

Lentil (*Lens culinaris*) Lipid Transfer Protein Len c 3: A Novel Legume Allergen

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Key Words

Food allergy · Len c 1 · Len c 2 · Lentil-allergic patients · Lipid transfer proteins

Abstract

Background: Lentils are increasingly consumed in many parts of the world. Two allergens, Len c 1 and 2, have been reported previously. Recently, peanut and green bean lipid transfer proteins (LTPs) have been identified as the first two members of an important group of allergens that might be associated with severe food allergies. **Objective:** To investigate lentil LTP as a potential new allergen. **Methods:** Efficacy of LTP extraction was monitored at different acidic pH values, using immunoblotting with cross-reactive anti-peach LTP antiserum. Natural LTP was purified from lentil extract and expressed as recombinant allergen in *Escherichia coli*. Sera from 10 lentil-allergic and/or -sensitized patients (Spain: 6, Italy: 1 and the Netherlands: 3) were used to further characterize lentil LTP. **Results:** Natural lentil LTP, purified from the homogenized germinated seeds and optimally extracted at pH 3, was identified and designated as allergen Len c 3. By CAP, 9/10 sera showed specific IgE to Len c 3. Recombinant (r) Len c 3 was successfully purified. The natural (n) Len c 3 CAP was completely inhibited by rLen c 3/rPru p 3. IgE binding to lentil pH 3 extract blot was completely inhibited

by rLen c 3. **Conclusion:** The availability of immunochemically active nLen/rLen c 3 as a novel legume allergen facilitates further development and implementation of a third (next to peanut and green bean) legume LTP in component-resolved diagnosis strategies and contributes to evaluate the clinical importance of legume LTPs. Preferential extraction of Len c 3 (pH 3) will affect the production of sensitive extract-based diagnostic tests.

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Introduction

The lentil (*Lens culinaris*) is a bushy plant of the legume family. Lentils are consumed in many countries, mainly because of their nutritional value and modest culturing requirements. Lentils rank among the healthiest foods in the world [1]. In addition to 26% protein (w/w), lentils contain dietary fiber, folate, vitamin B₁, minerals and just 1% fat. The prevalence of lentil hypersensitivity in the countries of the Mediterranean area where lentils are common food has been studied previously and showed that the symptoms of lentil allergy are ranging from mild

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Table 1. Patient characteristics

Serum	Gender	Age	Specific IgE, kU/l			Allergic symptoms after consumption of the indicated food	SPT
			lentil	nLen c 3	rPru p 3		
SP1	female	47	5.6	12.8	20.3	bean, pea: U	lentil+, bean+, pea+
SP2	female	29	10.8	1.4	NT	lentil: S	lentil+
SP3	female	6	13.6	13.9	41.0	lentil: OAS	lentil–
SP4	male	44	1.1	0.8	6.7	lentil: S	NP
SP5	female	28	7.8	8.2	NT	peach: OAS	peach+
SP6	female	26	1.8	6.6	NT	peach: U	peach+
IT1	female	30	3.0	4.0	NT	lentil: U	bean+, pea +
NL1	male	38	20.2	1.4	9.3	legumes ¹ : OAS	NP
NL2	male	33	27.0	0.2	6.9	lentil: S	peanut+
NL3	female	48	2.3	3.5	9.0	legumes ¹	NP

NT = Not tested; NP = not performed; U = urticaria; S = systemic reaction; OAS = oral allergy syndrome.

Allergic symptoms were defined as at least 1 allergic reaction after the consumption of the indicated food.

¹ Reported allergy for some legumes but nothing known for lentil.

to severe in both adult and pediatric populations [2, 3]. So far, Len c 1, a 47-kDa vicilin, and Len c 2, a seed-specific biotinylated 66-kDa protein, have been identified as lentil allergens. Also, other allergens have been detected by Western blotting, but their identity is still unknown [4]. Lentils are boiled prior to consumption, indicating that lentil allergens, at least some of them, are heat stable. Plant food lipid transfer proteins (LTPs) [5–7] are well-established risk factors for severe fruit and nut allergies [8]. LTPs are compact proteins with a molecular mass of ± 9 kDa, and their tertiary structures are maintained by 4 conserved disulfide bonds even under proteolytic conditions. These properties facilitate LTPs to reach the central parts of the gastrointestinal tract as fully allergenic compounds, from where they can initiate severe systemic reactions [5]. Recently, LTPs were also identified as an allergen in legumes, i.e. in peanut [9–11] and green bean [12, 13]. Possibly, the identification of legume LTPs has been delayed because commonly used diagnostic reagents for legume allergy, obtained by extraction at neutral pH, were shown to be devoid of LTPs. Only at acidic pH LTP was effectively extracted from peanut. Recently, LTP was purified and cloned from lentil, but its role as a putative allergen has not yet been investigated [14]. Here we report its production as a recombinant molecule and its identification as an allergen. The availability of the lentil LTP, identified as allergen Len c 3, might facilitate further research with respect to: lentil allergy prevalence/geographic distribution and allergic symptom-eliciting capacity.

Methods

Retrospectively Selected Serum Samples

Human sera were obtained from 10 patients sensitized for lentil. Patients (5/10) demonstrated clear allergic symptoms after lentil consumption ranging from mild to severe, whereas 3/10 patients showed symptoms after other legume consumption like peanuts or peas. From the latter group, no clear data were available on whether these patients were tolerant or not with respect to lentil consumption. Sera of 2 additional patients (SP5 and SP6, sensitized to lentil/Len c 3 but without reported lentil symptoms) were used for nLen c 3 CAP inhibition (table 1).

Patients were recruited from the Hospital Clínico San Carlos, Madrid, Spain; Clinica San Carlo, Paderno Dugnano, Italy, and from the Department of Dermatology and Allergy at the University Medical Center of Utrecht, The Netherlands. The study was reviewed and approved by the local ethical committees in Spain and Italy and by the Ethical Committee of the University Medical Center of Utrecht, The Netherlands.

Cross-reactive polyclonal antibodies against the peach LTP were obtained as described for rMal d 3 [15].

Lentil Protein Extract Preparation

Ground dry lentil seeds were extracted (10% w/v) in PBS or in distilled water at different pH levels, ranging from 2.0 to 6.0 [16]. Following addition of the lentil flour, the pH of the distilled water was adjusted again to the desired values. After 1 h of extraction, the protein-containing solution was centrifuged at 16,100 g. The supernatant was collected and subjected to protein determination (Pierce, Rockford, Ill., USA).

Purification of nLen c 3

Purification of natural lentil LTPs was performed as reported previously [14]. In brief, a crude protein preparation was obtained from the homogenized germinated lentil seeds after extraction, centrifugation and ammonium sulfate precipitation. Subsequent

ly, the resulting precipitate was dissolved in 200 mM ammonium acetate with 2 M urea (pH 7.0) and loaded onto a Sephadex G-75 column equilibrated with the same buffer. The fractions containing 7- to 9-kDa proteins were further purified using cation exchange chromatography on Mono S column and reverse-phase (RP)-HPLC. As a result, 2 LTP-containing fractions were obtained. These fractions were vacuum dried, dissolved in water, freeze dried, stored at -20°C and dissolved in water prior to usage. In the present study, a fraction containing the mixture of Lc-LTP2 and Lc-LTP4 (GenBank accession Nos. AY793554 and AY793558, respectively) with Lc-LTP2 as the major component was used. Both Lc-LTP2 and Lc-LTP4 consist of 93-amino-acid residues and differ from each other by the only residue at position 85 (Ser/Thr). The Allergen Nomenclature Committee of the International Union of Immunological Societies has recently designated lentil LTP as Len c 3 (namely Lc-LTP2 isoform as Len c 3.0101).

Len c 3 Heterologous Expression in Escherichia coli

Cloning of 6 isoforms of Len c 3 (Lc-LTP1–6) was reported previously [14]. Lc-LTP2 was selected as rLen c 3 isoform for expression and became available for immunological characterization. The expression cassette was composed of T7lac promoter, a ribosome binding site, a start codon (ATG), and a sequence encoding N-terminal histidine octamer, TrxL carrier protein, Asp-Pro-Met double cleavage site and the mature Lc-LTP2 sequence. It was assembled of several PCR amplicons spliced in the desired order and cloned in a high copy number pGEM-T vector (Promega, USA). Using PCR 2 Polymerase Mix (Clontech, USA), PCR products were separated in agarose gel and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA). The sequence of TrxL, representing modified *E. coli* thioredoxin A, was amplified from pET-32a(+) vector (Novagen, USA) by SOE PCR that introduced M37L mutation. In order to prevent the carrier protein fragmentation and simplify further purification, the internal methionine residue within the thioredoxin sequence was replaced with a leucine residue by site-directed mutagenesis (M37L). The Lc-LTP2 sequence was amplified from *L. culinaris* cDNA using gene-specific primers: GCGAGATCTGATCCGATGGCAATCTCATGTGGAGCT (forward) and GCGAATTCGCGGATCCTTAAAC-TTAACTGTATTA (reverse). The 790-bp expression cassette was excised from the cloning vector and ligated with the 5,251-bp BglII/XhoI fragment of pET-31b(–) vector (Novagen) containing pBR322 origin of replication, β -lactamase gene, LacI gene, and T7 terminator, resulting in the pET-His8-TrxL-Lc-LTP2 expression plasmid (6,041 bp). *E. coli* strain DH-10B (Life Technologies, USA) was used for cloning and plasmid preparation. *E. coli* BL-21 (DE3; Novagen) was chosen as an expression host. The culture of BL-21 (DE3)/pET-His8-TrxL-Lc-LTP2 cells was grown in Erlenmeyer flasks containing SOB or LB media with addition of 20 mM glucose and 100 $\mu\text{g}/\text{ml}$ ampicillin. After OD_{600} reached 0.6–0.7, the cells were induced with 0.2 mM isopropyl β -D-1-thiogalactopyranoside and further incubated at 37°C at 200 rpm for 4–6 h. The expression level and solubility of the fusion protein His8-TrxL-Lc-LTP2 were monitored using SDS-PAGE.

Recombinant Len c 3 Purification and Identity Confirmation

The cells harvested by centrifugation at 4,000 g were sonicated on ice in the binding buffer (50 mM Tris-HCl, pH 7.8, 0.5 M NaCl, 20 mM imidazole and 1 mM PMSF). After centrifugation for 20 min at 25,000 g, the supernatant containing soluble fusion protein

was collected and loaded onto a Ni-NTA (Qiagen, Germany) column prewashed with the binding buffer. The fusion protein was eluted with 0.5 M imidazole and dialyzed overnight against deionized water before lyophilization. The protein of interest was dissolved in 80% TFA (10–20 mg/ml) containing an equal mass of CNBr. The chemical cleavage was performed overnight at room temperature in the dark. Then the mixture was diluted 10-fold with distilled water, dried and dissolved in 80% TFA. The last step of rLen c 3 purification was performed on a Reprosil-Pur C₁₈-AQ RP column (5 μm , $250 \times 10 \text{ mm}$; Dr. Masch, Germany) using a linear gradient from 5 to 80% acetonitrile for 60 min with 0.1% TFA at a flow rate of 2 ml/min. After RP-HPLC, purified proteins were vacuum dried, dissolved in water, freeze dried and stored at -20°C . Purified rLen c 3 was analyzed by MALDI-TOF MS using a Reflex III mass spectrometer (Bruker, Germany) and by automated Edman degradation using the Procise cLC 491 Protein Sequencing System (Applied Biosystems, USA). Purified nLen and rLen c 3 were analyzed by SDS/PAGE followed by gel staining and immunoblotting. Antimicrobial activity of nLen and rLen c 3 towards the *Agrobacterium tumefaciens*, strain A281, and *Aspergillus niger*, VKM F-2259, was measured microspectrophotometrically using 96-well microplates as described [14]. In case of *A. niger*, the inhibition of spore germination was evaluated by measuring absorbance at 620 nm. *A. niger* was grown on potato glucose agar plates for 10 days until spores were abundantly produced. The spores of *A. niger* were collected, and the concentration was adjusted to 10^4 spores/ml using double-diluted potato glucose medium. Spore suspension (110 μl) was incubated in microtiter wells with the indicated amount of proteins (10 μl) for 48 h at 25°C . The IC_{50} was defined as the concentrations necessary for 50% growth inhibition.

Specific IgE Determination by ImmunoCAP

Specific IgE against lentil and peach (Pru p 3) LTP was measured by ImmunoCAP (Phadia, Uppsala, Sweden; f235 and f420, respectively) according to the manufacturer's instructions. In case of specific IgE against lentil LTP, purified nLen c 3 was biotinylated (molar ratio 1:10) using sulfo-NHS-LC-biotin according to the manufacturer's instructions (Pierce, USA). Subsequently, biotinylated nLen c 3 was coupled to a streptavidin-coated CAP (0.5 μg allergen/CAP) [17].

nLen c 3-CAP inhibition was performed by preincubation of 100 μl SP5 or SP6 serum with (serially diluted) 100 μl rLen c 3 or rPru p 3 for 1 h at room temperature.

SDS-PAGE/Immunoblotting

Lentil extracts prepared at different pH levels were applied onto 4–12% gradient SDS polyacrylamide gels (5.2 $\mu\text{g}/\text{lane}$) and subsequently Western blotted to nitrocellulose according to the manufacturer's instructions (Novex, Invitrogen, Calif., USA). After overnight incubation with 1 μl rabbit anti-peach LTP antibodies in a final volume of 12 ml PTE (PBS/0.1% Tween 20/0.5% skimmed milk powder), the blot was washed 5 times in PBS/0.1% Tween. Bound rabbit antibodies were detected using goat anti-rabbit HRP (KPL, Gaithersburg, Md., USA)-conjugated antibodies in conjunction with enhanced chemiluminescence (GE Healthcare, Life Sciences, Waukesha, Wisc., USA) and Fuji Medical X-ray film super RX.

For IgE binding analyses, Len c 3 immunoblot strips (5 μg LTP/3-mm-wide strip) were incubated with pooled serum (SP1–

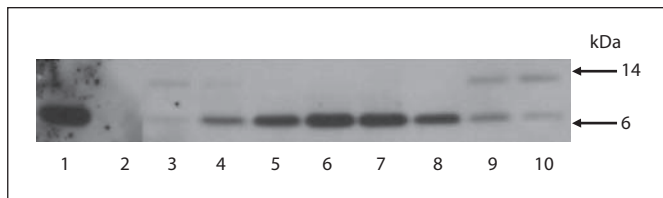


Fig. 1. Immunoblot of lentil proteins extracted at different pH levels. Lentil proteins were detected using rabbit anti-peach LTP antibodies. After washing, bound rabbit antibodies were detected using goat anti-rabbit antibodies conjugated to HRP. Lanes: 1 = nLen c 3; 2 = lentil PBS extract; 3 = H₂O; 4 = pH 2.0; 5 = pH 2.5; 6 = pH 3.0; 7 = pH 3.5; 8 = pH 4.0; 9 = pH 5.0; 10 = pH 6.0.

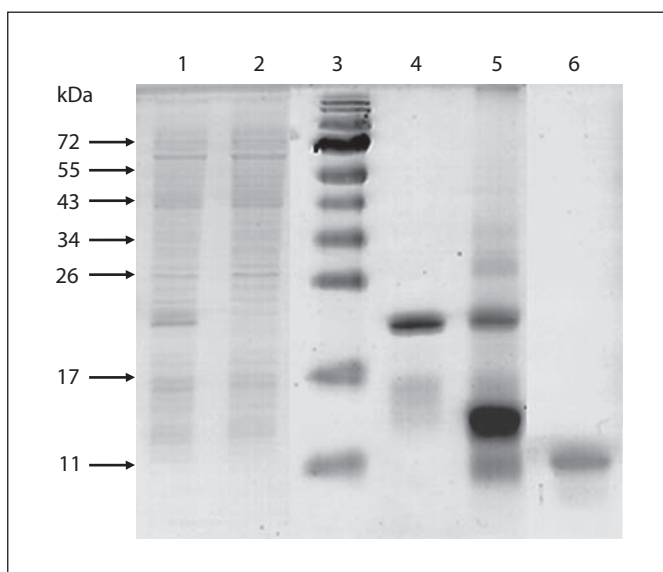


Fig. 2. SDS-PAGE analysis of the rLen c 3 expression and purification. Lanes: 1 = clarified lysate; 2 = flow-through from a Ni-NTA column; 3 = molecular mass standards; 4 = eluate from a Ni-NTA column; 5 = the fragments after CNBr cleavage; 6 = the LTP-containing fraction after RP-HPLC.

SP3). Bound IgE was detected as described previously [18]. After SDS-PAGE followed by immunoblotting, Len c 3 (3-mm-wide strip, 0.8 µg) was incubated overnight with 100 µl human serum samples in a final volume of 3 ml PTE. Bound IgE was detected using HRP-conjugated goat anti-human IgE (KPL). For lentil pH 3 immunoblot inhibition, SP1 was preincubated with 10 µg rLen c 3 before incubation with a blot. Bound IgE was detected using custom-made monoclonal antibody (1:10,000; anti-human IgE MH-25; Sanquin, Amsterdam, The Netherlands) conjugated to IRDye® 800 (LI-COR, Biosciences, Lincoln, N.E., USA) by Rockland Immunochemicals (Gilbertsville, Pa., USA). Bands were visualized using the infrared scanning equipment from Odyssey (LI-COR).

Results

Lentil Extract Preparation at Different pH Levels

Lentil protein extracts were prepared at different pH levels ranging from 2.0 to 6.0 and showed an optimum extraction of a protein, presumably LTP (~9 kDa) at pH 3.0, as was demonstrated by Coomassie staining (not shown) and immunoblotting with rabbit anti-Pru p 3 antiserum (fig. 1). Almost no signal at ~9 kDa was detected in the extracts obtained at neutral pH (PBS and water). A band of almost 14 kDa was recognized by the same rabbit antiserum at pH 5 and 6. This could be a protein that was already recognized by the pre-immune serum from the rabbit in lentil extracts of rather neutral pH.

Characterization of nLen and rLen c 3

Len c 3 was expressed in *E. coli* as a fusion protein with thioredoxin. Most of the fusion protein was expressed in soluble form. The expression level constituted up to 15–20% of the total cell protein after induction with IPTG, as determined by SDS-PAGE. The purification protocol of rLen c 3 included separation of cell-soluble proteins, Ni-NTA, affinity chromatography, the fusion protein cleavage with CNBr and subsequent RP-HPLC (fig. 2). This purification scheme resulted in a final yield of 1.5–2 mg of rLen c 3 per liter of starting culture.

nLen c 3 and rLen c 3 showed matching retention times under the same RP-HPLC conditions. The molecular mass of the recombinant Len c 3 (9,282.3 Da), determined by MALDI-TOF mass spectrometry, correlated well with the calculated molecular mass of Lc-LTP2 (9,282.7 Da) stabilized with 4 disulfide bonds. Homogeneity of the recombinant Len c 3 and its identity to the natural protein were also monitored by SDS-PAGE, automated microsequencing and antimicrobial assay. It was shown that rLen c 3 was identical to the natural protein in respect of their molecular masses, amino acid sequences and antimicrobial activities.

The purified rLen c 3 suppressed growth with the same efficiency as nLen c 3 of the Gram-negative bacterium *Agrobacterium tumefaciens*, strain A281, IC₅₀ 40.5 µM, and phytopathogenic fungus *A. niger*, VKMF-2259, IC₅₀ 17.5 µM. Altogether these experiments demonstrate that rLen c 3 closely resembles its natural homologue physically. It is worth mentioning that the electrophoretic patterns of both purified nLen and rLen c 3 sometimes showed an additional band around the 18-kDa region which corresponds to spontaneously formed protein dimer (data not shown).

CAP and Immunoblot Analyses

All 10 patients demonstrated lentil-specific IgE titers (ranging from 1.1 to 27.0 kU/l; table 1). Specific IgE against purified nLen c 3 was positive (>0.35 kU/l) in 9/10 cases. IgE antibody titers against peach LTP (rPru p 3) were significantly higher ($p = 0.03$) than against the natural Len c 3. The nLen c 3 CAP of Spanish patients 5 and 6 was completely inhibited by rLen c 3 and rPru p 3 (fig. 3). IgE binding to the 9-kDa band on a lentil pH 3 blot was completely inhibited with rLen c 3 (fig. 4).

On purified nLen c 3 blot, 7/7 patients recognized both monomer and dimer nLen c 3 (fig. 5a). Natural Len c 3 (including the dimer) and rLen c 3 were recognized by IgE at the same molecular weight level using serum pooled from Spanish patients 1–3 (fig. 5b) and by cross-reactive rabbit anti-Pru p 3 (data not shown).

Discussion

The most notorious legume allergy is undoubtedly allergy to peanut, both in terms of prevalence and severity [19, 20]. Another legume often associated with suspicion of food allergy is soy [21]. More recently, lupine has been reported as a potential source of food allergy, mainly as flour in composite foods [22]. Lentil has received rather limited attention as potentially allergenic food, but increased consumption of this healthy low-fat, protein-rich legume [1] may lead to an increased prevalence of allergic reactions.

For peanut, essentially three sources of sensitization have so far been described. Peanut allergy based on direct sensitization via oral intake (or via the skin [23]) of peanuts has been demonstrated to be dominated by several seed storage proteins, i.e. Ara h 1 (vicilin), Ara h 2 and 6 (2S albumins), and Ara h 3 (glycinin) [24]. Also in lentils, a vicilin-like 7S globulin has been identified as major allergen [4]. Peanut allergy can also originate from primary sensitization to pollen. In this case, homologues of Bet v 1 and profilin, Ara h 8 [18] and Ara h 5 [25], respectively, are implicated in the induction of peanut allergy. For lentils, these putative allergens have not yet been studied. Most recently, a third source of sensitization has been added. Already 10 years ago, Asero et al. [5] demonstrated that IgE antibodies to peach LTP cross reacted with peanuts. Food allergy to lentils was also reported in some of the patients. In the Mediterranean countries, like Spain and Italy, peanut allergy has now indeed been shown to be linked to LTP sensitization. In this case, peach LTP has been proposed to be the primary sensitizer leading to cross-reactivity to

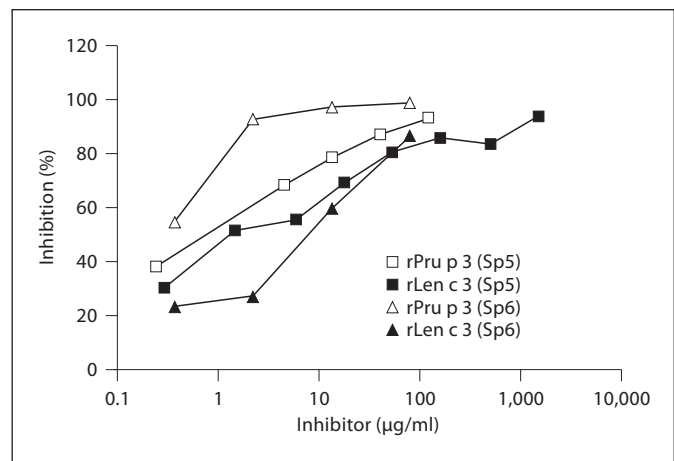


Fig. 3. nLen c 3 CAP inhibition. Sera (100 μ l) from 2 Spanish patients (SP5 and SP6) were incubated with serial dilutions (100 μ l) of rPru p 3 and rLen c 3 prior to addition to the nLen c 3 CAP.

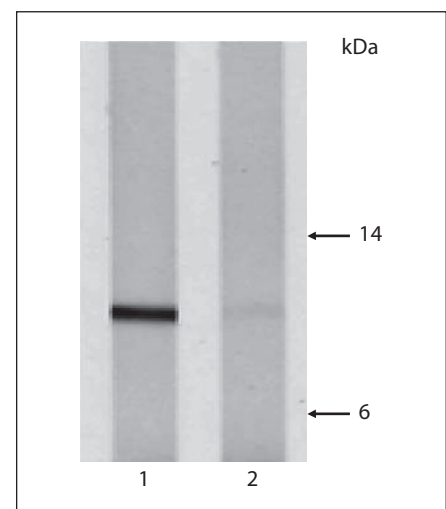
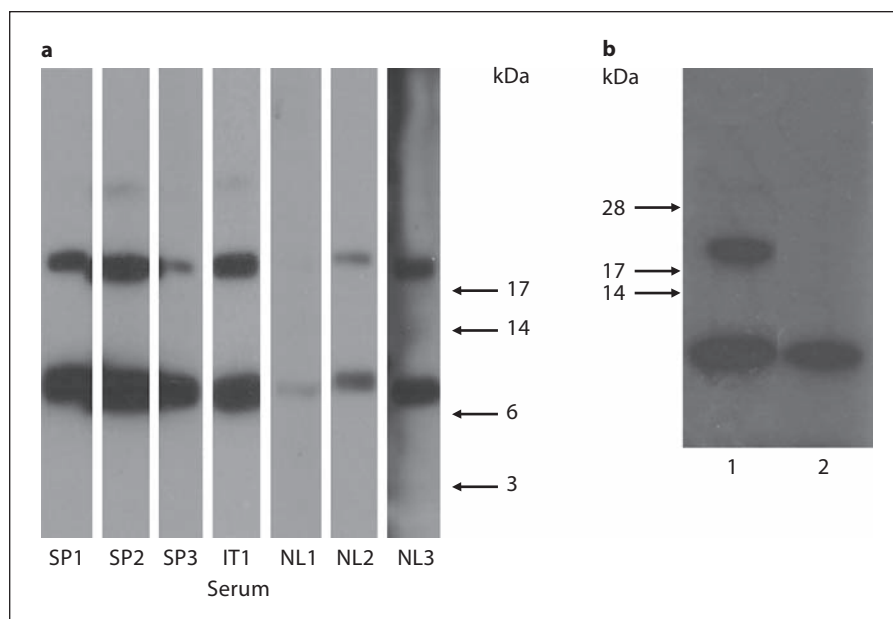


Fig. 4. Immunoblot inhibition assay. Blot strips of lentil extract prepared at pH 3.0 were incubated with Spanish serum 1 without or with 10 μ g rLen c 3. Bound IgE was detected using mAb anti-human IgE conjugated to IRDye800cw and visualized using the Odyssey equipment. Lanes: 1 = 100 μ l serum; 2 = 100 μ l serum plus 10 μ g rLen c 3 as inhibitor.

peanut LTP (Ara h 9) [9–11] and bean LTP (Pha v 3) [12, 13]. In the present study, we have now extended this to a role of LTP as a cross-reactive allergen in lentils, the third legume for which LTP is shown to be an allergen.

One of the possible reasons that legume LTPs have long escaped from attention is that extraction at neutral pH

Fig. 5. a Purified nLen c 3 immunoblot incubated with 3 Spanish (SP1–SP3) and 3 Dutch sera (NL1–NL3) and 1 Italian serum (IT1). Bound IgE was detected using goat anti-human IgE-HRP. **b** nLen c 3 (lane 1) compared with rLen c 3 (lane 2) using Spanish pooled serum (SP1–SP3). Bound IgE was detected by ^{125}I sheep anti-human IgE.



very ineffectively solubilizes these proteins. This was first demonstrated by Krause et al. [11] for peanuts and confirmed for lentils in the current study. Preferentially at acidic pH, peanut and lentil LTPs are very effectively extracted, as was shown by immunoblotting. These findings are important for improving sensitivity of extract-based diagnostic tests. Negative skin prick tests obtained with extracts that do not contain all relevant allergens might result in false-negative results (e.g. in SP3), which might subsequently jeopardize the patient's safety [26]. The purification of both nLen and rLen c 3 facilitated a thorough physiological characterization. Comparison of molecular weight, chromatographic retention times, automated microsequencing and antimicrobial activity showed a close resemblance between these artificial 'twin allergens'.

Also, the immunological characterization in general shows similar IgE binding properties to both nLen and rLen c 3. IgE of Spanish pooled sera detects both nLen and rLen c 3 (with an additional dimer in case of nLen c 3) with comparable intensity, although immunoblots are not the optimal technique to quantify IgE binding. The 9-kDa band obtained after extraction at pH 3.0 was completely inhibited after preincubation of Spanish serum 1 with rLen c 3, thereby indicating a close immunological resemblance and confirming that this band very likely represents Len c 3. nLen c 3 CAP inhibitions (using serum from Spanish lentil-sensitized patients 5 and 6) with rLen c 3 and rPrup 3 showed a stronger inhibition by rPrup 3 although rLen c 3 was also capable of complete inhibition.

As putative sensitizing allergen, rPrup 3 [7] was expected to be the better inhibitor. Thus, both rPrup 3 and rLen c 3 demonstrated complete inhibition indicating that rPrup 3 is cross reactive with Len c 3 and that rLen c 3 mimics its natural counterpart sufficiently. The differences observed for the inhibition curves in figure 3 between the 2 Spanish sera might indicate that different IgE binding epitopes are involved and/or epitope-specific IgE titers differ. Cloning and expression of additional isoforms of rLen c 3 might yield isoform(s) that cover all relevant IgE binding epitopes.

Future basophil histamine release experiments aim to estimate the biological activity of Len c 3. Unexpectedly, Dutch patients 1–3 demonstrated clearly elevated IgE anti-rPrup 3 titers. This is not very common in birch endemic areas and it is also not clear whether these patients can tolerate peach or not, but recent research in the Netherlands showed more Pru p 3 sensitization than expected (own observations).

In conclusion, it remains to be established whether lentil LTP for lentil allergy in Mediterranean countries is as important as fruit and tree nut LTPs. Currently this is also being investigated using serum samples from the EuroPrevall surveys on food allergy. Serum samples from 12 European cities, China, India and Western Siberia are being screened for IgE reactivity for lentils and lentil LTP. These studies will establish the importance of this novel legume LTP for lentil-allergic patients and highlight potential geographical differences.

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