

Len c 1, a major allergen and vicilin from lentil seeds: Protein isolation and cDNA cloning

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Background: Lentils are among the main plant foods causing allergic reactions in pediatric patients in the Mediterranean area and in many Asian communities. However, very few reports have been devoted to identifying lentil allergens. Seed storage proteins of the vicilin family have been characterized as major allergens in several seed legumes and tree nuts.

Objective: We sought to evaluate the role of lentil vicilins as food allergens.

Methods: A serum pool and individual sera from 22 patients with lentil allergy were used in different IgE-binding assays. Mature lentil vicilin was isolated by means of cation-exchange chromatography, followed by reverse-phase HPLC, and characterized by means of N-terminal amino acid sequencing, matrix-assisted laser desorption/ionization mass spectrometry (MALDI) analysis, complex asparagine-linked glycan detection, specific IgE immunodetection with individual sera, and ELISA inhibition assays. Complete cDNAs encoding lentil vicilin variants were isolated by means of PCR with primers based on the amino acid sequence of the allergen.

Results: A major IgE-binding component of approximately 50 kd was detected in lentil extracts. This component was isolated and characterized, showing a single N-terminal amino acid sequence homologous to those of legume vicilins and a broad peak (maximum at 48,613 d) in MALDI analysis. The purified allergen was recognized by 77% (17/22) of the individual sera from patients with lentil allergy and reached up to 65% inhibition of the IgE binding to the crude lentil extract. The allergen showed 3 isoforms varying in their degree of N-glycosylation. Two cDNA clones encoding different allergen variants were isolated. The amino acid sequences deduced from both clones (415 and 418 residues; 47.4 and 47.8 kd) showed greater than 50% identity with major peanut (Ara h 1) and soybean (conglutinin subunits) allergens belonging to the vicilin family. Furthermore, these sequences included those of the previously characterized lentil allergen Len c 1.02 (108 amino acid residues of the C-terminal domain) and those of a novel lentil IgE-binding protein of 26 kd.

Conclusion: The mature 48-kd lentil vicilin, designated Len c 1.01, is a major allergen. Two of its processing fragments, corresponding to subunits of 12 to 16 kd (previously named Len c 1) and 26 kd, are also relevant lentil IgE-binding proteins. The sequence homology of Len c 1.01 to those of major allergens from peanut, soybean, walnut, and cashew can help to investigate potential cross-reactions among these plant foods. (*J Allergy Clin Immunol* 2003;112:1208-15.)

Key words: Food allergy, lentil, vicilin, major allergen, cDNA cloning, Ara h 1, vicilin subunits

In the Mediterranean area and many Asian countries, lentils and chickpeas are among the most frequent foods associated with IgE-mediated hypersensitivity reactions, particularly in pediatric patients.¹⁻³ Thus 10% of children with food allergy have a convincing clinical history of allergy to lentils in the Spanish population.⁴ A high proportion (approximately 20%) of the subjects allergic to these legumes present with severe and systemic symptoms,¹⁻⁵ although isolated cutaneous reactions are most common. Moreover, several cases of bronchial asthma or anaphylaxis induced by inhaling vapors from cooking or by eating lentils or chickpeas have been reported.⁶⁻⁸ Cross-reactivity among lentil, chickpea, and pea has been detected.^{1-3,5} In other areas, such as the United States, the United Kingdom, and Japan, peanut and soybean are the 2 major legumes involved in food allergy, with lentils and chickpeas rarely associated with allergic reactions.^{9,10}

The major allergens from soybean, and particularly from peanut, have been extensively studied. Among them, Ara h 1, a 65-kd peanut glycoprotein belonging to the vicilin family, is one of the best-characterized plant food allergens at present.^{11,12} Interestingly, members of the vicilin family from other species, such as soybean,¹³ English walnut,¹⁴ cashew,¹⁵ and sesame,¹⁶ have been identified as relevant allergens. Vicilins are major seed storage proteins in most legume seeds. Mature polypeptides ranging from 40 to 70 kd and usually glycosylated are associated in vivo, forming trimers of 150 to 190 kd. However, different patterns of posttranslational processing, both by means of proteolysis and glycosylation, lead to a wide variation in the subunit composition of the oligomers.^{17,18} Several vicilins have been shown to be resistant to digestion and food processing, thus strengthening their role as potential allergenic proteins.¹⁹ Primary structure and conformational similarity among vicilins from different species suggest a putative role of this protein family as plant panallergens, but a strong link

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Abbreviations used

MALDI: Matrix-assisted laser desorption/ionization mass spectrometry

PVDF: Polyvinylidene difluoride

between clinical cross-reactivity and sequence identity does not seem to be established.¹⁹

Contrary to peanut and soybean, the research on lentil and chickpea allergens is surprisingly rare. No chickpea allergen has been identified, and only 2 lentil allergens have been isolated.²⁰ One of them, termed Len c 1 (hereafter Len c 1.02), corresponds to the γ -subunit (approximately 12-16 kd) of lentil vicilin, which is probably produced by means of posttranslational proteolytic processing of initially synthesized polypeptide chains of approximately 50 kd.¹⁸ In this context it should be mentioned that uncharacterized bands of approximately 50 kd, which bound IgE from greater than 70% of individual sera from allergic patients, have been detected in lentil extracts.^{1,5} We report on the purification and characterization of the protein responsible for such bands, as well as the isolation of its corresponding cDNA. This protein is a major lentil allergen identified as the mature vicilin chain precursor of Len c 1.02.

METHODS

Patients and sera

Twenty-two patients were selected from a population with allergy to legumes during a prospective study performed by the Servicio de Alergia of Hospital Infantil La Paz (Madrid, Spain). Informed consent was obtained from all participants. The selection was carried out on the basis of a convincing clinical history of allergic reactions after lentil ingestion, confirmed on the basis of either anaphylaxis or a positive open food challenge response, a positive skin prick test response, and high specific serum IgE levels (>3.90 kU/L) to lentils. Skin prick tests were performed by using standard methods, and IgE levels were quantified with the CAP-FEIA System (Pharmacia Diagnostic). Clinical and demographic data of the selected patients are summarized in Table I.

A serum pool (patients 1-17; specific IgE to lentil, 83 kU/L) or individual sera were used in immunodetection and ELISA inhibition assays. A pool of sera from 5 patients allergic to fish but not to plant foods or pollens was tested as a negative control.

Lentil extracts

Mature lentil seeds (*Lens culinaris* cultivar Guareña) were ground, defatted with cold acetone ($2 \times 1:10$ [wt/vol]) for 1 hour at 4°C, dried, and extracted with PBS buffer (0.1 mol/L sodium phosphate [pH 7.4] and 0.15 mol/L NaCl; $1 \times 1:5$ [wt/vol]) for 1 hour at 4°C. After centrifugation (10,000 rpm for 30 minutes at 4°C), the supernatant was dialyzed (cut-off point, 3.5 kd) against H₂O and freeze-dried. The extract from boiled lentils was obtained by means of the same protocol, except that seeds were previously boiled for 30 minutes in an H₂O bath and then freeze-dried.

Protein contents in both lentil extracts were determined according to the method of Bradford.²¹

Isolation of Len c 1.01

Len c 1.01 (the 48-kd lentil vicilin) was isolated from the crude lentil extract, redissolved in 0.5 mol/L NaCl, and salted out with

ammonium sulfate at 85% saturation (2 hours at 4°C). After centrifugation, the supernatant was dialyzed against H₂O and freeze-dried and then fractionated by means of anion-exchange chromatography on a Mono Q HR 5/5 column (Amersham Biosciences; 1.0-mL total volume) by using a linear gradient to 1 mol/L NaCl in 20 mmol/L ethanolamine (pH 9.0; 0%-100% in 120 minutes). The Len c 1.01 enriched fractions were further purified by means of reverse-phase HPLC on a Vydac-C4 column (2.2×25 cm; particle size, 10 μ m; The Separations Group), eluting with a 2-step linear gradient of acetonitrile in 0.1% trifluoroacetic acid (30%-40% in 30 minutes and 40%-85% in 220 minutes, 1.0 mL/min). Peaks containing Len c 1.01 in each chromatographic profile were identified by means of SDS-PAGE and immunodetection with the pool of sera from patients with lentil allergy. The isolated allergen, as well as the chromatographic fractions, were quantified by means of the bicinchoninic acid test.²²

SDS-PAGE was carried out according to the method of Laemmli²³ on Bio-RAD MiniProtein II System gels (15% polyacrylamide). Two-dimensional electrophoresis (isoelectrofocusing, ampholytes pH 5-7 $\times$ SDS-PAGE) to analyze purified Len c 1.01 (10 μ g) was performed on a Bio-RAD MiniProtein II 2-D cell, as described in the manufacturer's instructions.

N-terminal amino acid sequencing

N-terminal amino acid sequences were determined by using standard methods with an Applied Biosystems 477 A gas-phase sequencer, analyzing either a solution of the isolated allergen (Len c 1.01) or the SDS-PAGE band corresponding to the 26-kd fragment of Len c 1.01 electrotransferred to a polyvinylidene difluoride (PVDF) membrane.

Matrix-assisted desorption/ionization mass spectrometry analysis

The laser desorption/ionization analysis was performed on a BIFLEX III time-of-flight spectrometer (Bruker-Franzen Analytik). Samples were analyzed in the linear mode, and typically, 100 laser shots were summed into a simple mass spectrum. External calibration was performed with BSA as the standard. A saturated solution of sinapinic acid in acetonitrile/H₂O (1:2) with 0.1% trifluoroacetic acid was used as the matrix. Both the sample and the matrix solution were spotted onto the target and dried at room temperature.

Detection of complex asparagine-linked glycans

Purified Len c 1.01 was subjected to SDS-PAGE and then electrotransferred to PVDF membrane (see below). Blocked membrane was incubated with rabbit antisera to complex asparagine-linked glycans of plant glycoproteins²⁴ (1:80,000 dilution; kindly provided by Dr M. J. Chrispeels, University of California, San Diego), then with alkaline phosphatase-conjugated anti-rabbit IgG (1:500 dilution, Sigma), and finally with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution.

Immunodetection and immunoblot inhibition assays

Samples (20 μ g of protein of lentil extract or 3 μ g of purified Len c 1.01) were fractionated by means of SDS-PAGE and then electrotransferred onto PVDF membranes, as previously reported.²⁵ After washing and blocking, membranes were incubated with a serum pool or individual sera from patients with lentil allergy or with control sera (1:10 dilutions) and then with alkaline phosphatase-conjugated monoclonal anti-human IgE (clon GE-1, Sigma; 1:500 dilution) and revealed by adding 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution.

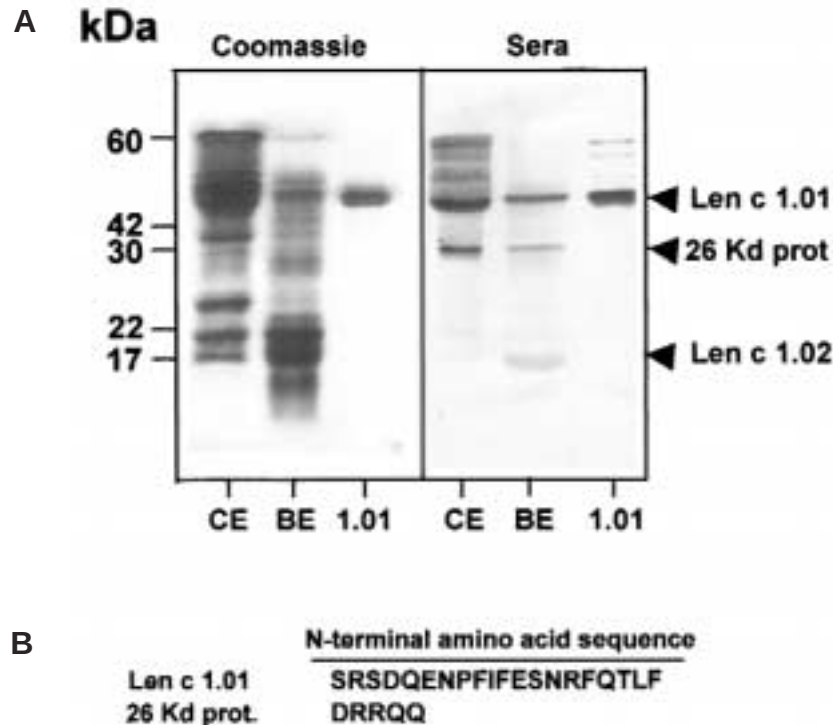


FIG 1. A, Protein staining (Coomassie) and IgE immunodetection (Sera) of crude (CE) and boiled (BE) extracts from lentil seeds and purified Len 1.01 (1.01). SDS-PAGE bands corresponding to Len c 1.01, the 26-kD protein, and Len c 1.02 are indicated. **B,** N-terminal amino acid sequences of purified Len c 1.01 and the 26-kD protein band.

Immunoblot inhibition assays were performed by using the same method, except that the serum pool was preincubated for 3 hours at 25°C with 7 µg/mL of each inhibitor (purified Len c 1.01, or BSA as negative control).

ELISA inhibition assays

Assays were carried out by using a method previously described²⁶ with a serum pool from patients with lentil allergy. Wells were coated with 50 µL of crude lentil extract at 5 µg/mL, blocked with 1% BSA, and then treated with 25 µL of the serum pool (1:9 dilution) previously preincubated with 25 µL of serial dilutions of the same lentil extract (range, 10.0-0.04 µg/mL) or of purified Len c 1.01 (range, 2.0-0.008 µg/mL) for 3 hours at 25°C. A peroxidase-labeled anti-human IgE (Dako A/S) and a peroxidase substrate buffer (Dako, code S2045) were finally used. PBS buffer with 1% BSA was tested as a negative control. All tests were performed in triplicate.

Isolation of cDNAs encoding Len c 1.01

Total RNA from mature seeds of lentil was obtained by using the method of Chang et al.²⁷ The reverse transcription was performed with 5 µg of total RNA by using the primer 5'-TCGACTCTAGAGATCCGAATTCAAGC(T)₁₅-3' and the First-strand cDNA Synthesis Kit (Amersham Biosciences).

The cDNA obtained was amplified by means of PCR with the oligonucleotide 5' primer 5'-CCTCGAATTCTCTAGATCCGATCAAGAGAACCCC-3' (the *Eco*RI restriction site is underlined), which is designed from the N-terminal amino acid sequence of Len c 1.01 and the 5' nucleotide sequence of a cDNA encoding pea vicilin (accession no. X67429). For the reverse direction, the 3' primer 5'-CTGCAGGTCGACTCTAGAGGATCC-3' (complementary to that used for reverse transcription) was applied. The amplification was carried out with Pwo polymerase (Roche Molecular Bio-

chemicals), as recommended by the manufacturer. The PCR program was 3 minutes of denaturation at 94°C, followed by 10 cycles each of 20 seconds of denaturation at 94°C, 30 seconds of annealing at 65°C, and 2 minutes of extension at 72°C; 10 cycles each of 20 seconds of denaturation at 94°C, 30 seconds of annealing at 65°C, and 2 minutes of extension at 72°C (increasing 5 seconds per cycle); and final extension for 7 minutes at 72°C.

The PCR products were digested with *Eco*RI and then inserted into pUC18 vector previously digested with *Eco*RI and *Hind*III. These constructs were used to transform *Escherichia coli* XL1-Blue cells, and the DNAs from several clones were sequenced by using the ABI PRISM Dye Terminator Kit and ABI 3100 sequencer (Perkin-Elmer Biosystem).

The 5' coding sequences of the cDNAs corresponding to Len c 1.01 variants were confirmed by using standard methods of 5' rapid amplification of cDNA ends and the Marathon cDNA Amplification Kit (Clontech).

RESULTS

The SDS-PAGE patterns of PBS extracts from crude and boiled lentils showed a complex mixture of protein components from 10 to 80 kD (Fig 1, A). Lentil seed boiling produced a strong increase of bands of less than 25 kD and a decrease of components of greater than 50 kD, which is in accordance with previous reports.²⁰ Immunodetection with a serum pool from patients with lentil allergy (Fig 1, A) revealed a major IgE-binding band of approximately 50 kD in both lentil extracts. In addition, main reactive bands of 26 and 16 kD were detected, with the latter especially shown in the boiled extract.

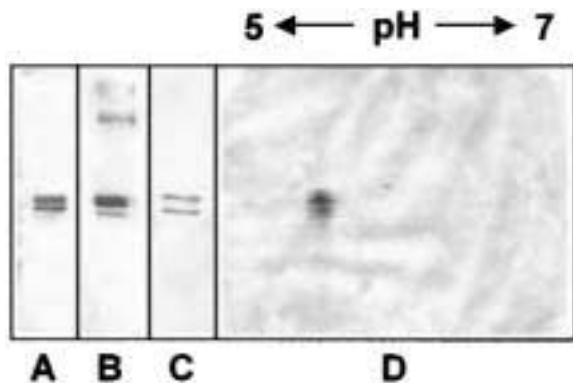


FIG 2. Analysis of purified Len c 1.01 after SDS-PAGE separation by means of protein staining (A), IgE immunodetection (B), and antibodies against complex asparagine-linked glycans (C). D, Two-dimensional separation and protein staining of purified Len c 1.01.

The 50-kD component, termed Len c 1.01, was isolated from the crude extract by means of differential precipitation with ammonium sulfate, followed by anion-exchange chromatography and reverse-phase HPLC on a Vydac-C4 column (see the “Methods” section). The isolated component behaved as a single wide band in SDS-PAGE and was strongly recognized by IgE from the serum pool from patients with lentil allergy (Fig 1, A). Moreover, immunodetection with 22 individual sera from patients with lentil allergy (Table 1) showed a positive response in 77% (17/22) for isolated Len c 1.01. Purified Len c 1.01 exhibited a single N-terminal amino acid sequence in its first 20 residues (Fig 1, B), which is homologous to the corresponding sequence region of other allergenic vicilins (see below). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) analysis showed a broad peak, with a maximum at a molecular mass of 48,614 d and a shoulder at 49,712 d (not shown). A further analysis of purified Len c 1.01 with 10% instead of 15% polyacrylamide gels (Fig 2, A) allowed us to resolve 3 different SDS-PAGE bands. All 3 bands reacted with the pool of sera from patients with lentil allergy (Fig 2, B), and 2 of them were recognized by rabbit polyclonal antibodies against complex asparagine-linked glycans of plant glycoproteins (Fig 2, C).²⁴ Two-dimensional electrophoresis (isoelectrofocusing × SDS-PAGE) confirmed the presence of 3 Len c 1.01 variants with similar isoelectric points around 5.5 (Fig 2, D).

A preliminary identification of the additionally reactive band of 26 kD (Fig 1, A; lane BE, sera) was carried out. The 26-kD protein from enriched anion-exchange chromatographic fractions was electrotransferred to PVDF membranes and subjected to N-terminal amino acid sequencing (Fig 1, B). The sequence obtained corresponded to that of an internal region (residues 186-190) deduced from cDNAs encoding Len c 1.01 variants (see below). The potential relationship among Len c 1.01 and the 16- and 26-kD reactive components was supported by immunoblot inhibition assays (Fig 3). A full IgE-binding inhibition to the 26- and 16-kD bands, as well as to components with apparent mo-

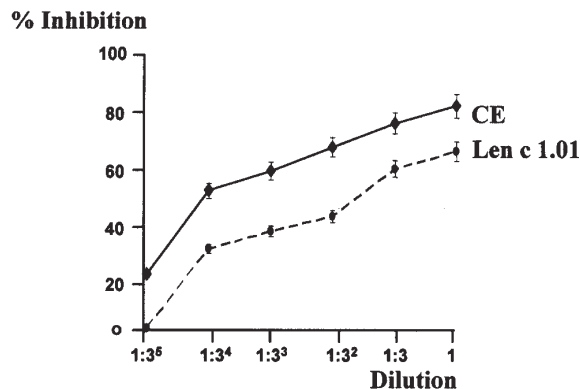


FIG 4. ELISA inhibition with crude lentil extract in the solid phase and the same extract (CE) and purified Len c 1.01 as an inhibitor. Means (n = 3) and SEs (bars) are represented.

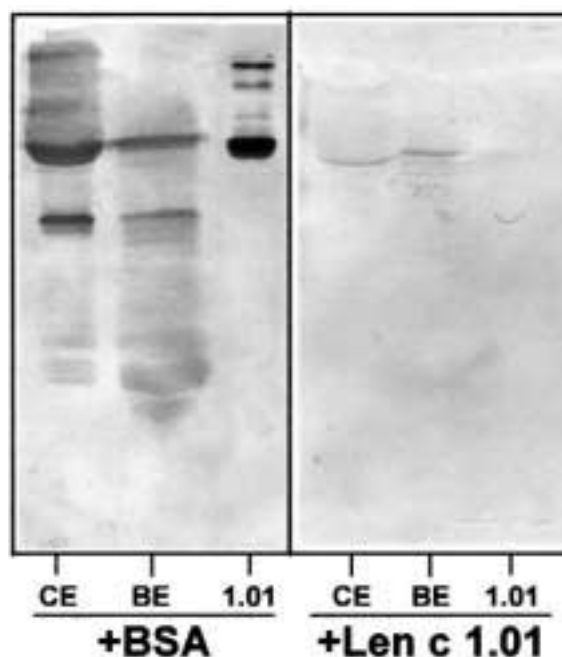


FIG 3. Immunoblot inhibition assays with BSA (negative control) and purified Len c 1.01. Crude (CE) and boiled (BE) lentil extracts (20 µg of protein) and purified Len c 1.01 (3 µg) separated by means of SDS-PAGE and electrotransferred to PVDF membranes were immunodetected with a serum pool from patients with lentil allergy preincubated with each inhibitor (7 µg/mL serum).

lecular sizes greater than that of Len c 1.01, was obtained by using purified Len c 1.01 as an inhibitor.

The relevance of Len c 1.01 as a lentil allergen was also highlighted by ELISA inhibition testing (Fig 4) with the crude lentil extract in the solid phase. The purified allergen reached progressive inhibition values of up to 65%.

To obtain further data on the complete primary structure of Len c 1.01 and its relationship with Len c 1.02 (previously named Len c 1²⁰), the 26-kD reactive protein and other allergenic members belonging to the vicilin family, nondegenerated oligonucleotide primers, were designed for PCR amplification of Len c 1.01 cDNAs.

L1.0101	tct aga tcc gat caa gag aac ccc ttt atc ttt aag tct aat agg ttt caa act att tat	60
L1.0102	a c c a	60
L1.0101	gag aat gaa aac ggt cat att cgt ctc ctc caa aga ttc gac aag cgt tcc aaa ata ttt	120
L1.0102	c g a t a t	120
L1.0101	gaa aat ctt caa aac tac cgt ctt ttg gaa tat aag tcc aaa cct cac acc att ttt ctt	180
L1.0102	t t a a t c	180
L1.0101	cca caa ttc acc gat gct gac ttc atc ctc gta gtc ctt agt gga aaa gcc att ctc aca	240
L1.0102	g a a c t t t g	240
L1.0101	gtg ttg aac tcc aat gat cga aac tcc ttc aat ctt gaa cgt ggt gat acc atc aaa ctc	300
L1.0102		300
L1.0101	cca gct ggc act att gct tat ttg gct aac cga gat gac aac gag gat ctc aga gta tta	360
L1.0102	t t	360
L1.0101	gat ctt gcc atc cca gta aac aga cca ggt caa ttg cag tct ttc tta tta tct gga act	420
L1.0102	c t ac c a g g	420
L1.0101	caa aat caa ccg tca ttc tta tct ggg ttc agc aag aat att cta gag gct gca ttc aat	480
L1.0102	t a g t	480
L1.0101	acc gaa tac gag gag ata gaa aag gtt ctc tta gaa gag cag gaa caa aag tca caa cac	540
L1.0102	t a t c g	540
L1.0101	cga aga agc ctt agg gat aag aga cag gag atc acc aat gaa gat gta ata gtc aaa gta	600
L1.0102	c c g a a a	600
L1.0101	tca agg gaa caa att gag gaa ttg agc aaa aat gca aag tcc agc tcg aaa aaa agt gta	660
L1.0102	a c g t c	660
L1.0101	tca tcg gaa tct gaa cca ttc aac ttg aga agt cgc aat cct atc tat tcc aat aaa ttt	720
L1.0102	c a c g	720
L1.0101	gga aaa ttc ttt gag atc acc ccg gag aaa aat cca caa ctt caa gac ttg gac ata ttt	780
L1.0102	c a	780
L1.0101	gtc aat tct gtg gag atc aag gag gga tct tta ttg tta cca aac tac aac tca agg gcc	840
L1.0102	t a g t	840
L1.0101	atc gtg ata gta act gtt aat gaa gga aaa gga gat ttc gaa ctt gtg ggt caa aga aat	900
L1.0102	t t t	900
L1.0101	gag aac caa caa gaa cag aga gaa gaa aat gat gag gaa gag gga caa gaa gaa gag aca	960
L1.0102	g --- --- --- c t a	951
L1.0101	acc aaa caa gtg caa agg tat aga gct aga ttg tct cca ggt gat gtt ctt gtg att cca	1020
L1.0102	g c g ag t g	1011
L1.0101	gca ggt cat cca gtt gcc ata aat gcc tcc tca gat ctc aat cta att gga ttt ggt atc	1080
L1.0102		1071
L1.0101	aat gcc aaa aac aat cag aga aac ttt ctg gca ggc gag gag gac aat gtc ata agc caa	1140
L1.0102		1131
L1.0101	ata cag aga ccg gtg aaa gag ctt gca ttt cct gga tct tct cgt gag gtt gat agg ctc	1200
L1.0102		1191
L1.0101	cta acg aat caa aaa cag tca cac ttt gca aat gct caa cct ctg caa ata gag taa	1257
L1.0102		1248

FIG 5. Nucleotide sequences of coding regions for the mature allergen variants Len c 1.0101 and Len c 1.0102. Only sequence differences between both cDNAs L1.0101 and L1.0102 are shown. Deletions are indicated (-).

The primers were deduced from the N-terminal amino acid sequence of Len c 1.01 (Fig 1, B), as well as from the nucleotide-encoding sequence of pea vicilin (accession no. X67429). Isolation and sequencing of selected

PCR products allowed us to identify 2 different cDNAs (Lc 1.0101 and Lc 1.0102) that encoded Len c 1.01 genetic variants (Figs 5 and 6). Lc 1.0101 (accession no. AJ551424) and Lc 1.0102 (accession no. AJ551425)

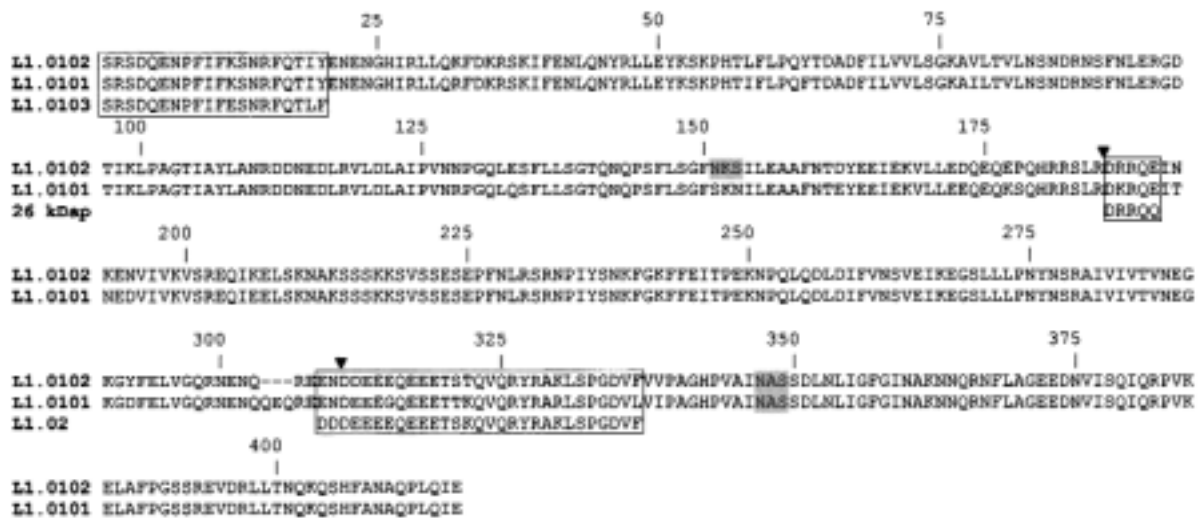


FIG 6. Deduced amino acid sequences for Len c 1.0101 and Len c 1.0102 aligned with N-terminal sequences of Len c 1.0103, the 26-kd reactive protein, and Len c 1.02.²⁰ Potential N-glycosylation sites are shaded. Subunit processing sites of the 47-kd pea provicilin are indicated by triangles.

TABLE I. Clinical and demographic data of selected patients with lentil allergy

Patient no.	Age (y)/sex	Symptoms*	Total IgE (kU/L)	Lentil-specific IgE (kU/L)	Specific IgE to Len c 1.01	Sensitization to other legumes†
1	3/F	OAS	710	3.95	+	cp, pe, sb
2	4/M	URT	168	30.40	+	cp, pe, sb, pn
3	28/F	URT	82	7.79	+	pn
4	46/F	URT	160	4.37	–	wb
5	19/F	A	226	9.39	+	pn
6	10/F	URT, AD	1274	8.08	–	pn
7	3/M	URT, AD	296	5.67	+	cp, pe, pn, wb
8	7/M	URT, AD	416	>100	+	cp, sb, pn, wb
9	3/M	URT, AD	332	7.38	–	cp, pe, sb, pn, wb
10	5/M	URT	880	22.10	+	cp, pe, sb, pn, wb
11	23/M	URT	634	>100	+	cp, pe, sb, pn
12	2/M	URT	3072	>100	+	cp, pe, sb, pn
13	7/F	URT	296	46.00	+	cp, pe, sb, pn, wb
14	12/M	URT	1726	18.70	+	cp, pe, sb, pn, wb
15	4/M	URT	1238	92.80	+	cp, pe, sb, pn, wb
16	5/M	URT, AD	780	30.30	+	cp, pe, pn
17	7/M	URT	546	8.26	–	cp, pe, sb, pn, wb
18	6/M	URT	330	6.06	–	cp, pe, sb, pn, wb
19	6/M	URT, AD	480	12.70	+	cp, pe, sb, pn, wb
20	10/M	URT	225	>100	+	cp, pe, sb, pn, wb
21	14/M	URT, AD, V	2290	46.00	+	cp, pe, pn
22	3/M	URT	1216	>100	+	cp, pe, sb, pn, wb

*OAS, Oral allergy syndrome; URT, urticaria-angioedema; A, anaphylaxis; AD, atopic dermatitis; V, vomiting.
†Specific IgE of greater than 0.35 kU/L. cp, Chickpea; pe, pea; sb, soybean; pn, peanut; wb, white bean.

included 1254- and 1245-bp open-reading frames (Fig 5) coding for 418 and 415 amino acid proteins (Fig 6) with theoretic molecular masses of 47,826 d and 47,464 d and isoelectric points of 5.34, respectively. The deduced amino acid sequences for mature Len c 1.0101 and Len c 1.0102 shared 93% of identical residues. Potential N-glycosylation sites were located in the 2 sequences (1 for Len c 1.0101 and 2 for Len c 1.0102).

Interestingly, both deduced amino acid sequences comprised those experimentally determined for the 50-kd lentil allergen Len c 1.01 (hereafter renamed as Len c 1.0103; 17/20 identical residues), the 26-kd reactive protein (4-3/5 identical residues), and Len c 1.02²⁰ (26-23/29 identical residues). Furthermore, the N-terminus of both Len c 1.02 and the 26-kd protein clearly matched the subunit processing sites of pea provicilin.¹⁸

Food and drug reactions
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On the other hand, the full amino acid sequences deduced for Len c 1.0101 (or Len c 1.0102) strongly confirmed the homology to several allergenic vicilins from other plants sources, such as peanut Ara h 1 (50% identity),¹² the β subunit of soybean conglycinin (56% identity),¹³ English walnut Jug r 2 (38% identity),¹⁴ cashew Ana o 1 (32% identity),¹⁵ and sesame Ses i 3 (31% identity).¹⁶

DISCUSSION

A relevant lentil allergen with an apparent molecular size on SDS-PAGE of approximately 50 kd has been purified and characterized. It probably corresponds to an unidentified major IgE-binding band with similar molecular weight described in previous reports,^{1,2} which was recognized by more than 75% of sera from patients with lentil allergy of the Spanish population. The isolated allergen included 3 different isoforms that showed the same N-terminal amino acid sequence and molecular masses ranging from 48.6 to 49.7 kd as determined with MALDI analysis. This heterogeneity can be explained in part by different glycosylation degrees (Fig 2). Complex asparagine-linked glycans were detected in 2 isoforms, a result in line with the described posttranslational processing of legume vicilins^{17,18} and with the N-glycosylation sites (2 in Len c 1.0102) located in the amino acid sequences deduced from the cDNAs encoding Len c 1.01 variants (Fig 6).

The potential allergenic relevance of Len c 1.01 was supported by 2 additional lines of experimental evidence. First, the purified allergen was recognized by a high percentage (77%) of individual sera from patients with lentil allergy. Second, immunoblot and ELISA inhibition testing showed its capacity to inhibit the IgE binding to the crude lentil extract (Figs 3 and 4). Up to 65% inhibition was reached with purified Len c 1.01 (at 2 μ g/mL) compared with the 82% inhibition exerted by the crude lentil extract (at 10 μ g/mL) in the ELISA inhibition assays. Interestingly, both samples showed parallel inhibition profiles, suggesting similarity in their allergenic composition. However, further *in vivo* studies should be performed to confirm the clinical relevance of Len c 1.01, as well as its contribution to the different symptoms.

The purified lentil allergen Len c 1.01 was identified as a mature vicilin chain on the basis of its amino acid sequences determined by means of direct N-terminal sequencing of the purified protein or deduced from the corresponding cDNA nucleotide sequences, as well as on the basis of its determined and calculated molecular size. These seed storage proteins are represented in most legume species by a heterogeneous group of closely related genetic variants encoded by a multigene family.^{17,18} Thus in lentils 4 different types of vicilin sequences gathered into 2 small multigene subfamilies have been described.²⁸ This fact, as expected, was reflected in the heterogeneity found for the isolated allergen, both by means of protein analysis and cDNA sequencing. Three genetic variants of the allergen have been detected attending to the determined amino acid sequences (1 from the N-terminal of the protein and 2

deduced from the complete cDNAs). The variants have been named Len c 1.0103, Len c 1.0101, and Len c 1.0102, according to the proposal of the International Committee of Allergen Nomenclature.

Amino acid sequence comparisons demonstrated a clear relationship between Len c 1.01 and several allergens of the vicilin family, such as Ara h 1,^{11,12} Jug r 2,¹⁴ Ana o 1,¹⁵ Ses i 3,¹⁶ and subunits of soybean conglycinin.¹³ Interestingly, sequence identities of approximately 50% have been uncovered between the complete sequences of Len c 1.01 variants and the corresponding regions of the peanut and soybean allergens mentioned above. This structural relationship represents a firm molecular basis for future studies on the cross-reaction among legumes, particularly peanut, lentil, chickpea, and pea. Preliminary results on the potential clinical significance of Ara h 1 and pea vicilin cross-reactions have been recently published.²⁹

Specific proteolytic cleavage is an additional post-translational modification of some vicilins that can be relevant for the identification of legume allergens. It is known that mature vicilin polypeptides of greater than 50 to 60 kd are processed to subunits (fragments) of 12 to 35 kd in some species, such as pea, whereas proteolysis virtually does not occur in others, such as French bean and peanut.^{17,18} This processing can generate an array of related allergens with different sizes if at least some of the produced subunits retain the IgE-binding capacity. This is the case with the IgE-reactive 16- and 26-kd bands detected in the lentil extracts by using the pool of sera from patients with lentil allergy (Fig 1). On the basis of its molecular size and its increase with boiling, the 16-kd band most probably corresponds to the previously described 12- to 16-kd allergen Len c 1,²⁰ here designated as Len c 1.02. The identification of both bands as fragments derived from mature Len c 1.01 chains is supported by the identity of their N-terminal amino acid sequences with those of internal regions of mature Len c 1.01 (Fig 6). Moreover, the N-terminal end of these regions matched the proteolytic cleavage sites described for pea vicilins,¹⁸ the member of the vicilin family closest to Len c 1.01 (>85% sequence identity). On the other hand, the theoretic molecular weights calculated for the corresponding fragments are in the range shown in SDS-PAGE by some Len c 1.02 isoforms²⁰ and the 26-kd reactive band. Similar proteolytic events can explain the presence of several IgE-binding bands from 15 to 33 kd in purified pea vicilin preparations.²⁹

Finally, the data presented here suggest a potential use of Len c 1.01 as a model allergen, complementary to Ara h 1, to study allergic reactions induced by ingestion of legumes scarcely consumed in the United States, Japan, and other countries but being a relevant source of food protein and an important agent of allergic symptoms in other extensive areas of the world. Thus the production of recombinant Len c 1.01 and its reactive fragments can help not only to standardize the diagnostic tools for lentil allergy but also to determine the location of the main structural regions involved in the IgE-vicilin interactions.

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