Isolation and characterization of relevant allergens from boiled lentils

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Background: Lentils seem to be the most common legume implicated in pediatric allergic patients in the Mediterranean area. However, no lentil allergen has been isolated and characterized.
Objective: We sought to purify and characterize relevant IgE-binding proteins from boiled lentil extracts.
Methods: IgE-binding proteins from crude and boiled lentil extracts were detected with a pool of sera from patients with lentil allergy. Allergens were isolated by gel-filtration chromatography followed by cation- and anion-exchange chromatography or by reverse-phase HPLC. Their characterization included N-terminal amino acid sequencing, complex asparagine-linked glycan detection, specific IgE immunodetection with 22 individual sera from allergic patients, and immunoblot and CAP inhibition assays.
Results: Heat treatment of lentils produced substantial changes in the SDS-PAGE patterns of whole extracts, mainly a strong increase of 12- to 16-kd bands and a decrease of 25- to 45-kd components. Major IgE-binding proteins from the boiled lentil extract were located in the 12- to 16-kd and 45- to 70-kd ranges. Two allergens of 16 kd, proteins L1 and L2, and another one of 12 kd, protein L3, were purified. N-terminal sequencing indicated that all 3 were related and allowed their identification as γ-vicilin subunits. Protein L1 was recognized by 68% of the individual sera tested and inhibited 64% of the IgE binding by commercial lentil CAPs. A second type of allergen of 66 kd, named protein H, was also isolated and identified as a seed-specific biotinylated protein. Protein H reacted with 41% of the individual sera and produced 45% inhibition in CAP inhibition assays.
Conclusions: Two different types of allergens have been identified in boiled lentils. Those of 12 to 16 kd, called Len c 1, correspond to γ-vicilin subunits, and those of 66 kd, designated Len c 2, correspond to seed-specific biotinylated protein. Homology with proteins from other legume species can explain potential cross-reactions among these foods. (J Allergy Clin Immunol 2000;106:955-61.)

Key words: *Lentil, allergen isolation, vicilin subunit, biotinylated protein, legumes*

Legumes are one of the most frequent plant foods associated with allergic reactions in children. Among food sources belonging to the Leguminoseae family, peanut and soybean are the two major ones involved in immediate hypersensitive responses in countries such as the United States, Japan, and the United Kingdom.[1-3] However, other legumes, such as lentils and chickpeas, seem to be an important cause of IgE-mediated hypersensitivity in the Mediterranean area and India. Niphadkar et al[4] have reported allergy to legumes as the most important food allergy in India, with lentils and chickpeas being relevant allergenic sources. Bronchial asthma has been induced by inhaling vapors from cooking chickpeas, beans, and lentils. Specific bronchial challenges with heated extracts of chickpeas and lentils have been shown to elicit isolated immediate responses.[5] More recently, Kalogeromitros et al[6] reported a case of severe type I hypersensitivity to lentils, which was associated with clinically relevant hypersensitivity to chickpeas. Our previous studies have shown that legumes, particularly lentils and chickpeas, are an important cause of allergic reactions in pediatric patients in our area.[7]

A wide array of reports has been focused on identifying and characterizing the relevant allergens from peanut and soybean in the last decade.[8-12] Interestingly, two of the characterized allergens, peanut Ara h 1[8,10] and soybean β-conglycinin,[11] are seed storage proteins included in the legume vicilin family of 7S proteins.[13] On the contrary, no allergen from lentils has yet been isolated and identified. Recent studies[14,15] have detected multiple specific IgE-binding bands in the immunoblotting patterns of crude lentil extracts obtained with sera from patients with lentil allergy. These bands range from 14 to 90 kd, with those around 54, 38, and 68 kd being the more frequently recognized.[14] A decrease in IgE binding has been reported,[15] mainly in the 20- to 70-kd range, when the crude extract is boiled.

Lentils are consumed after a heat treatment, usually boiling. Heating can produce inactivation, modification, or both of the putative allergens. Thus it seems more appropriate to study the relevant allergens of boiled lentils than those of the crude seeds, considering the clinical significance of the former. We report the isolation and characterization of relevant allergens from boiled lentils.

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Abbreviations used
PVDF: Polyvinylidene difluoride
RP-HPLC: Reverse-phase HPLC

METHODS

Patient sera

Serum samples were obtained from 22 pediatric patients with lentil allergy. The diagnosis was made on the basis of positive skin prick test responses and positive specific serum IgE to lentils, as well as a convincing clinical history of anaphylaxis after lentil ingestion or a positive open food challenge response.[14]

Specific IgE levels to lentils were quantified by using the CAP-FEIA System (Pharmacia Diagnostic, Uppsala, Sweden). All individual sera showed specific IgE levels above 5 kU/L, and the serum pool comprising the 22 sera had 155 kU/L. A serum pool from 10 patients allergic to fish but not allergic to pollen or plant food with similar total IgE levels to those of the lentil sera pool was used as the negative control.

Lentil extracts

Lentils (*Lens culinaris* syn. *esculenta* cv. Rubia Castellana) were purchased at a local market and carefully washed with distilled water. The seeds were then soaked for 1 hour in PBS buffer with 10 mmol/L ascorbic acid (1:5 wt/vol), ground, stirred for 18 hours at 4°C, and centrifuged (15,000*g* for 30 minutes at 4°C). The extract was dialyzed against distilled water (24 hours at 4°C; molecular weight cut-off point, <3500 d), filtered through a 0.45-μm filter (Millipore, Bedford, Mass), and lyophilized. Alternatively, the supernatant and precipitate after dialysis were separated by centrifugation (30,000*g* for 30 minutes at 4°C) and processed separately as described above.

Crude extract, as well as supernatant and precipitate after dialysis, from boiled lentils was obtained by a similar procedure, except that the samples were boiled for 15 minutes at 100°C after being soaked in PBS buffer.

Protein concentration in the crude and boiled lentil extracts was determined according to the method of Bradford.[16]

Isolation and characterization of allergens

The supernatant after dialysis of the crude extract from boiled lentils was used as starting material to purify allergens. The supernatant protein preparation (120 mg of protein) was fractionated by gel-filtration chromatography on a Sephacryl S200 column (2.5 × 85 cm; Pharmacia Biotech, Uppsala, Sweden) by using 0.1 mol/L ammonium acetate with 3 mol/L urea (pH 6.8) as the elution buffer (1 mL/min). After dialysis against water, fractions enriched in a 66-kd IgE-binding protein were further separated by cation-exchange chromatography on a Mono S HR 5/5 column (Pharmacia Biotech; 1.0 mL total volume) coupled to a fast-performance liquid chromatography system (Pharmacia Biotech). The column was equilibrated with 10 mmol/L sodium acetate (pH 5.4, buffer A) and eluted with a two-step linear salt gradient to 0.5 mol/L NaCl in the same buffer (buffer B; 0% to 50% of buffer B in 40 minutes and 50% to 100% of buffer B in 30 minutes; 0.5 mL/min). Fractions containing the 66-kd protein were pooled, freeze-dried, and rechromatographed on a Mono Q HR 5/5 anion-exchange column (Pharmacia Biotech; 1 mL of total volume). After equilibrating with 30 mmol/L ethanolamine (pH 9.5, buffer C), elution was carried out with a two-step salt linear gradient to 1.0 mol/L NaCl in the same buffer (buffer D; 0% to 50% of buffer D for 120 minutes and 50% to 100% of buffer D for 10 minutes; 0.5 mL/min).

To isolate 12- to 16-kd putative allergens, the appropriate gel-filtration fractions were fractionated by using reverse-phase (RP) HPLC on a Vydac-C4 column (2.2 × 25 cm; particle size, 10 μm; The Separations Group, Hesperia, Calif). Elution was performed with a 3-step linear gradient of acetonitrile in 0.1% trifluoroacetic acid (0% to 10% for 10 minutes, 10% to 50% for 190 minutes, 50% to 85% for 60 minutes, and 85% for 60 minutes; 1 mL/min).

Peaks containing the corresponding putative allergen in each chromatographic separation were identified by analyzing pooled fractions corresponding to each peak of the profiles by SDS-PAGE and immunodetection with the pool of sera from patients with lentil allergy. The chromatographic fractions and the isolated proteins were quantified by using the bicinchoninic acid test.[17]

SDS-PAGE was performed according to the method of Laemmli[18] on Bio-Rad Miniprotean II system gels (15% and 4% polyacrylamide in the separating and stacking gels, respectively).

N-terminal amino acid sequences were determined by standard methods by using an Applied Biosystems 477A gas-phase sequenator.

Enzymatic hydrolysis of the purified 66-kd protein was carried out by using endoproteinase Lys-C (EC3.4.99.30; Boehringer Mannheim, Mannheim, Germany). A 1:10 enzyme/protein ratio was used during 4 hours at 37°C in a 25 mmol/L TRIS-HCl with 1 mmol/L EDTA (pH 8.5) buffer. The resulting peptides were separated by RP-HPLC on a Nucleosil-C4 column (4.6 × 250 mm; particle size, 5 μm; Sugelabor, Madrid, Spain) by using a two-step linear gradient of acetonitrile in 0.1% trifluoroacetic acid (0% to 50% for 90 minutes and 50% to 100% for 30 minutes; 0.5 mL/min). The 3 major peaks obtained were subjected to N-terminal amino acid sequencing.

The presence of complex asparagine-linked glycans attached to lentil proteins was determined, as described by Diaz-Perales et al,[19] by using a previously tested specific serum[20] (kindly provided by Dr M. J. Chrispeels, University of California, San Diego) and goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma).

Immunodetection and immunoblot inhibition assays

Samples were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes, as previously reported.[19] After washing and blocking, membranes were incubated with a serum pool or individual sera from patients with lentil allergy (1:10 dilution) and then with alkaline phosphatase–conjugated monoclonal anti-human IgE (clone GE-1, Sigma; 1:500 dilution) and revealed by adding a 5-bromo-4-chloro-3 indoyl phosphate/nitro blue tetrazolium solution.

Immunoblot inhibition assays were performed as described above, except that the pool of sera was preincubated for 3 hours at room temperature with each of the inhibitors (purified low and high molecular size allergens from lentil and BSA as the negative control).

CAP inhibition assays

The assay was carried out in duplicate samples, and each one was tested in duplicate. The pool of sera (1:10 dilution) was premixed with the same volume of progressive dilutions of each inhibitor solution in PBS buffer and incubated for 1 hour at room temperature. After incubation, samples were centrifugated, lentil commercial Immuno-CAPs were then added, and specific IgE levels were assessed, according to the CAP System procedure. The boiled lentil extract and purified low- and high-molecular-weight allergens were used as inhibitors at concentration rates from 10 μg to 0.1 pg for each one. Amoxicillin was used as a negative control. Inhibitor-dilution relationships were calculated by plotting the mean inhibition values as a function of the log inhibitor dilution, and the assays were validated if the linear correlation coefficients were higher than 0.95.

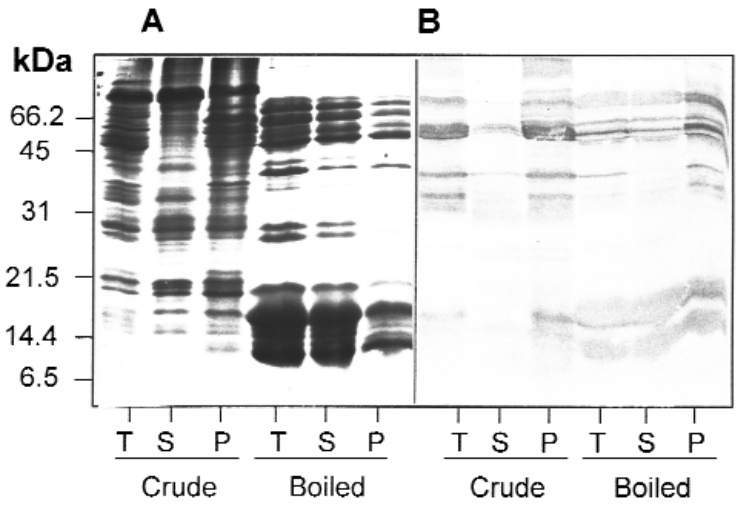


FIG 1. SDS-PAGE fractionation (15 μg of protein) of whole extract *(T)* and its supernatant *(S)* and precipitate *(P)* after dialysis against H_2O from crude and boiled lentils. Replica gels were stained with Coomassie blue **(A)** or electrotransferred to PVDF membranes and immunoblotted with a pool of sera from patients with lentil allergy **(B)**.

RESULTS

Whole extracts from crude lentils were obtained by using PBS buffer extraction and then separated by SDS-PAGE (Fig 1, *A*). A complex pattern of bands from 10 to 80 kd was shown. Most of these components appeared in the precipitate obtained after dialysis against water, thus indicating their globulin nature. Heat treatment of the lentil seeds produced drastic changes in the electrophoretic pattern, mainly a strong increase of low-molecular-weight bands of 12 to 16 kd and a decrease (or even disappearance) of bands in the 25- to 45-kd range. The 12- to 16-kd components, as well as major high-molecular-weight bands of 45 to 70 kd, were chiefly included in the supernatant obtained after dialysis of the whole extract from boiled lentils (Fig 1, *A*). A strong IgE-binding capacity by these two groups of bands was revealed when immunodetection with a serum pool from patients with lentil allergy was performed (Fig 1, *B*). Control sera from subjects not allergic to legumes did not react with any band of the lentil extracts tested.

To purify major IgE-binding proteins of both low and high molecular weights, the supernatant obtained after dialysis of the boiled lentil extract was fractionated by using gel-filtration chromatography (Fig 2, *A*). Fractions containing high-molecular-weight components that bound specific IgE (Fig 3, *A* and *B*) were further separated by using cation-exchange chromatography (Fig 2, *B*). A peak comprising mainly a 66-kd protein together with 45- to 50-kd contaminants was repurified with an anion-exchange column (Fig 2, *C*). The 3-step chromatographic procedure allowed us to obtain a highly enriched preparation of the 66-kd protein (Fig 3, *A*), hereafter referred to as protein H. The IgE-binding capacity of the protein was confirmed by specific IgE immunodetection (Fig 3, *B*).

Gel-filtration fractions containing IgE-binding components with low molecular weights (Fig 3, *C* and *D*) were subjected to RP-HPLC. Three protein preparations, proteins L1 to L3, were obtained by this method. Protein L3 was further rechromatographed by using the same RP-HPLC column to enrich it in the lower molecular weight component (Fig 3, *C* and *D*). Proteins L1 and L2 behaved as a single 16-kd band in SDS-PAGE, whereas protein L3 presented a major 12-kd band and a weak contaminant of 16 kd (Fig 3, *C*). All 3 proteins were clearly reactive when IgE was immunodetected with the serum pool from patients with lentil allergy (Fig 3, *D*).

N-terminal amino acid sequencing of the allergens was undertaken. The results obtained are summarized in Fig 4. Protein L1 rendered 3 different sequences (ie, DDDEE..., DDEE..., and DEE...) in a 1:1:1 ratio. In addition to the progressive shortening of the N-terminus, the 3 sequences were identical up to the 29 residue (Fig 4, *A*). The same was true for protein L2, except that a 1:1:4 ratio and just 14 residues were determined in this case. A single N-terminal sequence was produced by protein L3. The sequence data concerning L1 to L3 components indicate that they are related and also suggest that differential processing events could operate, affecting the N-terminus of proteins L1 and L2. Therefore protein L1 was chosen hereafter as the representative member of this allergen family from lentil seeds. A high similarity (25 of 29 identical residues with the 47-kd form; Fig 4, *B*) was found between the sequence of L1 protein and those corresponding to the γ-subunits of pea provicilins.[21] Interestingly, both the characteristics of the processing site between β- and γ-pea provicilin subunits[21,22] and the molecular size of the γ-subunit (16 kd) are fully in agreement with the N-terminal sequence and the size determined for protein L1.

No sequence was obtained for two different batches of purified protein H, indicating that the protein is probably N-terminal blocked. To gather sequence information, protein H was digested by endoproteinase Lys C. The resulting peptides were fractionated by RP-HPLC, and the 3

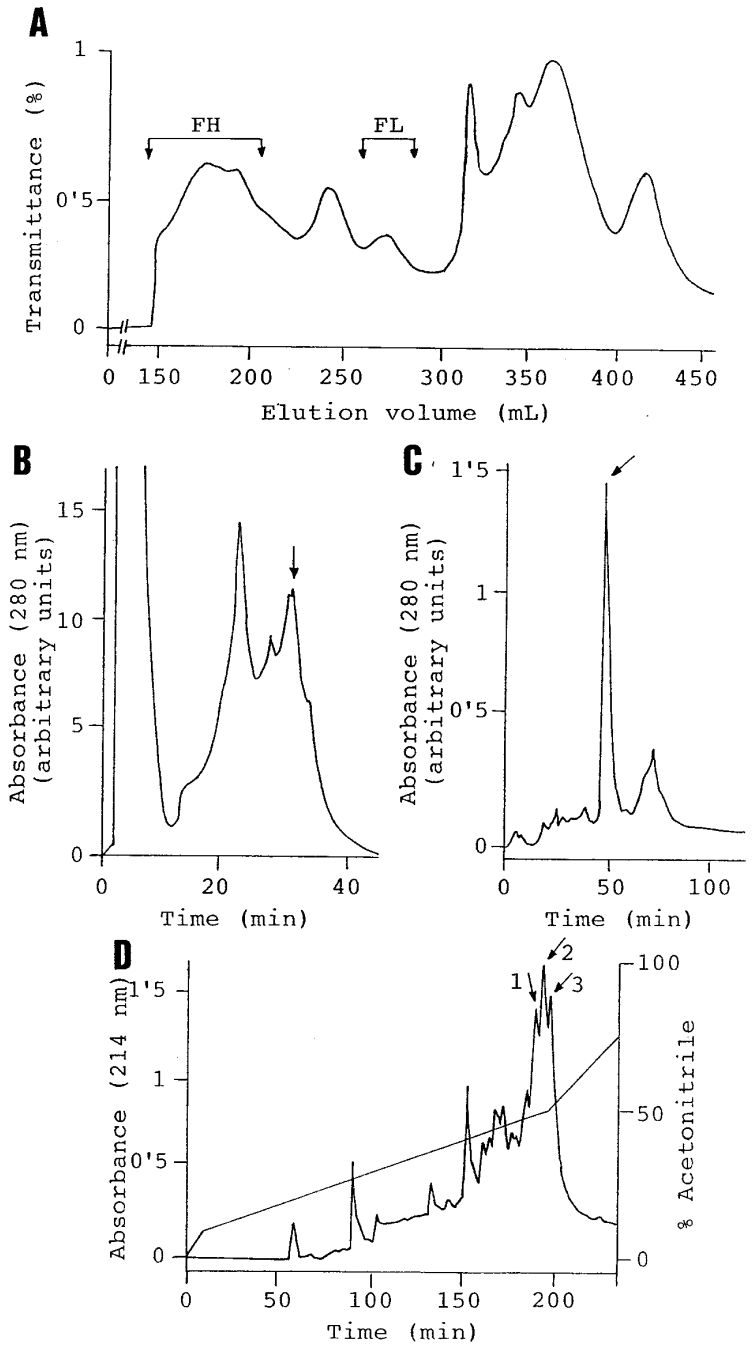


FIG 2. A, Gel filtration on Sephacryl S200 of the dialyzed supernatant of the extract from boiled lentils. Fractions further used to isolate high-molecular-weight *(FH)* and low-molecular-weight *(FL)* allergens are indicated. **B,** Cation-exchange chromatography on Mono S column of the gel-filtration high-molecular-weight fraction. **C,** Anion-exchange chromatography on Mono Q column of the proteins included in the peak marked in panel **B**. **D,** RP-HPLC separation of the gel-filtration low-molecular-weight fraction. Elution peaks of the purified high-molecular-weight **(C)** and low-molecular-weight **(D)** allergens are indicated.

major peptides were subjected to automatic sequencing (Fig 4, *C*). The 3 sequences obtained (42 overall residues) can be very well matched (35 of 42 identical residues) with 3 different sequence fragments of the major seed biotinylated protein from pea.[23] These data, together with the same apparent molecular weight of both proteins (around 65 kd), allowed us to identify protein H as the homologous biotionylated protein from lentil seeds.

Neither proteins L1 to L3 nor protein H reacted with a specific serum to N-linked complex glycans (not shown).[20]

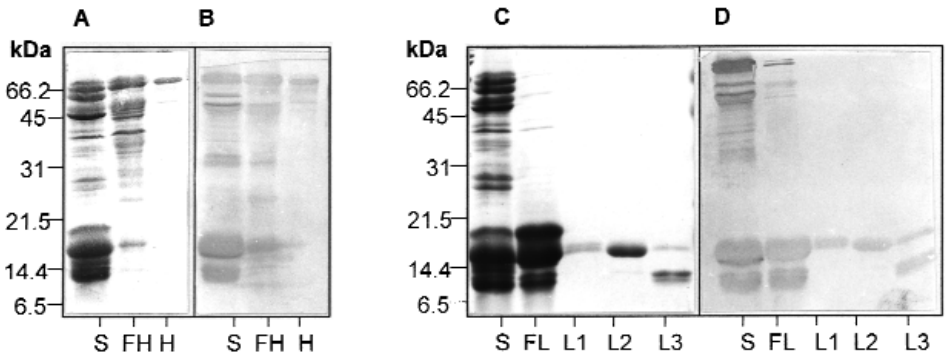


FIG 3. SDS-PAGE fractionation of the dialyzed supernatant of the extract from boiled lentils (*S*; 15 μg of protein); gel-filtration high-molecular-weight *(FH)* and low-molecular-weight *(FL)* fractions shown in Fig 2, *A* (10 μg of protein); and purified high-molecular-weight *(H)* and low-molecular weight *(L1-L3)* allergens (1 μg of each). Gels were stained with Coomassie blue (**A** and **C**) or electrotransferred to PVDF membranes and immunoblotted with a pool of sera from patients with lentil allergy (**B** and **D**).

A

Allergen	N-terminal amino acid sequence (1 … 29)
L1	D D D E E E E Q E E E T S K Q V Q R Y R A K L S P G D V F
L2	D D D E E E E Q E E E T **T** K
	G
L3	D E E E E Q E E E T **T** K Q V
	G

B

Allergen/protein	1 … 29 / 306 … 339
L1	D D D E E E E Q E E E T S K Q V Q R Y R A K L S P G D V F
Provicilin 47 kd	G K **E** **N**▲D **K** E E E Q E E E T S K Q V Q **L** Y R A K L S P G D V F V I
Provicilin 50 kd	R E **E** D D E E E E Q^R E E E T **K** **N** Q V **S** **Q** Y **K** A K L **T** P G D V F V I

C

Allergen/protein	326 … 441
H peptides	V A S V A G W T A … A A A N A V E G A V
SBP65	K E **K** A **T** V A G W T A S H Y A T Q L T V D G T R A A A N A V E G A V
H peptides	G Y V A P K
SBP65	G Y V A P K A S L A A K S V E T V K G L A A S A G E T A K E F T A R
H peptides	… V I
SBP65	K K E E S W R E Y E A K R A S Q L Q E G E E I L P S T G G I G K V **L**
H peptides	P S E E R I Q A E G T N H Q E
SBP65	P S **G** E R **T** Q A **Q** G T N **L** Q E K V

FIG 4. A, N-terminal amino acid sequences of low-molecular-weight allergens L1, L2, and L3. Heterogeneity in position 7 of L2 and position 5 of L3 (E/G in both cases) is indicated. **B,** Alignment of the N-terminal sequence of L1 with those of internal fragments of provicilins from pea *(Pisum sativum). Triangle,* β-γ Subunits processing site of the 47-kd provicilin. **C,** Alignment of 3 major endoproteinase Lys-C peptides of the high-molecular-weight lentil allergen H with that of the major biotinylated protein from pea seeds (residues 326-441).

The sequence and molecular size data described above indicate a close relationship among proteins L1 to L3 but not protein H. These facts were supported by immunoblot inhibition assays against the corresponding lentil extract by using the isolated proteins as inhibitors (Fig 5). Protein H fully inhibited the IgE binding by the 66-kd band of the extracts without affecting the reactivity of the 12- to 16-kd components. The opposite was roughly true when protein L1 was tested as an inhibitor.

Finally, two different types of experiments were performed to evaluate the relevance of the purified proteins as potential lentil allergens. IgE immunodetection with each of the 22 individual sera included in the serum pool showed a positive response in 68% (15/22) for protein L1 and 41% (9/22) of sera for protein H (not shown). CAP inhibition assays with a commercial crude lentil extract as the solid phase (Fig 6) rendered progressive inhibition values up to 80% for the boiled

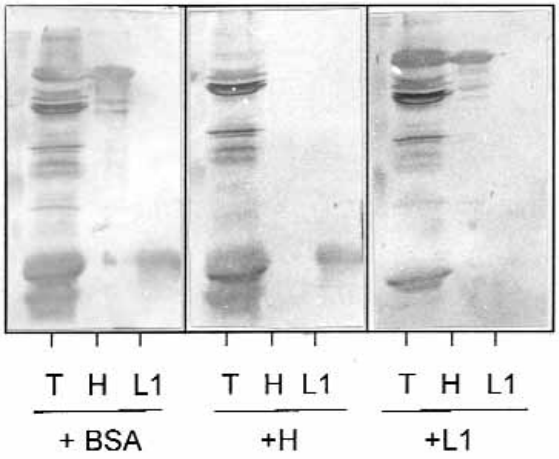


FIG 5. Immunoblot inhibition assays with purified allergens H and L1. Whole extracts from boiled lentils (*T*; 15 μg of protein) and isolated allergens H and L1 (1 μg of each) were separated by using SDS-PAGE, electrotransferred to PVDF membranes, and immunodetected with a pool of sera from patients with lentil allergy preincubated with isolated allergen H *(+H)*, L1 *(+L1)*, or BSA *(+BSA)* as a negative control. Final amounts of 3 μg of each inhibitor per gel lane were used.

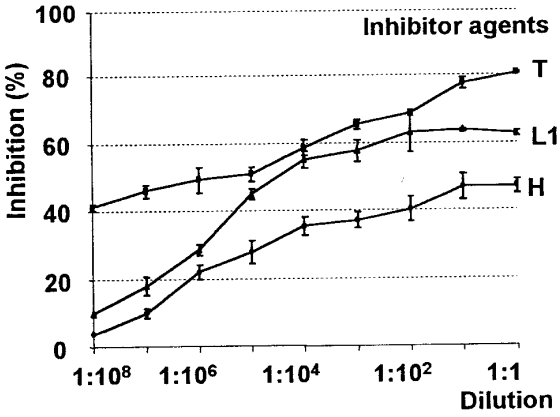


FIG 6. CAP inhibition assays of crude lentil by boiled lentil extract *(T)* and purified proteins L1 and H. A pool of sera from patients with lentil allergy was preincubated with progressive 1:10 dilutions of each inhibitor solution (10 μg/mL initial concentration). Values are means (n = 3), and *bars* indicate SE. No significant inhibition (<5%) was obtained by using amoxicillin as a negative control.

lentil extract and 64% and 45% for purified proteins L1 and H, respectively.

DISCUSSION

Comparison of the SDS-PAGE patterns of crude and heated lentils indicates that boiling produces substantial changes in the extractability of these proteins. An enrichment in 12- to 16-kd polypeptides identified as γ-vicilin subunits and a depletion in several components of 25 to 45 kd (some of them probably being vicilin-related proteins according to their solubilities and molecular sizes[13]) were the most prominent effects produced by heating. A diminished extractability from heated seeds has been recently described for Ara h 1, the major vicilin and allergen from peanut.[24] Vicilins represent one of the most abundant seed storage proteins in many legumes.[13] They are typically trimeric proteins of 150 to 190 kd that lack disulphide bonds. Their subunit composition varies considerably among legume species because of differences in the posttranslational processing by proteolysis and glycosylation of the initially synthesized polypeptide chains of around 50 kd. Thus in peas, some of these initial chains are processed to smaller polypeptides (α-, β-, and γ-subunits) of 12.5 to 33 kd. By contrast, in beans glycosylation is more extensive but proteolysis does not occur, and the processed subunits show molecular sizes in the 43- to 53-kd range.

Two different types of allergens have been characterized from boiled lentils. One type comprises proteins L1 and L2 of 16 kd and protein L3 of 12 kd, which are members of the same family according to their structural and immunochemical relationships. These allergens were identified as γ-vicilin subunits, showing protein L1 to have 86% of identical residues with the homologous subunit from pea throughout the determined sequence. The N-terminal fragment of proteins L1 to L3 includes an initial aspartic acid (D), followed by a stretch of 9 to 13 highly polar amino acids, predominantly acidic residues (mostly glutamic acid, E). Both features have been described as sequence characteristics of the β-γ processing sites of the 47- to 50-kd provicilins from pea.[21,22] Furthermore, the presence of one to three aspartic acid residues in the N-terminus of proteins L1 and L2 can be explained by an alternative cleavage in 3 different positions during the posttranslational processing. In addition to an N-terminal cleavage, a removed fragment from the C-terminal has been also found in some pea vicilin subunits.[13,21] This event could be responsible for the molecular size differences between proteins L1-L2 and L3. A band of around 50 kd, which strongly bound IgE (Figs 3 and 5), could represent the lentil provicilin (precursor of proteins L1-L3). Both its molecular size and reactivity are compatible with this hypothesis.

The L1 to L3 protein family seems to represent the main IgE-binding group in boiled lentil extracts. The relevance of protein L1 in lentil allergy was supported by its high percentage of recognition by individual sera from patients with lentil allergy (68%) and by its inhibitory capacity (64%) of IgE binding by commercial lentil CAPs. These properties led us to propose renaming it as Len c 1, according to the allergen nomenclature. Vicilin-like proteins have also been identified as allergens in peanut (Ara h 1), soybean, and walnut.[8,11,25] The determined sequence for Len c 1 shows a high degree of similarity (45% of identical residues) with the corresponding stretch of Ara h 1, including one of the IgE epitopes located in the peanut allergen.[8] The data discussed above regarding Len c 1 can be significant to study cross-reactions among legume foods and to establish molecular models for the manipulation of legume allergens by protein engineering.

The second type of allergen isolated from boiled lentils corresponds to protein H, an IgE-binding component of 66 kd. The purified protein was recognized by 41% of individual sera from allergic patients and inhibited the IgE binding of commercial lentil extracts by 45%. Protein H seems to be N-terminal blocked, but the isolation and sequencing of internal peptides allowed us to identify it as a seed-specific biotinylated protein, according to its close identity (83%) with a previously described pea biotinylated protein, SBP65.[23] Putative pea SBP65 homologs have been detected in soybean, castor bean, and carrot.[26] No protein belonging to this family had been previously described as a legume allergen. We suggest renaming protein H as Len c 2 according to the allergen nomenclature.

The identification of Len c 1 and Len c 2 may help in future studies on cross-reactions among legume foods, the effects of cooking on seed allergenicity, and the relationships among allergens from peanut, soybean, lentil, pea, and chickpea.

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