27]. The polypeptide chain of some of these proteins comprises, in addition to the barrel core in which usually resides the catalytic activity, one or more extra domains. In some cases, these complementary domains come close to the active site and can participate in substrate binding or catalysis as a regulatory module, as well as establishing contact between subunits and with membranes. CtD constitutes an independent domain, as demonstrated in this work, and could play a role in the biological function of the whole protein. Although Ole e 9 conserves the catalytic tetrad, its specific activity as a 1,3- β -glucanase is significantly lower than that of short-type glucanases [4]. Thus the CtD could be involved in a regulatory function. On the other hand, homology searches of the CtD amino acid sequence allowed us to find significant similarities with polypeptide segments (at around 360–460) of several yeast protein families encoded by the genes *GAS* (glycophospholipid-anchored surface) from *Saccharomyces cerevisiae*, *PHR* (pH-regulated) from *Candida albicans* and *EPD* (essential for pseudohyphal development) from *Candida maltosa*, whose expression is required for correct morphogenesis in yeast [28–31]. Popolo and Vai [32] have proposed the organization of Gelp family proteins and their sequentially related *GAS*, *PHR* and *EPD* gene products [33,34], into three domains, one of which contains highly conserved cysteine residues (Cys Box domain) and is the segment that was found to align with CtD. Amino acid sequence identities (and similarities) between CtD and the Cys Box from these gene products were 29% (45%), 29% (44%) and 26% (45%), respectively, and cysteine residues were located at equivalent positions. Popolo and Vai [32] speculate that the Cys Box is involved in binding ions and could be required for protein–protein interactions, for regulation of catalytic activity or for modulating the interaction or accessibility of the protein to its substrate. *GEL1*, *PHR1* and *GAS1* gene products display 1,3- β -glucanosyltransferase activity responsible for elongation of 1,3- β -glucan chains [33–35]. As mentioned above, the recombinant expression of CtD in *P. pastoris* produced an atypical glycosylated molecule. This fact could be related to a putative role of CtD within the glycosylation pathway or glycan metabolism of cells from higher organisms. Availability of rCtD can help to analyse the biochemical role of this domain of Ole e 9, not only as a part of the 1,3- β -glucanase enzyme, but as an independent module of a wide variety of proteins. Our results

could help the design and production of recombinant homologous molecules and chimaeras in order to allow functional studies.

On the other hand, the three-dimensional structure of Ole e 9 cannot be solved because it was obtained from pollen at very low yield [4], and has not been produced by recombinant technology. The independent production of both domains as pure and functional products can facilitate the analysis of its conformation. As there is a direct relationship between sequence and folding similarities [36], and the primary structure of CtD displays similarity not only with 1,3- β -glucanases but with 1,3- β -glucan-*osyl*transferases, the global folding of this domain could be assumed for a wider collection of polypeptide blocks. The elucidation of the disulphide pattern arrangement reported in this work could help to make out the spatial conformation of this domain of Ole e 9 and to establish possible architectural correlation with polypeptide segments possessing comparable sequences, despite different biochemical activities.

Ole e 9 is a significant allergen from olive pollen and thus it is a good candidate to be included in mixtures of pure allergens to be used in protocols for diagnosis and specific immunotherapy of olive pollen allergy. However, it is not yet possible to supply high amounts of the natural allergen. The CtD retains more than 50 % of the IgE-binding capability of the complete allergen and is recognized by all sera from patients allergic to Ole e 9. It constitutes a quarter of the whole protein, but its structural independence makes its high allergenicity feasible. This molecule seems to be an adequate substitute for the whole allergen for clinical purposes, because of the high-level production and facility of management. Moreover, its IgE reactivity could be more easily decreased by molecular engineering than that of the whole Ole e 9. Furthermore, the ubiquity of 1,3- β -glucanases in plant tissues suggests the possibility of these proteins being involved in IgE cross-reactivity between pollens, fruits and latex [4]. In this sense, latex of the rubber tree (*Hevea brasiliensis*) has been found to contain 1,3- β -glucanases able to induce allergy, and are strongly recognized by sera from patients allergic to tomato, potato and banana [37]. The availability of allergenic modules from these proteins will facilitate immunological and structural relationships studies between them.

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