ORIGINAL ARTICLE

The Allergenic Structure of the Thaumatin-like Protein Ole e 13 Is Degraded by Processing of Raw Olive Fruits

M Torres,[1] E Alvarez-García,[1] J Bartra,[2] M Alcántara,[3] O Palomares,[1] M Villalba,[1] R Rodríguez[1]

[1]Departament of Biochemistry and Molecular Biology, Faculty of Chemistry, Universidad Complutense de Madrid, Madrid, Spain
[2]Unitat d'Alèrgia Servei Pneumologia i Alèrgia Respiratòria, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain
[3]Unidad de Alergología del Complejo Hospitalario de Jaén, Jaén, Spain

Abstract

Background: The thaumatin-like protein (TLP) Ole e 13 in raw olive fruit is responsible for occupational allergy in olive oil mill workers. However, these workers do not experience allergic symptoms after ingestion of edible olive.
Objectives: To analyze the presence of IgE-reactive TLP in raw and edible olive fruit and to assess the allergenic potency of both sources.
Methods: The content of TLP in raw and edible olive fruit protein extracts was analyzed using immunoblotting with sera from allergic patients and with olive TLP–specific IgG. The structural and immunological stability of TLP were assayed using immunoblotting after treatment of both raw olive and purified TLP with 0.25 M NaOH solution for 24 hours. Olive pollen extract was investigated by immunoblotting for TLP content.
Results: The TLP contained in raw olive fruit was not present in edible olives as a result of maceration before human consumption. No TLP was detected in olive pollen using specific IgG or sera from patients allergic to olive fruit. Sera from patients allergic to olive pollen did not react with purified TLP.
Conclusions: IgE-reactive TLP is not present in edible olive, thus explaining the low number of patients allergic to this highly consumed fruit. Patients allergic to olive pollen are not sensitized to TLP and, therefore, not expected to be at risk of food allergy to olive fruit or TLP plant sources.

Key words: *Olea europaea*. Olive fruit. Thaumatin-like protein. Allergen.

Resumen

Introducción: La aceituna natural contiene una proteína de la familia de las taumatinas (TLP) que es responsable de la alergia ocupacional en trabajadores de molinos de aceite. Sin embargo, éstos no presentan síntomas cuando ingieren aceitunas comestibles.
Objetivos: Analizar la presencia de TLP en aceituna natural y comestible, y correlacionar sus niveles con la potencia alergénica de ambos productos.
Métodos: El contenido de TLP en los extractos proteicos de las aceitunas fue analizado por inmunotransferencia y tinción con sueros de pacientes alérgicos así como con antisuero específico para TLP de olivo. La estabilidad estructural e inmunológica de la TLP se ensayó mediante inmunotinción después del tratamiento del extracto de aceituna natural y de la TLP purificada con NaOH 0.25 M durante 24 h. También se analizó la presencia de TLP en el polen de olivo por inmunotinción.
Resultados: La TLP presente en la aceituna natural no se detecta en la comestible como consecuencia del tratamiento de maceración al que es sometida para obtener el producto apto para el consumo humano. No se observó TLP reactiva en el polen de olivo, ni con anticuerpos específicos ni con sueros de pacientes alérgicos a aceituna. Sueros de pacientes alérgicos al polen de olivo no reaccionan con la TLP purificada de aceituna.
Conclusiones: La TLP de olivo no está presente en las aceitunas comestibles lo que explica el escaso número de pacientes alérgicos a la aceituna. Además, los pacientes alérgicos al polen de olivo no están sensibilizados a TLP, por lo que no tendrían riesgo de sufrir alergia alimentaria a aceitunas o a fuentes vegetales de TLPs.

Palabras clave: *Olea europaea*. Aceituna. Taumatina. Alérgeno.

Introduction

The olive fruit is frequently consumed in Mediterranean countries as food, but it is also used as raw material to obtain olive oil, which has been demonstrated to prevent cardiovascular diseases, diabetes, and cancer [1,2]. Olive fruit derivatives are also used in cosmetic and health products. Allergies to olive fruit, olive oil, and derived products have been seldom reported despite widespread consumption of these products. Two cases of olive fruit allergy have been published. In the first, a 5-year-old boy experienced anaphylactic shock after eating olives [3], and in the second, a pizza chef experienced contact urticaria induced by olive fruit [4]. As for allergy to olive oil, most reported cases involve occupational allergy: patients, mainly cooks and masseurs, experienced contact eczema after overexposure to olive oil in the workplace [5-8]. The allergens were not identified in any of these cases. Olive pollen, on the other hand, frequently causes type I allergy [9,10] in countries where the olive tree (*Olea europaea*) is extensively cultivated, such as those of the Mediterranean coast, several areas of America (California, Chile, and Argentina), Australia, and South Africa. Thus, 12 allergens have been identified and characterized in olive pollen, and clinical significance varies for each, ranging from very high prevalence for Ole e 1 to very low prevalence for Ole e 8 [11,12]. Nevertheless, to date, none of these allergens have been reported in other olive tree tissues, including olive fruit.

Olive fruit was the material responsible for the occupational asthma experienced by an olive oil mill worker who was exposed daily to inhaled particles derived from the processing of olive fruit. However, the patient was asymptomatic when he ingested edible olives or inhaled pollen [13]. A putative thaumatin-like protein (TLP) from raw olive fruit extract was identified to be the elicitor of asthma in this case. TLPs have been reported to be food allergens in several fruits, such as apple, sweet cherry, pepper, kiwi, grape, peach, and banana, as well as in pollen from juniper, cypress, and cedar. The proteins of the TLP family are type 5 pathogenesis-related proteins, which defend plants against pathogens and consist of a panallergen family [14] whose members are involved in cross-reactivity processes [15]. Recently, TLP from wheat flour and from obeche dust were found to be responsible, respectively, for respiratory allergy in bakers [16] and occupational rhinitis and asthma in carpenters [17].

In this study, we searched for differences in the content and integrity of TLP in raw and edible olive fruit in order to explain the minor allergenic activity of the latter. We found that the exhaustive maceration of raw olives before they can be considered suitable for human consumption results in degradation of the TLP. Therefore, the IgE reactivity of olive TLP is abolished during processing. We analyzed the IgE reactivity to raw and edible olive fruit of patients sensitized through inhalation or ingestion. The presence of TLP in pollen was also analyzed, and the results discussed to determine the implication of this protein in the potential sensitivity of olive pollen–allergic patients.

Methods

Sera and Antibodies

The patients included in this study were recruited from the Complejo Hospitalario de Jaén, Jaen, Spain (n=1, patient J; and a pool of 10 sera from patients allergic to olive pollen) and Hospital Clínic, Barcelona, Spain (n=2, patients B1 and B2). Patient J (age 41) had a clinical history of occupational allergy to the flour of olive fruit when the olive oil mill was operating; the results of skin prick tests with purified TLP from olive fruit outside the harvesting period were positive at 1 µg/mL (9 mm^2) [13]. Testing was also performed with the pool of sera from 10 patients, who had a radioallergosorbent test score >3 to olive pollen extract, seasonal rhinitis and/or bronchial asthma from late April to June, and a positive skin prick test result with *O europaea* pollen extract (ALK-Abelló). Patient B1 (age 27; total IgE, 54.10 kU_A/L) experienced anaphylaxis to peach and hazelnut and had a positive prick test result to peach peel, apple, hazelnut, kiwi, lettuce, mustard, and corn; he was sensitized to *Artemisia* and *Salsola* pollen and had rhinoconjunctivitis to *Platanus acerifolia*. Patient B2 (age 20; total IgE, 95.40 kU_A/L) experienced anaphylactic shock after ingestion of nuts and had positive prick test results to mites, hazelnut, peach peel, and mustard; he was sensitized to *Parietaria* and *P acerifolia*. Patients B1 and B2 had anaphylaxis after ingesting olive fruit and positive prick-prick results to olive fruit (7 x 7 mm and 6 x 5 mm, respectively). Neither was sensitized to olive pollen. Written informed consent was obtained from all patients. The study was approved by the Ethics Committees of Universidad Complutense (Madrid, Spain), Complejo Hospitalario de Jaén (Jaén, Spain), and Hospital Clínic (Universitat de Barcelona, Barcelona, Spain).

Polyclonal antiserum against olive TLP was obtained by weekly intraperitoneal injections of BALBc mice with 1 µg of the purified allergen preincubated for 1 hour with $Al(OH)_3$. After 35 days of treatment, serum was obtained by centrifugation of the blood [18].

Olive Fruit and Pollen Extracts

Edible and raw olives were obtained, respectively, from a local food store and from an olive cultivar (both were *O europaea*, var. manzanilla). Olive pulp was manually removed from the pits, cut into pieces, and lyophilized. The material obtained was pulverized by grinding in a mortar in liquid air. The resulting powder was extracted 3 times with 4% (wt/vol) ether/ethanol (3:1). The pellet was air-desiccated, suspended in 5% phosphate-buffered saline (pH 7.0), and gently stirred for 1 hour. The supernatant was separated by centrifugation at 10 000 rpm for 30 minutes and dialyzed against 50 mM of ammonium bicarbonate (pH 8.0) for 16 hours. All the steps were performed at 4ºC. Supernatants were lyophilized and stored at –20ºC.

Protein extract from olive tree pollen was prepared as previously described [19], with minor modifications.

Purification of Thaumatin-Like Protein From Olive Fruit Pulp

Lyophilized olive pulp extract was dissolved in 0.2 M ammonium bicarbonate (pH 8.0) applied onto a Sephadex

G-50 column, and eluted with the same buffer. The fractions were tested for protein by staining of SDS-PAGE gels with Coomassie Brilliant Blue R-250 and for IgE reactivity with the serum of the patient with occupational asthma using Western blot on nitrocellulose membranes. Those fractions containing the IgE-reactive protein were pooled, lyophilized, and further resolved on a reverse-phase Nucleosil C-18 HPLC column eluted with a gradient (0-60%) of acetonitrile in 0.1% trifluoroacetic acid. Fractions with IgE reactivity were lyophilized and stored at –20ºC until use.

Analytical Procedures

SDS-PAGE was performed in 15% (wt/vol) polyacrylamide gels either under reducing conditions (in the presence of 2-mercaptoethanol [2-ME]) or under nonreducing conditions. Proteins were visualized using Coomassie Brilliant Blue R-250 or transferred to nitrocellulose membranes (Amersham Biosciences). Protein concentration was determined using the Lowry assay [20].

IgE and IgG Immunoblot Analyses

Purified TLP from olive fruit (1 µg per strip), olive pollen extract (40 µg of total protein per strip), and olive fruit pulp extracts (200 µg) were separated using SDS-PAGE under reducing or nonreducing conditions and blotted onto nitrocellulose membranes. Samples of pulp extracts from raw and edible olives (10 µg of total protein) were also assayed by dot blot. After incubation with blocking buffer, the membranes were probed with patients' sera (diluted 1:10). Bound IgE antibodies were detected by incubating first with mouse antihuman IgE monoclonal antibodies (diluted 1:5000; kindly donated by ALK-Abelló) and then with horseradish peroxidase–coupled goat antimouse IgG (diluted 1:2500; Pierce Chemical Co) [21]. The peroxidase reaction was developed using the ECL Western blot reagent (Amersham Biosciences) [22] and detected in an LAS3000 luminescent image analyzer (Fujifilm Life Sciences). Quantification was performed in triplicate using the computer program Multigauge V3.0.

IgG reactivity of purified protein or protein extracts was assayed with specific pAb obtained against purified olive TLP (diluted 1:500) and detected by horseradish peroxidase–labelled goat antimouse IgG (diluted 1:3000) as previously described [18]. The signal was developed and quantified (see above).

Preparation of Edible Olive Fruit

Raw olive fruit was immersed in 0.25 M of NaOH for 24 hours at room temperature, washed with distilled water, and left for 2 hours; washing was repeated at least twice. In most commercial procedures, this treatment is completed by adding spices (eg, oregano, thyme, rosemary, fennel, and bay leaf) or dressings to obtain different flavors. We avoided these products when treating the raw olive fruits, although they are generally used in the commercial olives obtained from food stores. Protein was then extracted for raw and edible olive fruit (see above).

Purified olive TLP underwent the same treatment.

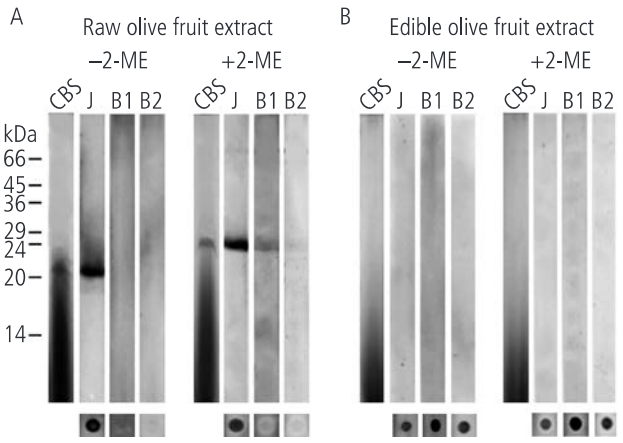

Figure 1. Comparison of raw olive (A) and edible olive (B) protein extracts (200 µg per lane) after separation by SDS-PAGE and staining with CBS or transfer to membranes and immunostaining with sera J, B1, and B2, in the absence (–) and in the presence (+) of 2-ME. Molecular weight markers are indicated in kDa. Immunostaining of the same samples (10 µg of total protein) by dot blot are shown at the bottom of the figure. CBS indicates Coomassie Brilliant Blue R-250; ME, 2-mercaptoethanol.

Results

TLP is Absent From Edible Olive Fruit

Protein extracts from raw and edible olive fruit were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (Figure 1). A 23-kDa protein band was detected in raw olive fruit under nonreducing conditions; this value increased to 27 kDa after treatment with 2-ME. The same bands were detected when the samples were transferred to nitrocellulose membranes and immunostained with the IgE antibodies of serum J, which belonged to the oil mill worker, indicating that the protein maintained IgE reactivity under reducing conditions. However, a faint signal (28% of the signal for serum J) was detected by the serum from patient B1, who was allergic to edible olive. The IgE-reactive protein corresponded to olive TLP, as previously described [13].

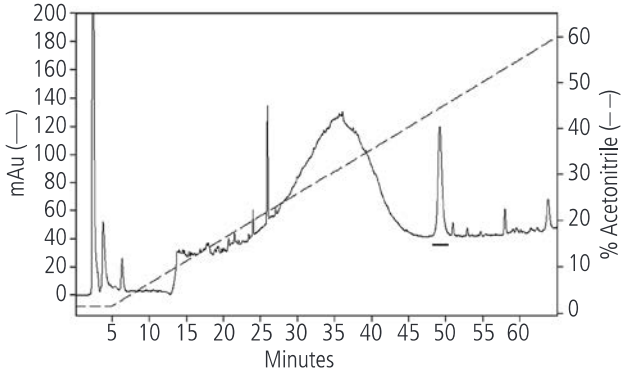

Figure 2. Last step of purification of TLP from raw olive fruit. Elution profile on a reverse-phase Nucleosil C-18 HPLC column using an acetonitrile gradient in 0.1% trifluoroacetic acid. The horizontal bar indicates the TLP position in the elution profile.

The results obtained by performing the assays using dot blot essentially agree with those obtained in SDS-PAGE. Furthermore, all traces of defined protein bands disappeared in the SDS-PAGE of protein extracts obtained from edible olive, and no IgE reactivity was detected for any sera. In the corresponding dot blot experiments, IgE reactivity was found for the 3 sera. Remarkably, sera B1 and B2 only reacted against edible olive in dot blot but not in immunoblotting, suggesting that low-molecular-weight IgE-reactive products added during the maceration of raw olives or remaining TLP-derived peptides could be present in edible olives.

Treatment With NaOH of TLP From Olives and Raw Olives: IgE Reactivity of the Products

The IgE-reactive protein was purified in 2 chromatographic steps, namely, size-exclusion chromatography in Sephadex G-50 and reverse-phase C-18 HPLC (Figure 2), which enabled removal of the high amount of pigments that were responsible for smearing in SDS-PAGE. The protein eluted at 53% of acetonitrile was identified as the IgE-reactive material Ole e 13. The purified protein was dissolved in 0.25 M NaOH, and raw olives were macerated in the same solvent. After 24 hours, the protein was dialyzed, and the olive was exhaustively rinsed in distilled water. Protein extract from olive fruit treated with NaOH was prepared for raw olive (see above) and lyophilized until use. Both samples underwent SDS-PAGE and were stained with Coomassie Brilliant Blue R-250 or transferred to membranes and immunostained with serum J (Figure 3). IgE reactivity disappeared in the samples treated with NaOH.

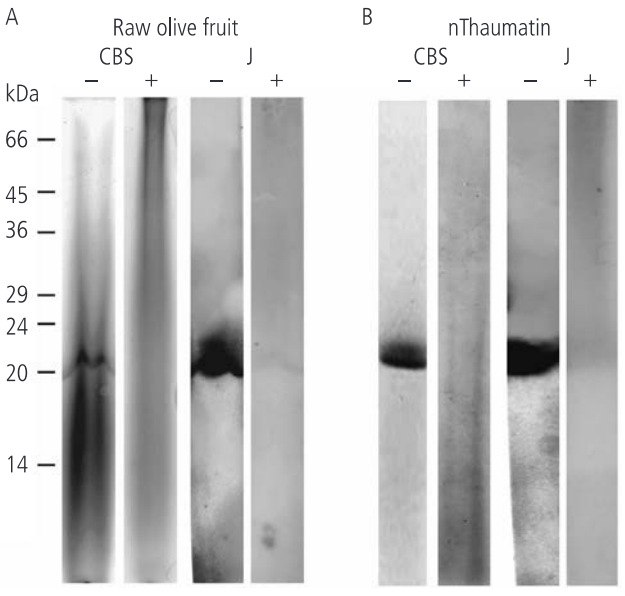

Figure 3. Treatment of raw olive fruit (A) and purified thaumatin (B) with NaOH. Staining with CBS and immunostaining with serum J after SDS-PAGE separation of the products obtained with (+) or without (–) NaOH. Molecular weight markers are indicated. CBS indicates Coomassie Brilliant Blue R-250.

Analysis of the Presence of TLP in Olive Tree Pollen

Immunoblot experiments were performed to investigate the presence of olive TLP in olive tree pollen. IgE antibodies from serum J directed to TLP from olive fruit did not react to olive pollen extract (Figure 4). In addition, specific polyclonal antibody to olive fruit TLP did not recognize any proteins in the pollen

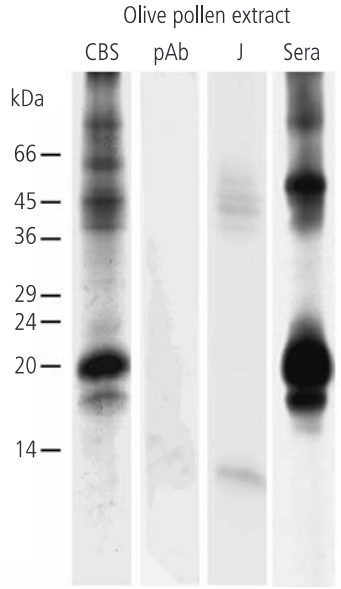

Figure 4. Western blot of olive pollen protein extract (40 µg total protein) with specific IgG and IgE antibodies to thaumatin-like protein. Pollen extract stained with CBS and immunostained with polyclonal antibody specific to olive thaumatin-like protein, or with serum J, and with a pool of sera from patients allergic to olive pollen. Molecular weight markers are shown. CBS indicates Coomassie Brilliant Blue R-250; pAb, polyclonal antibody.

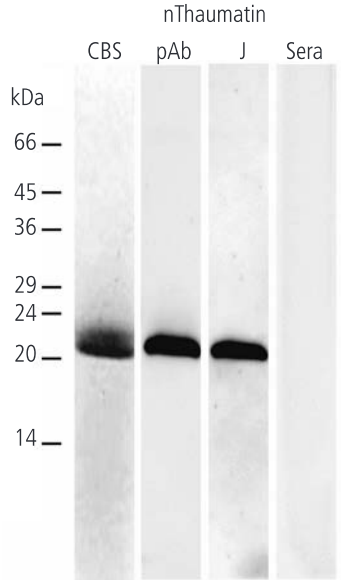

Figure 5. Assay of IgE reactivity of olive pollen allergic patients (lane Sera) to purified thaumatin (1 µg) in comparison to the reactivity of a specific polyclonal antibody and serum J. Molecular weight markers are shown. CBS indicates Coomassie Brilliant Blue R-250; pAb, polyclonal antibody.

extract. For comparison, olive pollen extract was immunostained with a pool of sera from olive pollen–allergic patients, and a positive response was observed for several proteins.

IgE Reactivity to Olive TLP of Sera From Olive Pollen–Allergic Patients

Purified olive TLP was analyzed to determine IgE reactivity against the same pool of sera from olive pollen–allergic patients, in comparison with the specific polyclonal antibody and serum J. The result of the immunoblotting assay was negative for the IgE reactivity of the sera from olive pollen–allergic patients (Figure 5), thus showing that TLP is not an allergen in this group.

Discussion

Olive fruit is widely consumed in Mediterranean countries. However, allergy to ingested olive fruit has received little attention in the literature [3]. Only a few cases of atopic dermatitis have been attributed to handling of this food or its oil [4-8]. Olive fruit allergen was not identified until 2008, when in vivo and in vitro analyses revealed a TLP to be responsible for bronchial and nasal allergic symptoms that an oil mill worker experienced in the workplace because of daily exposure to flours derived from ground olive fruits [13]. The causative allergen was found in the protein extract obtained from the raw olive fruit and is the only allergen identified to date. The lack of association between high consumption of olive and the very low prevalence of allergy to this fruit prompted us to explore differences in TLP protein content between raw and edible sources. Our results could provide the first explanation for the scarce number of patients allergic to edible olives, even though the food is widely consumed in Mediterranean countries and in other countries (albeit to a lesser extent). In addition, the observation that patients who are allergic to olive pollen do not show hypersensitivity to olive fruit could be explained by the absence of TLP in pollen.

TLP from raw olive fruit was well recognized by serum from patient J and did not lose IgE reactivity when treated with a reagent disrupting disulphide bridges, which are abundant in the native structure of TLP. However, sera from patients B1 and B2 (sensitive to edible olive fruit by ingestion) only reacted with TLP from the raw material under reducing conditions, and the intensity of IgE reactivity was very low. When the same assay was performed with commercial edible olives, no signal was detected under any conditions. This difference in the reaction of the patient's serum to TLP could be due to the route of access of the olive fruit, since it behaved as an aeroallergen for patient J but not for patients B1 and B2, who had a long history of food allergy. Many of the plant foods to which patients B1 and B2 were hypersensitive contained allergenic TLP. Therefore, the faint IgE reactivity observed against the 2-ME–treated raw olives (Figure 1A) could be explained by residual cross-reactivity between the members of this protein family, because reducing conditions could lead to exposure of the peptides responsible for traces of reactivity.

The IgE reactivity for any of the protein components present in the SDS-PAGE was lost for all the sera with the edible material, indicating that no TLP was present in the samples. The result of the dot blot assay with edible samples was positive, both in the absence and in the presence of 2-ME. There could be 2 possible explanations for this observation: (*a*) the existence in the sample of small but IgE-reactive peptides derived from protein components of the olive fruit disrupted by processing with NaOH to make olive fruits edible; or (*b*) nonprotein molecules derived from the condiments (mainly spices) added to the olive preparations in order to flavor the edible commercial products. Glycosylation cannot be involved in the positive reaction of the dot blot, since olive thaumatin is not glycosylated. Furthermore, patients B1 and B2 could be sensitive to lipophilic proteins removed during delipidation of the protein extract, thus supporting their clinical data.

The protein content of olive fruit is 1.3% to 1.8% of the dry weight, irrespective of the variety of fruit and stage of ripening [23]. TLP is the major protein component of the soluble saline protein extract of olive fruit after delipidation of the ground pulp, as deduced from staining with Coomassie Brilliant Blue R-250, in which no other defined protein bands were detected (Figure 1A). This result was consistent with those of our previous report [13] and with those of a recent study of olive pulp proteins in which SDS-PAGE revealed a major band of 24 kDa [24]. TLP from raw olive was purified by reverse-phase HPLC eluting at a high concentration of the organic solvent (Figure 2). Such a finding points to the stability of the protein in the raw olive fruit and a high lipid content. Treatment of olive fruit to remove oleuropein, which is the phenolic glycoside component that gives raw olive its bitter, pungent taste, requires the raw material to be maintained in an NaOH solution for 24-36 hours. This procedure was carried out with olive fruit and with purified TLP. In both cases, the protein and IgE reactivity disappeared from the samples. TLP are antifungal proteins of approximately 23 kDa that belong to type 5 pathogenesis-related proteins [14]. These proteins are generally resistant to proteolysis and denaturation by heat or changes in pH [25]. However, prolonged treatment with NaOH led to disruption of the protein (probably by hydrolysis) and loss of IgE recognition. This result is consistent with the lack of detection of TLP in edible olives and supports the scarce allergenicity of edible olives.

It is not unusual for crushed plant foods to cause allergy by inhalation. Soy seed powder and cereal flours are responsible for asthma in hypersensitive patients and are frequently involved in occupational allergy in individuals who handle ground foods (eg, baker's asthma). The allergens identified in these seeds are mainly members of the prolamin superfamily and include 2S albumins and a-amylase inhibitors. TLPs were recently reported to be involved in occupational allergy in bakers and carpenters caused by wheat flour and obeche dust [16,17]. TLP from olive fruit is a new finding and should be taken into account in the diagnosis of allergy to *O europaea*. Nevertheless, TLP cannot be a significant allergen from olive pollen, which is a typical source of aeroallergens, since TLP epitopes were detected neither by specific IgG nor by sera from olive pollen–allergic patients with high IgE titers. Therefore, the large number of patients who are hypersensitive to olive pollen in areas of intensive cultivation of olive trees do not

seem to be at risk of allergy to olive fruit or other plant sources of TLPs.

The TLPs described as allergenic components of plant foods include Mal d 2 from apple [26], Act d 2 from kiwi [27], Pru av 2 from sweet cherry [28], Pru p 2 from peach [29], and Mus a 4 from banana [30]. Major allergens of *Cupressus arizonica* and *Juniperus ashei* pollen have also been detected [31,32]. Putative IgE cross-reactivity of these allergens with olive TLP remains to be analyzed and requires in-depth study. The data we present indicate that patients who are allergic to TLP from plant foods might not be sensitive to edible olives through this family of proteins.

Finally, since TLP seems to be the main protein component of olives—and thus is potentially present in olive oil—we can speculate that it could also be involved in allergy to cosmetic or health products containing olive-derived materials, both by contact and through ingestion. Olive-derived materials are increasingly used in the manufacture of cosmetic and health products and should be taken into consideration when performing clinical assays.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Rosalía Rodríguez

Dpto. Bioquímica y Biología Molecular I
Facultad de Química
Universidad Complutense de Madrid
28040 Madrid, Spain
E-mail: rrg@bbm1.ucm.es