Purification, amino acid sequence and immunological characterization of Ole e 6, a cysteine-enriched allergen from olive tree pollen

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Abstract The Ole e 6 allergen from olive tree pollen has been isolated by combining gel permeation and reverse-phase chromatographies. It is a single and highly acidic (p*I* 4.2) polypeptide chain protein. Its NH_2-terminal amino acid sequence has been determined by Edman degradation. Total RNA from the olive tree pollen was isolated, and a specific cDNA was amplified by the polymerase chain reaction using a degenerate oligonucleotide primer designed according to the NH_2-terminal sequence of the protein. The nucleotide sequencing of the cDNA rendered an open reading frame encoding a 50 amino acid polypeptide chain, in which two sets of the sequential motif Cys-X_3-Cys-X_3-Cys are present. No sequence similarity has been found between this protein and other previously described polypeptides.

Key words: Allergen; Olive pollen; Ole e 6; Amino acid sequence

1. Introduction

An increasing proportion of the population of developed countries is affected by type-I allergy, suffering from respiratory, skin and intestinal disorders. Although the molecular basis of these IgE-mediated diseases are not well explained, their diagnosis and treatment require the preparation of large amounts of pure and well defined allergens, which can be mainly provided by means of cDNA cloning and expression.

Anemophilous pollens are responsible for one of the most frequent type-I allergy, the pollinosis, and in particular, the olive tree (*Olea europaea*) pollen is one of the main causes of pollinosis in countries of the Mediterranean area [1]. The major allergen present in this pollen, Ole e 1, is a protein of 20 kDa molecular mass, which has been cloned and expressed in *E. coli* [2,3]. It consists of a single 145 amino acid polypeptide chain and contains a N-linked glycosyl moiety [4]. Besides Ole e 1, several IgE-binding proteins have been detected in the saline extract of this pollen [5–7], and some of them have been purified and characterized [8,9]. To improve the diagnosis and therapy of this allergy, as well as to explain the observed cross-reactivities with other allergenic sources, mainly pollens and fruits, the analysis of the other IgE-binding proteins from olive tree pollen is required. In addition, the study of these proteins is necessary in understanding structure-allergenicity relationship. In this paper, we describe the isolation and characterization of a novel olive tree pollen allergen. Isolation and sequence of a cDNA clone encoding this allergen is also reported.

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2. Materials and methods

Pollen extract was prepared as previously described [8]. The lyophilized saline extract was dissolved in 0.2 M ammonium bicarbonate and subjected to gel filtration on a Sephadex G-75 column. IgE-binding activity of the fractions was assessed by using sera from patients allergic to olive tree pollen, by immunoblot analysis [8]. Active fractions were chromatographed on a Sephadex G-50 superfine column equilibrated in 0.2 M ammonium bicarbonate, and afterwards on a nucleosil C_{18} column (reverse-phase high performance liquid chromatography; RP-HPLC). An acetonitrile gradient (15–55%) in 0.1% trifluoroacetic acid was employed for the elution of the samples. Absorbance was measured at 214 nm, and the above immunoblot analysis was used to detect the allergenic protein.

NH_2-terminal Edman degradation of both native and alkylated protein were performed on an Applied Biosystems model 477A sequencer. The phenylthiohydantoin-amino acid derivatives were analysed by using a 120 A on-line analyzer and the standard Applied Biosystems program. SDS-PAGE was performed according to Laemmli [10] in 15% polyacrylamide gels. Molecular weight estimations were done according to Weber and Osborn [11] by using protein markers provided by Sigma (MW-SDS-70L). Alternatively, the samples were analysed after reduction at 80°C for 20 min. Isoelectrofocussing (IEF) was performed in a 3–9 pH gradient, with a 5% polyacrylamide gel containing Pharmalyte carrier ampholytes as described [8].

Sera from untreated donors with positive skin and radioallergosorbent tests (RAST) class 4–6 to *Olea europaea* allergen extract were used for immunoassays. Polyclonal antiserum against the purified antigen was prepared by immunizing New Zealand rabbit over a 8-week period by multidermal injections of the protein (50 μg) [12].

Protein bands in SDS-PAGE were transferred onto nitrocellulose membranes according to [13]. Immunoblot analyses were performed as described in [8], with minor modifications. Human sera were used diluted 10-fold, and the rabbit polyclonal antibody 5000-fold. The signal was developed by the ECL-Western-blotting reagent (Amersham). IgE-binding measurements were performed by densitometric readings of the immunoblotting analyses. ELISA titration was performed as described [8]. After coated with 100 μl antigen (1 μg/ml), the plates were incubated with the human sera diluted 10-fold followed by mouse anti-human IgE and horseradish-peroxidase-labelled goat anti-mouse IgG as described [8]. Alternatively, the antigen-coated wells were incubated with different dilutions of the rabbit polyclonal antibody obtained against the purified allergen, followed by horseradish peroxidase-labelled anti-rabbit IgG.

Total RNA from olive tree pollen was extracted as described [3]. Single-stranded cDNA was synthesized from total RNA by using a cDNA synthesis kit (Pharmacia Biotech) according to the manufacturer's instructions and using an oligo dT-adaptor as primer: 5′-GAGAATTCGGATCCATCGA$(T)_{17}$-3′. The specific oligonucleotide primer (OL4-1) was designed based on the NH_2-terminal amino acid sequence of the isolated protein, positions 1–8 (5′-CGGGATCC-GAYGARGCNCARTTYAARGARTG-3′), and containing a *Bam*HI restriction site. The cDNA template and 50 pmol of the primers, OL4-1 and adaptor, were denatured at 95°C for 15 min in a PCR mixture. After adding 2.5 U DynaZyme DNA polymerase (Finnzymes O*y*), the sample was subjected to PCR amplification following the procedure described by Villalba et al. [3]. Under these conditions, a single PCR product of about 440 bp was obtained. The fragment was purified by using the Magic PCR Prep kit (Promega), and digested with *Bam*HI endonuclease. The fragment was incorporated into a dephosphorylated *Bam*HI-digested pUC19 plasmid vector, and used

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for transformation of competent *E. coli* DH5αF′ cells, by standard procedures. pUC19 plasmid minipreps were used as template for sequencing, which was achieved by using the dideoxy chain termination method [14] and deoxy[α-^{35}S]ATP with the Sequenase 2.0 kit (U.S. Biochemical). Sequencing primers for both strands, M13mp18 universal and reverse, were from New England Biolabs. Sequencing was carried out according to the manufacturer's recommendations.

3. Results

3.1. Isolation and characterization of the antigen

The pure protein was obtained after three chromatographic steps: two size exclusion chromatographies on Sephadex G-75 and G-50 superfine, and a reverse-phase HPLC C_{18} column (Fig. 1). The analysis of the different elution profiles was performed by immunoblotting after SDS-PAGE. A pool of sera from patients allergic to olive tree pollen lacking reactivity against Ole e 1 was used for such a purpose. The isolated protein was homogeneous by SDS-PAGE, IEF and NH_2-terminal amino acid sequencing. It exhibits an apparent molecular mass of 10 kDa by SDS-PAGE (Fig. 2). IEF analysis of this protein renders an acidic p*I* of 4.2 (data not shown). The NH_2-terminal amino acid sequence, obtained by means of the Edman degradation, is DEAQFKECYDTCHKECSDKGNGFT, this result being identical for three different protein preparations. No microheterogeneity was detected at any of the 24 determined residues.

3.2. Antigenic activity

Immunoblotting analysis with 255 individual sera from patients allergic to olive tree pollen revealed that 40 of them contained IgE able to bind the purified protein. Although the obtained prevalence was around 15%, however differences are found depending on the geographic origin of the sera. Ten percent of the sera collected at the area of Madrid were positive, while this value increases up to 55% at the area of Córdoba (Andalucía). These facts correlates the major allergenic character of the purified protein with intensive olive tree culture. Most of the sera (90%) from patients allergic to olive pollen, which recognized the purified protein, also exhibited IgE-binding capability to other olive pollen proteins, such as Ole e 1 or profilin. For about 30% of them, the IgE antibodies specific to the isolated protein represented more than 40% of the total IgE response. We designated this protein as Ole e 6, in accordance with the approved nomenclature system of the International Union of Immunological Societies [15].

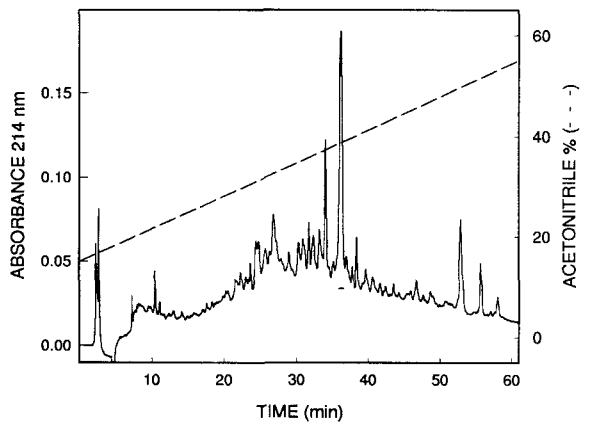

Fig. 1. Elution profile of the chromatography on a RP-HPLC nucleosil C-18 column by using a gradient (15–55%) of acetonitrile. The fractions containing the Ole e 6 allergen are denoted by an horizontal bar.

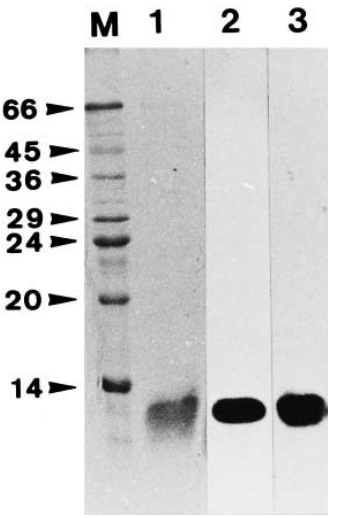

Fig. 2. SDS/PAGE analysis of Ole e 6. Lanes: 1, Coomassie blue staining of the 15% polyacrylamide gels; 2, IgE-binding analysis with a pool of sera of patients allergic to olive pollen; 3, IgG-binding analysis with a polyclonal antiserum raised against purified Ole e 6.

A pool of the above positive sera was used for the antigenic characterization of Ole e 6 in immunoblotting (Fig. 2). The IgE-binding affinity of the allergen was measured by ELISA titration by using the same pool of sera. The obtained results are shown in Fig. 3a, in comparison with the response of a pool of sera obtained from non-allergic individuals.

A specific polyclonal antibody was obtained by rabbit immunization with Ole e 6. This was used to analyze the IgG-binding response of the purified allergen by both immunoblotting (Fig. 2) and indirect ELISA titration (Fig. 3b). The allergen Ole e 1 was used as a negative control.

3.3. cDNA cloning and nucleotide sequence of Ole e 6

Total RNA was obtained from olive tree pollen and the single-stranded cDNA synthesized. A DNA fragment encoding this protein was amplified by means of the PCR, using a degenerate oligonucleotide primer, designed according to the

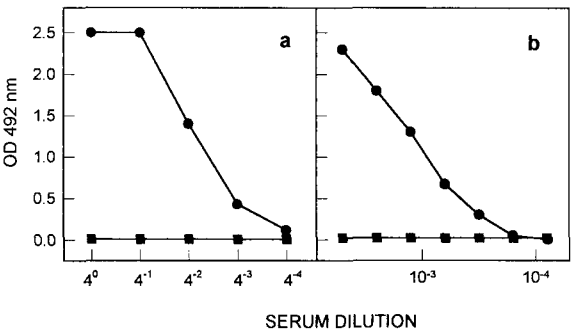

Fig. 3. ELISA titration of Ole e 6. (a) IgE-binding to purified Ole e 6 with a pool of sera from patients allergic to olive pollen (●), in comparison to the response obtained with the sera from non-allergic individuals (■). (b) IgG-binding analysis of Ole e 6 (●) with a specific rabbit polyclonal antiserum; Ole e 1 (■) was used as a negative control.

NH_2-terminal sequence of the allergen, and the adaptor primer that hybridized with the sequence linked to the oligodT primer used in the cDNA synthesis. A PCR fragment of about 340 bp was isolated and sequenced. Fig. 4 shows both the nucleotide sequence and the deduced amino acid sequence. It corresponds to a 50 residues polypeptide chain. Because of the small size of the deduced sequence compared to the apparent molecular mass of the purified protein, analysis of three different PCR amplifications were performed in order to confirm the stop codon position. They rendered identical nucleotide sequences.

4. Discussion

Pollens are common sources of allergy and frequently contain several allergenic proteins. A number of allergens have been detected and characterized from olive tree pollen extracts [2,3,8,9]. Now, the Ole e 6 allergen has been isolated. The protein consists of a single polypeptide chain of 50 amino acids, highly polar (22 charged residues). No evidence of different isoforms of Ole e 6 has been obtained from both the isolated protein (IEF, NH_2-terminal Edman degradation, HPLC-rechromatography) and nucleotide sequence analysis (different PCR amplifications from different cDNA preparations rendered identical deduced amino acid sequences). It is remarkable the presence of 6 Cys residues (12%) arranged in two sets of the sequential motif Cys-X_3-Cys-X_3-Cys, which may suggest a novel conformational element. The repetition of this Cys-enriched sequence motif could arise from a duplication process since the segments comprised between the positions 5–19 and 23–37 of the polypeptide chain display 40% sequence identity.

The molecular mass of the protein, calculated from the amino acid sequence, is 5830 Da, while the value obtained by SDS-PAGE is 10 kDa. The result of this analysis is strongly dependent on the conformation of the molecule for molecular mass below 15 kDa, and non-globular proteins give anomalous results. In addition, Ole e 6 is a small acidic protein and the binding of the negatively charged SDS molecules could be defective. The protein could also bear a molecular group covalently bound to the polypeptide chain. No conca-

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GAC GAG GCC CAG TTT AAG GAA TGT TAC GAC ACC TGC 36
Asp Glu Ala Gln Phe Lys Glu Cys Tyr Asp Thr Cys

CAC AAG GAG TGT TCT GAT AAG GGC AAT GGC TTC ACA 72
His Lys Glu Cys Ser Asp Lys Gly Asn Gly Phe Thr

TTC TGT GAG ATG AAG TGC GAC ACT GAT TGT AGC GTC 108
Phe Cys Glu Met Lys Cys Asp Thr Asp Cys Ser Val

AAA GAC GTT AAA GAG AAA CTC GAA AAC TAC AAG CCA 144
Lys Asp Val Lys Glu Lys Leu Glu Asn Tyr Lys Pro

AAA AAT TAA tggattgaagatagttgccacagattagaacagta 188
Lys Asn Stop
cggagaataaggtggataatagttacaattaggttcaagttttgggt 235
ccactcttgtattgcaatgaagtgaaacttattattgcatgaaaatt 282
ttgattcaataaatttaattaaac(a)15                    321
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Fig. 4. Nucleotide sequence of a cDNA clone encoding Ole e 6, and its deduced amino acid sequence. The primer used is underlined. The amino acid sequence obtained by Edman degradation is indicated in italic letters. Cystein residues are highlighted in bold letters. The polyadenylation signal is boxed. The nucleotide sequence reported has been submitted to the GenBank/EMBL Data Bank and will appear with accession No. U86342.

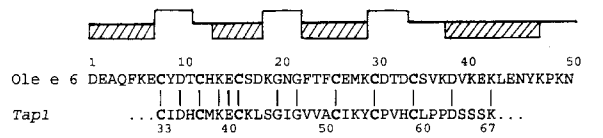

Fig. 5. Secondary structure prediction according to Ref. [18], of Ole e 6 (α-helix, shaded boxes; β-turns, upper line) and amino acid sequence alignment with the 33–67 region of the primary structure deduced from the stamen-specific gen *Tap1* from snapdragon (identities are indicated by vertical bars).

navalin A reaction product was detected for Ole e 6 (data not shown), and the protein does not contain any consensus N-linked sugar binding site; however, the presence of a Ser-bound sugar moiety cannot be totally discarded.

Ole e 6 is a major allergen clinically important in regions where the olive tree pollen constitutes a main cause of respiratory allergies since its prevalence among these populations is higher than 50%. The delay in finding this significant allergen may be related to its loss from the dialysis bags usually employed through the preparation of pollen extracts.

Searching for homologous polypeptides into nucleotide and amino acid sequence databases resulted unsuccessful, since no significant similarity has been found with any other known molecule. Interestingly, two sets of the Cys-X_3-Cys-X_3-Cys motif are also present in the amino acid sequence deduced from *Tap1*, a stamen-specific gene from snapdragon (*Antirrhinum majus*) [16], and the sequence length between these two Cys-motifs (9 residues) is identical to that of Ole e 6 (Fig. 5). However, *Tap1* cDNA contains an open reading frame of 107 amino acids, and the similarity of the whole sequences is not significant by using the criteria of Doolittle and Feng [17]. In addition, genes homologous to *Tap1* have been detected in other plants such as *Nicotiana tabacum*, by Southern blot analysis of genomic DNA with *Tap1*-specific cDNA under stringent conditions, which suggests an evolutionary conserved sequence for the protein from different sources. Therefore, although these proteins share 30% identity in the considered region, Ole e 6 is not expected to be the equivalent protein in the olive tree.

Finally, both small and Cys-enriched proteins, such as hevein, apamine, Amb t 5 or Amb f 5, have been also found to be allergens. The comparison of the molecular and physical properties of Ole e 6 with those of these allergens may inform about their allergenicity-structure relationships.

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