

RESEARCH ARTICLE

Identification of thaumatin-like protein and aspartyl protease as new major allergens in lettuce (*Lactuca sativa*)

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Scope: Today, about 2–8% of the population of Western countries exhibits some type of food allergy whose impact ranges from localized symptoms confined to the oral mucosa to severe anaphylactic reactions. Consumed worldwide, lettuce is a Compositae family vegetable that can elicit allergic reactions. To date, however, only one lipid transfer protein has been described in allergic reaction to lettuce. The aim of this study was to identify potential new allergens involved in lettuce allergy.

Methods and results: Sera from 42 Spanish lettuce-allergic patients were obtained from patients recruited at the outpatient clinic. IgE-binding proteins were detected by SDS-PAGE and immunoblotting. Molecular characterization of IgE-binding bands was performed by MS. Thaumatin was purified using the Agilent 3100 OFFGEL system. The IgE-binding bands recognized in the sera of more than 50% of patients were identified as lipid transfer protein (9 kDa), a thaumatin-like protein (26 kDa), and an aspartyl protease (35 and 45 kDa). ELISA inhibition studies were performed to confirm the IgE reactivity of the purified allergen.

Conclusion: Two new major lettuce allergens—a thaumatin-like protein and an aspartyl protease—have been identified and characterized. These allergens may be used to improve both diagnosis and treatment of lettuce-allergic patients.

Keywords:

Allergens / Aspartyl protease / Food allergy / Lettuce allergy / Thaumatin-like protein



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1 Introduction

The prevalence of food allergies has been increasing over the last 10 years. Up to 6% of young children and 3–4% of adults have adverse immune reactions to food, and this tendency has increased in recent years [1]. Among food allergens, plant-derived foods are a major cause of food allergy during adulthood in Mediterranean countries [2].

Although a large number of plant-derived food allergens have been characterized, most of them belong to a limited

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Abbreviations: CEFA, cofactor-enhanced food allergy; LTP, lipid transfer protein; MW, molecular weight; PBS-T, PBS containing 0.1% Tween-20; RT, room temperature; TLP, thaumatin-like protein

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number of protein families. Classifying allergens into protein families can help answering questions such as which allergens might be cross-reactive [3, 4].

Lettuce (*Lactuca sativa*), which belongs to the Compositae family, is a widely consumed vegetable in the Mediterranean area. Vegetables such as endive, escarole, artichoke, chicory and chard, as well as pollens such as mugwort, ragweed, and taraxacum are also included in this large plant family. Cross-reactivity between members of this family has been described [5]. Despite being a common dietary component worldwide—and more so in Mediterranean areas—few reports on lettuce allergy have been published to date [6–13]. However, in the last year it has been reported as a frequent cause of food allergy among patients suffering from cofactor-enhanced food allergy (CEFA) [14] and the so-called lipid transfer protein (LTP) syndrome [15].

With regard to lettuce-allergen characterization, several IgE-binding bands have been detected in the lettuce extract, but only one 9 kDa protein has been isolated and identified as a LTP. In 2003, San Miguel-Moncín et al. characterized the protein, naming it Lac s 1, a major allergen among patients with systemic reactions to lettuce [11]. It was later proved that Lac s 1 cross-reacted with other LTPs; it has an amino acid identity of 66% to Pru p 3, and 72% to the N-terminal peptide of plane tree pollen LTP, Pla a 3 [12].

In 1998, Vila et al. [9] described a 16 kDa protein in the lettuce extract that could correspond to a profilin but which has not been characterized subsequently as a lettuce allergen. Patients who had specific IgE to this 16 kDa protein developed local symptoms after eating lettuce. In the same study, three other IgE-binding proteins were found in the lettuce extract, with molecular weights (MWs) of about 50, 43, and 39 kDa, but they were not further identified. A 42–48 kDa IgE-binding band was also described by other authors [7, 8, 16], and Cadot et al. suggested it could be responsible for cross-reactivity between lettuce, endive, and chicory.

The aim of the study was to characterize new major allergens in lettuce extracts based on the study of a large sample of lettuce-allergic patients combined with a proteomic approach. In our study, we identified and characterized two major lettuce allergens, a 26 kDa protein which was found to be a member of the thaumatin-like family, and two proteins of MW around 35 and 45 kDa, which belong to the aspartyl protease family.

2 Materials and methods

2.1 Patient sera

Sera from 42 patients allergic to lettuce were selected from Hospital Vall d'Hebron (Barcelona, Spain) and Fundación Jiménez Díaz Hospital (Madrid, Spain). Lettuce allergy (inclusion criteria) was diagnosed in patients having a clear history of adverse reactions suggestive of IgE-mediated allergy, along with positive skin prick test and/or food challenge test, in ac-

cordance with the diagnostic algorithm described by Ibañez et al. [17]. Patients experiencing severe systemic reactions to lettuce, as well as patients with typical, recent, repeated, and unequivocal reactions, who had positive skin tests, did not undergo an oral challenge test to diagnose lettuce allergy. A panel of aeroallergens and plant food was tested in all patients in skin prick test (see Supporting Information Table 1). CEFA patients were diagnosed according to the algorithm proposed by Cardona et al. [14]. The protocol was approved by the Hospital Ethic Committee and patients provided informed consent to be included in the study.

2.2 Preparation of lettuce extracts

Lettuce extract was prepared from *L. sativa* L. var. *longifolia* and carried out as previously described [13] with some modifications. Briefly, 200 g of lettuce was homogenized in 100 mL of acetone at -60°C and stored overnight at -80°C . The sample was centrifuged at $4500 \times g$ for 15 min at 4°C . The pellet was then washed three times with acetone at -60°C , lyophilized, dissolved in PBS and extracted overnight at 4°C under constant magnetic stirring. After centrifugation at $14\,000 \times g$ for 45 min at 4°C , the supernatant was dialyzed against NH_4HCO_3 0.1 M, lyophilized and dissolved in PBS. For in vitro experiments, the protein concentration of lettuce extracts was adjusted to 1 mg/mL.

2.3 SDS-PAGE

SDS-PAGE was carried out according to Laemmli [18] using the Hoefer SE 600 electrophoresis system (GE Healthcare). Polyacrylamide concentrations of 14% w/v and 5% w/v were used for separating and stacking gels, respectively. A total of 20 μg of protein extract and 5 μg of purified protein were applied per lane. The samples were mixed with 0.1 M Tris, pH 6.8, containing 4% w/v SDS, 20% w/v glycerol, 10% w/v 2 β -mercaptoethanol, and 0.02% w/v bromophenol blue (under reducing conditions). To ensure proper protein separation and visualization, the gels were stained with PageBlue Protein Staining Solution (Fermentas International, Inc., Canada) or used for immunoblotting as described below.

2.4 Immunoblot analysis

Protein bands separated by SDS-PAGE were transferred by semidry blotting onto nitrocellulose sheets according to the method used by Towbin et al. [19]. Membranes were blocked with PBS containing 0.1% Tween-20 (PBS-T), 3% skimmed milk powder, and 3% BSA for 2 h at room temperature (RT) and incubated overnight at 4°C with sera diluted 1:10 in 50 mM Tris buffered saline, 150 mM NaCl, 5 mM EDTA, and 0.05% Triton X-100, pH 8.8. After washing them five times with PBS-T, the blots were incubated for

1 h at RT with goat antihuman IgE antibody conjugated with horseradish peroxidase (Nordic, CultiK) diluted 1: 5000 in PBS containing 0.05% Tween-20 and 1.5% skimmed milk powder. Finally, after five washings with PBS-T, the protein bands were visualized by enhanced chemiluminescence (GE Healthcare) following the instructions provided by the manufacturer. Immunoblot assays were performed with the 42 individual sera from lettuce-allergic patients. In addition, sera from five lettuce-allergic patients were used as a positive serum pool. The criteria for inclusion of patients in the serum pool were (i) high level of specific IgE to lettuce, (ii) serum availability, and (iii) lettuce-allergic patients with no peach allergy. Sera from five nonatopic patients were used as negative controls. For immunoblot inhibition assays, sera from allergic patients were preincubated for 4 h at RT with 100 µg of the inhibitor per milliliter of serum.

2.5 Protein identification and characterization by MS

Protein bands recognized by the sera of more than 50% of patients were selected for further study, and bands were extracted from the gel as previously described [20]. Proteins were identified by MS using LC-MS/MS, as previously described [21], in the Proteomic Service of the Complutense University of Madrid, a member of the ProteoRed Network.

2.6 Purification of native thaumatin-like protein (TLP)

Lettuce thaumatin was purified using the Agilent OffGel 3100 system. Briefly, isoelectric point-based protein separation was performed on the 3100 OFFGEL Fractionator and the OFFGEL Kit pH 4–7 (both Agilent Technologies) with a 12-well setup according to the protocol of the supplier. Fifteen minutes prior to sample loading, IPG gel strips with a linear pH gradient ranging from 4 to 7 were rehydrated in the assembled device with 0.7 mL of focusing buffer per well. A total of 1 mg of the lettuce extract was diluted in focusing buffer to a final volume of 1.8 mL, and 150 µL of sample was loaded in each well. The sample was focused with a maximum current of 50 µA. The experimental isoelectric point of lettuce thaumatin was around 5.50–6.00. Peach thaumatin was purified as previously described by Palacin et al. [22].

2.7 ELISA and ELISA inhibition studies

For ELISA and ELISA inhibition assays, 96-well flat-bottom plates were used (Immunolon 4HBX, Thermo). The plates were coated in duplicate overnight at 4°C with 1 µg per well of lettuce extract diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). The plates were blocked with PBS containing 0.05% Tween-20 and 2% BSA for 1 h at RT. After blocking, the plates were washed three times with PBS

containing 0.05% Tween-20. The plates were then incubated for 2 h at RT with patients' sera diluted 1:10 in blocking buffer. The plates were washed again and incubated with the secondary antibody (goat antihuman IgE-peroxidase, Nordic, CultiK) diluted 1:5000 with 2% BSA and 0.05% PBS-T for 1 h at RT. IgE reactivity was detected by adding 3,3',5,5'-tetramethylbenzidine (Chemicon) and measured at 620 nm. Sera from five nonatopic patients were used as negative controls. IgE reactivity was considered positive when a patient's OD at 620 nm was two times higher than a negative control's OD at the same absorbance. All tests were performed in duplicate. For ELISA inhibition assays, sera from allergic patients were preincubated for 4 h at RT with dilutions ranging from 100 to 0.001 µg of the inhibitor (purified protein) per millilitre of serum.

3 Results

3.1 Clinical features of lettuce-allergic patients

Characteristics of the patients included in the study are shown in Table 1. The median age of patients was 34 years (range 16–60) with a gender distribution of 14/42 males (33.33%) and 28/42 females (66.66%). The most frequent clinical symptom was co-factor-enhanced anaphylaxis (20/42, 47.61% of patients), followed by anaphylaxis (9/42, 21.42%), oral allergy syndrome (7/42, 16.66%), and urticaria (5/42, 11.9%).

In Table 1, sensitizations to pollens and plant foods are shown. Forty-seven patients had also concomitant pollen sensitization, mainly to *Platanus acerifolia*, *Artemisia vulgaris*, grass pollen mixture, and olive pollen. Most patients were sensitized to other plant-derived foods, mainly rosaceae fruits (80.95%) and nuts (73.8%).

3.2 SDS-PAGE and immunoblotting

SDS-PAGE of lettuce extract showed multiple protein bands with an apparent MW ranging from 8 to 115 kDa (Fig. 1). Figure 2 shows the binding of specific IgE in the sera of 42 lettuce-sensitized patients. The most frequently recognized allergens had MWs around 9, 26, 35, 45, 55, and 66 kDa (Fig. 2), detected by the sera of more than 50% of the patients. The frequency of recognition of protein bands is shown in Table 2. Control immunoblot assays with the pooled serum from nonatopic patients did not show any IgE-binding bands. Specificity of IgE-binding bands was proved by immunoblotting inhibition after preincubation with lettuce extracts (see Supporting Information Fig. 1).

3.3 Protein identification and characterization by MS

To identify the IgE binding proteins, we performed MS/MS and obtained the sequence of several internal peptides (Table 2). Protein identification was performed by searching

Table 1. Clinical features of lettuce-allergic patients

Patient	Age	Sex	Symptoms	CEFA symptoms/ cofactor	Aeroallergens sensitization	Sensitization to other foods
1	35	F	U/AE		Ar, Cu, G, Me, O, P, Phr, PI, Ptg	Ce, Fi, L, Me, N, Pe, Ro
2	43	M	CEFA	AF/NSAIDs	PI	Be, L, Ro
3	31	F	AF		Ar, G, PI	N, Ro, To
4	24	F	AF		Ar, PI	L, N
5	44	M	U/AE		Ar, G, O, PI	Be, N, Ro
6	29	F	OAS		Ar, O, PI, Ptg	Ki, L, Mu, N, On, Pe, Ro, To
7	30	F	CEFA	AF/NSAIDs-exercise	PI	Ki, N, Ro
8	56	M	CEFA	AF/Alcohol	Ar, PI	L, N, Ro,
9	39	F	AF		Ar, PI	Ce, L, N, Ro
10	44	M	OAS		Ar, P, PI	N, Ro
11	39	M	AF		-	L, N, Ro, To
12	29	F	OAS		Ar, PI	Ce, Mu, N, Ro, To,
13	38	F	U/AE		Ar, P, PI	Ki, N, Ro
14	23	F	CEFA	AF shock/exercise	Ar, PI	N, Ro, To
15	40	F	CEFA	AF/NSAIDs	Ar, G, P, PI, Sa	Gra, Me, Mu, N, Ro
16	37	F	U/AE		Ar, Cu, G, Me, O, P, Ptg, Phr, PI	Ce, L, Me, N, Pe Ro
17	22	F	OAS		PI	N, Ro, To
18	34	F	CEFA	AF/Alcohol-NSAIDs	Cu, O, PI	Ce
19	37	F	OAS		Ar, PI	Av, L, Me, N, Ro
20	19	F	AF		Cu, G, Phrg, PI, Ptg	Av, Ce, L, Mu, N, Ro
21	34	M	CEFA	AF/Exercise	Ar, G, P	Ce, N, Ro
22	21	F	CEFA	AF/Exercise	Ar, PI	Ki, N, Ro
23	42	F	CEFA	AF/Exercise	—	Ro
24	60	F	CEFA	AF/Alcohol-NSAIDs	P	Me, N, Pe, Ro
25	29	F	AF		PI	Ba, Be, Gra, Ro, To
26	24	M	OAS		Ar, P, PI	N, Ro
27	34	M	CEFA	AF/NSAIDs	Cyn, G, O, phrg	—
28	45	M	CEFA	AF/NSAIDs	Ar, G, O, PI, ptg	N, Ro
29	41	F	CEFA	AF/NSAIDs	Ar, PI	Me, N, Ro
30	22	F	CEFA	AF/Exercise	G	N, Ro
31	45	M	CEFA	AF/NSAIDs	PI	L, N, Ro, To
32	25	F	CEFA	AF/Exercise	Ar, G, O, P	Ba, Mu, N
33	30	M	CEFA	AF/ NSAIDs-exercise	Ar, Cu, G, O, PI	N, Ro
34	32	M	AF		G, O, PI, Ar, tx, be	—
35	25	F	OAS		PI, G, O, Sa	Av, Fi, Me, Ro
36	29	F	AF		PI, G, O	—
37	39	F	AF		PI, G, Cu	ki, Pr, Ro, Str
38	43	M	U/AE		PI, G, Cu, O, Sa, ptg	Ki, Me, Ro, Wa
39	55	M	VO		G, Sa	Av, Ki, Ma, Me, Wa
40	33	F	CEFA	AF/Exercise	PI, Ar	L, Mu, N, Ro
41	16	F	CEFA	AF/Exercise	PI, O, P, cyn, phag, Me	Be, Ce, Mu, N, To
42	23	F	CEFA	AF/Exercise	PI	Ce, Gra, Me, N, Ro

M: male; F: female. Symptoms: AF: anaphylaxis; CEFA: co-factor-enhanced food allergy; OAS: oral allergy syndrome; U: urticaria; AE: angioedema; VO: vomiting. *Cofactors*: NSAIDs: Nonsteroidal anti-inflammatory drugs. *Aeroallergen sensitization*: A: Artemisia; Be: Betula; Cu: Cupressus; Cyn: Cynodon; G: grass mix; Me: mercurialis; O: Olea; P: Parietaria; PI: plane tree; Ptg: Plantago. Phr: Phragmites; Sa: Salsola; Tx: Taraxacum. *Foods*: Av: avocado; Ba: banana; Be: green bean; Ca: carrot; Ce: cereals; Fi: fig; Gra: grape; Ki: kiwi; L: legume; Ma: mango; Me: melon; Mu: mustard; N: nuts; On: onion; Pe: green pepper; Pi: pineapple; Pr: pear; Ro: Rosaceae fruits; Str: strawberry; To: tomato; Wa: watermelon.

a nonredundant protein sequence database (NCBI) using the Mascot program (<http://www.matrixscience.com>). Research conducted with protein databases led to the identification of the 9 kDa protein band as a LTP. The peptide sequences of the 26 kDa protein yielded a high match with TLP from related species such as *Sambucus nigra*, *Isatis tinctoria* and *Brassica rapa* subsp. *pekinensis*. Match found between sequence of internal peptides of lettuce TLP and related TLP species was 100%. For 35 and 45 kDa IgE-binding band, peptide

sequences exhibited a high degree of homology with proteins from the aspartyl protease protein family. No significant homologies were found for 55 and 66 kDa IgE-binding proteins.

3.4 Purification of the allergenic TLP from lettuce

The purified lettuce 26 kDa protein was analyzed by means of MS/MS and identified as being a TLP according to the

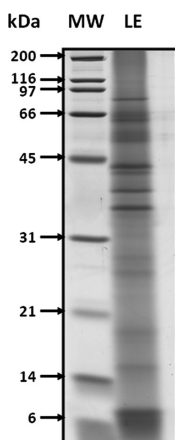


Figure 1. SDS-PAGE of lettuce extract under reducing conditions. MW: molecular weight. LE: lettuce extract.

sequence of internal peptides obtained. Peptides identified from purified lettuce thaumatin were the same as those previously identified together with an additional peptide (ASTMSCPGGTNYR) probably due to the higher purity of the purified TLP. The IgE-binding activity of the purified lettuce thaumatin was maintained throughout the whole process, as confirmed by immunoblots performed with a serum pool from lettuce-allergic patients (Fig. 3). To prove that its IgE-binding activity was the same as the natural thaumatin contained in the whole extract, ELISA inhibition assays were performed using lettuce extract in solid phase with a pool of sera recognizing the TLP in the immunoblotting studies. Purified thaumatin inhibited the IgE-binding capacity from pooled sera of lettuce-allergic patients in a dose-dependent manner, with the highest inhibition (37%) observed at 100 $\mu\text{g/mL}$ of inhibitor concentration (Fig. 4). No inhibitory effect was detected in the control experiment using nonatopic pool serum.

3.5 Cross-reactivity between lettuce and peach TLP

In order to study cross-reactivity between lettuce thaumatin and peach thaumatin, we performed immunoblotting using a purified peach TLP, Pru p 2.1, with serum pool from five lettuce-allergic patients not allergic to peach (Fig. 5). Peach thaumatins were recognized by the serum pool. To demonstrate cross-reactivity with lettuce thaumatins, immunoblot inhibition assays were performed using thaumatins in solid phase. Preabsorption of pool sera with lettuce extract and purified lettuce thaumatin resulted in complete inhibition of IgE binding (Fig. 5).

4 Discussion

In this article, we characterize two new lettuce allergens for the first time, which were shown to be two new major let-

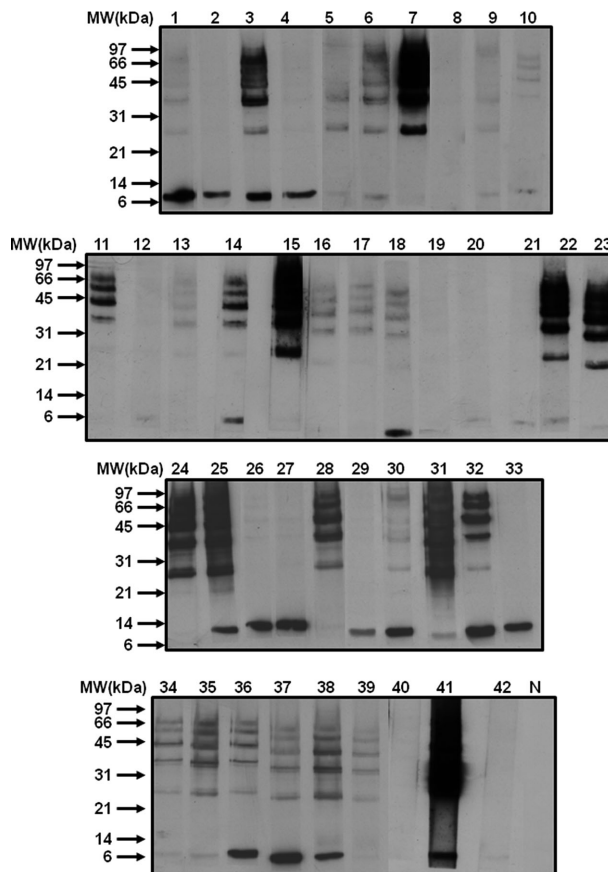


Figure 2. IgE-binding bands in lettuce extract by immunoblotting. Lanes 1 through 42 represent serum from 42 patients. Lane N represents a negative control of serum pool from nonatopic patients. MW: molecular weight markers (kDa).

tuce allergens: a TLP and an aspartyl protease. These findings are significant because these molecules can be used to improve standardization of lettuce extracts for in vivo or in vitro diagnosis, component-resolved diagnosis, biomarkers of allergy diseases, or future immunotherapy treatment of lettuce-allergic patients.

We have reached these conclusions by combining clinical and immunological studies as well as MS analysis. We believe some aspects of our findings are especially noteworthy. The first characteristic of note is seen in the clinical aspect of the study, considering both the inclusion criteria and the number of patients. On analyzing our patients, the most frequent allergic reactions to lettuce were CEFA reactions, which were diagnosed following the diagnostic algorithm previously reported by Cardona et al. [14]. In addition, patients included in this study constituted the largest series of lettuce-allergic patients assembled to date, thus making the percentage of IgE-binding bands more representative of major allergens than previous studies of its kind.

Lac s 1, the lettuce LTP, is the only allergen to be identified so far [11], and our findings are in agreement with it is a

Table 2. Analyzed IgE-binding protein bands from lettuce extract

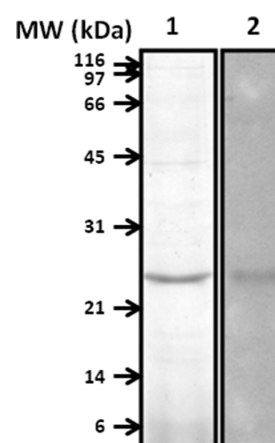
Estimated molecular weight by SDS-PAGE (kDa)	% Recognition of patients	Peptides	Identification
9	31/42 → 73.8%	NGGTPPQPCCTGVR	Lipid transfer protein
26	29/42 → 69%	TNCNFDASGR APGGCINNCTVFPR	Thaumatococcus-like protein
35	30/42 → 71.4%	AFDNPTGGGTLDSGTVFTR TTGSSIPPKGVLGIGR	Aspartyl protease family protein
45	30/42 → 71.4%	AFDNPTGGGTLDSGTVFTR LVDGAYTAVRDEFR VLFDLPSNR	Aspartyl protease family protein
55	27/42 → 64.3%	SSLSDILSR	No significant identity
66	27/42 → 64.3%	EELLTPYVSKNPR LAVIIGFR	No significant identity

Percentage of recognition by sera from lettuce-allergic patients in immunoblotting and identification of proteins by MS/MS.

major lettuce allergen, recognized by the 73% of our patients. In addition to that, our results found that Lac s 1 is also a major allergen among patients experiencing CEFA reactions, and this was shown to be the most frequent manifestation of lettuce allergy in our area.

Over the last decade, proteomic approaches have proved to be a powerful tool for molecular characterization of complex allergen sources. As a tool, it has allowed us to characterize two IgE-binding proteins from lettuce extracts: a TLP and an aspartyl protease, which were revealed to be new major lettuce allergens, recognized by the 69 and 71% of our patients, respectively. Noteworthy, 7/42 (16.67%) of the lettuce allergic patients in this study were sensitized either to the TLP or the aspartyl protease but not to LTP and thus, the inclusion of both allergens in the panel of lettuce allergens tested would improve its diagnostic sensitivity. The specificity of these IgE-binding bands from lettuce extracts was checked by immunoblotting inhibition assays.

Proteins of the thaumatin-like family have molecular masses of 20–30 kDa, with a very stable 3D structure that is maintained by six disulphide bridges. They have been described as plant defense proteins (PR-5) against pathogen attacks, especially of the fungal sort, and are generally resistant to proteases and pH or heat-induced denaturation. TLPs are panallergens and may be responsible for cross-reactivity between foods and pollen [23]. Several allergenic TLPs have been described in fruits, including peach (Pru p 2) [22], apple (Mal d 2) [24], sweet cherry (Pru a 2) [25], bell pepper [26], kiwi [27], grape [28], and in pollens such as cypress [29], olive [30], and plane tree [31]. Also, some TLPs have been described as glycoproteins and the presence of carbohydrates determinant for their allergenic capacity [32]. Although lettuce TLP is reported as an allergen in the present study, we used it in studies of cross-reactivity of TLPs. Indeed, in a recent study by Palacin et al. [31] evaluating the cross-reactivity of TLP, lettuce TLP was included along with 15 other TLPs from foods and pollens so as to study their role in cross-reactivity between fruits and pollens. The study reported TLPs are panaller-

**Figure 3.** Purified lettuce thaumatin. Lane 1: SDS-PAGE analysis of purified protein under reducing conditions. Lane 2: Immunoblot analysis of purified protein under reducing conditions with a serum pool from lettuce-allergic patients.

gens and described different patterns of TLP recognition. In the present study, cross-reactivity between lettuce and peach TLP was confirmed by immunoblot and ELISA-inhibition experiments.

Interestingly, the 35 and 45 kDa IgE-binding bands had virtually the same MS spectrum (fingerprinting) and the sequence of the most abundant peptide in both spectra obtained by MS/MS was identical. Thus, these two bands correspond to the same protein, as the 35 kDa protein is a truncated form of the 45 kDa protein because the majority of plant aspartic proteinases characterized are synthesized with a prepro-protein and subsequently converted to mature enzymes. The biological role of plant aspartyl proteases is not completely established, but their involvement in protein processing or degradation under different conditions suggests aspartic proteinases are components of the plant defense response. Some proteins from this family have been reported to be defense proteins in tomato and potato [33, 34]. These proteins

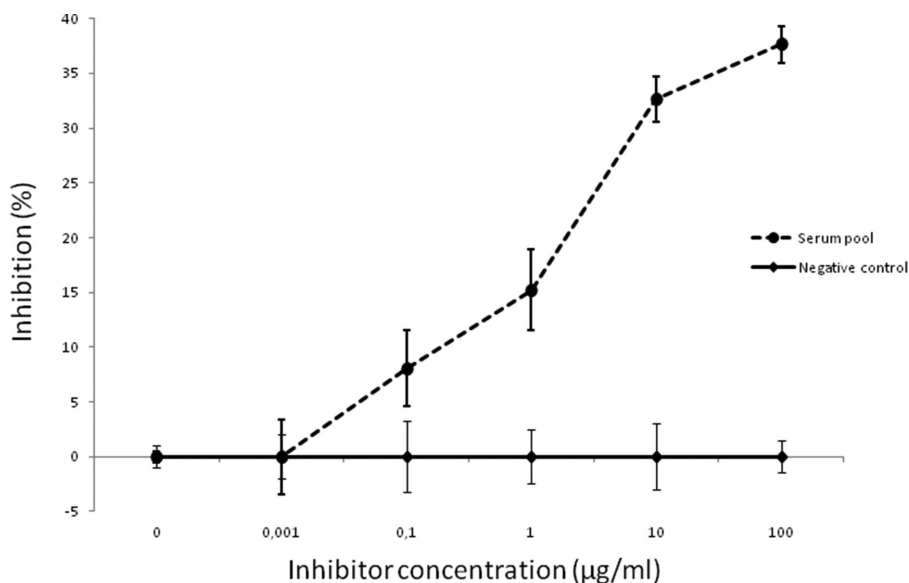


Figure 4. ELISA inhibition assay. Lettuce extract (10 µg) was used as solid phase and serum pool was preincubated with increasing concentrations of purified lettuce thaumatin (dotted line). Serum pool from nonatopic patients preincubated with increasing concentrations of purified lettuce thaumatin as negative control (solid line). All tests were performed in duplicate.

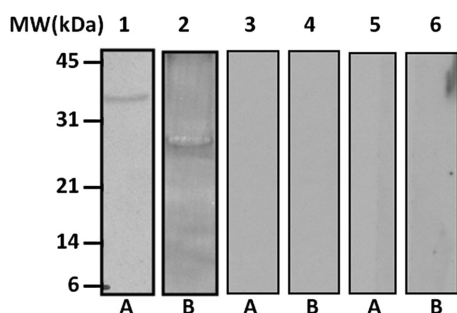


Figure 5. Immunoblot analysis of purified thaumatin (1 µg) with a serum pool from lettuce-allergic patients with no peach allergy. (A) Pru p 2 in solid phase. (B) Lettuce thaumatin in solid phase. Lanes 1 and 2: without preincubation. Lanes 3 and 4: preincubation with lettuce extract. Lanes 5 and 6: preincubation with purified lettuce thaumatin.

were recognized by the sera of more than 50% of patients and identified as proteins belonging to the aspartyl protease family. Both probably correspond to the 42–48 kDa protein found by other authors [7, 8, 16], since they are major allergens. It has been suggested that these IgE-binding bands are involved in the cross-reactivity between lettuce, endive, and chicory. Allergens belonging to this family have been found in extracts from cockroach (Bla g 2, Per a 2), fungi (Asp p 10, Rhi m AP), almond (Pru du conglutin), lupin (Lup a gamma conglutin), and *Cryptomer japonica* (www.allergome.org).

In addition to LTP, profilin has been reported to be a frequent allergen involved in plant food allergic patients from Spain [35]. Based on this, Vila et al. suggested that profilin was an allergen [9]. Our results have found that profilin was present in lettuce extracts used for immunological studies (see Supporting Information Fig. 2), but none of our patients was sensitized to profilin. Although it cannot be ruled out that

some patient might be sensitized to profilin, the results from other studies and ours both indicate that lettuce profilin is not a significant allergen among lettuce-allergic patients. In summary, we have identified and characterized two new major lettuce allergens—a TLP and an aspartyl protease—which may be used to improve the diagnosis and future treatment of lettuce-allergic patients.

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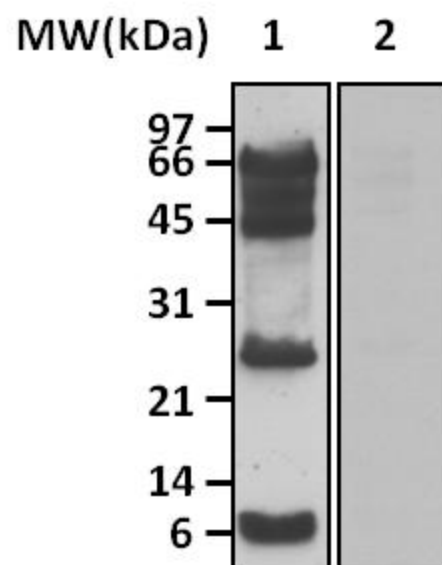
The authors have declared no conflict of interest.

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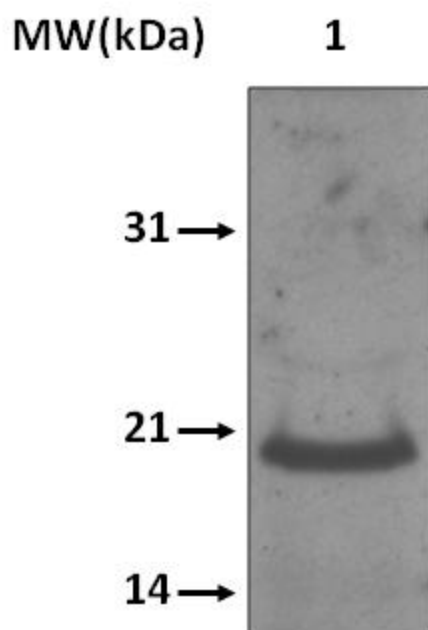
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Supporting Information Figure S1



Supporting Information Figure S1: IgE-binding bands in lettuce extract by immunoblotting (lane 1) and inhibition immunoblot by lettuce extract preadsorption (lane 2)

Supporting Information Figure S2



Supporting Information Figure S2: Immunoblot analysis of lettuce extract under reducing conditions with a rabbit anti-profilin antibody.

Supporting Information Table S1

Aeroallergens	Plant Food
<i>D. Pteronyssinus</i>	Almond
<i>D. Farinae</i>	Hazelnut
<i>Glyciphagus</i>	Peanut
<i>Alternaria</i>	Walnut
<i>Aspergillus</i>	Pinion
Cockroach	Pistachio
Tropomiosin	Chestnut
Dog Epithelium	Sunflower seed
Cat Epithelium	Strawberry
Horse Epithelium	Melon
Latex	Kiwi
<i>Pinus</i>	Apple
<i>Betula</i>	Peach (peel)
<i>Cupressus</i>	Banana
<i>Olea europea</i>	Avocado
<i>Platanus</i>	Lettuce
<i>Graminies</i>	Tomato
<i>Parietaria</i>	Green bean
<i>Artemisia</i>	Pea
<i>Plantago</i>	Lentil
<i>Chenopodium</i>	Lupine (flour)
<i>Cynodon</i>	Soy (flour)
<i>Phragmites</i>	Cereals
Profilin	Gluten
LTP	Gliadin
	Egg
	Cow milk
	Whitefish
	Bluefish
	Squid
	Prawn
	Mussel
	Pork
	Chicken
	Mustard
	Anisakis

Supporting Information Table S1: The panel of aeroallergens and plant food tested in skin prick test. Aeroallergens and plant food extracts were supplied by Leti SL (Madrid Spain), except LTP, supplied by Bial (Bial Aristegui) and ALK Abello (Madrid, Spain).